Obtention de nanoparticules à base de polymères : étude fondamentale et application au développement de nanocapsules à usage pédiatrique

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Obtention de nanoparticules à base de polymères: étude fondamentale et application au développement de nanocapsules à usage pédiatrique

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RESUME

L’objectif de ce travail de thèse est d’étudier la relation entre la méthode de préparation des nanoparticules, les propriétés colloïdales et l’encapsulation d’un principe actif à usage pédiatrique. Dans ce but, le diclofenac a été utilisé comme molécule modèle et les nanoparticules ont été préparées via la nanoprécipitation et l’émulsification-diffusion. Des études fondamentales et systématiques ont permis de mettre en évidence l'existence de différences notables entre les propriétés électrocinétiques et l'efficacité d’encapsulation en fonction du procédé utilisé pour la préparation des particules. Ces propriétés colloïdales et physico-chimiques sont primordiales pour la bonne stabilité des dispersions de nanosphères et des nanocapsules et pour le comportement de ces vecteurs lors d’utilisation *in vivo*. Cette étude a permis de proposer et de discuter le mécanisme de formation des nanoparticules en se basant sur le comportement des variables critiques du procédé et de la formulation, les propriétés de surface et l'efficacité d’encapsulation de l’actif modèle.

Mots clés: Nanoprécipitation, émulsification-diffusion, nanoparticules, encapsulation, libération, diclofenac.

ABSTRACT

The objective of this PhD thesis is to point out the relationship between the preparation method of the nanoparticles, the colloidal properties and the encapsulation efficiency of a given active molecule for paediatric purpose. In this direction, diclofenac was used as model molecule and the nanoparticles were prepared via the nanoprecipitation and the emulsification-diffusion processes. The conducted fundamental and systematic studies rend evident notable differences between the two processes, particularly in the electrokinetic properties of the particles and the effectiveness of the drug encapsulation. These colloidal and physicochemical properties are paramount for the good stability of the nanoparticles and their *in vivo* use. This research work made it possible to propose and to discuss the mechanism of nanoparticle formation from the behavior of key variables of the process and the recipe used, the surface properties of the particles and the effectiveness of encapsulation of the model drug.

Key words: Nanoprecipitation, emulsification-diffusion, nanoparticles, encapsulation, drug release, diclofenac.

SOMMAIRE

INTRODUCTION GÉNÉRALE

Dans le domaine pharmaceutique, les nanoparticules (nanosphères et nanocapsules) sont largement utilisées pour la vectorisation des principes actifs. Durant ces dernieres decenies, un nombre important de travaux de recherche ont été cités dans la litterature portant esentiellement sur la résolution des problèmes liés à la stabilité, à l'efficacité et à l'administration des molécules actives[1]. Ainsi, par exemple, ces particules peuvent protéger les actifs contre toute dégradation rapide (ou d'agents de dégradations présents dans le milieu de dispersion), contrôler leur profil de libération, augmenter leur absorption ou bien protéger l'organisme contre leurs effets secondaires tels que l'irritation gastro-intestinale.

Une application intéressante des nanoparticules comme vecteurs de molécules actives est leur utilisation dans le domaine pédiatrique. Actuellement, il y a un changement de paradigme par rapport à la thérapie avec médicaments chez l'enfant[2]. Normalement, pour ce groupe de patients, une quantité importante de thérapies sont adaptées à partir de médicaments développés et évalués par rapport à leur efficacité et sécurité chez l'adulte. Cependant, depuis quelques années, la meilleure compréhension scientifique de la physiologie des enfants, des aspects pharmacocinétiques et pharmacodynamiques liés à l'administration des substances actives ont rendu évidente la nécessité de développer des formulations innovatrices spécifiques aux enfants suivant un cahier des charges rigoureux afin de garantir une administration facile[3].

[1] C. Pinto, R.J. Neufeld, A.J. Ribeiro, F. Veiga, Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles, Nanomedicine: NBM. 2 (2006) 8-21; E. Fattal, C. Vauthier, Nanoparticles as drug delivery systems, in: J. Swarbrick, J.C. Boylan (Eds.), Encyclopedia of Pharmaceutical Technology, Marcel Dekker, New York, 2002, pp. 1864-1882; C. Vauthier, K. Bouchemal, Methods for the preparation and manufacture of polymeric nanoparticles, Pharm. Res. 26 (2009) 1025-1058; V. Lassalle, M. Luján, PLA nano and microparticles for drug delivery: An overview of the methods of preparation, Macromol. Biosci. 7 (2007) 767-783.

[2] K. Rose, Challenges in pediatric drug development, Pediatr. Drugs 11 (2009) 57-59.

[3] European Medicines Agency EMEA, Reflection paper: Formulations of choice for the paediatric population, London, 2006; J.F. Standing, C. Tuleu, Paediatric formulations. Getting to the heart of the

Par exemple, en premier lieu il faut prendre en compte que l'organisme de l'enfant est en constante évolution sous l'effet de la croissance et de la maturation, ce qui le rend particulièrement vulnérable aux agents étrangers tels que les médicaments et par conséquent une sélection adéquate des matières premières conforme aux normes de sécurité est exigée[4]. Par ailleurs, compte tenu que les doses des molécules actives doivent être normalement adaptées au poids de l'enfant, la quantité d'actif par unité de dosage devrait être optimisée afin de permettre une administration de différentes quantités de la molécule active sous des formes galéniques adéquates (par exemple, des gouttes). Un troisième aspect à considérer est lié aux propriétés organoleptiques du produit, car pour ce groupe de patients, elles contribuent à l'accomplissement des traitements. Finalement, comme pour tous les produits pharmaceutiques, les exigences de stabilité sont importantes afin de garantir la conservation de la qualité des médicaments dans le temps[5].

En 2006, le Laboratoire d'Automatique et de Génie des Procédés (LAGEP, UMR-5007) de l'Université Claude Bernard Lyon-1 a rapporté l'un des travaux pionniers dans le domaine du développement des nanoparticules à usage pédiatrique[6]. Cette recherche a été dédiée à la préparation et à la caractérisation des nanocapsules de spironolactone en utilisant la technique de nanoprécipitation. Des études à l'échelle de laboratoire (via le procédé classique) et la production à l'échelle pilote (en utilisant un contacteur membranaire), ont permis d'examiner l'effet de quelques variables liées à la formulation et au procédé d'obtention des nanocapsules.

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[5] World Health Organization, Development of paediatric medicines: Pharmaceutical development. Points to consider, QAS/08.257, Geneve, 2008; J.A. Mennella, G.K. Beauchamp, Optimizing oral medications for children, Clin. Ther. 30 (2008) 2120-2132; World Health Organization, Technical consultation on the use of pharmacokinetic analyses for paediatric medicine development, Geneve, 2009.

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Une autre substance active, présentant un intérêt pour la recherche des nouvelles alternatives thérapeutiques d'administration chez l'enfant est le diclofenac, qui est un anti-inflammatoire non-stéroïdien[7], indiqué pour le traitement du rhumatisme chronique infantile[8] et de la douleur postopératoire[9]. Dans ces cases, le dosage (normalement entre 2 et 3 mg/kg[10]) est adapté en utilisant des produits pharmaceutiques développés pour les adultes.

Le principal effet secondaire des traitements avec le diclofenac est l'irritation de la muqueuse gastro-intestinale après son administration par voie orale, due essentiellement au contact direct d'une concentration élevée de cette molécule avec les muqueuses. Egalement, mais de façon moindre, cette irritation peut avoir une origine systémique lors de la circulation entérohépatique du diclofenac. Pour cette raison, les formes galéniques solides disponibles, généralement sous forme de comprimé, ont un enrobage gastro-résistant, ce qui empêche leur fractionnement et par conséquent, limite l'usage de cet actif pour des patients moins de 12 ans. Il existe aussi des comprimés solubles, dispersibles, effervescents ainsi que des poudres solubles avec doses à partir de 0.15 mg[11]. Cependant, ces produits ont un goût amer, astringent et produisent une sévère irritation au niveau du larynx[11]. Quelques nouveaux développements ont été réalisés pour obtenir des solutions de préparation extemporanée contenant le diclofenac tout en réduisant les effets secondaires, comme celles basées sur le mélange de diclofenac avec des carbonates de soude et de magnésium[12].

[7] B. Chuasuwan, V. Binjesoh, J.E. Polli, H. Zhang, G.L. Amidon, H.E. Junginger, K.K. Midha, V.P. Shah, S. Stavchansky, J.B. Dressman, D.M. Barends, Biowaiver monographs for immediate release solid oral dosage forms: Diclofenac sodium and diclofenac potassium, J. Pharm. Sci. 98 (2009) 1206-1219.

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Des travaux initialement développés dans le Laboratoire de Pharmacie Galénique et Biopharmacie de l'Université Paris XI et poursuivis dans l'Universidade Federal do Rio Grande do Sul, au Brésil, montrent la faisabilité d'encapsuler le diclofenac dans des nanoparticules tout en réduisant l'irritation de la muqueuse digestive[13]. Ces travaux de recherches basés sur la méthode de nanoprécipitation ont été réalisés en examinant l'effet des matières premières impliquées dans la formulation (polymères, huiles et agents stabilisants). La stabilité colloïdale des dispersions[14], la libération de l'actif encapsulé[15] et la stabilisation des nanoparticules par des méthodes de séchage par atomisation[16] et de lyophilisation[17] ont été étudiés. Néanmoins, quelques problèmes de stabilité des dispersions aqueuses de nanoparticules contentant le diclofenac ont été rapportés et attribués à la présence d'une fraction du principe actif non encapsulé[18].

Dans la continuité de ces travaux et dans le but de contribuer à la bonne compréhension de la formation et de la stabilité des systèmes nanoparticulaires, l'objet principal de cette thèse est d'étudier la relation entre la méthode de preparation, les propriétés colloïdales des dispersions formulées et leurs comportements comme vecteurs de molécules actives. Dans ce travail de

[13] S.S. Guterres, H. Fessi, G. Barrat, J.P. Devissaguet, F. Puisieux, Poly(DL-lactide) nanocapsules containing diclofenac: I. Formulation and stability study, Int. J. Pharm. 113 (1995) 57-63; S.S. Guterres, H. Fessi, G. Barrat, F. Puisieux, J.P. Devissaguet, Poly(D,L-lactide) nanocapsules containing non-steroidal anti-inflammatory drugs: Gastrointestinal tolerance following intravenous and oral administration, Pharm. Res. 12 (1995) 1545-1547.

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[18] S.R. Schaffazick, A.R. Pohlmann, L.L. Freitas, S.S. Guterres, Caracterização e estudo de estabilidade de suspensões de nanocápsulas e de nanoesferas poliméricas contendo diclofenaco, Acta Farm. Bonaerense 21 (2002) 99-106.

recherche, seules les méthodes de préparations utilisant les polymères préformés ont été considérées et le diclofenac a été choisi comme molécule modèle.

Dans ce sens, l'objectif du *premier chapitre* est d'extraire de l'état de l'art une approche théorique et comparative des méthodes de préparation des nanoparticules biodegradables en identifiant les techniques et les matières premières les plus utilisées. Une attention particulière est portée sur la tendance générale des propriétés colloïdales selon la méthode de préparation et en particulier les propriétés qui pourraient être intéressantes pour le développement de produits pharmaceutiques tels que la taille et la charge de surface (ou le potentiel zêta) des particules comme l'efficacité d'encapsulation et la libération du principe actif.

Par ailleurs, l*e deuxième chapitre* a comme objectif d'étudier expérimentalement l'incidence de la méthode de préparation sur les propriétés des nanoparticules formulées. Pour ce faire, une étude systématique et comparative ainsi qu'une étude s'appuyant sur une méthode statistique de planification expérimentale ont été conduites pour l'obtention des particules neutres par nanoprécipitation et par émulsification-diffusion. Il est à noter que ces deux méthodes sont les plus utilisées pour la préparation de nanoparticules à partir de polymères préformés. Dans un premier temps, une étude sur l'influence de chaque variable de la formulation (pour un procédé donné) sur la taille et le potentiel zêta des particules a été développée. Les résultats obtenus ont été confrontés à ceux rapportés par d'autres groupes de recherches, ce qui a permis d'analyser les méthodes dans leur ensemble et d'approfondir la connaissance des aspects mécanistiques liés à la formation des nanoparticules.

Le *troisième chapitre*, enfin, a pour objet principal de déterminer l'effet de la méthode de préparation sur l'efficacité d'encapsulation du diclofenac en utilisant les deux méthodes privilégiées dans cette thèse (la nanoprécipitation et l'émulsification-diffusion). Comme démarche stratégique pour maximiser la quantité d'actif encapsulé, nous avons choisi d'examiner l'effet de l'huile comme agent favorisant la solubilité du diclofenac. Les particules obtenues ont été évaluées en examinant les propriétés physico-chimiques, colloïdales et la libération de l'actif.

Une conclusion est proposée en fin de cette thèse récapitulant l'ensemble des résultats obtenus. Une discussion comparative des deux procédés (la nanoprécipitation et l'émulsification-diffusion) basée sur les propriétés électrocinétiques, le profile de libération de

l’actif et les conséquences éventuelles sur les performances *in vivo* des dispersions formulées a été présentée. Le mécanisme de formation des nanoparticules a également été discuté en relation directe avec le procédé utilisé.

1. NANOPARTICULES POLYMERIQUES COMME VECTEURS DE MOLECULES ACTIVES

Les particules sont des systèmes divisés, qui présentent des tailles variables, allant de quelques nanomètres à des centaines de micromètres. Ces particules sont utilisées dans des domaines divers et en particulier, dans le domaine biomédical. Dans le domaine pharmaceutique où les particules de taille comprise entre 10 nm et 1 μm (taille submicronique) sont utilisées comme véhicules pour le transport de molécules actives, on parle de nanoparticules. Il existe deux types de nanoparticules: les nanosphères et les nanocapsules. Le premier est un système colloïdal du type matrice polymérique contenant le principe actif. Le second est un système de type vésiculaire où le principe actif est generalement confiné dans un réservoir liquide ou solide enveloppé par une couche polymérique.

Dans le domaine de la vectorisation par des nanoparticules, un grand nombre de molécules actives (les analgésiques, les anti-inflammatoires, les immunosuppressifs, les antinéoplasiques, les antigènes, les hormones, les antivirales, les anti-bactérieus, les antifongiques, les diurétiques, et les vitamines, entre autres) a été étudié. Cependant, l'efficacité d'encapsulation ainsi que les caractéristiques des nanoparticules obtenues dépendent de plusieurs facteurs, en particulier de la solubilité de la molécule active dans les solvants utilisés, de la formulation et de la technique de préparation utilisée. Ainsi, dans ce chapitre bibliographique, est présenté l'état de l'art sur les nanocapsules polymériques pour la libération de substances actives sous forme de revue.

Ce travail bibliographique discute les différentes méthodes de préparation de nanocapsules à partir de polymères préformés. Une attention particulière est portée sur la relation entre la méthode de préparation et les propriétés finales des nanocapsules comme la taille, la distribution en taille, le potentiel zêta, l'épaisseur de la membrane polymérique, l'efficacité d'encapsulation de substance active, la stabilité colloïdale, le profil de libération *in vitro* du

principe actif et le comportement *in vitro* ou *in vivo* des nanocapsules. Les principales conclusions émanant de cette étude bibliographique sont présentées ci-dessous.

1.1 Méthodes et matières premières pour la préparation des nanocapsules.

Les nanocapsules formulées à partir de polymères preformés peuvent être préparées en utilisant des techniques comme la nanoprécipitation, l'émulsification-diffusion, la double émulsification, l'émulsification-coacervation, l'émulsification-évaporation, etc. Le choix d'une de ces méthodes dépend de l'application ciblée, de la nature de la molécule active à libérer, du polymère et de ses propriétés physico-chimiques et enfin, de la formulation utilisée. Afin d'assurer une bonne stabilité colloïdale des particules finales, l'utilisation d'un agent stabilisant (tensioactif ou polymère) est généralement préconisée.

Il est à noter que chaque procédé de préparation de nanocapsules exige également le choix de solvants organiques appropriés, des électrolytes (salinité), un agent stabilisant, un pH de la phase aqueuse bien défini et dans certains cas, una température de préparation.

Le choix de la technique et des matières premières les plus adéquates dépend des caractéristiques physico-chimiques de la molécule active, principalement de sa nature hydrophile ou lipophile et de sa stabilité dans les conditions utilisées.

À ce jour, 90% des travaux de recherche dans ce domaine sont développés à l'échelle du laboratoire uniquement et montrent que le procédé basé sur la double émulsification est la technique la plus utilisée pour l'encapsulation de molécules hydrophiles. Les autres méthodes sont utilisées pour la vectorisation des principes actifs lipophiles. Parmi elles, se démarquent la nanoprécipitation et l'émulsification-diffusion. Enfin, la technique de synthèses couche-par-couche permet l'encapsulation de substances actives solides.

1.2 Caracteristiques des nanocapsules selon la méthode de préparation.

La nature chimique de la molécule active semble déterminer l'efficacité d'encapsulation, c'est-à-dire, la quantité de molécule active incluse à l'intérieur des nanocapsules ou adsorbée à la surface. Celle ci est estimée de manière indirecte à partir de la différence entre la quantité de substance active introduite et la quantité restante dans le milieu de dispersion ou de manière directe, par dosage spécifique de l'actif dans les nanocapsules après lavage. Ainsi, l'efficacité d'encapsulation des molécules hydrophiles peut atteindre des valeurs de l'ordre de 10%, tandis que le taux d'encapsulation des molécules lipophiles est largement supérieur à 70%. D'autre part, les méthodes qui comportent une étape d'émulsification dans leurs procédures de fabrication de nanoparticules permettent d'atteindre un taux d'encapsulation de principe actif très élevé. Il a été également rapporté que les particules préparées par ces techniques atteignent des concentrations comprises entre 12 et 50 mg d'actif/ml de dispersion de nanocapsules. Cet intervalle de valeurs est largement superieur à la valeur maximale de 6.5 mg d'actif/ml de dispersion rapportée dans le cas de la nanoprécipitation.

Concernant la taille des dispersions et la distribution en taille, elles dépendent à la fois du procédé utilisé et de la formulation. En général, les tailles les plus petites sont obtenues par la nanoprécipitation, tandis que les plus grandes, par les techniques où l'actif à l'état solide est encapsulé (couche-par-couche, par exemple). Cependant, à titre indicatif, l'intervalle de taille obtenu pour chaque procédé est le suivant: pour la nanoprécipitation, la taille est comprise entre 150 et 500 nm, pour l'émulsification-diffusion entre 250 et 600 nm, pour la double émulsification entre 200 et 500 nm et pour l'émulsification-coacervation entre 200 et 350 nm.

En ce qui concerne la charge de surface qui contribue à la stabilité colloïdale des particules, elle est très rarement déterminée directement. Dans une certaine mesure, la densité de charge de surface est supposée proportionnelle au potentiel zêta dont la mesure est rapide. Ce potentiel zêta dépend non seulement de la formulation et du procédé utilisés, mais aussi des conditions de mesure comme le pH et la salinité. Ainsi, la mesure de potentiel zêta dans des conditions spécifiques donne rapidement des informations sur la nature de la charge nette de la surface des particules. Dans le cas des nanocapsules, des particules de charge positive ou négative peuvent être obtenues par n'importe quelle méthode.

L'étude comparative de la libération *in vitro* d'une substance active en fonction de la méthode de préparation des nanocapsules, permet de dégager une tendance et non une généralité en raison de la quantité limitée des travaux publiés dans ce domaine. Ainsi, pour les molécules lipophiles, environ 75% d'actif encapsulé par émulsification-diffusion ou par émulsification-coacervation est libéré en 15 min, et 100% de libération est atteint dans la première heure de l'étude. Par ailleurs, les nanocapsules préparées par nanoprécipitation libèrent 75% de l'actif dans la première heure et 90% après 12 heures. En ce qui concerne les molécules hydrophiles encapsulées par la méthode de double émulsification, seulement 25% de l'actif est libéré en 2h et il est nécessaire d'attendre plus d'un jour pour avoir 75% de l'actif disponible.

Plusieurs facteurs peuvent avoir une incidence sur les propriétés physico-chimiques, colloïdales et la libération de la molécule active. Parmi ces paramètres, la répartition de la molécule active entre la phase huileuse (ou organique) et la phase aqueuse, l'existence des interactions entre le polymère et la molécule active, la concentration en tensioactif utilisé comme stabilisant et la taille des particules ont été étudiées. L'épaisseur de la couche polymérique (approximativement de 10 nm pour les nanocapsules préparées par nanoprécipitation et de 2 nm lorsque l'émulsification-diffusion est utilisée) paraît n'avoir aucune influence marquée sur la libération de l'actif. Des groupes de recherches affirment que la paroi polymérique des nanocapsules n'est pas suffisamment épaisse et rigide pour former une barrière limitant la diffusion des molécules. Cependant, d'autres recherches revendiquent que la nature et la concentration en polymère peuvent avoir un effet non négligeable sur la libération des actifs.

En ce qui concerne la stabilité colloïdale et physico-chimique de ces systèmes d'encapsulation, les données rapportées dans la littérature disponible portent malheureusement sur les nanocapsules préparées par nanoprécipitation uniquement. Ainsi, ce manque d'études comparatives nous ne permet pas d'aboutir à une conclusion tangible.

Néanmoins, cette information permet de montrer que la stabilité de ces systèmes colloïdaux est liée non seulement à la formulation mais aussi aux conditions de stockage, les propriétés physico-chimiques et la microstructure du polymère, la nature de l'huile et au pH de la dispersion. Comme la stabilité (agrégation, sédimentation et dégradation) de ces nanoparticules dans le milieu aqueux reste un problème majeur, des groupes de recherches ont proposé la lyophilisation et le séchage par atomisation comme solution pour assurer au moins

la stabilité chimique des nanocapsules. Cependant, la recherche dans ce domaine reste à ce jour préliminaire et nécessite des études systématiques et complémentaires pour optimiser le procédé.

L'étude bibliographique réalisée ici a permis de conclure que les principaux défis de l'administration de nanocapsules comme vecteurs de molécules actives consistent principalement: à atteindre les organes cibles, à permettre une action sélective, à minimiser les effets secondaires, à augmenter la biodisponibilité et à assurer une libération contrôlée. Les résultats rapportés dans la littérature sont prometteurs, car un grand nombre d'applications en découlent.

La vectorisation des principes actifs encapsulés dans les nanocapsules a permis d'augmenter sa biodisponibilité et de modifier sa biodistribution et sa pharmacocinétique. En effet, il a été observé l'augmentation des effets thérapeutiques, la diminution de l'hépatoxicité, la biocompatibilité avec la muqueuse oculaire et enfin, le franchissement de la barrière cutanée. Toutefois, quelques problèmes ont été soulevés quand les nanoparticules entrent dans le flux sanguin. Ces dernières sont capturées par le système phagocytaire. Par conséquent, la recherche sur les nanocapsules a été orientée vers la diminution de la taille et le contrôle des propriétés de surface afin de surmonter les difficultés d'opsonisation. Dans la même optique, le développement de particules capables de transporter le principe actif sélectivement aux cellules cibles en utilisant des marqueurs ou des récepteurs spécifiques, a attiré une attention particulière ces dernières années.

L'étude bibliographique réalisée sur les nanocapsules polymériques pour l'encapsulation et la vectorisation de médicaments a permis la publication de la revue suivante: C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, International Journal of Pharmaceutics 385 (2010) 113–142.

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Polymer-based nanocapsules for drug delivery

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ABSTRACT

A review of the state of knowledge on nanocapsules prepared from preformed polymers as active substances carriers is presented. This entails a general review of the different preparation methods: nanoprecipitation, emulsion–diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer, from the point of view of the methodological and mechanistic aspects involved, encapsulation of the active substance and the raw materials used. Similarly, a comparative analysis is given of the size, zeta-potential, dispersion pH, shell thickness, encapsulation efficiency, active substance release, stability and *in vivo* and *in vitro* pharmacological performances, using as basis the data reported in the different research works published. Consequently, the information obtained allows establishing criteria for selecting a method for preparation of nanocapsules according to its advantages, limitations and behaviours as a drug carrier.

Contents

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1. Introduction

Generally, nanoparticles are defined as solid colloidal particles that include both nanospheres and nanocapsules. They can be prepared by both polymerization methods and synthesis with preformed polymers (Fattal and Vauthier, 2002; Vauthier and Bouchemal, 2008). One of their fundamental characteristics is their size, which is generally taken to be around 5–10 nm with an upper size limit of ~1000 nm, although the range generally obtained is 100–500 nm (Quintanar et al., 1998a).

As asserted by different authors, nanoparticulated systems show promise as active vectors due to their capacity to release drugs (Cruz et al., 2006; Amaral et al., 2007); their subcellular size allows relatively higher intracellular uptake than other particulate systems (Furtado et al., 2001a,b); they can improve the stability of active substances (Ourique et al., 2008) and can be biocompatible with tissue and cells when synthesized from materials that are either biocompatible or biodegradable (Guinebretière et al., 2002).

Other advantages of nanoencapsulated systems as active substance carriers include high drug encapsulation efficiency due to optimized drug solubility in the core, low polymer content compared to other nanoparticulated systems such as nanospheres, drug polymeric shell protection against degradation factors like pH and light and the reduction of tissue irritation due to the polymeric shell (Pinto et al., 2006a; Anton et al., 2008).

Polymeric nanoparticles have been extensively studied as drug carriers in the pharmaceutical field (Legrand et al., 1999; Barratt, 2000; Chaubal, 2004; Sinha et al., 2004; Letchford and Burt, 2007) and different research teams have published reviews about the nanoparticle formation mechanisms (Quintanar et al., 1998a; Moinard-Checot et al., 2006), the classification of nanoparticulated systems (Letchford and Burt, 2007) and the techniques for preparation of nanocapsules (Moinard-Checot et al., 2006; Pinto et al., 2006a; Vauthier and Bouchemal, 2008). As a contribution to updating the state of knowledge, the present review focuses on nanocapsules obtained from preformed polymers, using prototype cases, among others, to provide illustrations. The aspects studied are mean size, zeta-potential, encapsulating efficiency, active release, nanodispersion stability and *in vivo* and *in vitro* pharmacological performance behaviours.

2. Nanocapsule definition

First of all the nanocapsules can be likened to vesicular systems in which a drug is confined in a cavity consisting of an inner liquid core surrounded by a polymeric membrane (Quintanar et al., 1998a). However, seen from a general level, they can be defined as nano-vesicular systems that exhibit a typical core-shell structure in which the drug is confined to a reservoir or within a cavity surrounded by a polymer membrane or coating (Letchford and Burt, 2007; Anton et al., 2008). The cavity can contain the active substance in liquid or solid form or as a molecular dispersion (Fessi et al., 1989; Devissaguet et al., 1991; Radtchenko et al., 2002b). Likewise, this reservoir can be lipophilic or hydrophobic according to the preparation method and raw materials used. Also, taking into account the operative limitations of preparation methods, nanocapsules can also carry the active substance on their surfaces or imbibed in the polymeric membrane (Khoee and Yaghoobian, 2008) (Fig. 1).

3. Methods for the preparation of nanocapsules and their fundamental mechanisms

Generally, there are six classical methods for the preparation of nanocapsules: nanoprecipitation, emulsion–diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer (Fig. 2). Nevertheless, other methods have been used such as emulsion–evaporation and the methodologies for the preparation of polymer liposomes.

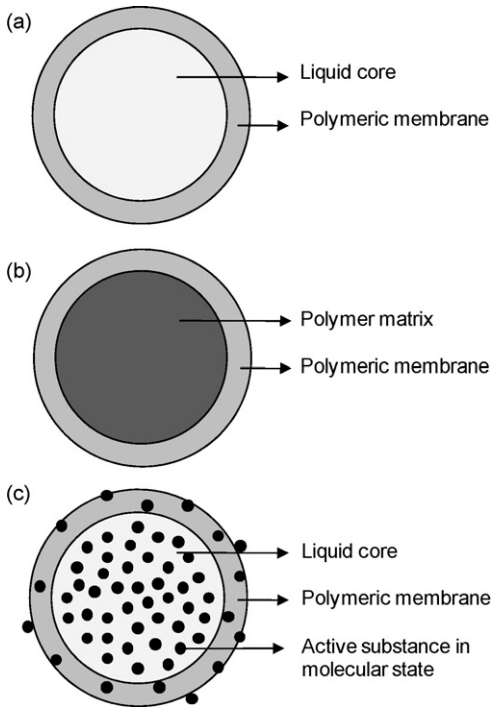

Fig. 1. Different nanocapsular structures: (a) liquid core, (b) polymer matrix and (c) active substance in molecular dispersion.

Regarding to the solvent emulsion–evaporation method, it has been used for the preparation of nanocapsules (Pisani et al., 2008). However, the latter research showed that several apparently different interfacial organizations coexist between the organic and aqueous phases at the same time within a single emulsion. Therefore the presence of compounds with high molecular weights, such as the polymers, can restrict solvent diffusion, which, when removed rapidly during the evaporation step, makes nanocapsule formation difficult.

Although Pisani et al. obtained preparation of nanocapsules by optimising the parameters of emulsion–evaporation process, according to Moinard-Chécot et al. (2008) this method is often performed using microencapsulation technology and is not recommended for nanoencapsulation. They suggest that the nanocapsules do not resist direct evaporation of the solvent, possibly due to the mechanical stress caused by the gas bubbles formed inside the aqueous suspension.

Thus, in agreement with the previous arguments, the emulsion–evaporation method is not currently recognized as feasible, thereby opening the path for other research works to provide options for nanocapsule synthesis.

On the other hand, regarding block copolymer-based vesicles, also called polymer-based liposomes or polymersomes, they appear to be promising for drug encapsulation because their double layer recalls the structure of lipids in membrane cells which could facilitate their biological performance and the design of targeted nanoparticles (Meng et al., 2005; Rodríguez-Hernández et al., 2005). They can be obtained from amphiphilic di-block, tri-block, graft or charged copolymers by means of self-assembled or covalently-assembled strategies. Among the copolymers used are PEG or PEO biodegradable derivatives, although researches has been developed using new materials as polypeptides and choles-

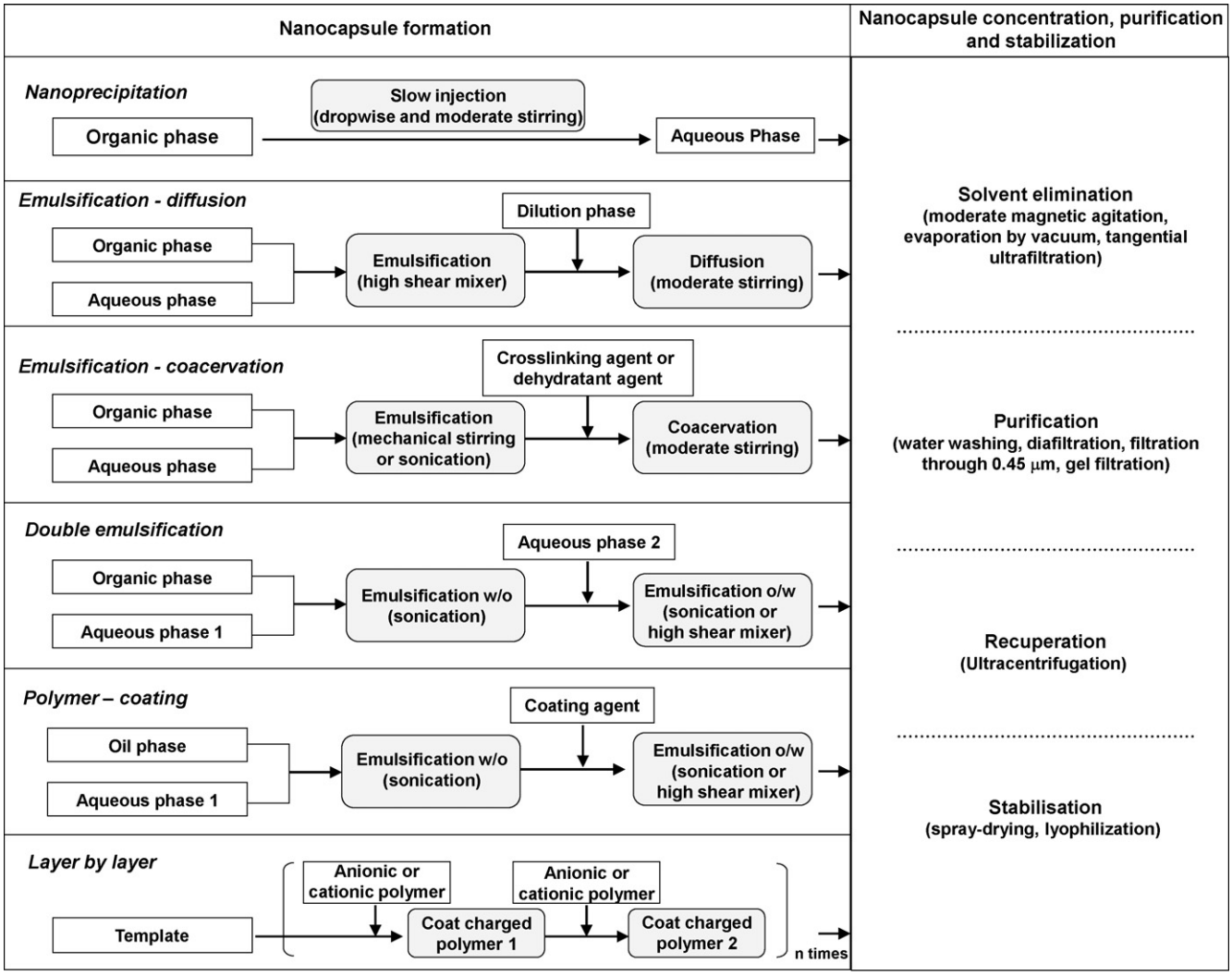

Fig. 2. General procedure of the different methods for the preparation of nanocapsules.

terol derivates (Chécot et al., 2003; Photos et al., 2003; Xu et al., 2005; Zhou et al., 2006).

Typically, the procedures for the polymersome preparation can be classified as solvent free and solvent displacement techniques. In the first method, the dried amphiphile polymer is brought in contact with the aqueous medium and then is hydrated to form vesicles. In the second method, the block copolymer is dissolved in organic solvents, then water is added and subsequently the organic solvent is eliminated. In order to reach monodisperse size distributions of the polymer vesicles, the obtained suspension can be treated by sonication, vortexing, extrusion or freeze-thaw cycles or a combination of these techniques (Kita-Tokarczyk et al., 2005). The cross-linking process of the block polymers allows optimizing the vesicular membrane properties associated with active substance protection and release effect (Chécot et al., 2003).

The encapsulation of active substances inside the polymer vesicles is obtained by incubation based techniques. The hydrophilic or lipophilic nature of the active molecule determines the choice of the polymersome core nature which in turn is obtained according to the block polymer chosen and to the assembly technique. Some examples of active substances encapsulated are mainly anticancer drugs as adriamycin (Xu et al., 2005), paclitaxel (Ahmed et al., 2006) and doxorubicin (Ahmed and Discher, 2004; Zheng et al., 2009), therapeutic proteins and antisense molecules for gene therapy (Christian et al., 2009; Kim et al., 2009).

Table 1
Suggested composition for preparation of nanocapsules by the nanoprecipitation method.

Material	Suggested composition
Active substance	10–25 mg
Polymer	0.2–0.5% of solvent
Oil	1.0–5.0% of solvent
w/o surfactant	0.2–0.5% of solvent
Solvent	25 ml
Stabilizer agent	0.2–0.5% of non-solvent
Non-solvent	50 ml

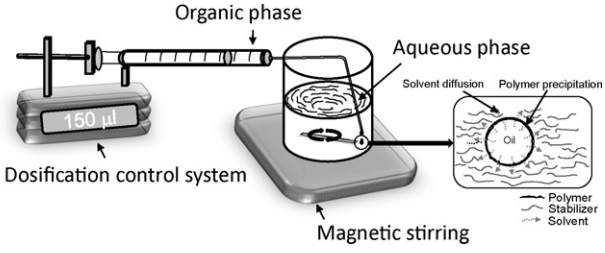

Fig. 3. Set-up used for preparation of nanocapsules by the nanoprecipitation method.

Table 2
Examples of raw materials used for preparation of nanocapsules by the nanoprecipitation method.

Active ingredient	Therapeutic activity	Polymer	Oil core	Solvent	Stabilizer agent	Non-solvent	Reference
Gemcitabine 4-(N)-stearoylgemcitabine 4-(N)-valeroylgemcitabine 4-(N)-lauroylgemcitabine	Antineoplastic	PACA or Poly[H_2NPEGCA-co-HDCA]	Caprylic/capric triglyceride	Acetone ethanol		Water	Stella et al. (2007)
		PLA[a]	Benzyl benzoate	Acetone	Poloxamer 188	Water	Fessi et al. (1989)
		PLA Mw 60 kDa PCL Mw 65 kDa PCL Mn 60 kDa	Phospholipids Capric/caprylic triglycerides Sorbitan monoestearate	Acetone	Polysorbate 80	Water	Fawaz et al. (1996) Pohlmann et al. (2008) Cattani et al. (2008)
Indomethacin	Anti-inflammatory, analgesic Selective cytotoxicity	PCL Mw 60 kDa or PLA[a]	Mineral oil Sorbitan monostearate	Acetone	Polysorbate 80	Water	Pohlmann et al. (2002)
		PCL Mw 40 kDa	Propylene glycol dicaprylate/dicaprate Lecithin	Acetone	Poloxamer 188 Chitosan	Water	Calvo et al. (1997)
		PCL Mw 40 kDa	Propylene glycol dicaprylate/dicaprate Lecithin	Acetone	Poloxamer 188	Water	Calvo et al. (1997)
Indomethacin ethyl ester	Anti-inflammatory, analgesic	PCL Mw 65 kDa	Capric/caprylic triglycerides Sorbitan monostearate	Acetone	Polysorbate 80	Water	Cruz et al. (2006) Cattani et al. (2008) Poletto et al. (2008a,b)
		PLA Mw 200 kDa, PCL Mw 65 or 100 kDa, PLGA Mw 40 kDa	Benzyl benzoate Soybean lecithine	Acetone	Poloxamer 188	Water	Cauchetier et al. (2003)
Atovaquone	Antipneumocystic		Capric/caprylic triglycerides Benzyl benzoate				
		PLA Mw 88 kDa	Caprylic/capric triglycerides PEG-4 complex Oleic acid Phospholipids Capric/caprylic triglycerides Benzyl benzoate	Acetone	Poloxamer 188	Water	Dalençon et al. (1997)
Rifabutine	Antibacterial (tuberculostatic)	PLA Mw 88 kDa	Caprylic/capric triglycerides PEG-4 complex Phospholipids	Acetone	Poloxamer 188	Water	Dalençon et al. (1997)
Tretinoin	Topical treatment of different skin diseases (acne vulgaris, ichtiosys, psoriasis), antineoplastic (hormonal)	PCL[a]	Capric/caprylic triglycerides Sunflower seed oil. Sorbitan monooleate	Acetone	Polysorbate 80	Water	Ourique et al. (2008)
Fluconazole labeled with ^{99m}Technetium	Antifungal	PLA Mw 75 kDa or PLA–PEG (90% PLA Mw 49 kDa–10% PEG Mw 5 kDa)	Capryc/caprylic triglycerides Soybean lecithin	Methanol Acetone	Poloxamer 188	Water	Nogueira de Assis et al. (2008)
Primidone	Anticonvulsant	PCL Mw 64 kDa	Benzyl alcohol	Acetone	Poloxamer 188	Water	Ferranti et al. (1999)
Vitamin E	Vitamin antioxidant	PCL Mn 10 kDa		Acetone	Polysorbate 20	Water	Charcosset and Fessi (2005)
Spironolactone	Diuretic	PCL Mw 10 and 80 kDa	Caprylic/capric triglycerides PEG-4 complex Sorbitan monooleate Sorbitan monolaurate	Acetone	Poloxamer 188 Polysorbate 80 Polysorbate 20	Water	Limayem et al. (2006)
Griseofulvine	Antifungal	PCL Mw 80 kDa	Benzyl benzoate Sorbitan monooleate	Acetone	Polysorbate 80	Water	Zili et al. (2005)
^{99m}Tc-HMPAO complex	Radiotracer	PLA MW/5 kDa or PLA–PLG (90% PLA Mw 49 kDa–10% PEG Mw 5 kDa)	Capric/caprylic triglycerides Soybean lecithin	Acetone	Poloxamer 188	Water	Pereira et al. (2008)
Melatonin	Antioxidant	Eudragit S100	Capric/caprylic triglyceride Sorbitan monooleate	Acetone	Polysorbate 80	Water	Schaffazick et al. (2008)

Diclofenac	Anti-inflammatory	PCL Mw 80 or Eudragit S90	Capric/caprylic triglyceride Benzyl benzoate Sorbitan monostearate	Acetone	Polysorbate 80	Water	Schaffazick et al. (2003)
Diclofenac	Anti-inflammatory	PLA Mw 88 kDa	Benzyl benzoate Capric/caprylic triglyceride Phospholipids	Acetone	Poloxamer 188	Water	Guterres et al. (1995)
Benzathine penicillin G	An bacterial	PLGA 50/50[a]	Sunflower oil Soybean oil Capric/caprylic triglyceride Benzyl benzoate Soy phosphatidylcholine	Acetone	Poloxamer 188	Phosphate buffer solution (pH 7.4)	Santos-Magalhães et al. (2000)
Xanthone 3-methoxyxanthone	Antiinflamatory Antitumoral	PLGA 50/50 Mw 50–75PLA	Soybean lecithine Capric/caprylic acid trygliceride	Acetone	Poloxamer 188	Water	Texeira et al. (2005)
Usnic acid	Antineoplastic	PLGA 50/50[a]	Soybean oil Soy phosphatidylcholine	Acetone	Poloxamer 188 Trehalose	Phosphate buffer solution (pH 7.4)	Pereira et al. (2006)
Tacrolimus	Immunosuppressant	Eudragit RS or Eudragit L100-55	Argan oil Oleoyl polyoxyglycerides	Acetone Absolute ethanol		Water	Nassar et al. (2009)
RU58668	Antiestrogen	PLA Mw 42 kDa PLGA Mw 75 kDa PCL Mw 40 kDa PLA–PEG (45–5 and 45–20 kDa) PLGA-PEG (45–5 kDa) PCL-PEG (40–5 kDa)	Capric/caprylic triglycerides Soy phosphatidylcholine	Acetone	Poloxamer 188	Water	Ameller et al. (2003)
Muramyltripeptide cholesterol (MTP-Chol)	Immunomodulator	PLA Mw 100 kDa	Soybean lecithin Ethyl oleate	Acetone	Poloxamer 188	Water	Seyler et al. (1999)
Benzazole dyes. O-aminophenol 1,2-phenylenediamine 5-aminosalicylic acid 4-aminosalicylic acid		Poly(N-acryloylamide) or Poly(vinylene) or Poly(methyl methacrylate)	Capric/caprylic triglyceride Sorbitan mo n oestea rate	Acetone	Polysorbate 80	Water	Jäger et al. (2007)
		PCL Mn 42.5 kDa	Capric/caprylic triglycerides Sorbitan monostearate	Acetone	Polysorbate 80	Water	Tewa-Tagne et al. (2006) Tewa-Tagne et al. (2007a)
		PCL Mn 42.5 kDa	Capric/caprylic triglycerides Sorbitan monostearate	Acetone	Polysorbate 80	Water	Tewa-Tagne et al. (2007b)
		PLA Mw 42 kDa, PLGA 75/25 Mw 75–120 kDa, PCL Mw 42.5 kDa, PLA–PEG 45-5 kDa, PLGA-PEG 45–20 kDa or PCL-PEG 45-5 kDa.	Capric/caprylic triglycerides Lecithin	Acetone	Poloxamer 188	Water	Furtado et al. (2001a,b)
		PLA Mw 9 kDa	Capric/caprylic triglycerides	Acetone	Poloxamer 188	Water	Rübe et al. (2005)

PACA: poly(alkylcyanoacrylate) derivate; [poly(H2NPEGCA-co-HDCA]: poly[aminopoly(ethylene glycol)cyanoacrylate-co-hexadecyl cyanoacrylate]; PLA: poly(lactide); PCL: poly(e-caprolactone); PLGA: poly(lactide-co-glycolide); PEG: poly(ethylene glycol); HPMC: hydroxypropylmethylcellulose; HPC: hydroxypropylcellulose; PVP: polyvinyl pyrrolidone.

[a] Molecular weight (Mw) non-specified.

In the current review polymer vesicles are not included though active substances have been encapsulated and polymersomes promising to be versatile nanocarriers. They are considered as new polymer therapeutics with profitable and triggered biopharmaceutic behaviours, which are more comparable with liposomal systems (Batrakova et al., 2006; Betancourt et al., 2007).

In what follows, a general review is provided of the methodologies, raw materials and mechanistic fundamentals of each classical method for the preparation of nanocapsules. Furthermore, considerations on aspects regarding the purification, concentration and stabilization of nanoencapsulated systems will be given.

3.1. Nanoprecipitation method

The nanoprecipitation method is also called solvent displacement or interfacial deposition. According to Fessi et al. (1988), the nanocapsule synthesis needs both solvent and non-solvent phases. The solvent phase essentially consisting of a solution in a solvent or in a mixture of solvents (i.e. ethanol, acetone, hexane, methylene chloride or dioxane) of a film-forming substance such as a polymer (synthetic, semi-synthetic or naturally occurring polymer), the active substance, oil, a lipophilic tensioactive and an active substance solvent or oil solvent if these are needed. On the other hand, the non-solvent phase consisting of a non-solvent or a mixture of non-solvents for the film-forming substance, supplemented with one or more naturally occurring or synthetic surfactants.

In most cases, the solvent and non-solvent phases are called organic and aqueous phases, respectively. As a general tendency, the solvent is an organic medium, while the non-solvent is mainly water. However, it is possible to use either two organic phases or two aqueous phases as long as solubility, insolubility and miscibility conditions are satisfied.

A composition base for 150–200 nm preparation of nanocapsules at laboratory-scale using the nanoprecipitation method is shown in Table 1. Likewise, Table 2 shows different examples of solvents, non-solvents, polymers, oils, surfactants and stabilizer agents used in this method. As it can be seen, although an extensive range of raw materials (Devissaguet et al., 1991) can be used in theory, in practice research has been performed with only a limited number of them.

The polymers commonly used are biodegradable polyesters, especially poly-e-caprolactone (PCL), poly(lactide) (PLA) and poly(lactide-co-glicolide) (PLGA). Eudragit can also be used as may other polymers such as poly(alkylcyanoacrylate) (PACA). Synthetic polymers have higher purity and better reproducibility than natural polymers (Khoee and Yaghoobian, 2008). On the other hand, some polymers are PEG copolymerized in order to decrease nanocapsule recognition by the mononuclear phagocyte system (Nogueira de Assis et al., 2008).

Besides the lipophilic active substance, the nanocapsule core is composed by a w/o surfactant and oil chosen having as criterion the highest possible drug solubility, absence of toxicity, low solubility of oil in the polymer and vice-versa, and the absence of risk of polymer degradation (Limayem et al., 2006). It is emphasized that the different capric/caprylic triglyceride types are often used because of their wide range of solubility for active substances. Although other oils such as benzyl benzoate, benzyl alcohol, oleic acid, ethyl oleate, argan oil, sunflower seed oil and soybean oil have not been used frequently, they can nonetheless give good results. Regarding w/o surfactants, sorbitan esters and phospholipids are preferred.

Regarding the polymer solvent, acetone is chosen in all cases. Other solvents such as ethanol are used in order for active substance or oil dissolution. Water or buffer solutions can be used as the non-solvent while the stabilizer agent is poloxamer 188 or polysorbate 80.

Table 3
Suggested composition for preparation of nanocapsules by emulsion–diffusion method.

Material	Suggested composition
Active substance	10–50 mg
Polymer	1.0–2.0% of inner phase solvent
Oil	2.5–5.0% of inner phase solvent
Inner phase solvent	10 ml
Stabilizer agent	2.0–5.0% of external phase solvent
External phase solvent	40 ml
Dilution phase	200 ml

In the nanoprecipitation method, the nanocapsules are obtained as a colloidal suspension formed when the organic phase is added slowly and with moderate stirring to the aqueous phase (Fig. 3). The key variables of the procedure are those associated with the conditions of adding the organic phase to the aqueous phase, such as organic phase injection rate, aqueous phase agitation rate, the method of organic phase addition and the organic phase/aqueous phase ratio. Likewise, nanocapsule characteristics are influenced by the nature and concentration of their components (Plasari et al., 1997; Chorny et al., 2002; Legrand et al., 2007; Lince et al., 2008).

Although disagreement exists regarding the mechanism of nanocapsule formation using this technique, research into polymer precipitation (Lince et al., 2008) and solvent diffusion (Quintanar et al., 1998a) have proved useful in this regard.

On the basis of Sugimoto's theory on polymer precipitation (Sugimoto, 1987), Lince et al. (2008) indicated that the process of particle formation in the nanoprecipitation method comprises three stages: nucleation, growth and aggregation. The rate of each step determines the particle size and the driving force of these phenomena is supersaturation, which is defined as the ratio of polymer concentration over the solubility of the polymer in the solvent mixture. The separation between the nucleation and the growth stages is the key factor for uniform particle formation. Ideally, operating conditions should allow a high nucleation rate strongly dependent on supersaturation and low growth rate.

On the other hand, in line with the research carried out by Davies on mass transfer between two liquids and the Gibbs–Marangoni effect (McManamey et al., 1973; Davies, 1975), Quintanar et al. explained rapid nanoparticle formation as a process due to differences in surface tension. Since a liquid with a high surface tension (aqueous phase) pulls more strongly on the surrounding liquid than one with a low surface tension (organic phase solvent). This difference between surface tensions causes interfacial turbulence and thermal inequalities in the system, leading to the continuous formation of eddies of solvent at the interface of both liquids. Consequently, violent spreading is observed due to mutual miscibility between the solvents, the solvent flows away from regions of low surface tension and the polymer tends to aggregate on the oil surface and forms nanocapsules. According to this explanation, nanocapsule formation is due to polymer aggregation in stabilized emulsion droplets, while apparently the nucleation and growth steps are not involved.

3.2. Emulsion–diffusion method

According to Quintanar et al. (1998b, 2005), preparation of nanocapsules by the emulsion–diffusion method allows both lipophilic and hydrophilic active substance nanoencapsulation. The experimental procedure performed to achieve this requires three phases: organic, aqueous and dilution.

When the objective is the nanoencapsulation of a lipophilic active substance, the organic phase contains the polymer, the active substance, oil and an organic solvent partially miscible with water, which should be water-satured. This organic medium acts

Table 4
Examples of raw materials used for preparation of nanocapsules by the emulsification – diffusion method – oil core.

Active ingredient	Therapeutic activity	Inner phase (active ingredient + polymer + core + solvent 1)			External phase		Dilution phase	Reference
		Polymer	Core	Solvent 1	Stabilizer agent	Solvent 2		
		PCL Mw 80 kDa	Capric/caprylic triglyceride	Ethyl acetate	PVA Poloxamer 188	Water	Water	Guinebretière et al. (2002)
Indomethacine	Anti-inflamatory	PCL Mw 10 and 80 kDa	Capric/caprylic triglyceride	Ethyl acetate	PVA	Water	Water	Limayem et al. (2004)
	Analgesic	PLA[a] Eudragit E	Capric/caprylic triglyceride	Ethyl acetate, propylene carbonate or benzyl alcohol	PVA	Water	Water	Quintanar et al. (1998b)
Progesterone Estradiol ChlorambucilClofibrateVitamin E	Progestogen Estrogen Antineoplastic Antilipemic Vitamin antioxidant	PLA[a] Eudragit E	Capric/caprylic triglyceride	Ethyl acetate, propylene carbonate or benzyl alcohol	PVA	Water	Water	Quintanar et al. (1998b)
Eugenol	Analgesic	PCL Mw 80 kDa		Ethyl acetate	Poloxamer 188	Water	Water	Choi et al. (2009)
Hinokitiol	Antibacterial	PCL Mw 40–60 kDa	Octyl salicylate	Ethyl acetate	SLS or CTAC or CTAC:gelatin	Water	Water	Joo et al. (2008)
4-Nitroanisole		PLA 70:30 Mw 1500 kDa	Hexane	DCM Acetone	PVA	Water	PVA aqueous solution	Romero-Cano and Vincent (2002)
Sudan III		PLA[a] Eudragit E PCL[a]	Capric/caprylic triglycerides	Ethyl acetate, propylene carbonate or benzyl alcohol	PVA	Water	Water	Quintanar et al. (1998b)
		PCL Mw 80 kDa	Capric/caprylic triglyceride	Ethyl acetate	PVA	Water	Water	Moinard-Chécot et al. (2008)
		PHBHV Mw 23 or 300 kDa	Caprylic/capric triglyceride or mineral oil	Chloroform:ethanol	PVA	Water	PVA aqueous solution	Poletto et al. (2008a,b)
		PCL Mw 14 kDa	Capric/caprylic triglycerides	Ethyl acetate	PVA	Water		Abdelwahed et al. (2006a,b,c)

PLA: poly(lactide); PCL: poly(e-caprolactone); PHBHV: poly(hydroxybutyrate-co-hydroxyvalerate); DCM: dichloromethane; PVA: poly(vinyl alcohol); SLS: sodium lauryl sulfate; CTAC: cetyltrimethylammonium chloride; PVP: polyvinyl pyrrolidone.

[a] Molecular weight (Mw) non-specified.

as solvent for the different components of the organic phase. If it is required, the organic phase can also include an active substance solvent or oil solvent. The aqueous phase comprises the aqueous dispersion of a stabilizing agent that is prepared using solvent-saturated water while the dilution phase is usually water.

A prototype composition for preparation of nanocapsules at laboratory-scale using the emulsion–diffusion method is shown in Table 3 (nanocapsule size: approximately 150–200 nm). Likewise, Table 4 shows different examples of polymers, oils, inner phase solvent, stabilizer agent, external phase solvent and dilution phase used in nanoencapsulation research with this method. As with the nanoprecipitation method, although an extensive range of raw materials can be used in theory (Quintanar et al., 2005), research has been performed with a only limited number of them in practice.

As can be observed, the polymers commonly used are biodegradable polyesters, especially PCL, PLA and eudragit. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBHV) may also be used. The inner phase contains the oil in addition to the active substance and solvent. In line with what has been mentioned previously about nanoprecipitation method, also different capric/caprylic triglyceride types are frequently used. Regarding the solvents, ethyl acetate is the first option, though propylene carbonate, benzyl alcohol and dichloromethane can also be chosen.

In regarding to the external phase, the solvent used is water and poly(vinyl alcohol) (PVA) is preferred as the stabilizing agent. Other stabilizing agents such as poloxamer and ionic emulsifiers have been used. The dilution phase is often water; nevertheless, in order to obtain better nanodispersion stability stabilizer agents may be used in diluted solutions.

For preparation of nanocapsules using the emulsion–diffusion method, the organic phase is emulsified under vigorous agitation in the aqueous phase (Fig. 4). The subsequent addition of water to the system causes the diffusion of the solvent into the external phase, resulting in nanocapsule formation. This can be eliminated by distillation or cross-flow filtration depending on the boiling point of the solvent. It has been shown that nanocapsule size is related to the shear rate used in the emulsification process, the chemical composition of the organic phase, the polymer concentration, the oil-to-polymer ratio and the drop size of the primary emulsion (Guinebretière, 2001; Moinard-Chécot et al., 2008).

The nanocapsule formation mechanism suggested by Quintanar et al. (1998a) is based on the theory that each emulsion droplet produces several nanocapsules and that these are formed by the combination of polymer precipitation and interfacial phenomena during solvent diffusion. Consequently, solvent diffusion from the globules carries molecules into the aqueous phase forming local regions of supersaturation from which new globules or polymer aggregates (not totally desolvated) are formed and stabilized by the stabilizer agent which prevents their coalescence and the formation of agglomerates. Then, if the stabilizer remains at the liquid–liquid interface during the diffusion process and if its protective effect is adequate, the nanocapsules will be formed after the complete diffusion of the solvent.

Guinebretière et al. (2002) demonstrated that mean nanocapsule size is always smaller than that of the emulsion droplets, in agreement with the diffusion theory proposed by Quintanar. In this sense, nanocapsule formation is a dynamic process associated with the diffusion of the solvent from the droplet to the external phase caused by the addition of water to the emulsion and resulting in the transformation of each droplet into a particle of smaller size.

In order to better understand nanocapsule formation, Hassou (2007) and Moinard-Chécot et al. (2008) had modeled the different intermediate states that take place during solvent diffusion at the dilution stage, by a step-by-step diffusion study and determined its duration by using the stopped-flow technique. According to these researches, diffusion of the solvent from the droplets takes place too fast (duration less than 20 ms) and as a continuous process. There are no discontinuities that reveal a transition from homogeneous droplets to heterogeneous nanocapsules.

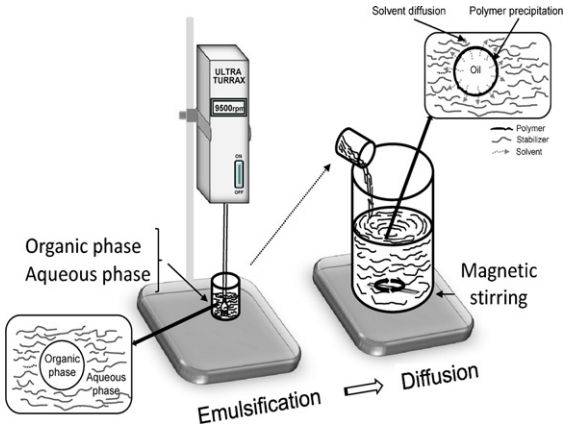

Fig. 4. Set-up used for preparation of nanocapsules by the emulsion–diffusion method.

Perez et al. (2001) and Ma et al. (2001) have modified the process proposed by Quintanar et al. (2005) in order to nanoencapsulate hydrophilic active substances. In this case, a stabilizer agent such as PVA or poly(vinylpirrolidone) (PVP) is present in the aqueous inner phase in addition to the active substance (Table 5), while the external phase is composed of the polymer and an organic solvent (methylene chloride or acetone). The dilution of the emulsion is made first by solvent addition (ethanol) which leads to organic solvent migration. Then, water addition is made in order to facilitate the collection of the particles. The aqueous dilution phase may or may not include a stabilizer agent.

3.3. Double emulsification method

Double emulsions are complex heterodisperse systems called "emulsions of emulsions", that can be classified into two major types: water-oil-water emulsion (w/o/w) and oil-water-oil emulsion (o/w/o) (Garti, 1997; Grigoriev and Miller, 2009). Thus the dispersed phase is itself an emulsion and the inner dispersed globule/droplet is separated from the outer liquid phase by a layer of another phase. Double emulsions are usually prepared in a two-step emulsification process using two surfactants: a hydrophobic one designed to stabilize the interface of the w/o internal emulsion and a hydrophilic one to stabilize the external interface of the oil globules for w/o/w emulsions.

For preparation of nanocapsules, the principle of double emulsion formation, specifically of the w/o/w type, is associated with the principles of both nanoprecipitation and emulsion–diffusion methods. In this case, in the primary w/o emulsion the oil is changed by an organic phase containing a solvent that is totally or partially miscible in water, the film-formed polymer and a w/o surfactant. Then the water containing a stabilizing agent is added to the system to obtain the water in organic in water emulsion. However in this step, particle hardening is obtained through solvent diffusion and polymer precipitation (Bilati et al., 2005c; Khoee and Yaghoobian, 2008). Water is frequently added to the double emulsion in order to achieve full solvent diffusion.

According to Khoee and Yaghoobian (2008), surfactants play a dual role in emulsions: as a film former and a barrier to drug release at the internal interface, and as a steric stabilizer on the

Table 5
Examples of raw materials used for preparation of nanocapsules by the emulsion–diffusion method—aqueous core.

Active ingredient	Therapeutic activity	Inner phase		External phase		Dilution phase	Reference
		Core	Solvent 1	Polymer	Solvent 2		
Plasmid DNA plasmid DNA–PVA plasmid DNA–PVP	Gene therapy	Active ingredient PVA or PVP (stabilizer agent)	Water	PLA–PEG46–5 kDa	Methylene chloride	Ethanol Water	Perez et al. (2001)
Insulin	Antidiabetic	Active ingredient	Hydrochloric acid	PLA–PEG–PLA copolymers (PLA from 2 to 45 kDa; PEG variable PLA (Mn 32 kDa) in glycerol trioleate	Acetone	Polysorbate 20, dextrin and water	Ma et al. (2001)

DNA: deoxyribonucleic acid; PLA: poly(lactide); PVA: poly(vinyl alcohol); PEG: poly(ethylene glycol); PVP: polyvinyl pyrrolidone.

Table 6
Suggested composition for preparation of nanocapsules by the double emulsification method.

Material	Suggested composition
Inner aqueous phase	
Active substance	Variable (0.5–25 mg)
Water	0.15–0.5 ml
Organic phase	
Polymer	5–10% of organic phase solvent
w/o surfactant	5–7% of organic phase solvent
Solvent	1.5–5 ml
External aqueous phase	
Stabilizer agent	1–5% of external aqueous phase solvent
Water	2–5 ml
Dilution phase (optional)	
Stabilizer agent	1–5% of dilution phase solvent
Water	50–100 ml

external interface. It was found that drug encapsulation efficiency and average particle size are affected by changing the type and concentration of both the w/o emulsion and the stabilizing agent.

A composition base for preparation of nanocapsules at laboratory-scale by the double emulsification method (size about 150–200 nm) is provided in Table 6.

As can be seen in Table 7, at present, the inner aqueous phase is composed only for the active substance, in some cases forming complexes, and water. In the organic phase, ethyl acetate, methylene chloride and dichloromethane have been used as solvents and biodegradable polyesters, such as PCL, PLA and PLGA have been frequently used. Regarding o/w surfactants, sorbitan esters are preferred.

Regarding the external aqueous phase, the stabilizing agents most frequently used are PVA and polysorbates. To contribute to nanocapsule dispersion, the same external aqueous phase composition is used for the dilution phase if the procedure used involves a final dilution stage.

In a typical procedure for preparation of nanocapsules by double emulsification, the primary emulsion is formed by ultrasound and the w/o surfactant stabilizes the interface of the w/o internal emulsion (Fig. 5). The second emulsion is also formed by ultrasound and nanocapsule dispersion is stabilized by the addition of the stabilizing agent. Finally, the solvents are removed by evaporation or extraction by vacuum, leaving hardened nanocapsules in an aqueous medium. As mentioned previously, as an optional step, nanocapsule dispersion can be diluted before extraction under vacuum to ensure full solvent diffusion.

On the other hand, Bilati et al. (2005a) (Table 8), showed that it is possible to obtain solid-organic–water systems by following the same method.

3.4. Emulsion-coacervation method

The emulsion-coacervation process is mainly presented as a strategy for nanocapsules preparation from naturally occurring polymeric materials. Up to now, sodium alginate and gelatin have been used though synthetic polymeric materials could be used for this purpose.

