ONCOLOGY NANOMEDICINE: Study of Interactions Between Nanoparticles Activated by External Electromagnetic Energy Sources and Cancer Cells forEnhancement of the Therapeutic Window

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Sujet de la thèse :

ONCOLOGY NANOMEDICINE:
Study of Interactions Between Nanoparticles Activated by External Electromagnetic Energy Sources and Cancer Cells for Enhancement of the Therapeutic Window

Soutenue le 3 décembre 2009 à Paris devant le jury composé de :

Michel MORANGE	Président du Jury
Alex DUVAL	Directeur de thèse
Julie MARILL	Co-directeur
Véronique ROSILIO	Rapporteur
Eric DEUTSCH	Rapporteur

“Un coeur né pour la liberté ne se laisse jamais traiter en esclave”

Mozart, L’énlèvement au sérail.

A ma famille

A Tristan

- J’adresse tous mes remerciements

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ABSTRACT

The understanding of interactions between nanomaterials and biological entities is fundamental to develop nanoproducts in oncology. NBTXR3 (hafnium oxide inert nanoparticle) and protoporphyrin IX (Pp IX) nanocarrier (silica nanoparticle encapsulating Pp IX, the monomer of Photofrin®) are nanoproducts, issued from Nanobiotix platforms, aim to enlarge the therapeutic window of oncology treatment, using external energy sources to carry out “on” / “off” activation. In the first part of this work, we have studied interaction between NBTXR3 and human colon cancer cells. NBTXR3 penetrates by endocytosis and presents long residence within endo-lysosomal compartment. Significantly enhance of tumour cell radiation response is demonstrated after NBTXR3 activation with ionizing radiation. Compared to controls, irradiated cells having internalized NBTXR3, offer a particular morphology which is described. In the second part, we present the synthesis of a new hybrid nanoproduct for photodynamic therapy (PDT), the Pp IX nanocarrier, and the study of its interaction *in vitro* on six human cancer cell lines and *in vivo* on nude mice model xenografted with three different cancer types. *In vitro,* Pp IX nanocarrier activated at 630 nm is more efficient than free Pp IX. Further, this new hybrid nanoproduct enhances biodistribution of Pp IX, with different kinetic of tumour accumulation between models. Ultimately, based on these new understanding of interactions between nanoparticles and cancer cells, both NBTXR3 nanoparticle and Pp IX nanocarrier, offer a breakthrough approach to create efficient pathways to cancer therapy.

Keywords:

Nanoparticles, Cancer, Nanomedicine, Tumour Cells, Nanoparticles Interaction, Cell Trafficking, Endosome Residence, Electromagnetic Energy Sources, Therapeutic Window, Pharmacokinetic, Biodistribution, Radiotherapy, Hafnium Oxide Nanoparticles, Gold Nanoparticles, Ionizing Radiation, Photodynamic Therapy (PDT), Protoporphyrin IX (Pp IX) Silica Nanoparticles, Pp IX Nanocarriers, Photosensitizer, Photofrin®, Light Excitation

NANOMEDECINE EN ONCOLOGIE :

Etude des Interactions Entre les Nanoparticules Activables par des Sources d'Energie Electromagnétique Externes et les Cellules Cancéreuses pour Elargir la Fenêtre Thérapeutique

La compréhension des interactions entre les nanomatériaux et les entités biologiques est fondamentale pour développer des nanoproduits en oncologie. NBTXR3 (nanoparticule inerte d'oxyde d'hafnium) et protoporphyrine IX (Pp IX) nanotransporteur (nanoparticule de silice encapsulant le Pp IX, le monomère du Photofrin®) sont des nanoproduits, issus des plateformes Nanobiotix, développés pour élargir la fenêtre thérapeutique des traitements du cancer en utilisant des sources d'activation externe de type « on » / « off ». La première partie de ce travail présente l'étude de l'interaction de NBTXR3 avec des cellules tumorales coliques humaines. NBTXR3 pénètre par endocytose et persiste dans le compartiment endo-lysosomial. Une augmentation significative de la réponse cellulaire à la radiothérapie est démontrée après activation de NBTXR3 par des radiations ionisantes. L'irradiation des cellules traitées par NBTXR3 entraîne des modifications spécifiques de leur morphologie par rapport aux contrôles; elles sont décrites ici. Une deuxième partie présente la synthèse d'un nouvel hybride pour la thérapie photodynamique, le Pp IX nanotransporteur, et étudie son interaction *in vitro* avec six lignées de cellules tumorales humaines et *in vivo* dans un modèle de souris nude xénogreffée avec trois types tumoraux. *In vitro*, le Pp IX nanotransporteur activé à 630 nm est plus efficace que le Pp IX libre. De plus, ce nouvel hybride favorise la biodistribution du Pp IX, avec une cinétique d'accumulation tumorale différente entre les modèles. La compréhension des interactions entre nanoparticules et cellules cancéreuses, apportée par ce travail, contribue à la création de nanothérapies innovantes.

Mots clefs:

Nanoparticules, Cancer, Nanomédecine, Cellules Tumorales, Interaction des Nanoparticules, Trafic cellulaire, Persistance dans les Endosomes, Sources d'Energie Electromagnétique, Fenêtre Thérapeutique, Pharmacocinétique, Biodistribution, Radiothérapie, Nanoparticules d'Oxyde d'Hafnium, Nanoparticules d'Or, Radiation Ionisante, Thérapie Photodynamique, Nanoparticules de Silice Encapsulant la Protoporphyrine IX (Pp IX), Pp IX Nanotransporteur, Photosensibilisant, Photofrin®, Excitation par la Lumière

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ABREVIATIONS

3D-CRT: Three-Dimensional Conformal Radiation Therapy
5-ALA: 5-AminoLaevulinic Acid
AET: 2-AminoEthaneThiol
AFM: Atomic Force Microscopy
AIDS: Acquired ImmunoDeficiency Syndrome
AUC: Area Under Curve
BDSA: 9,10-bis[4'-(4''-aminostyryl)styryl]anthracene
BSA: Bovine Serum Albumin
CAM-DR: Cell Adhesion-Mediated Drug Resistance
CdS: Cadmium Sulfide
CHART: Continuous Hyperfractionated Accelerated Radiation Therapy
C_{max} : maximum Concentration
CVD: CardioVascular Diseases
DDS: Drug-Delivery Systems
DEF: Dose Enhancement Factor
DHBA: 3,5-DiHydroxyBenzylAlcohol
DNA: Desoxy-riboNucleic Acid
EBRT: External Beam Radiation Therapy
EC_{50}: 50% Effective Concentration
ECM: ExtraCellular Matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EMDR: Environment-Mediated Drug Resistance
EMEA: Europe, Middle East and Africa
EPR: Enhanced Permeability and Retention
ER: Endoplasmic Reticulum
FAP: Familial Adenomatous Polyposis
FCS: Fetal Calf Serum
FGF: Fibroblast Growth Factor
FRET: Fluorescence Resonance Energy Transfer
GNP(s): Gold NanoParticle(s)
Gy: Gray
Hb: Hemoglobin
HER: Human Epidermal Receptor
HGF: Hepatocyte Growth Factor
HIFU: High Intensity Focused Ultrasound
HIV: Human Immunodeficiency Virus
HNPCC: Hereditary Non-Polyposis Colorectal Cancer

HP: HaematoPorphyrin
HPPH: 2-devinyl-2-(1-Hexyloxyethyl) PyroPheopHorbide
HT: Hyperthermia Therapy
i.p: intraperitoneal
i.v: intravenous
ICP-MS: Inductively Coupled Plasma-Mass Spectrometry
id/g: injected dose per gram
IgG: Immunoglobulin G
IGRT: Image Guided Radiation Therapy
IL: InterLeukin
IMRT: Intensity Modulated Radiation Therapy
IR: infra-red
Ir: Iridium
LD_{50}: Lethal Dose 50
LDL: Low Density Lipoprotein
LITT: Laser-induced Interstitial ThermoTherapy
mAb: monoclonal Antibodies
MMP: Matrix MetalloProteinases
MPS: Mononuclear Phagocyte System
MRD: Minimal Residual Disease
MRI: Magnetic Resonance Imaging
MRT: Mean Resident Time
m-THPC: meta-Tetra(HydroxyPhenyl)-Chlorin
MTS: 3-(4,5-diMethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium inner Salt
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
MW: Molecular Weight
NCI: National Cancer Institute
NE: Nuclear Envelope
NGF: Neurotrophin Growth Factor
NNI: National Nanotechnology Initiative
NO: Nitric Oxide
NP(s): NanoParticle(s)
NSCLC: Non-Small Cell Lung Cancer
OMS: Organisation Mondiale de la Santé
ORMOSIL: ORganically MOdified SILicate
Pc4: Phthalocyanine 4
PDGF: Platelet-Derived Growth Factor
PDT: PhotoDynamic Therapy
PEG: PolyEthylene Glycol
PG: ProstaGlandin
pK: PharmacoKinetic
PLGA: Poly(D,L -Lactide-co-Glycolide) Acid
QCM: Quartz Crystal Microbalance

QD: Quantum Dots
RES: ReticuloEndothelial System
RF: Radio Frequency
RNA: RiboNucleic Acid
ROS: Reactive oxygen species
RTK: Receptor Tyrosine Kinases
SBRT: Stereotactic Body Radiation Therapy
SCLC: Small Cell Lung Cancer
SEM: Scanning Electron Microscopy
SFM-DR: Soluble Factor Mediated Drug Resistance
SME: Small and Medium Enterprises
SPIO: SuperParamagnetic Iron Oxide
SWCNT: Single-Walled Carbon NanoTubes
$t_{1/2}$: half-life
TEM: Transmission Electron Microscopy
TNF: Tumour Necrosis Factor
TPA: Two Photon Absorption
US: United States
USPIO: Ultra Small SuperParamagnetic Iron Oxide
UV: UltraViolet
VEGF: Vascular Endothelial Growth Factor
WHO: World Health Organization
WST-1: (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)
Z: atomic number

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PART 1: NANOMEDICAL RESEARCH

1. NANOMEDICINE: nanotechnology for health

1.1. When “nano” meet medicine: the importance of being small

A key date for nanotechnology and more specifically for nanotechnology applied to medicine is the year 1959, where R. Feynman in its historic lecture, ‘There’s Plenty of Room at the Bottom, An Invitation to Enter a New Field of Physics”, given at the California Institute of Technology, pointed out the promises and potentials of nanotechnology: *“When we get to the very, very small world---say circuits of seven atoms---we have a lot of new things that would happen that represent completely new opportunities for design. Atoms on a small scale behave like nothing on a large scale, for they satisfy the laws of quantum mechanics. So, as we go down and fiddle around with the atoms down there, we are working with different laws, and we can expect to do different things. We can manufacture in different ways. We can use, not just circuits, but some system involving the quantized energy levels, or the interactions of quantized spins, ...”*

His discussion may be considered as the earliest vision of nanotechnology applied to medicine as he raised the question and encouraged the vision of “*manufacture of an object that can manoeuvre at the level of biological cells*” (Webster et al., 2007).