The procedure involves the o/w emulsification of an organic phase (oil, active substance and active substance solvent if necessary) with an aqueous phase (water, polymer, stabilizing agent) by mechanical stirring or ultrasound. Then, a simple coacervation process is performed by using either electrolytes as done by Lertsutthiwong et al. (2008a,b) with a sodium alginate–calcium chloride system, by the addition of a water miscible non-solvent or a dehydration agent as done by Krause and Rohdewald (1985) with a gelatin–isopropanol–sodium sulfate system or by temperature

Table 7
Examples of raw materials used for preparation of nanocapsules by the double emulsification method—liquid core.

Active ingredient	Therapeutic activity	W1 phase	Organic phase	W2 phase	Reference
Insulin	Antidiabetic	Active ingredient Water	PLA Mw 10 kDa DCM Sorbitan monooleate Sorbitan monostearate Sorbitan monolaurate	Polysorbate 80, 60 or 20 glycerin:water (1:1)	Zhu et al. (2005)
		Protein–SLS 0.1 M HCl solution	PLA Mw 28 kDa or PLGA 50/50 Mw 34 kDa Ethyl acetate or methylene chloride	PVA Water	Bilati et al. (2005a)
Ciprofloxacin.HCl	Antibacterial	Active ingredient Water	PLGA[a] DCM	PVA Water	Jeong et al. (2008)
Bovine serum albumin	Protein	Protein Water	PLA Mw 16 and 51 kDa or PCL–PEO block copolymer 60/40 Mw 79 Kd Sorbitan monooleate Methylene chloride	Polysorbate 80 glycerin:water (1:1)	Lu et al. (1999)
		Protein Water	PLGA Mw 40 kDa or PCL Mw 42 kDa Methylene choride	PVA Water	Lamprecht et al. (2000)
Penicillin G	Antibacterial	Active ingredient Water	PBA Mw 10 kDa Sorbitan esters 60 or 20. DCM	Polysorbate 60 or 20. glycerin:water (1:1)	Khoee and Yaghoobian (2008)
Plasmid DNA Plasmid DNA–PVAPlasmid DNA–PVP	Gene therapy	Active ingredient PVP or PVA Water	PLA 46 kDa–PEG 5 kDa Ethyl acetate:methylene chloride (1:1)	PVA Water	Perez et al. (2001)
Tetanus toxoid Lysozyme	Antigen Mucolytic enzyme Antiviral	Protein Water Protein–sodium oleate Water	PLA Mw 28 kDa or PLGA Mw 34 kDa Ethyl acetate or methylene chloride	PVA Water	Bilati et al. (2005a)

DNA: deoxyribonucleic acid; PVA: poly(vinyl alcohol); PVP: polyvinyl pyrrolidone; SLS: sodium lauryl sulfate; HCl: hydrochloric acid; PLA: poly(lactide); DCM: dichloromethane; PLGA: poly(lactide-co-glycolide); PEG: poly(ethylene glycol); PCL: poly(e-caprolactone); PEO: poly(ethylene oxide); PBA: polybutyl adipate.

[a] Molecular weight (Mw) non-specified.

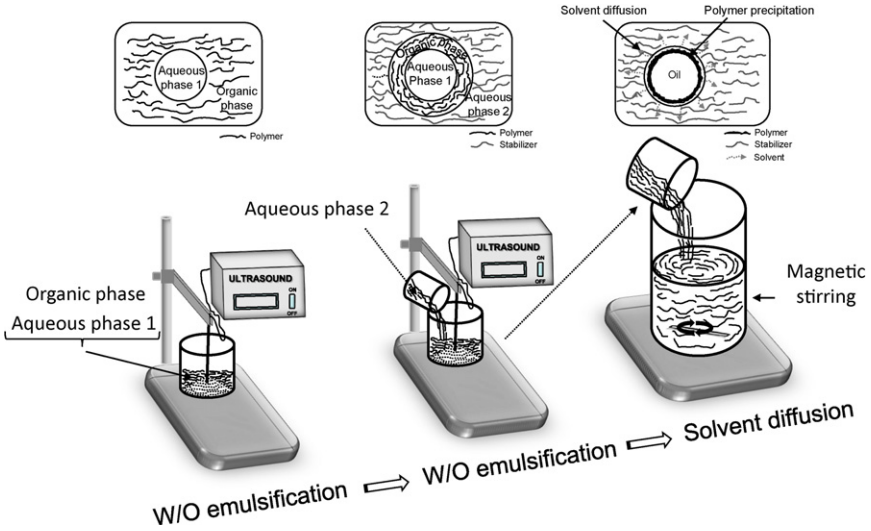

Fig. 5. Set-up used for preparation of nanocapsules by the double emulsification method.

modification as done by Lutter et al. (2008) with the application of triblock terpolymer in gold nanocapsule synthesis. Finally, the coacervation process is complemented with additional cross-linked steps that make it possible to obtain a rigid nanocapsule shell structure (Fig. 6).

Nanocapsule formation by the emulsion-coacervation method uses the emulsion as a template phase and the formation of a coacervate phase that causes polymer precipitation from the continuous emulsion-phase to form a film on the template forming the nanocapsule. Additionally, it can be stabilized by physical intermolecular or covalent cross-linking, which typically can be achieved by altering pH or temperature, or by adding a cross-linking agent.

Probably the critical stage in preparation of nanocapsules by the emulsion-coacervation method is coacervate phase formation. As explained by Gander et al. (2002), the polymer dissolved in water is enclosed by water molecules that solvate its functional groups, typically through hydrogen-bonding and van der Waals forces that prevent attraction among chain segments in close proximity by interchain H-bonds, or van der Waals or opposing ionic forces. Thus, the coacervating agents lower the solvation of dissolved polymers and induce thin solvated shell. It may also allow the attraction among contiguous chains via secondary valence bonds to form an entangled network or even non-covalent weak cross-links as the polymer concentration gradually increases in the coacervated phase.

The use of electrolytes for polymer desolvation is known as salting-out and the electrolytic efficiency for this process follows the Hofmeister or lyotropic series, which arranges ions in increasing order according to their capacity to immobilize water molecules in solvation in the ternary polymer–water–salt system. A practice demonstration of polymer coacervation behaviour according to the lyotropic series was performed by Yin et al. (2008) in their work on konjac glucomannan.

On the other hand, in the case where a dehydrating agent is used, the ternary system formed (polymer – dehydrating agent – water) allows the increase of polymer concentration due to solvent–solvation competition process. This results in the desolvation of the polymer chains, leading to phase separation.

Regarding the use of temperature changes to trigger polymer precipitation, it is essential to bear in mind the theories of Flory and Huggins on the interaction of parameter χ, which predicts that a polymer will dissolve in a solvent only if the interaction parameter is lower than a critical value χ_c, which, at a given temperature, depends on the degree of polymerization of the polymer.

Although electrolytes, dehydration and temperature modification are frequently used to reduce polymer solvation, other factors such as changing pH and adding other materials that are incompatible with the polymer solution can also be used.

Table 9 gives a non-exhaustive list of different raw materials used in research using emulsion-coacervation for preparation of nanocapsules. It is noteworthy that research conducted by Lutter et al. (2008) which, contrary to work done elsewhere, used the principle of emulsion-coacervation to prepare aqueous core nanocapsules.

Taking into account the limited amount of research and particularly the different methodological strategies followed by each team, it appears premature to establish general criteria regarding the materials and compositions that can be employed.

3.5. Polymer-coating method

References on the use of the polymer-coating method for preparation of nanocapsules are provided in Table 10. As can be seen, different methodological strategies can be used to deposit a thin layer of polymer on the nanoparticle surface. This can be achieved by adsorbing the polymer onto the preformed uncoated

Table 8
Examples of raw materials used for preparation of nanocapsules by the double emulsification method—solid core.

Active ingredient	Therapeutic activity	S phase	Organic phase	W phase	Reference
Tetanus toxoid Lysozyme Insulin	Antigen Mucolytic enzyme Antiviral Antidiabetic	Protein Protein–sodium oleato Protein–SLS	PLA Mw 28 kDa or PLGA Mw 34 kDa Ethyl acetate or methylene chloride	PVA Water	Bilati et al. (2005a)

Mw: molecular weight; SLS: sodium lauryl sulfate; PLA: poly(lactide); PLGA: poly(lactide-co-glycolide); PVA: poly(vinyl alcohol).

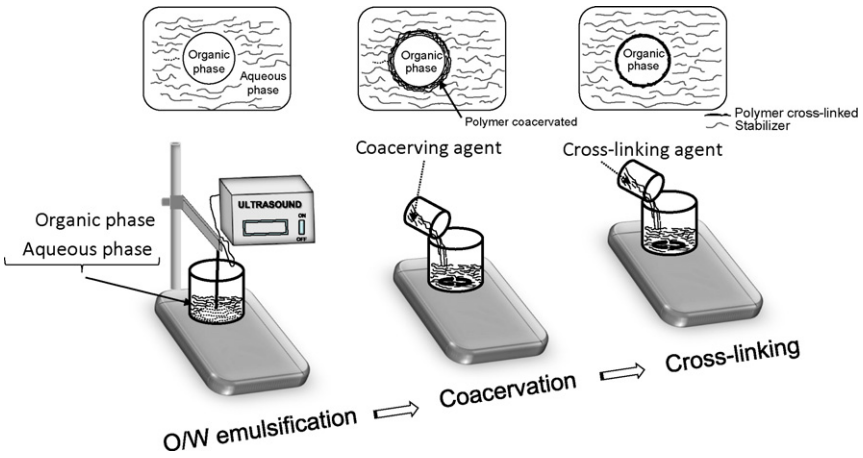

Fig. 6. Set-up used for preparation of nanocapsules by the emulsion-coacervation method.

nanocapsules when the latter are incubated in polymer dispersion under predetermined stirring and time conditions (Calvo et al., 1997).

Likewise, layer-formed polymer can be added during the final stage of conventional methods for the preparation of nanocapsules such as nanoprecipitation and double emulsification. Thus, these methods have been modified in order to add a layer of polymer to the external aqueous medium and allow to simultaneous layer formation due to the precipitation of the charged polymer (mainly negatively in nature) and to the diffusion of the solvent (Calvo et al., 1997; Vila et al., 2002).

On the other hand, Prego et al. (2006) propose a polymer-coating method in which the first step is to prepare the nanoemulsion template and then coat it by polymer deposition on the water/oil nanoemulsion surface. The polymers are added in the continuous phase and their precipitation onto the nanoemulsion droplets is triggered by solvent evaporation, as opposed to the emulsion-coacervation method.

Prego et al. (2006) have encapsulated salmon calcitonin using chitosan and PEG chitosan. In their procedure (Fig. 7), they start from an organic phase composed of the active substance, oil, surfactant (lecithin) and acetone as solvent; an aqueous phase containing the stabilizing agent and an aqueous polymer-coating solution. The organic and aqueous phases are mixed under moderate stirring and the o/w nanoemulsion is formed by solvent displacement. The solvents are subsequently evaporated under vacuum until reaching a specific volume and the nanoemulsion is finally coated by the polymer by simple incubation in the polymer solution.

The nanocapsule formation mechanism is mediated by the ionic interaction between the negatively charged phospholipids and the positively charged chitosan molecules. As established by Prego et al. (2006), the use of high lecithin concentrations affects the amount of chitosan associated with the surface of the nanocapsules while the chain length of chitosan molecules determines nanocapsule size.

Likewise, Anton et al. (2008) report a method used by Paiphansiri et al., based on the formation by sonication of a w/o nanoemulsion followed by coating with a solution composed of polymer and dichloromethane gradually added in the continuous organic phase of the nanoemulsion. The layer-formed polymers used by them are poly(methyl methacrylate) (PMMA), poly(methacrylate) (PMA) and PCL. Nanocapsule formation is based on the mechanism of engulfment in three-phase systems (Torza and Mason, 1970). When two drops of liquids miscible with each other are brought together in a third liquid phase that forms a film between them, the third phase drains until a hole suddenly forms in the same way as when two identical drops coalesce to form one drop. Since one of the drops comprises the polymer, when the two drops fuse a third interface is formed at the expanding hole and engulfment occurs via a combination of simultaneous penetration processes driven by the difference of capillary pressure between the two drops and the spreading of the polymer phase over the aqueous phase. Thus, when the solvent is finally evaporated, the polymer precipitates onto the nanoemulsion water droplets to form the nanocapsules.

As in the emulsion-coacervation method, taking into account the limited amount of research and their different methodological strategies, it is premature to establish general criteria for the materials and compositions that could be employed.

3.6. Layer-by-layer method

The layer-by-layer assembly process developed by Sukhorukov et al. (1998) for colloidal particle preparation makes it possible to obtain vesicular particles, called polyelectrolyte capsules, with well-defined chemical and structural properties. To sum up, the mechanism of nanocapsule formation is based on irreversible electrostatic attraction that leads to polyelectrolyte adsorption at supersaturating bulk polyelectrolyte concentrations.

This method requires a colloidal template onto which is adsorbed a polymer layer either by incubation in the polymer solution, subsequently washed, or by decreasing polymer solubility by drop-wise addition of a miscible solvent (Radtchenko et al., 2002a). This procedure is then repeated with a second polymer and multiple polymer layers are deposited sequentially, one after another.

As shown in Tables 11 and 12, the solid form of the active substance can be used as a template (Chen et al., 2009; Agarwal et al., 2008), as can inorganic particles and biological cells (Krol et al., 2004). The use of dyes, compact forms of DNA, protein aggregates and gel beads (Radtchenko et al., 2002b) have also been reported.

Likewise, the adsorption of oppositely charged polyelectrolytes can be done on the surface of colloidal particles with subsequent core dissolution. The hollow nanocapsules are then loaded with the substance of interest (Antipov et al., 2002; Fan et al., 2002; Radtchenko et al., 2002b; Ai and Gao, 2004; Krol et al., 2004; Cui et al., 2009).

According to Radtchenko et al. (2002b), "large macromolecules cannot penetrate polyelectrolyte multilayers whereas small solutes like ions or drug molecules can do so readily. As a result the presence of macromolecules only inside the capsules leads to a difference in physicochemical properties between the bulk and capsule interior and makes it possible to establish a polarity gradient across the capsule wall that could be used to precipitate poorly

Table 9
Examples of raw materials used for preparation of nanocapsules by the emulsion-coacervation method.

Active ingredient	Therapeutic activity	Polymer	Core	Organic phase	Aqueous phase	Cross-linking agent	Other components	Reference
Turmeric oil	Antifungal Antibacterial Antioxidant Antimutagenic Anticarcinogenic	Sodium alginate Mw 80–120 kDa	Organic	Turmeric oil ethanol or acetone	Sodium alginate Polysorbate 80 Water			Lertsuttiwong et al. (2008a)
		Sodium alginate Mw 80–120 kDa – chitosan Mn 41 and 72 kDa	Organic	Turmeric oil ethanol	Sodium alginate Polysorbate 80 Water Chitosan acetic acid Water	Calcium chloride		Lertsutthiwong et al. (2008b)
Triamcinolone acetonide	Glucocorticoid Antiasthmatic Antiallergic	Swine skin gelatin type II	Organic	Chloroform	Desolvation agents: sodium sulfate and isopropanol	Glutaraldehyde	Sodium metabisulfate	Krause and Rohdewald (1985)
Hydrogen tetrachloroaureate $HAuCl_4$.[a]		Poly (1,4 butadiene) (PB)-block-polystyrene (PS)-block-poly(ethylene oxide) (PEO) triblock terpolymer Mn 76–86 kDa	Aqueous	w/o microemulsion of the pseudo-ternary system water/SDS/xylene–pentanol		Sodium borohydride		Lutter et al. (2008)

[a] Precursor gold nanoparticle synthesis. Mw: molecular weight.

Table 10
Examples of raw materials used for preparation of nanocapsules by the polymer-coating method.

Active ingredient	Therapeutic activity	Organic phase	Aqueous phase	Coating	Reference
Nanoemulsion					
Salmon calcitonin	Calcium regulator	Active ingredient capric/caprylic triglycerides Ethanol Soybean lecithin Acetone	Poloxamer188 Water	Chitosan oligomers[a] or medium molecular weight chitosan[a] Water	Prego et al. (2006)
Modified nanoprecipitation					
Indomethacin	Anti-inflammatory Analgesic	PCL Mw 40 kDa Capric/caprylic triglycerides Lecithin Acetone	Poloxamer 188Water	Chitosan[a] or Poly-L-lysine[a]	Calvo et al. (1997)
Modified double emulsification					
Tetanus toxoid	Antigen	Aqueous phase 1: active ingredient/water Organic phase: PLA Mw 28 kDa/lecithin/ethyl acetate or PLGA[a]/lecithin/ethyl acetate Aqueous phase 2: PVA/water		PEG Mn 5 kDa or Chitosan Mw Mn >50 kDa	Vila et al., 2002

PCL: poly(e-caprolactone); PEG: poly(ethylene glycol); PLA: poly(lactic acid); PLGA: poly(lactic acid-glycolic acid); PVA: poly(vinyl alcohol).
[a] Molecular weight (Mw) non-specified.

water-soluble materials (like most drugs) within them". In line with this approach, the permeability properties of hollow polyelectrolyte multilayer nanocapsules as a function of pH and the reversible behaviour of the open and closed states of the capsule wall have been demonstrated (Antipov et al., 2002). Also, this shift from "open" to "closed" nanocapsule and vice-versa, may happen through changes in environmental conditions such as temperature or the presence of organic solvents (Ai and Gao, 2004).

On the other hand, Preetz et al. (2008) have made methodological modifications in order to prepare oil-loaded polyelectrolyte nanocapsules (Fig. 8). Firstly, an emulsion containing modified starch (octenyl succinic anhydride-modified starch) and oil was prepared by high-pressure homogenization. The modified starch was used both as an emulsifier of the oily phase and as the first negatively charged polyelectrolyte layer of the shell. Then, the solution of the second polyelectrolyte was added under stirring and when adsorption had terminated, a solution of a third polyelectrolyte was injected into the system under the same conditions. Once the polyelectrolyte addition had ended, nanocapsule dispersion was again treated by high-pressure homogenization and the dispersion was finally centrifuged.

As reported in different research works, the layer-by-layer method makes used of polycations such as polylysine, chitosan, gelatin B, poly(allylamine) (PAA) poly(ethyleneimine) (PEI), aminidextran and protamine sulfate. The following polyanions are used: poly(styrene sulfonate) (PSS), sodium alginate, poly(acrylic acid), dextran sulfate, carboxymethyl cellulose, hyaluronic acid, gelatin A, chondroitin and heparin (Agarwal et al., 2008).

According to Radtchenko et al. (2000), the key issue of layer-by-layer assembly is the need for surface recharging at each adsorption step. The molecules employed for assembly should have a sufficient number of charged groups to provide stable adsorption on an oppositely charged surface and non-compensated charges exposed to the exterior. Nevertheless, taking into account energetic considerations, the possibility that the sequential adsorption of the following polyelectrolyte may remove the contrapolyion deposited instead of adsorbing onto it cannot be excluded (Sukhorukov et al., 1998).

Furthermore, this method raises other difficulties such as the formation of contraion aggregates, the separation of the remaining free polyelectrolyte from the particles prior to the next deposition cycle and polyelectrolyte-induced bridging during centrifugation. Close particle–particle encounters may cause unfavorable inter-

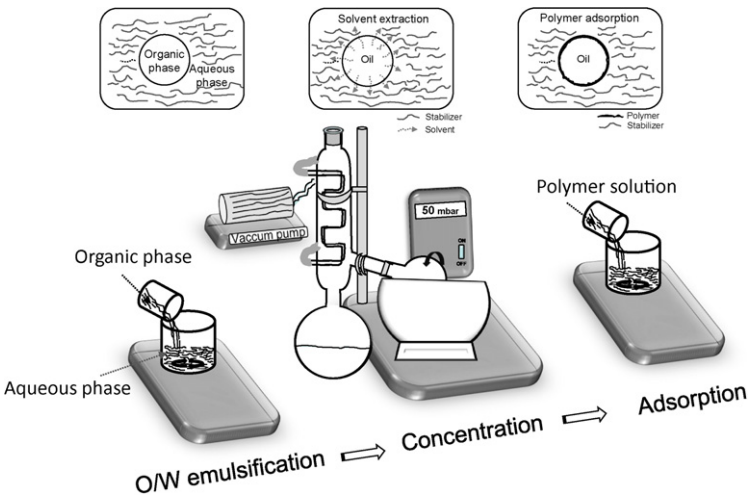

Fig. 7. Set-up used for preparation of nanocapsules by the polymer-coating method.

Table 11
Examples of raw materials used for preparation of nanocapsules by the layer-by-layer method—non-removable template.

Active ingredient	Therapeutic activity	Core	Cationic polymer	Anionic polymer	Solvent LbL procedure	Reference
Artemisinin	Antineoplastic	Artemisinin solid	Chitosan Mw 250 kDa, PDDA Mw 200 kDa or gelatin Mw 500 kDa	Sodium alginate Mw 70 kDa	Water	Chen et al. (2009)
Tamoxifen	Antineoplastic	Tamoxifen solid	PAH[a]	PSS[a]	Water and PBS	Agarwal et al. (2008)
Paclitaxel	Antineoplastic	Paclitaxel solid	PDDA[a]			
Sacharomyces cerevisiae *Neurospora crassa*		*Sacharomyces cerevisiae* *Neurospora crassa*	PAH Mw15 kDa	PSS Mw 70 kDa	NaCl aqueous solution	Krol et al. (2004)
		Medium-chain triglycerides OSA starch	Chitosan Mw 400 g/mol	Lambda-carrageenan[a]	Acetate buffer (pH 4.5) for cationic polymer Water for anionic polymer	Preetz et al. (2008)

OSA starch: octenyl succinic anhydride-modified starch; PAH: poly(allylamine hydrochloride); PDDA: poly(dimethyldiallylamide ammonium chloride); PSS: sodium poly(styrene sulphonate); PBS: sodium phosphate buffer.
[a] Molecular weight (Mw) non-specified.

actions with the polyelectrolyte films, possibly leading to film destruction and aggregate formation (Sukhorukov et al., 1998).

In addition, another difficulty is the particle sizes obtained which are higher than 500 nm (Sukhorukov et al., 1998; Chen et al., 2009). Although these particle sizes are at submicronic scale, they are obviously larger than the size commonly accepted for nanocapsules. However, this problem has been overcome by ultrasonic treatment of aqueous suspensions to decrease the size of individual drug particles to nano-scale (100–200 nm). They are then stabilized in solution by applying layer-by-layer coating by ultrasonic treatment and thin polyelectrolyte shells are assembled on their surfaces (Agarwal et al., 2008).

Consequently, although research using this strategy has greatly improved the technique, it is acknowledged that the high number of assembly steps involved is quite complex and time consuming, particularly for the synthesis of thick walled polymer nanocapsules (Sablon, 2008). In addition, taking into account that research into this method of nanoencapsulation of active substances has only just begun, it is not possible to propose formulations that can be used as a model.

3.7. Strategies for the concentration, purification and stabilization of nanoencapsulated systems

There are different reasons for ensuring the concentration, purification and stabilization of nanocapsule dispersions. In relation to the need of concentration, the different methods used for preparation of nanocapsules frequently produce dispersions with low drug carrying contents which is a serious disadvantage when the aim is to obtain therapeutic concentrations. This information is limited in reviews of research so it is difficult to make comparisons between works due to the different volumes used and the different encapsulation efficiencies reported by each team. Table 13 shows an approximation of dispersion concentrations before and after their concentration.

With regard to the need for purification, the initial nanocapsule dispersions obtained from preformed polymers can be contaminated by solvents, salts, stabilizers and cross-linking agents that must be eliminated in order to guarantee the purity required for in vivo nanocapsule administration.

Likewise, regarding stabilization, although nanocapsule dispersions are catalogued as stable systems due to Brownian motion, they can be subject to non-stability phenomena due to, among other things, polymer degradation, migration of the active substance from the inner liquid and microbiological contamination of aqueous systems. Indeed, one of the things limiting the industrial development of polymeric nanocapsule suspensions as drug delivery systems is the problem encountered in maintaining the stability of suspensions (Pohlmann et al., 2002).

As shown in Fig. 2, different options exist for the concentration, purification and stabilization of nanoencapsulated systems that can be used independently or combined sequentially. Evapo-

Table 12
Examples of raw materials used for preparation of nanocapsules by the layer-by-layer method—removable template.

Template	Cationic polymer	Anionic polymer	Solvent LbL procedure	Core removed solvent	References
Polystyrene latex particles	PHA Mw 8–11 kDa	PSS Mw 70 kDa	NaCl aqueous solution		Sukhorukov et al. (1998)
$CaCO_3$ particles	PHA Mw 15 kDa	PSS Mw 70 kDa	NaCl aqueous solution	HCl solution	Krol et al. (2004)
$CdCO_3$ particles	PHA Mw 50 kDa	PSS Mw 70 kDa	NaCl aqueous solution	HCl solution	Antipov et al. (2002)
MFparticles	PDDA Mw 200 kDa in water	Gelatin negatively charged Mw 50 kDa	PBS (pH 7.4)	HCl solution	Ai and Gao (2004)
MFparticles	PHA Mw 50 kDa	PSS Mw 70 kDa	NaCl aqueous solution	HCl solution	Radtchenko et al. (2002a)
$PSS^{-}/7Y^{3+}$ complex-MFparticles	PHA Mw 50 kDa	PSS Mw 70 kDa	NaCl aqueous solution	HCl solution and NaCl/EDTA	Radtchenko et al. (2002a)

MFparticles: melamine formaldehyde colloidal particles; PSS^{-}/Y^{3+} complex-MFparticles: poly(styrene sulfonate)/Yttrium3+ ions complex onto the surface of the melamine formaldehyde colloidal particles; PAH: poly(allylamine hydrochloride); PDDA: poly(dimethyldiallylamide ammonium chloride); PSS: sodium poly(styrene sulphonate); PBS: sodium phosphate buffer; EDTA: ethylenediaminetetraacetic acid.

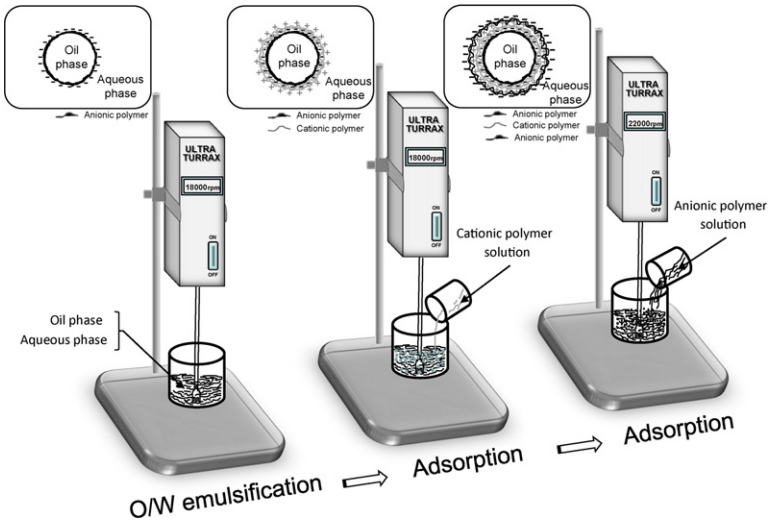

Fig. 8. Set-up used for preparation of nanocapsules by the layer-by-layer method.

ration under reduced pressure, water washing, ultracentrifugation and lyophilization are undoubtedly the methods used most. However, they are often inapplicable due to the aggregates formed (Duclairoir et al., 1998; Vauthier et al., 2008) and they are currently only adapted for purifying small batches (Limayem et al., 2004).

Among the strategies used for nanocapsule purification, the literature reports the use of dialysis against water (Schaffazick et al., 2003; Stella et al., 2007), dialysis against a polymer solution (Vauthier et al., 2008), filtration through 0.45 μm (Stella et al., 2007), cross-flow microfiltration and diafiltration, which efficiently eliminates surfactants and solvents (Limayem et al., 2004). Nevertheless, it is important to note that techniques such as filtration, dialysis, and ultracentrifugation do not provide efficient separation for small nanocapsule sizes (80–150 nm). In these cases, methods such as gel permeation chromatography have proved to be efficient (Ma et al., 2001).

Likewise, in an attempt to find alternatives for nanocapsule stabilization, the spray-drying technique using lactose or colloidal silicon dioxide as nanocapsule protectors has been proposed instead of lyophilization (Pohlmann et al., 2002; Tewa-Tagne et al., 2007a,b). However, research into optimizing the latter technique is still in progress and the use of cryoprotectants and lyoprotectants is necessary since the thin polymeric envelope of the nanocapsules may not withstand the stress of this process. Nanocapsules can be destabilized by the crystallization during freezing, dessication or storage of certain cryoprotectants such as mannitol, sucrose or glucose (Abdelwahed et al., 2006c). However, the behaviour of other protectants such as povidone and colloidal silicon dioxide appears to be acceptable (Schaffazick et al., 2003; Abdelwahed et al., 2006b). Table 14 provides a summary of research into nanocapsule lyophilization and spray-drying.

4. Behaviour of nanocapsules as drug delivery systems

The current section of this review will focus on the behaviour of nanocapsules in relation to their size, zeta-potential, dispersion pH, shell thickness, encapsulation efficiency, drug release, stability and *in vivo* and *in vitro* performances as a function of their preparation method. These properties have been chosen because they are those most frequently sought.

To this end, more than seventy research works available in electronic databases (Science direct® and Springerlink®) have been studied. The data analysis performed was confined to the comparison of methods and identification of trends in order to contribute to the state of knowledge. Hence, it is clear that comparing data from the literature is difficult when differences exist in the experimental methods used and in the specific aims of each research team. Likewise, generalizations are limited because the studies chosen represent only a sample of the universe of research performed in this field as many works may remain unpublished or hard to obtain.

4.1. Mean nanocapsule size

The mean particle sizes of nanocapsules prepared from preformed polymers are in general between 250 and 500 nm (Fig. 9). Exceptions stem from research in which the solid active substance has been encapsulated directly (s/o/w emulsification and layer-by-layer methods). However, as mentioned previously, in these cases it is possible to obtain low mean particle sizes by using ultrasound in the initial steps of the procedure.

Fig. 9 shows the range of sizes that can be obtained by each method while an explanation is provided in Table 15. This table summarizes research illustrating the impact of changes made to composition parameters on nanocapsule sizes. As can be seen, such changes are significant for most nanoencapsulation methods. For example, in regarding to nanoprecipitation, the nature and concentration of the polymer in the organic phase, solvent polarities, the nature and ratio of internal/external phases and the nature and concentration of surfactants are essential factors in determining nanocapsule size (Santos-Magalhães et al., 2000; Zili et al., 2005).

With regard to emulsion–diffusion method, parameters such as the nature and the volume of the organic and aqueous phase, the nature and concentration of surfactants and polymers have rele-

Table 13
Drug encapsulation in diluted and concentrated dispersions as a function of nanoencapsulation method.

Method	Drug concentration in diluted dispersions (mg/ml)	Drug concentration in concentrated dispersions (mg/ml)
Nanoprecipitation	0.002–0.09	0.15–6.5
Emulsification–diffusion	~0.2	~50
Double emulsification	2–5	20–50
Emulsification–coacervation	~0.24	~12

This data corresponds to a general estimate taking as base different information available in the researcher works that supported this review.

Table 14
Summary of research into the stabilization of nanoencapsulated systems by lyophilization and spray-drying.

Method	Material evaluated	Conclusion	Reference
Spray-drying	Colloidal silicon dioxide	Yield about 70%. The nanocapsule drug recovery and their morphological characteristics presented stable after 5 months of storage at room temperature.	Pohlmann et al. (2002)
Spray-drying	Colloidal silicon dioxide	The concentrations of both NC and excipient, and the mixing procedure are crucial parameters for the NC spray-drying. Nanocapsule concentration suggest: 1% (w/v). Excipient concentration suggest: 10% (w/v).	Tewa-Tagne et al. (2006, 2007a)
Spray-drying	Lactose, mannitol, dextrose, maltodextrine, PVP, HPC, HPMC	Lactose allows a desirable powder morphology and favouring NC suspension reconstitution with only ~2% of agglomerates. Mannitol and PVP allow the particle redispersion in the range of the original particle size	Tewa-Tagne et al. (2007b)
Lyophilization	Colloidal silicon dioxide	It is required an excipient for the successful NC lyophilization. The microparticle surface of the freeze-dried powders showed NC with size range similar to that observed for the corresponding original suspensions with SiO_2.	Schaffazick et al. (2003)
Lyophilization	HPbCD, sucrose, glucose, anhydrous glucose, trehalose, mannitol, PVP	Nanocapsule aggregation and the formation of macroscopic particles were noticed after the freeze-drying without cryoprotectant. Nanocapsule sizes are conserved after freeze-drying when sucrose, HPbCD, glucose and PVP are used. Nanocapsule freeze-drying with mannitol produces aggregates.	Abdelwahed et al. (2006a,b,c)

PVP: poly(vinylpyrrolidone); HPC: hydroxypropylcellulose; HPMC: hydroxypropylmethylcellulose; SiO_2: colloidal silicon dioxide; HPbCD: hydroxypropylbeta-cyclodextrine

vant implications on particle size distribution. Likewise, the control of nanocapsule mean diameter can be achieved by the intensity and duration of homogenization, in other words, the shear rate of the emulsification process (Ma et al., 2001; Joo et al., 2008; Moinard-Chécot et al., 2008).

Research into the double emulsification method has concluded that particle size depends on the balance between the types and concentrations of the internal and external surfactants that determine droplet size, the interactions at the interface and the structural conformation of the nanocapsule wall (Khoee and Yaghoobian, 2008).

On the other hand, it has been observed that the nature of concentration of drugs does not appear to influence the size of nanocapsules when the latter are prepared by nanoprecipitation or emulsion–diffusion methods (Guterres et al., 1995; Pereira et al., 2006; Joo et al., 2008). However, research elsewhere has reported contrasting conclusions (Fessi et al., 1989; Dalençon et al., 1997; Quintanar et al., 1998b; Stella et al., 2007).

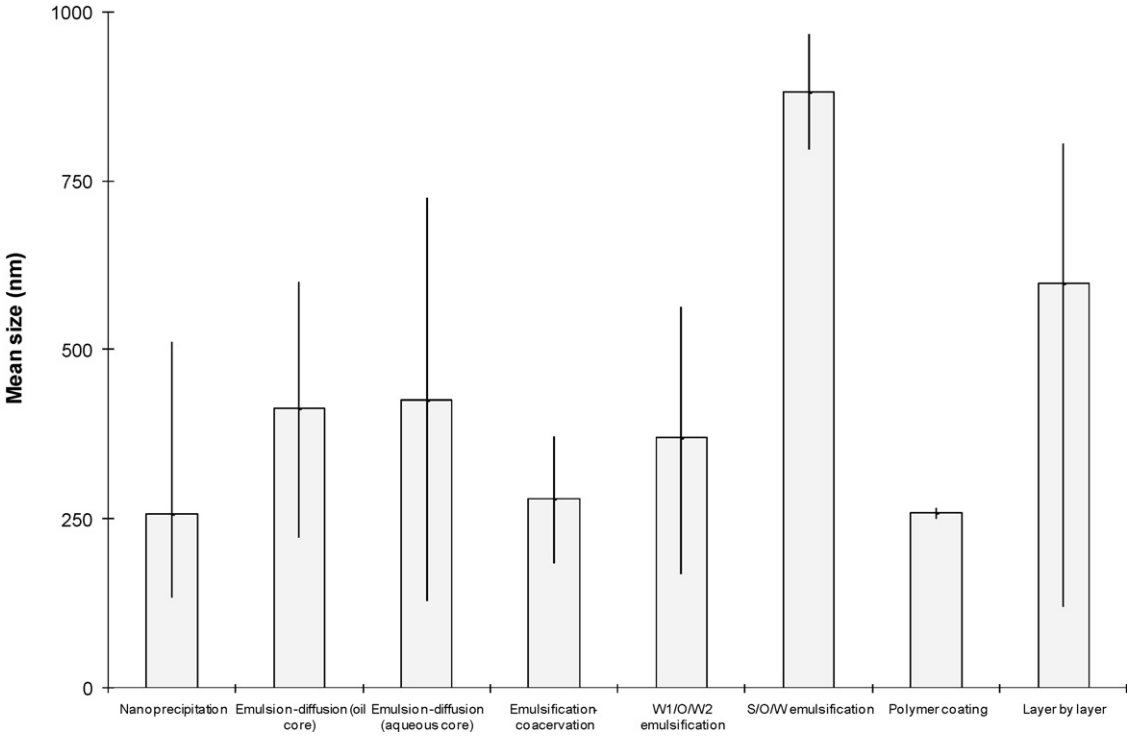

Fig. 9. Size behaviour obtained as a function of method for preparation of nanocapsules.

Table 15
The effect of various parameters on the size of formed nanocapsules.

Variable	Method	Evaluated materials	Work conditions	Mean size range (nm)	Behaviour*	Reference
Active principle nature	Nanoprecipitation	Gemcitabine derivates	Variable	182–301	Significant	Stella et al. (2007)
		Taxol, dexamethasone, vitamin K	Variable	260–300	Significant	Fessi et al. (1989)
		DNA, DNA–PVP, DNA–PVA	Variable	127–132		Perez et al. (2001)
	Emulsion–diffusion	Indomethacine – Progesterone – Estradiol	20 mg	335–510	Significant	Quintanar et al. (1998b)
	Double emulsification	DNA, DNA–PVP, DNA–PVA	Variable	272–296		Perez et al. (2001)
Active principle concentration	Nanoprecipitation	Rifabutine	0.32–0.8 mg/ml solvent	205–512	Significant	Dalençon et al. (1997)
Oil nature	Nanoprecipitation	Capric/caprylic triglycerides–benzyl benzoate	0.012 ml/ml acetone	225–202		Schaffazick et al. (2003)
	Emulsion–diffusion	Mineral oil–capric/caprylic triglycerides	0.25 ml/ml AcEt	303–340	Significant	Moinard-Chécot et al. (2008)
	Layer-by-layer	Capric/caprylic triglycerides, sesame oil, olive oil	5%	150–200		Preetz et al. (2008)
Oil concentration	Emulsion–diffusion	Capric/caprylic triglycerides	5–25%	360–483	Significant	Moinard-Chécot et al. (2008)
	Emulsion-coacervation	Turmeric oil	0.5–10%	87–739	Significant	Lertsutthiwong et al. (2008a)
Oil viscosity	Emulsion–diffusion	Capric/caprylic triglycerides	Viscosity variable	358–702	Significant	Moinard-Chécot et al. (2008)
		PCL, Eudragit	3.7 mg/ml acetone	327–225	Significant	Schaffazick et al. (2003)
		PLA, PLA–PEG	Variable	218–277	Significant	Furtado et al. (2001b)
	Nanoprecipitation	PLA, PCL	3.7 mg/ml acetone	197–182		Pohlmann et al. (2002)
		PLA, PCL	4 mg/ml acetone	228–241		Cauchetier et al. (2003)
		PLA, PLGA	4 mg/ml acetone	206–205		Dalençon et al. (1997)
Polymer nature		PLGA, PLA, PCL, PEG–PLGA, PEG–PLA, PEG–PCL	5 mg/ml acetone	210–287	Significant	Ameller et al. (2003)
	Emulsion–diffusion	PLA-Tone P-700	10 mg/ml AcEt	340–346		Fessi et al., 1989
		PLA, PLA–PEG	Variable	726–133	Significant	Ma et al. (2001)
	Double emulsification	PLA, PCL	Variable	890–317	Significant	Lu et al., 1999
Polymer concentration	Nanoprecipitation	PCL 10000	4–10 mg/ml acetone	741–924	Significant	Limayem et al., 2006
	Emulsion–diffusion	PCL 80000	20–50 mg/ml	585–1329	Significant	Guinebretière et al. (2002)
		PCL 80000	10–80 mg/ml AcEt	465–483		Moinard-Chécot et al. (2008)
		PLA	6.25–30 mg/ml AcEt	319–614	Significant	Quintanar et al. (1998b)
		PLA	20–35 mg/ml solvent	549–601	Significant	Romero-Cano and Vincent (2002)
	Double emulsification	PLGA or PCL	Variable	300–600	Significant	Lamprecht et al. (2000)
		PLGA	25–51 mg/ml DCM	130–353	Significant	Jeong et al. (2008)
Polymer molecular weight	Nanoprecipitation	PCL	10,000–80,000	741–924	Significant	Limayem et al. (2006)
	Emulsion–diffusion	PCL	14,000–80,000	420–483	Significant	Moinard-Chécot et al. (2008)
	Double emulsification	PLA	16,000–51,000	563–890	Significant	Lu et al. (1999)
Surfactant nature	Double emulsification	Sorbitan esters–Polysorbates (20, 60, 80)	Variable	169–254	Significant	Zhu et al. (2005)
		Sorbitan esters–Polysorbates (20, 60, 80)	Variable	75–621	Significant	Khoee and Yaghoobian (2008)
Surfactant concentration	Double emulsification	Sorbitan esters–polysorbates (20, 60, 80)	Variable	75–621	Significant	Khoee and Yaghoobian (2008)
Solvent nature	Emulsion–diffusion	Ethyl acetate–propylene carbonate–benzyl alcohol	20 ml	332–239	Significant	Quintanar et al. (1998b)
Solvent volume	Nanoprecipitation	Acetone	Solvent/water ratio: 0.5–0.8	352–308	Significant	Ferranti et al. (1999)
	Double emulsification	Ethyl acetate–methylene chloride	Water/organic solvent ratio: 1:2; 1:7	425–1402	Significant	Bilati et al. (2005a)
Stabilizer nature	Nanoprecipitation	Polysorbate 20, Polysorbate 80, PLX 188	Variables	320–825	Significant	Moinard-Chécot et al. (2008)
	Emulsion–diffusion	SDS, CTAC: gelatin, CTAC	0.20%	223–598	Significant	Joo et al. (2008)
Stabilizer concentration	Nanoprecipitation	PLX 188	0.1–0.5% of aqueous phase	814–725	Significant	Limayem et al. (2006)
	Emulsion–diffusion	PVA 88000	0.5–3.75% of aqueous phase	365–1247	Significant	Moinard-Chécot et al. (2008)
	Double emulsification	PVA	0–0.4%	300–275	Significant	Lamprecht et al. (2000)
Stabilizer molecular weight	Emulsion–diffusion	PVA	31,000–88,000	456–483	Significant	Moinard-Chécot et al. (2008)
Water volume	Nanoprecipitation	Water	Ratio solvent/water: 1:2–1:4	320–536	Significant	Limayem et al. (2006)

* Significant behaviour exists when the nanoparticle size difference among evaluated conditions is greater than 20 nm.

Table 16
Zeta-potential of nanoencapsules as a function of preparation method.

Polymer	Stabilizer agent	Z-Potential (mV)	Reference
Nanoprecipitation			
PCL	Polysorbate 80	−50.7	Cruz et al. (2006)
PCL	Polysorbate 80	−7.3	Ourique et al. (2008)
PCL	Polysorbate 80	−31	Tewa-Tagne et al. (2007a)
PCL	Polysorbate 80	−27.9	Tewa-Tagne et al. (2006)
PCL	Poloxamer 188	−39.9	Calvo et al. (1997)
PCL	Poloxamer 188/Chitosan	37.1	Calvo et al. (1997)
PLA	Poloxamer 188	−62.0	Pereira et al. (2008)
PLA–PEG	Poloxamer 188	−60.3	Pereira et al. (2008)
PLGA	Poloxamer 188	−39.5	Texeira et al. (2005)
PLGA	Poloxamer 188 + trehalose	−28.4	Pereira et al. (2006)
Eudragit	Polysorbate 80	−33	Schaffazick et al. (2008)
Emulsion-diffusion aqueous core			
PCL	Poloxamer 188	−5.9	Choi et al. (2009)
Aqueous core			
	PVA or PVP	30.9	Perez et al. (2001)
Emulsion-coacervation			
Sodium alginate	Sodium alginate/polysorbate 80 calcium chloride—cross-linking agent	−17.4	Lertsutthiwong et al. (2008a)
Double emulsification (W/O/W)			
PLA	Polysorbate 80/glycerin	−38.9	Zhu et al. (2005)
PLA–PEG	PVA	−18.6	Perez et al. (2001)
PLGA	PVA chitosan	21.8	Vila et al. (2002)
Polymer-coating			
Chitosan	Poloxamer 188	+34.8	Prego et al. (2006)
Poly-L-lysine	Poloxamer 188	27.9	Calvo et al. (1997)
Layer-by-layer			
Chitosan/Alginate		−30	Chen et al. (2009)
Chitosan/lambda-carraaeenan		−21.1	Preetz et al. (2008)

All measures have been realized "after adequate dilution of an aliquot of the suspension in water".

4.2. Nanocapsule zeta-potential

No specific trend regarding nanocapsule zeta-potential behaviour has been brought to light as yet (Table 16). Taking into account the author's experience, nanocapsule zeta-potential mainly depends on the chemical nature of the polymer, the chemical nature of the stabilizing agent and pH of the medium. Therefore when nanocapsules are prepared from polyester polymers or methacrylate derivates using non-ionic stabilizing agents, negative zeta-potential values are obtained due to the presence of polymer terminal carboxylic groups. Likewise, positive zeta-potential values are obtained when cationic polymers and non-ionic stabilizing agents are used.

On the other hand, when nanocapsules are prepared by using negatively charged polymers and negatively charged stabilizing agents (i.e. sodium lauryl sulphate), negative zeta-potential values are obtained with absolute values higher than when non-charged stabilizers are used. Similarly, the zeta-potential is positive if a positively charged stabilizing agent is chosen. This behaviour is due to the adsorption of the stabilizing agent onto the nanocapsule surface, which, for example in the case of PCL, can be explained by its hydrophobic nature. Consequently, the hydrocarbon chains of the

Table 17
The effect of various parameters on the zeta-potential of the formed nanocapsules.

Variable	Method	Materials evaluated	Work conditions	Z-Potential range (mV)	Behaviour*	Reference
Active principle nature	Double emulsification	DNA, DNA–PVP, DNA–PVA	Variable	(−29)–(−34)		Perez et al. (2001)
Active principle concentration	Nanoprecipitation	4-(N)-stearoylgemcitabine	100–1000 mcg/ml	27–44	Significant	Stella et al. (2007)
Oil nature	Layer-by-layer	Medium-chain triglycerides, sesame oil, olive oil	5%	(−12.7)–(−21.1)		Preetz et al. (2008).
Oil concentration	Emulsion-coacervation	Turmeric oil	0.5–10%	(−17)–(−19)		Lertsutthiwong et al. (2008a)
Polymer nature	Nanoprecipitation	PLA, PLA–PEG	Composition variable	(−50)–(−56)		Furtado et al. (2001a)
		PLGA, PLA, PCL, PEG–PLGA, PEG–PLA, PEG–PCL	Variable	(−42)–(−57)		Ameller et al. (2003)
	Emulsion–diffusion	DNA, DNA–PVP, DNA–PVA	Composition variable	(−29)–(−33)		Perez et al. (2001)
Polymer concentration	Emulsion–diffusion	PLA	6.25–30 mg/ml AcEt	(−15)–(−20)		Quintanar et al. (1998b)
Stabilizer nature	Double emulsification	Sorbitan esters–polysorbates (20, 60, 80)	Variable	(−34)–(−39)		Zhu et al. (2005)

* Significant behaviour exists when the Z-potential difference among evaluated conditions is greater than 15 mV.

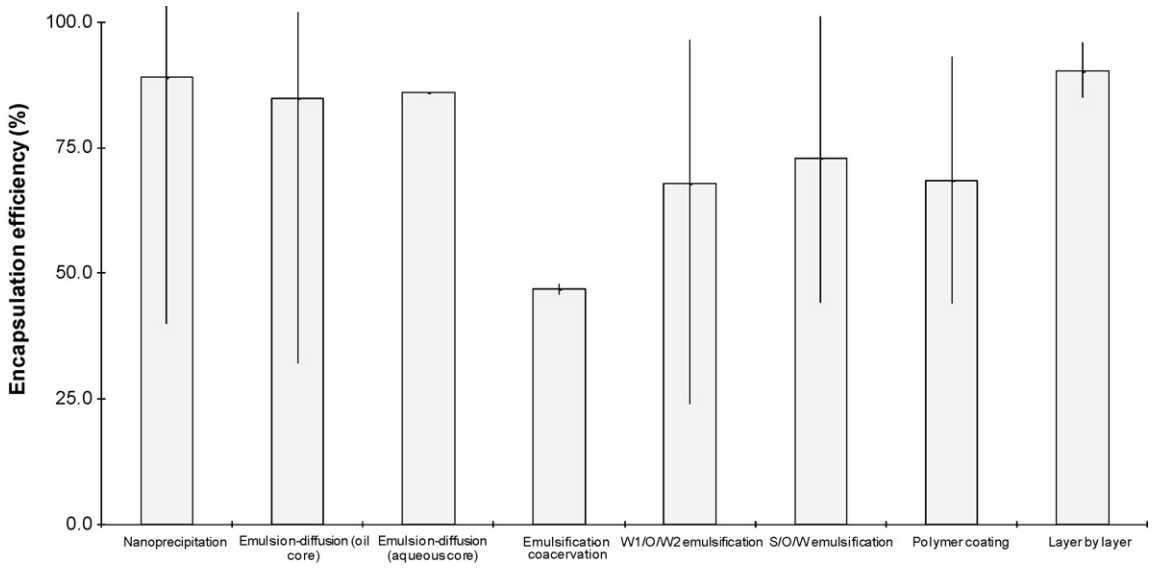

Fig. 10. Encapsulation efficiency behaviour obtained as a function of method for preparation of nanocapsules.

surfactant interact with the hydrophobic regions of the PCL wall and the surfactant head facing aqueous phase, which induces negative or positive zeta-potentials depending on its chemical nature (Joo et al., 2008).

In addition, the magnitude of the zeta-potential depends on the dispersion pH regardless of the nature of the stabilizing agent (Joo et al., 2008). Unfortunately, the literature reports no specific value for zeta-potential measurement, which is frequently expressed as "all measurements have been performed after adequate dilution of an aliquot of the suspension in water". With unknown pH and salinity, it is difficult to propose general behaviour. However, it can be stated that in most cases, zeta-potential values lower than −10 mV (usually between −25 and −30 mV, Table 16) are reported, which allows predicting good colloidal stability due to the high-energy barrier between particles.

Furthermore, the studies reported in Table 17 which were developed with 4-(N)-stearoylgemcitabine nanocapsules prepared by nanoprecipitation (Stella et al., 2007), indomethacine and DNA nanocapsules obtained by emulsion–diffusion (Quintanar et al., 1998b; Perez et al., 2001) and turmeric oil and DNA nanocapsules prepared by the emulsion-coacervation and double emulsification methods, respectively (Perez et al., 2001; Lertsutthiwong et al., 2008a), suggest that the zeta-potential of the nanocapsules shows no dependence on the nature of the active molecule, polymer concentration or stabilizer concentration. According to the conclusions of these studies and taking into account that the active substance may be entrapped within the nanocapsule core, the resulting zeta-potential probably depends on the combination of materials and maybe on certain process conditions such as those that determine molecular organization when the polymer is re-precipitated.

4.3. Nanocapsule dispersion pH

In general terms, nanocapsule dispersion pH-values fall within a range of 3.0–7.5 when nanoprecipitation, emulsion–diffusion or layer-by-layer methods are applied. No information is available in the literature for the other methods for preparation of nanocapsules.

As mentioned previously, dispersion pH determines the zeta-potential of colloidal dispersions which can impact on their stability. For example, it has been reported that PLA hydrolysis is non-enzymatic and depends on the temperature and pH of the medium, accelerated under both acidic and basic conditions. Therefore when PLA nanocapsules were prepared with benzyl benzoate, pH-dispersion was more acidic than with capric/caprylic triglycerides, probably because of traces of free acids in the central oil core. The stability study of these nanocapsule dispersions shows considerable polymer degradation in the formulations with benzyl benzoate after 8 months storage, whereas minimal PLA breakdown was seen in the preparations containing capric/caprylic triglycerides (Guterres et al., 1995; Dalençon et al., 1997).

The pH of the dispersion medium seems to be a key factor controlling the size of nanoparticles and thus their biodistribution. In fact the nanoparticles in the circulation can leak from endothelial barrier openings named fenestrations (Gaumet et al., 2008). Unfortunately in the current review, it was not possible to identify studies illustrating the impact of pH on nanocapsules biodistribution.

4.4. Nanocapsule shell thickness

As will be discussed later, in the case of nanocapsules the polymeric shell plays a predominant role in protecting the active substances incorporated and probably in the release profile (Rübe et al., 2005; Poletto et al., 2008a). According to different authors, shell thickness values are about 10 nm (Rübe et al., 2005) and 20 nm (Cauchetier et al., 2003) when PCL is selected as polymer by the nanoprecipitation method and 10 nm when PLGA is chosen (Nassar et al., 2009). The differences observed between studies are probably due to the methods used for each one. Whereas Cauchetier et al. (2003) make theoretical approaches based on the hypothesis that the polymer is the unique component of the nanocapsules wall, Rübe et al. (2005) and Nassar et al. (2009) estimate shell thickness by using TEM photomicrographs of nanocapsules. The over-estimation of shell thickness obtained by Cauchetier et al. (2003) suggests that probably not all the polymer forms nanocapsules, meaning that nanosphere formation may also occur.

For nanocapsules prepared by emulsion–diffusion method had been reported shell thickness values between 1.5 and 2 nm (Guinebretière et al., 2002). At present, there is not enough experimental evidence to explain the huge difference between the shell thicknesses obtained when nanoprecipitation and emulsion–diffusion methods are used.

On the other hand, it has been reported that in both nanoprecipitation and emulsion–diffusion methods, the higher polymer

Table 18
The effect of various parameters on the encapsulation efficiency of the formed nanocapsules.

Active	Polymer	Oil	Nanocapsule preparation method	Variable of interest	Work conditions	Encapsulation efficiency (%)	Reference
Primidone	PCL	Benzyl alcohol	Nanoprecipitation	Ratio solvent/water	0.5–0.8	75–67	Ferranti et al. (1999)
Spironolactone	PCL	Capric/caprylic triglyceride PEG-4 esters	Nanoprecipitation	Ratio solvent/water	0.25–0.5	16–96	Limayem et al. (2006)
Xanthone	PLGA	Capric/caprylic triglyceride	Nanoprecipitation	Active concentration	200–600 mcg/ml	85–89	Texeira et al. (2005)
3-Methylxhantone	PLGA	Capric/caprylic triglyceride	Nanoprecipitation	Active concentration	1000–1400 mcg/ml	77–89	Texeira et al. (2005)
RU 58668	PLA, PLGA, PCL, PEG–PLA, PEG–PLGA, PEG–PCL	Capric/caprylic triglyceride	Nanoprecipitation	Polymer type	5 mg/ml acetone	94–99	Ameller et al. (2003)
	PLA		Emulsion–diffusion	Polymer type	PLA	18.5	
Insuline	PLA–PEG copolymers				PLA–PEG	32–38	Ma et al. (2001)
DNA	PLA–PEG copolymer		Emulsion–diffusion	Complex active-polymer	DNA	87	Perez et al. (2001)
					DNA–PVP or DNA–PVA	82–89	
Insulin	PLA		Double emulsification	Tensioactive—stabilizer ratio, tensioactive type, stabilizer type, stabilizer concentration	Sorbitan ester 80: Polysorbate 80, low concentration. Sorbitan ester 80: Polysorbate 80, high concentration.	34 66	Zhu et al. (2005)
Bovine serum albumin	PLA or PCL–PEO		Double emulsification	Polymer type	PLA	51–60	Lu et al. (1999)
					PCL–PEO	29	
Penicillin G	PBA		Double emulsification	Tensioactive—stabilizer ratio, tensioactive type, stabilizer type, stabilizer concentration	Sorbitan ester 60: Polysorbate 60, 1:2.8 ratio, low concentration. Sorbitan ester 60: Polysorbate 60, 1:3.8 ratio, high concentration.	22 76	Khoee and Yaghoobian (2008)
DNA	PLA–PEG copolymer		Double emulsification	Complex active-polymer	DNA	72	Perez et al. (2001)
					DNA–PVP or DNA–PVA	79–59	

concentration in the oil phase leads to an increase in the shell thickness of the nanocapsules obtained (Romero-Cano and Vincent, 2002; Cauchetier et al., 2003).

Regarding shell thickness for nanocapsules prepared by the layer-by-layer method, it depends on the number of layers, the measurement conditions and possibly the conditions for preparation of nanocapsules. Consequently, the value estimated is between 1.5 and 1.7 nm per polycation/polyanion bilayer in dry state (Radtchenko et al., 2002a; Agarwal et al., 2008). Furthermore, the research performed by Agarwal et al. (2008) shows that shell thickness is almost twice these values when the measurements are carried out in water. According to other studies, the mean increase of the particle diameter per cationic/anionic layer is 5 nm; however, the first layer has an apparent thickness of 8–11 nm (Sukhorukov et al., 1998).

Unfortunately, there does not appear to any information about this parameter for nanocapsules prepared by double emulsification, emulsion-coacervation and polymer-coating, which makes a global comparison of all the methods problematic.

4.5. Nanocapsule encapsulation efficiency

As shown in Fig. 10, nanoprecipitation, emulsion–diffusion and layer-by-layer methods currently give the best results for nanocapsule encapsulation (80% or more). In the case of the layer-by-layer method the fact that the solid drug is the template ensures high encapsulation efficiency. Nevertheless, as shown in Table 18, for the nanoprecipitation and emulsion–diffusion methods, different determinant factors of drug encapsulation efficiency exist. For example, the active chemical nature of the drug and its polarity in particular, determine encapsulation efficiency. In this sense, hydrophilic drugs can reach maximum values of 10% and in cases of lipophilic compounds major encapsulation efficiency is getting (higher than 70%) (Ma et al., 2001; Stella et al., 2007).

On the other hand, as mentioned previously, in these methods (nanoprecipitation and emulsion–diffusion) the maximum solubility of the active substance in oil is one of the criteria for oil selection and defining initial concentration when starting preparation of nanocapsules. Therefore it is logical to assume that systems in which the concentration of the active substance is close to the saturation concentration can give better results. However, it is necessary take into account that when using saturation concentrations, the active substance may precipitate easily due to process conditions. Consequently, drug nanocrystals can be present in the drug-loaded polymeric nanocapsule aqueous suspensions. This phenomenon can have a big impact on the drug release profile (Pohlmann et al., 2008).

Regarding the double emulsification method, it was found that drug mean encapsulation efficiency ranges from 65% to 75% (Fig. 10). This parameter may well be influenced by both the polymers and the surfactants used. Therefore when polymers are used with hydrophilic groups in their structure, for example the polycaprolactone-poly(ethylene oxide) block copolymer, these groups tend to enter the aqueous phase which might facilitate leakage of the drug from the nanocapsule to the outer aqueous solution and, as a result, provide the lowest encapsulation efficiency (Lu et al., 1999).

With regard to the surfactant effect when the double emulsification method is performed, it has been evaluated for sorbitan ester–poly(ethylene oxide) ester systems whose aggregation is controlled by a balanced molecular geometry determined by the packing parameter of each surfactant. Thus systems with good packing between the pair of surfactant, high emulsifying power and a high concentration, give better encapsulation efficiency results since they contribute towards obtaining more tightly sealed barrier structures with an inner aqueous phase capable of improving drug residence (Zhu et al., 2005; Khoee and Yaghoobian, 2008).

Finally, as shown in Fig. 10, regarding the other nanoencapsulation methods, the encapsulation efficiency obtained with the polymer-coating method is within the ranges obtained when using nanoprecipitation or double emulsification, depending on the method used for nanocapsule template preparation. In relation to the emulsion-coacervation method, its encapsulation efficiency is obviously low in comparison to other nanoencapsulation methods. According to the scanning electron microphotography of nanocapsules obtained by this method, holes due to solvent migration from the inner core can be seen at their surface. These holes probably allow drug leakage (Krause and Rohdewald, 1985).

4.6. Nanocapsule active substance release

It is rash to make generalizations about active substance release as a function of preparation method due to the limited number of available case studies. However, by way of illustration, Fig. 11 shows the results obtained by different studies while Table 19 provides a comparative summary of the results of different methods.

As can be observed, active substance release is the faster from nanocapsules prepared by emulsion–diffusion and emulsification coacervation methods. They are followed in descending order by nanoprecipitation, polymer-coating, layer-by-layer and double emulsification.

Some cases can be considered as exceptions because of their marked difference from the overall data. They are atovaquone nanocapsules prepared by nanoprecipitation and 4-nitroanisole nanocapsules obtained by emulsification diffusion. In the case of atovaquone, only between 20% and 25% of active substance was released within 4 months. This was assumed by researchers to be due to the capacity of the polymer or phospholipids to retain the active substance (Cauchetier et al., 2003). On the other hand, with regard to 4-nitroanisole, the results of slow release allow observing the effect of the nature and concentration of the polymer, likewise with the influence of the organic phase composition, which in this case is PLA, the active substance, hexane and DCM (Romero-Cano and Vincent, 2002).

In vitro active substance release behaviours of nanocapsules depends on a great variety of factors, such as the concentration and physicochemical characteristics of the active substance (particularly its solubility and oil/water partition coefficient); the nature, degradability, molecular weight and concentration of the polymer; the polymer solid microstructure when re-precipitated, the nature of the oil, nanocapsule size, the conditions of the *in vitro* release test (medium pH, temperature, contact time, among others) and the conditions of the preparation method. Therefore, the different active release behaviours seen in Fig. 11 are determined by the conditions established for carrying out each study. Likewise, each study has provided explanations for the behaviours observed in relation to the underlying theory used and additional tests carried out in the framework of the same research. Consequently this review compiles these explanations in order to provide better understanding of the general behaviours obtained.