Since, nanotechnologies have created a “nanoworld” and are today able to generate systems, from the very simple to a more complex and elaborated one, as illustrated by the emergence of supramolecular chemistry which is based on molecular recognition and self-assembly processes (Lehn Jean-Marie, 1995). Nanomaterials, due to their very specific small size are able to interact with excessively small systems; and biological systems are today the playground for nanomaterials applications having for ambition to address unmet needs in biology. When materials size matches the size of biological entities, pathways between the two systems may be established, the nanomaterials being able to gain access and even to operate within cells. Indeed, controlling and manipulating things at the nanometer scale allows exploring and interacting at the cellular level with unprecedented fashion.

When looking at the nanometer scale, one speaks of objects on the order of one billion of a meter. To put this size in perspective, a human hair is approximately 80.000 nm wide, and a red blood cell is approximately 7.000 nm wide. Atoms are smaller than 1 nm, whereas many molecules including some proteins range between 1 nm and larger (Sahoo et al., 2007). Generally, the sizes of nanomaterials are comparable to those of viruses, desoxy-ribonucleic

acid (DNA), and proteins, while microparticles are comparable to cells, organelles, and larger physiological structures (Buzea et al., 2007). Figure 1 and Figure 2 give an illustration of the structures down to the nanometer scale.

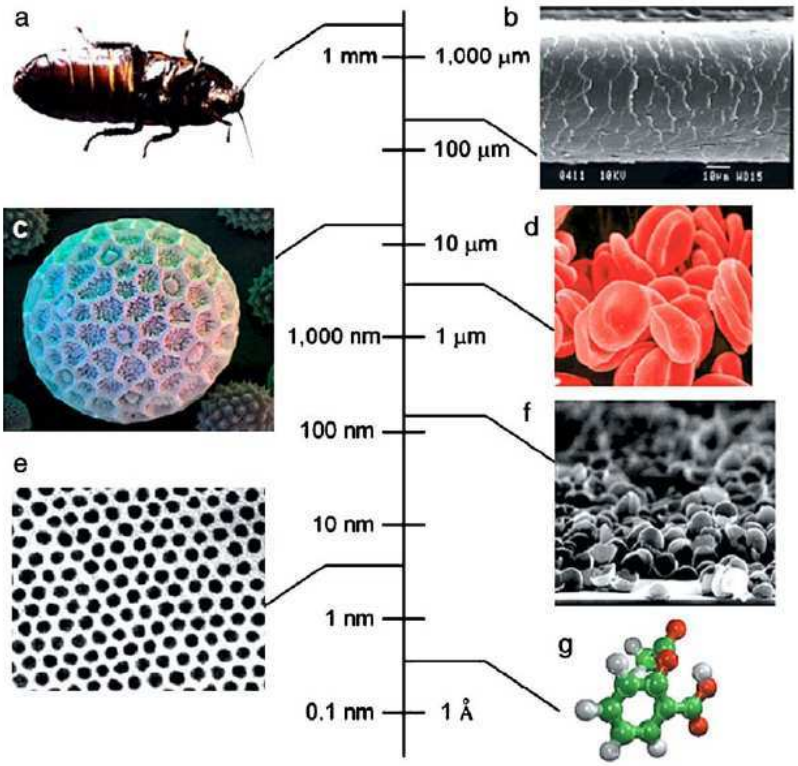


Figure 1**:** Relative sizes of small objects including (a) cockroach, (b) human hair, (c) pollen grain, (d) red blood cells, (e) aggregates of half shells of palladium, (f) superlattice of cobalt nanocrystals, and (g) aspirin molecule (Emerich et al., 2006)

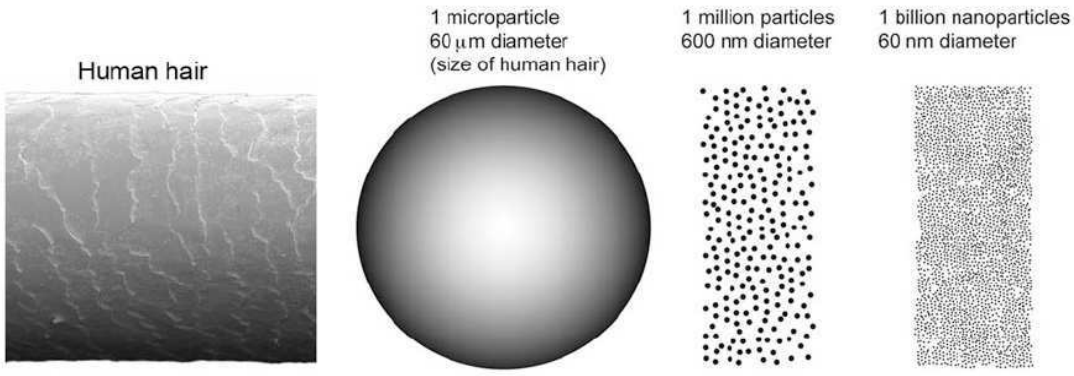


Figure 2**:** Schematics illustrating a microparticle of 60 µm diameter, about the size of a human hair (shown in the left at scale) and the number of NPs with diameter of 600 nm and 60 nm having the same mass as microparticle of 60 µm diameter (Buzea et al., 2007)

The strength of nanomaterial in medicine lies in its ability to operate on the same small scale as all the intimate biochemical functions involved in the growth, development and ageing of the human body (Figure 3). It is expected to provide a new framework for diagnosing, treating and preventing disease.

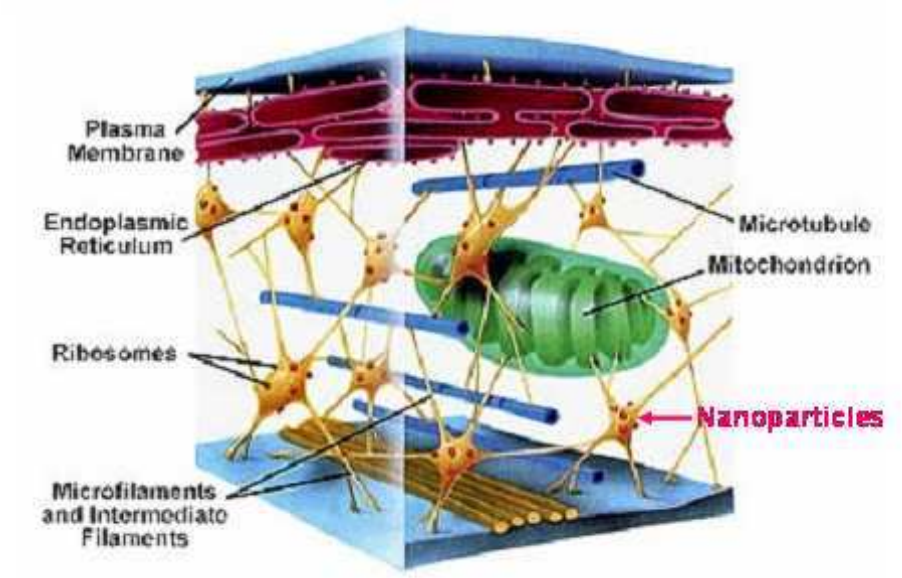


Figure 3: Sub-cellular nanoparticles size

Nanomaterials in medicine, are function-oriented: they may bring an enabling function – drug delivery systems for instance, aiming to improve the benefice over risk ratio comparatively to free drugs – or they may bring new modalities, due to their specific properties arising at the nanometer scale, which are expected to address breakthrough potential for patient care.

Anyhow, those nanomaterials originated from the technological world have to enter the biological world if they have to realize all their promises. Indeed, design of nanomaterials matching the size of most biological entities, is the foundation for creating pathways and effective interactions between the two systems. But lot needs to be done to establish proper recognition and create effective cross-talk between engineered nanomaterials and biological entities for the safe and efficient use of “nano” in medicine.

1.2. What is nanomedicine

The term nanomedicine can be traced back to the late 1990s; according to the Science Citation Index (Institute for Scientific Information, Thompson, Philadelphia, PA, United States; US) the first research publications that use this term appeared in the year 2000. With

research programs, conferences and journals focusing on nanomedicine for a number of years now, it has become clear that nanomedicine is more than a semantic fashion, though it was difficult to find a precise definition for this field with its blurred borderlines encompassing biotech and microsystems technology.

In general, two concepts can be distinguished. Some experts define nanomedicine very broadly as a technology that uses molecular tools and knowledge of the human body for medical diagnosis and treatment. Others prefer an emphasis on the original meaning of nanotechnology as one that makes use of physical effects occurring in nanoscale objects that exist at the interface between the molecular and macroscopic world in which quantum mechanics still reigns. This second concept is commonly adopted and nanomedicine may be defined as the use of nanoscale or nanostructured materials in medicine that according to their structure have unique medical effects, for example, the ability to cross biological barriers or the passive targeting of tissues (Wagner et al., 2006).

Such medical effects are not strictly limited to a size range below 100 nanometers (nm). Therefore, unlike the physical definition of nanotechnology, which is restricted to objects with dimensions in the range of 1 nm to 100 nm, structures and objects up to 1.000 nm in size are included. Such a definition also seems to be justified from a technical point of view because the control of materials in this size range not only results in new medical effects but also requires novel, scientifically demanding chemistry and manufacturing techniques.

1.3. A brief history of nanomedicine

Important events have contributed to the emergence of nanomedicine and to its expansion to the point we stand today. Some of them are highlighted in the followings.