Firstly, there is evidence of either modification of the release effect attributed to nanoencapsulation or its effect as a dissolution enhancer. Therefore, when the release profiles of non-encapsulated active substances are compared with those of the same active substance encapsulated by nanoprecipitation or layer-by-layer, a significant reduction of amounts released by unit of time is displayed from nanoencapsulated systems. This is because the presence of oil may increase the half-life of the sustained phase (Ferranti et al., 1999; Texeira et al., 2005; Agarwal et al., 2008; Poletto et al., 2008a). Likewise, the drug release behaviour observed when polymer-coating and double emulsification methods are per-

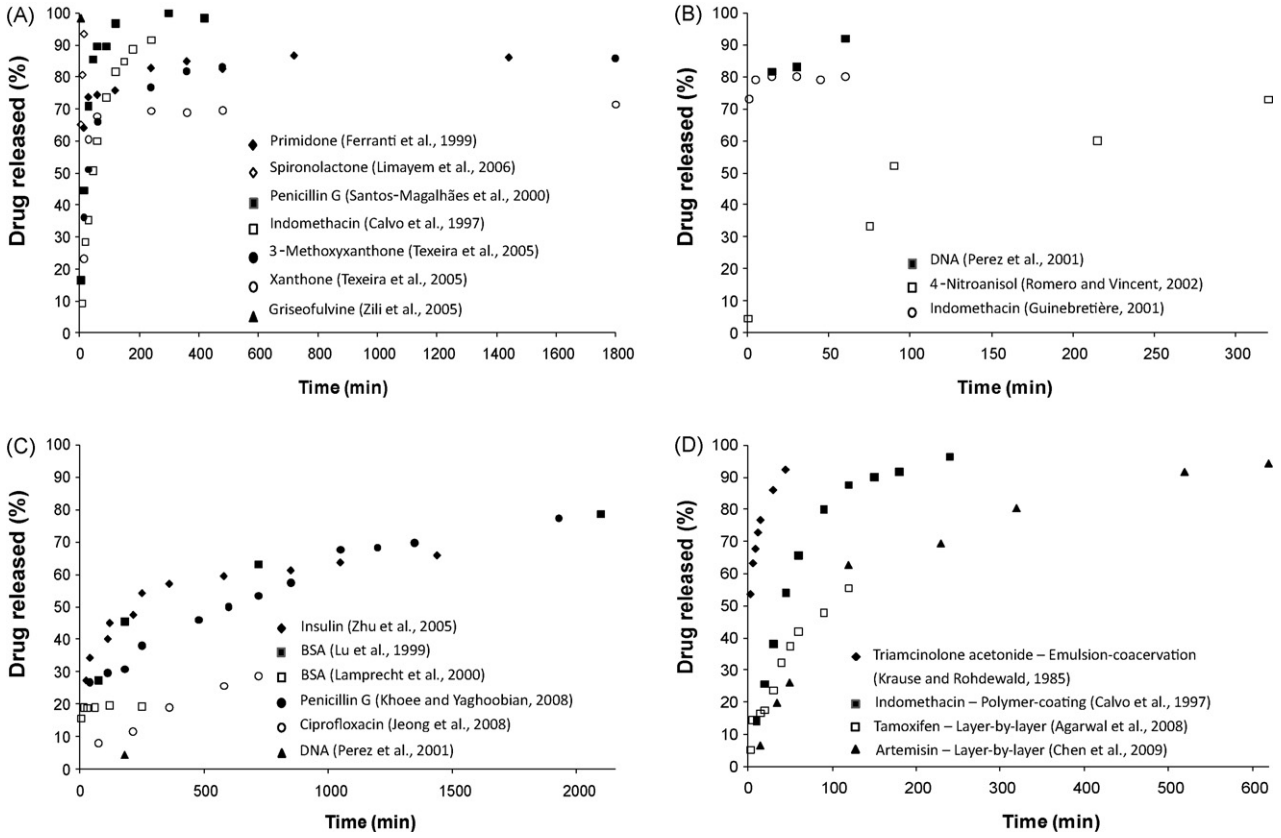

Fig. 11. Drug release behaviour of nanocapsules obtained by: (A) nanoprecipitation, (B) emulsion–diffusion, (C) double emulsification, and (D) emulsion-coacervation, polymer-coating and layer-by-layer.

formed demonstrates modified release (Lamprecht et al., 2000; Prego et al., 2006). On the other hand, it has also been reported that active substance dissolution rate is enhanced by encapsulation (Zili et al., 2005).

Furthermore, it has been proposed that nanocapsules obtained by nanoprecipitation, emulsion–diffusion, emulsion-coacervation and polymer-coating are biphasic systems with a fast initial release phase followed by a slower second release phase (Fig. 11A, B and D) (Cauchetier et al., 2003). The initial phase, called burst effect, can be attributed either to desorption of the drug located on the nanocapsule surface (Ferranti et al., 1999; Perez et al., 2001; Cruz et al., 2006), or to the degradation of the thin polymeric membrane (Cauchetier et al., 2003). Its behaviour is exhibited by apparent zero order kinetics (Santos-Magalhães et al., 2000).

The second phase corresponds to the diffusion of the drug molecules from the inner compartment, the reservoir core, to the outer phase. This diffusion process seem to be determined by the partition coefficient of the drug between the oily core and the aqueous external medium, the relative volumes of both phases, the existence of active substance-polymer interactions and the concentration of surfactants (Calvo et al., 1997; Zili et al., 2005; Texeira et al., 2005; Limayem et al., 2006).

In this diffusion process, the drug diffusion rate through the thin polymeric barrier does not seem to be a limiting factor (Krause and Rohdewald, 1985; Calvo et al., 1997; Zili et al., 2005). Nevertheless, it has been demonstrated that increasing the amount of polymer used significantly reduces the release rate (Romero-Cano and Vincent, 2002) and in these cases, the possibility that the polymer erosion could contribute to facilitating drug release has been considered by some researchers (Poletto et al., 2008a). This apparently contradiction could be explained by the fact that at low polymer concentrations (between 0.5% and 1% of the organic phase), the polymer-coating of nanocapsules does not form a consistent polymer wall but rather a thin polymer film possibly without impact on drug release (Cruz et al., 2006). It is probable that the walls of polymers at increased concentrations and high molecular weights, as in the case of the studies carried out by Romero-Cano and Vincent (2002), are more consistent, thereby having an impact on the release of the active substance.

On the other hand, nanoparticle size can influence the nanocapsule dissolution rate which increases as particle size decreases, due to an increase of available surface area (Zili et al., 2005). Likewise, the incomplete active substance release observed in most cases may be attributed to the retention capacity of the active substance by the polymer or surfactants such as phospholipids (Cauchetier et al., 2003).

With regard to the double emulsification process, which is the method preferred for water-soluble active substance nanoencapsulation, the drug release behaviour of the nanocapsules was different from that described for the other methods. According to Fig. 11C,

Table 19
General trend of active substance released from nanocapsules as a function of preparation method.

Method	Active substance release time (min)[a]			
	25%	50%	75%	90%
Nanoprecipitation	10	45	75	750
Emulsion–diffusion	<2	<2	10	60
Emulsion-coacervation	<4	<4	15	45
Double emulsification	145	1000	>2000	>2000
Polymer-coating	20	40	60	150
Layer-by-layer	40	85	320	510

[a] Time and percentaje release values estimated taking into account the data general trend.

the profiles show active substance releases higher than 70% within 30 h of beginning the test. According to some researchers, the active substance release follows a typical biphasic release model. The first phase is probably due to surface molecules and to molecule diffusion through the aqueous pores or channels created during particle preparation. The second phase corresponds to the release following the degradation–erosion of the particles (Perez et al., 2001). However, other researchers have proposed a model with three phases for drug release: an initial burst release, a plateau phase for a certain period resulting from the diffusion of the drug dispersed in the polymer matrix and, finally, a constant sustained release of the drug due to drug diffusion through the polymer wall and the erosion of the latter (Lamprecht et al., 2000).

According to Perez et al., and bearing in mind that the polymer concentration used for preparation of nanocapsules by double emulsification is higher than that used for the other methods (concentrations suggested in relation to the solvent used: Nanoprecipitation: 0.2–0.5%; emulsion–diffusion: 1–2% and double emulsification: 5–10%) it seems that nanocapsules prepared by double emulsification may have a compact structure so release is mainly controlled by the degradation and erosion of the polymer.

Therefore, release behaviour can be determined by parameters such as polymer molecular weight, nanocapsule inner core composition and particularly the nature of the w/o surfactant (Lu et al., 1999; Perez et al., 2001; Zhu et al., 2005). Moreover, it is important to take into account that drug encapsulation efficiency with double emulsification is lower than that obtained by the nanoprecipitation and emulsion–diffusion methods, which can also influence active substance release (Lu et al., 1999). Differences of particle size and drug content do not seem to affect the kinetic release of nanocapsules (Perez et al., 2001; Jeong et al., 2008).

4.7. Nanocapsule stability

Many factors, combined with nanocapsule composition, the parameters used in the preparation method and nanocapsule storage conditions, may affect the stability of nanoencapsulated systems. Therefore in most cases, it is difficult to identify specific determinants and the behaviours observed are the consequences of combinations that necessarily lead to general conclusions.

Consequently, researchers have focused on studying the stability of nanoencapsulated systems and seek to identify properties recognized as "instability tracers". Thus visual appearance can highlight advanced instability and particle size can reflect presence of aggregation while pH and active molecule quantification can permit the detection of chemical degradation for example.

In general terms, from the point of view of visual appearance and nanocapsule size, there are no variations under the different conditions studied (Cauchetier et al., 2003; Zili et al., 2005; Pereira et al., 2006; Limayem et al., 2006; Pohlmann et al., 2008; Lertsutthiwong et al., 2008a,b). In cases where variation has been detected 6 months after starting the study due to unknown storage conditions, polymer degradation is given as the reason (Dalençon et al., 1997).

In relation to pH variations, these have been detected in some cases when PLA or PCL are used (Pohlmann et al., 2002; Cauchetier et al., 2003) and this behaviour has been attributed to polymer degradation. Thus it has been reported that hydrolytic degradation of low molecular weight PLA polymers starts within a few days, whereas for high molecular weights this takes much longer (Romero-Cano and Vincent, 2002).

Table 20 summarises the results of stability studies developed with nanocapsules prepared by the nanoprecipitation method (only available information) taking as "instability tracer" the variation of the active substance concentration. As can be seen, storage of nanocapsules dispersion under high temperature conditions (above 40 °C) affects the stability of the system. Probably it is due to weakness of the polymeric structure, which facilitates the migration of the active substance from the inner core oil.

Likewise, studies of atovaquone, indomethacine, tretinoin and diclofenac nanocapsules have illustrated the impact of variables such as polymer molecular weight, active substance concentration, polymer nature and oil nature. Thus as an example, the photodegradation study of tretinoin nanocapsules shows the importance of the polymer in preventing active photodegradation. In this case, according to the researchers, the better protection obtained could be due to the crystallinity of the polymer, as it can reflect and scatter UV radiation. In the same study it was concluded that the use of different oily phases did not show any effect in this respect (Ourique et al., 2008).

In addition, a study of rifabutine nanocapsule stability exemplified another common instability factor of nanoencapsulated systems. Here, drug instability had been explained by the relative solubility of its ionized form in water and the suspension pH which increased rifabutine migration from the nanocapsule oily core to the aqueous medium (Dalençon et al., 1997).

4.8. Nanocapsule performance evaluation

Among the main challenges of administering nanocapsules as carriers of active molecules are the targeting of specific organs, allowing site-selective action of the compounds, minimizing their side effects, and providing sustained drug delivery in order to increase therapeutic availability, modification of tissue drug distribution, transmucosal delivery, gastrointestinal mucosal protection and simply to obtain significant therapeutic activity (Fawaz et al., 1996; De Jaeghere et al., 1999; Whelan, 2001; Prego et al., 2005; Pinto et al., 2006b; Singh and Lillard, 2009; De Martimprey et al., 2009).

Indeed, these objectives are not easy to achieve because when the nanocapsules enter the blood, they are quickly removed by the action of the mononuclear phagocytic system (MPS). Also, the extent and nature of nanocapsule opsonization, which is the first step of phagocytosis, depends on nanocapsule physicochemical properties such as size, surface charge and surface hydrophobicity. Consequently, the opzonization preferentially occurs in hydrophobic rather than hydrophilic surfaces, the negative surface charge increases the clearance of nanocapsules in relation to neutral or positively charged surfaces and particles less than 100 nm can leave the circulation through gaps or fenestrations in the endothelial cells lining the blood vessels (De Jaeghere et al., 1999).

Taking the above into consideration, some researchers have advanced towards the corroboration of their research expectations by using *in vitro* or *in vivo* models. A summary of the conclusions obtained is shown in Table 21. As can be seen, the results are promising. The role of nanocapsules used as active substance carriers is highlighted in drug pharmacokinetic modification (Fawaz et al., 1996; Furtado et al., 2001b; Vila et al., 2002; Prego et al., 2006; Jeong et al., 2008), increased drug bioavailability (Calvo et al., 1997; Vila et al., 2002; Nassar et al., 2009), modification of drug biodistribution (Furtado et al., 2001b; Vila et al., 2002), the capacity to increase therapeutic effects (Dalençon et al., 1997; Vila et al., 2002; Prego et al., 2006; Pereira et al., 2006; Jeong et al., 2008; Schaffazick et al., 2008), the hepatotoxicity reduction (Pereira et al., 2006), biocompatibility with ocular mucosa (Calvo et al., 1997) and skin-barrier permeation (Joo et al., 2008). Likewise, surface modification achieved by hydrophilic copolymers shows a reduction of opsonization (Furtado et al., 2001a) whereas size reduction facilitates phagocytosis in view to attacking tumor cells (Seyler et al., 1999).

On the other hand, the results of the above mentioned research has also shown limitations of nanocapsules such as their lim-

Table 20
Nanocapsule stability studies as a function of preparation method.

Active	Polymer	Oil	Stability study conditions		Active concentration variation (%)	Reference
			Storage temperature	Sampling (months)		
Nanoprecipitation						
Ato-vaquone	PLA 200000	Benzyl benzoate	4 °C	0 and 4	27	Cauchetier et al. (2003)
	PLGA 40000			0 and 3	18	
	PCL 65000			0 and 4	10	
	PCL100000			0 and 4	3	
Indomethacin (1 mg/ml)	PCL 65000	Capric/caprylic triglycerides	Room temperature and protected from light	0 to 5	0	Pohlmann et al. (2008)
Indomethacin (3 mg/ml)				0 to 0.6	52	
Indomethacin (1.5 mg/ml)	PCL 60000	Mineral oil	Room temperature	0 to 3	5	Pohlmann et al. (2002)
			50 °C	0 to 3	50	
	PLA[a]		Room temperature	0 to3	10	
			50 °C	0 to 3	30	
Tretinoin	PCL[a]	Capric/caprylic triglycerides	UV radiation exposition	1 h	32	Ourique et al. (2008)
		Sunflower oil	UV radiation exposition	1 h	34	
Spironolactone	PCL 10000	Capric/caprylic triglycerides PEG esters	25 °C	0 and 6	0	Limayem et al. (2006)
Griseofulvine	PCL 80000	Benzyl benzoate	4 °C	0 and 6	0	Zili et al. (2005)
Diclofenac	PLA 88000	Benzyl benzoate	Room temperature and protected from light	0 to 8	10	Guterres et al. (1995)
		Capric/caprylic triglycerides			40	
Emulsion–diffusion						
Indomethacin	PCL 80000	Capric/caprylic triglycerides	25 °C	0–2.5	Remain stable	Limayem et al. (2004)
Eugenol	PCL 80000			0–2	Remain stable	Choi et al. (2009)
	PCL 80000	Capric/caprylic triglycerides	Protected from light 4 °C and 40 °C	0–5	Remain stable	Moinard-Chécot et al. (2008)

[a] Polymer molecular weight non-specified.

Table 21
In vitro and *in vivo* performance of nanocapsules.

Active	Test	Conclusion	Reference
Indomethacin	Pharmacokinetic study and potential irritant effect on the rectal mucosa in rabbits.	Nanocapsules enhance the extravascular distribution by enhancing the capture of the colloidal carrier by the liver and at the same time, increases the active elimination rates compared to active solutions. A limited protective effect on the rectal mucosa was shown.	Fawaz et al. (1996)
Indomethacin	Active ocular distribution and acute ocular tolerance studies in rabbits.	The nanocapsules displayed a good ocular tolerancy and an ocular bioavailability of indomethacin higher than for control solution.	Calvo et al. (1997)
Atovaquone	Antiparasitic activity.	Nanocapsules increases the therapeutic effect compared with active suspension.	Dalençon et al. (1997)
Muramyltripeptide cholesterol (MTP-Chol)	Immunomodulating capacity towards a mouse macrophage cell line *in vitro*.	MTP-chol included within biodegradable polymeric nanocapsules can activate mouse macrophages.	Seyler et al. (1999)
–	Biodistribution studies in mice of PEG–PLA nanocapsules against PLA nanocapsules.	Covalent attachment of PEG to the nanocapsule surface led to significant changes in the body distribution of the particles, the AUC and the mean residence time are higher that PLA nanocapsules.	Furtado et al. (2001b)
Tetanus toxoid	Absorption, biodistribution and inmunologic test in mice after oral and nasal administration.	PEG or chitosan coated nanocapsules were able to enhance the behaviour of absorption, biodistribution and inmunologic responses than PLA nanocapsules.	Vila et al. (2002)
Salmon calcitonin	Hypocalcemic effect in rats.	Pulsatile pharmacological profile and enhancement of the hypocalcemic effect when compared to the peptide solution.	Prego et al. (2006)
Usnic acid	Antitumor activity in Sarcoma 180-bearing mice and subchronic toxicity in healthy animals.	Nanoencapsulation was able to maintain and improve the usnic acid antitumor activity and considerably reduce the hepatotoxicity of this drug.	Pereira et al. (2006)
4-(N)-stearoylgemcitabine	Cytotoxic activity on human cancer cell lines.	Active incorporation in nanocapsules did not change the IC_{50} compared to the free active.	Stella et al. (2007)
Indomethacin ethyl ester	Antiedematogenic activity study in rats.	Nanoencapsulation was not able to target the pro-drug to the site of action and the antiedematogenic effect observed was exclusively due the metabolite formed *in vivo*.	Cattani et al. (2008)
Hinikitiol	Permeation study in hairless mice.	The active nanoencapsulated was more skin-permeable than active in propyleneglycol.	Joo et al. (2008)
Ciprofloxacin	*In vitro* and *in vivo* antibacterial activity test with *E. coli*.	*In vitro*, antibacterial activity of active nanoencapsulated shows less cytotoxic response than that of active free due probably to the nanocapsules sustained release behaviour. *In vivo*, the active nanoencapsulate can inhibit the growth of bacteria for a longer period rather than active free.	Jeong et al. (2008)
Melatonin	Acute antioxidant effect of intra-peritoneal administration in mice.	Increase in the antioxidant effect of the melatonin-loaded nanocapsules.	Schaffazick et al. (2008)
Tacrolimus	Pharmacokinetic study in rats and pigs.	Nanocapsules yielded significantly higher drug levels than an active emulsion, resulting in a more enhanced bioavailability.	Nassar et al. (2009)

ited protective effect on rectal mucosa (Fawaz et al., 1996); the non-reduction of certain toxic effects (Stella et al., 2007) and the non-achievement of expectations regarding their drug targeting performance (Cattani et al., 2008). Obviously, as has been mentioned, these results should be considered within the context of each research.

5. Discussion and concluding remarks

Nanoencapsulation is an attractive strategy for the vectorization of a variety of active substances. As is shown in Table 2, although with different objectives, research has been focused on antineoplastics, antiinflammatories, immunosupresants, antigens,

hormones, antivirals, antibacterials, antifungals, diuretics, antipneumocystics and vitamins, among others.

According to different authors, nanocapsules used as drug carriers can mask unpleasant tastes, provide controlled release properties and protect vulnerable molecules from degradation by external factors such as light or by enzymatic attack in their transit through the digestive tract (Furtado et al., 2001b; Whelan, 2001; Ourique et al., 2008). Likewise, they can increase the therapeutic efficacy of active molecules because their biodistribution follows that of the carrier, rather than depending on the physicochemical properties of the active molecule itself (Barratt, 2000). Additionally, although nanoencapsulated systems have a relatively higher intracellular uptake compared with microparticles, this behaviour can be modified depending on nanocapsule surface charges and the hydrophilic or hydrophobic nature of the polymer used in shell formation (Pinto et al., 2006a).

Therefore, research into nanocapsules obtained by nanoprecipitation, emulsion–diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer methods support some of these assertions. There is evidence of increased therapeutic efficacy and the role of nanoencapsulation in both drug release modification and absorption enhancement. What is more, it has been shown that strategies such as polymer modification in order to obtain more hydrophilic surfaces or polymer coatings to obtain positively charged surfaces could provide better *in vivo* performance. In addition, some studies have verified favorable behaviour regarding active substance stability in the case of encapsulation. Unfortunately, no experimental data on important aspects such as nanocapsule behaviour in masking unpleasant tastes was found in the literature.

Also, as with all nanoparticulated delivery systems, the nanosize range obtained for nanocapsules produced by all methods except layer-by-layer (all method between 250 and 500 nm, layer-by-layer upper 500 nm) allows their administration by different routes: oral, rectal, transdermal, ocular, nasal, subcutaneous, intraperitoneal and intramuscular and they can be injected directly into the systemic circulation without the risk of blocking blood vessels as suggested by some researchers (Barratt, 2000; Fattal and Vauthier, 2002; Letchford and Burt, 2007). However, it has been asserted that nanocapsules reduce the systemic toxicity of active substances (Whelan, 2001) and numerous reviews focusing on the state of knowledge of their behaviour and interaction with biological systems have been published and much concern remains on this subject (FDA, 2007).

On the other hand, bearing in mind that there are different alternatives for nanocapsule synthesis by using preformed polymers, the choice of a specific method is usually determined by the drug's physicochemical characteristics, particularly its solubility and the therapeutic objective of nanocapsule administration, for example the route chosen and drug release profile. Nevertheless, it is important to take into account that the method chosen should also considerer other aspects such as active substance stability under operational conditions, particularly stirring, encapsulation efficiency, method feasibility, the generation of contaminants and the need for subsequent purification steps, solvent nature, the water volume required and time consumption. Likewise, the feasibility of scaling-up and cost should be considered. However at the moment, there is not enough information to back up judgement on this matter.

Table 22 shows a comparative analysis of some of the criteria mentioned previously taking into account the author's experience and the information on nanoencapsulation research available in databases. Most of the research has been done at laboratory-scale.

As can be observed, there is no ideal method because each one has its advantages and limitations. In general terms, for example, all the methods allow lipophilic active substance encapsulation, excluding the double emulsification method which had been developed for hydrophilic active substances such as proteins. In their majority, all procedures can be used with solvents with low toxic potential and without the addition of other chemical substances that allow an easy purification. However, emulsion-coacervation is excluded and the polymer-coating and layer-by-layer methods require particular considerations on their procedure. From the point of view of water consumption, emulsion–diffusion is undoubtedly disadvantageous. Nevertheless this condition represents an advantage in terms of purification steps.

In relation to method feasibility and time consumption, it is only possible to make an approximation taking into account laboratory experiment and pilot scales. In principle, all the methods are feasible at laboratory scale and as is logical, some difficulties are predictable in their scaling-up. Nevertheless, since the time for assembly preparation is approximately the same for all the methods, nanoprecipitation, which requires the slow addition of the organic phase, provides poor results in terms of time consumption. Consequently, research into the use of a membrane contactor at the pilot scale is being performed to find a more efficient alternative (Charcosset and Fessi, 2005; Limayem et al., 2006). In spite of the method's advantages and limitations mentioned above, it is possible to identify trends in research into nanoencapsulation method selection. Therefore, taking into account a general review of the available information in electronic databases (Science direct® and Springerlink®) on nanoencapsulation research, the nanoprecipitation method patented by Fessi et al. (1988) is the most used (Fig. 12). It is valued for the simplicity of its procedure, low cost, reproducible carrier size and high encapsulation efficiency (Leroueil-Le Verger et al., 1998; Lamprecht et al., 2001; Chorny et al., 2002; Cauchetier et al., 2003; Pinto et al., 2006a). Approximately 50% of research has been developed in line with this method followed by emulsion–diffusion and double emulsification methods. Nevertheless, it is important to take into account that if the objective of research is hydrosoluble molecule encapsulation, the method preferred is double emulsification.

In view to obtaining the best results as a function of the target design of the nanocapsules, besides the researches developed on polymeric vesicles or polymersomes, the classical methods can be modified or combined as described in the methodologies proposed by Calvo et al. (1997), Bilati et al. (2005a,b,c) and Nassar et al. (2009) on nanoprecipitation method; Ma et al. (2001) and Perez et al. (2001) on emulsion–diffusion method and Perez et al. (2001), Romero-Cano and Vincent (2002), Vila et al. (2002) and Béduneau et al. (2006) on modified double emulsification methods. Likewise, the literature reports research on scaling-up nanocapsule production using membrane contactor based on the nanoprecipitation principle, after substantial modification of operational conditions (Charcosset and Fessi, 2005; Limayem et al., 2006).

The other methodologies are not used very often. Emulsion-coacervation historically was the first methodological approximation for preparation of nanocapsules through the research done by Krause and Rohdewald (1985) on triamcinolone acetonide nanoencapsulation using gelatine as a polymer. However, as already

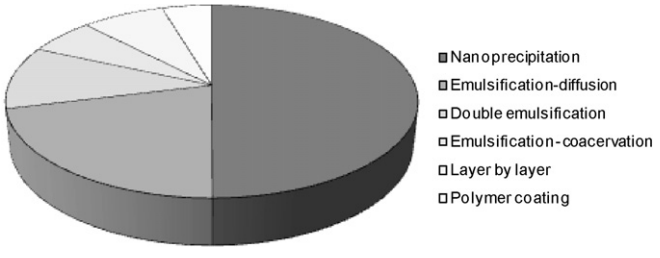

Fig. 12. Method selection trends in nanocapsule research.

Table 22
Comparative analysis of criteria suggested for the selection of nanoencapsulation methods.

Criterions	Nanoprecipitation	Emulsion–diffusion	Double emulsification	Emulsion-coacervation	Polymer-coating	Layer-by-layer
Active substance nature	Oil core: lipophilic	Oil core: lipophilic Aqueous core: hydrophilic	Aqueous core: hydrophilic Solid core: solid	Oil core: lipophilic Aqueous core: hydrophilic	Oil core: lipophilic	Oil core: lipophilic Solid core: solid
Active substance stability	High	High	Proteins can be denatured by high shear rate.	High	High	High
Solvent nature	Class 3	Class 3	Class 3/Class2	Class 3	Class 3	No required
Water volume consumption	Moderate	High	Moderate	Moderate	Moderate	Moderate
Method feasibility	High	High	High	High	High	High
Generation of contaminants	Low	Low	Low	Moderate	Low	Low
Purification steps	Low	Low	Low	High	Moderate	Moderate
Time consuming	High	Moderate	Low	Moderate	No reference available	No reference available

mentioned, this method requires an exhaustive purification process due to its inherent generation of nanocapsule dispersion contaminants, which is a major disadvantage in comparison with other alternatives.

On the other hand, the nanoencapsulation strategies such as polymer-coating and the layer-by-layer technique have shown interesting results, particularly in relation to *in vivo* nanocapsule behaviours since the final nanocapsule positive charge reduces their enzymatic degradation (Calvo et al., 1997). Such method is promising but needs more systematic and fundamental investigations.

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2. PREPARATION DE NANOPARTICULES PAR NANOPRECIPITATION ET PAR EMULSIFICATION-DIFFUSION

ETUDE COMPARATIVE DES METHODES

Comme présenté dans le précédent chapitre, les nanoparticules élaborées à partir de polymères préformés peuvent être formulées par différents procédés. Il n'existe pas de technique idéale, car chacune a ses avantages et ses inconvénients et choisir la plus adéquate, dépend de plusieurs facteurs : (i) l'application envisagée, (ii) la nature de la molécule active, (iii) le cahier des charges défini par l'application ciblée et (iv) les particularités associées à la technique de préparation.

Cependant, même si la sélection de la méthode appropriée est réalisée, les résultats peuvent ne pas répondre totalement au cahier des charges imposé suite à de nombreux problèmes liés à la formulation, les conditions opératoires et également à la nature des matières premières utilisées. Ceci détermine les caractéristiques physico-chimiques et colloïdales des particules telles que la taille, la distribution en taille, les propriétés électrocinétiques, la stabilité colloïdale et également la libération de principe actif encapsulé et par conséquent la performance de ces nanoparticules in vivo.

Ainsi, l'obtention d'un procédé robuste et reproductible pour préparer des nanoparticules nécessite des études fondamentales, systématiques et exhaustives afin de mieux connaître l'influence de chaque paramètre qui peut avoir de loin ou de près un effet mineur ou majeur sur leurs propriétés. Par conséquent, la première partie de ce chapitre est consacré à une étude comparative et fondamentale des deux méthodes les plus utilisées dans le domaine de la vectorisation de médicaments, mais mal expliquées à ce jour : la nanoprécipitation (aussi appelée déplacement du solvant) et l'émulsification-diffusion, qui permettent la préparation des nanosphères et des nanocapsules. D'un autre côté, dans la deuxième partie du chapitre, sera illustrée l'utilisation d'une méthode statistique de planification expérimentale comme outil pour l'identification des variables clés des procedès de préparation de nanoparticules.

2.1 Etude systématique et fondamentale de la préparation de nanoparticules par nanoprécipitation et par émulsification-diffusion.

Dans le cadre de notre travail, nous avons choisi des nanosphères comme particules modèles pour la réalisation de l'étude fondamentale et systématique des deux méthodes, car les matières premières (polymère, agent stabilisant, solvant organique et l'eau) utilisées pour la préparation restent les mêmes indépendamment de la méthode adoptée. L'analyse détaillée des résultats expérimentaux de la littérature a permis de conduire une étude complète, comparative et compréhensive sur l'effet des paramètres impliqués dans chaque procédé afin d'approfondir l'aspect mécanistique gouvernant la formation des nanoparticules.

2.1.1 Incidence des variables de procédé sur la taille des nanosphères.

En règle générale, la nanoprécipitation nécessite l'utilisation de deux solvants parfaitement miscibles. Bien qu'il existe plusieurs stratégies pour mener à bien cette méthode, le point de départ exige, (i) la disolution du polymère choisi dans un bon solvant organique (appelée phase organique), et (ii) un non-solvant du polymère contenant un agent stabilisant (tensioactif ou polymère) pour assurer une bonne stabilité colloïdale des particules lors de la formation (appelée ici phase aqueuse). Lorsque les deux phases (phase organique et phase aqueuse) sont mélangées, le polymère se trouve dans un mauvais solvant ce qui induit sa précipitation instantanée et par conséquent conduit à la formation de nanoparticules. Le mécanisme de formation via la nanoprécipitation se déroule en trois étapes en accord avec la théorie classique de la précipitation (nucléation-croissance et agrégation) ou via l'effet Gibbs-Marangoni. Dans ce dernier cas, la différence des tensions superficielles entre les deux phases organique et aqueuse conduit à la fragmentation de la phase organique dans la phase aqueuse en gouttelettes ou nanogouttelettes, de taille nanométrique. Il est à noter que le polymère précipite rapidement dès que son milieu est perturbé via la diffusion du solvant organique vers la phase aqueuse et vice versa.

Dans cette direction, les résultats émanant de notre étude systématique montrent que pour une solution de polymère (poly-ε-caprolactone dans l'acétone) et une solution aqueuse contentant le poloxamer (polyoxy éthylène –polyoxy propylène co-polymère) comme agent stabilisant, le

rapport volumique de ces deux phases gouverne l'effet des autres variables du procédé sur la taille des particules. Pour les petites quantités de phase organique (donc des faibles concentrations du polymère), ni la vitesse d'agitation du système lors de la réalisation de mélange, ni la façon dont le mélange des deux phases est réalisé a une incidence drastique sur les propriétés colloïdales. En revanche, pour des volumes de phase organique plus élevés (c'est-à-dire plus du polymère à précipiter), la taille des particules est affectée par la vitesse d'agitation, la vitesse d'addition de la phase organique et également la façon dont le mélange des phases est réalisé. Par conséquent, à notre avis, les deux approches mécanistiques sont possibles et une domine l'autre selon la concentration du polymère dans la phase organique et le rapport volumique des deux phases (organique et aqueuse).

Concernant la méthode d'émulsification-diffusion, les deux phases (organique et aqueuse) sont nécessaires comme pour la nanoprécipitation. Toutefois il est à noter dans ce cas, que le solvant de la phase organique est partiellement miscible avec l'eau. Ainsi, pour préparer les deux phases, chacune est saturée avec le solvant de l'autre (saturation mutuelle). Lorsque les phases sont mélangées, il est possible d'obtenir une nanoémulsion en utilisant une vitesse d'émulsification importante (comme dans le cas de la mini-émulsion). L'émulsion est diluée dans une grande quantité d'eau pour faciliter la miscibilité de tout le solvant organique, ce qui induit l'insolubilité du polymère et favorise l'obtention de nanoparticules.

La recherche dans ce domaine de l'émulsification-diffusion propose comme mécanismes, la formation de particules via l'effet Gibbs-Marangoni ou via la possibilité que chaque gouttelette d'émulsion soit transformée en nanoparticule. Il est tout à fait évident qu'il existe une relation entre la taille des gouttelettes formant la dispersion et la taille finale de la nanoémulsion. En plus, les études rapportées dans la littérature et dans nos derniers résultats démontrent que la vitesse et le temps d'émulsification sont les variables du procédé les plus critiques contrôlant la taille finale et la distribution en taille des particules. Il semblerait que le seconde approche mécanistique (chaque nanoparticule est formée à partir d'une nanogouttelette) est la plus probable pour expliquer la formation des nanoparticules préparées par le procédé d'émulsification-diffusion.

Néanmoins, nous avons remarqué que le rapport volumique des deux phases (organique et aqueuse) a un faible effet sur la taille des particules, et en particulier pour des faibles valeurs de ce rapport. Ceci est probablement dû à l'effet Gibbs-Marangoni, attribué à la différence de

tensions superficielles entre les phases. À notre avis, les phases sont mutuellement saturées et par conséquent, les différences de tensions superficielles ne seraient pas suffisamment importantes pour disperser la phase organique (la phase polymère) jusqu'à l'obtention de gouttelettes de taille nanométrique. Ainsi, notre approche propose que l'effet Gibbs-Marangoni pourrait être produit pour la libération de l'énergie thermique cumulée dans le système lors de l'étape d'émulsification.

2.1.2 Effet des matières premières sur les propriétés des nanosphères.

Dans le cas de l'étude réalisée sur l'effet des matières premières utilisées pour préparer les nanoparticules via les deux méthodes, notre recherche s'est focalisée sur l'effet des polymères, des agents stabilisants et des solvants organiques sur la taille et le potentiel zêta des particules formées.

a. Effet sur la taille des nanosphères

En ce qui concerne la taille hydrodynamique moyenne des dispersions préparées, la tendance des résultats est résumée ci-dessous:

- La nature du polymère constitue un facteur déterminant, car son caractère amorphe ou semi-cristallin conduit à des particules de tailles différentes. En effet, les plus petites tailles sont observées pour le polymère cristallin.

- La taille des particules préparées par nanoprécipitation dépend de la concentration du polymère. La taille est plus grande pour une forte teneur en polymère. De même pour le procédé émulsification-diffusion, la taille des particules suit la même tendance pour un taux du polymère supérieur à 2.5% (massique), tandis qu'en dessous de cette valeur, la concentration du polymère semble n'avoir aucune influence.

- Indépendamment de la méthode de préparation, la taille des particules est associée à la nature de l'agent stabilisant utilisé. Ainsi, les tensioactifs ioniques conduisent à des tailles petites par rapport à l'utilisation des tensioactifs non-ioniques. Ceci étant probablement

dû aux mécanismes de stabilisation électrostatique, stérique ou électro-stérique qui pourraient prédominer lors de la formation des particules.

- La concentration de l'agent stabilisant n'a aucun effet marqué sur la taille des particules préparées par nanoprécipitation. En revanche, dans le cas de l'émulsification-diffusion, la taille des particules diminue avec l'augmentation de la concentration en agent stabilisant.

- Lors de la nanoprécipitation, il semblerait que les propriétés physico-chimiques des solvants organiques et les interactions entre le solvant organique, le polymère et l'eau, ne gouvernent la taille finale des particules préparées. Ce comportement est en accord avec la théorie classique de la précipitation comme mécanisme de formation des particules. Cependant, comme déjà mentionné plus haut, pour un rapport volumique important (phase organique / phase aqueuse), la formation des particules est gouvernée par le mécanisme basé sur l'effet Gibbs-Marangoni.

- Dans le cas de l'émulsification-diffusion, il y a une corrélation notable entre la taille des particules et l'interaction polymère–solvant organique et aussi avec les propriétés physico-chimiques de solvant organique utilisé. Il paraît que la plus grande interaction entre le polymère et le solvant conduit à une diminution de la vitesse de diffusion du solvant organique de la gouttelette organique vers la phase aqueuse, ce qui conduirait à des tailles de particule plus grandes. Il est à noter que les plus grandes valeurs de viscosité, de densité et de tension superficielle des solvants organiques diminuent l'efficacité de la fragmentation du système et par conséquent, contribuent à la formation de particules de tailles plus élevées.

b. Effet sur le potentiel zeta des nanosphères

Le potentiel zêta est une propriété intéressante, car elle permet d'avoir des informations sur la charge de surface, sa variation en fonction du pH et également sur la stabilité colloïdale des dispersions. La tendance générale des résultats est présentée ci-dessous:

- La concentration locale en groupe carboxylique originaire de l'hydrolyse partielle des polymères type polyesters détermine la magnitude du potentiel zeta. Ainsi, plus la

concentration surfacique de groupes carboxyliques est importante, plus la valeur absolue du potentiel zêta est importante à pH basique.

- La nature cationique ou anionique de l'agent stabilisant détermine le signe du potentiel zêta des particules.

- Le potentiel zêta des particules préparées en utilisant le poly-ε-caprolactone et des agents stabilisants non-ioniques est significativement dépendant de la méthode utilisée.

Cette dernière observation, a particulièrement attiré notre attention en raison de son incidence possible sur la stabilité colloïdale des dispersions ainsi que sur leur comportement in vivo, et par conséquent sur l'effet phagocytose. Dans l'état de l'art, ce phénomène a été largement marginalisé. En effet, les études rapportées dans la littérature sont principalement focalisées sur la mesure du potentiel zêta à pH fixe et sans aucune comparaison des deux systèmes ni d'études systématiques.

Ainsi, nous avons conduit une étude systématique de physico-chimie colloïdale pour examiner l'origine et la nature de la charge de surface des nanoparticules préparées par nanoprécipitation et par émulsification-diffusion. Nous avons également examiné l'effet de l'agent stabilisant sur la détection et la quantification des groupes carboxyliques à la surface des particules. L'estimation de la densité de charge superficielle est réalisée par dosage conductimétrique ou par approximation via la mesure de la mobilité électrophorétique. Une attention particulière est portée à l'effet de la polarité du solvant organique et du pH de la phase aqueuse sur le potentiel zêta des nanoparticules.

Les résultats émanant de cette étude montrent que le potentiel zêta des nanoparticules préparées par émulsification-diffusion est gouverné par un réarrangement interfacial du polymère lors de l'étape d'émulsification. En effet, la phase aqueuse étant saturée en solvant, induit probablement une reconformation via une restructuration des parties polaires et non-polaires des chaînes polymères soit en solution, soit à l'interface phase continue - polymère. Ce réarrangement suit probablement le déplacement (ou la dilution) du solvant organique de l'intérieur de la particule (en cours de formation) vers l'extérieur (phase continue composée majoritairement d'eau). Par conséquent, il semblerait que la polarité du solvant organique

détermine le potentiel zêta des particules et donc dans une certaine mesure, la charge de surface.

Concernant la nanoprécipitation, la polarité des solvants organiques semble n'avoir aucune incidence drastique sur le potentiel zêta des particules. Comme la précipitation du polymère est instantanée lors de mélange des deux phases (organique et aqueuse), la conformation du polymère lors de sa précipitation favorisera l'exposition des groupes carboxyliques à la surface, puisqu'elle se produit dans une phase aqueuse de polarité supérieure à 50. Ainsi, la force ionique et le pH de la phase aqueuse utilisée pour la préparation des nanoparticules affectent drastiquement les propriétés électrocinétiques de la dispersion finale.

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Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification–diffusion methods Critical comparison

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ABSTRACT

Solvent displacement and emulsification–diffusion are the methods used most often for preparing biodegradable submicron particles. The major difference between them is the procedure, which results from the total or partial water miscibility of the organic solvents used. This review is devoted to a critical and a comparative analysis based on the mechanistic aspects of particle formation and reported data on the influence of operating conditions, polymers, stabilizing agents and solvents on the size and zeta-potential of particles. In addition, a systematic study was carried out experimentally in order to obtain experimental data not previously reported and compare the data pertaining to the different methods. Thus the discussion of the behaviors reported in the light of the results obtained from the literature takes into account a wide range of theoretical and practical information. This leads to discussion on the formation mechanism of the particles and provides criteria for selecting the adequate method and raw materials for satisfying specific objectives in submicron particle design.

Contents

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1. Introduction

Nowadays, several methods for preparing submicron particles from preformed polymers are available. They can be categorized into two groups depending on the steps involved in their procedure [1]. Examples of the first group are emulsification–diffusion (also called emulsification–solvent displacement), emulsification–evaporation and emulsification–coacervation which are based on two steps: the first is the preparation of an emulsified while the second is based on particle formation by polymer precipitation or cross-linking. The second group of methods does not require emulsion preparation prior to obtaining the particles. They are based on polymer precipitation under conditions of spontaneous dispersion and particle formation from a polymer solution or the self-assembly of macromolecules, or the synthesis of polyelectrolyte complexes. Examples of this type of procedure include solvent displacement (also termed nanoprecipitation, solvent diffusion or interfacial deposition), polymersome preparation and the layer-by-layer technique.

Regardless of the method chosen, the development of biodegradable submicron particles and the assurance of a robust production process require exhaustive knowledge of the process and materials to be used. Consequently, extensive studies have been carried out and different research teams have published reviews on the techniques and initial materials for preparing submicron particles and on particle formation mechanisms [1–11]. As a contribution to updating the state of knowledge, this review provides an in-depth study on the incidence of operating conditions and formulation variables on particle characteristics when particles are prepared by either the solvent displacement technique or emulsification–diffusion method. These methods have been chosen as representative examples of the two major groups mentioned previously for preparing submicron particles since they are those used most often [10,12,13]. In addition, they are characterized by procedural simplicity, high encapsulation efficiency, high reproducibility, low possible contaminant content, low cost and easy up-scaling [9,10,14–18]. Another advantage is that they use preformed polymers as starting materials rather than monomers and toxic solvents [7,19].

Our first aim in this review is to establish an updated view of the two preparation methods for providing readers with consolidated information on research trends in the domain of submicron particle synthesis by using the solvent displacement technique and the emulsification–diffusion method.

We also focus on comparing methods, taking into account the behaviors obtained for the different variables studied. This provides criteria for making decisions on the best starting materials, preparation method and operating variables according to expectations regarding particle performance. Thus the results and conclusions reported by various authors form the starting point and are described in this review through comparisons made using data deduced from the reported results. Taking into account that the information available comes from works carried out with different objectives or reported from a qualitative standpoint, such fragmentation makes it difficult to obtain a complete, comparable and comprehensive survey of all the key variables required to ensure robust process design. To overcome this problem, the results from a systematic study carried out by the authors are included. Submicron spheres have been chosen as model particles to facilitate comparing the methods, since similar materials are used for both particle preparations. Size and zeta-potential have been chosen as the particle characteristics to be studied as they provide simple illustrations of particle behavior. Particle size is a critical parameter as it is directly linked to stability, cellular uptake, biodistribution and drug release [20–22] and the zeta-potential value can influence the stability of particle dispersion as well as particle mucoadhesivity [23–26].

Furthermore, this review is aimed at identifying major advances in the domain to provide understanding of the mechanistic aspects associated with particle formation obtained by each method. However, these research works highlight correlations with particle formation mechanisms, particle characteristics and variables that are limited to the particular experimental conditions used in each work. Thus, in this work, the correlations reported were verified in as many cases as possible in order to investigate their general applicability.

2. Solvent displacement and emulsification–diffusion as methods for preparing biodegradable submicron particles

Submicron biodegradable particles may be defined as solid colloidal particles with a size smaller than 1 μm that contain an active substance [10,27]. However, in the field of pharmaceuticals, there is good agreement that particle size should be in the middle or lower submicronic range (100 to 500 nm) [1–11]. Submicron particles include both spheres and capsules. Submicron spheres can be defined as matrix-type colloidal particles in which a drug is dissolved, entrapped, chemically bound or adsorbed to the constituent polymer matrix [27] while submicron capsules can be defined as vesicular systems that exhibit a typical core–shell structure in which the drug is mainly confined to a reservoir or within a cavity surrounded by a polymer membrane. It can also be carried on the capsule surface or imbibed in the polymeric membrane [13].

The typical procedures for preparing submicron particles by the solvent displacement technique and emulsification–diffusion method were firstly developed by Fessi et al. [19] and Leroux et al. [28], respectively. An outline of the main steps for each method is shown in Figs. 1 and 2, taking sphere preparation as an example (see also Supplementary data for additional illustrations on the procedures).

Sphere synthesis by solvent displacement requires both solvent and nonsolvent phases. The solvent phase essentially consists of a solution of the drug and the polymer. The nonsolvent phase is a nonsolvent or a mixture of nonsolvents for the polymer, supplemented with one or more naturally occurring or synthetic surfactants. In most cases, solvent and nonsolvent phases are respectively called organic and aqueous phases, because the solvent is an organic medium, while the nonsolvent is mainly water. However, it is possible to use either two organic phases or two aqueous phases as long as solubility, insolubility and miscibility conditions are satisfied. Regarding particle preparation, the organic phase is mixed with the stirred aqueous phase in one shot, stepwise, dropwise or by controlled addition rate. Submicron spheres are formed instantaneously and the solvent is removed from the system by using evaporation under reduced pressure.

The method for preparing submicron particles by emulsification–diffusion requires three phases: organic, aqueous and dilution. The organic phase is a solution of the polymer and the active substance in an organic solvent partially miscible with water, that has previously been water-saturated. The aqueous phase comprises the aqueous dispersion of a stabilizing agent prepared by using solvent-saturated water while the dilution phase is usually water.

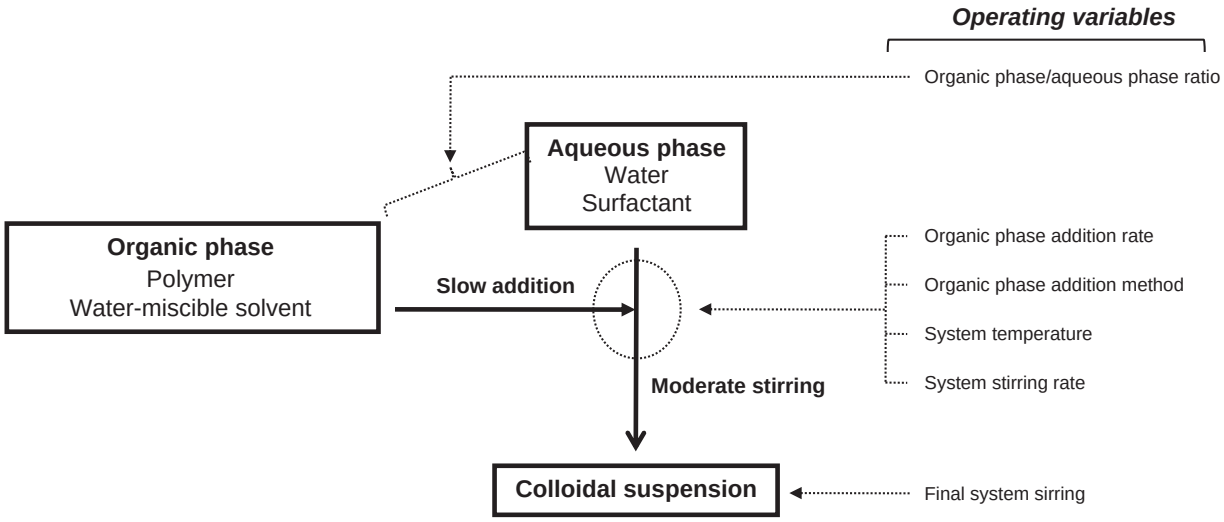

Fig. 1. Preparation of submicron particles by solvent displacement method: schematic procedure and operating variables.

To prepare the particles, as shown in Fig. 2, the organic phase is added rapidly (in less than 5 s) to the aqueous phase and an o/w emulsion is formed immediately by stirring at high speed. The emulsion formed is diluted into the dilution phase by mechanical stirring in order to allow the migration of the organic solvent in the water, leading to particle formation. The solvent and part of the water are then removed by evaporation under reduced pressure.

It must be emphasized that the two methods require mixing two phases (organic and aqueous) as their starting point. Likewise, in both of them the migration of organic solvent in water leads to instantaneous submicron particle formation. However, the procedure differs due to the miscibility of organic solvent in water. Thus the solvent displacement technique (one step procedure) simply requires phase mixing to obtain particles since the organic solvent used is totally miscible in water. Regarding the emulsification–diffusion method (two-step procedure), it requires partially water miscible solvents that must be water saturated. Phase mixing forms a submicron emulsion (first step). Then, the addition of water to dilute the emulsion leads to particle formation (second step). As can be concluded, the simplicity of this procedure and its set-up, the lower consumptions of energy, time and water, and the mild shear forces required by the solvent displacement technique, represent advantages in comparison to the emulsification–diffusion method.

Sahana et al. [22] and Hariharan et al. [26] used a modified emulsification–diffusion method, keeping the basic principles of the Leroux et al. method. Thus the organic and aqueous phases are prepared by using a non-saturated organic solvent and water, by stirring for 3 h to form irregular-sized globules in equilibrium with a continuous phase. The o/w emulsion is formed by high speed stirring and the dilution step is carried out by the addition of water into the emulsion with constant stirring in a water bath set at 40 °C.

With respect to the raw materials used for sphere preparation, Tables 1 and 2 provide a compilation of polymers, stabilizing agents, organic solvents, active substances and other materials reported by

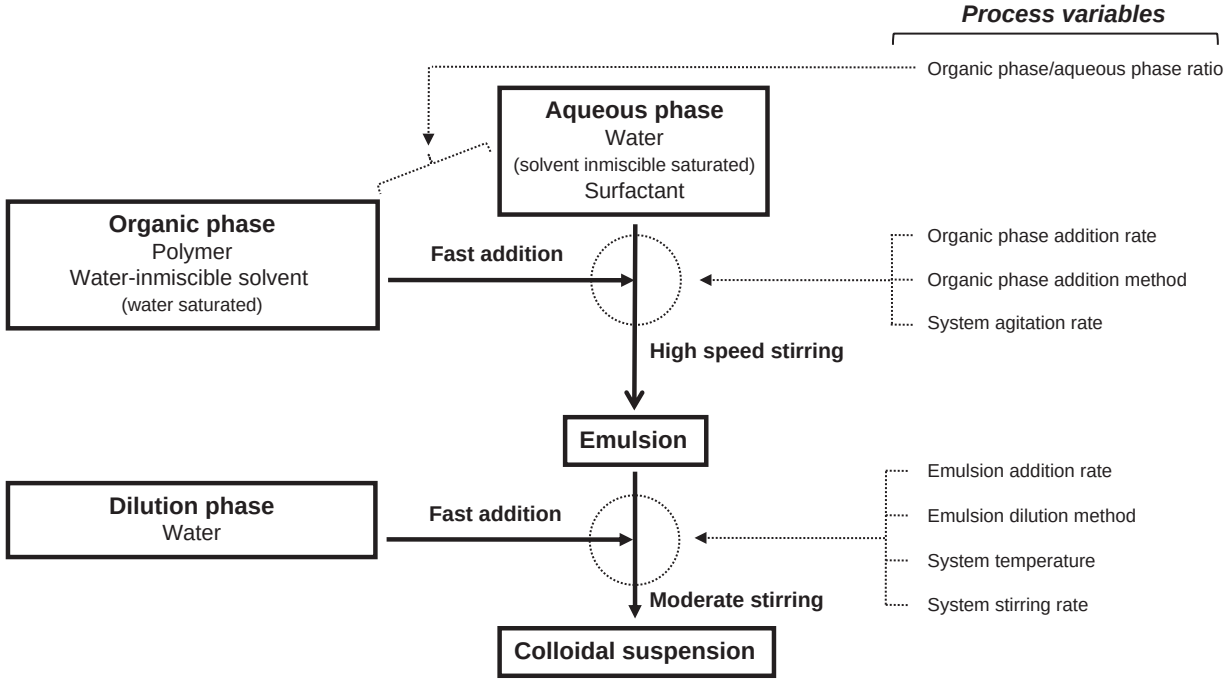

Fig. 2. Preparation of submicron particles by emulsification–diffusion method: schematic procedure and operating variables.

Table 1
Solvent displacement method: examples of raw materials and work conditions used and size and zeta-potential of submicron spheres obtained.

Organic phase			Stabilizing agent/Nonsolvent phase	Work conditions		Size (nm)	Zeta potential (mV)	Drug loading (%)	Drug entrapment efficiency (%)	Reference
Polymer	Solvent	Other		Way of organic phase addition	System stirring					
PCL PDLLA100 PLGA (85:15; 50:50) (0.125 g)	Acetone (20 ml)	Isradipine (2 mg)	PLX 188 0.5% (50 ml)	nr.	nr.	200–210	−20 to −35	nr.	74–97	[14]
PCL Mw: 10 kDa (125 mg)	Acetone:EtOH 6:4 (10 ml)	Rolipram (20 mg)	PVA Mw: 20 kDa; 80% Hydrolyzed, sodium cholate Mw: 430.6 Da (0.1–10%) (100 ml)	Stepwise	400 rpm	150–345	−5 to −55	nr.	2–20	[15]
PDLLA Mw: 90–120 g/mol (100–300 mg)	Acetone, DCM, EtOH (20 ml)	Tyrphostin AG-1295 (0–3 mg)	PLX 188 (20–50 mg) (40 ml)	nr.	Moderate stirring	50–140	nr.	nr.	70	[16]
PLGA 50:50 (Mw: 7, 14, 24, 48 and 63 kDa) PLGA 65:35 (Mw: 114 kDa) PLGA 75:25 (Mw: 92 kDa) PDLLA (Mw: 109 kDa)	Acetone (5 ml)	Haloperidol (0.5 mg/ml)	PVA (Mw: 25 kDa; 88% hydrolyzed) 1% (50 ml)	nr.	nr.	170–230	nr.	0.25–4	nr.	[18]
PDLLA Mw:88 kDa (150 mg)	Acetone (40 ml)	Primaquine (7.5 mg) Soy phospholipid mixture (150 mg)	PLX 188 0.14–0.3%, pH 9.0 (80 ml)	nr.	Magnetic stirring	150–165	−35 to +1.5	nr.	85–94	[29]
PCL Mw: 14 kDa (0.5–15%)	Acetone	nr.	W	nr.	nr.	150–200	nr.	nr.	nr.	[30]
PLGA 50:50 (100–200 mg)	Acetone (20–40 ml)	Cyclosporin A (100 μg/ml)	PVA, PLX 188 (100 mg) (20–40 ml)	Addition rate: 0.1–0.6 ml/s	500 rpm	45–145	nr.	nr.	82–98	[31]
Gliadin (100 mg)	EtOH:W 7:3, MetOH:W 8:2, Acetone:W 5:5, Propan-1-ol:W 5:5, Propan-2-ol:W 5.5:4.5 (20 ml)	All-trans-retinoic acid	PLX 188 (0.5% w/w) in Physiological saline solution (0.9% NaCl) (40 ml)	nr.	250 rpm	460–1000	nr.	0.5–8	75–97	[32]
PCL Mw: 78 kDa, 29.4 kDa; PDLLA 50 Mw: 81.9 kDa; PLGA 75:25 Mw: 96.8 kDa; PLGA 75:25 Mw: 71.6 kDa; PLGA 50:50 Mw: 51.5 kDa (0.5/0.6% of final product)	Acetone	nr.	PLX 188	nr.	Moderate magnetic stirring	110–235	nr.	nr.	nr.	[33]
PDLLA Mw: 90,000 PCL-Mw: 128,000 PLGA 50:75	Acetone (15 ml)	nr.	PLX 188 (38 mg) (15 ml)	nr.	Magnetic stirring	115–220	−20 to −35	nr.	nr.	[34]
PCL (40.75–159.25 mg)	Acetone (8.10–31.89 ml)	Cyclosporin A (1 mg)	PLX (40.75–159.25 mg) (40 ml)	Addition rate: 23 ml/min	Magnetic stirring	110–215	nr.	nr.	90–98	[35]
PLGA 85:15 Mw: 47 kDa (200 mg)	Acetone:DCM (25:0.5 ml)	nr.	PVA different degrees of hydrolyzation (4%w/w) (50 ml)	Addition rate: 2 ml/min	400 rpm	190–315	nr.	nr.	nr.	[36]
PDLLA Mw: 200 kDa (125 mg)	Acetone (25 ml)	Pentamidine (0.5 mg/ml) Soy bean lecithin (0.6–1.25%)	PLX 188 (1.25–3% w/v, pH 7.5–8)	nr.	Magnetic stirring	130–155	nr.	nr.	40–76	[37]
Ethylcellulose (83 kDa)	EtOH	nr.	W	nr.	300–3000 rpm	60–100	nr.	nr.	nr.	[38]
HP55 (1–4%)	Acetone:W (different proportions) (10 ml)	nr.	W (20 ml)	nr.	Moderate magnetic stirring	~300	nr.	nr.	nr.	[39]
Gliadin (0.5% w/v)	EtOH:W (20 ml)	nr.	PLX 188 (0.5% w/w) in physiological saline solution (0.9% NaCl) (40 ml)	Aqueous phase slowly added into organic phase	500 rpm	170–370	nr.	nr.	nr.	[40]
PLGA 75:25 (Mw: 10 kDa) (75 mg)	Acetone (5 ml)	Vancomycin, phenobarbital, valproic acid, cyclosporin A, indomethacin, and ketoprofen (2.5 mg)	PLX 188 (75 mg) (15 ml)	nr.	Moderate stirring	155–170	nr.	nr.	5–94	[41]

(continued on next page)

Table 1 (*continued*)

Organic phase			Stabilizing agent/Nonsolvent phase	Work conditions		Size (nm)	Zeta potential (mV)	Drug loading (%)	Drug entrapment efficiency (%)	Reference
Polymer	Solvent	Other		Way of organic phase addition	System stirring					
PLGA 50:50 Mw: 10 kDa (50 mg)	ACN (5 ml)	Procaine hydrochloride (0–10%)	W pH 9.3 (15 ml)	Dropwise	Magnetic stirring	155–210	−50 to −55	0.2–4.5	28–62	[42]
PLA Mw: 28 kDa PLA-PEG (different molecular weight) (1–20 mg/ml)	Acetone, ACN (5 ml)	Procaine hydrochloride (2–20%)	W (15 ml)	nr.	nr.	50–150	−6 to −50	0.2–3.5	nr.	[43]
PCL Mn: 42.5 kDa PCLLA PDLLA (100 mg)	Acetone (20 ml)	Nimodipine (10 mg)	PLX 188 0.2% (50 ml)	Dropwise	Moderate stirring	80–135	nr.	3.5–9	20–90	[44]
PLA-PEG copolymers (50 mg)	ACN (5 ml)	Procaine hydrochloride (0–20% w/w)	W pH 5.8 (15 ml)	nr.	Magnetic stirring	50–175	−5 to −30	0.2–0.3	6–11	[45]
PLGA 50:50 Mw: 40,000 g/mol, PVA-g-PLGA, SBPVA-g-PLGA (100 mg)	Acetone:EtAc (0–32.5%) (10 ml)	nr.	PLX 188 0.1% w/w (50 ml)	Addition rate: 10 ml/min	250 rpm	100–120	−3 to −25	nr.	nr.	[46]
PLGA 85:15, 75:25, 50:50 PLA 05, 10, 20. (5 g)	Acetone, ACN, EtOH (125 ml)	nr.	PVA 4% w/w (300 ml)	Addition rate: 10 ml/min	400 rpm	200–270	nr.	nr.	nr.	[47]
PLGA (Mw: 22 kDa) PLGA-mPEG (Mw: 37 kDa)	Acetone	^{125}I-CA	Sodium cholate (12 mM) in PBS, pH 7.4	Dropwise	Stirred solution	110–160	−4.0 to −45	nr.	>70	[48]
PLGA 75:25 (25 mg)	Acetone (5 ml)	Rose Bengal (2.5–10 mg)	W (10 ml)	Dropwise	Gentle magnetic stirring.	135–150	−40 to −55	0.3–0.8	1–1.6	[49]
PVM/MA Mw: 200 kDa (100 mg)	Acetone (5 ml)	nr.	EtOH:W (1:1) 10 ml	nr.	nr.	250–280	−25 to +30	nr.	nr.	[50]
PLGA 50:50 (Mw: 6 and 14.5 kDa), 75:25 Mw: 63.3 kDa (100 mg)	Acetone (10 ml)	Paclitaxel (0.4–1 mg)	PLX 188 0.25% (10 or 20 ml)	nr.	Magnetic stirring	115–160	−20 to −35	nr.	15–100	[51]
PLA Mw: 2 kDa (25 mg)	Acetone, EtOH or MetOH (0.3 ml) CHCl3 (1.2–2.0 ml)	Sodium cromoglycate (2.5 mg) PG (150 mg)	EtOH (70%) (5 ml)	nr.	Mixing	200–270	−4 to −8	nr.	nr.	[52]
PCL Mw: 60 kDa PDLLA (1 g) PLGA Mw (75 kDa), PDLLA50 Mw: 42 kDa, PCL	Acetone (270 ml)	Indomethacin (0.150 g)	Polysorbate 80 (0.766 g) (530 ml)	nr.	Moderate magnetic stirring	170–180	nr.	nr.	100	[53]
Mw: 40 kDa, PEG5-PLGA, PEG20-PDLLA, PEG5-PDLLA, PEG5-PCL (20 mg)	Acetone (1 ml)	Antiestrogen RU58668 (2×10^{-5}–10^{-3} M)	PLX 188 (1%) or W (2 ml)	Rapidly dispersed	nr.	75–265	−10 to −65	3.1–3.3	94–100	[54]
PLGA, PLGA-mPEG (different molecular weight)	Acetone	nr.	Sodium cholate (12 mM)	Dropwise	Stirred solution	55–135	−5 to −55	nr.	nr.	[55]
PDLLA Mw: 16 kDa; 109 kDa; 209 kDa (75 mg)	Acetone (20 ml)	Acyclovir (165 mg)	Brij 96, PLX 188, Triton X100 or Polysorbate 80 (0.25–2%) in EtOH:W (1:1 v/v) (40 ml)	nr.	Magnetic stirring	105–265	−10 to −35	2–8	1–3.5	[56]
PCL Mw: 80 kDa PMMA (1 g) PDLLA50 Mw: 42 kDa, PLGA Mw: 75 kDa, PCL	Acetone (267 ml)	Diclofenac (0.1 g)	Polysorbate 80 (0.766 g) (533 ml)	nr.	Moderate magnetic stirring	85–195	nr.	nr.	100	[57]
Mw: 40 kDa, PEG-PDLLA, PEG-PLGA, PEG-PCL (20 mg)	Acetone (1 ml)	Antiestrogen RU 58668 (2×10^{-5} to 10^{-3} M)	PLX 188 1% or W (2 ml)	Rapidly dispersion	nr.	95–260	−5 to −65	3.1–3.3	94–100	[58]
PMMA type C NF/USP (Eudragit L100-55) (360–810 mg)	Acetone, DMSO, Isopropyl alcohol, EtOH, Ethyl lactate (25 ml)	nr.	PVA Mw: 26,000, 88% hydrolyzed (0.4% w/w) (50 ml)	nr.	Stirred magnetically	95–325	nr.	nr.	nr.	[59]
PCL-PEG diblock copolymer	Acetone, THF (4 ml)	All-trans-retinoic acid	W (10 ml)	nr.	nr.	70–460	nr.	2.2–10.8	68–97	[60]

PLGA 74:26 Mw: 50 kDa, PLGA 73:27 Mw: 20 kDa (1% of the organic phase)	Acetone (2–10 ml)	5-Fluorouracil (10 mg)	PLX 188 (1%), PLX F127 (1%), PVA (10%)	nr.	Moderate stirring	75–255	nr.	nr.	66–78	[61]
PMMA type C NF/USP (Eudragit L100-55) (1.44% w/w)	Acetone (25 ml)	Ibuprofen (1.4%)	PVA Mw: 26,000, 88% hydrolyzed (0.8% w/w) (50 ml)	nr.	Stirred magnetically	105–145	nr.	3.2–4.5	40–50	[62]
PCL Mw: 14.8 kDa (1% w/v)	Acetone (50 ml)	Tamoxifen	PLX 188, PLX F108 (0.1–0.5%)	Addition rate: 1 ml/min	Magnetic stirring	180–800	−15 to +25	20	> 90	[63]
PLGA 50:50 Mw: 50–75 kDa (63 mg)	Acetone (10 ml)	XAN or 3-MeOXAN (60 μg/ml)	PLX 0.25% (10 ml)	nr.	Magnetic stirring	150–165	−35 to −40	nr.	26–40	[64]
PCL Mw: 80 kDa (138 mg)	Acetone (25 ml)	Griseofulvin (0–13.8 mg) Span 80 (50 mg)	Polysorbate 80 (100 mg) (50 ml)	Addition rate: 48 ml/min	Magnetic stirring	250–325	nr.	1–7	78–98	[65]
PDLLA Mw: 16 kDa; 109 kDa; 209 kDa PLGA 50:50 (75 mg)	Acetone (20 ml)	Docetaxel (0.5–1% in weight drug/polymer)	Polysorbate 80 0.5% in W:EtOH (1:1 v/v) (40 ml)	nr.	Magnetic stirring	95–175	−2 to −40	nr.	10–23	[66]
PLGA 50:50 Mw: 8 kDa (500 mg)	ACN:EtOH (60:40) (12 ml)	Zinc phthalocyanine (0.5 mg)	PLX 407 5% w/w (50 ml)	nr.	500 rpm	200–210	nr.	nr.	70	[67]
PLGA 75:25 Mw: 98 kDa (90 mg)	Acetone (25 ml)	Flurbiprofen (0.16–1.84 mg/ml)	PLX 188 (6.6–23.4 mg/ml) (50 ml)	nr.	Moderate stirring	150–290	−25 to −30	nr.	74–97	[68]
Hydrophobic derivatives of dextran DexPx, DexC6x, DexC10x (8.5–25%)	THF (1 ml)	nr.	DexP15 (0 and 0.5%) 10 ml	Dropwise	Vigorous magnetic stirring	150–300	nr.	nr.	nr.	[69]
PLGA-b-PEG-COOH (5–50 mg/ml)	Acetone, ACN, DMF, THF	Docetaxel (0–10% of the polymer)	W (2 x organic phase volume)	Dropwise	Stirring	65–295	nr.	nr.	nr.	[70]
PDLLA Mw = 22,600 to 124,800 g/mol (5–20 mg/ml)	THF, Acetone (4.5 ml)	nr.	W (9 ml)	Addition rate: 4.5 ml/min	300 rpm	75–325	nr.	nr.	nr.	[71]
Poly(H2NPEGCA-co-HDCA) (40 mg)	Acetone: EtOH (4 ml)	4-(N)-acyl-gemcitabine derivatives	W (8 ml)	nr.	nr.	150–190	+25 to +35	0.3–10	6–100	[72]
PDLLA Mw: 3 kDa	Acetone:EtOH	Oridonin	PLX 188	Dropwise	400 rpm	105–195	~−15	2.3	92	[73]
PDLLA 0.20 dl/g (25 mg)	Acetone (2 ml)	nr.	PLX 188 (4 ml)	nr.	Mild stirring	240–290	−15 to −40	nr.	nr.	[74]
PCL Mw: 14 kDa (0.25–10 mg/ml) PCL 80 kDa (0.03–4 mg/ml)	Acetone	nr.	W	Addition rate: 3–120 ml/min	nr.	130–630	nr.	nr.	nr.	[75]
PLGA 50:50 (25–150 mg)	Acetone, ACN (5 ml)	Coenzyme Q10 (1/10 mg)	W or PBS (50 ml)	nr.	Magnetic stirring	125–260	~−40	1–19	49–72	[76]
PES (125 mg)	THF (10 ml)	Silymarin (25 mg)	PVA, PLX 188 or Polysorbate 80 in W (25 ml)	Dropwise	Continuous stirring	200–1000	nr.	nr.	20–62	[77]
PLGA (125–175 mg)	Acetone (10 ml)	Carvedilol (5–10 mg)	PLX 188 (250–350 mg) (20 ml)	Addition rate: 10 ml/min	Stirring	130–235	nr.	1.2–4.5	41–56	[78]
PLGA (RG502H, RG503H, RG504H) P(VS-VA)-g-PLGA-4-10 (1–10 mg/ml)	Acetone, ACN, THF	Salbutamol	PLX 188	Addition rate: 10 ml/min	500 rpm	60–190	−25 to −45	1.4–3.2	nr.	[79]
PLA, DexC10$_{164}$ (10–25 g/l of organic phase)	THF, Acetone (4.5 ml)	nr.	DexP20 (2–10 g/l)	Dropwise	Vigorous magnetic stirring	140–240	nr.	nr.	nr.	[80]

nr: non-reported; PLGA: poly(D,L-lactide-co-glycolide); P(VS-VA)-g-PLGA: poly(vinyl sulfonate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide); ACN: Acetonitrile; THF: Tetrahydrofuran; 125I-CA-labeled: 125I bound to cholesterylaniline; PLGA-mPEG: poly(lactide-coglycolide) monomethoxy(polyethyleneglycol); LA: D,L-lactide; GA: glycolide; PBS: phosphate buffered saline; PEG: poly(ethylene glycol); PCL: Poly-e-caprolactone; PLGA-b-PEG-COOH: carboxy-terminated poly(D,L-lactide-co-glycolide)-block-poly(ethylene glycol); Brij 96: decaethylenglycol oleyl ether; PES: polyethylene sebacate; PCL-PEG diblock copolymer: poly(e-caprolactone)/poly(ethylene glycol); SB-PVA-g-PLGA: poly(2-sulfobutyl-vinyl alcohol)-g-poly(lactide-co-glycolide); CHCl3: Chloroform; PG: Propylene glycol; Poly(H2NPEGCA-co-HDCA): poly[aminopoly(ethylene glycol)cyanoacrylate-co-hexadecyl cyanoacrylate]; XAN: Xanthone; 3-MeOXAN: 3-methoxyxanthone; HP55: hydroxypropyl methylcellulose phthalate; PVM/MA: Poly(methyl vinyl ether-co-maleic anhydride); DexPx, DexC6x, DexC10x: hydrophobic derivatives of dextran where x is the substitution ratio, i.e. the average number of grafted phenoxy, C6 or C10 alkyl chains respectively per 100 glucose units; PCLLA: copolymer of e-caprolactone and L-lactide; PDLLA: poly(D,L-lactic acid); PLA: poly(L-lactic acid); PMMA: Poly(methyl methacrylate); W: Table 2. Emulsification–diffusion method: examples of raw materials and work conditions used and size and zeta-potential of submicron spheres obtained.

Table 2
Emulsification–diffusion method: examples of raw materials and work conditions used and size and zeta-potential of submicron spheres obtained.

Organic phase			Stabilizing agent–aqueous phase	Dilution phase	Work conditions		Size (nm)	Zeta potential (mV)	Drug loading (%)	Drug entrapment efficiency (%)	Reference
Polymer	Solvent	Other			Emulsification	Diffusion					
PLGA 50:50 (50 mg)	Acetone, CHCl3, DCM, EtAc (2.5 ml)	Estradiol (5 mg)	DMAB, PVA Mw: 30 kDa (1%, 5 ml)	W	15,000 rpm, 5 min	Constant stirring	95–585	+70 to +95	nr.	48–95	[22]
PLGA 50:50 (50 mg)	EtAc (2.5 ml)	Estradiol (5 mg)	DMBA, PVA (5 ml)	W	15,000 rpm, 5 min	Constant stirring on a water bath set at 40 °C	100–655	−1 to +70	nr.	46–73	[26]
PDLLA 100DL, PLGA 85:15, PCL, PMMA S100 (3 g)	BA (21 g)	Chlorambucil	PVA 26 kDa, gelatin (10–28%, 40 g)	W or buffer (660 g)	1200 rpm, 10 min	nr.	70–1000	nr.	5.5–8.5	60–63	[28]
PMMA L100-55 (3 g)	BA (21 g)	nr.	PVA Mw: 26 kDa (7–21%, 30 g)	W (660 g)	2000 rpm, 15 min	nr.	105–715	nr.	nr.	nr.	[59]
PMMA L100-55 (3 g)	BA (21 g)	Ibuprofen (1.4%)	PVA Mw: 26 kDa (12%, 40 g)	W (660 g)	2000 rpm, 15 min	nr.	310–430	nr.	5.5–8	62–86	[62]
PDLLA100 (200 mg)	PC (10 ml)	nr.	PVA Mw: 26 kDa, 30–70 kDa, PLX 188 (5%, 20 ml)	W (80 ml)	8000 rpm, 10 min	Stirring	100–450	nr.	nr.	nr.	[81]
PDLLA100 (200 mg)	EtAc (20 ml)	nr.	PVA Mw: 26 kDa (5%, 20 ml)	W (200 ml)	8000 rpm, 10 min	Stirring	~174	nr.	nr.	nr.	[82]
PLGA 75:25 Mw: 75–120 kDa (1–4 mg)	PC (10 ml)	17b-estradiol benzoate (3 mg)	DMAB (1.0–4.0%), PVA Mw: 30–70 kDa (2.5–10%, 20 ml)	W (80 ml)	4800–15,000 rpm, 7 min	Moderate magnetic stirring	75–350	nr.	nr.	67	[83]
PLGA 50:50 Mw: 12 kDa, PMMA S100 (5%)	BA (2.1 g)	Enalaprilat (42 mg)	PVA Mw: 27 kDa (10–20%, 4 g)	W (66 g)	15,000 rpm, 5 min	nr.	180–615	−30 to −60	7–13	24–46	[84]
PLGA 75:25 Mw: 75–120 kDa (200 mg)	MEK, EtAc, PC, BA (10 ml)	nr.	PLX 188 (20 ml)	W (500 ml)	12,000 rpm, 7 min	Moderate magnetic stirring	120–270	nr.	nr.	nr.	[85]
PLGA 50:50 Mw: 12 kDa, PLGA 75:25 Mw: 12 kDa, PDLLA Mw: 22 kDa	BA (6 g)	p-THPP (0–20%)	PVA 4-88 Mw: 26 kDa (17%, 8 g)	W (500 ml)	2000 rpm, 15 min	2000 rpm	90–160	−4 to −8	3.5–13	47–91	[86]
PDLLA50 Mw: 30 kDa (0.4–2 g)	EtAC (20 ml)	nr.	PLX 188 (0.5–5%, 40 ml)	W (215 ml)	8000 rpm, 5 min	Moderate stirring	230–560	nr.	nr.	nr.	[87]
PLGA 70:30 (200 mg)	EtAc (10 ml)	nr.	PVA (100 mg) and chitosan (30 mg) (10 ml)	W	13,500 rpm, 10 min	Stirring	100–180	+10 to +30	nr.	nr.	[88]
PLGA RG502 Mw: 8 kDa, PDLLA Mw: 2 kDa, CAP Mw: 2.5 kDa (400 mg)	EtAc, MEK (20 ml)	Triclosan (0–33%)	PVA (5%, 40 ml)	W (160 ml)	1700 rpm, 10 min	nr.	175–450	nr.	0.8–24	63–89	[89]
PLGA 75:25 Mw: 75–120 kDa (100 mg)	DCM, EtAc, PC, Acetone (10 ml)	nr.	DMAB, PVA Mw: 9–10 kDa, PLX 188 (1%, 20 ml)	W (80 ml)	1 min, sonicator operating 40% amplitude intensity	Moderate magnetic stirring	50–460	nr.	nr.	nr.	[90]
PCL Mw: 42.5 kDa (0.5 g)	EtAc (10 ml)	Magnetite (0.3 g), gemcitabine.HCl (150 mg)	PVA 15–20 kDa (20 ml)	W	Probe sonicator at 200 W, 10 min	Moderate stirring	130–170	nr.	0.1–0.7	3.4–8	[91]
PLGA 50:50 (50 mg)	EtAc, DCM, CHCl3, EtAc:DCM 20:80 (2 ml)	Cyclosporine (10 mg)	DMAB (0.1%, 3 ml)	W (30 ml)	Sonication, 1 min	1000 rpm	60–270	nr.	nr.	16–23	[92]
PLGA 50:50 (Mw: 14.5; 45; 85; 137; 213 kDa)											
PLGA 65:35 (Mw: 97 kDa), PLGA 85:15 (Mw: 87 kDa) (50 mg)	EtAc (10 ml)	Estradiol (5 mg)	DMAB (1%, 20 ml)	W (80 ml)	15,000 rpm, 5 min	Constant stirring	90–155	+70 to +105	nr.	35–68	[93]
PHBHV Mw: 23, 300 kDa. (20 mg)	$CHCl_3$:EtOH (different proportions) (4 ml)	nr.	PVA Mw: 200 kDa (0.025%, 80 ml)	PVA 0.01% (200 ml)	17,500 rpm, 5 min	Moderate magnetic stirring	250–890	nr.	nr.	nr.	[94]
Propyl-starch derivatives (degrees of substitution: 1.05 and 1.45) (1 mg)	EtAc (1 ml)	nr.	PVA (0–1%, 4 ml)	W (5 ml)	14,000 rpm, 15 min	nr.	150–185	−5 to −9	nr.	nr.	[95]
PLGA 50:50 Mw 5–70 kDa, PLGA-mPEG (10 mg/ml)	EtAc (10 ml)	Tacrolimus (10 mg)	PLX 188 (2%, 20 ml)	W (90 ml)	20,000 rpm, 10 min.	Magnetic stirring	215–220	−20 to −30	nr.	50–60	[96]

nr: non-reported; BA: Benzyl alcohol; CAP: cellulose acetate phthalate; DMAB: didodecyldimethyl ammonium bromide; EtAc: Ethyl acetate; MEK: Methyl ethyl ketone; PC: Propylene carbonate; PHBHV: poly(3-hydroxybutyrate-co-hydroxyvalerate); PDLLA: poly(D,L-lactic acid); PLA-PEG: methoxy PEG-(D,L-lactide); PLGA: Poly(D,L-lactide-co-glycolide); PLGA-mPEG: Poly(lactide-co-glycolide)-methoxy poly(ethylene glycol); PLX 188: Poloxamer 188; PMMA: poly (methyl methacrylate); p-THPP: Mesotetra(p-hydroxyphenyl)porphyrin; PVA: polyvinyl alcohol; W: Water.

different authors. The quantity used of each material, the operating conditions worked and the results of size and zeta-potential obtained in each study are also included. Briefly, poly-ε-caprolactone (PCL), poly(D,L-lactic acid) (PDLLA) and poly(D,L-lactic-co-glycolic acid) (PLGA) are the polymers used most often. However, alternatives such as starch and cellulose derivatives, polyethylene sebacate, hydrophobic dextranes, poly(methyl methacrylates), poly(methyl vinyl ether-co maleic anhydride), poly-cyanoacrylates, poly(3-hydroxybutyrate-co-hydroxyvalerate) and copolymers based on polyethylene glycol or polyvinyl alcohol have also been investigated. The use of poloxamer and polyvinyl alcohol as stabilizing agents predominates. Certain research works have reported the use of sodium cholate, polysorbate 80, Brij 96, triton and hydrophilic dextrans, and a few studies have been performed without stabilizing agent or using buffering agents. The organic solvents are chosen for each method as a function of their specific solvent requirements. Thus water-miscible solvents such as acetone, tetrahydrofuran, acetonitrile, methanol, ethanol, isopropanol, ethyl lactate, dimethyl sulfoxide and dimethyl formamide have been used for particle preparation by the solvent displacement method. Also, partially water-miscible solvents such as ethyl acetate, benzyl alcohol, propylene carbonate and methyl ethyl ketone have been chosen in studies of the emulsification–diffusion method. In addition, water is the dilution phase commonly used in this latter method while buffer solutions or stabilizing agent solutions at low concentration have been used with this purpose, though more rarely.

It can be concluded that solvent displacement and emulsification diffusion are versatile methods from the standpoint of the polymers and stabilizing agents that can be used. Thus synthetic, semi-synthetic and natural starting materials can be investigated. However, research into new materials is limited to those soluble in the few organic solvents capable of satisfying total or partial water miscibility requirements. This variety is even more limited when submicron particles are intended as carriers of active molecules due to the safety requirements for organic solvents, such as their low toxicity. On the other hand, it is interesting to note that research on new starting materials and on the encapsulation of active molecules is more intensive for the solvent displacement technique than for emulsification–diffusion method. This is probably due to the advantages associated with the ease of implementing this method. In addition, low amounts of stabilizing agent are used which can facilitate subsequent purification steps.

Lipophilic-like active substances are generally used when submicron spheres are prepared by the two methods (Tables 1 and 2). However, they have also been modified for loading hydrophilic molecules such as peptides and proteins by the solvent displacement technique [97–99] or for using other starting materials such as lipid substances, in order to obtain solid lipid particles by emulsification–diffusion [100,101].

The two methods allow active substance loading higher than 10% and entrapment efficiencies higher than 70% (Tables 1 and 2). However, wide ranges are also reported for these parameters in both solvent displacement [15,41,42,44,51,72,77] and emulsification–diffusion studies [22,86,93]. Unfortunately, there are no works have been published providing comparisons of the two methods when the same active substance is used. In addition, as shown in Table 3, contradictory conclusions have been obtained by researchers when the same variable was investigated, possibly due to the purification and concentration of the particles after their preparation (e.g., washing [15,26,32,56,63,66,67,73,93], dialysis [72,83], ultrafiltration–centrifugation [29,37,53,57,68], ultracentrifugation [31,35,41–45,54,58,92], cross-flow filtration [28,86], filtration by 0.1 μm filter [65,84], centrifugation [22,62,64,76,77,89,91,96] and separation by gel filtration [14,48]).

Despite this, an all-embracing view of the conclusions reported by different authors on the effect of operating variables and starting materials on the entrapment of active molecules (Table 3) allows us extending the general statements suggested by Sahana et al. [22] in the case of emulsification–diffusion method to the solvent displacement technique. Thus, the highest entrapment efficiency is reached at the lowest molecule solubility in the aqueous phase, the fastest rate of polymer precipitation/solidification, the largest solid-state solubility of the molecule in the polymer and the highest affinity between the organic solvents and the aqueous phase. Consequently, the nature and concentration of the stabilizing agent, the pH of the aqueous phase,

Table 3
Influence of operating variables and starting materials on the entrapment efficiency and on the loading of active substances into submicron spheres prepared by solvent displacement and emulsification–diffusion methods.

Variable	Solvent displacement	Emulsification–diffusion
Operating variables		
Stirring rate	The lowest stirring rate the largest EE [31]	nr.
Method for preparing organic phase	There is influence on EE [51]	
Aqueous to organic phase volume	The lowest aqueous phase volume the smallest EE [16,18] The highest organic/aqueous phase ratio the lowest EE [77]	
Starting materials		
Drug nature	Hydrophilic molecules show the lowest EE [45,79]	nr.
Drug initial amount	EE increases as drug initial amount increases till a maximum value. After drug precipitation occurs.	The largest drug initial amount the largest EE [26,91].
	Therefore, it is common that the largest initial concentration the largest DL but, the largest initial concentration the lowest EE [18,29,42,43,49,64,65,68,76].	There is influence but without a particular trend [89]
	Drug/polymer ratio has incidence on EE [32,77,78]	The largest drug initial amount the largest DL but, the largest initial concentration the lowest EE [86].
	Non significant influence on EE [16]	Non significant influence on EE [28]
Polymer nature	The best drug–polymer affinity the largest EE [41,44,56,60,66,72,79].	The best drug–polymer affinity the largest EE [84,89,93].
	Non significant influence on EE [14,51,54,58,64,66].	Non significant influence on EE [86,96].
Polymer concentration	The largest polymer concentration the largest EE [31,35,68,77].	Non significant influence on EE [93].
	Non significant influence on EE [56,79].	
Stabilizing agent nature	Non significant influence on EE [15,77].	There is influence on EE [22]
Stabilizing agent concentration	The largest stabilizing agent concentration the largest EE [15].	The largest stabilizing agent concentration the largest EE [26].
	The largest stabilizing agent concentration the lowest EE [35].	The lowest stabilizing agent concentration the largest EE [92].
	Non significant influence on EE [18].	Non significant influence on EE [84,96]
Aqueous phase pH	Significant influence on EE [42,68].	Non significant influence on EE [28]
Solvent nature	Significant influence on EE [32,60].	The highest solvent water solubility the largest EE [22].
		Non significant influence on EE [92]

EE: Entrapment efficiency; DL: Drug loading; nr.: None reported information.

and the natures of the polymer and the solvent prove to be the key variables in governing the entrapment of active substances. Also, the initial amount of active substance turns out to be particularly important when the solvent displacement technique is used, which in turn might be linked to active substance–polymer affinity.

3. Mechanistic aspects related to particle formation by solvent displacement and emulsification–diffusion methods: The state of the art

First of all knowledge of the mechanistic aspects related to particle formation is necessary in order to obtain deeper understanding of the factors influencing the characteristics of submicron particles prepared by the solvent displacement technique and emulsification–diffusion method. Following this, the different approaches taken by each of the methods will be discussed.

3.1. Solvent displacement technique

Different approaches derived from the spontaneous emulsification process have been proposed in order to explain particle formation when the solvent displacement technique is used. Stainmesse et al. demonstrated in 1995 that submicron particles only can be formed at certain proportions of polymer, solvent and nonsolvent, characterized by a low concentration of polymer and small amount of organic solvent [30]. Afterwards, in 1998, Quintanar et al. proposed a mechanistic approach based on interfacial phenomena due to variations of surface tension between solvent/nonsolvent phases [3] while more recently, in 2005, Ganachaud and Katz [102] correlated the findings of Stainmesse with the "ouzo effect" proposed by Vitale and Katz [103] for homogeneous liquid–liquid nucleation.

In this review the two main approaches taken up-to-now are grouped as those based on mechanical mechanisms (dispersion mechanisms or spinodal decomposition) and those due to system chemical instability (condensation mechanism or nucleation), which is in line with the categorization adopted by other researchers [4,104–106].

Fig. 3 provides an illustration from a ternary phase diagram for the polymer/solvent/nonsolvent system. The particle dispersions are formed in the metastable region located between the binodal (miscibility-limit curve) and the spinodal (stability-limit curve) compositions. The mechanical mechanisms involve all the phenomena occurring from the spinodal region towards the metastable region and the nucleation approach ranges from the binodal curve towards the metastable region.

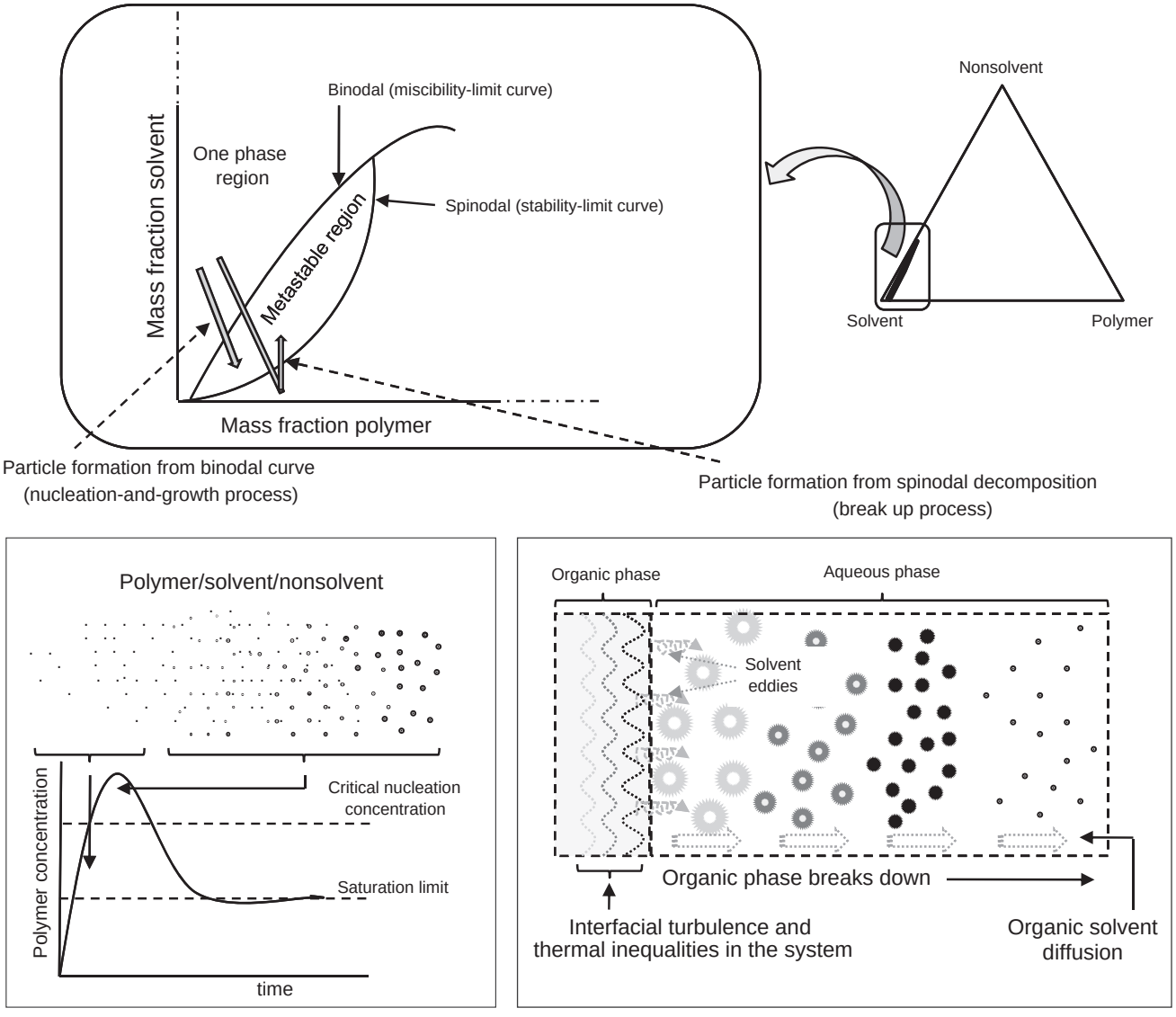

Fig. 3. Schematic representation of the mechanistic aspects related to the particle formation by solvent displacement method.

3.1.1. Mechanical mechanisms

The mechanical mechanisms for particle formation involve breaking up of the organic phase and dispersing it as drops in the aqueous phase. Thus Quintanar et al. and Galindo et al. [3,59] proposed the formation of submicron particles via interfacial turbulence or the Gibbs–Marangoni effect, taking into account the differences in surface tension between the solvent and nonsolvent used. Since a liquid with a high surface tension (aqueous solvent) pulls more strongly on the surrounding liquid than one with a low surface tension (organic solvent), this difference between surface tensions causes interfacial turbulence and thermal inequalities in the system, leading to the continuous formation of eddies of solvent at the interface of both liquids which generates interfacial convective flows. These flows contribute towards renewing the interfacial surface and are capable of sharply increasing the mass-exchange rate between the phases [107,108]. Consequently, violent spreading is observed due to mutual miscibility between the solvents which breaks down the organic phase into small droplets which again break down into smaller droplets and so on until forming "submicron droplets". Then the solvent flows away from regions of low surface tension and the polymer precipitates, forming submicron particles (Fig. 3).

The intensity of the interfacial tension gradients can be estimated by the Marangoni number (*Ma*). To guarantee system instability, *Ma* must be larger than a critical value that is specific for each solvent/nonsolvent system [109,110]. In the particular case where the surface tension gradient is caused by concentration gradients, the Marangoni number can be defined as follows [111]:

$$Ma = \frac{\Delta\gamma \cdot \Delta C}{\eta \cdot D_{AB}} \tag{1}$$

where: $\Delta\gamma$ is the rate of change of interfacial tension; ΔC is the concentration gradient, η is the viscosity of the organic phase and D_{AB} the diffusion coefficient of the organic phase in the aqueous phase. Thus it is obvious that in addition to surface tension, the viscosity of the aqueous phase plays a critical role. Research by Ostrovsky and Ostrovsky [108] showed that $\Delta\gamma$ decreases as the concentration of organic solvent in water increases. This reduces the intensity of the pulsations and, as a consequence, the Marangoni effect. Also, they highlighted differences in the intensity and frequency of the pulsations according to the organic solvent/aqueous phase system.

Although the Marangoni effect appears to be the most popular mechanical approach in view of the experimental research carried out, another mechanism for breaking up the organic phase into the aqueous phase was proposed by Montasser et al. [4]. This is a theoretical approach based on the results of spontaneous emulsification previously reported for ternary systems: toluene/ethanol/water or toluene/ethanol/water-surfactant. In this case, the driving force for breaking up the organic phase is the development of transient negative values of interfacial tensions that cause spontaneous interfacial expansion, generating a crowd of solvent droplets in the nonsolvent. The main argument in favor of this mechanism is the fact that the stabilizing agent used could inhibit the Marangoni effect without having any impact on spontaneous emulsification.

Research on spontaneous emulsification for oil–water systems has highlighted other kinds of instabilities possibly involved in spinodal decomposition [112]. For instance, Miller referred to Rayleigh–Taylor instability or the phase fragmentation phenomena governed by the drop curvature of the dispersed phase [105]. In fact, according to Ostrovsky and Ostrovsky [108] it has been demonstrated that mass transfer by Marangoni effect could not show total agreement with dependence on $\Delta\gamma$. In these cases, the intensity of the mixing process is also influenced by natural convection and forced mixing [108]. The Rayleigh number ($\overline{R}$) describes the natural convection intensity, which is proportional to both the mass transfer coefficient for a stable surface (K_D) and the expression on the right:

$$\overline{R} \approx K_D \approx (d\rho / dc)\Delta C\left(\sqrt{D_{AB}} / \eta\right) \tag{2}$$

where $d\rho/dc$ is the change in density of the aqueous phase with the concentration of organic phase added; ΔC is the change of concentration at the surface of the organic/aqueous phase; D_{AB} is the diffusion coefficient of the organic phase in the aqueous phase and η the organic phase viscosity [108]. As can be seen, the interaction between organic and aqueous phases and mixture density could also influence the efficiency of phase mixing.

3.1.2. Mechanism based on the chemical instability of the system

Chemical instability in polymer precipitation has been investigated by Beck et al. [79], Ganachaud and Katz [102] and Aubry et al. [106] who took into consideration that particles are formed both with and without surfactant. This suggests that interfacial tension variations that support Gibbs–Marangoni theory may not be critical for particle formation [79]. In this case, when the polymer solution is in contact with water, the solvent diffuses into the aqueous phase, creating a local supersaturation of polymer molecules which leads to spontaneous nucleation in the form of small particles ("protoparticles") that grow with time (nucleation-and-growth process) [105] (Fig. 3). This scenario presumes that the blending rate and the associated process of molecular diffusion are extremely rapid, in comparison to the nucleation rate [113]. Thus, when phases are mixed the free energy of the system changes in such a way that phase separation is energetically more favorable and the polymer molecules coalesce forming nuclei [5].

As shown by Lince et al. [75] in the case of solvent displacement process, the nucleation rate (J) can be calculated by the following expression:

$$J = \frac{2D}{d^5}\exp\left(-\frac{16\pi\gamma^3\,\tilde{v}^2}{3k_B^3T^3[\ln(S)]^2}\right) \tag{3}$$

where D is the molecular diffusion of the polymer molecule, d is its molecular diameter, k_B and T are the Boltzmann constant and absolute temperature respectively, γ is the interfacial tension between the already formed particles and the solution, $\tilde{v}$ is the polymer molecular volume and S is the super-saturation defined as the ratio of the actual polymer concentration and the solubility of the polymer in the solvent mixture.

If the nuclei radius is higher than the critical nucleus radius (r^*), the "protoparticle" can grow until the system reaches equilibrium (Fig. 3). r^* depends on the surface tension between the two phases (γ) and their difference in free energy per unit volume [5]:

$$r^* = -\frac{2\gamma}{\Delta g_v} \tag{4}$$

The particle growth rate is governed by the molecular weight of the polymer (M_w), its density (ρ), the mass transfer coefficient (k_m), the polymer concentration (c) and the super-saturation as follows [75]:

$$G = \frac{2k_m M_w c}{\rho}(S-1) \tag{5}$$

Particle aggregation can also occur via the Ostwald ripening phenomenon as explained by Horn and Rieger [5]. However, Lince et al. and Aubry et al. [75,106] stated that the aggregation phenomena depend on the size of the particles and their probability of encounters due to Brownian motion (perikinetic aggregation) and fluid motion (orthokinetic aggregation). The rate at which perikinetic aggregation occurs can be estimated from the dynamic viscosity of the dispersive medium (η), temperature (T), the Boltzman constant and the radii of the colliding particles (a_i, a_j) [114]:

$$k = \frac{8k_BT}{3\eta}\frac{\left(a_i + a_j\right)^2}{a_i a_j} \tag{6}$$

In turn, orthokinetic aggregation is influenced by particle size and shear rate (velocity gradient, G). Thus the collision rate coefficient is:

$$k_{ij} = \frac{4}{3}G\left(a_i + a_j\right)^3 \tag{7}$$

Also the number of aggregates can be estimated from the mass fraction of the solvent (f_s), the initial mass fraction of the polymer in the solvent (f_p^i), the densities of the dispersive medium and the particles (ρ_{sol} and ρ_p respectively), and the mean particle diameter (d) by the expression:

$$n = \frac{6 f_s f_p^i \rho_{sol}}{\rho_p \pi d^3} \tag{8}$$

Finally, it is possible to know the variation of particle size as a function of aggregation time, as shown by Aubry et al. [106]:

$$d^3 = \frac{8 k_B T \rho_{sol} f_s f_p^i}{\pi \rho_p \eta} \times t. \tag{9}$$

As can be seen, according to the nucleation-and-growth mechanism final particle size is governed by the growing process, the aggregation phenomena and the performance of the stabilizing agent during the nucleation process. In addition, since polymer precipitation obeys the classical nucleation theory, it can be either homogeneous or heterogeneous depending on the composition of the system [5]. Thus in the particular case of submicron particles as carriers of active substances, the interaction between the polymer and active substance may play an important role during particle formation.

Cluster structure could also affect nucleation rate, as was reported by Ruckenstein et al. [115]. Clusters are built by successively adding layers around the central molecule. Thus, for example, icosahedral configurations are recognized as being more preferred, energetically, by small clusters than amorphous or face-centered cubic (fcc) configurations. This is due to the number of bonds of the surface molecules located on the vertices, edges and facets of each structure, the way the cluster is formed and the number of nearest neighbors for interaction. The local molecular order in the icosahedral cluster exhibits an almost crystalline structure.

It is noteworthy that the main difference between mechanical mechanisms in particular, the Gibbs–Marangoni effect, and the nucleation-and-growth process is the driving force underlying particle formation. The Gibbs–Marangoni effect is referred to as "surface tension-driven flow" and, as mentioned above, variations in interfacial tension at the solvent/nonsolvent interface cause disturbances in mechanical equilibrium, resulting in low free energy [3]. As quantitative description of this phenomenon shows, the factors governing particle formation are the physicochemical properties of the organic phase and its interaction with the aqueous medium. Furthermore, nucleation and growth is a spontaneous process that is strongly dependent on the composition of the polymer/solvent/nonsolvent system, the interaction between the particles formed and the physicochemical properties of the dispersive medium [75,116].

At present, there is not enough experimental evidence with permission that favors a specific mechanistic approach. It appears that particle formation by using the solvent displacement technique occurs via the nucleation and growth process at low organic/aqueous phase ratios and low polymer concentrations. It is probable that the Gibbs–Marangoni effect is the prevailing mechanism when precipitation occurs at the highest polymer concentration and organic/aqueous phase ratio (between the composition ranges leading to efficient particle formation by using this method, as demonstrated by Stainmesse et al. [30]).

3.2. Emulsification–diffusion method

For this method, the first step of particle preparation is organic phase dispersion of globules in aqueous phase at high stirring speed. Taking into account that the organic solvent used is partially water soluble, mutual saturation of the phases is required in order to obtain an emulsion in thermodynamic equilibrium. Once the emulsion is formed, the submicron droplets are then diluted in water and the interaction between the emulsion droplets and the dilution phase is referred to as a "modification of phase equilibrium and solvent diffusion", which leads to polymer precipitation since the polymer is in poor solvent [3,83]. Two approaches to particle formation can be taken with this method (Fig. 4). The first is based on the Marangoni

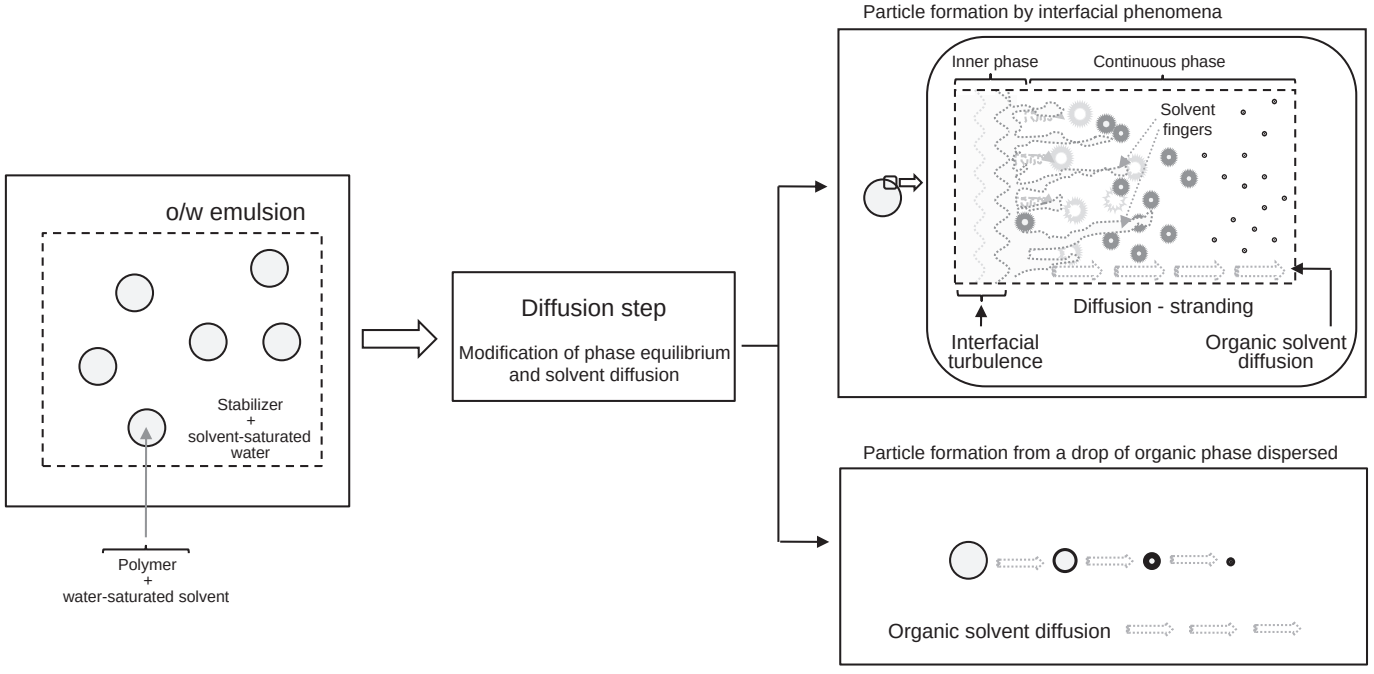

Fig. 4. Schematic representation of the mechanistic aspects related to the particle formation by emulsification–diffusion method.

effect (mechanical mechanism) and the second involves particle formation from droplets of emulsion.

3.2.1. Mechanical mechanism

The mechanical approach to particle preparation by using the emulsification–diffusion method was proposed by Quintanar et al. [3], based on polymer precipitation theories and interfacial phenomena, as explained previously for the solvent displacement method. However, in this case strong interfacial tension gradients cannot be driven by variations of interfacial concentrations since the solvent is partially water-miscible and it is water-saturated beforehand in order to maintain thermodynamic equilibrium during the emulsion step. In addition, higher stabilizing agent concentrations are used for the emulsification–diffusion method than for the solvent displacement procedure (usually 1.0% and 0.25%, respectively) which could drastically reduce the interfacial phenomena that govern the breakup of emulsion globules.

In addition, it is important to note that the interface between organic and aqueous phases was subjected to shear force during the emulsification step. According to Sternling and Scriven [109], energy will be dissipated because the molecules must be reoriented and that energy increases with the rate of shearing and the presence of surface-active agents. Thus, for the emulsification–diffusion method it might be expected that surface tension gradients can be due to the thermal effects associated with heat transport during organic solvent diffusion. Typically, the thermal Marangoni effect results in fingering instability [117] where the low interfacial tension difference and the drop curvature less than its spontaneous one allow that the flexible drop surface develops multiple undulations generating long fingers as the organic solvent diffuses towards the aqueous medium. In this process, the solvent carries polymer molecules into the aqueous phase. Then, if spontaneous curvature favors an organic phase-in-water arrangement, it could be expected that many drops of smaller diameter detach from the fingers and become dispersed in the aqueous phase. Thus new globules or polymer aggregates (not totally desolvated) are formed and stabilized by the stabilizing agent (protoparticles). The submicron particles will be formed after the complete diffusion of the solvent, if the stabilizing agent remains at the liquid–liquid interface during the diffusion process and if its protective effect is adequate (Fig. 4). The works of Moinard et al. [9] suggest that solvent diffusion from the droplets takes place too quickly (duration less than 20 ms), leading to the rapid formation of particles. The theoretical analysis of similar phenomena carried out for Miller supports this mechanistic approach [105].

As in the solvent displacement technique, the intensity of interfacial tension gradients can be estimated by the Marangoni number, but in this case thermal *Ma* is defined by the expression [111]:

$$Ma = \left|\frac{\partial\gamma}{\partial T}\right| \frac{\Delta\gamma \cdot \Delta T}{\eta \cdot \alpha} \tag{10}$$

where: $\left|\frac{\partial\gamma}{\partial T}\right|$ is the temperature coefficient of surface tension, $\Delta\gamma$ is the rate of change of interfacial tension; ΔT is the temperature gradient, η is the viscosity and α the thermal diffusivity. System instability occurs in this way if *Ma* is higher than the critical Marangoni number which is specific for each system [109].

3.2.2. Mechanism based on particle formation from an emulsion droplet

The second approach to submicron particle formation by using the emulsification–diffusion method is supported by the research performed by Guinebretière et al., Galindo et al., Moinard et al. and Hassou et al. [9,62,118,119]. It is strongly suggested that particles prepared after solvent diffusion are formed from an emulsion droplet (Fig. 4). Moinard et al. [118] demonstrated that mean particle size is always smaller than that of the emulsion droplets. Thus emulsion droplet size governs final particle size and consequently, it is directly influenced by all the operating variables linked to the preparation of the emulsion and their colloidal properties. Although the mathematical model developed by Moinard et al. [118] takes into account submicron capsules as model particles, a similar approach could be taken for submicron spheres. Thus the ratio between mean particle diameter (d_p) and mean diameter of the primary emulsion drop (d_{ed}) is determined by particle volume (V_p) and emulsion drop volume (V_{ed}), as in the following:

$$\frac{d_p}{d_{ed}} = \left[\frac{V_p}{V_{ed}}\right]^{\frac{1}{3}} \tag{11}$$

The way droplets in the organic phase are formed can be explained by binary break-up or by capillary break-up mechanisms [120]. With the binary break-up mechanism, droplets are continuously broken up into two fragments, until the drop size is small enough to survive the prevailing hydrodynamic conditions. With the capillary break-up mechanism, the droplet is stretched to produce a long filament that will fragment due to the action of capillary waves into a relatively large number of fragments during a single break-up. The prevalence of a particular mechanism depends on the capillary number (*Ca*) which is the ratio between the viscous stress that causes the droplet fragmentation and the restoring stress from surface forces [118]. *Ca* can be defined by the expression [120]:

$$Ca = \frac{r_{ed}\eta\dot{\gamma}}{2\gamma} \tag{12}$$

where r_{ed} is the drop emulsion radius, η is the aqueous phase viscosity, $\dot{\gamma}$ the shear stress and γ the interfacial tension between the organic and aqueous phases. The organic phase fragmentation occurs at a critical value of *Ca*, which in turn depends on the viscosity ratio between the organic phase and aqueous phases and on the presence of other components such as surfactants, as reported by Briscoe et al. [120]. Then, if the operating conditions or the physicochemical properties of the liquids lead to a capillary number just above the critical capillary number, the droplet breaks up via the binary break-up mechanism. If the capillary number is increased to a value well above the critical value, the capillary break-up mechanism prevails [120].

According to Galindo et al. [62], the maximum stable drop size of the droplets ($d_{ed\ max}$) depends on the stirring rate ($r_{stirring}$), the stirrer diameter ($d_{stirrer}$), the interfacial tension (γ) and the density of the aqueous phase (ρ) as follows:

$$d_{ed\,\max} \approx r_{stirring}^{-6/5} d_{stirrer}^{-4/5} \gamma^{3/5} \rho^{-3/5} \tag{13}$$

From the latter, it has been possible to express the evolution of the droplet mean size according to the stirring rate and establish their relationship with mean particle size [62].

Up-to-now, experimental research focused on mechanistic aspects associated with the emulsification–diffusion method are based on the assumption that particle formation stems from emulsion droplets [62,118]. In fact, the high shear stress due to the emulsification step may guarantee submicron droplet formation. In this phase, physicochemical properties and system stirring govern both the ease with which the emulsion is formed and its stability. There is no reported works evidencing that the Marangoni effect is the driving force during particle formation. However, although Galindo et al. [62] demonstrated the relationship between stirring rate and mean particle size, they reported discrepancies between the theoretical model and experimental data. From our standpoint, the high thermal energy of the emulsification process released in the aqueous phase during the dilution step, may have an impact on particle formation, thereby practically explaining these deviations.

As can be concluded from the discussion of mechanistic aspects related to the solvent displacement and emulsification–diffusion techniques, the theoretical and experimental evidence suggests that submicron particle formation depends on the successful combination of operational conditions and starting materials. In the following, this review will focus in these aspects, by making a comparative analysis between the preparation methods.

4. Influence of the operating conditions on submicron sphere size

The study of the operating conditions related to the submicron particle preparation methods can be investigated from different angles such as their influence on the up-scaling procedure [38,62,75] or on particle characteristics, in particular size [81,121]. In this review, we have adopted the second approach to compile useful information for handling variables to obtain specific particle sizes and discuss the behaviors obtained from the mechanistic aspects of the particle formation described previously for each method.

4.1. Solvent displacement process

When considering particle formation mechanisms, the organic/aqueous phase ratio, the organic phase addition method, the stirring system, the temperature and the final stirring time prove to be interesting operating variables for studying the solvent displacement method (Fig. 1).

As is shown in Table 4, the research performed up to now has focused on the phase mixing method, the organic phase addition rate, the organic/aqueous phase ratio, the type of stirring used, the system stirring rate and the system temperature. Nevertheless, contradictory behaviors are reported regarding the organic phase addition rate and the organic/aqueous phase ratio which might be due to differences in experimental conditions or in the materials used. To overcome this problem, Fig. 5 summarizes a controlled study of the solvent displacement method in which the following operating variables were investigated: organic/aqueous phase ratio, organic phase injection rate, method of organic phase addition (dropwise-out and dropwise-in continuous medium), system stirring rate, experimental temperature and final stirring time (for methodological aspects to see Supplementary data).

An all-embracing view of the results reported in the literature and those from the systematic study allows explaining the previously mentioned conflicting results. Thus the organic phase addition rate can influence particle size but is dependent on the organic/aqueous phase ratio (Fig. 5B). The highest injection rate produces the largest particle mean size, particularly at the highest organic/aqueous phase ratios. This behavior suggests that submicron particle formation due to solvent diffusion is time-dependant. Therefore, the particles can continue to grow if the diffusion time is insufficient due to an excessively fast organic phase injection rate. However, this increase in particulate growth can be offset by increasing the continuous medium stirring rate until phase mixing is significantly faster than particle formation. This was demonstrated in an additional study in which the operating conditions of the organic phase injection rate — system stirring rate were 300 μl/min–825 rpm, 300 μl/min–750 rpm and, 225 μl/min–750 rpm. In these cases particle sizes ranged from 160 to 190 nm.

Table 4
Summary of reported studies on the influence of operating variables on the size of submicron spheres prepared by solvent displacement method.

Variable	System composition		Work conditions	Particle size (nm)	Reference
	Organic phase	Aqueous phase			
Organic phase addition rate	PCL–acetone	PLX–W	6–270 ml/min	100–165 nm	[35]
	PCL–acetone	W	3 ml/min	611	[75]
			40 ml/min	474	
			60 ml/min	363	
			80 ml/min	312	
			120 ml/min	340	
	PLGA–acetone	PLX–W	3.5 ml/min	142	[79]
			10.6 ml/min	121	
Organic/aqueous phase ratio	PCL–acetone	W	0.1	115	[30]
			0.2	130	
			0.3	150	
	Ethylcellulose–EtOH	W	0.12–0.34	70–85	[38]
	PLGA–acetone	PVA–W	0.2	181	[18]
			0.4	220	
			0.8	270	
	PLGA-b-PEG–acetone	W	0.1	118	[70]
			0.2	115	
			0.5	122	
			1.0	149	
	PLGA–ACN	W	0.1	126	[76]
			0.2	155	
			0.6	164	
	PES–THF	PLX–W	0.2	581	[77]
			0.4	298	
			0.6	258	
	PLGA–acetone	PLX–W	0.1	149	[79]
			0.2	145	
Type of stirrer	Ethylcellulose–EtOH	W	Rushton turbine	75–90	[38]
			Four pitched 45° blade turbine	70–90	
System stirring	PCL–acetone	W	Flow rate on the organic and aqueous phases: 3–120 ml/min	Smaller particle sizes are promoted working the largest conditions on phases flow rate.	[75]
Method of phases mixing	PMMA–acetone PMMA–THF	W	Adding in one shot the aqueous phase into the organic phase	PMMA–acetone: 74 PMMA–THF: 131	[105]
			Dropwise addition of the aqueous phase to the organic phase	PMMA–acetone: 116 PMMA–THF: 183	
			Dropwise addition of the organic phase to the aqueous phase	PMMA–acetone: 129 PMMA–THF: 142	
Temperature	Ethylcellulose–EtOH	W	10–40 °C	80–90	[38]

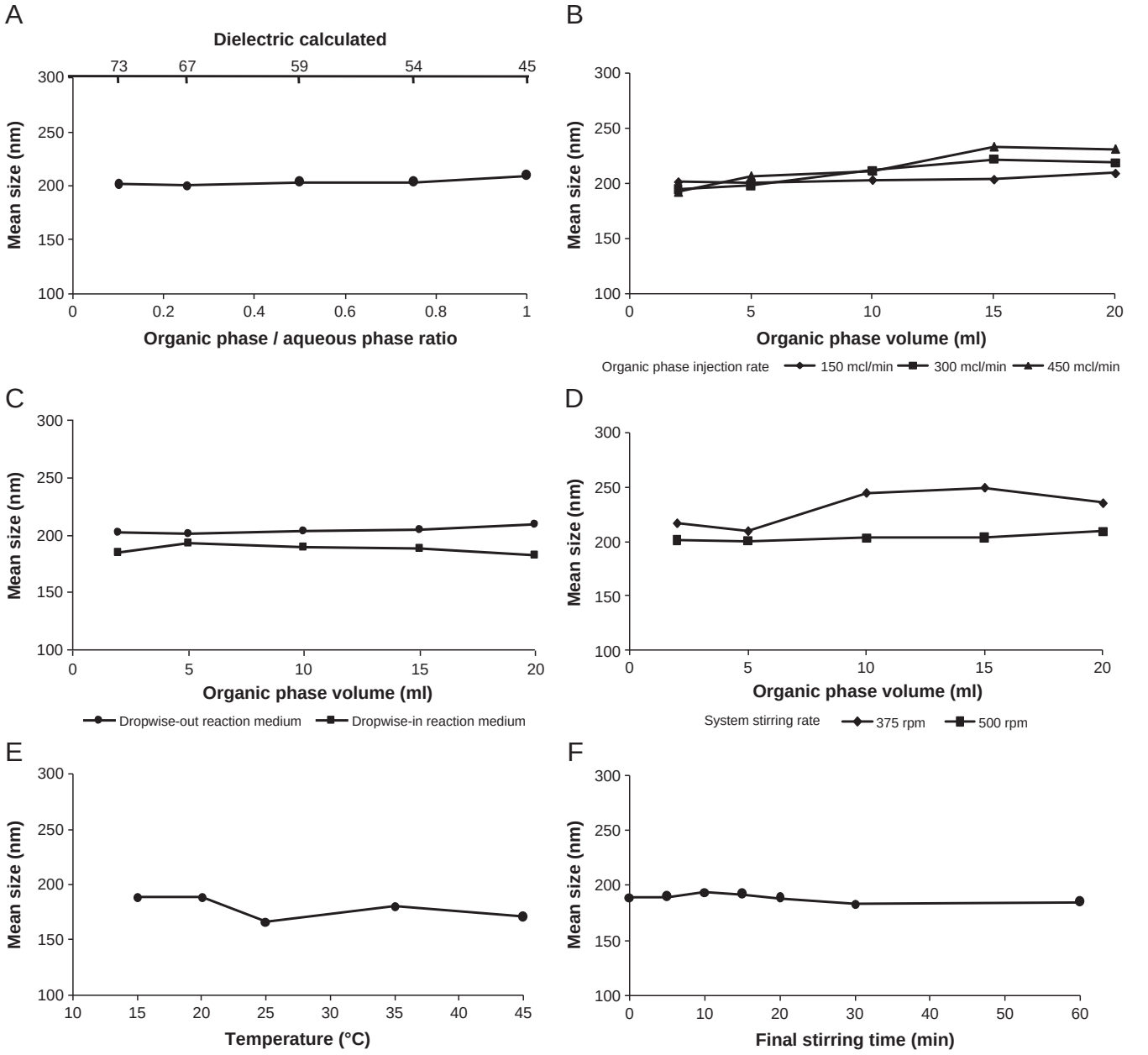

Fig. 5. Preparation of submicron particles by solvent displacement method: influence on mean size of operating variables. A. Organic phase/aqueous phase ratio; B. organic phase injection rate; C. method of organic phase addition; D. system stirring rate; E. system temperature; and F. final stirring time.

Aubry et al. [106] reported that the organic phase addition method can also influence particle size. This might depend on the order of phase mixing (organic phase into aqueous phase or vice versa) or the nature of the organic solvent. In our experiments, for example, when the organic phase is added dropwise into a continuous medium (i.e. organic phase added drop by drop into the continuous medium) instead of dropwise-out of a continuous medium, the particle size obtained is smaller (Fig. 5C). An initial approximation allows stating that drop size is smaller when the dropwise into continuous medium is used due to stirring shear strength. This facilitates the nucleation of smaller particles and consequently smaller particle sizes are obtained.

It was found that the system stirring rate is another factor influencing particle size but it depends on the volume of the organic phase (Fig. 5D). This may clarify the conclusions reported by Lince et al. on the smaller particle size obtained at the highest stirring rates of organic and aqueous phases [75]. In addition, it shows that close attention is needed for setting stirring conditions precisely when submicron particles are prepared by the solvent displacement process. As shown in Table 1, terms such as “moderate stirring”, “magnetic stirring” and “gentle magnetic stirring” are frequently used to refer to the stirring rate of the system, omitting its effect on fluid dynamics and neglecting its possible influence on polymer supersaturation phenomena, solvent migration, system micromixing and particle aggregation [122].

The absence of impact of the organic/aqueous phase ratio (Fig. 5A), system temperature (Fig. 5E) and final stirring time (Fig. 5F) on particle mean size was confirmed. The relative standard deviations (RSD) of the particle sizes obtained in these cases are between 3 and 8% which is common for submicron particle dispersions prepared by the solvent displacement process [38,75]. The organic/aqueous phase ratio was studied using an organic phase addition method with an organic injection rate of 150 μl/min, a system stirring rate of 500 rpm and a dropwise-in continuous medium. Adequately balanced operating conditions can be achieved in this case, leading to efficient solvent

diffusion and particle nucleation. On the other hand, the non-effect of temperature suggests that this variable is not significant if maximum solvent diffusion is achieved. With regard to the behavior observed when the final stirring time was examined, this suggests that particle formation is associated with stirring speed during organic phase addition. Therefore additional stirring is not necessary.

4.2. Emulsification–diffusion method

Table 5 shows published data on the impact of operating variables on the size of submicron particles prepared by the emulsification–diffusion method, which is in good agreement with the results obtained in our systematic study (Fig. 6).

In general terms, the emulsification–diffusion technique is a robust process and the emulsification rate governs particle size (Fig. 6B). The highest values of this variable lead to exhaustive fragmentation in the organic phase, forming small emulsion droplets. Consequently, smaller particle sizes are obtained. Also, as reported by Leroux et al. and Poletto et al. [28,94], the organic/aqueous phase ratio appears to have an influence on particle size, highlighting non-homogeneity in the emulsion when low phase ratios are used (Fig. 6A). In addition, emulsification time has less effect than emulsification speed, while the phase ratio (Fig. 6C) and organic phase/aqueous phase mixing method do not have any effect (198 ± 1.4 and 199 ± 7.3 nm for controlled addition at 1.25 ml/min and for total addition in one step, respectively).

The operating variables related to the solvent diffusion step do not seem to affect particle size (Table 5, Fig. 6D–H). Indeed, unlike the solvent displacement process, the operating conditions of the emulsification–diffusion method guarantee free solvent diffusion as long as the organic solvent solubility condition is satisfied. This explains the seemingly contradictory results reported by Song et al. [90] in which the highest particle size is obtained at the lowest volumes of water for dilution In their study, the lowest volumes of water used did not lead to complete solubility of the organic solvent. In addition, difficulty in solvent diffusion can be expected due to the barrier effect of the stabilizing agent on the emulsion droplet. This could explain the data

Table 5
Summary of reported studies on the influence of operating variables on the size of spheres prepared by emulsification–diffusion method.

Variable	System composition		Work conditions	Particle size (nm)	Reference
	Organic phase	Aqueous phase			
External/internal phase ratio	PMMA–BA	PVA–W	1.4	178	[28]
			2.8	162	
			4.7	153	
			8.5	139	
	PHBHV–$CHCl_3$	PVA–W	0.25	896	[94]
			0.35	691	
			0.4	606	
			0.5	629	
			0.6	481	
			0.8	458	
Emulsification stirring rate	PMMA–BA	PVA–W	1200 rpm	244	[28]
			1200 rpm and concomitant sonication	244	
			5000 rpm	141	
	PDLLA–PC	PLX–W	1500–2460 rpm	>1000	[81]
			9000 rpm	166	
			13,500 rpm	149	
	PMMA–BA	PVA–W	1000 rpm	427	[62]
			1250 rpm	375	
			1500 rpm	351	
			1750 rpm	323	
			2000 rpm	312	
	PLGA–PC	PVA–W	4800 rpm	348	[83]
			8000 rpm	285	
			11,200 rpm	205	
			13,600 rpm	200	
			15,000 rpm	197	
Type of stirrer	PDLLA–PC	PLX–W	High speed homogenizer (9000 rpm)	166	[81]
			Propeller stirrer (2500 rpm)	211	
Volume of water for dilution	PLGA–EtAc	DMAB–W	20 ml	190	[90]
			40 ml	106	
			80 ml	67	
			160 ml	56	
	PLGA–PC	DMAB–W	20 ml	194	[90]
			40 ml	63	
			80 ml	46	
			160 ml	41	
Temperature of adding water	PLGA–PC	PVA–W	25 °C	204	[83]
			47 °C	173	
			60 °C	170	
	PLGA–PC	DMAB–W	25 °C	78	[83]
			47 °C	68	
			60 °C	65	
Adding rate of water for dilution	PLGA–PC	PVA–W	0.03 ml/s	220	[83]
			16 ml/s	204	
	PLGA–PC	DMAB–W	0.03 ml/s	76	[83]
			16 ml/s	78	
Stirring rate for the dilution	PLGA–PC	PVA–W	0 (arbitrary units)	206	[83]
			2 (arbitrary units)	204	
			8 (arbitrary units)	195	
			10 (arbitrary units)	193	

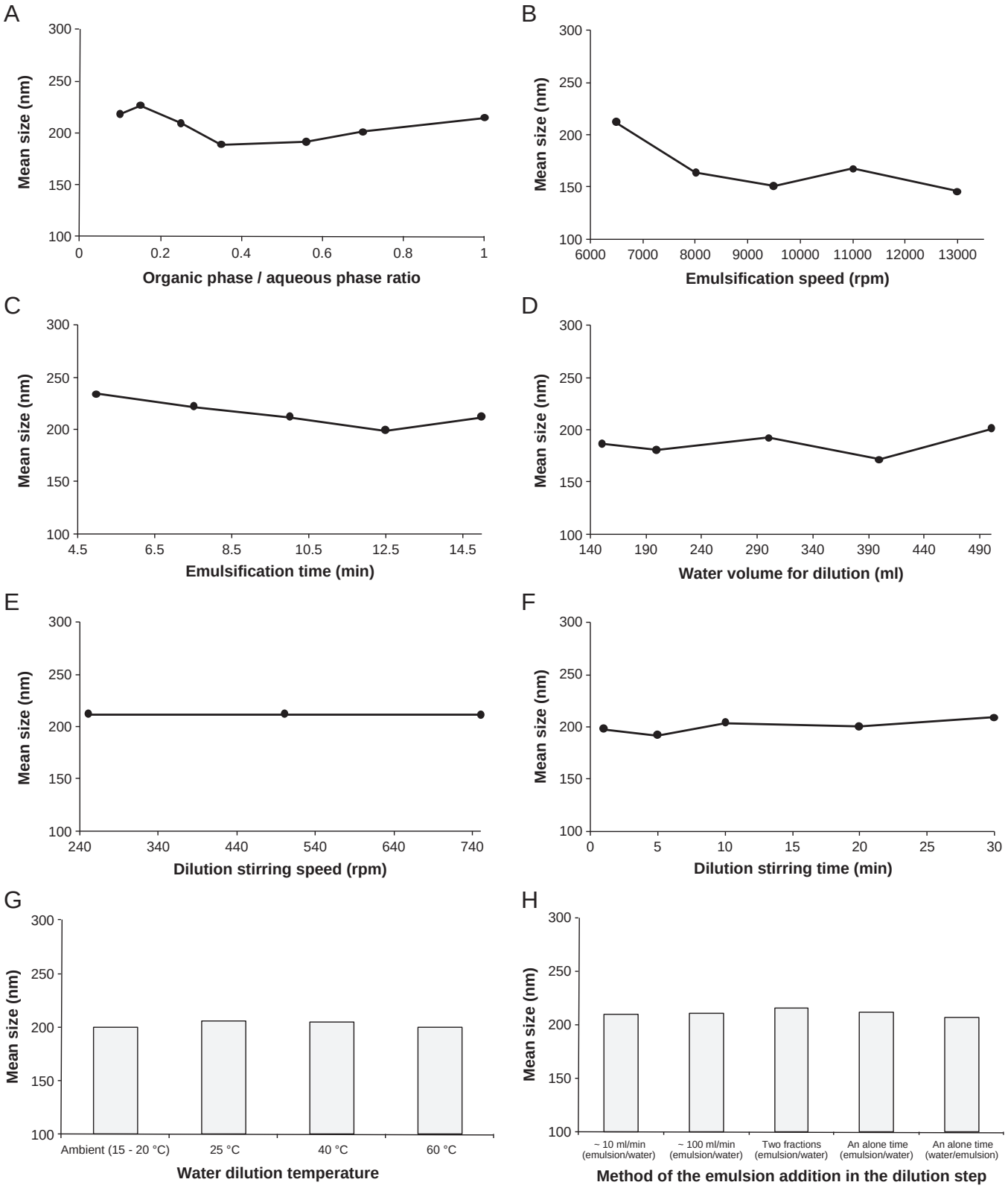

Fig. 6. Preparation of submicron particles by emulsification–diffusion method: influence on mean size of operating variables. A. Organic phase/aqueous phase ratio; B. emulsification stirring speed; C. emulsification time; D. water volume for dilution; E. dilution stirring speed; F. dilution stirring time; G. water dilution temperature; and H. method of emulsion addition in the dilution step.

reported by Kwon et al. where the size of submicron particles prepared using PVA as a stabilizing agent is influenced by the temperature of the dilution water [83]. In this case, reducing the viscosity of the external phase could facilitate solvent diffusion.