In 1986, the Atomic Force Microscopy (AFM) was invented which had subsequently allowed for unprecedented control over nanomaterials design and characterization.

In 1987, the first university symposium “Exploring Nanotechnology” was opened at the Massachusetts Institute of Technology, Cambridge, MA, US.

In 1988, the first university course, “Nanotechnology and Exploratory Engineering”, was created at Stanford University, Palo Alto, CA, US.

In 1990, the first journal called *Nanotechnology* was edited.

In 1996, the first "nano-bio" conference by the International Business Communications "Biological Approaches and Novel Applications for Molecular Nanotechnology" hold in San Diego, CA, US.

In 2000, US President Clinton announced the creation of the US National Nanotechnology Initiative (NNI). NNI is a multi-agency umbrella programs to build, to characterize, and to understand nanoscale devices. The NNI lists medicine, manufacturing, material sciences, information technology, energy, and environmental sciences as target beneficiaries. The program slated to spend nearly $1 billion in fiscal year 2005 as compared with $464 million in 2001 (Sahoo et al., 2007).

In 2003, **Robert Freitas Jr.**, who was one of the earliest contemporary proponents of nanoscience, in his book *Nanomedicine*, envisaged the future of nanoscience as it applies to medicine in *"three overlapping and progressively more powerful technologies; the first, in the immediate future, refers to addressing medical problems by using nanostructures: materials that can be manufactured today; then, in the near future, he anticipated advances in molecular medicine and botics; and, finally, in the long term, molecular machine systems and nanorobots joining the medical armamentarium"* (Asiyanbola et al., 2008).

In 2003, **Pr. Paras N. Prasad**, the executive director of the Institute for Lasers, Photonics and Biophotonics (State University of New York) and one of the world's leading authorities on nanotechnology, in his book *Introduction to biophotonics* covered current and future applications based on light-activated therapies. Since, Biophotonics offer exciting opportunities for fundamental research to probe intercellular interactions as well as to produce novel biotechnology to advance human health, particularly in the new field of nanomedicine.

In 2006, the first international journal in nanomedicine, *International Journal of Nanomedicine*, was edited.

In 2008, **Nanobiotix** company, a spin-off of the State University of New York (SUNY) at Buffalo that has been incorporated in 2003, announced that the European service of patents have delivered their patent for new nanoXray^{TM} nanoparticles activated by X-ray for cancer care. NanoXray^{TM} offers a dramatic innovation in cancer therapy, based on a technology that is designed to allow destruction of cancer cells by inert particles.

In 2009, **Mauro Ferrari**, a specialist in the field of nanomedicine, gave an interesting vision when answering the following question: How do you think the nanomedicine field will influence the treatment of disease? *"I think medicine is really going to change in five to ten years, with much greater emphasis on early detection so that we can catch diseases before they develop to the point where it's very hard and expensive and many times impossible to*

control. And that will be enabled by nanotechnology. We will be able to develop personalized approaches for cancer and for other diseases, and, again, nanotechnology will be a necessary lever of all of that. Everything is changing, and luckily for the better" (Ferrari et al., 2009).

Nowadays, nanomedicine is perceived as embracing five main sub-disciplines that in many ways are overlapping and underpinned by the following common technical issues: analytical tools; nanoparticles-imaging; nanomaterials and nanodevices; novel therapeutics and drug delivery systems; regulatory, clinical and toxicological issues.

1.4. Focus on nanomaterials in health care

1.4.1. Survey of the main diseases around the world

Figure 4 shows main causes of human death around the world and according to continental area. Among them, main diseases leading to human deaths are highlighted. In Sub-Saharan Africa, infectious and parasitic diseases, including measles and malaria, are more frequent causes of death than elsewhere. Respiratory infection disproportionately affects people living in Southeast Asia and Sub-Saharan African. These two regions are also particularly hit by maternal conditions and perinatal conditions. The Asia and the West Pacific region have a rate of non-communicable respiratory diseases, such as chronic bronchitis and emphysema, which is nearly 2.5 times higher than the rest of the world. Western Europe has a greater proportion of deaths due to heart (cardiovascular) disease and cancer (malignant and other neoplasms).

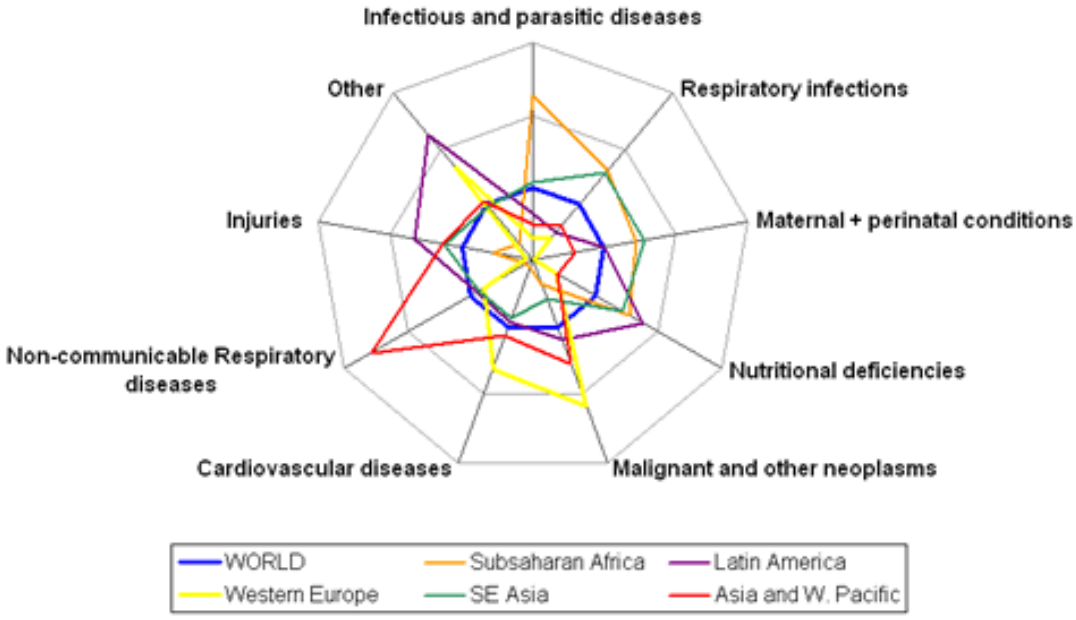


Figure 4 : What people die? (UC Atlas of Global Inequality)

In developing nations, inferior sanitary conditions and lack of access to modern medical technology make death from infectious diseases more common than in developed countries.

In the world, over 30.000 children under the age of five die each day from preventable causes related to conditions of extreme poverty. Malnutrition is estimated to contribute to more than one third of all child deaths, although it is rarely listed as the direct cause. Infection – particularly frequent or persistent diarrhoea, pneumonia, measles and malaria – also undermines a child's nutritional status.

The main causes of mortality in the world for adults aged between 15 and 60 are acquired immunodeficiency syndrome (AIDS), cardiovascular disease and tuberculosis. For people aged more than 60 the highest causes of mortality are cardiovascular diseases, cancer and diabetes (Figure 5 and Figure 6).

The main diseases are briefly summarized in the following; cancer will be reviewed in a specific chapter.

Mortality-Adults Aged 15-59

Rank	Cause	Deaths (000)
1	HIV/AIDS	2279
2	Ischaemic heart disease	1332
3	Tuberculosis	1036
4	Road traffic injuries	814
5	Cerebrovascular disease	783
6	Self-inflicted injuries	672
7	Violence	473
8	Cirrhosis of the liver	382
9	Lower respiratory infections	352
10	Chronic obstructive pulmonary disease	343

Mortality-Adults Aged 60+

Rank	Cause	Deaths (000)
1	Ischaemic heart disease	5825
2	Cerebrovascular disease	4689
3	Chronic obstructive pulmonary disease	2399
4	Lower respiratory infections	1396
5	Trachea, bronchus, lung cancers	928
6	Diabetes mellitus	754
7	Hypertensive heart disease	735
8	Stomach cancer	605
9	Tuberculosis	495
10	Colon and rectum cancers	477

Figure 5 : Leading causes of mortality among adults into the world in 2002 (OMS, 2009)

A. Diabetes and cardiovascular disease

Diabetes

Diabetes is a chronic disease which occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. In 2000, at least 171 million people worldwide had diabetes. This figure is likely to more than double by 2030 (366 million estimated). In developing countries the number of people with diabetes will increase by 150 % in the next 25 years. The global increase in diabetes will occur because of

population ageing and growth, and because of increasing trends towards obesity, unhealthy diets and sedentary lifestyles. In developed countries most people with diabetes are above the age of retirement, whereas in developing countries those most frequently affected are aged between 35 and 64 (WHO world health report, 2009).

Cardiovascular diseases (CVD)

CVD are a group of disorders of the heart and blood vessels and include coronary heart, cerebrovascular, peripherical arterial, rheumatic heart, congenital heart disease and deep vein thrombosis and pulmonary embolism. CVD are the number one cause of death globally: more people die annually from CVD than from any other cause. An estimated 17.5 million people died from CVD in 2005, representing 30% of all global deaths. Of these deaths, an estimated 7.6 million were due to coronary heart disease and 5.7 million were due to stroke. Over 80% of CVD deaths take place in low and middle income countries and occur almost equally in men and women. By 2015, almost 20 million people will die from CVD, mainly from heart disease and stroke. CVD are projected to remain the single leading causes of death (WHO world health report, 2009).

B. Infectious disease

AIDS

Acquired immune deficiency syndrome or acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV). 33 million people were living with HIV in 2007 all around the world with 2 million of deaths and 2.7 million newly infected (UNAIDS, 2009).

Malaria

Malaria is caused by a parasite called Plasmodium, which is transmitted via the bite of infected mosquitoes. In the human body, the parasites multiply in the liver, and then infect red blood cells. There were 247 million cases of malaria in 2006, causing nearly one million deaths, mostly among African children. Approximately half of the world's population is at risk of malaria, particularly those living in lower-income countries (WHO world health report, 2009).

Measles

Measles is a leading cause of death among young children even though a safe and cost-effective vaccine is available to prevent the disease. In 2007, there were 197 000 measles deaths globally. More than 95% of measles deaths occur in low income countries with weak health infrastructures. Measles vaccination efforts have reaped major public health gains, resulting in a 74% drop in measles deaths between 2000 and 2007 worldwide - with a drop of about 90% in the eastern Mediterranean and Africa regions (WHO world health report, 2009).

C. Others

In developed countries with an ageing population, there is rising prevalence in diseases of the central nervous system. Parkinson's disease, senile dementia or Alzheimer's disease, arthritis and ocular diseases are also important mortality causes.

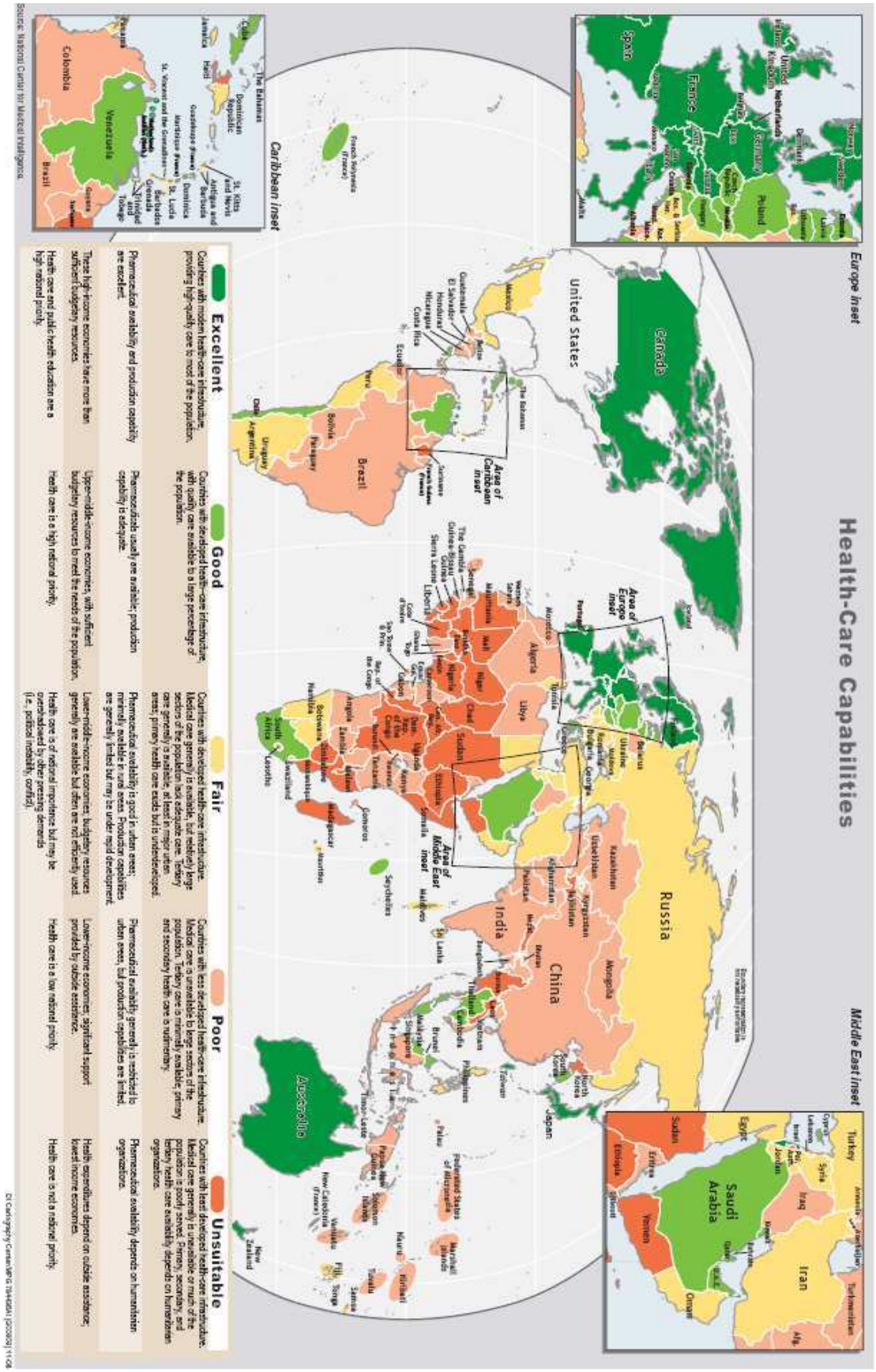


Figure 6: Health map world in 2008
http://www.dni.gov/nic/PDF_GIF_otherprod/ICA_Global_Health_2008_foldout.pdf

1.4.2. Where nanotechnology could play a role in health care

A. Prevention

In medicine, prevention is the action taken to decrease the chance of getting a disease or condition. For example, cancer prevention includes avoiding risk factors (such as smoking, obesity, lack of exercise, and radiation exposure) and increasing protective factors (such as getting regular physical activity, staying at a healthy weight, and having a healthy diet) (NCI).

New diagnostic tests making use of nanotechnology to quantify disease-related biomarkers could offer an earlier and more personalised risk assessment before symptoms show up. Supported by such an analysis and bioinformatics, health professionals could advise patients with an increased risk to take up a personalised prevention program. People with an increased risk for a certain disease could benefit from regular personalised check-ups to monitor changes in the pattern of their biomarkers. Nanotechnology could improve *in vitro* diagnostic tests by providing more sensitive detection technologies or by providing better nano-labels that can be detected with high sensitivity once they bind to disease-specific molecules present in the sample. Diseases with no secretion of biomarkers into blood or urine will require imaging procedures of high specificity for their early detection. One well-known example used already is X-ray mammography for the early detection of breast cancer. Novel targeted imaging agents, precisely homing in on diseased cells, promise a much higher sensitivity than today's imaging procedures, making possible the detecting of disease at an even earlier stage.

B. Diagnosis

Diagnosis is the process of identifying a disease, such as cancer, from its signs and symptoms (NCI).

If a medical check-up had found an indication or a hint of symptoms for a disease, it is important that “false positives” are excluded by applying more specific diagnostic procedures. In this case, molecular imaging, which makes use of specific targeted agents, plays a crucial role for localisation and staging of a disease, or – equally important – for ascertaining the health of a patient. Here, nanotechnology could help to design a plethora of very specific imaging agents over the next ten years. Conceptually novel methods, combining biochemical techniques with advanced imaging and spectroscopy provide insight to the behaviour of single diseased cells and their microenvironment for the individual patient. This could lead to

personalised treatment and medication tailored to the specific needs of a patient. The main advantage of nanomedicine on quality of life and on costs for healthcare is earlier detection of a disease, leading to less severe and costly therapeutic demands, and an improved clinical result. However, once a disease is diagnosed, therapeutic action is required. A decision needs to be taken as to which cure offers the best therapeutic ratio (risk/benefit) for the patient. Here, diagnostic imaging procedures provide crucial input for clinical decision taking and therapy planning.

C. Therapy

In many cases, therapy will not be restricted to medication only but requires more severe therapeutic action such as surgery or radiation treatment. Planning of therapeutic interventions will be based on imaging, or may be performed under image guidance. Here, nanotechnology will lead to a miniaturisation of devices that enable minimally invasive procedures and new ways of treatment. The possibilities range from minimally invasive catheter-based interventions to implantable devices. Targeted delivery systems and nanotechnology-assisted regenerative medicine will play the central role in future therapy. Targeted delivery agents will, as example, allow a localised therapy which targets only the diseased cells, thereby increasing efficacy while reducing unwanted side effects. Thanks to nanotechnology, pluripotent stem cells and bioactive signalling factors will be essential components of smart, multi-functional implants which can react to the surrounding micro-environment and facilitate site-specific, endogenous tissue regeneration (making lifelong immune-suppressing medication obsolete). Imaging and biochemical assay techniques will be used to monitor drug release or to follow the therapy progress. This therapeutic logic will lead to the development of novel, disease modifying treatments that will not only significantly increase quality of life but also dramatically reduce societal and economic costs related to the management of permanent disabilities.

D. Follow-up monitoring

Medical reasons may call for an ongoing monitoring of the patient after completing the acute therapy. This might be a regular check for reoccurrence, or, in the case of chronic diseases, a frequent assessment of the actual disease status and medication planning. Continuous medication could be made more convenient by implants, which release drugs in a controlled way over an extended length of time. *In vitro* diagnostic techniques and molecular

imaging play an important role in this part of the care-process, as well. Biomarkers could be systematically monitored to pick up early signs of reoccurrence, complemented by molecular imaging where necessary. Oncology is one of the areas where these techniques are already being evaluated today. Some types of tumours can be controlled by continuous medication extending life expectancy. However, in the case of drug resistance, signs of disease progression can be immediately picked up and alternative treatments can be prescribed.

1.4.3 Which nanomaterials could play a role in health care and why

Due to the advance in nanotechnology, the preparation of a large variety of nanomaterials is today achievable. Those nanomaterials offer various unique properties that are able to meet important medical needs.

Nanomaterials may be classified in two main groups, depending on the core of the material: the inorganic and the organic nanomaterials.

Their way of production may be rationalized within two mains approaches. The Top-Down approach involves creating smaller materials by using larger ones by for example moulding or etching. Nanolithography is a typical example of the top-down approach. The Bottom-Up approach involves arranging small components such as atoms or molecules in more complex structures. Molecular self-assembly is a typical example of the bottom-up approach.

A. Organic-based nanomaterials

Organic-based nanomaterials encompass a large variety of structures having as common feature the ability to carry and eventually deliver molecules in a controlled way. The most advanced nanomaterials for clinical applications are the liposomes, the micelles, the polymeric nanoparticles and the dendrimers. A brief overview of each type of structure is proposed in the following.