Particle suspension concentration under reduced pressure was examined and found to have no effect on particle size (mean size differences less than 10 nm). This may be attributed to total solvent diffusion from the emulsion droplet during the diffusion stage and,

consequently, the complete formation of submicron particles during this step. However, additional discussion on this subject from the standpoint of polymer–solvent interaction will be included below.

4.3. Mechanistic approaches and operating conditions: Comparative analysis between methods

As was shown above, the data reported highlights that particles prepared by the solvent displacement technique can be formed via either nucleation and growth or the Marangoni effect. Therefore the non effect of operating conditions on particle size when the lowest phase ratios are used show that nucleation and growth is the prevailing mechanism. Furthermore, the incidence of the stirring rate on particle size and the method used for adding the organic phase reveal that the Marangoni effect is predominant for particle formation, but only when the highest phase ratios are investigated.

On the other hand, the formation of submicron particles by the emulsification–diffusion method is governed by the emulsion step, particularly the rate and time of emulsification. At first sight, this is in agreement with the mechanistic approach based on the formation of a particle from an emulsion drop. However, the modest effect of the phase ratio suggests that additional mechanistic considerations should be included. It is risky assume that thermal Marangoni effect fully explains this behavior, because, as mentioned above, an effect of the solvent concentration may be present. Whatever the case, it is a potential starting point for investigating unknown factors in further studies.

From a comparative standpoint, the emulsification–diffusion method appears to be robust. Basically two variables determine particle size, which supposes easy up-scaling. However, difficulties can be expected with the emulsion dilution step, which has to take place very quickly, since Ostwald ripening phenomenon may occur. On the other hand, the solvent displacement technique does not allow general statements on its robustness. According to our previous discussions, if particle formation is obtained by the nucleation and growth mechanism the robustness of the method should be better than that of emulsification–diffusion. In fact, particle size is no affected by any operating variable. However, if particles are formed via the Marangoni effect, their size depends on a complex combination of variables which can make up-scaling difficult.

5. Influence of the materials from which the submicron spheres are prepared

The technical literature regarding the preparation of submicron particles by solvent displacement and emulsification–diffusion methods provides many examples illustrating the incidence of different composition variables on particle characteristics, such as their morphology, size, size distribution and zeta-potential. Thus our aim under this subheading is to perform a comparative analysis of the methods described in the literature and those used in our experimental study, taking into consideration how the particularities of the different polymers, stabilizing agents and solvents employed determine particle behavior, and how can this behavior determine decision-making regarding the development of products based on submicron particles.

5.1. Influence of polymer

Two points are usually recognized as critical with respect to the influence of the polymer on the size and zeta-potential of the submicron particles, namely the nature and the concentration used.

5.1.1. Behavior of the nature of polymer

Data reporting the influence of the nature of the polymer used on the size and zeta-potential of submicron particles is summarized in Tables 6 and 7. In general terms, different conclusions can be drawn from the information reported: (1) the particle size obtained by the two methods is in the same range (50–300 nm); (2) submicron

Table 6
Summary of reported studies on the influence of polymer nature on the size of submicron spheres prepared by solvent displacement and emulsification–diffusion methods.

Polymer order according to the particle size	Particle size range (nm)	Organic solvent	Reference
Solvent displacement method			
$PLGA_{50:50} < PLGA_{75:25} < PDLLA < PCL$	110–235	Acetone	[33]
$PLGA < PDLLA \ll PCL$	118–220	Acetone	[34]
$PDLLA < PLGA_{50:50} = PLGA_{85:15} \ll PCL$	109–208	Acetone	[14]
$PLA:PEG_{15:5} < PLA:PEG_{45:5} < PLA:PEG_{75:5} = PLA < PLA:PEG_{110:5}$	50–157	Acetone	[43]
$PCL:LA_{6:4} = PCL:LA_{2:8} < PDLLA < PCL$	81–132	Acetone	[44]
$SB\text{-}PVA\text{-}g\text{-}PLGA = PVA\text{-}g\text{-}PLGA = PLGA$	104–120	Acetone	[46]
$PLGA_{50:50\ 6\ kDa} = PLGA_{50:50\ 14.5\ kDa} < PLGA_{75:25}$	117–159	Acetone	[51]
$PDLLA = PCL$	169–182	Acetone	[53]
$PMMA \ll PCL$	84–195	Acetone	[57]
$PEG:PLGA < PEG\text{-}PCL < PEG\text{-}PLA < PCL = PDLLA < PLGA$	78–262	Acetone	[58]
$PLGA\text{-}PEG_{34} = PLGA\text{-}PEG_{70} < PLGA\text{-}PEG_{495} < PLGA$	58–134	Acetone	[55]
$PDLLA_{209\ kDa} < PDLLA_{109\ kDa} < PDLLA_{16\ kDa}$	51–131	Acetone	[56]
$PLGA = PDLLAR_{203} < PDLLAR_{207}$	98–138	Acetone	[66]
$PLGA_{50:50\ 7\ kDa} = PLGA_{5050\ 63\ kDa} = PLGA_{65:35} = PLGA_{75:25} = PDLLA$	175–194	Acetone	[18]
$PDLLA_{22\ kDa} = PDLLA_{52.3\ kDa} < PDLLA_{124.8\ kDa}$	185–260	Acetone	[71]
$PCL_{14\ kDa} \ll PCL_{80\ kDa}$	295–395	Acetone	[75]
$PLA_{05} = PLA_{20} < PLGA_{75:25} = PLGA_{85:15}$	201–258	Acetone:EtOH	[47]
$PCL\text{-}PEG_1 = PCL\text{-}PEG_2 < PCL\text{-}PEG_3$	71–93	THF	[60]
$DexP_{130} = DexC_{6\text{–}85} < DexC_{6\text{–}300} < DexP_{210} \ll DexC_{10\text{–}52}$	145–300	THF	[69]
$PDLLA_{22.6\ kDa} = PDLLA_{32.1\ kDa} < PDLLA_{52.3\ kDa} \lll PDLLA_{124.8\ kDa}$	104–322	THF	[71]
$PLA\text{-}PEG_{15:5} = PLA\text{-}PEG_{30:5} < PLA\text{-}PEG_{75:5} < PLA:PEG_{110:5}$	55–152	ACN	[45]
Emulsification–diffusion method			
$PLGA < PLGA$	176–219	EtAc	[89]
$Propyl\text{-}starch_{subst.\ 1.05} < Propyl\text{-}starch_{subst.\ 1.45}$	150–183	EtAc	[95]
$PLGA = PLGA\text{-}PEG$	218–220	EtAc	[96]
$PMMA \ll PLGA = PCL$	140–265	BA	[28]
$PLGA_{50:50} = PLGA_{75:25} = PDLLA$	112–132	BA	[86]

Criterion for classifying the nanoparticle size difference: =: difference smaller than 20 nm; <: difference between 21 and 70 nm; ≪: difference between 71 and 120 nm; ⋘: difference larger than 121 nm.

Table 7
Summary of reported studies on the influence of polymer nature on the zeta-potential of submicron spheres prepared by solvent displacement and emulsification–diffusion methods.

Polymer	Organic solvent	Zeta potential (mV)	Reference
Solvent displacement method			
PCL	Acetone	−22 to −29	[14,54,58]
PLA	Acetone	−6 to −50	[43]
PDLLA	Acetone	−20.3 to −67	[14,54,58,66]
PDLLA R206	Acetone	−6 to −10	[66]
PLGA 85:15	Acetone	−23 to −54	[14,51,55]
PLGA 50:50	Acetone	−6 to −10	[46,54,58,66]
PCL-PEG	Acetone	−11	[54,58]
PLA:PEG (low PLA:PEG ratios)	Acetone or ACN	−6 to −14	[43,45]
PLA:PEG (high PLA:PEG ratios)	Acetone or ACN	−18 to −28	[43,45,54,58]
PLGA-PEG (different PLGA:PEG)	Acetone	−4 to− 9	[54,55,58]
PVA-g-PLGA	Acetone	−3.2	[46]
SB-PVA-g-PLGA 10 (different)	Acetone	−18	[46]
Emulsification–diffusion method			
PDLLA	BA	−6	[86]
PLGA 50:50	BA	−5	[86]
PLGA 50:50	EtAc	−28	[96]
PLGA-PEG	EtAc	−24	[96]
Propyl-starch	EtAc	−5 to −8.3	[95]

particles are spherical (Fig. 7 shows typical TEM and AFM micrographs); (3) regardless of the preparation method, the zeta-potential of particles prepared using non-ionic stabilizing agents is always negative due to the presence of terminal carboxylic groups in the polymer molecule; (4) the nature of the polymer influences the size and zeta-potential of particles prepared either by solvent displacement or by emulsification–diffusion; and (5) the size and zeta-potential of particles prepared from the same polymer or the same series of polymers is influenced by other starting materials and operating conditions. The latter conclusion makes it difficult to perform in-depth analysis and make general statements on influence of the polymer on size and zeta-potential of submicron particles on the basis of the data reported. Therefore we performed a controlled study of the impact of the nature of the polymer used on particle size and zeta-potential. The polymers chosen were those commonly used in submicron sphere preparation (PCL, PLGA, and PDLLA).

5.1.1.1. Influence of the nature of polymer on particle size. The nature of polymer influences particle size (Fig. 8). The differences found could be explained by the crystalline and amorphous character of the polymers when re-precipitated after having been solubilised in organic solvent. According to the X-ray diffraction and differential scanning calorimetry studies performed by Leroueil-Le Verger et al. [14], the precipitation by solvent displacement of PLGA and PDLLA exhibit amorphous character while PCL exhibits amorphous as well as crystalline domains.

Although crystalline grade and particle structure depend on specific precipitation conditions (taking into account the analysis carried out by Rastogi and Terry [123] on the behavior of other polymers like polyesters and poly-hydroxy alkanoates), it can be assumed that the semi-crystalline behavior of PCL will produce a larger precipitation nucleus than that obtained from amorphous polymers (PLGA and PDLLA). This is mainly due to the molecular ordering of semicrystalline and amorphous structures. In the case of PCL, the thin crystalline lamellae are separated by amorphous regions and the chains that emerge at the crystalline surfaces with a high degree of molecular alignment must either fold back into the crystallite or stay in the amorphous matrix. This results in a three-phase model consisting of the crystalline and the rigid and mobile amorphous fractions, where the specific volume of the rigid amorphous phase is larger than that of the mobile amorphous phase.

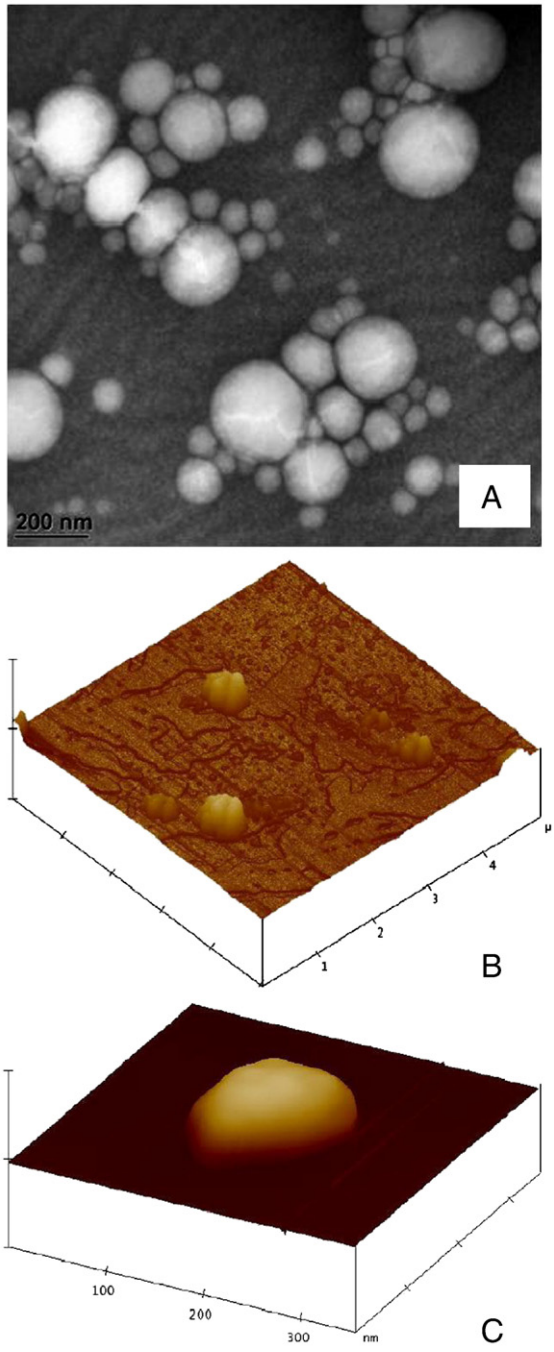

Fig. 7. TEM micrograph of typical PCL spheres prepared by solvent displacement process (A); AFM micrograph of typical spheres prepared by emulsification–diffusion method: from PDLLA (B), from PLGA (C).

It is important to note that when PCL is used for submicron particle preparation by solvent displacement, there is no difference between sphere sizes when different molecular weights are used (Fig. 8A). This is in agreement with the results reported by Lince et al. [75] in their research relating to PCL. Likewise, this conclusion could be inferred for PLGA, in agreement with the results reported by Leroueil-Le Verger et al. [14] for different PLGA (PDLLA85GA15 and PDLLA50GA50) when using acetone as a solvent and PLX 188 0.5% as a stabilizing agent. This behavior suggests that the semi-crystalline or amorphous nature of the re-crystallized polymers predominates more than the difference in polymer molecular weight when the solvent displacement method is used.

Regarding the emulsification–diffusion method, DSC analysis of PDLLA and PLGA submicron particles shows the precipitation of the polymers in

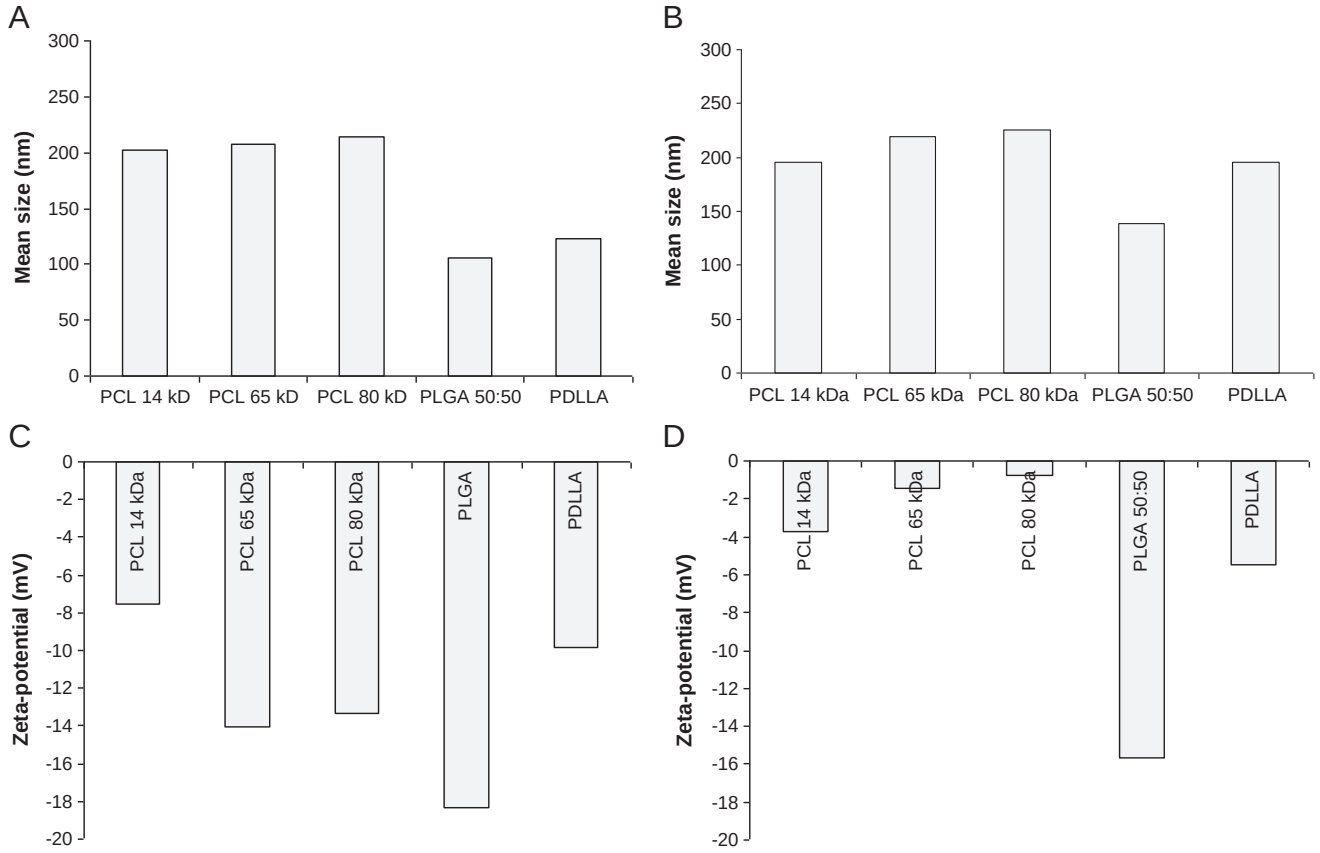

Fig. 8. Influence of polymer nature on the size and the zeta-potential of submicron spheres. A and C: spheres prepared by solvent displacement: polymer concentration 3.5 mg/ml, stabilizing agent PLX 0.4%; B and D: spheres prepared by emulsification–diffusion: polymer concentration 10 mg/ml, stabilizing agent PLX 1%.

amorphous state. However, certain crystallization phenomena are associated with PDLLA precipitation due to the formation of polymer crystallites after 24–72 h [89]. This might explain the larger mean size of PDLLA particles in comparison to PLGA particles. Unfortunately, to our knowledge no works have been reported in which PCL is re-precipitated from ethyl acetate by the emulsification–diffusion method, thus it is not possible to express any opinion on the influence of polymeric arrangements on particle size.

As shown in Fig. 8B, the behavior trends of particle size using different polymers are more marked when the emulsification–diffusion method is used. This is probably due to the polymer concentration used, which is almost three times that used in the solvent displacement method. Thus, when the polymer concentration is high, different behaviors can be observed as a function of the molecular weight of PCL due to molecule size and molecular arrangement during polymer precipitation. Additional study of this aspect showed that mean particle size is similar when using the same polymer concentration regardless of the preparation method used (Fig. 9). Aggregate formation by using the solvent displacement method highlights its limitation regarding the maximum polymer concentration to be used. This was predicted by Stainmesse et al. [30] and explains why comparison between methods is not adequate under the same conditions of polymer concentration. In addition, this limitation entails a disadvantage for the solvent displacement method as the presence of aggregates implies difficulties related to particle yield and purity.

5.1.1.2. Influence of nature of polymer on particle zeta-potential. In general terms, the absolute values of the particle zeta-potential follow the order: PLGA > PDLLA > PCL, which is directly linked to the carboxylic group/alkyl chain ratio per polymer monomer unit. Fig. 8C and D show that the zeta-potential values obtained for submicron particles prepared by solvent displacement are more negative than those obtained from particles prepared by the emulsification–diffusion method. Although these conclusions can be logically supported by the study of the stabilizing agent, as will be discussed below, they only appear valid for polymers such as PCL. The research by Trimaille et al. and Hirsjärvi et al. using the emulsification–diffusion method and the solvent displacement technique respectively [74,87], showed that PDLLA behaves differently. The zeta-potential of particles prepared by solvent displacement is always the lowest. This suggests that the hydrophilic/hydrophobic moiety ratio of the polymeric molecule could influence the electrostatic behavior of particles.

5.1.2. Behavior of polymer concentration

The influence of polymer concentration on submicron particle size is of considerable importance. Fig. 10 shows the behavior of polymer

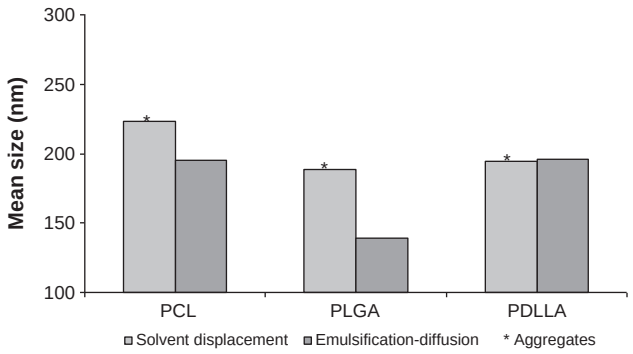

Fig. 9. Influence of polymer nature on size of submicron spheres prepared by solvent displacement and emulsification–diffusion methods (polymer and the stabilizing agent concentrations: 10 mg/ml and 1% respectively).

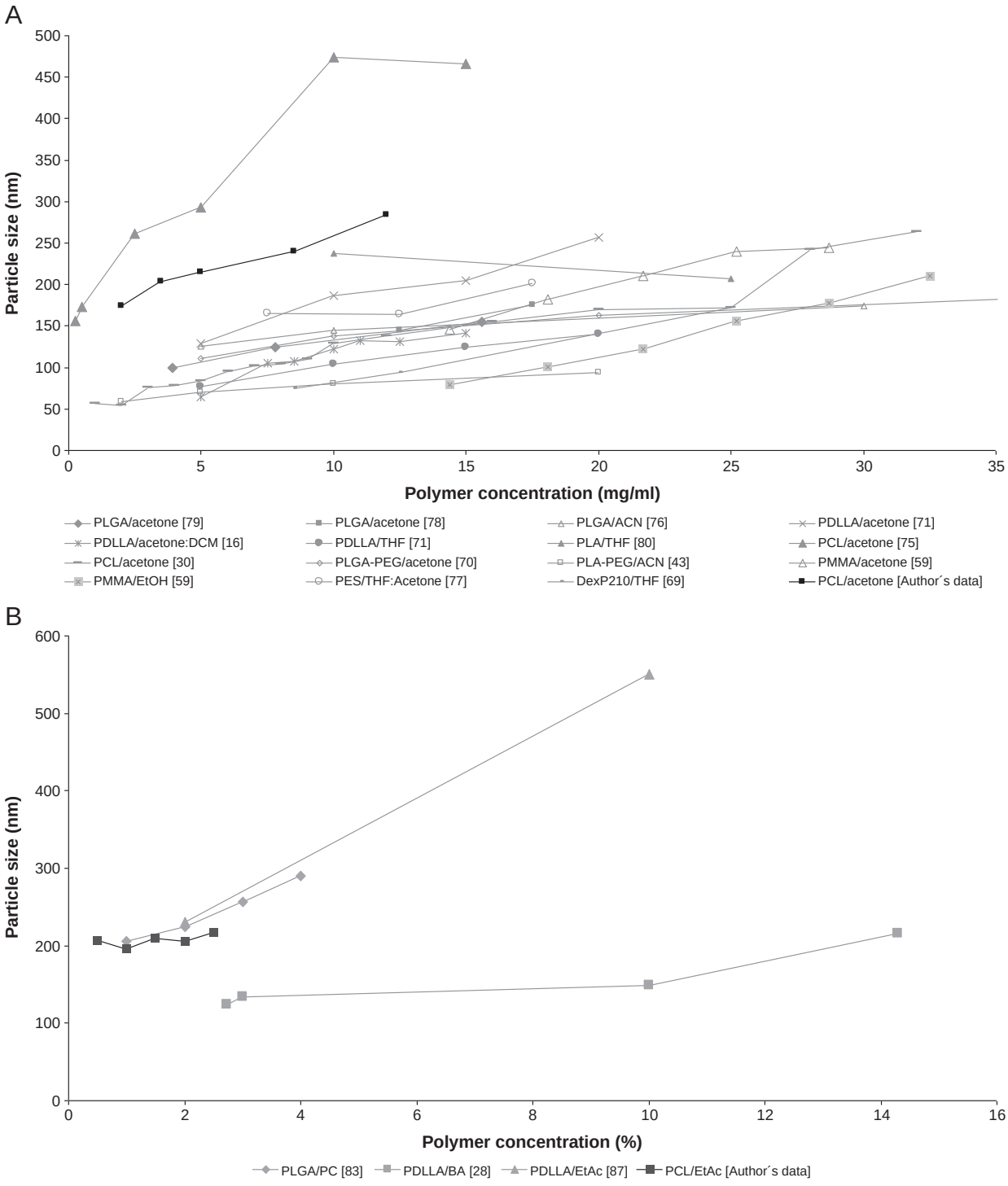

Fig. 10. Influence of polymer concentration on size of submicron spheres prepared by A. solvent displacement process; B. emulsification–diffusion method.

concentration according to sphere size taken from published data and those obtained from our experimental study.

It is obvious that the solvent displacement process is highly sensitive to changes in polymer concentration regardless of the nature of polymer, the other initial materials used or the operating conditions (e.g., in our study, particle sizes are ~170 and 300 nm for the lowest and highest polymer concentrations, respectively). On the other hand, the size of particles prepared by emulsification–diffusion does not undergo significant variations at concentrations lower than 2.5%. Above this value, particle size increases as polymer concentration increases.

Particle size behavior obtained as a function of the method used can be interpreted from two angles: droplet formation and particle formation.

Regarding droplet formation, in the emulsification–diffusion method this depends on high-shear stirring that guarantees droplet formation regardless of the composition of the organic phase. However, if the polymer solution is too concentrated, it can impede solvent diffusion due to higher viscosity in the organic phase and promote the Ostwald ripening phenomenon in the emulsion, leading to an increase in particle size.

Also, in the solvent displacement method the viscosity of the organic phase is highly dependent on polymer concentration even at the lowest

Table 8
Summary of reported studies on the influence of stabilizing agent nature on the size of submicron spheres prepared by solvent displacement and emulsification–diffusion methods.

Stabilizing agent order according to the particle size	Particle size range (nm)	Organic phase	Reference
Solvent displacement method			
$PVA_{98.5\%\ Hydrolyzed} < PVA_{88\%\ Hydrolyzed} < PVA_{80\%\ Hydrolyzed}$	225–290	PLGA:acetone	[36]
Polysorbate 80 < PLX 188 = Triton X100 < Brij 96	131–194	PLA:acetone	[56]
$PLX_{F68} = PLX_{F108}$	180–190	PCL:acetone	[63]
Polysorbate 80 ≪ PVA = PLX 188	220–300	PES:THF	[77]
Emulsification–diffusion method			
DMAB ⋘ PVA	102–260	PLGA:EtAc	[22]
DMAB ⋘ PVA	145–410	PLGA:EtAc	[26]
DMAB < PLX 188 ≪ PVA	67–213	PLGA:EtAc	[90]
PVA ⋘ gelatin	270–730	PMMA:BA	[28]
$PVA_{26\ kDa} < PLX\ 188 = PVA_{30–70\ kDa}$	123–179	PLA:PC	[81]

Criterion for classifying the nanoparticle size difference: =: difference smaller than 20 nm; >: difference between 21 and 70 nm; ≫: difference between 71 and 120 nm; ⋙: difference larger than 121 nm.

values. This has been demonstrated by Thioune et al. [39] and might be explained by the increase in polymer chain association as the polymer concentration increases. However, since solvent displacement is a spontaneous process without additional mechanical energy, polymer chain association could govern nucleation and growth rates. In addition, rapid solvent diffusion towards the aqueous phase could be hindered.

5.2. Influence of the stabilizing agent

Usually, stabilizing agents are recognized as key factors for guaranteeing the physical stability of dispersions of submicron particles; however, this depends on their properties and their role in particle synthesis. Therefore in the following, we consider the performance of the stabilizing agent from the standpoint of the particle preparation method, paying great attention to the impact of typical variables such as the nature and the concentration used on the size and zeta-potential of the particles.

5.2.1. Behavior of the nature of stabilizing agent

Table 8 summarizes data taken from the literature on the effect of the nature of stabilizing agent on the size of submicron particles prepared by the solvent displacement technique and the emulsification–diffusion method. In general terms, these conclusions are in agreement with our experimental results in which PCL was chosen as polymer while PVA, PLX and polysorbate 80 (non-ionic surfactants), and SDS and DTAB (negatively and positively charged surfactants respectively) were the stabilizing agents investigated (Fig. 11). Although poly(ethylene glycol) (2000, 4600 and 10,000) and dextran (T500 and T2000) have been used as steric stabilizing agents for obtaining poly(alkylcyanoacrylates) (PACA) particles [34], they did not exhibit any stabilizing effects in our study. They perhaps require a higher concentration or synergistic effect with another steric or "electro-steric" stabilizing agent.

5.2.1.1. Influence of the nature of stabilizing agent on particle size. The mean sizes of spheres are significantly dependent on the nature of stabilizing agent, with similar trends for the two preparation methods. In addition, in all cases adequate stabilization was obtained for the particles as aggregates were not detected.

It should be taken into account that the role of the stabilizing agent differs as a function of preparation method. In the solvent displacement method, the stabilizing agent prevents aggregation during particle formation without significantly affecting droplet formation. This is due to the high initial spreading coefficient of the organic solvent (e.g., 42.4 dyn/cm at 20 °C for acetone [124]) which

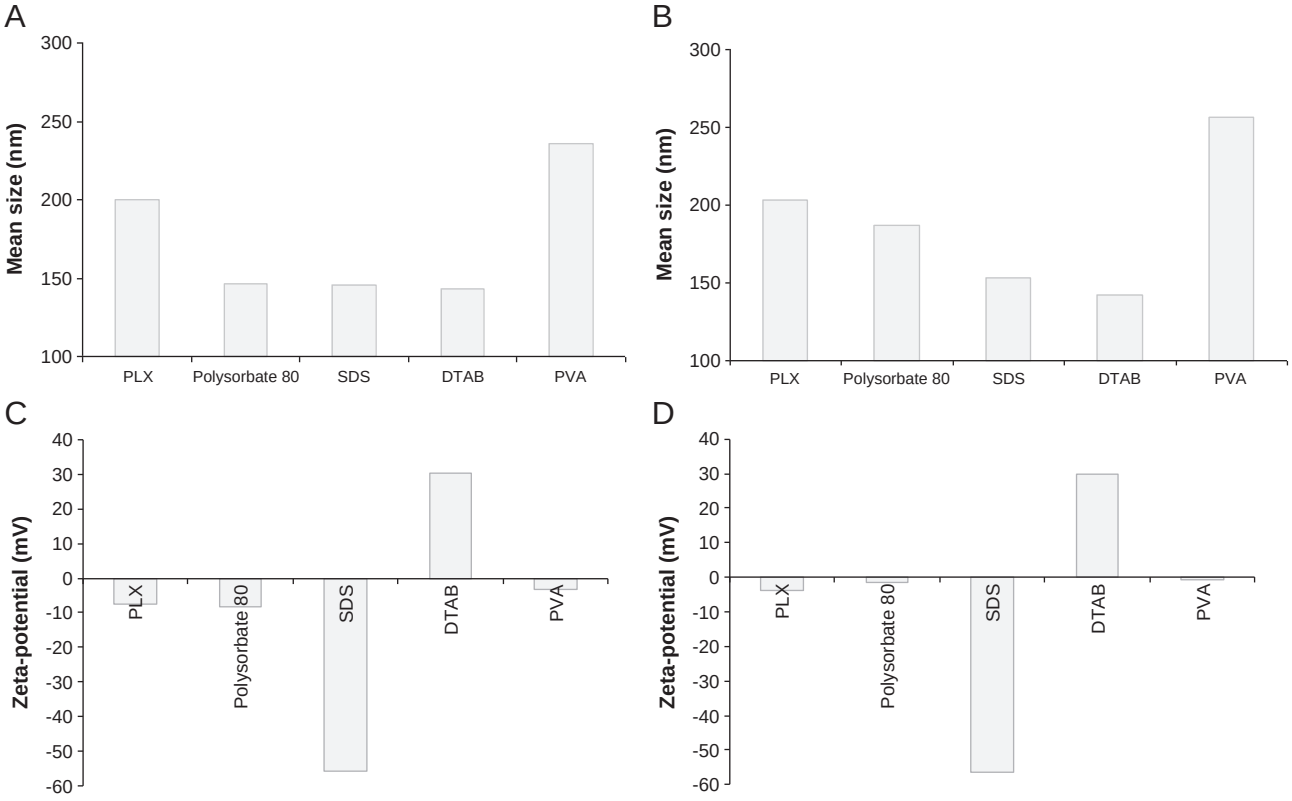

Fig. 11. Influence of the stabilizing agent nature on mean size and zeta-potential of submicron spheres. A and C: spheres prepared by solvent displacement: polymer concentration 3.5 mg/ml, stabilizing agent 1%; B and D: spheres prepared by emulsification–diffusion: polymer concentration 10 mg/ml, stabilizing agent 1%.

guarantees efficient solvent–water interaction when organic and aqueous phases are brought into contact.

Therefore the performance of stabilizing agents is governed by their electrostatic, steric and electro-steric effects [125]. As shown in Fig. 11, the size of the particles prepared by solvent displacement decreases, following the order PVA > PLX > Polysorbate 80 = SDS = DTAB. PVA, PLX and polysorbate 80 have a predominantly steric effect, whereas SDS and DTAB exhibit an electro-steric effect. This suggests that stabilizing agents with an electro-steric effect are adequate for obtaining smaller particle sizes. In addition, the steric effect might delay solvent diffusion, thereby favoring particle growth. In fact, in the particular case of PVA, Murakami et al. [36] suggest the localized gelatinization of PVA due to a kind of acetone–PVA interaction. Such interaction occurs preferentially on the surface of particles, delaying solvent migration.

Unlike the solvent displacement process, the stabilizing agent in the emulsification–diffusion method acts as a surfactant in droplet formation and as a stabilizer of particles during their formation. Thus the stabilizing agent is adsorbed on the solvent–water interfacial area formed during the emulsification step, while the remaining quantity contributes towards preventing particle aggregation in the dilution step. Consequently, the performance of a stabilizing agent is governed by its ability to lower the interfacial tension between aqueous and organic phases, which in turn depends on the ability of the hydrophobic moiety of the molecule to bind to the organic phase and on that of its hydrophilic part to remain in the aqueous medium. In addition, the steric, electrostatic and electro-steric effects are also important for preventing polymer aggregation. The behavior of the submicron particles obtained confirms that efficient reduction of interfacial tension combined with electro-steric effects permits obtaining smaller particle sizes. Therefore particle size decreases as follow: PVA > PLX > Polysorbate 80 > SDS = DTAB.

Regardless of the method for preparing submicron particles and taking into account the surfactant absorption mechanisms studied by Zhang and Somasundaran [126], the hydrophobic segment of the polymer and the hydrophobic moieties of the stabilizing agent interact via hydrophobic interaction when the stabilizing agents are non-ionic (PLX, polysorbate 80 and PVA) or negatively charged (SDS). Positively charged molecules such as DTAB exhibit both attractive electrostatic and hydrophobic interactions with the polymer.

5.2.1.2. Influence of the nature of stabilizing agent on particle zeta-potential. The data in the literature leads to the sole assumption that non-ionic stabilizing agents have no impact on the zeta-potential of particles prepared by solvent displacement (Table 9). However, comparative analyses between the methods for the particular case of particles prepared from PCL can be established on the basis of our experimental work (Fig. 11C and D). The values obtained from the submicron spheres prepared by the solvent displacement process by using non-ionic stabilizing agents are more negative than those obtained from the emulsification–diffusion method. As mentioned above, a similar result was obtained when investigating the nature of the polymer (Fig. 8). There are two possible explanations for this. The first is based on the polymer–stabilizing agent ratio while the second takes into account the role of the stabilizing agent in the droplet formation as a function of the preparation method.

Table 9
Summary of reported studies on the influence of stabilizing agent nature on the zeta-potential of submicron spheres prepared by solvent displacement and emulsification–diffusion methods.

Stabilizing agent	Organic phase	Zeta potential (mV)	Reference
Solvent displacement method			
PLX 188	PLA:acetone or PCL:acetone	−15 to −34.4	[56,63]
PLX 188	PLA:acetone	−25	[74]
Without stabilizer		−34	
Triton X100	PLA:acetone	~−32.9	[56]
Brij 96	PLA:acetone	~−30.4	[56]
Polysorbate 80	PLA:acetone	~−31.3	[56]
Emulsification–diffusion method			
PVA	PLGA:EtAc	−1.4 to −5.8	[22,26]
PVA	Eudragit S100:BA	~−50	[84]
DMAB	PLGA:EtAc	+75 to +80	[22,26]

It was found that the polymer–stabilizing agent ratio was 1:2.3 for the solvent displacement method and 1:4 for the emulsification–diffusion process. It is known that stabilizing agents such as PLX and PVA are adsorbed on the particles, stabilizing the polymer–water interface during preparation [87,127,128] and the charge and potential distribution of the electric double layer surrounding the particle may be affected by the presence of the polymer adlayer [127]. The work reported by Hirsjärvi et al. [74] highlights a difference in zeta-potential between stabilizing agent–free PDLLA particles and those prepared from PLX aqueous dispersion (Table 9). Thus the higher quantity of stabilizing agent used in the emulsification–diffusion method in comparison to the solvent displacement process could form a dense steric barrier making it difficult to detect the negative polymer charge.

By taking into consideration the role of the stabilizing agent as a function of the preparation method in the emulsification–diffusion method, as mentioned above, it can be seen that the stabilizing agent takes part in droplet formation, perhaps leading to stronger polymer–stabilizing agent interaction. It can be presumed that as a result of this interaction, some stabilizing agent molecules can be mechanically trapped by the structure of the particle, particularly at its surface, masking the negatively charged polymer and reducing the negative electrical behavior of the particles. When the solvent displacement method is used, polymer–stabilizing agent interaction is probably less due to the major role of the stabilizing agent which is to prevent particle aggregation. Thus, the negative polymer groups can be highly exhibited. Additional considerations regarding this point will be expressed below from the standpoint of the influence of the solvent on particle zeta-potential.

5.2.2. Behavior of stabilizing agent concentration

Although submicron particles can be prepared without stabilizing agents as they are stabilized by the electrostatic repulsion of their surface charge [74,97], the use of a stabilizing agent is strongly advised since it prevents aggregate formation and contributes to system stability [34,58,129]. Consequently, determining the optimal concentration becomes a variable of interest in the study of solvent displacement and emulsification–diffusion methods.

As shown in Fig. 12, it is clear that the concentration of the stabilizing agent does not have a significant effect on the mean size of particles when the solvent displacement process is used. On the contrary, the concentration of the stabilizing agent affects particle size when using the emulsification–diffusion method.

Once again, these results might be due to the role of the stabilizing agent as a function of the droplet formation mechanism and the extent to which the stabilizing agent participates in it. Since the stabilizing agent does not take part in droplet formation by solvent displacement, its effect on sphere size is neglected. However, regarding the emulsification–diffusion method, the extent to which the stabilizing agent takes part in emulsion formation might be governed by the extent of organic phase–stabilizing agent affinity, in addition to the emulsifying capacity of the stabilizing agent. For instance, Quintanar et al. [81] reported drastic particle size reduction (from 450 nm up 160 nm) as a function of stabilizing agent concentration in a system composed of PDLLA as polymer, propylene carbonate as organic solvent and PLX (0.5–15%) as stabilizing agent, at 8000 rpm for 10 min in the emulsification step. Nevertheless, in our results using PCL/PLX (0.5–5%)/EtAc, particle size reduction was only from 140 nm up 90 nm. Unlike ethyl acetate, propylene carbonate has a major solubility parameter (δ_{EtAc}: 18.2 $MPa^{1/2}$; δ_{PC}: 27.2 $MPa^{1/2}$

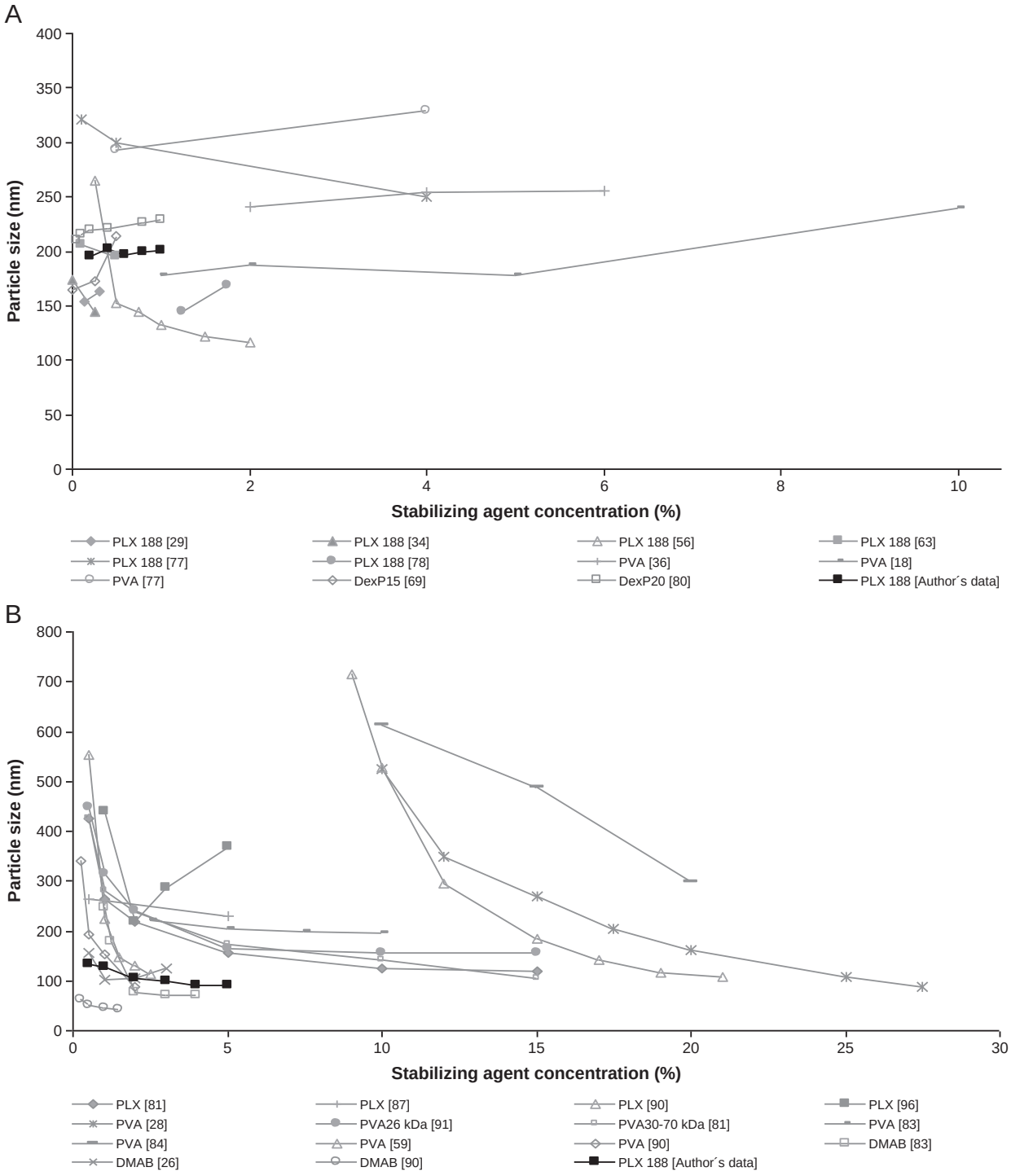

Fig. 12. Influence of stabilizing agent concentration on size of submicron spheres prepared by: A. solvent displacement method; B. emulsification–diffusion method.

[130]), which may facilitate solvent–stabilizing agent interactions and make the emulsification process more efficient.

Leroux et al. [28] also reported drastic reduction of particle sizes (from 500 nm to 100 nm) when the emulsion was prepared from PDLLA/benzyl alcohol/PVA (10–30%) at 1200 rpm stirring speed. In this case, the low emulsion stirring speed could be compensated by both the solvent–stabilizing agent interactions favored ($\delta_{\text{Benzyl alcohol}}$: 23.7 $MPa^{1/2}$ [130]) and the high concentration of stabilizing agent, which reduces the interfacial tension between the organic and aqueous phases [62]. Other examples of this can be found in the works reported by Kwon et al. [83] who used a PLGA–propylene carbonate–PVA (2.5–10%) system at a high emulsification rate (non-specified) for 7 min; Galindo et al. [62], who used PMMA L100-55–benzyl alcohol–PVA (8–20%) at 2000 rpm for 15 min; and Song et al. [90] who used PLGA–propylene carbonate and PVA or PLX as stabilizing agents (0.5–2.5%) and an emulsification process using the ultrasound technique.

5.3. Influence of the solvent

In this review the study of the solvent's influence on the size and zeta-potential of submicron particles has taken a global view of the

polymer/stabilizing agent/solvent system. In what follows, despite certain constraints, this approach provides us with very interesting evidence that contributes to elucidating the mechanistic aspects relating to particle formation.

5.3.1. Influence of the nature of solvent on particle size

Different approaches from the physicochemical point of view have been investigated in order to understanding the particle size behavior obtained when polymer, organic phase and water interact during the particle preparation. For instance, for the solvent displacement method, Stainmesse et al. used the organic solvent dielectric constant [30]; Galindo et al. used the solvent/water interactions [62]; Ganachaud and Katz used solvent/water solubility parameter difference [102], and Legrand et al. and Thioune et al. used polymer–solvent interactions [39,71]. Regarding the emulsification–diffusion method, the effect of solvents was analyzed from the standpoint of solvent–water solubility [90], solvent–polymer interactions, the solvent–water diffusion coefficient [85] and from that of molecular descriptors of solvent hydrophilicity [131]. In both solvent displacement and emulsification–diffusion methods, researchers have found certain correlations between particle size and the physicochemical parameters chosen. In addition, Murakami et al. [132] demonstrated that polymer–solvent affinity influences the size and yield of particles, by using a method that combines solvent displacement and emulsification–diffusion.

In this review we have chosen polymer/solvent/nonsolvent interactions and the physicochemical properties of organic solvent to obtain an overall view of the data available in the literature. Both solubility parameter difference ($\Delta\delta$) and interaction parameter (χ) were used for illustrating the behaviors reported in the largest number of cases possible. We are aware that this approach may lead to misinterpretations of behavior, mainly due to the different experimental conditions and formulations used in each study reported and to the assumption that polymers/solvents/nonsolvents are the most important components, whereas the effect of other starting materials such as the active substance or the stabilizing agent are omitted. However, we run this risk because it is offset by the possibility of obtaining evidence on the influence of thermodynamic properties on submicron particle characteristics, thus making a contribution to the discussion on the mechanisms proposed for particle formation.

The solubility parameters of the solvents (δ), solvent mixtures and polymers were searched in the literature or recalculated. From these values, $\Delta\delta$ and χ were estimated for the pair polymer–solvent and solvent–water (Tables 10 and 11). The method of group contribution proposed by van Krevelen for determining the polymer solubility parameter [133,134], the calculation of the solubility parameter of solvent mixtures assuming additive behavior, as proposed by Martin and Bustamente [135], and the calculation of the interaction parameter according to Peppas procedure [116], were used in this review. The approximations involved in each of these methods have been shown to be valid and are good tools for obtaining practical information. The results are shown graphically to facilitate analysis (Figs. 13 and 14). Certain differences were detected between our results and those reported by other teams. They are due to differences in the bibliographical sources used for data such as solubility parameters and do not substantially modify the general conclusions reported.

As can be seen in Figs. 13A–B and 14A–B, $\Delta\delta_{\text{polymer–solvent}}$ between 1 and 15 $MPa^{1/2}$ and $\Delta\delta_{\text{solvent–water}}$ between 20 and 40 $MPa^{1/2}$ can be used for preparing submicron particles by the two methods. It seems that the thermodynamic criterion required for polymer solubility and solvent diffusion are satisfied in these wide ranges of $\Delta\delta$. However, none of these physicochemical parameters clearly interacts with particle size.

Regarding χ, a theoretical view is necessary in order to facilitate the interpretation of the results. Lower $\chi_{\text{solvent–water}}$ values mean better solvent–water affinity which is favorable for solvent diffusion. On the other hand, higher $\chi_{\text{polymer–solvent}}$ values can also facilitate solvent diffusion. According to the above, we could expect that in terms of particle size, lower $\chi_{\text{solvent–water}}$ values and higher $\chi_{\text{polymer–solvent}}$ values lead to the smallest size.

As shown in Fig. 13C, the behavior of $\chi_{\text{polymer–solvent}}$ estimated for the systems used by the solvent displacement technique do not correlate clearly with particle size, probably because of the lower polymer concentration commonly used by this method. On the other hand, in some cases $\chi_{\text{solvent–water}}$ displays interaction with particle size, although this trend generally does not highlight any correlation (Fig. 13D). Indeed, it was difficult to suggest any mechanistic interpretation. Some explanation could be given in terms of total solvent–water miscibility which guarantees fast phase mixing making the impact of solvent diffusion irrelevant. Therefore, particle size is governed by parameters related to the polymer and stabilizing agent as mentioned above. However, taking into account that particle size depends on the organic phase/aqueous phase ratio, particularly at the highest values of this variable (see Section 4.1), it can also be suggested that in these cases, the ease of solvent diffusion is a critical factor. Thus, given that the nature of the solvent has no influence in some cases, whereas the amount of solvent does have an impact in others, it is possible to propose once again that particle formation by the solvent displacement technique could be carried out simultaneously via the two mechanistic approaches (i.e. nucleation and mechanically), the most relevant mechanism depends on the phase ratio and the composition of the system.

In the emulsification–diffusion method, both $\chi_{\text{polymer–solvent}}$ and $\chi_{\text{solvent–water}}$ appear to maintain a correlation with particle size (Fig. 14C and D). Thus major polymer–solvent and solvent–water affinities lead to the largest particle sizes. This appears logical from the point of view of the mechanistic approach which proposes particle formation from one emulsion droplet. Therefore higher polymer–solvent affinity causes solvent diffusion difficulties that might lead to incomplete solvent migration towards the external phase. Consequently, the particle sizes are the largest. Coincidentally, the two largest particle sizes seen in the charts were obtained by using DCM as a solvent. Its very low water solubility compared with other solvents can make complete solvent dissolution in water difficult, promoting the Ostwald ripening phenomenon. If these results are removed, it is can be seen that polymer–solvent interactions govern particle size without major influence on solvent–water interaction.

In addition, the influence of the solvent on the size of submicron particles prepared by the two methods can also be analyzed through the physicochemical properties of the organic solvent. To this end the data obtained in our systematic study was used to guarantee that organic solvent was the sole experimental variable and that the effect of the polymer could be neglected due to its constant concentration in the organic phase. Table 12 compiles the solvent properties that can affect particle formation (density, viscosity, surface tension and water solubility) and includes a preliminary qualitative analysis facilitating discussion. As can be seen, particle size does not correlate with solvent properties when the solvent displacement method is used. However, good agreement between solvent physicochemical properties and particle size is observed for the emulsification–diffusion method. Thus the lowest values of density, viscosity and surface tension provide the smallest particle sizes. For the particular case of PC, its water solubility can overcome the difficulties associated with high density, viscosity and surface tension values. Therefore these results further support the idea that solvent physicochemical properties do not have a critical impact on particle formation when using the solvent displacement procedure and working with low phase ratios. It also supports the hypothesis that the ease of emulsion formation is the critical factor for obtaining

Table 10
Some physicochemical parameters related to the solvent/polymer/nonsolvent systems used for the sphere preparation by solvent displacement method.

Solvent	Polymer	Solvent molar volume (ml/mol)[a]	Solvent solubility parameters ($MPa^{1/2}$)[b]				Polymer solubility parameters ($MPa^{1/2}$)[c]				$\Delta\delta_{polymer-solvent}$ ($MPa^{1/2}$)[d]	$\Delta\delta_{solvent-water}$ ($MPa^{1/2}$)[d]	$\chi_{polymer-solvent}$[e]	$\chi_{solvent-water}$[e]	Size (nm)	Reference
			δ	δd	δd	δh	δ	δd	δd	δh						
Acetone:EtOH (99.5:0.5)	PCL 14 kDa		20.1	15.5	10.4	7.1					6.0	35.8			100	[30]
Acetone:EtOH (97:3)			20.3	15.5	10.4	7.4					5.9	35.5			99	
Acetone:EtOH (93:7)			20.6	15.5	10.3	7.9					5.8	35.0			98	
Acetone:EtOH (90:10)			20.8	15.5	10.2	8.2	19.7	17.2	4.8	8.3	5.7	34.6			105	
Acetone:EtOH (85:15)			21.1	15.5	10.2	8.9					5.6	34.0			115	
Acetone:EtOH (80:20)			21.4	15.6	10.1	9.5					5.7	33.4			162	
Acetone:EtOH (75:25)			21.7	15.6	10.0	10.1					5.7	32.9			215	
EtOH:W (7/3 v/v)	Gliadin		33.0	15.7	11.0	26.3									470	[32]
MetOH:W (8/2 v/v)			33.3	15.2	13.0	26.3									742	
Acetone:W (5/5 v/v)			34.0	15.5	13.2	24.	34.5[f]								466	
Propan-1-ol:W (5/5 v/v)			36.3	15.8	11.4	29.9									1000	
Propan-2-ol:W (5.5/4.5 v/v)			34.5	15.7	10.6	28.1									772	
EtOH : W Mixture solubility parameter	Gliadin		32.9				34.5[f]								173	[40]
			34.0												182	
			34.5												157	
			35.0												338	
			36.0												278	
			37.8												374	
Acetone	SB-PVA-g-PLGA	74.0	20.1	15.5	10.4	7.0									88	[46]
Acetone:EtAc (99:1)			20.1												75	
Acetone:EtAc (98:2)			20.1												73	
Acetone:EtAc (95:5)			20.0												75	
Acetone:EtAc (91:9)			20.0												88	
Acetone:EtAc (84:16)			19.9												108	
Acetone:EtAc (75:25)			19.7												138	
Acetone:EtAc (72:28)			19.7												293	
Acetone:EtAc (70:30)			19.6												402	
Acetone:EtAc (67:33)			19.6												550	
Acetone:EtOH (6:4)	PLGA 85:15		22.7	15.6	9.8	12.0					1.1	31.1			261	[47]
Acetone:MetOH (6:4)			23.9	15.3	11.2	13.1					1.7	29.7			266	
ACN:EtOH (50:50)			21.0	16.9	7.5	22.0	23.0	16.5	10.4	12.2	10.2	22.2			244	
ACN:EtOH (60:40)			19.8	17.1	7.2	22.5					10.8	21.8			240	
ACN:EtOH (70:30)			18.7	17.3	6.9	23.0					11.4	21.5			247	

Acetone: DCM (19.5:0.5)	PDLLA			20.1	15.6	10.3	7.0					4.6	35.9			114	[16]
Acetone:DCM:EtOH (19.0:0.5:0.5)				20.3	15.6	10.3	7.3					4.3	35.6			105	
Acetone:DCM:EtOH (18.5:0.5:1.0)				20.4	15.6	10.2	7.6					4.0	35.3			97	
Acetone:DCM:EtOH (17.5:0.5:2.0)				20.8	15.6	10.1	8.2					3.4	34.7			90	
Acetone:DCM:EtOH (16.5:0.5:3.0)				21.1	15.6	10.1	8.8					2.8	34.1			64	
Acetone:DCM:EtOH (15.5:0.5:4.0)				21.4	15.6	10.0	9.5					2.2	33.5			54	
CHCl3: acetone	PLA			24.0	17.0	6.5	12.0					2.7	31.9			260	[52]
CHCl3:MetOH				25.6	17.0	6.8	14.6	21.7	16.2	8.0	11.3	4.0	29.3			200	
CHCL3:EtOH				25.1	17.1	6.2	14.1					4.0	30.0			270	
Ethyl lactate	PMMA	115.0		21.7	16.0	7.6	12.5							31.1	32.2	178	[59]
Acetone		74.0		20.1	15.5	10.4	7.0							35.8	23.4	146	
Isopropyl alcohol		76.8		23.5	15.8	6.1	16.4	18.6 to 26.4						27.8	18.8	101	
DMSO		71.3		26.6	18.4	16.4	10.2							32.3	13.4	97	
EtOH		58.5		26.6	15.8	8.8	19.4							24.1	11.1	79	
Acetone	PCL-PEG	74.0		20.1	15.5	10.4	7.0					6.2	35.8	0.4	23.4	336	[60]
THF		81.7		19.4	16.8	5.7	8.0	19.6	17.1	4.6	8.5	1.2	38.9	0.4	27.1	458	
DMF		77.0		24.8	17.4	13.7	11.3					9.5	31.2	1.2	16.9	276	
DMF	PLGA-b-PEG	77.0		24.8	17.4	13.7	11.3					5.0	31.2	0.5	16.9	83	[70]
Acetone		74.0		20.1	15.5	10.4	7.0	22.7	17.3	8.7	11.8	5.4	35.8	0.6	23.4	138	
ACN		52.6		24.6	15.3	18.0	6.1					11.1	36.4	0.4	11.9	165	
THF		81.7		19.4	16.8	5.7	8.0					4.9	35.9	0.7	27.1	144	
Acetone	PLGA 50:50	74.0		20.1	15.5	10.4	7.0	23.0	16.5	10.4	12.2	5.3	35.8	0.6	23.4	165	[76]
ACN		52.6		24.6	15.3	18.0	6.1					9.8	36.4	0.4	11.9	164	
Acetone	PLGA	74.0		20.1	15.5	10.4	7.0					5.3	35.8	0.6	23.4	140	[79]
ACN		52.6		24.6	15.3	18.0	6.1	23.0	16.5	10.4	12.2	9.8	36.4	0.4	11.9	148	
THF	PLA	81.7		19.4	16.8	5.7	8.0					6.3	35.9	0.8	27.1	185	[80]
THF		81.7		19.4	16.8	5.7	8.0	21.7	16.2	8.9	11.3	4.6	35.9	0.5	27.1	237	
Acetone		74.0		20.1	15.5	10.4	7.0					4.6	35.8	0.4	23.4	220	
Acetone	PCL	74.0		20.1	15.5	10.4	7.0					6.0	35.8	0.4	23.4	203	[Author's data]
THF		81.7		19.4	16.8	5.7	8.0					1.0	35.9	0.4	27.1	192	
ACN		52.6		24.6	15.3	18.0	6.1	19.7	17.2	4.8	8.3	13.5	36.4	0.9	11.9	197	
DMF		77.0		24.8	17.4	13.7	11.3					9.4	31.2	1.2	16.9	180	

[a] Reference: Van Krevelen and te Nijenhuis [133].

[b] Data for pure solvents from Grulke [130]; data for solvent mixtures $= \sum(f_s\ \delta_s)$; where f_s: volume solvent fraction and δ_s: solubility parameter of solvent.

[c] Solubility parameter calculated by group contribution method according to van Krevelen–Hoftyzer procedure [133].

[d] Solubility parameter difference between substanceA and substanceB $(\Delta\delta) = [(\delta_{d,A} - \delta_{d,B})^2 + (\delta_{p,A} - \delta_{p,B})^2 + (\delta_{h,A} - \delta_{h,B})^2]^{1/2}$ where substanceA and substanceB refer to any polymer, solvent or water that correspond.

[e] Interaction parameter $\chi_{\text{substanceA}-\text{substanceB}} = 0.35 + [V_{\text{solvent}}/(RT)](\delta_{\text{substanceA}-\text{dsubstanceB}})^2$ where substanceA and substanceB refer to any polymer, solvent or water as correspond, V is the molar volume of the organic solvent, R is the gas constant, T is the temperature, and $\delta_{\text{substanceA}}$ and $\delta_{\text{substanceB}}$ are the total solubility parameters of any polymer, solvent or water that correspond.

[f] Data reported by Duclaroir et al. [40].

Table 11
Some physicochemical parameters related to the solvent/polymer/nonsolvent systems used for the sphere preparation by emulsification–diffusion method.