LIPOSOMES

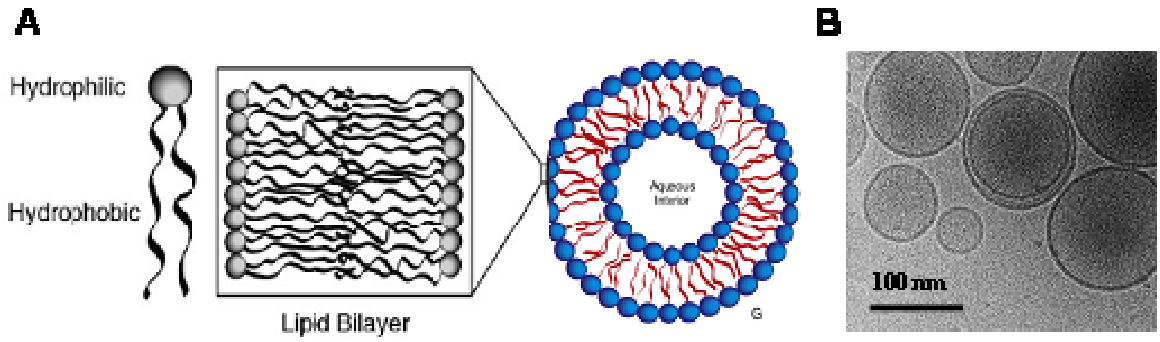


Figure 7 : Liposomes structures with phospholipid bilayer enclosing a hydrophilic core. A- Schematic diagram of lipids incorporated into a bilayer membrane to form a liposome (Huang et al., 2008 and Husseini et al., 2008). B- Cryo-TEM images of liposomes (Li et al., 1998)

Liposomes are vesicles which consist of one to several, chemically-active lipid bilayers. Drug molecules can be encapsulated and/or solubilised within the bilayers according to their hydrophilic/lipophilic balance. Drugs incorporated within a liposome are effectively protected from premature degradation. Drug delivery may be triggered by different stimuli (temperature, pressure for instance) according to the composition of the lipid bilayer. “Channel” proteins can also be incorporated in liposome membrane to act as size-selective filters allowing the diffusion of small solutes such as ions, nutrients and antibiotics. The drug molecule, however, is able to diffuse through the channel, driven by the concentration difference between the interior and the exterior of the liposome.

Main applications: Nanocarriers

Drug Delivery Systems

MICELLES

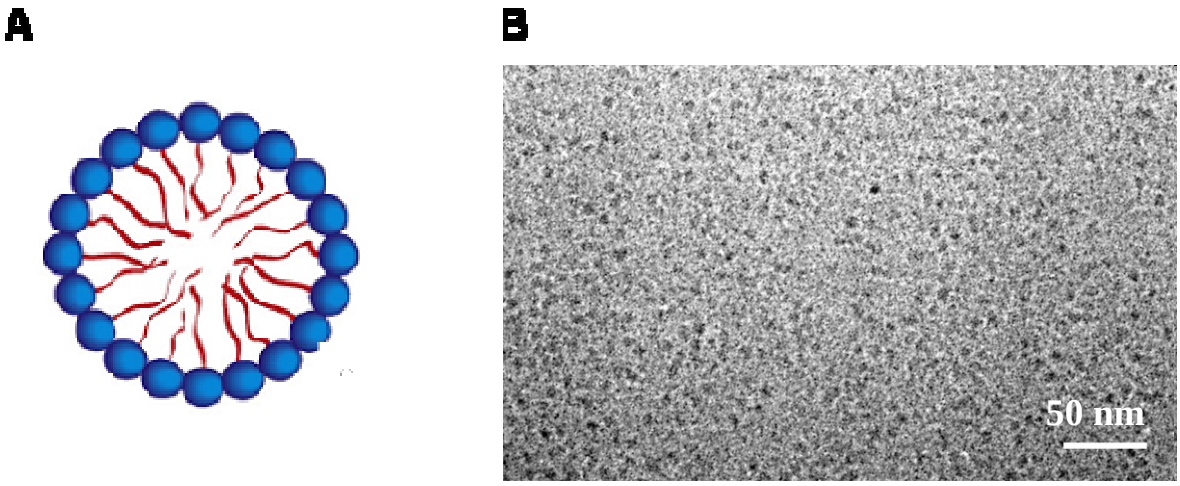


Figure 8 : A- Micelles structures with phospholipids monolayer enclosing a hydrophobic core (Husseini et al., 2008) B- CryoTEM images of mixed micellar structures

Micelles are vesicles formed from aggregation of surfactant molecules. In aqueous solution (oil-in-water micelles), the hydrophilic "head" of surfactant molecules are in contact with the surrounding solvent, sequestering the hydrophobic tail region in the micelle core. Drugs, particularly hydrophobic drugs, can be trapped in the core of a micelle and transported at concentrations even greater than their intrinsic water solubility. A further feature which makes micelles attractive is that their size and shape can be changed by, for example, addition of co-surfactant, temperature, surfactant concentration. Chemical techniques using cross-linking molecules can improve the stability of the micelles and their temporal control.

Main applications: Nanocarriers
Drug Delivery Systems

POLYMERIC NANOPARTICLES

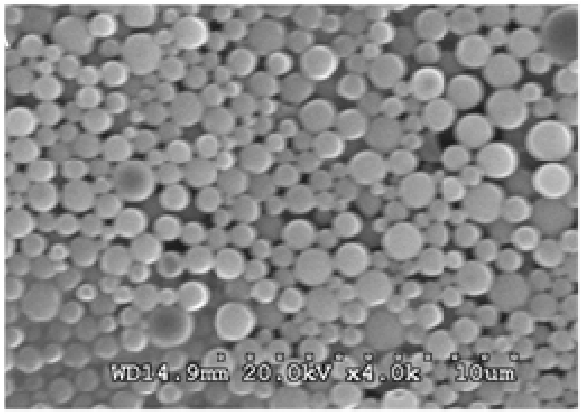


Figure 9 : Scanning electron microscopy (SEM) image of poly(D,L -lactide-co-glycolide) (PLGA) spheres (Zhao et al., 2007)

Polymeric nanoparticles encompass a large variety of structures such as nanogels, albumin, polymer, and the lipid-like systems, that all can serve in the transport process. The polymeric nanoparticles typically function to enclose or encapsulate a drug with a controlled release time. Ideally, polymeric nanoparticles should be biodegradable, like those made of polylactic acid, polyalkylcyanoacrylate, polyglycolic acid, or polyethylene oxide, among others (Brewer et al., 2007).

Main applications: Nanocarriers
Drug Delivery Systems

DENDRIMERS

A B

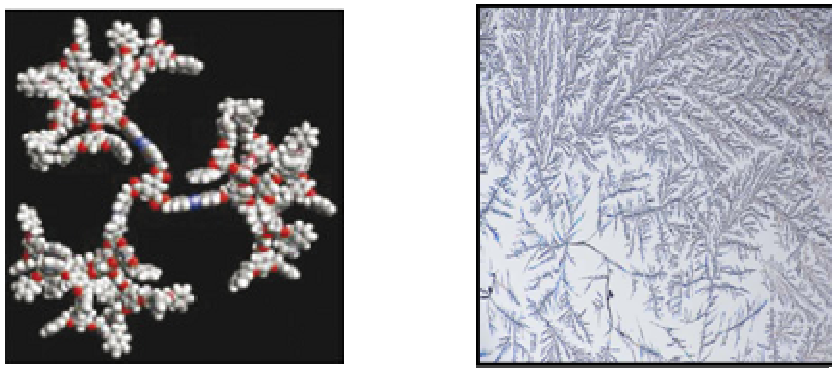


Figure 10 : A-Dendrimers structures (Sanvicens et al., 2008) B- Photograph of 3,5-dihydroxybenzylalcohol (DHBA) based dendrimers on silicon substrate. Scale: 5×5 mm (Vassilieff et al., 2006)

Dendrimers are a major architectural class of nanoscale chemical polymers. The term dendrimer describes a large, synthetically produced precisely defined polymer in which the atoms are arranged in many branches and sub-branches radiating out from a central core. Dendrimers are built from a starting atom, for example nitrogen, to which carbon and other elements are added by a repeating series of chemical reactions that produce a spherical branching structure. The core chemistry determines the solubilizing properties of the cavity within the core, whereas external chemical groups determine the solubility and chemical behaviour of the dendrimer itself.

Main applications: Nanocarriers

Drug Delivery Systems

B. Inorganic-based nanomaterials

Inorganic-based nanomaterials are of particular relevance for medical applications. Indeed, exclusive properties arise from the core of the materials specifically at the nanometer scale due to the so called quantum-size effect. As the surface-area-to-volume ratio increases when reducing the size of the materials, new electronic or magnetic properties may be observed and exploited for specific medical applications. Examples of the most developed inorganic nanoparticles for nanomedicine are reported below.

SUPERPARAMAGNETIC NANOPARTICLES

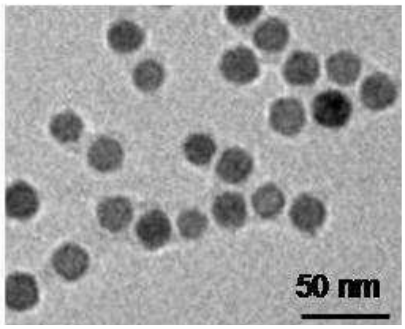


Figure 11 : Transmission electron microscopy (TEM) image of superparamagnetic ion oxide (SPIO) Nanoparticles

Small iron oxide nanoparticles (SPIO) or ultra-small iron oxide nanoparticles (USPIO) are the most exploited superparamagnetic oxide nanoparticles in medicine. They adopt the magnetite (Fe_3O_4) or maghemite (Fe_2O_3) spinelle structure. When particles size goes down 10 nm, superparamagnetic property occurs. Such property is of particular relevance for imaging using standard magnetic resonance imaging (MRI) technology due to changes in the spin-spin relaxations of neighbouring water molecules. Such nanoparticles are also developed for activation by high frequency alternative magnetic field for hyperthermia therapy.