Solvent	Polymer	Solvent molar volume (ml/mol)[a]	Solvent solubility parameters ($MPa^{1/2}$)[b]				Polymer solubility parameters ($MPa^{1/2}$)[c]				$\Delta\delta_{polymer-solvent}$ ($MPa^{1/2}$)[d]	$\Delta\delta_{solvent-water}$ ($MPa^{1/2}$)[d]	$\chi_{polymer-solvent}$[e]	$\chi_{solvent-water}$[e]	Size (nm)	Reference
			δ	δd	δp	δh	δ	δd	δp	δh						
BA	PLGA 50:50	103.6	23.7	18.4	6.3	13.7	23.0	16.5	10.4	12.2	4.8	30.4	0.4	24.8	263	[85]
PC		85.0	27.2	20.1	18.0	4.1					11.7	38.6	1.0	15.1	152	
MEK		90.1	19.0	16.0	9.0	5.1					7.3	38.0	0.9	30.7	126	
EtAc		98.5	18.2	15.8	5.3	7.2					7.2	36.8	1.3	35.4	119	
EtAc	PLGA 50:50	98.5	18.2	15.8	5.3	7.2	23.0	16.5	10.4	12.2	7.2	36.8	1.3	35.4	64	[92]
DCM		63.9	20.3	18.2	6.3	6.1					7.5	37.7	0.5	20.0	502	
CHCl3		80.7	19.0	17.8	3.1	5.7					9.9	39.0	0.9	27.6	201	
EtAc:DCM (20:80)			19.9	17.7	6.1	6.3					7.4	37.5			266	
PC	PLGA 75:25	85.0	27.2	20.1	18.0	4.1	23.0	16.5	10.4	12.2	11.7	38.6	1.0	15.1	213	[90]
EtAc		98.5	18.2	15.8	5.3	7.2					7.2	36.8	1.3	35.4	117	
Acetone		74.0	20.1	15.5	10.4	7.0					5.3	35.8	0.6	23.4	221	
DCM		63.9	20.3	18.2	6.3	6.1					7.5	37.7	0.5	20.0	461	
CHCl3:EtOH (100:0)	PHBHV 23 kDa.		19.0	17.8	3.1	5.7	20.1	16.6	6.3	9.5	5.1	39.0			896	[94]
CHCl3:EtOH (90:10)			19.8	17.6	3.7	7.1					3.7	37.5			780	
CHCl3:EtOH (70:30)			21.3	17.2	4.8	9.8					1.6	34.5			540	
CHCl3:EtOH (60:40)			22.0	17.0	5.4	11.2					2.0	33.0			421	
CHCl3:EtOH (50:50)			22.8	16.8	6.0	12.6					3.1	31.5			356	
CHCl3:EtOH (40:60)			23.6	16.6	6.5	13.9					4.4	30.0			335	
CHCl3:EtOH (30:70)			24.3	16.4	7.1	15.3					5.8	28.6			253	
EtAc	PLGA 50:50	98.5	18.2	15.8	5.3	7.2	23.0	16.5	10.4	12.2	7.2	36.8	1.3	35.4	257	[22]
DCM:EtAc (50:50)			19.3	17.0	5.8	6.7					7.2	37.2			414	
DCM:EtAc (60:40)			19.5	17.2	5.9	6.5					7.3	37.3			452	
CHL:EtAc (50:50)			18.6	16.8	4.2	6.5					8.5	37.9			372	
Acetone:EtAc (50:50)			19.2	15.7	7.9	7.1					5.8	36.2			230	
Acetone:EtAc (60:40)			19.3	15.6	8.4	7.1					5.6	36.1			255	
EtAc	PCL	98.5	18.2	15.8	5.3	7.2	19.7	17.2	4.8	8.3	1.8	36.8	0.4	35.4	203	[Author's data]
PC		85.0	27.2	20.1	18.0	4.1					14.2	38.6	2.3	15.1	215	
EMK		90.1	19.0	16.0	9.0	5.1					5.4	38.0	0.4	30.7	115	
Water		18.0	47.9	15.5	16.0	42.4										

[a] Reference: Van Krevelen and te Nijenhuis [133].
[b] Data for pure solvents from Grulke [130]; data for solvent mixtures $= \sum(f_s\ \delta_s)$; where f_s: volume solvent fraction and δs: solubility parameter of solvent.
[c] Solubility parameter calculated by group contribution method according to van Krevelen-Hoftyzer procedure [133].
[d] Solubility parameter difference between substanceA and substanceB $(\Delta_d) = [(\delta_{d,A} - \delta_{d,B})^2 + (\delta_{p,A} - \delta_{p,B})^2 + (\delta_{h,A} - \delta_{h,B})2]^{1/2}$ where substanceA and substanceB refer to any polymer, solvent or water that correspond.
[e] Interaction parameter $\chi_{substanceA-substanceB} = 0.35 + [V_{solvent}/(RT)](\delta_{substanceA} - \delta_{substanceB})^2$ where substanceA and substanceB refer to any polymer, solvent or water that correspond, V is the molar volume of the organic solvent, R is the gas constant, T is the temperature, and $\delta_{substanceA}$ and $\delta_{substanceB}$ are the total solubility parameters of any polymer, solvent or water that correspond.

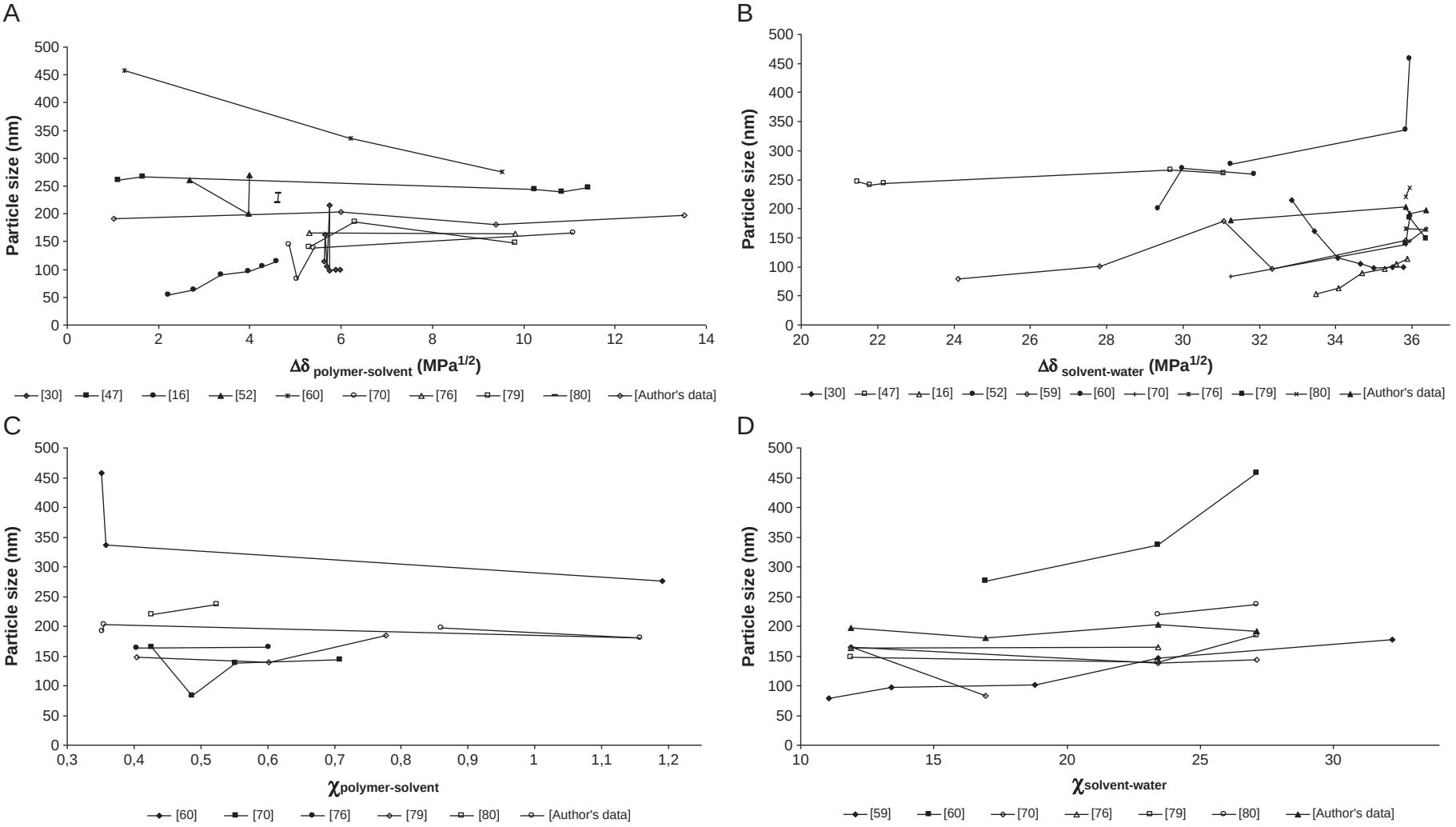

Fig. 13. Influence of some physicochemical parameters related to the solvent/polymer/nonsolvent systems on the size of particles prepared by solvent displacement.

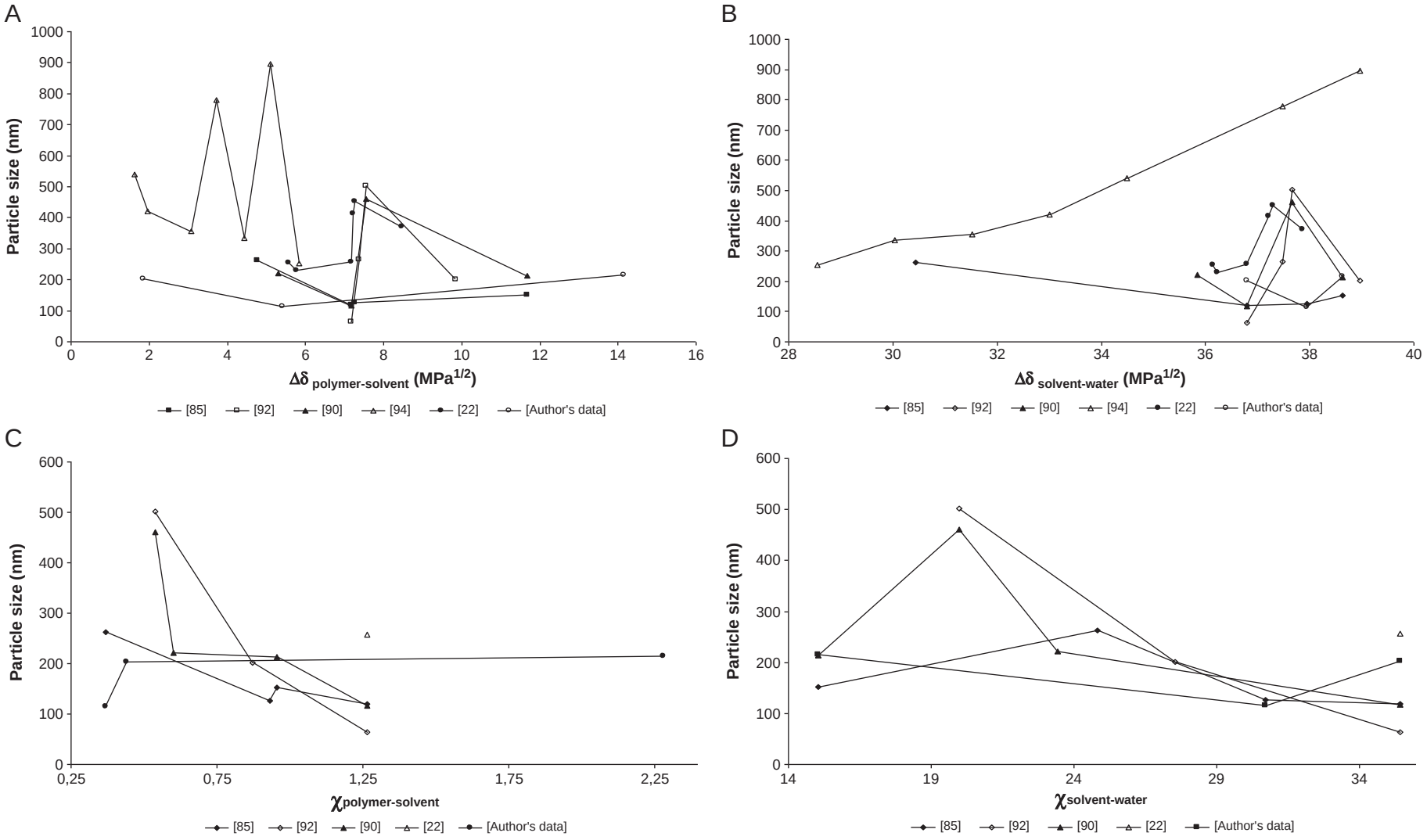

Fig. 14. Influence of some physicochemical parameters related to the solvent/polymer/nonsolvent systems on the size of particles prepared by emulsification–diffusion.

Table 12
Properties of the solvents commonly used in the solvent displacement and emulsification–diffusion methods and preliminary comparative analysis respect to the particle size behavior.

	Solvent displacement method				Emulsion–diffusion method		
	Acetone	THF	ACN	DMF	EtAc	PC	MEK
ρ (g/cm^3; 20 °C)[a]	0.792	0.888	0.783	0.949	0.901	1.201	0.805
η (mPa s; 25 °C)[a]	0.32	0.36	0.35	0.80	0.44	2.8	0.42
γ (10^{-3}; N/m; 20 °C)[a]	23.7	26.4	29.3	~36.76/~38	23.9	40.5	~24.3
Water solubility (%, 25 °C)	Miscible[b]	Miscible[b]	Miscible[b]	Miscible[b]	8.2[c]	21.7[c]	27.5[b]
ρ solvent order	DMF>THF>ACN≈Acetone				PC>EtAc>MEK		
η solvent order	DMF>THF≈ACN≈Acetone				PC>>EtAc≈MEK		
γ solvent order	DMF≫ACN>THF>Acetone				PC>>EtAc≈MEK		
Particle size order	Acetone≈ACN≈THF≈DMF				PC>EtAc≈MEK		

[a] Reference: [133].
[b] Reference: [136].
[c] Reference: [137].

specific particle sizes when the emulsification–diffusion method is used.

5.3.2. Influence of the nature of solvent on particle zeta-potential

The results compiled under the subheadings devoted to the analysis of natures of polymer and stabilizing agent suggest that the nature of the organic solvent might play a role in the electrostatic behavior of submicron particles, particularly when PCL is used as the polymer. Therefore Fig. 15 shows the PCL particle zeta potential as a function of the solvent and the preparation method. It is clear that the solvent influences the particle zeta-potential, particularly when the emulsification–diffusion method is used. One possible explanation for this is that the monomer structure of PCL has one hydrophilic carboxylic group and one hydrophobic alkyl chain (five monomer units). Thus different molecular arrangements of the polymeric chains could be obtained, depending on the nature of the solvent used to ensure re-precipitation. When PCL is precipitated from PC, a hydrophilic solvent (dielectric constant: 64.8 at 25 °C [138]), during the solvent diffusion to water phase, the polar parts of the PCL are predominantly exhibited in the vicinity of the water–polymer interface. Taking into consideration the randomness and speed of polymer precipitation, it is possible that only a few alkyl chains are positioned facing the aqueous phase.

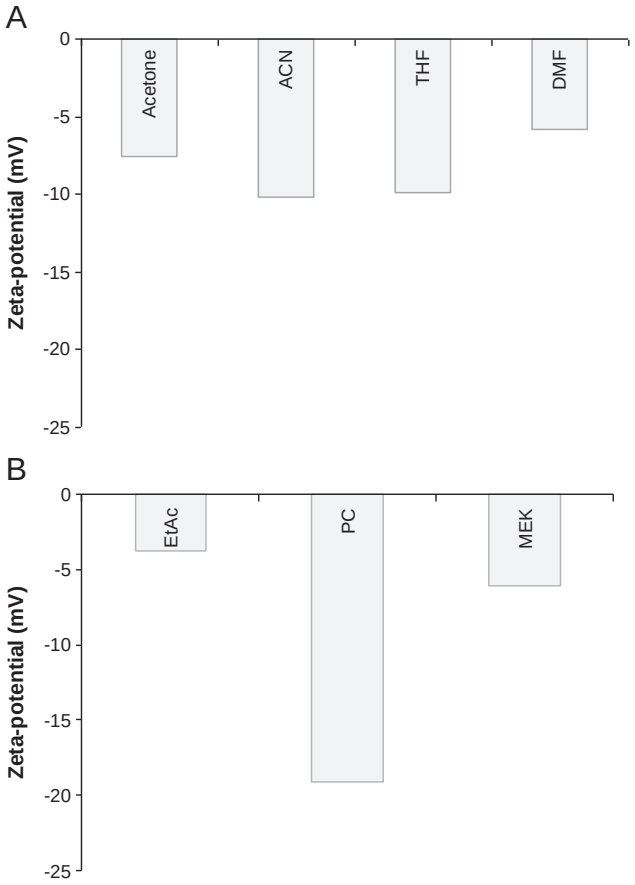

Fig. 15. Influence of solvent nature on the zeta-potential of particles. A: Prepared by solvent displacement: PCL 14 kDa (3.5 mg/ml), stabilizing agent PLX 0.4%; B: prepared by emulsification–diffusion: PCL 14 kDa (10 mg/ml), stabilizing agent PLX 1%.

In the same way, when EtAc is used as a solvent due to its hydrophobic nature (dielectric constant: 6.27 at 20 °C [133]), the PCL alkyl chains are located near the particle surface in the solvent diffusion step and carboxylic groups can be positioned facing the external phase. MEK, a solvent with intermediate polarity (15.45 to 18.51 [133]), permits the preparation of particles with intermediate zeta-potential.

The investigations of the surface properties of PCL films performed by Tang et al. [139] lend credence to this approach to particle formation. Their results from contact angle, surface morphology and attenuated total reflection–Fourier transform infrared spectroscopy (ATR–FTIR) prove that polymer arrangement depends on the nature of the solvent.

Regarding the solvent displacement method, in spite of the significant difference between the dielectric constants of the solvents used for submicron particle preparation, there is no tangible difference between the solvents with respect to particle zeta-potential. This might be because the lower concentration of the polymer used did not permit the detection of a clear tendency. However, seen from another angle, the difference observed between the two methods might suggest a critical influence on particle electrostatic behavior of the organic solvent present in the solvent-saturated aqueous phase when using emulsification–diffusion method. Unfortunately, to our knowledge there is no experimental evidence to support this approach, thus it is not possible to deal with this issue in-depth.

6. Concluding remarks

There is increasing interest in investigating submicron particles due to their potential capacity for carrying drugs, targeting systems and overcoming the typical problems of conventional drug delivery systems due to the stability, dissolution, gastrointestinal mucosa irritation or the disagreeable organoleptic properties of the active substances used. Consequently, the preparation method is a key step for ensuring that particles behave according to the use intended. As can be seen in this review devoted to the study of the solvent displacement procedure and emulsification–diffusion technique, the operating variables and starting materials used influence the size and

zeta-potential of particles as well as their capacity to entrap and load active molecules.

The study of mechanistic aspects reveals that the formation of submicron particles depends on the combination of operating conditions, the composition of the organic and aqueous phases (since it determines their physicochemical properties) and the physicochemical interactions between phases. The extent of their participation is unclear at present, though it appears that one prevails over another depending on their interrelationship.

The emulsification–diffusion technique is the more robust method from the experimental standpoint. In this case, the emulsification rate and time are the key variables for obtaining specific particle size without any influence being introduced by the dilution step. On the contrary, the size of submicron particles prepared by solvent displacement is strongly determined by the interrelated effects of operating variables such as the method for mixing the organic and aqueous phases, the system stirring rate and the organic phase volume. Nevertheless, the use of large volumes of material, i.e. water for dilution, and high power consumption due to emulsification through high mechanical shear strength, are disadvantages for the emulsification–diffusion method that contrast with the very simple procedure and assembly required for the solvent displacement technique.

The size of submicron particles can be influenced by the materials used in their preparation. Thus the nature of the polymer and the stabilizing agent influences the size of the particles prepared by the two methods. Likewise, polymer concentration is a critical factor for obtaining specific particle size by solvent displacement, whereas the concentration of the stabilizing agent may influence the sizes of particles prepared by emulsification–diffusion. The nature of the organic solvent also plays a key role but only in the emulsification–diffusion method.

Regarding zeta-potential, it is influenced by the nature of the polymer and stabilizing agent chosen. Zeta-potential behavior depending on solvent polarity was observed for PCL submicron particles prepared by the emulsification–diffusion method. This opens the door for new research work focusing on, for example, the molecular ordering of polymeric chains depending on the ratio of hydrophilic and hydrophobic groups and its incidence on particle properties.

The literature suggests, that regardless of the method, the efficient entrapment of active molecules depends on their partition between the aqueous and the organic phases, molecule–polymer affinity and polymer precipitation rate. Therefore, taking into consideration and comparing the mechanisms governing particle formation by each method, it might be expected that molecule–polymer affinity would be more critical when the solvent displacement technique is used and molecule partition between phases would be the predominant factor in the emulsification–diffusion method. As particles are formed immediately phases are mixed, the solubility of the active substance in the polymer governs its entrapment by the solvent displacement technique. Regarding the emulsification–diffusion method, the emulsification step could facilitate the migration of the active molecule towards the aqueous phase, thus it governs the amount of active substance to be encapsulated. Therefore additional factors associated with the starting materials used for preparing the particles, such as operating conditions, might influence the loading and entrapment of active substances. Unfortunately, the literature does not allow in-depth analysis or making general statements on this subject.

On the other hand, in addition to the identification of general trends and correlations between variables and particle behavior, studying the method requires taking into account the mechanistic aspects related to particle formation. Thus this review contributes to discussion by analyzing the available data from the physicochemical standpoint, i.e. polymer/solvent/water molecular interactions and organic solvent properties.

In brief, taking into consideration that neither solvent–polymer–water interactions nor solvent physicochemical properties seem to govern the size of particles prepared by the solvent displacement technique, it is possible that nucleation and mechanical phenomena occur simultaneously. Apparently, their respective importance as the main mechanisms for particle formation depends on the organic phase/aqueous phase ratio. Thus mechanical phenomena predominate at the highest phase ratios.

For the emulsification–diffusion method, particle size has some correlation with $\chi_{\text{polymer–solvent}}$ which suggests that particle formation from a drop of emulsion is the most convincing mechanistic approach. In addition, there is good agreement between the density, viscosity and surface tension of the solvents and particle size, thereby supporting the hypothesis that emulsion formation is the critical factor for obtaining specific particle sizes.

Furthermore, these findings allow us to explain the main drawback of the solvent displacement procedure compared with the emulsification–diffusion method. Since particle formation by the emulsification–diffusion method is governed by the emulsion step, the high mechanical force used for obtaining the emulsion facilitates processing larger quantities of polymer for obtaining particles of a specific size. On the contrary, the solvent displacement technique can only be carried out with low polymer concentrations, due to the limitation of working in the “metastable region”, which means low particle yields. Research on this subject is underway to determine the industrial applicability of this procedure [62,75,140,141].

In conclusion, the results compiled and analyzed in this review give a complete background on the incidence of the method variable on submicron particle properties when the solvent displacement and emulsification–diffusion methods are used. By taking a logical approach from the outset, they can be used as the starting points for defining the work conditions and choosing the starting materials according to the aims of each research team. Also, they can be used for implementing versatile strategies for submicron particle production as the statistical design of experiments [31,35,61,142,143]. However, the comprehensive study carried out highlights the importance of making progress regarding research into the mechanistic aspects related to particle formation. Basic understanding of how particles are formed leads to flexible process manipulation achieved by varying process parameters and using suitable starting materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cis.2011.02.005.

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Supplementary data

Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification-diffusion methods: Critical comparison

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Materials and methods used in the systematic study of the solvent displacement and emulsification-diffusion methods.

Materials

Poly(ε-caprolactone) (PCL) (Mw: 14 kDa, 65 kDa and 80 kDa) were obtained from Sigma–Aldrich, poly(L-lactic acid) (PDLLA) Purasorb® PDL 05, Mw: 60 kDa, from PuracBiomaterials, poly(lactic-co-glycolic acid) (PLGA) Medisorb® 50:50, Mw: 7 kDa, from Alkermes, poloxamer 188 (PLX 188) (Synperonic PE®/F68) from Fluka, poly(vinyl alcohol) (PVA) (Mowiol 4-88 Mw: 31000 g/mol) was obtained from Aldrich, polysorbate 80 (Tween® 80) from Uniquema, sodium dodecyl sulphate (SDS) from Prolabo, and dodecyl trimethyl ammonium bromide (99%) (DTAB) from Aldrich. Acetone, tetrahydrofurane (THF), acetonitrile (ACN), N-dimethyl formamide (DMF), ethyl acetate (EtAc), propylene carbonate (PC), methyl ethyl ketone (MEK), NaCl, NaOH and HCl were analytical grade. Deionised water from Milli-Q system was used in all experiments.

Methods

Preparation of submicron particles by solvent displacement method.

Submicron particle suspensions were prepared by solvent displacement method [19]. As is shown in Figure A1, an organic solution was added continuously, drop by drop (Harvard Apparatus Syringe

Infusion Pump 22), to a stirred (MSC Basic C, Yellow line TC3) aqueous solution containing the stabilizing agent. The organic solvent was removed from the system during the final magnetic stirring period.

In order to investigate the influence of different operating parameters on the particle sizes they were changed during the study as shown in Table AI-1. For this purpose submicron spheres were prepared from PCL 14 kDa (0.035 g) in acetone (10 ml) as organic phase, and PLX (0.08 g) in water (20 ml) as aqueous phase. On the other hand, in order to study the starting material influence on mean size and zeta-potential of the particles, submicron spheres were prepared using the best operating conditions for obtaining smaller particles (organic phase volume: 10 ml; aqueous phase volume: 20 ml; organic phase injection rate: 150 μl/min; way of the organic phase addition: dropwise-in continuous medium; system stirring rate: 500 rpm; system temperature: ambient (15 – 20 °C); system final stirring: 30 min). Table AI-2 summarizes the different condition for the study of each variable.

Preparation of submicron particles by emulsification-diffusion method.

The preparation of submicron particles by emulsification-diffusion method was performed according to Leroux et al. [28]. In this case, as is shown in Figure A2, the organic phase was added rapidly (less than 5 s) on the aqueous phase and immediately, an emulsion o/w was formed by stirring at high speed (Ultraturrax stirrer T25 IKA). At the end of the stirring step, the formed emulsion was added to water under mechanical stirring (Heidolph RZR 2102) in order to allow the organic solvent diffusion into the water, leading to the formation of the particles. The solvent and part of the water were thereafter removed by evaporation under reduced pressure (Büchi Rotavapor R-124).

The influence of the operating parameters on the mean size of particles was investigated by the preparation of submicron spheres from PCL 14 kDa (0.1 g) in 10 ml of water-saturated ethyl acetate as organic phase and PLX (0.4 g) in 40 ml of ethyl acetate-saturated water. 200 ml of water were used for the diffusion step. The starting material influence on the particle mean size and zeta-potential was investigated. In these cases, the basic operating conditions were identified as the best ones leading to particle mean size nearest to 200 nm: organic phase volume 10 ml, aqueous phase volume 40 ml, emulsification stirring rate 6500 rpm, emulsification stirring time 10 min, water for dilution volume 200 ml, dilution stirring rate 500 rpm and dilution stirring time 15 min. Table AI-3 and AI-4 summarize the different work conditions and composition variables examined.

Particle characterization.

The particle size was measured by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments), 5 measures/sample, 5 runs of 10 s/measure at 25 °C, after adequate dilution about 1:6 in deionised water (pH about 7.0) of a suspension aliquot. Zeta-potential in 1 mM NaCl solution of pH 5.0, was determined using the same measuring instrument. In order to verifier the mean size of particles, the morphological examination of representative samples of particles was performed by Transmission Electron Microscopy (TEM) (Philips CM120 microscope) following negative staining with phosphotungstic acid solution (0.5%). Likewise, particles were examined by Atomic Force Microscopy. For this purpose 5 ml of the particle suspension were deposited on freshly cleaved muscovite mica and 5 min after its preparation the still wet sample was observed on a multimode-Veeco AFM (tapping mode).

Table AI-1. Operating conditions for the systematic study of solvent displacement method.

Variable	Method operation conditions modified
Organic phase / aqueous phase ratio	Organic phase volume: 2, 5, 10, 15, 20 ml. Aqueous phase volume: 20 ml.
Organic phase injection rate	150, 300, 450 μl/min.
Way of the organic phase addition	Dropwise-out reaction medium Dropwise-in reaction medium
System stirring rate	375 and 500 rpm.
System temperature	Ambient (15 – 20 °C), 20 ± 1°C , 25 ± 1°C, 35 ± 1°C and 45 ± 1°C.
Final stirring	0, 5, 10, 15, 20, 30 and 60 min.

Table AI-2. Composition conditions for the systematic study of solvent displacement method.

Variable	Composition conditions modified
Polymer nature	PCL 14 kDa, PCL 65 kDa, PCL 80 kDa, PDLLA, PLGA 50:50, Concentration: 3.5 mg/ml.
Polymer concentration	PCL organic phase concentration: 2, 3.5, 5, 8.5 and 12 mg/ml.
Stabilizing agent nature	PLX 188, Polysorbate 80, SDS, DTAB, PVA, PEG (2000, 4600 and 10000) and Dextran (T500 and T2000). Concentration: 1%.
Stabilizing agent concentration	Poloxamer 188 or PVA concentration: 0, 0.2, 0.4, 0.6, 0.8 and 1.0 %.
Solvent	Acetone, ACN, THF and DMF.

Table AI-3. Operating conditions for the emulsification-diffusion method study.

Variable	Method conditions modified
Way of the organic phase / aqueous phase mixing	Rate of organic phase addition: 1.25 ml/min. Both phases at the same time (organic phase on aqueous phase).
Organic phase / aqueous phase ratio	0.1, 0.15, 0.25, 0.35, 0.40, 0.55, 0.70 and 1.0.
Emulsification stirring speed	6500, 8000, 9500, 11000 and 13000 rpm.
Emulsification time	5, 7.5, 10, 12.5 and 15 min.
Way of the dilution step	~ 10 ml/min emulsion on water; ~ 100 ml/min emulsion on water; two equal fractions emulsion on water; emulsion an alone time on water; water an alone time on emulsion.
Dilution stirring type	Magnetic and Helix. Stirring speed: 500 rpm.
Dilution stirring speed	250, 500 and 750 rpm.
Dilution stirring time	1, 5, 10, 20 and 30 min.
Water volume for dilution	150, 200, 300, 400 and 500 ml.
Temperature of water dilution	Ambient (15 – 20), 25, 40 and 60 °C.

Table AI-4. Composition conditions for the emulsification-diffusion method study.

Variable	Composition conditions modified
Polymer nature	PCL 14 kDa, PCL 65 kDa, PCL 80 kDa, PDLLA, PLGA 50:50. Concentration: 1.0%.
Polymer concentration	PCL organic phase concentration: 0.5, 1, 2, 3, 4 and 5 %.
Stabilizing agent nature	PLX 188, Polysorbate 80, SDS, DTAB, PVA. Concentration: 1%.
Stabilizing agent concentration	Poloxamer 188 or PVA concentration: 1, 2, 3, 4 and 5 %. Polymers: PCL 14 Kd 1.0%.
Solvent	EtAc, PC, MEK.

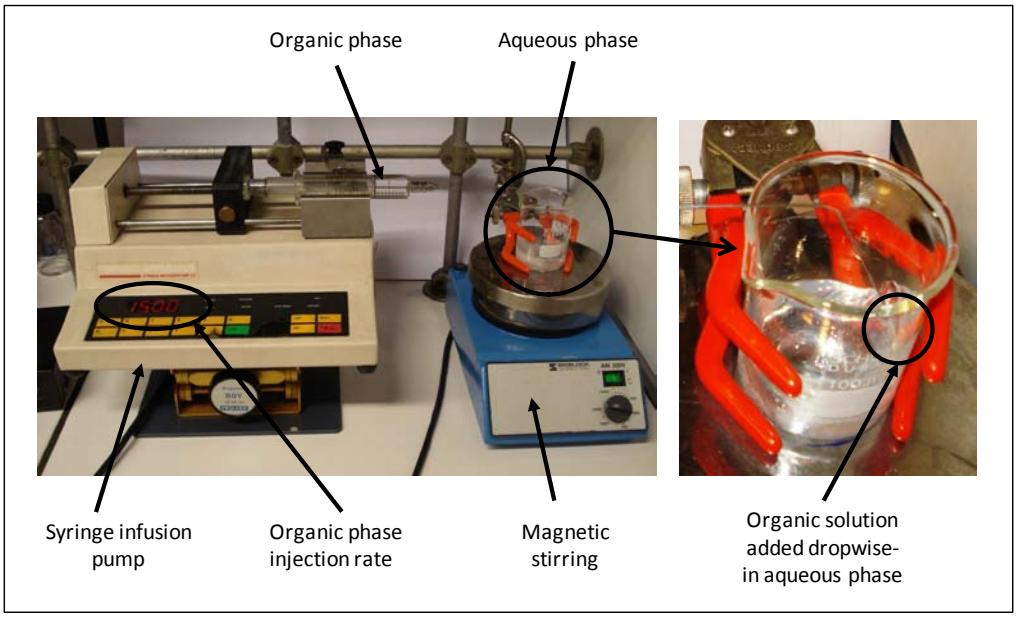

Figure A1. Set-up for preparing submicron particles by solvent displacement method.

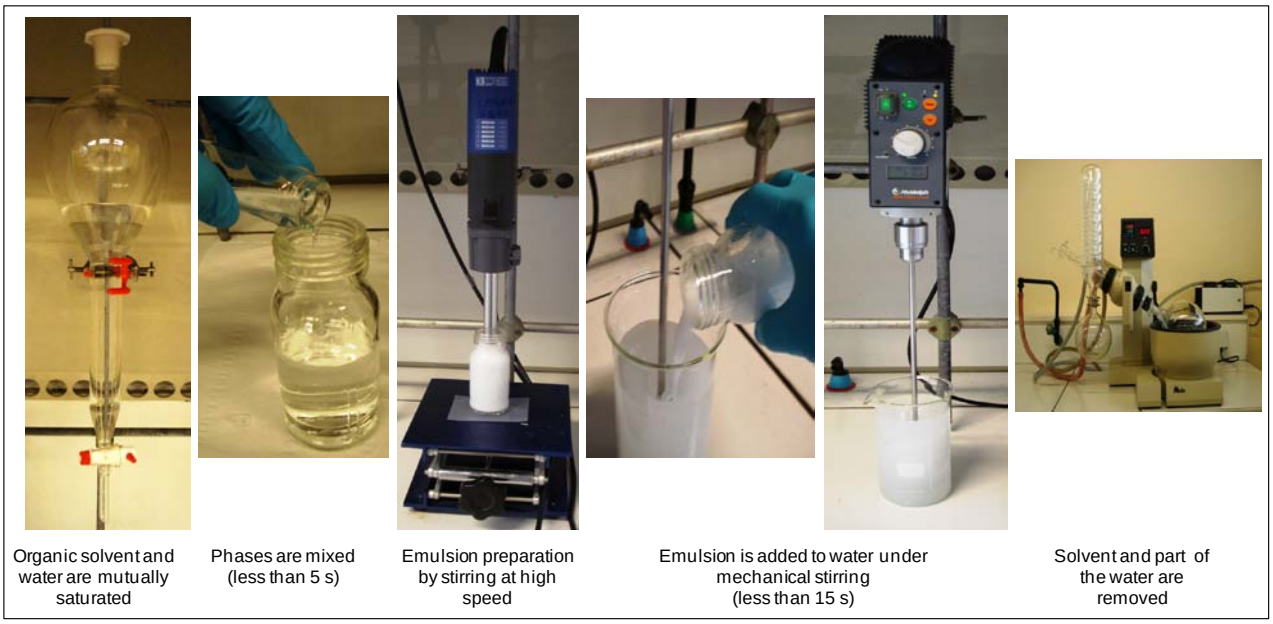

Figure A2. Set-up for preparing submicron particles by emulsification-diffusion method.

Electrokinetic properties of poly-ε-caprolactone-based nanoparticles prepared by nanoprecipitation and emulsification-diffusion methods: a comparative study

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Abstract The aim of this work is to investigate the influence of the preparation method on the surface charge and electrokinetic properties of poly-ε-caprolactone-based particles by using poloxamer 188 as stabilizing agent. To target such objective, two processes (the nanoprecipitation and the emulsification-diffusion) are used to prepare well-defined nanospheres. The effect of the materials used on the particle zeta potential is systematically studied in order to compare the two preparation methods. The polarity of the organic solvent directly affects the zeta potential of particles prepared via the emulsification-diffusion method. The results obtained suggest that the aqueous phase used for preparing particles affects the possible re-arrangement of polymers during the emulsification step. As the aqueous phase is saturated with the organic solvent, the polar and the non-polar moieties of the polymer chains might be reconformed following organic solvent diffusion from the particle core to the continuous phase. Regarding the nanoprecipitation process, the electrokinetic properties of the particles were found to be organic solvent independent, but principally affected by the pH and the salinity of the aqueous phase used during the particle preparation.

Keywords *Nanoparticle; Nanoprecipitation; Emulsification-diffusion; Zeta potential; Surface charge; Electrokinetic.*

Introduction

Nanoparticles (nanocapsules and nanospheres) are usually characterized in terms of particle size, size distribution and zeta potential measurements as widely reported (Fattal and Vauthier 2002; Mora et al. 2010; Pinto et al. 2006; Vauthier and Bouchemal 2009). In particular, the zeta potential and the surface charge density are particle characteristics that can be directly related to colloidal stability and to their in vivo performance (Calvo et al. 1997; Couvreur et al. 2002; Einarson and Berg 1993; Furtado et al. 2001; Gessner et al. 2002; Hariharan et al. 2006; Lertsutthiwong et al. 2008; Lück et al. 1998; Owens and Peppas 2006; Prego et al. 2006). Up-to-now, the pH and the ionic strength of the dispersion medium have been reported as factors affecting the electrokinetic properties of charged particles (Ishikawa et al. 2005; Sahoo et al. 2002). The nature and concentrations of the polymer and stabilizing agent, have been also reported to influence the surface charge of nanoparticles (Ahlin et al. 2002; Ameller et al. 2003; Ameller et al. 2004; Avgoustakis et al. 2003; Fonseca et al. 2002; Giannavola et al. 2003; Govender et al. 2000; Hariharan et al. 2006; Hirsjärvi et al. 2008; Joo et al. 2008; Jung et al. 2000; Konan et al. 2003; Leroueil et al. 1998; Musumeci et al. 2006; Quintanar et al. 1998a; Riley et al. 1999; Sahana et al. 2008; Santander et al. 2010; Shin et al. 2010; Zhu et al. 2005). Usually, the results reported have been mainly related to the used recipes rather than to the real interfacial properties and the reported zeta potential values have only been discussed at fixed pH (Govender et al. 2000; Hariharan et al. 2006; Konan et al. 2003; Musumeci 2006; Sahana et al. 2008) or as a function of pH (Hirsjärvi et al. 2008; Joo et al. 2008). The zeta potential values reported for both preparation methods generally tend to be negative and in some cases particles exhibit low negatively charges irrespective of pH (Legrand et al. 1999; Mora et al. 2010).

However, to our knowledge, the electrokinetic properties of such colloidal particles have not been investigated and discussed in terms of charge density as a function of preparation method. Thus the aim of this research work is to report for the first time a comparative electrokinetic study of poly-ε-caprolactone (PCL) containing particles prepared by nanoprecipitation and by emulsification-diffusion, the methods most used for this purpose (Fattal and Vauthier 2002; Mora et al. 2010; Pinto et al. 2006; Vauthier and Bouchemal 2009).

Table 1 provides a summarized comparison of the processes and materials used for both preparation methods. The key difference between them is the requirement for total or partial miscibility of phases A and B. This leads to particle formation in one step when nanoprecipitation method is used, because the mixing of miscible phases leads to a poor solvent in order to induce polymer precipitation (i.e. polymer in poor solvent) (Fessi et al. 1989). In turn, the partial miscibility of the two phases and the fact that they are mutually saturated when used in the emulsification-diffusion technique, leads to the preparation of an emulsion in the initial step by high mechanical shear. Particle formation then occurs during emulsion dilution, controlled by the migration of the organic solvent towards the water rich domain (Leroux et al. 1995).

Table 1 Summary of the starting materials and procedures used for nanosphere preparation by using the nanoprecipitation technique and the emulsification-diffusion method

		Nanoprecipitation	**Emulsification-diffusion**
Starting materials	Phase A (so-called organic phase)	Polymer Organic solvent totally miscible with phase B	Polymer Organic solvent partially water miscible which is previously water-saturated
	Phase B (so-called aqueous phase)	Stabilizing agent Solvent totally miscible with phase A	Stabilizing agent Organic solvent-saturated water
	Phase C (so-called dilution phase)	-	Water
Procedure		The organic phase is mixing with the stirred aqueous phase in one shot, stepwise, dropwise or by addition rate controlled	Emulsion step: the organic phase is added rapidly (less than 5 s) on the aqueous phase and immediately, an emulsion o/w is formed by stirring at high speed. Dilution step: The formed emulsion is diluted into the dilution phase under mechanical stirring.

From the standpoint of the recipe for preparing particles by each method, some important remarks should be made regarding our reviews on this subject (Mora et al. 2010; Mora et al. 2011). Usually, in the emulsification-diffusion method, the polymer concentrations are higher

than those used in the nanoprecipitation technique (1.0 to 2.0% of the organic solvent for emulsification-diffusion vs. 0.2 to 0.5% in the case of nanoprecipitation). Likewise, this method requires considerable stabilizing agent concentration (from 0.5 to 5.0% in polymer nonsolvent for emulsification-diffusion and from 0.2 to 0.5% in polymer nonsolvent in the case of nanoprecipitation).

Experimental

Materials

Poly-ε-caprolactone (PCL) (Mw: 14 kDa) was from Sigma–Aldrich and poloxamer 188 (PLX) (Lutrol® F68) from Basf. Acetone, tetrahydrofurane (THF), acetonitrile (ACN), N-dimethyl formamide (DMF), ethyl acetate (EtAc), propylene carbonate (PC), methyl ethyl ketone (MEK), NaCl, NaOH and HCl were analytical grade. Deionised water from Milli-Q system was used in all experiments.

Methods

Nanoparticle preparation

The preparation process of particle suspensions by nanoprecipitation was performed as first reported by Fessi et al. (1989). The organic phase was a solution of PCL (0.070 g) in organic solvent (20 ml). The organic phase was added (48 ml/h) into an aqueous solution of PLX (0.25%, 40 ml) stirred at 375 rpm. Particles of sizes ranging between 180 nm and 210 nm were formed instantaneously.

The preparation of particles by the emulsification-diffusion method was performed as reported by Leroux et al. (1995). The organic phase was a solution of PCL (0.1 g) in water-saturated organic solvent (10 ml). The organic phase was emulsified with 40 ml of a solvent-saturated aqueous phase containing PLX (1%), by using a high speed homogenizer (Ultraturrax stirrer T25 IKA; 6500 rpm for 10 min). The emulsion was added in one shot to

water (200 ml) under mechanical stirring (500 rpm, Heidolph RZR 2102), leading to the formation of particles with sizes ranging from 100 nm to 200 nm.

The solvent and part of the water of the particle dispersions were removed by evaporation under reduced pressure and 40°C (Büchi Rotavapor R-124), resulting in a final volume of 10 ml particles dispersion. For particular experiments, the particles were ultracentrifugated at 40 000 rpm and 20°C for 30 min (Ultracentrifuge Optima™ Max-XP) to remove free surfactant and residual solvent traces.

Nanoparticle characterization

The particle size was measured by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments), 5 measurements/sample, 5 runs of 10 s/measurement at 25 °C, after adequate dilution of a suspension aliquot in deionised water (water pH between 6 and 7). Zeta potential was deduced from electrophoretic mobility measurement by using a Zetasizer Nanoseries (5 measurements/sample, 5 runs/measurement at 25°C). Particle dispersion was highly diluted in a 1 mM NaCl solution of different pHs (2.5 – 9.0). The pH of the samples was adjusted with NaOH or HCl and controlled by a potentiometer (TIM856 Titralab®).

Determination of the surface charge density

The amount of carboxyl groups on the particle surface layer was determined directly by using the conductimetric titration method (Conductivity meter CDM210 MeterLab™; cell type CDC745-9). To perform such experiment, 20 ml of 0.01 N HCl solution were added to 30 ml particle dispersions containing around 1.5 g of PCL. Titration was carried out by using 0.01 N NaOH solution. The particles used in this experiment were first washed using deionised water and then redispersed in water before filtration using a 0.45 μm regenerated cellulose filter.

Results and discussion

Generally speaking, the zeta potential (i.e., the potential at the shear plan position of a given surface) is related to surface properties such as ionized groups and the presence of charged or uncharged polymers in the vicinity of the surface. In fact, the changes in the zeta potential depend on (i) the accessible surface charge density originated from acid-base dissociations of ionisable surface functional groups; and (ii) the possible adsorption of charged species (i.e. molecules, macromolecules and polymers) present in the medium (Hiemenz and Rajagopalan 1997; Hunter 1981).

In the particular case of particles prepared from polyester polymers such as PCL and non-ionic stabilizing agents like PLX, it is expected that the dissociation of terminal carboxylic groups depending on pH, is the most probable mechanism explaining the exhibited negative surface charge of such particles. However, as shown in Figure 1, particles prepared by the nanoprecipitation technique exhibit more significant negative zeta potential (-15mV at pH 7) than those obtained by the emulsification-diffusion method (-4 mV at pH 7).

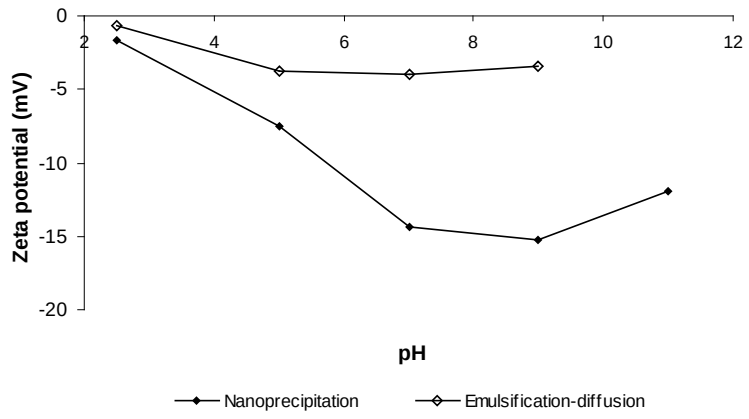

Figure 1. Zeta-potential behavior of nanospheres prepared by the nanoprecipitation process and the emulsification-diffusion method

Estimation of the particle surface density

Surface charge density and the electrophoretic mobility of PCL nanoparticle dispersions have never been investigated in-deep although different approaches have used for classical polystyrene latex particles (Brooks and Seaman 1973; Elimelech and O'melia 1990; Fernández et al. 1994; Schulz et al. 1994; van den Hoven and Busterbosch 1987), magnetic particles (Tourinho et al. 2002), gold nanoparticles (Makino and Oshima 2010) and complex colloidal particles (Nabzar et al. 1998). Firstly, in this study we target the estimation of surface charge density of the PCL-based particles. It seems that surface charge density is a more characteristic quantity than zeta potential because, for particles with a constant surface charge density, zeta potential is not a constant quantity but depends on the concentration of the electrolyte (Makino and Oshima 2010).

Conductimetric titration of each dispersion was carried out to determine directly the amount of carboxylic groups on the surface of the particles, thus the surface charge density (σ) is:

$$\sigma = d.meq.N_A.e.\frac{\rho_{PCL}}{6} \qquad (1)$$

where ρ_{PCL} is the PCL density, d the particle diameter, meq the number of microequivalents of NaOH per gram of polymer, N_A the Avogadro number and e the elementary electric charge. Figure 2 shows the titration curve of particles prepared by the nanoprecipitation technique and the profile obtained is in agreement with the classical titration of carboxylic acid containing polystyrene particles (Zwetsloot and Leyte 1994). The charge density deduced from the titration by using the equation 1, corresponds to 9.1 $\mu C/cm^2$. Unfortunately, in the case of emulsification-diffusion, the amount of carboxylic groups cannot be quantified via direct conductimetric titration method due to the flat titration curve obtained as shown in Figure 2. This behaviour is likely attributable to the small number of accessible carboxylic groups on the surfaces of the particles or to the possible effect of residual organic solvent traces which could be hydrolyzed at the basic conditions of the titration experiment (Keusch 2003).

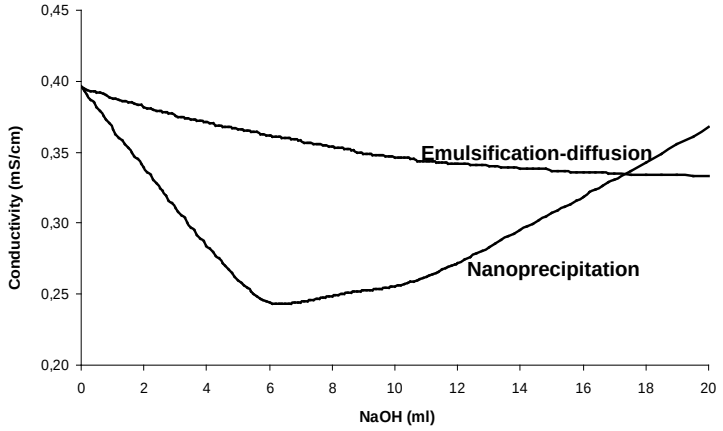

Figure 2. Conductimetric titration of PCL nanoparticles prepared by the nanoprecipitation technique and the emulsification-diffusion method

To overcome the titration problem in the emulsification-diffusion process and be able to compare the surface charge density between particles prepared by nanoprecipitation and emulsification-diffusion, the following theoretical approach based on the transformation of electrophoretic mobility to surface charge density as first approximation was used initially (Gadzinowski et al. 2000):

$$\sigma \approx \kappa . \eta . \mu_e \qquad (2)$$

where μ_e is the electrophoretic mobility, η the viscosity of the medium and κ the Debye-Hückel parameter, defined in SI units as:

$$\kappa = \left(\frac{2e^2 n z^2}{\varepsilon_0 \varepsilon_r k_B T} \right)^{1/2} \qquad (3)$$

here, *n* denotes the electrolyte concentration expressed as ions/m^3 of medium, *z* is the valence number of ions, ε_0 the permittivity of vacuum, ε_r the dielectric constant of the medium, k_B the Boltzman constant and *T* the temperature.

Likewise, the Grahame equation (eq. 4) based on the Gouy-Chapman theory (Hiemenz and Rajagopalan 1997), which predicts the surface charge density for flat plates, and the Debye-Hückel theory developed for spherical particles (eq. 5) (Schumacher and van de Ven 1987) were used:

$$\sigma = \varepsilon_0 \varepsilon_r \left(2k_B T n / \varepsilon_0 \varepsilon_r\right)^{1/2} \left[\exp\left(ze\psi_0 / 2k_B T\right) - \exp\left(-ze\psi_0 / 2k_B T\right)\right] \qquad (4)$$

$$\sigma = \varepsilon_0 \varepsilon_r \frac{1+\kappa a}{a} \psi_0 \qquad (5)$$

where ψ_0 is the surface potential, which was assumed in this work to be Smoluchowski zeta potential (ζ) of the particle, deduced from the electrophoretic mobility measured by using the following equation:

$$\mu_e \approx \frac{\varepsilon_0 \varepsilon_r}{\eta} \zeta \qquad (6)$$

In addition, the empirical relationship derived by Loeb et al. (eq. 7) (Hunter 1981; Makino and Oshima 2010):

$$\sigma = 4\pi\varepsilon_0 \varepsilon_r \frac{k_B T}{ze} \kappa a^2 \left[2\sinh\left(\frac{ze\psi_0}{2k_B T}\right) + \frac{4}{\kappa a} \tanh\left(\frac{ze\psi_0}{4k_B T}\right)\right] \qquad (7)$$

and the approximated expression proposed by Oshima et al. (1982) (eq. 8) for estimating the surface charge density of spherical colloidal particles with low zeta potential and with a constant surface charge density, were used.

$$\sigma = \frac{2\varepsilon_0\varepsilon_r\kappa k_B T}{ze}\sinh\left(\frac{ze\zeta}{2k_B T}\right)$$
$$\left[1+\frac{1}{\kappa a}\frac{2}{\cosh^2\left(ze\zeta/4k_B T\right)}+\frac{1}{(\kappa a)^2}\frac{8\ln\left[\cosh\left(ze\zeta/4k_B T\right)\right]}{\sinh^2\left(ze\zeta/2k_B T\right)}\right]^{1/2} \quad (8)$$

In the latter case, the zeta potential was deduced from the electrophoretic mobility by using the following equation as first used by Oshima et al. (1982) (eq. 9):

$$\mu = \frac{2\varepsilon_0\varepsilon_r\zeta}{3\eta}\left(1+\frac{1}{2\left[1+2.5/\left\{\kappa a\left(1+2e^{-\kappa a}\right)\right\}\right]^3}\right)$$
$$-\frac{2\varepsilon_0\varepsilon_r\zeta}{3\eta}\left(\frac{ze\zeta}{k_B T}\right)^2\left[\frac{\kappa a\left\{\kappa a+1.3\exp\left(-0.18\kappa a\right)+2.5\right\}}{2\left\{\kappa a+1.2\exp\left(-7.4\kappa a\right)+4.8\right\}^3}+\left(\frac{m_+ + m_-}{2}\right)\frac{9\kappa a\left\{\kappa a+5.2\exp\left(-3.9\kappa a\right)+5.6\right\}}{8\left\{\kappa a-1.55\exp\left(-0.32\kappa a\right)+6.02\right\}^3}\right] \quad (9)$$

here, the ionic drag coefficients m_+ and m_- were calculated from equations 10 and 11 by using the limiting molar conductance values ($\Lambda^0_\pm$) for Na^+ and Cl^- ions reported by Schumacher and van de Ven (1987) (50.11×10^2 and 73.34×10^2 $\Omega^{-1}m^{-1}equiv^{-1}$, respectively):

$$m_\pm = \frac{2\varepsilon_0\varepsilon_r N_a k_B T}{3\eta z\Lambda^0_\pm} \quad (10)$$

Surface charge density ($\mu C/cm^2$) values (experimentally determined or theoretically estimated) of nanospheres prepared by solvent displacement and emulsification-diffusion methods using the above mentioned approaches are reported in table 2. As can be deduced from this table, the surface charge densities of particles prepared by nanoprecipitation technique are higher than those obtained by emulsification-diffusion. In addition, there are not significant differences between the results theoretically estimated. The surface charge density of the representative particles size (200 nm, in 1 mM NaCl) were calculated as a function of the zeta potential using various theoretical approaches and the obtained variations are reported in Figure 3. As can be seen, the surface charge density versus zeta potential was

found to be linear below 25 mV zeta potential value. In fact, when the zeta potential is low than 25 mV all approaches are asymptotic since various phenomena can be neglected such as particle-particle interactions or distortion of the surrounding ions atmosphere by the external field as widely reported (Dukhin 1991; Egorova 2001; Hiemenz and Rajagopalan 1997; Hunter 1981). Whereas, above 25mV, the used approaches diverge totally and this can be attributed to the non validity of used approximation for a given theory such as κa domain, particle size and size distribution, surface roughness, surface charge distribution, etc.

Table 2 Surface charge density ($\mu C/cm^2$) of nanospheres prepared by solvent displacement and emulsification-diffusion methods

	Nanoprecipitation	**Emulsification-diffusion**
Conductimetric titration	-9.1	nd.
From electrophoretic mobility	-0.103	-0.040
Debye-Hückel theory for spherical particles	-0.104	-0.041
Grahame equation – Gouy – Chapman theory	-0.106	-0.041
Loeb et al. equation	-0.116	-0.045
Oshima et al. equation	-0.114	-0.044

nd.: Non-determined.

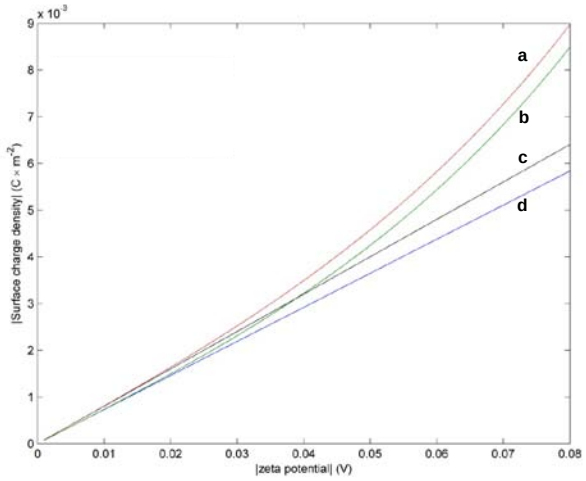

Figure 3. Surface charge density calculation of nanoparticles of radius 100 nm in a 1 mM aqueous NaCl solution as a function of the zeta potential. Theoretical behaviors obtained from: a: Loeb's equation and Oshima's equation (they are superposed); b: Grahame equation; c: Debye-Hückel equation; d: Oshima et al. equation.

On the other hand, the κa values for our PCL-based particles are in between 8 and 10, which are sufficiently large than unit (i.e., >>1) for correctly applying Smoluchowski's equation for estimating the zeta potential. Likewise, this permits the use of approximate expressions based on electrophoretic mobility or those reported approaches by the Debye-Hückel theory or by Loeb et al. (Hunter 1981; Makino and Oshima 2010). Moreover, it seems that models developed for planar surfaces might be also used (e.g., Grahame equation). Refined methods such as that reported by Oshima et al. (1982) are not necessary for estimating surface charge density in the particular case of our PCL-based nanoparticles, although it has been shown to be particularly applicable for the smallest colloidal particles (κa <1) (Makino and Oshima 2010).

On the contrary, the surface charge density results obtained experimentally by conductimetric titration are significantly higher than those estimated theoretically. This can be attributed to heterogeneous distribution of carboxylic groups at the inner layers under the particle surface, which makes the conductimetric titration more complicated. In addition, the dissociation of acidic groups may affect the polymer matrix rigidity (i.e., interfacial structure at the particle surface), rendering the inner layers accessible (Gadzinowski et al. 2000). In fact, as reported by Siparsky et al. (1998), PCL hydrolysis might occur at the beginning of the chemical titration process due to the addition of HCl. It is also interesting to note that the use of zeta potential instead of surface potential may induce differences between the experimental and the calculated surface charge density.

For explaining the different behavior of the zeta potential of PCL-based particles and of the estimated surface charge density, depending on the preparation method, the effect of the stabilizing agent concentration, the nature of the organic solvent and the nature of the aqueous medium from which the polymer is re-precipitated are examined.

Effect of stabilizing agent on the zeta potential of PCL-based nanoparticles

The first approach in attempting to explain the electrokinetic behaviour of PCL-based nanoparticles could be related to the stabilizing agent amount (i.e., used in the recipe) required

in each process in order to maintain good colloidal stability of the formed particle dispersion. As suggested by Santander et al. (2006), the colloidal stability of PLX-coated PLGA particles is related and governed by the interfacial PLX concentration (i.e., adsorbed PLX amount). In this work, our typical recipes used for preparing the particles require a higher quantity of stabilizing agent in the case of the emulsification-diffusion method (around 1%) compared to the nanoprecipitation technique (in the range of 0.25%). Then, the presence of adsorbed non-charged stabilizing agent induces shift in the slipping plan position toward far from the particle surface. Consequently, the zeta potential decreases. It is interesting to notice, that the shift in the shear plan position in which the zeta potential is measured is directly related to the adsorbed amount of the non-charged stabilizing agent, the interfacial thickness layer, the molecular weight and the microstructure of the stabilizing agent used and its interfacial conformation (Brooks 1973; Hunter 1981). Thus, the real interpretation of such a complex interface is questionable as reported by various authors (Nabzar et al. 1998; Zhang et al. 2010) and the general tendency as a consequence of the presence of non-charged adsorbed polymer is that the zeta potential (in absolute value) decreases and in some cases reaches zero irrespective of pH and salinity (Einarson and Berg 1993; Shakesheff et al. 1997; Trimaille et al. 2003).

Different experiments were performed to validate this approach. Firstly, particles prepared by the two methods were cleaned by repetitive centrifugation and redispersion cycles by using ultracentrifugation and deionised water in order to remove the excess and the poorly adsorbed non-charged stabilizing agent (PLX). Regardless of the preparation method used, the zeta potential of purified particles was found to be higher than for non-cleaned (crude) particles. This is in agreement with the expected reported tendency. Nevertheless, the difference in particles zeta potential depends on the preparation method as seen in Table 3.

On the other hand, a second experiment was carried out to investigate the possible effect of the stabilizing agent quantity on the zeta potential. In this case, the particles were prepared by nanoprecipitation using 1% PLX, similar to the emulsification-diffusion method. Comparably, particles were prepared by emulsification-diffusion using 0.25% PLX. The zeta potential performed at fixed pH and salinity shows the following trend; -17 mV (nanoprecipitation) and -8 mV (emulsification-diffusion). These results show that the concentrations of stabilizing agent used in each preparation process have not marked impact on the negative charge of the particle. In brief, this approach based on the stabilizing agent

quantity was found to be inappropriate for explaining the effect of the preparation method on the zeta potential of PCL-based particles.

Table 3 Characteristics of nanospheres prepared by the nanoprecipitation technique and the emulsification-diffusion method

	Nanoprecipitation		**Emulsification-diffusion**	
	Before washing	**After washing**	**Before washing**	**After washing**
Particle size (nm)	195	165	200	162
Zeta potential (mV)	-14.3	-24.3	-5.6	-16.6
Maximal electrophoretic mobility (μm.cm/V.s)	-1.124	-1.903	-0.439	-1.303

Effect of the polarity of the organic solvent

From another standpoint, the zeta potential behavior of PCL-based particles according to the preparation method could be a result of PCL polymer chains conformation during particle formation due to the polarity of the organic solvent. Tang et al. (2004) reported different surface properties of PCL films depending on the nature of the solvent used during polymer precipitation process. In our study, the nanoprecipitation technique uses a semi-polar solvent (acetone, dielectric constant ε= 20.7) (van Krevelen and te Nijenhuis 2009) as organic solvent and emulsification-diffusion a non-polar solvent (ethyl acetate, dielectric constant ε=6) (van Krevelen and te Nijenhuis 2009). Then, during particle formation the solvent must migrate from the pure organic solvent phase containing polymer to the aqueous phase leading to particle formation occurring as fast as the polymer is located in poor solvent. The polar groups of the PCL chains could be rearranged for being exhibited onto the particle surface, face to more polar medium (i.e., in the vicinity of water phase) when high polar organic solvent (i.e. acetone) is used. In turn, when a non-polar solvent is used (i.e. ethyl acetate) the PCL can be reconformed in order to exhibit the predominant hydrophobic moiety of PCL on the particle surface, as schematically illustrated in Figure 4. Thus, it is expected that particles prepared by using the nanoprecipitation technique exhibit more ionizable groups on their surface than those obtained by the emulsification-diffusion method.

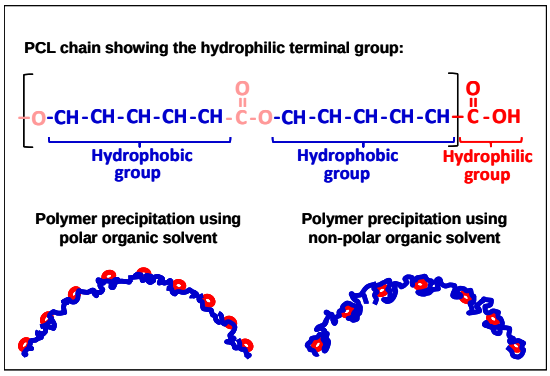

Figure 4. Schematic illustration of PCL polymer chain arrangements on the surface of nanoarticles when polar or non-polar organic solvents are used

The behaviour of particle zeta potential as a function of the pH, shown in Figure 1, appears to be the first argument corroborating this approach. The presence of an acidic functional on the particle surface leads to negative zeta potential which increases with increasing the pH until a plateau region is reached, indicating the complete dissociation of the carboxylic groups (Bellmann et al. 2002; Campagne et al. 2002; Inglesby et al. 2005; Jacobasch 1989). As can be seen, the amount of carboxylic groups on the surface of particles prepared by the nanoprecipitation technique is higher than the particles prepared via the emulsification-diffusion. Therefore, to emphasise the relationship between the nature of the solvent and surface properties, different organic solvents bearing different polarities were studied.