Main applications:

Contrast Agents,

Therapeutic Agents,

Nanocarriers

QUANTUM DOTS

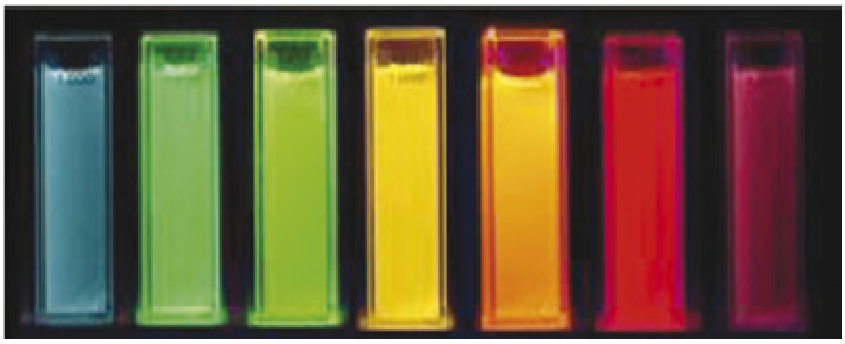


Figure 12 : Quantum dots (QD) optical properties (Sanvicens et al., 2008)

Quantum dots (QD) are colloidal fluorescent semiconductor nanocrystals with size ranging from about 2 nm up to 10 nm. The central core of QD consists of combination of elements from groups II–VI (CdSe, CdTe, CdS, PbSe, ZnS and ZnSe) or III–V (GaAs, GaN, InP and InAs) of the periodic table. The nanoparticle core is generally "overcoated" with a layer of ZnS. QD show size and composition-tuneable emission spectra and high quantum yield. They are resistant to photobleaching and show exceptional resistance to photo and chemical degradation. All these characteristics make QD excellent contrast agents for imaging and labels for bioassays.

Main applications: Contrast Agents,
Nanocarriers
Labels for Bioassays

METALLIC NANOPARTICLES

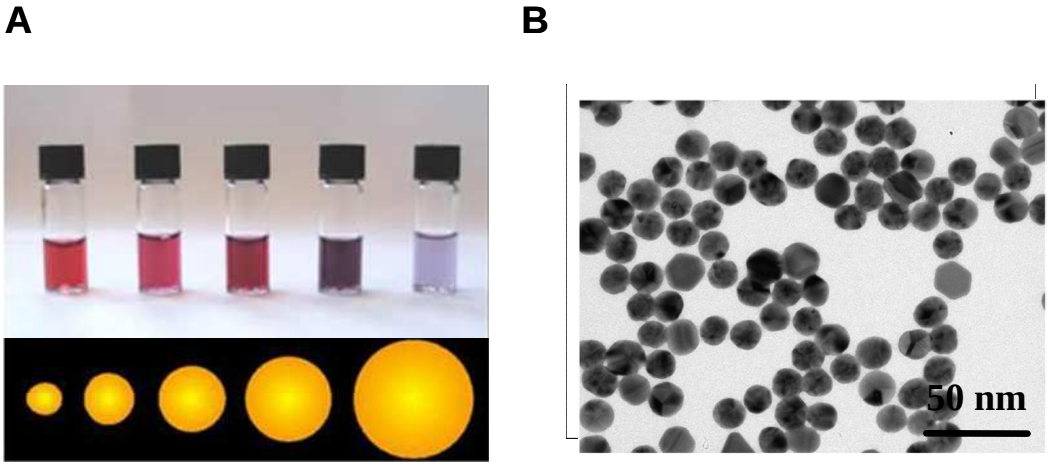


Figure 13 : A-Gold nanoparticles. B-TEM Images

Both gold and silver nanoparticles have received renewed interest because of their fascinating localized surface plasmon resonance properties, which can generate a strong electromagnetic field in the vicinity of a particle surface on irradiation with light. This light-induced field can enhance the intensity of Raman scattering by up to a billion times, enabling the development of optical probes for detecting biomarkers indicative of specific diseases at low level.

These nanoparticles have been further developed into colorimetric sensors, contrast agents for imaging modalities based on optical coherence, photoacoustics, two-photon fluorescence; and photothermal agents for cancer treatment and controlled release of drugs.

Main applications: Contrast Agents,
Therapeutic Agents
Nanocarriers
Labels for Bioassays

1.5. The nanomedicine market

A characterizing feature of nanotechnology today, is its enabling function to add new functionality to existing products, making them more competitive. As measuring the added value of nanotechnology to a product is not possible, it has become common praxis in nanotechnology business studies to take the total sales of nanotechnology-enhanced products as a measure of the economic importance of nanotechnology in an industrial sector.

Data published in 2006 (Wagner et al.) proved that commercialization efforts were already significant, with more than 150 start-ups and small and medium enterprises (SME) pursuing focused nanomedicine research and development projects and 38 nanotechnology-enabled products currently on the market were reported with total sales valued at $6.8 billion. Taking into account the pipeline of nanomedicine products that were in an advanced development stage, it was predicted that the market will grow to around $12 billion in the year 2012. Figure 14 summarizes some of nanomedicine products on the market (Wagner et al., 2006) and Figure 15 reports a list of product entering preclinical or clinical phase (Zhang et al., 2007).

Currently, one can see that nanomedicine is dominated by drug delivery systems using organic-based nanomaterials as nanocarriers. Inorganic-based nanomaterials are mostly developed for other health care applications such as *in vitro* diagnostic and *in vivo* imaging. In the field of *in vitro* diagnostic, the most widely used nanotechnology product is colloidal gold in lateral flow assays, which is used in rapid tests for pregnancy, ovulation, HIV and other indications. Further magnetic nanoparticles are used for cell sorting applications in clinical diagnostics. Nanotechnology-based contrast agents, albeit often cited as important examples for nanomedicine, are a niche market and all of the marketed contrast agents consist of SPIO nanoparticles for MRI.

Interestingly, nanomaterials are used to develop novel therapies or drugs in which the nanomaterial plays the pivotal therapeutic role. Here, the specific property offered by the materials at the nanometer scale is fundamental. A prominent example for such a nanomedicine is nanoparticles-based magnetic hyperthermia being developed for the treatment of cancer by the startup Magforce (Berlin). In this treatment, aminosilane-coated magnetic nanoparticles are injected into the tumour and subsequently heated with a newly developed magnetic field applicator. Nanoparticles coated with aminosilane are taken up faster by tumour cells than by normal cells. The tumour cells are destroyed by the heat generated by the nanoparticles upon activation.

Application	NP Composition	Indication	Compagny
Drug delivery			
Abelcet	Amphotericin B/ lipid complex	Fungal infections	Enzon, US
Amphotec	Amphotericin B/ lipid colloidal	Fungal infections	InterMune, US
Ambisome	Liposomal amphotericin B	Fungal infections	Gilead, US and Fujisawa, JP
DaunoXome	Liposomal daunorubicin	Kaposi sarcoma	Gilead
Doxil	Liposomal doxorubicin	Cancer, Kaposi sarcoma	Ortho Biotech, US and Schering-Plough, US
Depocyt	Liposomal cytarabine	Cancer	SkyePharma, GB and Enzon
Epaxal Berna	Virosomal hapatitis vaccine	Hepatitis A	Berna Biotech, CH
Inflexal V Berna	Virosomal influenza vaccine	Influenza	Berna Biotech
Myocet	Liposomal doxorubicin	Breast cancer	Zeneus Pharma, GB
Visudyne	Liposomal verteporfin	Macular degeneration	QLT, CA and Novartis, CH
Estrasorb	Estradiol in micellar NP	Mesopausal therapy	Novavax, US
Adagen	PEG-adenosine deaminase	Immunodeficiency disease	Enzon
Neulasta	PEG-G-CSF	Febrile neutropenia	Amgen, US
Oncaspar	PEG-asparaginase	Leukemia	Enzon
Pegasys	PEG-α-interferon 2a	Hepatitis C	Nektar, US and Hoffmann-La Roche, CH
PEG-Intron	PEG-α-interferon 2b	Hepatitis C	Enzon, Schering-Plough OSI Pharmaceuticals and Pfizer, US
Macugen	Pegylated anti-VEGF aptamer	Macular degeneration	Nektar, Pfizer
Somavert	PEG-HGH	Acromegaly	TEVA Pharmaceuticals, IL
Copaxone	Copplymer of alanine, lysine, glutaminic acid and tyrosine	Multiple sclerosis	Genzyme, US
Renagel	Crosslinked polyresin	Chronic kidney disease	Elan Drug Delivery and Merck, US
Emend	NP aprepitant	Antiemetic	Elan Drug Delivery, Wyeth Pharmaceuticals, US
MegaceES	NP megesterol acetate	Eating disorders	
Rapamune	NP megesterol sirolimus	Immunosuppressant	Elan Drug Delivery, Abbott, US
Tricor	NP fenofibrate	Lipid regulation	SkyePharma, First Horizon Pharmaceuticals, US
Triglide	NP fenofibrate	Lipid regulation	
Abraxane	Paclitaxel protein bound NP	cancer	Abraxis Bioscience, US and AstraZeneca, GB
Biomaterials			
Ceram X duo	NP composite	Dental filling material	Dentspley, GB
Filtek Supreme	NP composite	Dental filling material	3M Espe, DE
Mondial	NP-containing dental prothesis	Dental restoration	Heraeus Kulzer, DE
Premise	NP composite	Dental repair	Sybron Dental Specialties, US
Tetric EvoCeram	NP composite	Dental repair	Ivoclar Vivadent, LI
Ostim	NP-hydroxyapatite	Bone defects	Osartis, DE
Perossal	NP-hydroxyapatite	Bone defects	Aap Implantate, DE
Vitoss	NP-hydroxyapatite	Bone defects	Orthovita, US
Acticoat	Silver NP	Antimicrobial wound care	Nucryst, US
In vivo imaging			
Resovist	Iron NP	Liver cancer	Schering, DE
Feridex/Endorem	Iron NP	Liver cancer	Advanced Magnetics, US and Guerbet, FR
Gastromark/Lumirem	Iron NP	Imaging of abdominal structures	Advanced Magnetics, Guerbet
In vitro diagnostics			
Lateral flow tests	Colloidal NP	Pregnancy, ovulation, HIV…	British Biocell and Amersham, BG; Nymox, US
Clinical cell separation	Iron NP	Immunodiagnostics	Dynal/Invitrogen, NO; Miltenyl Biotec, DE; Immunicon, US
Active implants			
Pacemaker	Fractal electrodes	Heart failure	Biotronik (Berlin)

Figure 14 : Nanomedicine products on the market (Wagner et al., 2006)

Composition	Therapeutic	Indication
Polymeric micelles		
Antibody-enzyme-conjugated nanoparticles (immunoenzymosomes)	Antibody-directed enzyme prodrug therapy	Ovarian cancer
Biotinylated antibody-conjugated polymeric micelles	Daunomycin	Brain targeting
Pluronic block copolymers	Doxorubicin	Various cancers
Polymer–lipid hybrid nanoparticles	Doxorubicin	Solid tumors
Polymersomes	Hemoglobin	Oxygen carrier
Poly(lactic-co-glycolic acid)-block-poly(ethylene glycol)	Docetaxel	Prostate cancers
Poly(vinyl alcohol) polymeric micelles	PVA polymer antitumor activity	Neuroblastoma, melanoma
Dendrimers		
Folic acid-PAMAM dendrimers	Methotrexate	Epithelial cancer
Ligand-conjugated PEG-poly-L-lysine dendrimers	Chloroquine phosphate	Malaria
Polypropyleneimine dendrimers	Efavirenz	HIV infection
Poly(glycerol-succinic acid) dendrimers	Camptothecin	Various cancers
Albumin-based nanoparticles		
Albumin-bound nanoparticles	Doxorubicin, methotrexate	Various cancers
Cationic albumin-PEG nanoparticles	NC-1900 vasopressin fragment analog	Scopolamine-induced memory deficits
Polysaccharide-based nanoparticles		
Aerosol OT (AOT)-alginate nanoparticles	Doxorubicin	Breast cancer
Glycol chitosan nanoparticles	Doxorubicin	Solid tumors
Virus-based nanoparticles		
Cowpea mosaic virus PEG nanoparticles	Gene therapy	Various purposes
Gold-conjugated cytomegalovirus nanoparticles	Phototherapy, gene therapy	Solid tumors
Metallic nanoparticles		
Anti-HER2 antibody-targeted gold/silicon nanoparticles	Nanoshell-assisted infrared photothermal therapy	Metastatic breast cancer
Aminosilane-coated iron oxide nanoparticles	Thermotherapy	Brain tumors
Starch-coated iron oxide nanoparticles	Magnetically guided mitoxantrone	Tumor angiogenesis
Ceramic nanoparticles		
Silica-based nanoparticles	Photodynamic therapy	Various cancers
Silica crosslinked block copolymer micelles	Imaging agents, chemotherapies	Imaging, chemotherapy

Figure 15: Examples of the variety of nanoparticles-based therapeutics in preclinical development (Zhang et al., 2007)

1.6. The future of nanomedicine: health care needs with promising nano-enabled technology impact

An investment strategy to accelerate nanoscience into nano-enabled technology for medicine and health should reflect both health need (technology pull) as well as science push. Within this context, science and engineering of nanoscale structures are expected to make major contributions across the entire medicine and health spectrum ranging from mortality rate, morbidity an illness imposes on a patient, disease prevalence, and general societal burden (Murday et al., 2009). The following examples illustrate potential economic and therapeutic impacts, even if nano-enabled technologies only contribute partial solutions:

- The direct medical cost for cancer in the US for 2007 was about $90 billion. The National Cancer Institute (NCI) has recognized the importance of nanostructures in the diagnosis and treatment of cancer in its Alliance for Nanotechnology in Cancer. Nanotechnology approaches are progressing rapidly in early diagnosis, nano-enabled contrast agents for *in vivo* imaging, nanoscale reformulations of chemotherapy agents for smaller quantities of drug, targeted delivery for smaller side effects, and new treatments such as nanoparticle-mediated tumour ablation.
- The direct medical cost for diabetes in the US for 2007 was about $116 billion. Nanotechnology approaches to monitors of glucose levels and production of insulin are being explored.
- The annual medical care cost for spinal cord injury in the US is about $1.5 billion; the full costs are estimated as about $10 billion/year. There are promising nano-enabled approaches to the regeneration of spinal neurons, a capability once thought impossible.
- In the US, so as to remain physically active, approximately 200.000 people receive hip implants and 300.000 people receive knee implants. The average lifetime of current orthopedic implants is only 10-15 years; revision surgeries and their recoveries are not as successful as the first operation. The cost of an implant varies but is roughly $20.000. Nano-enabled innovations in bone cement and composite structures are opening new possibilities for improvements in implants.

The 2006 “Nanomedicine: Nanotechnology for Health” publication of the European Technology Platform Strategic Research Agenda for Nanomedicine presented additional examples of expected nanomedicine impact. The workshop participants were polled for their

opinion of pressing clinical needs amenable to nano-enabled technology and identified the following as illustrating the wealth of opportunities:

- Intelligent nanobiomaterials for cell therapy to improve heart function
- Safe and affordable therapeutic strategies to regenerate neural tissues
- Kidney-hollow fiber membranes
- Detoxification implants-correction of metabolic disorders
- Cochlear and retinal implants
- New power source technology for implants
- Repair of articular cartilage and regaining of homeostasis with the joint
- Skin regeneration
- Antimicrobials
- Drug delivery with:
    - Targeted pharmacotherapy-tissue/organ
    - Therapeutic DNA transfer vectors
    - Nanoparticles to carry a therapeutic payload across the blood-brain barrier
    - Transfection devices for therapeutic uses.
    - Controlled release (especially long term, continuous, and programmed)
    - Transient application sonoporation and electroporation

The considerable investment in nanoscience across the world has been leading to new discoveries (Figure 16). In the second 5 years of the US NNI, but also, as proposed by the European Union through its European technology platform, there is a growing effort to identify potential applications for those discoveries and to accelerate their transition into innovative technology solutions to societal problems.

Research issues and opportunities will embrace the following priorities:

- *In vitro* and *in vivo* diagnostics,
- Drugs delivery and therapy,
- Implants and tissues regeneration,
- Biological systems engineering
- Innovations in medical instrumentation and devices

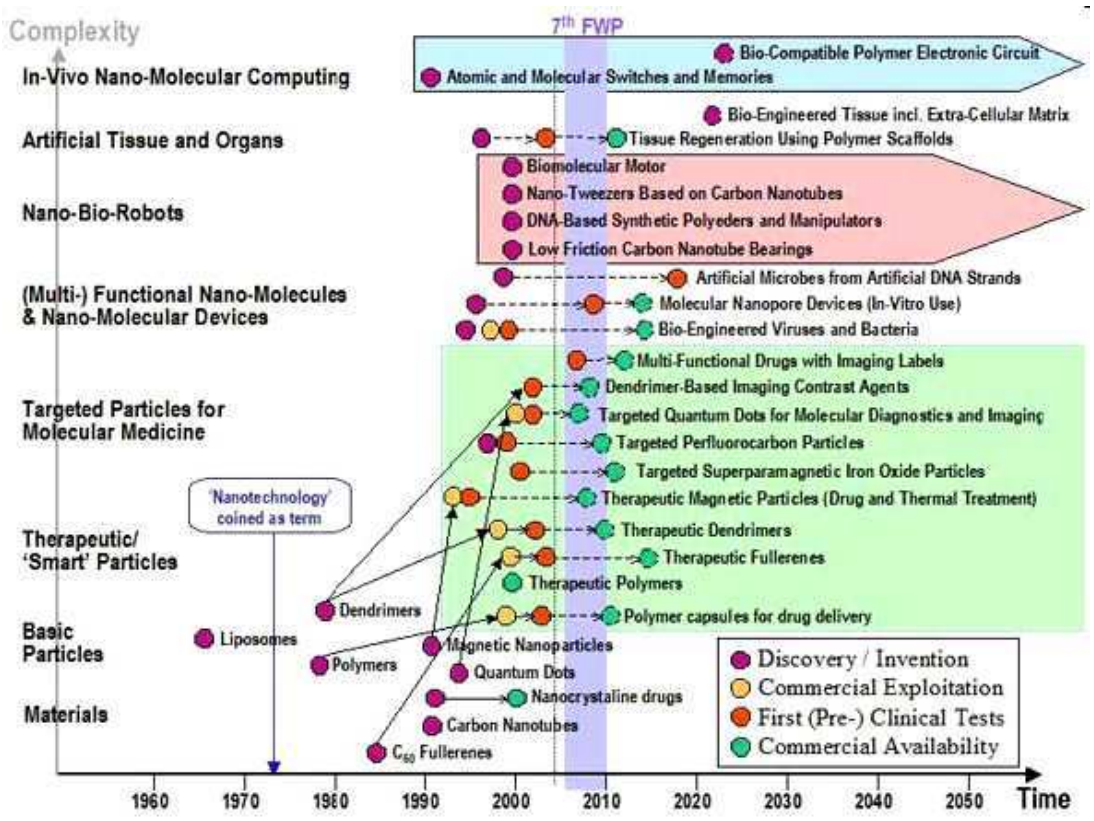


Figure 16 : Future nanomedicine timeline (nanowerk, 2008)

2. Focus on Cancer Diseases

2.1. Cancer

2.1.1. Epidemiology

Cancer remains one of the world's most devastating diseases, with more than 10 million new cases every year. Cancer is one of the principal causes of death today in the developed countries: much more than 150 000 cases per year in France. Cancer diseases killed more than 6.7 million people around the world in 2002, especially in developing countries (by order relative to the population density): Europe, US, Australia, Asia, Russia and South America. This tendency is not going to decrease in the next decade. The WHO predicted that cancer could kill 10.3 million people per year until 2020, especially in newly and non-industrial countries. It was predicted that 16 million new cases will appear until 2020 (50% of increase). Figure 17 indicates the mortality at 5 years for the most current cancer.

Cancers	ovary	pancreas	colorectal	bladder	kidney	breast	stomach	liver	prostate	lung	oesophagus	thyroid
Mortality at 5 years	68%	96%	54%	36%	52%	27%	79%	95%	44%	86%	92%	25%

Figure 17: Cancer statistic with mortality at 5 years (Simon et al., 2007)

2.1.2. Four most frequent cancers

A. Lung cancer

Lung cancer is the second most common cancer with an estimated 1.2 million new cases being diagnosed every year on a global basis. The “epidemic” of lung cancer mortality has been identified as a major health issue confronting both developed and developing countries, with the disease featuring high mortality rates across all countries. Lung cancer is one of the predominant causes of cancer related deaths in the seven major markets, causing up to 3 million deaths annually. Lung cancer usually begins in one lung from where it moves to the lymph nodes and tissues in the other lung. There are two main types of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the diagnosis of which can be ascertained by histopathological studies. SCLC is more invasive but comprises only 15-20% of all diagnosed lung cancer cases, while NSCLC represents an estimated 80-85% of the classifications and comprises a group of slower growing and therefore more treatable cancers.