The zeta potential results showed good agreement with organic solvent polarity when the emulsification-diffusion method was used. In fact, the higher the organic solvent polarity, the higher the negative zeta potential of the particles (Figure 5). However, an additional experiment was necessary to eliminate the effect of the residual organic solvent traces in the final aqueous dispersion of particles prepared by this method. For example, EtAc cannot be completely removed by reduced pressure from EtAc-water mixtures because of their azeotropic mixture (Bera et al. 2006) and then EtAc traces remain after the particle purification by diafiltration (Limayem et al. 2004). Likewise, PC is difficult to remove from particle suspensions by using reduced pressure or centrifugation because of its high boiling point (242°C) (van Krevelen and te Nijenhuis 2009) and water solubility (21.7% to 25°C)

(Góral et al. 2009). It might be possible that, rearrangements of polymer chains occur due to dispersion medium polarity or to the changes of the constant of double-layer dielectric as a function of the quantity of the residual organic solvent (Seebergh and Berg 1997). Thus, the particle surface charge might be affected, which implies the modification of both the surface charge density at the shear plane position and consequently, the electrokinetic properties of such surfaces.

Based on the solvent effect mentioned above, particle dispersions of low negative zeta potential were voluntarily contaminated with increasing quantities of PC (the most polar solvent). After 8 hours incubation under stirring, the zeta potential of the dispersions was measured at pHs 6 and 9, and no marked difference was observed irrespective of the PC traces. Then it is possible to conclude that once the particles are formed by polymer precipitation, the presence of possible traces of solvent in the aqueous medium of the dispersion do not affect the dielectric constant and the electrokinetic properties of the particles.

In addition, as shown in Figure 5, the zeta potential values obtained from the dispersion prepared by the emulsification-diffusion method using MEK and PC as organic solvents were similar or higher than those obtained when prepared via the nanoprecipitation technique. This shows that the high negative zeta potential of particles prepared by emulsification-diffusion can be detected irrespective of the quantity of non charged stabilizing agent, which contributes to cancelling our first approach explaining that, the particle zeta potential differences depend on the preparation method due to the PLX concentration.

On the other hand, there is no convincing argument for explaining the behaviors observed in Figure 5 when different organic solvents were used for preparing particles by the nanoprecipitation technique. There is no significant difference in zeta potential despite the broad range of polarities investigated.

Effect of polymer re-precipitation conditions

Additional studies were carried out to facilitate the understanding of the zeta potential behaviour of nanoparticles prepared via nanoprecipitation. Initially, we saw that the pH of the solvent saturated aqueous phases used for preparing particles by emulsification-diffusion is in good agreement with the behavior of particle zeta potential (i.e., the higher the aqueous phase pH, the higher the negative zeta potential, as shown in Figure 5). In turn, regardless of the organic solvent, the same aqueous phase was used when the nanoprecipitation method was performed. Consequently, the zeta potential of the particles exhibited the same behavior.

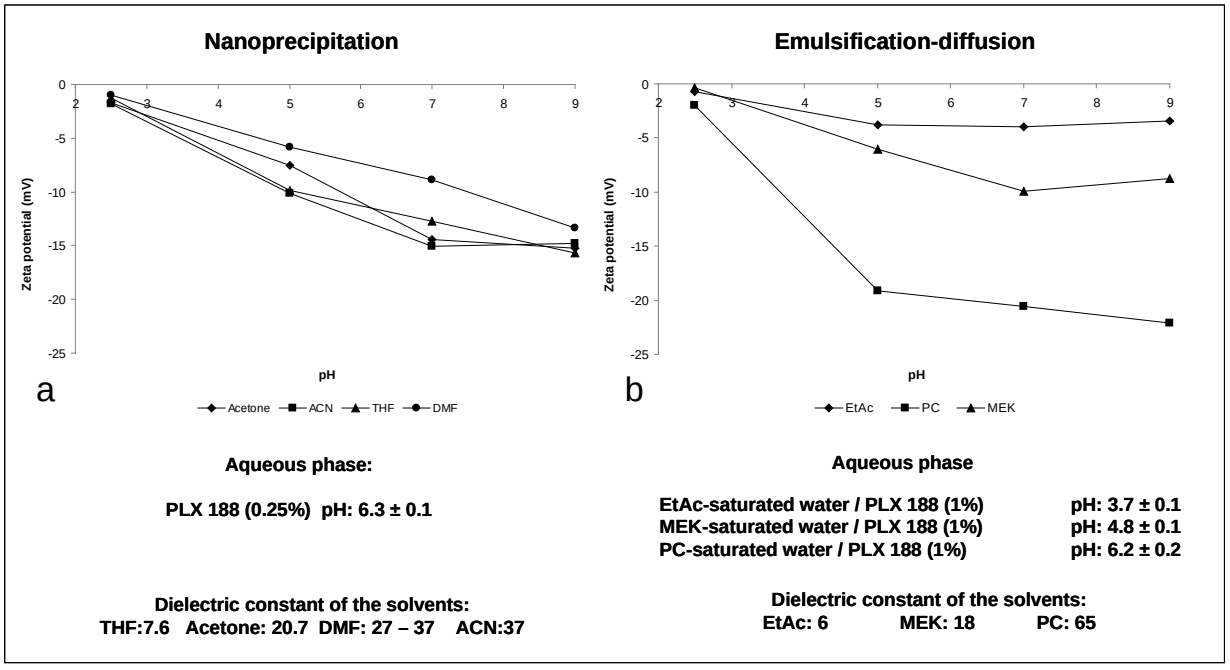

Figure 5. The influence of the nature of the solvent on the zeta potential of nanospheres. a: prepared by the nanoprecipitation procedure; b: prepared by the emulsification-diffusion method (dielectric constant values from van Krevelen and te Nijenhuis (2009))

In this direction, the nature of the aqueous phase (at a given pH) used for preparing nanoparticles by the nanoprecipitation technique was investigated. The emulsification-

diffusion method was not studied because the aqueous phase must be saturated by organic solvent. This condition makes it difficult to adjust the pH, because larger amounts of NaOH are required, which strongly affects ionic strength or produces the ethyl acetate esterification which might lead to misinterpretations of the behaviors.

As shown in Figure 6, all the particles prepared by the nanoprecipitation technique (using acetone as organic solvent) exhibit similar behaviors regardless of the pH of the aqueous phase used, except for those prepared at pH 3 which seem to exhibit slight increases in zeta potential, from zero at pH 3 to -10mV at pH 9. Presumably, this behaviour could be a consequence of the low degree of dissociation of the PCL carboxylic groups because pH of precipitation medium is close to the pH of zero charge of the polymer, which was found to be pH 2.5 according to our experimental data (Figure 6) and around pH 2.9 as reported by Gadzinowski et al. (2000). Then it is likely that non-dissociated carboxylic groups tend to buried and hydrophobic character predominates the particle surface.

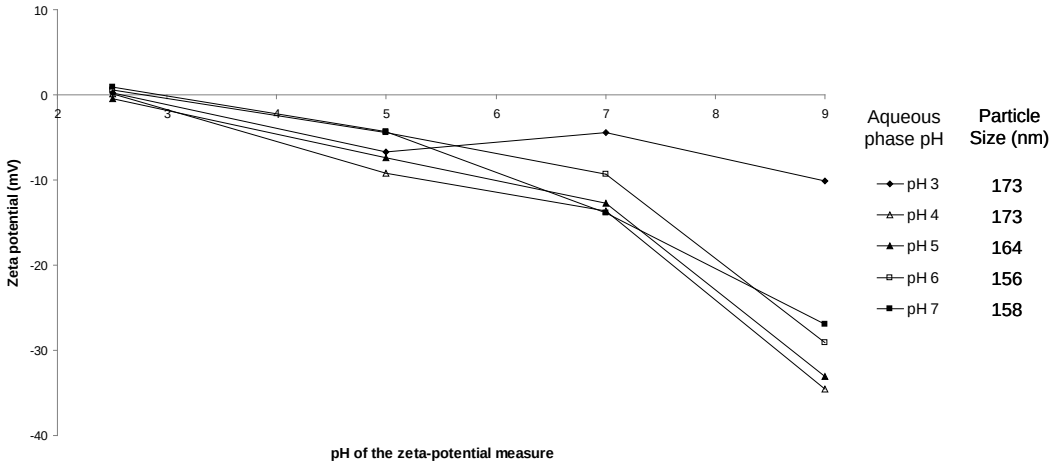

Figure 6. Influence of the aqueous phase pH on the zeta-potential of nanospheres prepared by the nanoprecipitation technique

Ionic strength could also have an effect. In terms of salinity, pH 3 is equivalent to 1 mM NaCl solution. Thus as shown in Figure 6 for pH 3, a similar trend of zeta potential versus

pH was also observed when the aqueous phase was prepared at pH 6 in the presence of 10 mM NaCl.

Discussion

Up-to-now, our findings allow suggesting the following conclusions: (1) aqueous phase of pH values higher than 4 does not govern the zeta potential of particles prepared by the nanoprecipitation technique; (2) the ionic strength of the aqueous phase used for the particle preparation might influence the zeta potential of particles prepared by the nanoprecipitation procedure; (3) organic solvent polarity governs the zeta potential of particles prepared by the emulsification-diffusion method; and (4) organic solvent polarity is not the critical factor affecting the zeta potential of particles prepared by the nanoprecipitation technique. In addition, it is noticed that particles prepared via nanoprecipitation using acetone as organic solvent and an aqueous phase of pH 3 (Figure 6) exhibit a similar behaviour of those prepared via emulsification-diffusion using ethyl acetate as organic solvent and an aqueous phase of pH around 3 (Figure 1).

Thus we proposed that the difference in behaviours of particle zeta potentials as a function of the preparation method could be explained from both the dissociation grade of PCL according to the nature of the aqueous medium from which it is re-precipitated and from the methodological aspects associated to each procedure.

Respect to the nature of the aqueous medium, low charged particles could be obtained when polymer is re-precipitated from aqueous phases of pHs around the PCL point of zero charge, regardless of the preparation method. Because of zeta potential of these particles is not influenced by the pH of the zeta potential measurement medium (Figures 1 and 6), it appears that the low dissociation of carboxylic groups favors a polymer conformation where non polar moieties of PCL are predominantly exhibited on the particle surface.

On the other hand, if the aqueous medium conditions govern the dissociation of the PCL carboxylic groups, it is likely that the methodological aspects associated to each procedure being critical for determining the electrokinetic behaviour of nanoparticles. Thus when the emulsification-diffusion is used, the first step is the organic phase dispersion as globules, within the aqueous phase at high stirring speed (6500 rpm during 10 min). Once the emulsion is formed, it is then diluted in water, causing organic solvent migration and polymer precipitation (Kwon et al. 2001; Moinard et al. 2008; Quintanar et al. 1998b). It is then possible that the close interaction between the polymer and the organic solvent present in the aqueous phase (because it is organic solvent-saturated) facilitates the arrangement of the polymer chains according to the polarity of the organic solvent during the solvent diffusion step (as illustrate in Figure 4).

On the contrary, when the nanoprecipitation method is used, the nanospheres are obtained instantaneously by the addition of the organic phase to the aqueous phase (Montasser et al. 2000; Quintanar et al. 1998b; Vauthier and Bouchemal 2009). It is unlikely that the fast mixing of the two phases leads to a particular polymer arrangement as a function of organic solvent polarity. Here, the PCL chains could form a random arrangement favoring the presence of polar groups at the particle surface when aqueous medium pH is higher than PCL point of charge zero, because the polymer precipitates instantaneously from the "organic solvent rich domain" to the water phase. The starting precipitation point have gradient chemical composition as the precipitation process progress, but mixture dielectric constants remain larger than 50.

Conclusion

This research dealt with the surface charge behavior of PCL-based nanoparticles prepared by the nanoprecipitation technique and by the emulsification-diffusion method. As a general rule, particles prepared from PCL and PLX by the nanoprecipitation method exhibited a negative zeta potential higher than the spheres prepared by the emulsification-diffusion process. In order to understanding why, a systematic study of several variables associated with particle synthesis was carried out. Thus the effect of the amount of stabilizing agent used was dismissed as explanation. Our evidence suggests that PCL polymer chains could

adopt a specific conformation depending on both the pH of the aqueous phase and the polymer interaction with the aqueous phase from which particles are formed. Thus fast polymer precipitation during nanoparticle formation by the nanoprecipitation technique, leads to polar groups of PCL forming on the particle surface, regardless of the nature of the organic solvent used. In addition, the study of the emulsification-diffusion process showed that interaction of the polymer with the organic solvent-saturated aqueous phase during the emulsification step seems to govern polymer rearrangement as a function of organic solvent polarity. Consequently, PCL particles prepared by the nanoprecipitation technique will always have negative zeta potentials whose magnitudes depend on the amount of dissociated carboxylic groups as soon as polymer re-precipitates. For PCL particles obtained by the emulsification-diffusion process, the amount of dissociable carboxylic groups on the particle surface depends on the nature of the organic solvent used in their formulation.

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2.2 Utilisation d'une méthode statistique de planification expérimentale pour l'identification des variables clés des procédés de préparation de nanoparticules.

Notre étude systématique de chacune des méthodes présentées dans la première partie de ce chapitre est réalisée en utilisant des nanosphères comme exemple. Il est important de noter que la préparation des nanocapsules par nanoprécipitation utilise une huile et un tensioactif lipophile en plus des composants déjà décrits pour les nanosphères. Egalement, pour la préparation des nanocapsules par la méthode d'émulsification-diffusion, il faut inclure l'huile dans la formulation. Ces nouvelles matières premières pourraient avoir un effet sur la taille et le potentiel zeta des nanocapsules obtenues. Ainsi, il est recommandé d'examiner l'effet des variables critiques de chaque méthode de préparation sur les caractéristiques des nanocapsules.

Pour ce faire, une méthode de planification expérimentale du type Plackett-Burman est utilisée. Les Tableaux 1 et 2 présentent une synthèse des variables étudiées avec leurs deux niveaux choisis, supérieur et inférieur. En résumé, la poly-ε-caprolactone et le poly(d,l-lactique) ont été utilisés comme polymères; le poloxamer 188 et l'alcool polyvinylique 4-88 comme agents stabilisants et les triglycerides caprylique/caprique PEG-4 (Labrafac hydro 1219®; HLB: 4.5) et le benzoate de benzyle (HLB: 6) comme exemples d'huiles. En plus, pour la préparation des nanocapsules par nanoprécipitation, le monopalmitate de sorbitane (HLB: 6.7) et le monolaurate de sorbitane (HLB: 8.6) ont été sélectionnés comme tensioactifs lipophiles. D'un autre côte, les variables des procédés qui ont une incidence sur la taille des particules selon l'étude réalisée dans la première partie de ce deuxième chapitre, ont été considérées. Ainsi, le rapport volumique entre les phases organique et aqueuse, la vitesse d'addition de la phase organique sur la phase aqueuse et la vitesse d'agitation du système ont été examinés dans le cas de la nanoprecipitation, alors que la vitesse d'émulsification et le temps d'émulsification ont été étudiés dans le cas de l'emulsification-diffusion.

Tableau 1. Variables sélectionnées pour l'étude de la méthode de nanoprécipitation en utilisant un plan des traitements du type Plackett-Burman.

	Factor name	Factor setting	
		-	+
A	Organic phase volume (ml)	10	20
B	Organic phase injection rate (μl/min)	150	400
C	System stirring rate (rpm)	350	500
D	PCL concentration (% organic phase)	0.5	1.0
E	PLA concentration (% organic phase)	0.5	1.0
F	PLX concentration (% aqueous phase)	0.5	1.25
G	PVA concentration (% aqueous phase)	0.5	1.25
H	Capric/caprylic triglyceride PEG-4 concentration (% organic phase)	0.5	1.5
I	BnBzO concentration (% organic phase)	0.5	1.5
J	Sorbitan monopalmitate concentration (% organic phase)	0.1	0.4
K	Sorbitan monolaurate concentration (% organic phase)	0.1	0.4

Les dispersions de nanocapsules ont été préparées en utilisant les procédés et les formulations déjà décrites pour la préparation des nanosphères. Cependant, quelques modifications selon chaque plan des traitements ont été faites (Tableaux 3 et 4). Par ailleurs, la taille et le potentiel zeta des nanocapsules ont été mesurés par les mêmes techniques et dans les mêmes conditions que l'étude de la préparation des nanosphères.

Tableau 2. Variables du procédé et de la formulation sélectionnées pour l'étude de la méthode d'émulsification-diffusion en utilisant un plan des traitements du type Plackett-Burman.

	Factor name	Factor setting	
		-	+
A	Emulsification rate (rpm)	6500	9500
B	Emulsification time (min)	5	10
C	PCL concentration (% organic phase)	0.5	1.0
D	PLA concentration (% organic phase)	0.5	1.0
E	PLX concentration (% aqueous phase)	0.5	1.25
F	PVA concentration (% aqueous phase)	0.5	1.25
G	Capric/caprylic triglyceride PEG-4 concentration (% organic phase)	0.5	1.5
H	BnBzO concentration (% organic phase)	0.5	1.5

Tableau 3. Matrice du type Plackett-Burman utilisée pour étudier la préparation des nanocapsules par nanoprécipitation. Les variables évaluées et les variables réponse.

Run	Factors											Output values	
	Organic phase volume	Organic phase addition rate	System stirring rate	PCL concentration	PLA concentration	PLX concentration	PVA concentration	Capric/caprylic triglyceride PEG-4 concentration	BnBzO concentration	Sorbitan monopalmitate concentration	Sorbitan monolaurate concentration	Size (nm)	Zeta-potential (mV)
1	+	-	+	+	-	-	+	+	-	-	+	372	-10
2	+	+	-	+	+	-	-	+	+	-	-	545	-7
3	-	+	+	-	+	+	-	-	+	+	-	266	-33
4	-	-	+	+	-	+	+	-	-	+	+	296	-32
5	+	-	-	+	+	-	+	+	-	-	+	399	-12
6	+	+	-	-	+	+	-	+	+	-	-	516	-7
7	-	+	+	-	-	+	+	-	+	+	-	306	-32
8	-	-	+	+	-	-	+	+	-	+	+	318	-36
9	+	-	-	+	+	-	-	+	+	-	+	456	-11
10	+	+	-	-	+	+	-	-	+	+	-	332	-26
11	-	+	+	-	-	+	+	-	-	+	+	298	-35
12	-	-	-	-	-	-	-	-	-	-	-	348	-24

Tableau 4. Matrice du type Plackett-Burman utilisée pour étudier la préparation des nanocapsules par émulsification-diffusion. Les variables évaluées et les variables réponse.

Run	Factors								Output variables	
	Emulification rate	Emulsification time	PCL concentration	PLA concentration	PLX concentration	PVA concentration	Capric/caprylic triglyceride PEG-4 concentration	BnBzO concentration	Size (nm)	Zeta-potential (mV)
1	+	-	+	+	-	-	+	+	224	-4
2	+	+	-	+	+	-	-	+	188	-3
3	-	+	+	-	+	+	-	-	249	-2
4	-	-	+	+	-	+	+	-	298	-3
5	+	-	-	+	+	-	+	+	192	-5
6	+	+	-	-	+	+	-	+	187	-4
7	-	+	+	-	-	+	+	-	243	-3
8	-	-	+	+	-	-	+	+	301	-3
9	+	-	-	+	+	-	-	+	181	-3
10	+	+	-	-	+	+	-	-	206	-2
11	-	+	+	-	-	+	+	-	262	-3
12	-	-	-	-	-	-	-	-	270	-2

Les résultats obtenus sont compilés dans les Tableaux 3 et 4 et l'effet de chacune des variables étudiées a été calculé (E_i), selon l'équation suivante (1):

$$E_i = \frac{\sum_{e=1}^{12} R_{(+)}}{(n_e/2)} - \frac{\sum_{e=1}^{12} R_{(-)}}{(n_e/2)} \qquad (1)$$

où $\Sigma R_{(+)}$ et $\Sigma R_{(-)}$ correspondent à la somme des résultats quand dans les 12 expériences la variable *i* est à son niveau supérieur (signe positif) ou inférieur (signe négatif) respectivement. n_e est le nombre d'expériences, c'est-à-dire 12. Les résultats de l'effet de chaque variable sont regroupés dans les Tableaux 5 et 6, et sont présentés de façon graphique sur les Figures 1 et 2. La valeur positive de l'effet pour une variable donnée signifie que quand la variable est utilisée à ses valeurs les plus élevées, la variable réponse (la taille, par exemple) augmente. De la même façon, la valeur négative de l'effet pour une variable spécifique, signifie que quand cette variable est utilisée à ses valeurs les plus faibles, la variable réponse augmente. Pour le cas du potentiel zeta, les calculs ont été réalisés à partir des valeurs absolues. Les résultats du potentiel zeta pour les nanocapsules préparées par la méthode d'émulsification-diffusion ne sont pas inclus dans les analyses des effets parce que les variations entre les expériences se trouvent dans un intervalle étroit (-2 mV à -5 mV) et on pourra donc supposer que les variables étudiées n'ont aucun effet sur le potentiel zeta des particules.

D'un autre côté, la signifiance des effets a été estimée à partir de l'équation (2) :

$$t_i = \frac{E_i (n_v - 1)^{1/2}}{s} \qquad (2)$$

où E_i est l'effet de chaque variable, n_v le nombre de variables et *s* la déviation standard calculée par l'équation (3).

$$s = \left[\sum_{i=A}^{K} \frac{(E_i)^2}{n_v} \right]^{1/2} \qquad (3)$$

La valeur de $t_{critique}$ (t*) correspond à celle reportée dans les tableaux $t_{student}$ pour n_v-1 degrés de liberté et un niveau de confiance de 95%.

Tableau 7. Résultats des effets et de leurs significations pour les nanocapsules préparées par nanoprécipitation en utilisant une planification expérimentale du type Plackett-Burman.

	Org. Ph. volume	Org. ph. inject. rate	Syst. Agit. Rate	PCL	PLA	PLX	PVA	Labrafac	BnBzO	Span 40	Span 20
						Particle size					
Σ+	2619,6	2264,1	1856,1	2385,3	2514,1	2015,0	1988,6	2605,1	2421,7	1816,5	2137,9
Σ-	1832,7	2188,3	2596,2	2067,0	1938,2	2437,3	2463,7	1847,3	2030,6	2635,9	2314,5
Σ+ - Σ-	786,9	75,8	-740,1	318,4	575,8	-422,3	-475,0	757,8	391,2	-819,4	-176,6
Effect	131,1	12,6	-123,4	53,1	96,0	-70,4	-79,2	126,3	65,2	-136,6	-29,4
Effect significance											
s	93,2										
t	4,450	0,429	-4,186	1,800	3,257	-2,388	-2,686	4,286	2,212	-4,634	-0,999
						Particle zeta potential					
Σ+	71,5	139,4	177,5	106,7	94,9	165,0	156,3	81,1	115,4	194,0	135,2
Σ-	192,3	124,4	86,3	157,1	169,0	98,9	107,5	182,7	148,5	69,8	128,7
Σ+ - Σ-	-120,7	15,0	91,2	-50,4	-74,1	66,1	48,7	-101,6	-33,1	124,1	6,5
Effect	-20,1	2,5	15,2	-8,4	-12,3	11,0	8,1	-16,9	-5,5	20,7	1,1
Effect significance											
s	12,8										
t	-4,976	0,619	3,758	-2,078	-3,053	2,725	2,008	-4,187	-1,365	5,116	0,269
t* (α=0.05; gl=10)	2,228										

Tableau 6. Résultats des effets et de leurs signifiancations pour les nanocapsules préparées par émulsification-diffusion en utilisant une planification expérimentale du type Plackett-Burman.

	Emulsif. rate	Emulsif. time	PCL	PLA	PLX	PVA	Labrafac	BnBzO
				Particle size				
Σ+	1178,7	1336,0	1577,8	1383,8	1203,9	1446,0	1520,6	1273,6
Σ-	1623,5	1466,2	1224,4	1418,4	1598,3	1356,2	1281,6	1528,6
Σ+ - Σ-	-444,8	-130,2	353,4	-34,6	-394,4	89,8	238,9	-255,0
Effect	-74,1	-21,7	58,9	-5,8	-65,7	15,0	39,8	-42,5
Effect significance								
s	46,6							
t	-4,205	-1,231	3,341	-0,327	-3,729	0,849	2,259	-2,410
t* (α=0.05; gl=7)	2,365							

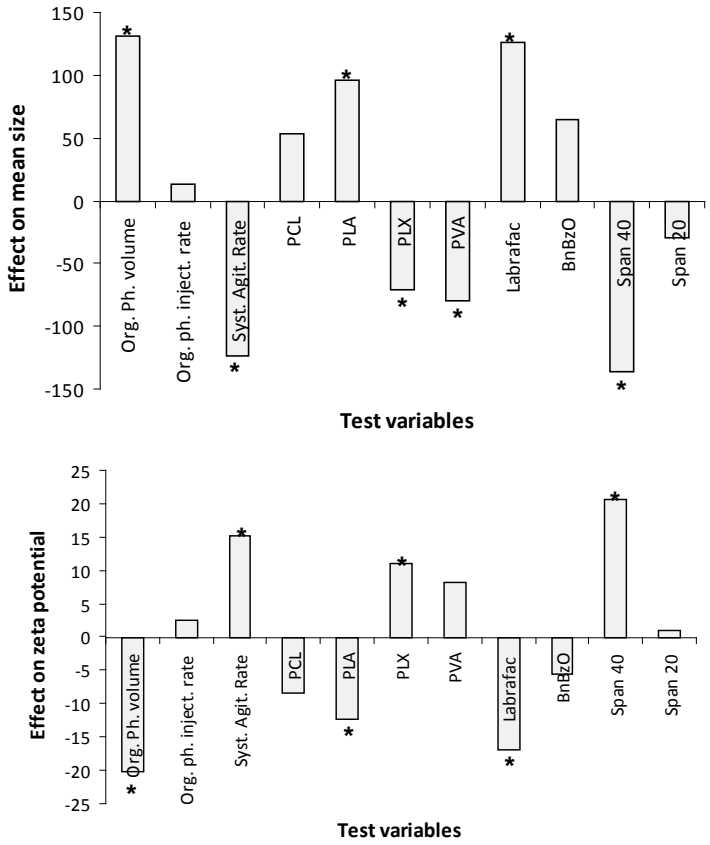

Figure 1. Effets des variables sur la taille et le potential zeta des nanocapsules préparées par nanoprécipitation en utilisant une planification expérimentale du type Plackett-Burman (marquées avec * les variables avec un effet significatif).

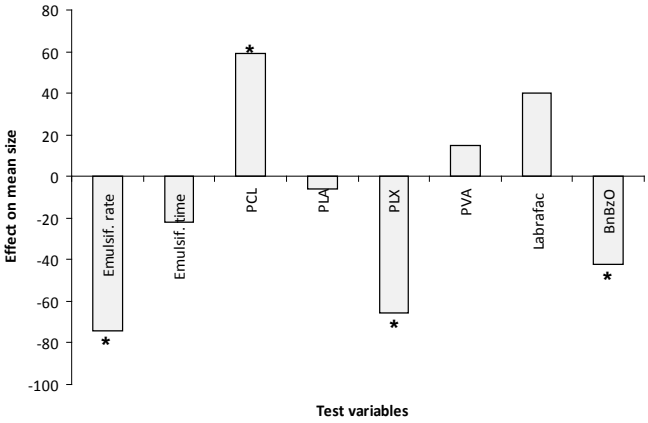

Figure 2. Effets des variables sur la taille des nanocapsules préparées par émulsification-diffusion en utilisant une planification expérimentale du type Plackett-Burman (marquées avec * les variables avec un effet significatif).

De façon générale, concernant la méthode d'émulsification-diffusion, les résultats de cette étude en utilisant une méthode statistique de planification expérimentale suggèrent que la vitesse d'émulsification détermine la taille des nanocapsules. Aussi, comme il a été observé dans le cas des nanosphères, le temps d'émulsification a un modeste effet sur les caractéristiques des particules. Par rapport à la méthode de nanoprécipitation, dans les conditions de notre étude, la vitesse d'addition de la phase organique paraît n'avoir aucun effet sur les propriétés des nanocapsules. Cependant, le rapport volumique entre les deux phases (organique et aqueuse) et la vitesse d'agitation du système, sont les variables du procédé les plus critiques affectant la taille et le potentiel zeta des nanocapsules préparées via la nanoprécipitation. En effet, quand la vitesse d'agitation du système est la plus faible, la taille des particules est la plus grande, ceci est probablement due à la difficulté d'obtenir un mélange homogène des phases comme expliqué pour la préparation des nanosphères. Dans cette même direction, la forte vitesse d'agitation du mélange favorise la formation des nanocapsules de petites tailles. Par conséquent, l'aire superficielle est la plus importante, ce qui peut expliquer l'augmentation signifiant du potentiel zeta. Des lectures similaires peuvent être faites à partir des résultats obtenus pour les autres variables étudiées et les comportements observés pourraient être dus aux mêmes raisons exposées dans l'étude sur les nanosphères.

D'un autre côte, l'effet de la nature et de la concentration des matières premières utilisées sur les propriétés des nanocapsules dépend de la méthode de préparation. D'après les résultats présentés sur la Figure 1, le type de polymère, d'huile et de tensioactif lipophile détermine la taille et le potentiel zeta des particules préparées par nanoprécipitation. Cependant, seulement les concentrations de PLA, de PLX, de PVA, des triglycerides caprylique/caprique ou de monopalmitate de sorbitane montrent une influence significative sur la taille des particules.

Dans le cas de l'émulsification-diffusion, la nature des matières premières gouverne drastiquement le comportement de la taille des nanocapsules. D'ailleurs, comme observé sur la Figure 2, la taille pourrait être contrôlée via la concentration de PCL, de PLX et de benzoate de benzyle. Comme déjà expliqué plus haut, ni la nature ni la concentration des matières premières utilisées dans cette étude ne présentent un effet sur le potentiel zeta des capsules préparées par émulsification-diffusion.

En guise de conclusion, le travail de recherche sur la comparaison des deux méthodes; nanoprécipitation et émulsification-diffusion, réalisé soit via une étude fondamentale et systématique soit par l'utilisation d'une méthode statistique de planification expérimentale, a permis l'identification des variables du procédé et des matières premières clés pour contrôler la taille et le potentiel zeta des nanocapsules et des nanosphères. En plus, les conclusions obtenues à partir des deux études sont cohérentes et permettent d'avoir une vue de l'ensemble des paramètres affectant les propriétés des nanoparticules.

D'autre part, la compréhension des mécanismes de formation des particules à travers l'étude approfondie et systématique de chaque variable de la formulation et pour chaque procédé de préparation, permet une bonne planification des traitements en utilisant une méthode statistique de planification expérimentale et une exploitation optimale des résultats. Néanmoins, des études systématiques nécessitent des ressources et du temps, par rapport à la méthodologie de recherche par planification expérimentale.

Dans cette étude notre intérêt a été d'illustrer ces deux approches pour la recherche sur la préparation de nanoparticules. Comme résultat, nous avons fourni une base conceptuelle solide sur les mécanismes de formation des nanoparticules fondée sur des études physico-chimiques et aussi, nous avons montré l'applicabilité en ce type de recherches, d'une méthode de planification expérimentale.

3. DEVELOPPEMENT DES NANOPARTICULES CONTENANT LE DICLOFENAC: ETUDE COMPARATIVE ENTRE LA NANOPRECIPITATION ET L'EMULSIFICATION-DIFFUSION

Ce troisième chapitre est consacré au développement des nanoparticules contenant le diclofenac comme principe actif en utilisant la nanoprecipitation et l'émulsification-diffusion. Une analyse de l'état de l'art sur l'encapsulation des molécules actives par ces deux techniques, soit dans les nanosphères soit dans les nanocapsules, montre que la quantité de substance active utilisée pour la préparation des particules est la même quelque soit la méthode ou le type de nanoparticule (Tableaux 7 et 8). Cependant, les meilleurs taux d'encapsulation des substances actives sont obtenus par la préparation des nanocapsules avec des valeurs supérieures à 50% pour la majorité des cas, tandis que l'efficience d'encapsulation par nanosphères est très variable et souvent très faible (entre 20% et 40%). En nous basant sur cette tendance, nous avons choisi l'élaboration de nanocapsules contenant le diclofenac comme molécule d'étude.

3.1 Critères de sélection des matières premières.

Des études développées dans le chapitre précédent concernant l'incidence des matières premières sur les propriétés des nanocapsules, montrent que le type du polymère, d'agent stabilisant et d'huile détermine la taille et le potentiel zeta des particules finales. Par conséquent, pour développer des nanocapsules contenant le diclofenac, la stratégie choisie a été de fixer les conditions opératoires pour chaque technique de préparation, d'utiliser

Tableau 7. Efficacité d'encapsulation de quelques substances actives, dans les nanocapsules et dans les nanosphères, par la méthode de nanoprécipitation.

Active molecule	% w/v of the organic phase	Encapsulation efficiency (%)	Reference[*1]	Active molecule	% w/v of the organic phase	Encapsulation efficiency (%)	Reference[*1]
Nanocapsules				**Nanosphères**			
Indomethacin	0.05	100	Fessi et al., 1989.	Indomethacin	0.06	100	Pohlmann et al., 2002.
Indomethacin	0.05	nr.	Pohlmann et al.,2002.	Primaquine	0.02	85 - 94	Rodrigues et al., 1995.
Indomethacin	0.04	100	Cruz et al., 2006.	Cyclosporin A	0.01	82 - 98	Chacon et al., 1996.
Atovaquone	0.04	100	Cauchetier et al., 2003.	Cyclosporin A	0.003	90 - 98	Molpeceres et al., 1996.
Atovaquone	0.10	100	Dalençon et al., 1997.	Pentamidine	0.05	40 - 76	Paul et al., 1997.
Rifabutine	0.31	93	Dalençon et al., 1997.	Isradipine	0.01	74 - 97	Leroueil et al., 1998.
Tretinoin	0.02	100	Ourique et al., 2008.	Nimodipine	0.05	20 - 90	Ge et al., 2000.
Indomethacin ethyl ester	0.04	nr.	Poletto et al., 2008.	Rolipram	0.20	2 - 20	Lamprecht et al., 2001.
Primidone	0.10	75 - 67	Ferranti et al., 1999.	Tyrphostin AG-1295	0.02	70	Chorny et al., 2002.
Vitamin E	2.50	nr.	Charcosset and Fessi, 2005.	Paclitaxel	0.01	15 - 100	Fonseca et al., 2002.
Spironolactone	0.06	76 - 96	Limayem et al., 2006.	Sodium cromoglycate	0.10	nr.	Peltonen et al., 2002.
Griseofulvine	0.03	78 - 99	Zili et al., 2005.	Acyclovir	0.82	1 - 3.5	Giannavola et al., 2003.
Melatonin	0.04	50	Schaffazick et al., 2008.	Diclofenac	0.04	100	Schaffazick et al., 2003.
Diclofenac	0.04	97 - 100	Schaffazick et al., 2003.	Ibuprofen	1.40	40 - 50	Galindo et al., 2005.
Diclofenac	0.06	100	Guterres et al., 1995.	Xanthone	0.006	26 - 40	Teixeira et al., 2005.
Tacrolimus	0.02	nr.	Nassar et al., 2009.	Griseofulvin	0.06	78 - 98	Zili et al., 2005.
Xanthone	0.01	85 - 89	Teixeira et al., 2005.	Docetaxel	0.004	10 - 23	Musumeci et al., 2006.
All-trans retinoic acid	0.05	68 - 97	Jeong et al., 2004.	Zinc phthalocyanine	0.04	70	Ricci et al., 2006.
				Flurbiprofen	0.18	74 - 97	Vega et al., 2006.
				Haloperidol	0.05	0,25 - 4	Budhian et al., 2007.
				Coenzyme Q10	0.20	49 - 72	Nehilla et al., 2008.
				Silymarin	0.25	20 - 62	Guhagarkar et al., 2009.
				Carvedilol	0.10	41 - 56	Jawahar et al., 2009.
				Phenobarbital and others molecules	0.04	5 - 94	Barichello et al., 1999.

nr. Non-reported value.

[*1] References available in: C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, Int. J. Pharm. 385 (2010) 113-142.

[*2] References available in: C.E. Mora-Huertas, H. Fessi, A. Elaissari, Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification-diffusion methods. Critical comparison, Adv. Colloid Interface Sci. 163 (2011) 90-122.

Tableau 8. Efficacité d'encapsulation de quelques substances actives, dans les nanocapsules et dans les nanosphères, par la méthode d'émulsification-diffusion.

Active molecule	% w/v of the organic phase	Encapsulation efficiency (%)	Reference[*1]	Active molecule	% w/v of the organic phase	Encapsulation efficiency (%)	Reference[*2]
Nanocapsules				**Nanosphères**			
Indomethacine	0.25	nr.	Guinebretière et al., 2002.	17b-estradiol benzoate	0.03	67	Kwon et al., 2001.
Indomethacine	0.11	nr.	Limayem et al., 2004.	Enalaprilat	2.00	24 - 46	Ahlin et al., 2002.
Indomethacine	0.10	100	Quintanar et al., 1998	Ibuprofen	0.20	62 - 86	Galindo et al., 2005.
Vitamin E	2.35	92	Quintanar et al., 1998	Estradiol	0.20	46 - 73	Hariharan et al., 2006.
Progesterone	0.10	99	Quintanar et al., 1998	Cyclosporine	0.50	16 - 23	Italia et al., 2007.
Estradiol	0.10	52	Quintanar et al., 1998	Estradiol	0.05	35 - 68	Mittal et al., 2007.
Chlorambucil	0.10	32	Quintanar et al., 1998	Estradiol	0.20	48 - 95	Sahana et al., 2008.
Eugenol	2.50	nr.	Choi et al., 2009.	Tacrolimus	0.10	50 - 60	Shin et al., 2010.
Hinokitol	0.80	92 - 94	Joo et al., 2008.				
4-nitroanisole	0.07	nr.	Romero and Vincent, 2002.				

nr. Non-reported value.

[*1] References available in: C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, Int. J. Pharm. 385 (2010) 113-142.

[*2] References available in: C.E. Mora-Huertas, H. Fessi, A. Elaissari, Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification-diffusion methods. Critical comparison, Adv. Colloid Interface Sci. 163 (2011) 90-122.

des formulations développées lors des travaux précédents de notre groupe de recherche[19] et de sélectionner les matières premières en considérant quelques aspects pratiques et la possible application pédiatrique du produit final.

Par conséquent, la poly-ε-caprolactone (PCL) a été sélectionnée comme polymère et son prix est avantageux par rapport au poly(D,L-lactique) ou poly(lactique-co-glycolique)[20]. Le poloxamer 188 (PLX) a été choisi comme agent stabilisant. Contrairement aux polysorbates communément utilisés dans la formulation des nanocapsules, le poloxamer n'a aucune restriction pour usage pédiatrique[21] et à la différence de l'alcool polyvinylique, il peut être utilisé pour les applications intraveineuses, ce que lui confére une confiance pour son administration aux enfants[22]. D'un autre coté, la facilité de mise en solution du poloxamer présente un avantage par rapport au PVA[22]. Pour la préparation des nanocapsules par nanoprécipitation, la lécithine de soja a été choisie comme tensioactif lipophile. Elle est utilisée dans la formulation des produits à usage intraveineuse et comme supplément nutritionnel important pour les enfants[22], lequel est plus conseillé par rapport aux esters de sorbitane. En raison de l'insolubilité de la lécithine dans l'acétone, un mélange d'éthanol : acétone (1 :3) a été utilisé comme solvant de la phase organique pour la technique de nanoprecipitation. L'acétate d'éthyle a été le solvant organique lors de la préparation des nanocapsules par émulsification-diffusion. Ces solvants sont classés de Type III, traduisant qu'ils ont une toxicité basse selon la Conférence Internationale d'Harmonisation - ICH[23].

[19] Formulation pour nanocapsules préparées par nanoprécipitation : Polymère (0.160 g) ; huile (0.4 ml); tensioactif H/L (0.060g) ; solvant organique (20 ml) ; agent stabilisant (0.1 g) ; eau (40 ml). Référence : H. Fessi, communication personnelle (Avril, 2010) ; Formulation pour nanocapsules

préparées par émulsification-diffusion : Polymere (0.1 g) ; huile (0.4 ml) ; solvant organique saturé d'eau (10 ml) ; agent stabilisant (0.4 g) ; eau saturée de solvant organique (40 ml). Référence : Guinebretière, S., 2001. Nanocapsules par emulsion-diffusion de solvant: Obtention, caracterisation et mecanisme de formation. Ph.D Thesis, Université Claude Bernard-Lyon 1, Francia.

[20] Prix de vente Sigma-Aldrich: poly-ε-caprolactone : 5g, ≈ 1.90 euros; poly(D,L-lactique): 5g, ≈ 120 euros; poly(lactique-co-glicolique): 5g, ≈ 270 euros.

[21] World Health Organization. Development of paediatric medicines: Pharmaceutical development. Points to consider. Geneve. 2008.

[22] R.C. Rowe, P.J. Sheskey, S.C. Owen, Handbook of Pharmaceutical Excipients, fifth edition, Pharmaceutical Press, Great Britain, 2006.

[23] International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use, Impurities: Guideline for Residual Solvents, ICH Harmonised Tripartite Guideline, 2005.

Selon l'état de l'art, la quantité de molécule active encapsulée dans les nanocapsules est en relation étroite avec sa solubilité dans l'huile[24]. Par conséquent, l'optimisation des nanocapsules comme vecteurs du diclofenac pourrait être envisagée après sélection de l'huile compatible avec la formulation. Dans cette optique, différentes huiles d'intérêt pharmaceutique, telles que les huiles végétales (huile de maïs et huile d'amande), des huiles semi-synthétiques (triglycerides des acides caprylique et caprique et leurs dérivés -miglyol 810®, miglyol 812® et miglyol 829®, labrafac PG®, labrafac lipophile®, capryol PGMC® et capryol 90®-) et des huiles auto-émulsifiantes (labrafil M1944CS® et labrafil M2125CS®), ont été étudiées. Toutes ces huiles ont été caractérisées en termes de leur densité, leur tension superficielle, leur polarité, leur viscosité et leur performance à solubiliser le diclofenac.

Dans la première étape de cette étude, ces huiles ont été sélectionnées selon leur capacité de former des dispersions stables de nanocapsules et leur compatibilité avec le polymère utilisée. Pour ce dernier, nous avons utilisé la technique de calorimétrie différentielle à balayage comme outil de caractérisation afin d'évaluer le comportement des dispersions de concentration croissante du polymère dans l'huile. Ces études préliminaires ont permis d'éliminer des composantes huileuses connues comme auto-émulsifiantes en raison de l'instabilité de leurs dispersions. D'un autre côté, la solubilité majeure du PCL dans le capryol 90® et dans le capryol PGMC® par rapport aux autres huiles, a été constatée clairement. Par conséquent, elles n'ont pas été utilisées pour la suite de notre étude.

3.2 Comportement des dispersions de nanocapsules.

Dans la seconde étape de cette étude, les dispersions stables de nanocapsules ont été caractérisées en termes de taille et de potentiel zeta des particules. Les résultats montrent l'effet de la méthode de préparation sur la taille des particules. En général, la taille la plus

[24]I. Limayem, C. Charcosset, H. Fessi, Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use, Int. J. Pharm. 325 (2006) 124-131; D. Moinard, Y. Chevalier, S. Briançon, H. Fessi, S. Guinebretière, Nanoparticles for drug delivery : Review of the formulation and process difficulties illustrated by the emulsion-diffusion process, J. Nanosci. Nanotech. 6 (2006) 2664-2681; P. Legrand, G. Barrat, V. Mosqueira, H. Fessi, J.P. Devissaguet, Polymeric nanocapsules as drug delivery systems, S.T.P. Pharma Sci. 9 (1999) 411-418; P. Couvreur, G. Barrat, E. Fattal, P. Legrand, C. Vauthier, Nanocapsule technology: A review, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 99-134.

grande est obtenue par la méthode d'émulsification-diffusion. Cependant, la nature de l'huile pourrait aussi déterminer la taille des nanocapsules préparées par la technique de nanoprécipitation. Ce comportement est probablement dû à la précipitation des acides gras à longue chaine, ce qui pourrait augmenter le volume du noyau huileux et par conséquent, la taille des particules.

D'un autre côté, la charge superficielle des nanoparticules varie en fonction de la méthode de préparation. Les nanocapsules obtenues par nanoprécipitation reflètent un potentiel zeta très négatif, dont la magnitude augmente grâce à la présence de la lécithine dans la formulation. Comme il a déjà été discuté dans le chapitre précèdent, cette différence de charge superficielle pourrait être liée à la conformation du polymère à la surface, ce qui pourrait affecter l'interaction entre le polymère et l'agent stabilisant. Par conséquent, la stabilité des dispersions de nanocapsules pourrait aussi être différente selon la méthode de préparation utilisée.

Pour approfondir ce dernier point, une étude sur l'effet de l'électrolyte sur la stabilité colloïdale des nanocapsules a été réalisée. Les résultats obtenus montrent que les nanoparticules préparées par nanoprécipitation sont stabilisées par effet électrostatique et celles obtenues par la méthode d'émulsification-diffusion montrent une stabilisation par via stérique. Ainsi, il est fort probable que l'interaction polymère – agent stabilisant soit plus importante dans le cas de nanocapsules préparées par émulsification-diffusion. Par conséquent, l'épaisseur de la couche polymérique et la densité surfacique en agent stabilisant sur les particules pourraient être importantes pour les particules obtenues via cette méthode.

Par ailleurs, la stabilité colloïdale des dispersions de nanocapsules a été également évaluée par rapport à sa performance lorsqu'elles sont soumises à des cycles répétitifs de chauffage-congélation-décongélation. Les nanocapsules préparées par émulsification-diffusion ont montré une bonne stabilité, ce qui a permis de suggérer un éventuel effet protecteur de la couche d'agent stabilisant contre les changements thermiques. Ce résultat permet de contribuer à la prise de décision sur l'utilisation de la lyophilisation comme stratégie pour la préparation de dispersions solides de nanocapsules. Ainsi, cette technique peut être adéquate pour stabiliser les nanocapsules préparées par émulsification-diffusion, mais moins indiqué pour les nanocapsules obtenues par nanoprecipitation.

L'étape finale du développement des nanocapsules dans cette recherche concernait l'étude de leur comportement lors de la dissolution. En premier lieu, la tendance générale de nos résultats montre des différences selon la méthode de préparation. Ainsi, lorsque l'émulsification-diffusion est utilisée, la totalité de l'actif est disponible dans le milieu de dissolution au bout des 15 minutes. En revanche, seulement 60% du diclofenac est libéré au bout des 48h lorsque la technique de nanoprecipitation est utilisée. Ammoury et al.[25] avaient déjà remarqué l'incomplète libération de la substance active en préparant des nanocapsules d'indomethacine par nanoprécipitation. Leur recherche sur ce sujet, fondée sur des études de dissolution, suggère la présence d'autres types de nanocolloïdes, tels que les liposomes et les nanoémulsions, formés lors de la préparation des nanocapsules.

De même, nous avons vérifié la faible libération du diclofenac à partir de différents nanovecteurs préparés par nanoprécipitation. Notre analyse théorique de l'interaction entre les composants de la formulation, suggère que la formation des nanoémulsions pourrait prédominer par rapport à la formation des autres colloïdes. En plus, nous pensons que certains de ces nanovecteurs sont probablement instables au pH acide du milieu de dispersion, ce qui explique la libération de 80% du diclofenac au bout de 15 min, lorsque les dispersions sont examinées 20 jours après leur préparation.

D'un autre côté, nous avons cherché aussi la possibilité de préparer d'autres colloïdes en utilisant la méthode d'émulsification-diffusion. Nos résultats démontrent que les nanoémulsions ont un profil de libération du diclofenac identique à celui des dispersions que nous avons appelé jusqu'à maintenant, « des nanocapsules ».

Malheureusement, la séparation de ces colloïdes n'est pas évidente, car ils ont une taille et un potentiel zeta similaires. En effet, la quantité de l'actif détecté dans le milieu de dispersion après le traitement des systèmes colloïdaux par la technique d'ultrafiltration-centrifugation, n'est pas significative. Cependant, afin de vérifier si l'actif était vraiment encapsulé à l'intérieur des nanocapsules, nous avons cherché à identifier le diclofenac avec l'acide nitrique via la formation d'un dérivé nitré dont la coloration varie du jaune au rouge selon la

[25] N. Ammoury, H. Fessi, J.P. Devissaguet, F. Puisieux, S. Benita, In vitro release kinetic pattern of indomethacin from poly(D,L-lactide) nanocapsules, J. Pharm. Sci. 79 (1990) 763-767.

concentration de la molécule active[26]. Les résultats obtenus confirment l'encapsulation incomplète du diclofenac dans les nanocapsules. En plus les dispersions colloïdales préparées par nanoprécipitation donnent une coloration jaune tandis que celles obtenues par émulsification-diffusion donnent une coloration rouge. Par conséquent, ces résultats suggèrent qu'en utilisant des formulations similaires, la technique de nanoprécipitation permet d'avoir les meilleurs taux d'encapsulation de l'actif par rapport à la méthode d'émulsification-diffusion.

L'étude complète relative à la préparation des nanocapsules de diclofenac par nanoprécipitation et par émulsification-diffusion est présentée dans le manuscrit suivant: C.E. Mora-Huertas, O. Garrigues, H. Fessi, A. Elaissari, The incidence of the oil nature on the behavior of nanocapsules prepared via nanoprecipitation and via emulsification-diffusion (2011).

[26] Essai d'identification pour diclofenac, Committe on Japanese Pharmacopoeia, Ministry of Health, Labour and Welfare, The Japanese Pharmacopoeia, Fifteenth edition, Japan's Pharmaceutical and Medical Devices Agency, Japan, 2006; A.A. Matin, M.A. Farajzadeh, A. Jouyban, A simple spectrophotometric method for determination of sodium diclofenac in pharmaceutical formulations, Il Farmaco 60 (2005) 855-858.

Influence of the oil nature on the colloidal properties of nanocapsules prepared via nanoprecipitation and emulsification-diffusion methods: Comparative study

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Abstract

The encapsulation of hydrophobic drugs has been widely investigated using mainly oil phase in order to enhance the encapsulation efficiency. However, the effect of the oil nature on the colloidal properties of the final nanocapsules irrespective of the elaboration process has been neglected and the hydrophobic drug location in the disperse media has not been completely elucidated. Therefore this paper describes the effect of the oil and the preparation method on the behavior of nanocapsules prepared via nanoprecipitation and via emulsification-diffusion. The colloidal stability of the final dispersions, drug location and the drug release are preparation method and oil nature dependent. The preparation method, direct affects the electrokinetic properties (i.e. zeta potential) of the nanocapsules and thus their colloidal stability. Oil nature governs drug encapsulation and it appears that oil polarity could affect the stability of the final dispersions.

Keywords: nanocapsule; nanoparticle; diclofenac; oil; nanoprecipitation; emulsification-diffusion.

1. Introduction

In pharmaceutics, nanocapsules based on biodegradable polymers have been widely developed as drug carriers by using preparation methods such as nanoprecipitation, emulsification-diffusion, emulsification-coacervation, double emulsification, polymer coating and layer-by-layer technique [1,2]. This allows to protect the active substance from degradation by oxidation [3,4], hydrolysis [5] or enzymatic attack [6], reduce their gastrointestinal side-effects such as irritation and mucosal damage [7-9], and increase their therapeutic efficacy by optimizing the bioavailability of the active ingredient [10-13] or drug targeting [14-16].

Different actives such as hydrophilic small therapeutic molecules, proteins and nucleic acids have been encapsulated. Also, much attention has been focused on the encapsulation of less or non water-soluble active molecules [2]. In this case, oil containing the active substance is usually chosen as the core of the nanocapsules, as widely reported in the literature. However, few studies have been dedicated to the relationship between the process and the physicochemical properties of the resulting nanocapsules. Consequently, the aim of this work is to emphasize the relationship between the particle elaboration process, the nature of the oil (used in the recipe), the colloidal stability and the encapsulated drug release. To this end, eleven oils were investigated to identify their possible use in diclofenac nanocapsule preparation by using both the nanoprecipitation technique and the emulsification-diffusion method. In addition to the typical oil characterization reported by various authors, oil polarity and oil-polymer compatibility were also investigated. Once the nanocapsules were prepared, hydrodynamic size and zeta potential were measured and evaluated and colloidal stability was carefully investigated. Finally, encapsulation efficiency and drug release were evaluated.

2. Materials and methods

2.1 Materials

Poly(ε-caprolactone) (PCL) (Mw: 14 kDa) was obtained from Sigma–Aldrich, poloxamer 188 (PLX) (Lutrol® F68) from Basf, soy lecithin (Lipoid® S75) from Lipoid GmbH, caprylic/capric tryglicerides (Miglyol® 810, Miglyol® 812 and Miglyol 829) from Condea Chemie, corn oil from Sigma and almond oil from Fluka. Labrafac PG, Labrafac lipophile WL1349, Labrafil M1944CS, Labrafil M2125CS, Capryol 90 and Capryol PGMC were kindly given by Gattefossé (France). Diclofenac sodium salt was kindly supplied by O4CP-Institut Villemin, France. Acetone, ethanol, ethyl acetate (EtAc) and all other chemicals and solvents used were analytical grade. Deionised water from Milli-Q system was used in all experiments.

2.2 Methods

2.2.1 Preparation of diclofenac free acid

A 1% diclofenac sodium aqueous solution (100 ml) was treated with 1N HCl (5 ml). The precipitated was filtered and washed with deionised water to remove chloride ions. It was verified by a specific limit test for chloride, using a silver nitrate solution. Then, precipitated was drying (45°C) during 72 h and purified by twice re-crystallization from ethanol-water solution (80:20, 200 ml) yielding white crystals with fusion range between 173°C and 182°C.

2.2.2 Characterization of oil physicochemical properties

Density was measured at 20 °C ± 2°C using a pycnometer; weights were known to ± 0.1 mg. Interfacial characterization (oil/water and oil/air) was made at 20 °C ± 2°C by the pendant drop method using a Drop Shape Analysis System DSA 10Mk2 (Krüss, Germany). For

oil/water interfacial tension, the classical sessile drop method (top-to-bottom) or the bottom-to-top sessile drop method was chosen according to the oil density. The viscosity of oils was measured at 20°C using a viscosimeter R180 (Lamy, France) equipped with a mobile system No. 11 rotating at 200 s^{-1} shear rate.

2.2.3 Diclofenac solubility in oils

To approximately 2.5 g of oil contained in a vial, diclofenac acid was added and the sample was hermetically sealed and magnetically stirred in a thermostate at 40°C ± 2°C (Ika-Ret Basic, 500 rpm). Addition of solute was repeated until solid material was remaining after stirring for 24h. Then, samples were kept in standby at 20°C ± 2°C during 48 h and supernatant was filtered by 0.45 μm PVDF filter. A known quantity of the supernatant was dissolved in EtOH for UV determination at 270 nm (Varian Cary 50 Probe UV-VIS spectrophotometer) using a validated method (r=0.9856, range 5 – 20 mcg/ml). The reference solution consisted of an identical amount of the pure oil in EtOH.

2.2.4 Polymer – oil compatibility

Calorimetric measurements were performed with a DSC Q200 equipped with a refrigerated cooling system RCS90 (TA Instruments) and calibrated with indium metal as standard. Dry nitrogen was used as purge gas at a rate of 50 ml/min. The temperature was known at ± 0.1°C and the samples were weighted to ± 0.01 mg. Samples of variable composition polymer – oil and of final weight between 12 mg and 15 mg were analysed at a temperature gradient of 2°C/min.

2.2.5 Preparation of nanocapsule dispersions

The preparation process of nanocapsules by nanoprecipitation followed the procedure proposed by Fessi et al. [17]. First, 160 mg of PCL and 60 mg of soy lecithin were dissolved in EtOH:acetone 1:3 (20 ml). Then, 0.4 ml of a diclofenac acid-saturated solution in oil was added to the acetonic solution. The resulting organic solution was added (Harvard Apparatus

Syringe Infusion Pump 22, 48 ml/h) into an aqueous solution of PLX (0.25%, pH 3.8, 40 ml) magnetically stirred (Ika-Ret Basic, 375 rpm). Particles were instantaneously formed.

The preparation of particles by emulsification-diffusion method was performed as reported by Quintanar et al. [18]. Briefly, PCL (0.1 g) was dissolved in 10 ml of water-saturated EtAc. Then, 0.4 ml of a diclofenac acid-saturated solution in oil was added to the polymer solution. The resulting organic phase was emulsified with 40 ml of a solvent-saturated aqueous phase containing PLX (1%), by using a high speed homogenizer (Ultraturrax stirrer T25 IKA; 6500 rpm for 10 min). The emulsion was added in one shot to water (200 ml) under mechanical stirring (500 rpm, Heidolph RZR 2102), leading to the formation of the particles.

The solvent and part of the water of the particle dispersions were removed by evaporation under reduced pressure and 40°C (Büchi Rotavapor R-124) until a final volume of 10 ml.

2.2.6 Size and zeta potential of submicron particles

The nanocapsule size was measured by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments), 5 measures/sample, 5 runs of 10 s/measure at 25 °C, after adequate dilution of a suspension aliquot in deionised water (water pH between 6 and 7). Zeta potential was deduced from the electrophoretic mobility measurement by using the Zetasizer Nanoseries (5 measures/sample, 5 runs/measure at 25°C). The particle dispersion was highly diluted in pH 6.0 1 mM NaCl solution.

2.2.7 Evaluation of the colloidal stability

The colloidal stability of the nanocapsules was evaluated by induced aggregation with Na_2SO_4 and with NaCl, and by thermal-freeze-thaw cycles. The induced aggregation with Na_2SO_4 was studied following the protocol proposed by Avgoustakis et al. [19], which was slightly modified. Thus, 0.1 ml of nanocapsule dispersion was adding to 2.5 ml of salt solutions of varying concentrations (0 – 1 M) stirred to 100 rpm and incubated at 37°C in a water bath. After 10 min, samples were shaken and the transmittance was measured at a wavelength of 564 nm (UVmini-1240 UV-VIS Spectrophotometer, Shimadzu). On the other

hand, induced aggregation of nanocapsules was promoted by adding 0.05 ml of nanocapsule dispersion to 2.5 ml of 10 mM NaCl solution adjusted at pH 3 with HCl. Particle size was measured at time cero and samples were kept in stand-by at room temperature for 24 h. Then, particle size was measured once again. For evaluating the nanocapsule stability by thermal-freeze-thaw cycles, samples of 2 ml of particle dispersion were stored in glass vials and subjected over and over again to the following treatment: 24h at 40°C; 24h at room temperature; 2h at -22°C; 24h at room temperature. Aggregate formation was measured using the Zetasizer Nanoseries, Malvern Instruments at the same work conditions as for size determination.

2.2.8 Qualitative assay for diclofenac

Diclofenac nonencapsulated presented in the dispersion medium was determined in qualitative way by adding 5 drops of nitric acid (68%) to 1.5 ml of nanocapsule dispersion. Yellowish to dark red colorations are developed depending on the nonencapsulated diclofenac concentration. This method was adapted from the identification test for sodium diclofenac reported in the official monograph of the Japanese Pharmacopoeia and the procedure for diclofenac quantification followed by Matin et al. [20,21].

2.2.9 Quantitative assay for diclofenac

Diclofenac was assayed by high-performance liquid chromatography (Thermo Separation, Spectra System SCM1000 pump, AS3000 autosampler and UV6000 LP detector) following the protocol proposed by Guterres et al. [22], which was slightly modified. A C18 column (Nova-Pak®, Waters, 4 μm, 3.9x300 mm) was used and the mobile phase consisted of acetonitrile:water (65:35% v/v) adjusted to pH 4 with glacial acetic acid. The work conditions were: sample volume injected: 20 μl, mobile phase flow: 1 ml/min, wavelength of diclofenac detection: 280 nm. The range of linear response was 0.2 μg/ml to 20 μg/ml with r = 0.9992. Free drug was determined in the clear supernatant following separation of nanocapsules from aqueous medium by a combined ultrafiltration and centrifugation

technique (Amicon® Ultra, Millipore, regenerated cellulose, 10 kDa MWCO). Total diclofenac was measured following complete dissolution of the nanocapsules in acetonitrile.

2.2.10 In vitro drug release

For investigating the diclofenac release, 0.5 ml of nanocapsule dispersion were added into 9 ml of phosphate buffer (pH 6.8) maintained to 37°C ± 2°C with slow magnetic stirring (≤ 25 rpm). Samples of 0.5 ml were collected at 3, 15, 30 and 60 min, submitted to ultrafiltration/centrifugation and assayed for diclofenac by HPLC as mentioned above. Sample volume was replaced immediately after each sampling.

All described experiments in the method subhead were carried out at least in triplicate.

3. Results and discussion

According to different authors, a critical aspect of formulating drug containing nanocapsules is apropriate selection of the oil used [23,24]. In fact, this starting material limits the quantity of active substance carried and can have a considerable impact on particle characteristics [25,26]. Thus we examine, the behavior of eleven oily solvents as candidats for preparing nanocapsules was examined. Some of them are classical oils used for the nanocapsule preparation, such as the capric/caprylic triglycerides and their derivatives (miglyol 810, miglyol 812, miglyol 829, labrafac lipophile and labrafac PG). To our knowledge, the others are non-reported alternatives [2], such as corn oil, almond oil, capryol PGMC and capryol 90. Likewise, oily surfactants like labrafils (M1944CS and M2125CS) were included to verify their potential use in the preparation of this kind of drug colloidal vectors. We think that tensioactive properties of oil could facilitate nanocapsule formation by the nanoprecipitation and the emulsification-diffusion, since the former involves spontaneous emulsification while the second includes an emulsification step.

3.1 Preliminary studies

Table 1 summarizes the composition and the physicochemical properties of the oils investigated. As can be seen, miglyol 829 has the highest density value, higher than that one of water. Almond oil and labrafil 1944CS have the highest surface tension values. Oil viscosities vary between 11 mPa.s and 70 mPa.s, and miglyol 829 has a significantly high viscosity outside this range. As reported by El-Mahrab et al. [27], oil/water interfacial tension matches well with oil polarity. Thus the most polar oils are miglyol 829, capryol PGMC and capryol 90, which exhibit the lowest values for this property. They are also be the best solvents for diclofenac.

Firstly, screening of the oils concerned their performance in the synthesis of nanocapsule dispersions by using our typical recipes and guaranteeing the same volume of oil in the formulation, regardless of the preparation method. Surfactant type oily compounds do not allow stable nanoparticle dispersions. Twelve hours after their preparation, the Ostwald ripening phenomenon was evident in nanocapsule dispersions using labrafils M1944CS and M2125CS. Thus our hypothesis on the applicability of oily surfactants as versatile starting materials for preparing nanocapsules via nanoprecipitation and via emulsification-diffusion method is not confirmed.

Secondly, polymer solubility in the oil was investigated. It has been reported that polymer oil solubility affects the long term stability of nanoencapsulated systems [29]. Considering the research of Ferrari et al. on olive oil authentication [30], in this work we propose the DSC method as a strategy for evaluating polymer solubility in oil. Figure 1 illustrates representative examples of polymer – oil systems and Table 1 gives the results obtained for all the oils investigated. As can be seen, the differences are evident in the thermograms obtained for almond oil and capryol 90. While the PCL signal is clearly detected at concentrations of about 0.5% when dispersed in almond oil, it is only noticeable at concentrations of about 2% when capryol 90 is examined. Thus, although capryol 90 could be used for preparing nanocapsules, the polymer concentrations in the formulation must be higher than those used when alternatives such as almond oil are chosen, in order to guarantee good long term stability.

Table 1. Physicochemical properties of the oils.

Oil	**Composition**	**Density** (g/ml; 20 ± 2°C)	**Surface tension** (mN/m; 20 ± 2°C)	**Oil/water interfacial tension** (mN/m; 20 ± 2°C)	**Viscosity** (mPa.s, 20°C)	**Acid number** (mg KOH/g)	**Diclofenac solubility** (%w/w, 20 ± 2°C)	**Polymer solubility** (%w/w)	**Diclofenac partition coefficient** Oil / pH 6.8 buffer (20 ± 2°C)
Corn oil	Triglycerides of linoleic acid (58,9%), oleic acid (25,8%), palmitic acid (11,0%), stearic acid (1,7%), linolenic acid (1,1%)	0.918 (0.03)	23.1 (3.5)	25.4 (1.3)	53.6 (0.8)	max. 0.5[*1]	0.34 (0.1)	< 0.5	34.0 (0.5)
Almond oil	Glycerides of oleic acid (64 - 82%), linoleic acid (8 - 28%), palmitic acid (6 - 8%)	0.914 (0.01)	30.2 (1.8)	23.5 (1.3)	69.0 (1.1)	max. 2.0[*1]	0.41 (0.1)	< 0.5	52.1 (0.9)
Miglyol 810	Medium-chain triglycerides: caprylic C8 70-80%, capric C10 18-28%	0.947 (0.03)	27.4 (1.0)	25.8 (0.9)	28.6 (1.2)	max. 0.1[*2]	0.82 (0.1)	< 0.5	65.9 (3.4)
Miglyol 812	Medium-chain triglycerides: caprylic C8 50-65%, capric C10 30-45%	0.944 (0.01)	25.0 (1.2)	25.2 (1.7)	29.1 (1.9)	max. 0.1[*2]	0.73 (0.1)	< 0.5	48.4 (6.6)
Miglyol 829	Caprylic/capric/succinic triglycerides: caprylic C8 45-55%, capric C10 30-40%, succinic acid 15 - 20%	1.008 (0.02)	25.5 (1.2)	5.4 (17.2)	149.9 (2.6)	max. 1.0[*2]	1.15 (0.1)	0.5 - 1	nd.
Labrafac PG	Propylene glycol dicaprylate/dicaprate	0.918 (0.01)	27.4 (1.1)	21.7 (1.0)	11.8 (0.5)	max. 0.2[*2]	0.93 (0.1)	0.5 - 1	55.2 (6.0)
Labrafac lipophile	Medium-chain triglycerides: caprylic C8 50-80%, capric C10 20-50%	0.945 (0.02)	24.6 (2.0)	24.1 (1.6)	25.5 (1.8)	max. 0.2[*2]	0.85 (0.1)	< 0.5	40.0 (8.0)
Labrafil M1944CS	Oleoyl polyoxyl-6 glycerides: (mono-unsatured + PEG) (Apricot kernel oil PEG-6 esters)	0.939 (0.02)	31.1 (0.2)	nd.	48.6 (5.1)	max. 2.0[*2]	1.60 (0.1)	0.5 - 1	nd.
Labrafil M2125CS	Linoleoyl polyoxyl-6 glycerides: (di-unsatured + PEG) (Corn oil PEG-6 esters)	0.941 (0.02)	24.1 (1.4)	nd.	46.1 (3.5)	max. 2.0[*2]	1.97 (0.2)	0.5 - 1	nd.
Capryol PGMC	Propylene glycol monocaprylate (Type I): monoesters 45-70%, diesters 30-55%, caprylic acid >90%	0.935 (0.01)	27.6 (0.3)	6.8 (1.0)	11.5 (1.8)	max. 0.5[*2]	2.91 (0.2)	1 - 5	48.3 (6.7)
Capryol 90	Propylene glycol monocaprylate (Type II): monoesters > 90%, diesters < 10%, caprylic acid >90%	0.940 (0.02)	24.0 (1.5)	5.5 (1.3)	13.5 (0.7)	max. 1.0[*2]	3.35 (0.1)	1 - 2	54.2 (3.3)

* in parenthesis RSD. nd.: Nondetermined measure.

[*1] PhEur 2005 [28].

[*2] Supplier quality control specification.

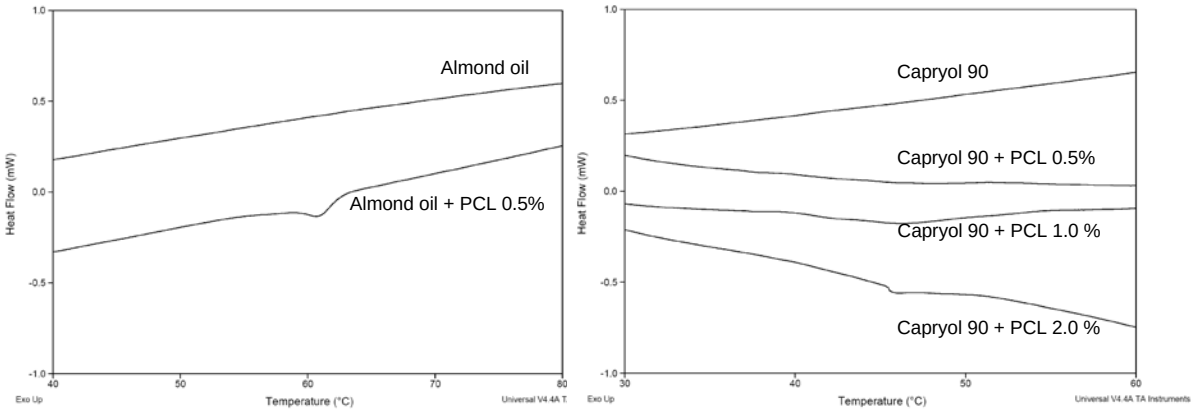

Figure 1. Differential scanning calorimetric heating curves of almond oil, capryol 90 and their mixtures with PCL.

3.2 Behavior of the size of nanocapsules

Generally, particles prepared by emulsification-diffusion method are larger than those obtained by the nanoprecipitation technique (Figure 2). Likewise, the type of oil has a more significant effect on size when nanocapsules are prepared by nanoprecipitation. It is noteworthy that the nanoprecipitation and emulsification-diffusion methods have very different procedures, making the interpretation of the interaction between starting materials during particle formation complex [31]. Therefore, our discussion on the behavior of nanocapsule size will focus only on the influence of the oil, taking a comparative standpoint between the preparation methods.

None of the physicochemical properties of oil (density, viscosity, surface tension or polarity) correlates with the size of nanocapsules, in either the nanoprecipitation technique or the emulsification-diffusion method. However, regarding emulsification-diffusion, Moinard et al. [32] demonstrate good agreement between the size of nanocapsules and the size of the "mother" nanoemulsion droplets from which they are formed. Also, our previous studies on the influence of formulation variables on the size of nanoparticles prepared by this method, suggest that physicochemical properties of the organic phase may influence particle size, because the viscosity, density and surface tension of the organic phase may govern the drop size of the nanoemulsion formed during the emulsification step) [31]. Thus an all-embracing study of oil properties and their impact on nanocapsule size was performed. To do this, radial

graphics were built from the data of oil physicochemical properties and the area of each polygon was estimated (Figure 3A). After, the particle size obtained was depicted against the estimated area for the polygon of each of the oils (Figure 3B).

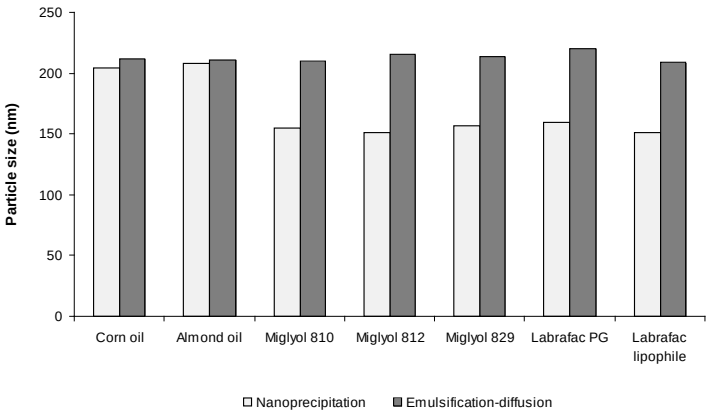

Figure 2. Size of nanocapsules prepared by nanoprecipitation and emulsification-diffusion methods using different oils.

As shown in Figure 3B, polygon area (i.e., oil physicochemical properties) has no incidence on the size of nanocapsules prepared by the emulsification-diffusion method. Moreover, particles prepared from oils having similar polygon areas but different individual physicochemical properties (corn oil and miglyol 810) exhibit similar sizes. Perhaps, the low oil concentration used (4% v/v of the organic phase) does not affect the physicochemical properties of the organic phase and, consequently, the size of the nanodroplets is not influenced by this starting material.

However, Figure 3B shows significant differences in size when corn oil and miglyol 810 are used for preparing nanocapsules via nanoprecipitation. In addition, it appears that oil composition has some effect on nanocapsule size as the highest particle sizes were obtained by using almond oil and corn oil, which contain low percentage of long-chain saturated fatty acids (8 – 13%) and significant quantities of mono and di long-chain unsaturated fatty acids (85 – 95%) [33]. The smallest nanocapsule sizes were obtained by using medium-chain triglycerides containing not less than 95% saturated fatty acids [33].

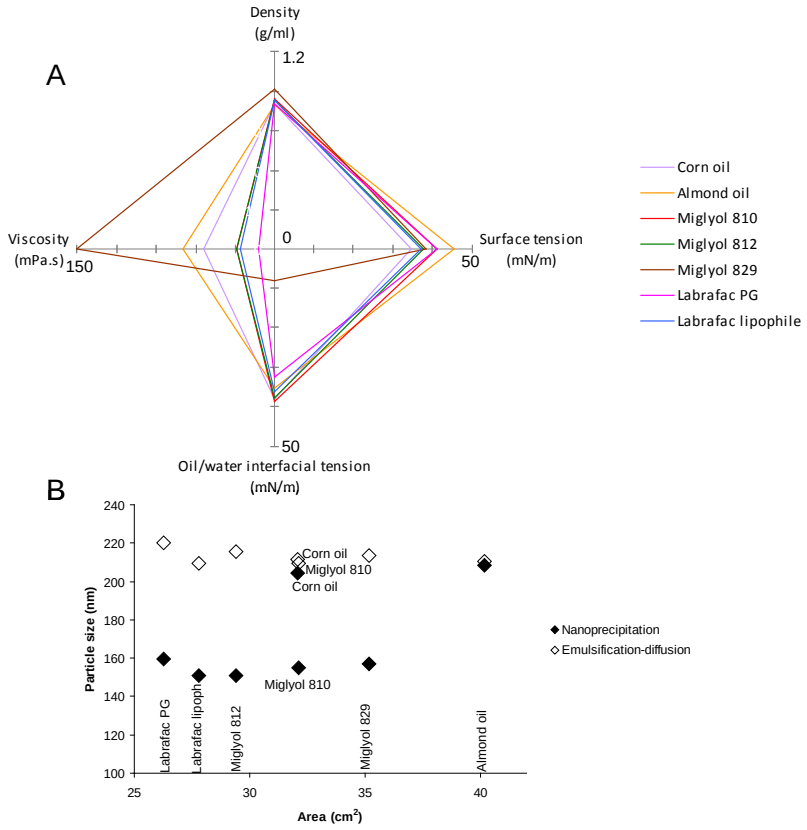

Figure 3. A. Radial graphs integrating the physicochemical properties of the oils used for preparing nanocapsules by nanoprecipitation and emulsification-diffusion methods. B. Behavior of nanocapsule size as a function of the area of the polygon for each of the oils and the preparation method.

One explanation for these results may be the precipitation of certain components of the oils during the nanocapsule formation. According to Bouchemal et al. [34], the solid phase in the core of the emulsion droplets is formed when nanoemulsions are prepared via spontaneous emulsification. On the other hand, the study carried out by Coupland [35] shows that the structure of the crystals within the droplets (dispersed phase) of the emulsions depends on other variables and on the type of fatty acids present in the oil. In addition, Maeda et al. [36] report binodal curves of two liquid phases and solid-liquid equilibrium for the ternary water + acetone + fatty acids (lauric, myristic or palmitic acids), showing that the solubility of fatty acids in aqueous solutions containing acetone decreases as length chain increases, leading to their separation by precipitation. Thus it might occur that certain fatty acids in corn oil and almond oil crystallize during the nanoprecipitation process, leading to bigger particle sizes.

Indeed, there is no convincing argument on why oil composition does not influence the size of nanocapsules prepared by the emulsification-diffusion method. However, it is important to remain readers of the mechanism involved in nanocapsule formation by the two methods [2,31-32,37-40]. Nanocapsule preparation via nanoprecipitation results from physicochemical phenomena associated with the Gibbs-Marangoni effect or to the ouzo effect, depending on the composition of the organic phase. Thus, particle size could be easily influenced by, among others factors, the arrangement of molecules when the compounds re-precipitate. In turn, the size of nanocapsules prepared via the emulsification-diffusion method seems to be governed by the size of the "mother" nanoemulsion droplet obtained through high mechanical strength. This implies that it is the physicochemical properties of the organic phase rather than the properties or the composition of the oil that determine nanocapsule size.

3.3 Behavior of the zeta potential of nanocapsules

As shown in Figure 4, the nature of the oil has not significant influence on the surface charge behavior of nanocapsules prepared by the emulsification-diffusion method. However, a slight effect of oil is observed when particles are prepared by the nanoprecipitation technique that could be linked to their acid number (i.e., the measurement of the amount of carboxylic acid groups in the oil) reported in Table 1. As a general trend, Figure 4 shows that oils characterized by the highest acid numbers lead to the formation of particles exhibiting the highest particle zeta potentials. Oils do not influence zeta potential because they are confined in the nanocapsule core, as proposed by Müller et al. following their DSC studies of nanocapsules prepared via nanoprecipitation [41]. However, Jäger et al. [42], based on their results on the distribution of fluorescent dyes in colloidal systems prepared by this same method, suggest that nanocapsules are composed of three pseudo-phases: oil, interface and aqueous phase. At the interface, the polymer is interacts molecularly with the other two phases and as a consequence, it might be possible to detect the oil, surfactants, polymer and water. According to this explanation for nanocapsule structure, the acidic character of the oil is evident, explaining our results.

From another standpoint, different zeta potentials have been reported when comparing oils for preparing nanoemulsions by spontaneous emulsification [43,44]. According to them, the assumptions proposed by Ammoury et al. [45] and Guterres et al. [22] on the formation of nanoemulsions and nanocapsules could be considered. Thus the zeta potential behaviors obtained in our experiments could correspond to nanoemulsions rather than nanocapsules. This point will be discussed in detail under the drug release subheading.

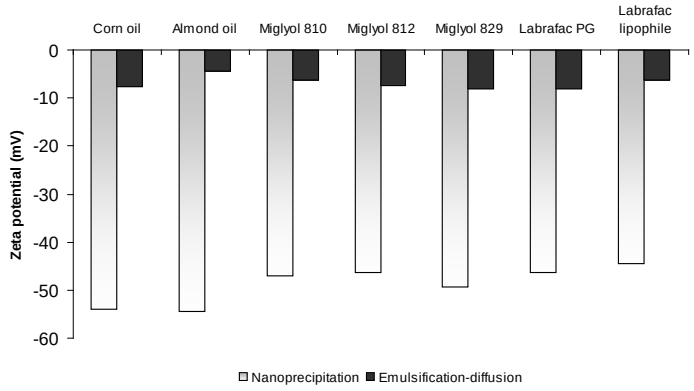

Figure 4. Zeta potential of nanocapsules prepared by nanoprecipitation and emulsification-diffusion methods by using different oils.

On the other hand, the nanocapsule zeta potential depends on the preparation method. Thus, particles obtained by nanoprecipitation always have more negative surface charge values than those prepared by the emulsification-diffusion method. Initially, it is possible to explain that behavior may be based on the recipe used by each method. Unlike the emulsification-diffusion technique, the nanoprecipitation method involves an amphoteric w/o surfactant (soybean lecithin, isoelectrical point:≈ 3.5 [33]) for preparing nanocapsules which could be imbibed in the polymeric membrane, as proposed by Jäger et al [42]. Consequently, a significant surface charge is exhibited on the particle surface.

However, our previous research works performed by our group on the zeta potential behavior of PCL nanospheres prepared by the nanoprecipitation technique and by the emulsification-diffusion method, and in which the same starting materials were used regardless of the method, showed differences in particle zeta potentials depending on the preparation technique (zeta potential values of≈ 15 and ≈ 5 for nanopheres prepared by nanoprecipitation and

emulsification-diffusion, respectively) [31]. Our investigation suggests that this behavior could be associated to the nature of the aqueous phase from which the polymer is re-precipitated and to the methodological aspects specific to each procedure. Nanocapsules obtained by nanoprecipitation are formed instantaneously when the organic phase of semipolar nature is in contact with the aqueous phase (because acetone is used as solvent, ε=20.7 [46]). Thus, as polymer always precipitates from a polar medium (because the dielectric constants of acetone-water mixtures remain higher than 60), perhaps the PCL chains are rearranged during their precipitation and the polar groups are present on the particle surface.

The preparation of nanocapsules by using the emulsification-diffusion method uses ethyl acetate as an organic solvent. Considering the non-polar nature of ethyl acetate (ε=6 [46]) and that the aqueous phase is solvent-saturated for the preparation of the primary emulsion, perhaps the polymer chains rearrange during the emulsification step and the hydrophobic moiety of PCL predominates on the particle surface when it precipitates, which explains the smaller zeta potential values of particles prepared by this technique.

3.4 Colloidal stability of the nanocapsules

One of the most important aspects of nanocapsule dispersion studies is to understand nanocapsule stability. Our zeta potential results and their interpretation based on different conformations of the PCL polymer chains depending on the aqueous phase from which the polymer is re-precipitated, suggest that the interaction between the stabilizing agent (PLX) and the nanocapsule surface could be different. As explained above, the key difference between nanocapsules obtained by the two preparation methods could be their hydrophobic/hydrophilic degree at the surface (i.e., the ratio of hydrophobic to hydrophilic groups on the particle surface). Thus particles prepared by emulsification-diffusion, exhibiting mostly hydrophobic PCL groups on the surface, will have a higher hydrophobic/hydrophilic degree than those prepared by nanoprecipitation, where carboxylic groups will predominate on the particle surface. On the other hand, the stabilizing agent PLX is an A-B-A block copolymer of structure $H(OCH_2CH_2)_{75}(OCHCH_3CH_2)_{30}(OCH_2CH_2)_{75}OH$; in short: POE-PPO-POE.

Then, from a theoretical standpoint, for nanocapsules prepared by emulsification-diffusion, a high density of PPO segments anchored on the particle would be expected because the hydrophobic moieties of PCL predominate on the surface; favoring hydrophobic interactions between PCL and the stabilizing agent. Consequently, the overlap of the adsorbed PLX layers when two particles approach result in strong repulsion due to the solvated POE end chains because they are subject to good solvency conditions [47,48]. This repulsion, called steric stabilization, is due to both the increase in the osmotic pressure in the overlap region (osmotic repulsion) and the reduction of the configurational entropy of the chains (chains compression phenomenon) in the interaction zone, as explained by Tadros [49].

In turn, the low hydrophobic/hydrophilic degree at the surface of nanocapsules prepared by nanoprecipitation will hinder the adsoption of PLX on the particle, rendering a low density of anchored PLX molecules and a high surface charge density [50]. Thus particles are stabilized via the electrosteric effect governed by the interdependence of steric and electric double-layer interactions, i.e., PLX adsorption and conformation is mostly affected by the electric double layer, while the surface charge may be affected by the presence of the PLX adlayer [48]. Considering the high zeta potential values exhibited by nanocapsules prepared by nanoprecipitation, the electrostatic effect might predominate in this case.

These particle stabilization mechanisms (occurring via a steric effect or via electrostatic repulsion) can be highlighted by the behavior of dispersions in the presence of electrolytes. According to Einarson and Berg [48], Hunter [51] and Hiemenz and Rajagopalan [52], electrostatic repulsion is sensitive to added electrolytes, whereas steric repulsion is sensitive to changes in the solubility of the stabilizing agent adlayer. To investigate the stabilization mechanism of PCL particles prepared by nanoprecipitation and emulsification-diffusion methods, two sets of induced aggregation experiments were performed with NaCl and with Na_2SO_4.

As shown in Figure 5, monovalente ions from NaCl cause the aggregation of nanocapsules prepared via nanoprecipitation, which is underlined by the significant change in particle mean size. This behavior depends on the nature of the oils which could be linked to their acid number values and the particle zeta potential. As mentioned above, the higher the acid number value of the oil, the higher the absolute zeta potential and, consequently, the greater the effect of the electrolytes on particle aggregation. In turn, the size of nanocapsules

prepared via emulsification-diffusion is not influenced by monovalent ions in spite of their low zeta potential.

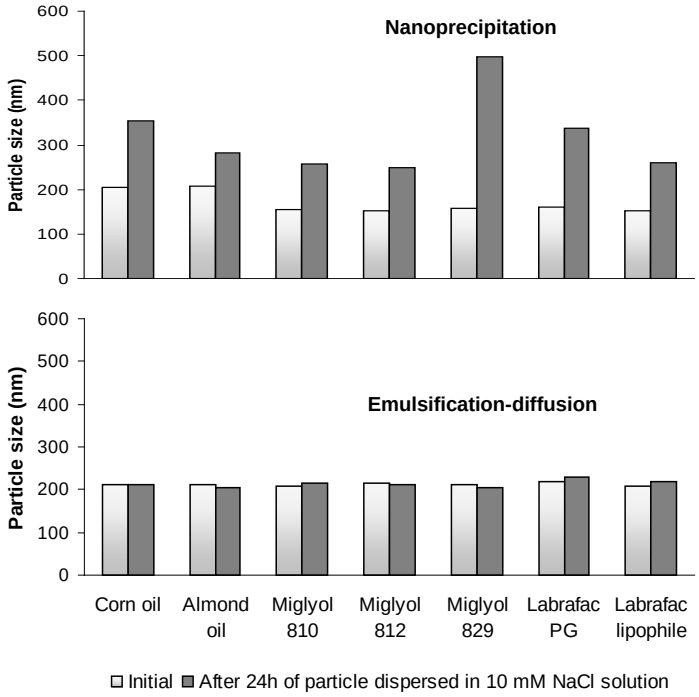

Figure 5. NaCl induced aggregation of nanocapsules prepared by nanoprecipitation and emulsification-diffusion methods using different oils.

Thus, nanocapsules prepared by nanoprecipitation exhibit the typical stabilization behavior through electrostatic repulsion while those obtained by emulsification-diffusion exhibit stabilization through the steric effect. The repulsive electrostatic force that results when the electrical double layers of nanocapsules prepared by nanoprecipitation overlap, then counteracts the attraction due to van der Waals force [52]. The addition of electrolytes to this type of dispersions follows the DLVO theory (B. Derjaguin, L.D. Landau, E.J.W. Verway and T.Th.G. Overbeek theory) and coagulation phenomena are possible under specific experimental conditions such as those used in this investigation. On the other hand, the poloxamer conformation on the particle surface shields the attractive force between nanocapsules prepared via emulsification-diffusion while the repulsive force due to the steric effect prevents coagulation phenomena in low-electrolyte environments.

The influence of Na_2SO_4 solutions of different molar concentrations on the stability behavior of nanocapsule dispersions, complement our conclusions deduced from the NaCl electrolyte experiment. Since the increase in the concentration of Na^+ ions reduces the double layer thickness and the zeta potential drops [51-53], it is possible to promote the rapid and strong aggregation of the particles increasing their settling rate. As shown in Figure 6, nanocapsule aggregation is evident for particles prepared by the two preparations methods and the critical coagulation concentrations are between 0.6 M and 0.7 M of Na_2SO_4.

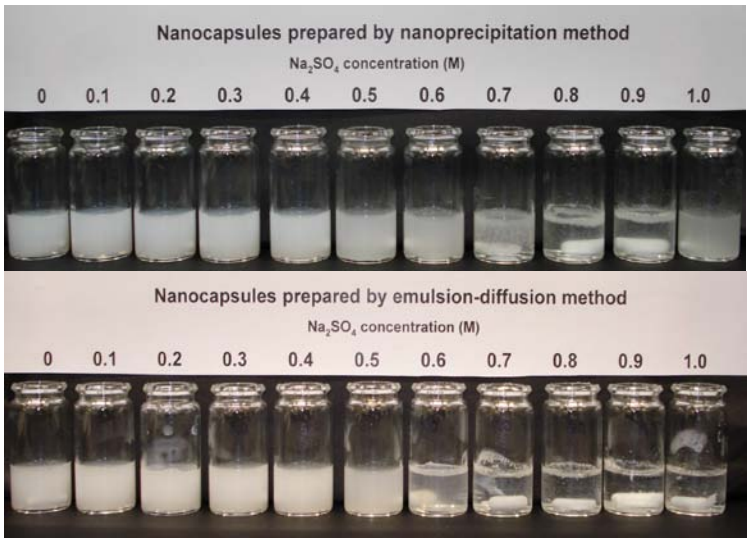

Figure 6. Na_2SO_4 induced aggregation of nanocapsules prepared by nanoprecipitation and emulsification-diffusion methods using different oils (representative behavior: Miglyol 810 sample). Aspect of dispersions before shaking.

However, once the nanocapsules have settled, their redispersion by gentle shaking, expressed as the dispersion transmittance percentage (Figure 7), can only be obtained from colloidal systems resulting from nanoprecipitation due to electrostatic repulsion phenomena. Moreover, as can be deduced from the visual aspect of these nanocapsule dispersions treated with 1 M Na_2SO_4 solution (Figure 6), the re-stabilization of the dispersion could occur as predicted by the DLVO theory [50].

On the other hand, drastic aggregation of the nanocapsules prepared by using emulsification-diffusion method was observed at Na_2SO_4 concentrations higher than the critical coagulation concentration. This could be due to a competition phenomenon between Na^+ ions and

hydrophilic POE poloxamer chains for the water available in the double layer. As Na^+ ions hydration could be favored, the water activity in the double layer decreases and the hydrophilic end chains of PLX could be partially dehydrated. Consequently, the steric effect was minimized and the polymer induced stability was not possible. As particles did not exhibit significant surface charge, aggregates could not be re-dispersed by simple shaking.

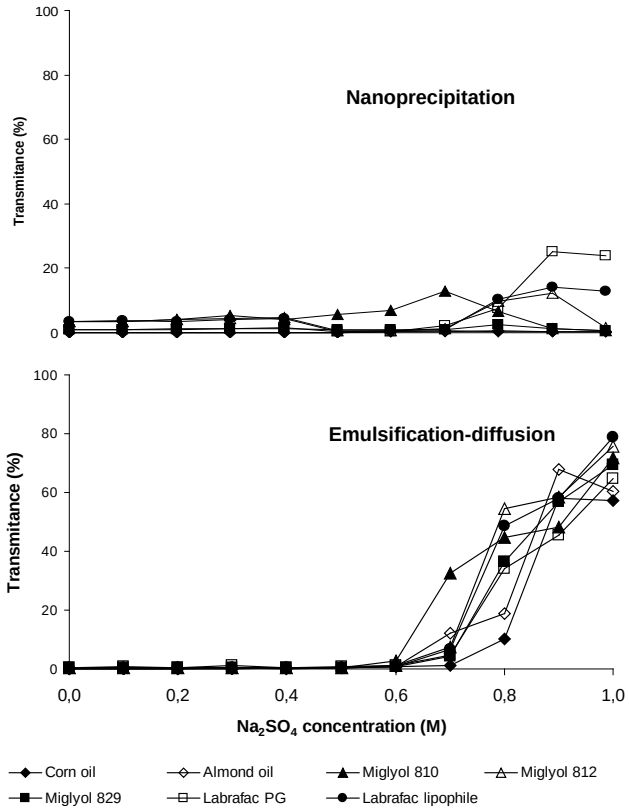

Figure 7. Na_2SO_4 induced aggregation of nanocapsules prepared by nanoprecipitation and emulsification-diffusion methods using different oils. Percentage of transmitance of the dispersions after simple shaking.

The colloidal stability of nanocapsule dispersions was also investigated by thermal-freeze-thaw cycles. This experiment could highlight other aspects of colloidal stability such as nanocapsule wall strength and predict the effect of thermal changes on the integrity of the nanocapsules. As shown in Figure 8, once again differences are underlined according to the preparation method. Nanoencapsulated systems obtained by emulsification-diffusion support up to five thermal-freeze-thaw cycles without significant formation of aggregates, whereas

those prepared by nanoprecipitation technique exhibit dramatic aggregation phenomena from the first cycle.

To analyze these behaviors, the influence of the core composition (mainly the nature of the oil), wall thickness and surface structure of the nanocapsules should be considered. As shown in Figure 8, oil had no clear incidence on particle dispersion instability. As the freezing condition used (-22°C) was always lower than the freezing temperature of the oils (corn oil: -18 to -10°C; almond oil: -18°C, caprylic/capric triglycerides: -5°C [33]), the nanocapsule behavior during the experiment was similar.

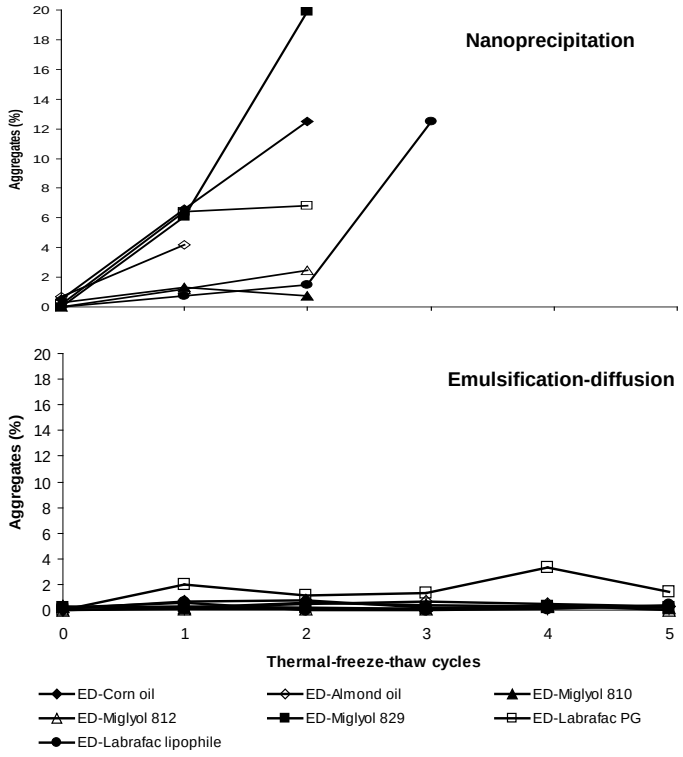

Figure 8. Thermal-freeze-thaw study for nanocapsule dispersions prepared by nanoprecipitation and emulsification-diffusion methods using different oils.

Regarding wall thickness, Table 2 shows comparatively the wall thickness for nanocapsules obtained by the two preparation methods, estimated from the theoretical approximation proposed by Moinard et al. [32]. The calculation was based on the experimental diameter of the nanocapsules and the composition of the organic phase. It was assumed that particle sizes

exhibit unimodal distribution, that the wall was only formed by the polymer and that the thickness of the polymer was homogeneous. As can be seen, nanocapsules prepared by nanoprecipitation had the thinnest thickness of polymeric wall. Thus it could be assumed that the strength of the PCL membrane was lower and that the volume expansion of the nanocapsule core during the freezing step led to the collapse of the capsular structure. In addition, as mentioned previously, the theoretical estimation assumes that the nanocapsule wall is essentially polymeric, but our zeta potential results suggest that lecithin could be trapped in its structure, which, perhaps, could reduce the rigidity of the nanocapsule polymeric wall, rendering it even more fragile. In conclusion, nanocapsules prepared by the nanoprecipitation technique are destroyed more easily than those prepared by the emulsification-diffusion method.

Table 2. Estimated wall thickness (nm) for nanocapsules prepared by nanoprecipitation and emulsification-diffusion method.

	Used oil						
	Corn oil	**Almond oil**	**Miglyol 810**	**Miglyol 812**	**Miglyol 829**	**Labrafac PG**	**Labrafac lipophile**
Nanoprecipitation	8.7	8.8	6.6	6.4	6.6	6.8	6.4
Emulsification-diffusion	10.1	10.0	9.9	10.2	10.1	10.4	9.9

* Wall thickness = $r[1-(V_{core}/V_{particle})^{1/3}]$ where r is the nanocapsule radius; V_{core} was estimated as the total of the oil, drug and soybean lecithin volumes which were obtained from the corresponding density values; and $V_{particle}$ was estimated from the calculus of the sphere volume using the experimental diameter of the nanocapsules.

From the standpoint of surface composition, the behaviors of nanocapsule dispersions in the thermal-freeze-thaw cycle experiment could be associated with our assumption on the amount of PLX molecules anchored on the particle surface, as has been discussed already. Thus the higher the amount of PLX actually fixed on the PCL polymeric wall of the nanocapsules, the larger the protecting stabilizing agent adlayer. Then, the contact between the particle surfaces is minimized, rendering more stable colloidal systems, as observed for nanoparticles prepared with the emulsification-diffusion method.

From our experimental evidence, it was not possible to opt for either wall thickness or surface composition as the correct explanation for nanocapsule dispersion behaviors observed during

thermal-freeze-thaw cycles. However, these results suggest that nanocapsules prepared via emulsification-diffusion could support additional processes such as freeze-drying, much better than those prepared by nanoprecipitation. Also, it is possible to hypothesise that PLX would be a good cryoprotectant agent and that the ability of the stabilizing agent to anchor to the particle surface is critical to obtain an efficient cryoprotectant effect.

These findings might be used in the research work on strategies for improving the stability of the nanoencapsulated systems. It is one of the most challenging objectives related to the use of the nanocapsules in the preparation of finished products, e.g., pharmaceuticals [3,4,22,23,29,32,54-62].

3.5 In vitro drug release

I*n vitro* drug release experiments during the development stages of nanoencapsulated systems allow predicting, provisionally, their performance as carriers of active substances. Figure 9 shows our results for diclofenac release from PCL based nanocapsules using miglyol 810 or labrafac PG as oil models. As can be seen, different behaviors are highlighted as a function of the preparation method. The total amount of diclofenac encapsulated by emulsification-diffusion is released within 15 min, whereas about 60% of the active substance encapsulated by nanoprecipitation is available in the release medium for as long as 48 hours after the beginning of the experiment. These results do not agree with those reported by Michalowski et al. [63] for PCL nanocapsules containing diclofenac and prepared by nanoprecipitation, where 100% of the drug was released within the first 5 min of the dissolution study.

Unfortunately, to our knowledge, comparative studies on this aspect have not been reported up-to-now so no reference was found on nanocapsule drug release as a function of preparation method. Indomethacin is the sole molecule encapsulated by the nanoprecipitation technique and by the emulsification-diffusion method by different research teams. This provides a comparative view in spite of the differences in the recipes, the methods for evaluating drug release and the experimental conditions. Ammoury et al. [45] report that 62% of the indomethacin encapsulated by nanoprecipitation was released from PLA nanocapsules within 24 h. These results are not in good agreement with those reported by Calvo et al. [10] where 60% of the indomethacin diffused from the PCL nanocapsules within the first hour and 90%

within 4 h. On the other hand, when using the emulsification-diffusion method, 80% of the indomethacin encapsulated by this technique was released within 5 min [64]. As can be seen, as with diclofenac, the behaviors of indomethacin delivery from nanocapsules lead to contradictory conclusions.

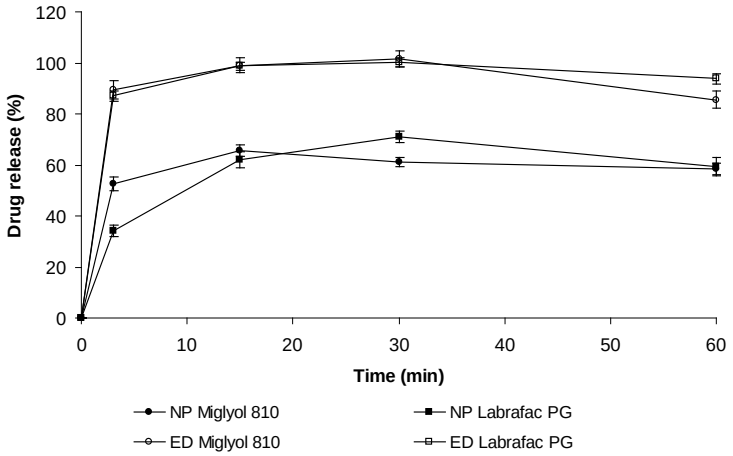

Figure 9. The drug release behavior of nanocapsules prepared by the nanoprecipitation technique (NP) and by the emulsification-diffusion method (ED) using Miglyol 810 and Labrafac PG as oils.

Density gradient studies have demonstrated that the nanoprecipitation technique [65] and the emulsification-diffusion method [18,66] yield nanocapsules exclusively. However, according to the experimental evidence provided by Ammoury et al. [45], the slow and incomplete drug release from colloidal suspensions prepared by using nanoprecipitation technique could be attributed to the retention capacity of other nanocarriers, such as liposomes and nanoemulsions formed at the same time as when nanocapsules are prepared. Accordingly, we assume that nanoemulsions may also be formed when nanocapsules are prepared by emulsification-diffusion method. In addition, there is possible that PLX micelles/aggregates are formed by the two preparation methods, because the PLX concentrations used (0.25% and 1% for nanoprecipitation and for emulsification-diffusion, respectively) are above the critical PLX micelle concentration (0.1%) [22,45,67-69]. Thus the quantity of diclofenac encapsulated by each one of these possible colloidal carriers depends on the multiple drug partition coefficients between the different phases occurring during nanocarrier formation.

Indeed, it is difficult to advance an opinion on which nanocarriers are formed when preparing nanocapsules by the nanoprecipitation and emulsification-diffusion methods. Speculation can be made from a physicochemical point of view. Consequently, we use the Teas graph to deduce the interactions between molecules. In spite of its empirical basis, this graph has been adopted in previous studies showing good agreement with the interaction of many compounds [70-74].

As can be seen in Figure 10, each molecule involved in nanocapsule preparation, i.e., drug, polymer, oil and surfactant, is represented by a single point in the Teas graph that reflects the contribution of the dispersion, polar and hydrogen forces according to its chemical structure. The closer the relative position of the points in the ternary diagram, the higher the affinity between the molecules. Figure 10 illustrates the case of nanocarriers prepared by using labrafac PG as oil. Considering that labrafac PG and soy lecithin are mixtures of various substances, their main components are depicted in the diagram. As can be seen, high affinity is predicted between the different molecules and perhaps nanoemulsion formation might be slightly favored with respect to the formation of nanocapsules or diclofenac micellization. Concerning this, Venturini et al. [65] state that different kinds of colloids could be obtained by varying the proportions of the raw materials in the organic phase. For example, a high oil concentration renders nanoemulsion simultaneously with nanocapsules.

On the other hand, to verify drug release behavior as a function of the nanocarrier formed, diclofenac nanocarriers were prepared via nanoprecipitation and via emulsification-diffusion by modifying our typical recipes as indicated in Table 3. Figure 11 shows that any of the colloids obtained via nanoprecipitation leads to the complete release of diclofenac. A maximum of 40% of the drug was released both from liposomes and from nanocapsules prepared without PLX, and the dissolution behavior of the nanoemulsions was slightly better than that of nanocapsules prepared with PLX, releasing a maximum of about 80% of the active substance. These results confirm the findings reported by Ammoury et al. [45] on the drug release from nanocarriers prepared by the nanoprecipitation technique and show good agreement with those reported by Li et al. [75] and Rubio et al. [76] on diclofenac release from liposomes. Regarding the emulsification-diffusion method, nanoemulsions and nanocapsules exhibited the same dissolution pattern, releasing 100% of the diclofenac during the first 15 min of the dissolution study.

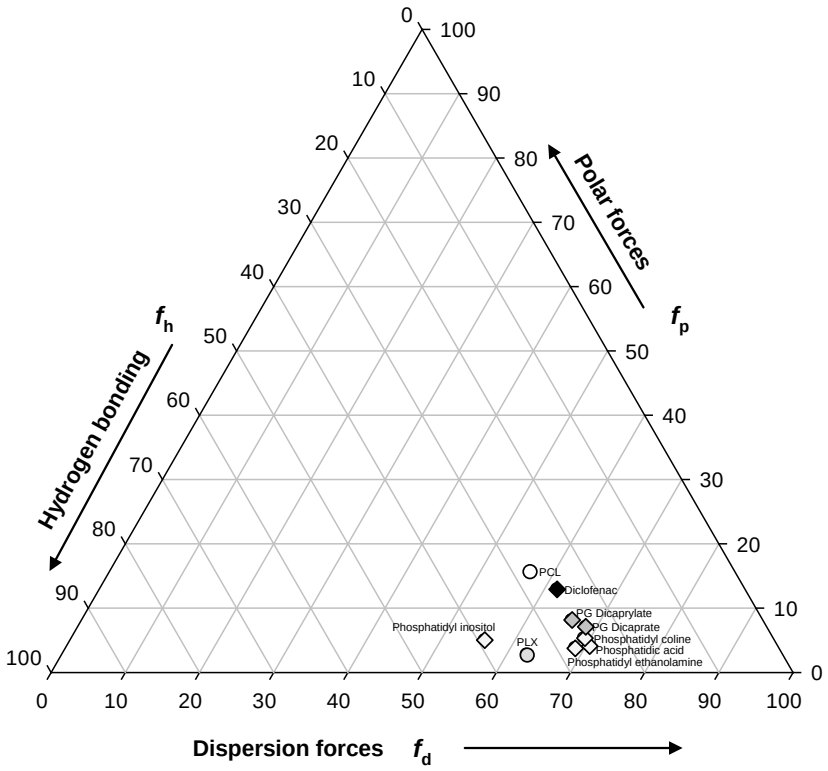

Figure 10. Teas graph for the materials used in the preparation of nanocapsules by the nanoprecipitation technique and the emulsification-diffusion method. The Hansen solubility parameters (δ_d, δ_p and δ_h) were calculated by using the group contribution method proposed by van Krevelen [46]. Then they were expressed as fractional cohesion parameters f_d, f_p, and f_h by $f_i = 100\delta_i/(\delta_d+\delta_p+\delta_h)$ where i corresponds to δ_d, δ_p or δ_h. Propyleneglycol dicaprylate and propyleneglycol dicaprate are the main components of Labrafac PG; phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidic acid are the main components of soy lecithin [33].

The dissolution behavior of diclofenac micelles was not investigated in this research work considering the fast drug release obtained from colloids prepared via emulsification-diffusion. In fact, the recipe used by this method contains a PLX concentration higher than that used by nanoprecipitation, which could facilitate the diclofenac micellization. Then, it is expected that regardless of the preparation method, diclofenac into micelles will be released beginning the dissolution test. This assumption is also supported by the reported evidence on the improved bioavailability of diclofenac solubilised with PLX [77].

It was not possible to prepare nanocarriers without any surfactant, either by nanoprecipitation technique or by the emulsification-diffusion method, in order to know real diclofenac encapsulation in PCL nanocapsules. In addition, it was not possible to separate the different carriers by ultrafiltration/centrifugation because nanocapsules and nanoemulsions have similar sizes; also liposomes could form agglomerates. Moreover, the amount of active substance in the aqueous supernatant of the different colloidal systems suggests that diclofenac can be efficiently captured by any of the carriers (Table 3).

Table 3. Characterization of different nanocarriers prepared via nanoprecipitation technique and via emulsification-diffusion method.

	Starting materials[*1]					**Size (nm)**	**Zeta potential (mV)**	**Drug in supernatant[*2] (%)**
	Diclofenac	**PCL**	**Labrafac PG**	**Soy lecithin**	**PLX**			
Nanocarriers prepared via nanoprecipitation technique								
Liposomes	x			x	X	42	-45	2.72 ± 0.42
Nanoemulsion	x		x	x	X	137	-37	0.19 ± 0.09
Nanocapsules without PLX	x	x	x	x		152	-50	0.13 ± 0.08
Nanocarriers from typical recipe	x	x	x	x	X	160	-46	0.26 ± 0.05
Nanocarriers prepared via emulsification-diffusion method								
Nanoemulsion	x		x		x	186	-10	0.08 ± 0.02
Nanocarriers from typical recipe	x	x	x		x	220	-8	0.04 ± 0.01

[*1]Acetone, ethyl acetate and water were the used solvents according to the procedures previously described in the method subhead.

[*2]Surnatant obtained after the ultrafiltration/centrifugation of samples.

Once again, our evidence does not lead to formulating precise explanations on diclofenac encapsulation via the preparation methods under study. Therefore, two additional experiments on this respect were carried out. The first is the drug release behavior exhibited by nanocapsule dispersions prepared by the nanoprecipitation technique after 20 days storage at room temperature (Figure 12). As can be seen, the amount of diclofenac found in the dissolution medium increases, reaching values up to 80%. This finding could lend credibility to the hypothesis that other nanocarriers are formed when attempting to prepare nanocapsules

by the nanoprecipitation technique because it suggests that some of the carriers are easily broken down. In fact, the pH of nanocapsule dispersions is about 3.8, promoting efficient diclofenac encapsulation by this method because the molecule is in non-dissociated form remaining in the oil phase. However, this pH is close to the isoelectric point of lecithin (≈ 3.5 [33]) and as a consequence, liposomes and nanoemulsions could easily be made unstable, thereby releasing the active substance into the aqueous medium of the particle dispersion. As nanocapsules have a polymeric envelope, higher stability versus pH was expected, which explains why 100% of drug release was not achieved in this experiment.

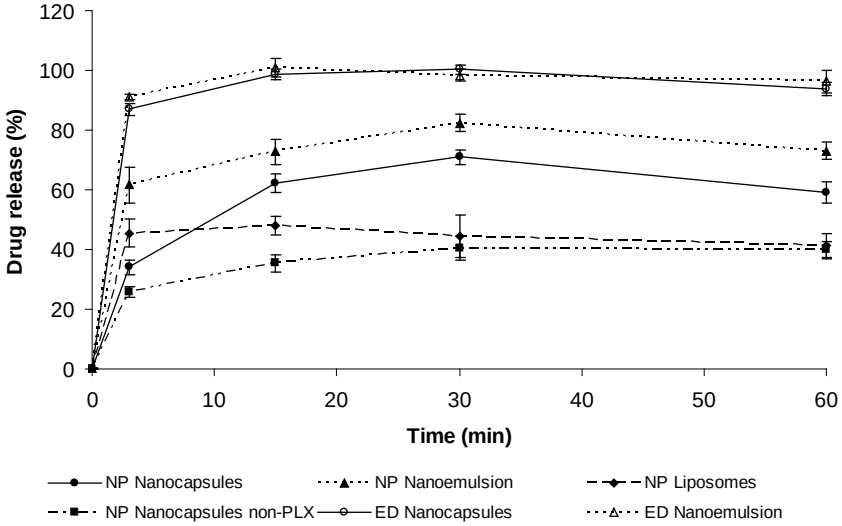

Figure 11. Drug release behavior of different nanocarriers: Labrafac PG nanocapsules prepared by nanoprecipitation (NP) and by emulsification-diffusion (ED) methods, labrafac PG nanoemulsions prepared by nanoprecipitation and by emulsification-diffusion methods, liposome prepared by nanoprecipitation and labrafac PG nanocapsules prepared by nanoprecipitation without stabilizing agent (PLX).

Reports exist of evidence of liposome unstability occurring in recipes similar to those used in our investigation. According to Li et al. [75], who study diclofenac liposomes stability, after 30 days the particle size of liposomes significantly increased and drug encapsulation efficiency fell due to the hydrolysis and oxidation of the lipids at room temperature that then led to the decomposition and aggregation of the liposome vesicles. On the other hand, Kostarelos et al. [78] state that soybean lecithin liposomes can be stabilized by low amounts

of PLX. However, high concentrations of the block copolymer (soybean lecithin:PLX ratio above 1:2.5) lead to drastic reduction of liposome size possibly due to solubilisation of the bilayer and the ultimate break down of the vesicles.

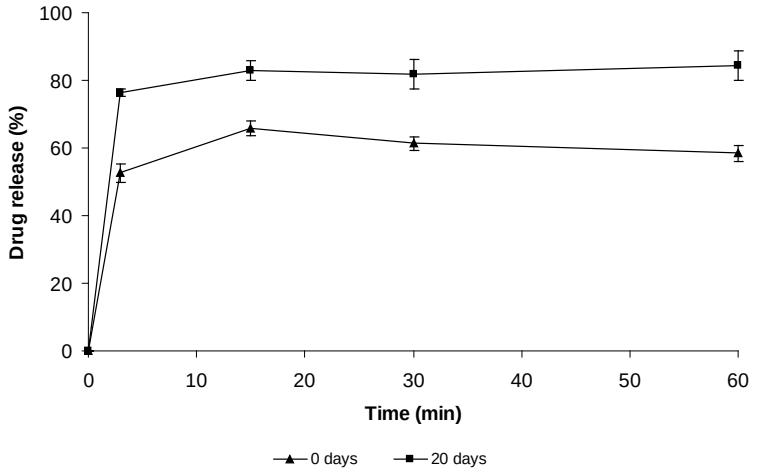

Figure 12. Drug release behavior of nanocarriers after 20 days preparation via the nanoprecipitation technique using miglyol 810 as oil.

The second experiment performed to ascertain whether the drug is inside the core of the nanocapsules, is based on the diclofenac identification test of the Japanese Pharmacopoeia XV [20]. The addition of concentrated HNO_3 to nanoparticle dispersion promotes the formation of diclofenac nitro derivatives developing yellowish to dark red colorations depending on the free diclofenac concentration in the medium.

Our results shown in Figure 13 suggest that the nanoprecipitation technique seems to be more efficient than the emulsification-diffusion method for facilitating the formation of polymeric nanocapsules, because yellow color and red colorations were exhibited by nanocarrier dispersions prepared from certain oils. Likewise, this experiment revealed that the nature of the oil had an impact on the partition of diclofenac between the different carriers, i.e., the amount of diclofenac encapsulated in each type of carrier. Thus, for example, miglyol 829, the most polar oil, seemed to facilitate the formation of nanoemulsions rather than nanocapsules. Nanocarriers prepared by nanoprecipitation by using almond oil led to the best encapsulation of the active substance. It is noteworthy that the efficiency of drug

encapsulation does not have an effect on drug release. After 20 days storage at room temperature of the samples prepared by using almond oil, 100% of the active substance was released within the 15 min after the beginning of the dissolution test.

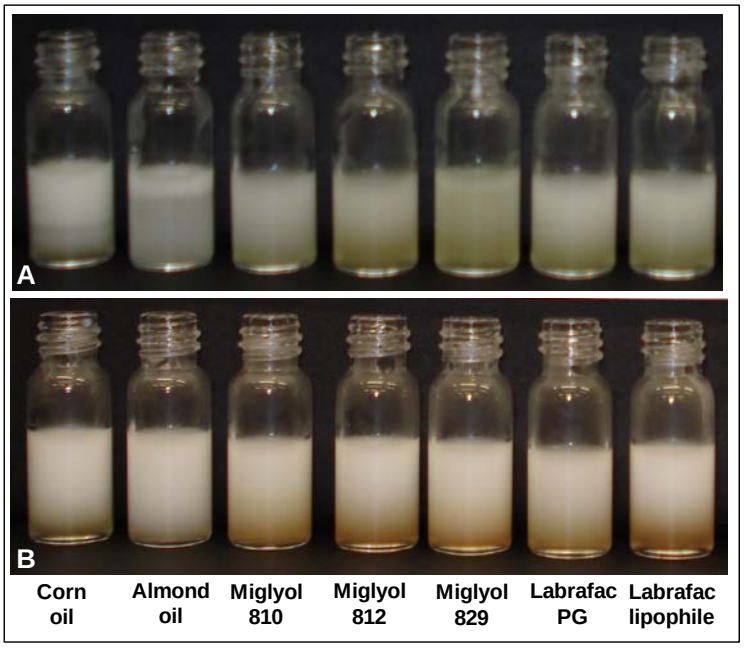

Figure 13. Qualitative diclofenac test for nanocapsule dispersions prepared by: A. the nanoprecipitation technique; B. the emulsification-diffusion method.

Conclusions

Up-to-now, research works on nanocapsules based on biodegradable polymers have focused on different preparation methods. Usually, the variables of the process and the recipes used are investigated with respect to their incidence on size, zeta-potential, drug encapsulation efficiency, drug release and stability, among others characteristics, of the resulting dispersed systems. However, experimental comparative studies showing the advantages and disadvantages of the preparation techniques are very scarce and conclusions on the subject must be deduced from published reviews making it difficult to decide on the most advisable method specifically aimed at nanocapsule design. This investigation contributed to this aim by describing a systematic study on the preparation of diclofenac nanocapsules via the nanoprecipitation technique and via the emulsification-diffusion method, with emphasis

placed on the incidence of the type of oil used in the recipe. Our results show the incidence of the preparation method on the size and zeta potential of the nanocapsules. Thus, the smallest particle size and the largest absolute values of zeta potential were obtained by the nanoprecipitation technique. The particle zeta potential determines the stabilization mechanism of the colloidal dispersion, as demonstrated by the induced aggregation experiments carried out. Thus, the nanoparticles obtained by nanoprecipitation were mainly stabilized via an electrostatic effect while those prepared by emulsification-diffusion exhibited steric stabilization. On the other hand, unlike the emulsification-diffusion method, the nature of the oil, particularly its polarity and its composition, governed particle size and stability against electrolytes, of particle dispersions prepared by nanoprecipitation. The amount of drug encapsulated was also influenced by the type of oil regardless of the preparation technique. Regarding the studies on the *in vitro* release of diclofenac, it is clear the different behaviors of the nanoparticle dispersions depending on the method used. The active substance from nanodispersions obtained by emulsification-diffusion was completely available in the dissolution medium 15 min from beginning of the experiment while only 60% of diclofenac encapsulated by nanoprecipitation was released, even after 48 h. Our investigation revealed interesting evidence on the efficiency of nanocapsule formation using recipes that could simultaneously lead to the formation of nanoemulsiones, liposomes and micelles. It seems that different nanocarriers are formed when preparing nanocapsules and their degradation determines the drug dissolution behavior of the dispersion of mixed nanocarriers. Finally, from a practical standpoint, our results suggest that nanoprecipitation technique produces nanocarriers for use in the preparation of redispersible pharmaceutical dosage forms and in the formulation of extended-release products. On the other hand, the emulsification-diffusion method produces nanocarriers for developing immediate-release dosage forms that could be efficiently stabilized by treatments such as the freeze-drying.

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4. CONCLUSIONS ET PERSPECTIVES

Cette thèse fondamentale porte sur une étude comparative entre la nanoprécipitation et l'émulsion-diffusion et leur utilisation pour l'encapsulation du diclofenac pour application pédiatrique.

Dans un premier temps, nous avons mené une étude systématique en examinant les variables capables d'affecter les procédés et les formulations utilisées pour l'obtention de nanosphères comme modèle de nanoparticules. Les résultats obtenus ont été examinés dans le cas des nanocapsules en utilisant une méthode statistique de planification expérimentale.

Quelle que soit l'approche ou la méthode de préparation, les résultats montrent que la taille des particules est déterminée par la nature et la concentration des matières premières utilisées. D'un autre côte, quelques variables du procédé montrent une incidence sur la taille des particules, comme le rapport volumique entre la phase organique et la phase aqueuse et la vitesse d'agitation du système lorsque la technique de nanoprécipitation est utilisée, ou la vitesse et le temps d'émulsification dans le cas de la méthode d'émulsification-diffusion.

Nous avons essayé de trouver une explication tangible à la relation entre la taille des particules et les variables de la formulation à partir des paramètres physico-chimiques associés aux interactions moléculaires et aux propriétés physico-chimiques des deux phases organique et aqueuse. Les données expérimentales ainsi que celles de nombreux exemples de la littérature nous ont permis d'avoir une vision plus générale et un point de vue sur les mécanismes de formation des nanoparticules via les deux méthodes. En ce qui concerne la nanoprécipitation, le mécanisme impliqué dans la formation des particules peut être expliqué soit par la théorie classique de la précipitation, soit par l'effet Gibbs-Marangoni (via des gradients de concentration). La dominance d'un mécanisme par rapport à l'autre est dépendante de la concentration du polymère dans la phase aqueuse et le rapport volumique des deux phases (organique et aqueuse). Concernant la formation des particules par la méthode d'émulsification-diffusion, le mécanisme le plus probable est celui qui repose sur la transformation de chaque gouttelette ou nanogouttelette d'émulsion en nanoparticules. Il est à

noter que quelques déviations des tendances générales pourraient êtres dûes à l'effet Gibbs-Maragoni (via des gradients de température).

D'un autre côté, notre étude portée sur la préparation des nanoparticules (nanocapsules et nanosphères) a montré l'influence de la méthode de préparation sur les propriétés électrocinétiques (le potentiel zêta) des particules finales. Ce point est discuté en considérant la reconformation du polymère à la surface des particules. Cette différence de conformation, ou de distribution des groupes polaires et non-polaires du polymère à la surface des particules, pourrait être fonction de la polarité du solvant à partir duquel ce dernier est précipité. Par conséquent, ce changement de distribution de groupements polaires à la surface des particules d'un procédé à l'autre, pourrait aussi expliquer le mécanisme de stabilisation des dispersions. Les résultats issus de cette étude révèlent une stabilisation à dominance électrostatique dans le cas de la nanoprécipitation et une stabilité à dominance stérique lorsque l'émulsification-diffusion est utilisée.

L'effet de l'encapsulation du diclofenac sur les propriétés physico-chimiques et colloïdales des nanoparticules préparées par les deux méthodes est également discuté. De cette étude, nous avons conclu que lors de l'encapsulation du diclofenac, plusieurs formes de 'nano-objets ou nanovecteurs' cohabitent avec les nanocapsules. Bien que cette observation avait déjà été rapportée dans la littérature, notre étude a eu pour objectif d'apporter des preuves expérimentales et une évidence théorique sur la présence de ces nanovecteurs, basées sur des études physico-chimiques, des profils de dissolution et des études sur l'efficacité d'encapsulation de l'actif. Il se dégage de cette analyse systématique et approfondie que, quelle que soit la méthode de préparation utilisée, la formation de nanoémulsion est favorisée par rapport à la formation de nanocapsules et que la nanoprécipitation offre des taux d'encapsulations d'actifs plus importants comparé à l'émulsification-diffusion. Ainsi, les nanovecteurs préparés via l'émulsification-diffusion pourraient être utilisés pour la libération immédiate alors que la nanoprécipitation peut être utilisée pour la préparation de nanovecteurs pour une libération prolongée.

Cette étude sur l'élaboration de nanoparticules et l'encapsulation de molécules actives nous a permis: (i) la connaissance des variables affectant chaque procédé, (ii) les mécanismes possibles impliqués dans la formation des particules et (iii) la bonne connaissance des

nanoparticules ainsi que leurs comportements et leurs performances comme vecteurs de molécules actives.

Cependant, l'encapsulation du diclofenac directement via les formulations classiques n'a pas permis l'obtention des résultats escomptés. Notre hypothèse d'encapsuler une quantité importante de substance active via la sélection d'une huile qui favorise une bonne solubilité de l'actif, ne semble pas la bonne solution. L'utilisation d'une huile saturée de molécule active ne permet pas d'avoir de bons rendements d'encapsulation de l'actif. En effet, la concentration maximal atteinte est d'environ 0.5 mg d'actif par ml de dispersion, laquelle ne semble pas d'un grand intérêt pour l'élaboration d'un produit pédiatrique.

Afin d'obtenir des concentrations plus importantes de molécules encapsulées pour la préparation des produits pharmaceutiques, nous proposons comme perspective de rechercher d'autres stratégies comme la formation des complexes polymère-substance active lors de la formulation des nanoparticules par précipitation. Cette idée a été explorée lors de la préparation des complexes métalliques insolubles de bethametasone, ce qui permet une meilleure interaction substance active – polymère[27] ou par la synthèse chimique de dérivés fluorescents des polymers (poly(methyl methacrylate) marqué avec benzazole)[28]. Toutefois, quelques groupes de recherche ont rapporté la formation des complexes du type polyélectrolyte-substance active, par exemple en contentant diclofenac comme actif[29]. Ces complexes peuvent permettre la libération immédiate ou prolongée de l'actif en fonction des matières premières choisies et ont été considerés prometteurs pour la préparation des matrices solides type comprimé, en raison de la taille des particules comprise entre 150 mm et 250 mm[30]. Dans cette mesure, si les conditions de précipitation favorisent l'interaction polymère - actif et si en plus, la formulation est bien adéquate pour maximiser l'encapsulation de l'actif et pour lui donner un profil de libération spécifique, alors les nanoparticules formulées

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peuvent encapsuler plus d'actif et seront par conséquent, compétitives comme vecteurs de molécules actives.

Dans la même direction que les complexes polymère – substance active, la formation des complexes huile – substance active pourrait être envisagée. A titre d'exemple, Piao et al.[31] ont étudié la préparation des complexes diclofenac - huile de soja – albumine sérique bovine. Les résultats rapportés montrent des dispersions avec une concentration de diclofenac de 15 mg/ml, une très faible libération de la molécule active en milieu acide et une libération de 60% de l'actif en 3 h dans un tampon phosphate (pH 6.8). Il serait intéressant de trouver une stratégie innovante afin d'optimiser l'encapsulation des molécules actives dans le noyau des nanocapsules.

Par ailleurs, il serait aussi intéressant de développer des outils pour l'étude *in silico* des nanoparticules, ce qui permettrait d'optimiser les étapes initiales de leur formulation. Des progrés sont déjà réalisés dans cette direction. En effet, la modélisation à partir des paramètres physico-chimiques de la membrane polymérique à l'interface de nanocapsules préparées par émulsification-diffusion est rapportée dans la littérature[32].

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« La théorie, c'est quand on sait tout et que rien ne fonctionne. La pratique, c'est quand tout fonctionne et que personne ne sait pourquoi. Ici, nous avons réuni théorie et pratique : Rien ne fonctionne... et personne ne sait pourquoi !»

Albert Einstein