B. Colorectal cancer

Colorectal cancer, also called colon cancer or bowel cancer, includes cancerous growths in the colon, rectum, and appendix. It is an extremely common form of cancer and overall, the second leading cause of death among cancers in the western world. Colon cancer is 2.5 times more common than rectal cancer. Approximately 145 300 new cases of colorectal cancer were diagnosed in the US during 2005. Known hereditary syndromes accounted for at least 10% of those, and it is likely that more subtle susceptibility factors contributed to the pathogenesis of many more. Colorectal cancer is a disease that originates in the epithelial cells lining the gastrointestinal tract. Cancer of the colon occurs when there are abnormal cells that result in a tumour. Many of the abnormal cells first develop as polyps inside the colon or rectum, which with time can become cancerous and metastasize. The incidence of colon

cancer increases with age, generally occurring in the sixth or seventh decade of life. While age is a major factor in the incidence of colorectal cancer, sedentary lifestyle, low fiber diets, diets rich in red and processed meat, excessive alcohol intake, inflammatory bowel conditions, radiation exposure, and related medical conditions are associated with an increased risk. A genetic basis for colorectal cancer has been observed in those who have had a family history of colon cancer or have a family history of familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC).

C. Breast cancer

Breast cancer is the most common cancer in women and the leading cause of death for women aged 40-44. It is second to lung cancer as the leading cause of all cancer deaths in women and it is estimated to have accounted for 211 240 new cancer diagnoses and 40 410 deaths in 2005 in US (American Cancer Society). The incidence of breast cancer increases rapidly with age until menopause, after which time it increases more slowly with advancing years. The majority (over 75%) of breast cancer begins in the (milk) ducts within the breast; the next most common site is the lobules – the glandular tissue that makes milk. Most breast cancers are slow-growing and by the time a lump can be felt, it may have been increasing in size for 5 or 10 years. Breast cancer is a malignant tumour that develops from the uncontrolled growth of breast tissue cells. These cells can then metastasize to other areas of the body via the lymph nodes and later spread beyond regional nodes to distant sites in the body. Though almost entirely found in women, a very small percentage of the cases (under 1%) are discovered in men. Age is a predetermining factor in most incidences of breast cancer, with sex hormones, genetics and ethnicity being important factors. Notably, women of European and African origin have been noted to have higher incidence rates of breast cancer. In the US, one woman in eight will develop breast cancer in her lifetime, but some families will be affected at even higher rates. Family breast cancer accounts for 5 to 10% for all breast cancer, and a substantial number of these cases can be linked to mutations in the genes BRCA1 or BRCA2. Other established risk factors include having no children, delaying first childbirth, not breastfeeding, early menarche (the first menstrual period), late menopause and some hormone replacement therapies, as well as diets that feature a high fat and red meat content. The probability of breast cancer rises with age but breast cancer tends to be more aggressive when it occurs in younger women, hence there is a need for screening procedures across all segments of the population. Several studies have also confirmed an association with

alcoholic intake and an increased risk of developing breast cancer. Perceptible decline mortality has occurred since 1992, most likely due to therapy with tamoxifen and other forms of chemotherapy, including therapy that targets human epidermal receptor (HER)2/neu. Early breast cancer usually also has no symptoms and the earlier a tumour is found, the better the chance of survival. Low rates of mortality are also attributed to the fact that women in the major markets are more likely to participate in mammography screenings and are more likely to be diagnosed with breast cancer during the early stages of the disease.

D. Prostate cancer

Prostate cancer is a malignant tumour, or group of cancerous cells, which arises in the prostate gland, a gland in the male reproductive system located below the urinary bladder and in front of the rectum. Prostate cancer tends to be slow-growing compared to other cancers, and as many as 90% of all prostate cancers can remain dormant and clinically unimportant for decades. As with other cancers, if it is advanced or left untreated in early stages, it can eventually metastasize through the blood and lymph fluid to other adjacent tissues and organs. Prostate cancer is the most frequently diagnosed cancer in American men, with an estimated 232 090 cases in 2005. It is also the second leading cause of cancer deaths in males, exceeded only by lung cancer, with approximately 30 000 deaths from prostate cancer occurring annually. A plethora of contradictory information exists regarding the cause of prostate cancer, although it is agreed that the most important factor is age. Prostate cancer occurs almost exclusively in men over the age of 50, and it is estimated that about 65% men over the age of 70 have microscopic evidence of prostate cancer. Hereditary factors, diets that have too much red meat content and environmental factors affecting the male hormones (androgen), are also implicated in the etiology of prostate cancer.

2.1.3. Molecular features of cancer

A. Cancer generation

Cancer is not a single disease but a wide range of different diseases of which there are over a hundred types. Cancers can be classified into two broad types: hematological (malignancies of the blood) or solid tumours. When the body's cells become abnormal and duplicate out of control a tumour is formed, these may be cancerous/malignant (spreading/metastasizing) or benign (non-cancerous).

The unique characteristic of cancer is the proliferation of cells of a type different from the normal complement of the organism. A cancer cell does not obey the complex rules of architecture and function that govern the usual placement and behaviour of cells within a tissue. Cancer is distinguished from other abnormal cellular growths that lead to benign tumours in its characteristic independence from the restrictions present in normal tissues. Benign tumours expand and compress, but do not attack or invade adjacent tissues. Through chemical and mechanical means, the cancer cell insinuates itself between and into space of the normal cells, killing them. Cancer is a genetic disease (Holland et al., 2006). The evolution of multicellular organisms has involved the development of intercellular communication required for such processes as embryonic development, tissue differentiation, and as systemic responses to wounds and infections. Proteomics is increasingly recognized as a method by which to decipher the molecular mechanisms underlying cancer cell growth and metastasis. The objectives are twofold: to understand the basic mechanisms of cancer initiation and progression, and to identify new therapeutic targets. However, cancer is a multifactorial disease so diverse that a great deal of time and effort will be necessary to define its associated proteome modifications and to translate these into practical applications for the clinic. Some information about the activity of oncogenic and tumour suppressor proteins has been obtained through proteomics.

These complex signaling networks are in large part mediated by growth factors, cytokines, and hormones. Growth factors mediate their diverse biologic responses by binding to and activating cell-surface receptors with intrinsic protein kinase-surface receptors with intrinsic protein kinase activity. To date, more than 50 receptor tyrosine kinases (RTK), which belong to at least 18 different receptors families, have been identified. Examples of growth factors: platelet-derived growth factor (PDGF) family, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) family, fibroblast growth factor (FGF) family, the insulin family, hepatocyte growth factor (HGF), neurotrophin growth factor (NGF) family. The first oncogenes were discovered through the study of retroviruses, ribonucleic acid (RNA) tumour viruses whose genomes are reverse-transcribed into DNA in infected animal cells. Proto-oncogenes encode proteins that are involved in the control of cell growth. Alteration of the structure and/or expression of proto-oncogene can activate them to become oncogenes capable of inducing in susceptible cells the neoplasmic phenotype. Oncogenes can be classified into 5 groups based on the functional and biochemical properties of protein products of their normal counterparts: growth factors, growth factors receptors, signal transducers, transcription factors and others including programmed cell death regulators. The

activation of oncogenes involves genetic changes to cellular proto-oncogenes. The consequence of these genetic alterations is to confer a growth advantage to the cell. Four genetic mechanisms activate oncogenes in human neoplasms: mutation, gene amplification, chromosome rearrangements and overexpression. The first three mechanisms resulted in either an alteration of proto-oncogene structure or an increase in proto-oncogene expression. As proto-oncogenes, approximately 30 tumour suppressor genes have been identified and definitively implicated in cancer development. Defects in genomic stability genes have also been implicated in a broad spectrum of human cancers. Like other tumour suppressor genes, the genomic stability genes are inactivated in human cancers. However, unlike the mutations in tumour suppressor genes, mutations in genomic stability genes are much more often inherited in mutant form. For example, DNA mismatch repair gene defects and HNPCC cancer, genes that contain repetitive DNA sequences, such as microsatellite tracts, might be expected to be targets of mutation in these cancers (Hamelin et al., 2008).

Due to the large number of cancers, there are, perhaps unsurprisingly, multiple risk factors implicated in the development of cancer. The influence of these risk factors varies substantially according to the type of tumor, with the etiology of some cancers being predominantly influenced by environmental factors, and other cancers by factors related to infectious disease or hereditary factors.

B. Metastasis

Despite improvements in cancer therapies over the past 50 years, metastatic solid cancers remain largely incurable, and the survival for patients with these malignancies is often measured in months. Approximately 30% of cancer patients have clinically detectable metastasis at the time of initial diagnosis and 40% harbour occult metastasis. Therefore, in this era of targeted therapies, substantial efforts are being made to identify the optimal target for each type of cancer in order to succeed to stop dissemination.

Tumour progression towards metastasis is often depicted as a multistage process in which malignant cells spread from the tumour of origin to colonize distant organs. However, these basic steps occur in the context of different organs, emerge at different rates and are clinically managed in different ways depending on the type of cancer.

The genetic basis of tumourigenesis can vary greatly; the steps required for metastasis are similar for all tumour cells. The detached cells from the primary site use lymphatic vessels

and the bloodstream for transport. Extracellular matrix (ECM) receptors, invasion of the basal-cell membrane by proteolytic enzymes, entry into the vasculature, and motility factors of organs are essential for tumour-cell migration. The development of micro-colonies in the secondary organ is facilitated by growth factors. Angiogenic factors are also essential to development of metastasis. Invasion and metastasis are the most insidious and life-threatening aspects of cancer. The capacity for invasion may not be expressed initially or in all tumours. Once the neoplasm becomes invasive, it can disseminate via the lymphatics and/or vascular channels that it induces through tumour-stimulated lymphangiogenesis and angiogenesis and other perturbations of the local environment. Invasion and metastasis kill hosts through two processes: local invasion and distant organ colonization and injury.

The diverse temporal courses of metastasis in different types of cancer and patient populations are evident from clinical observations. As the kinetics of disease progression and distinct physiological barriers can dictate the latency between the infiltrating and colonizing steps of metastasis, each clinical course has different implications for the organ-selective evolution of metastatic cell populations. In oestrogen receptor-positive breast cancer, prostate cancer and ocular melanoma, metastasis might become manifest decades after the removal of even a small primary malignancy. The absence of immediate clinical relapse implies that these tumour cells are not fully competent to overtake organs immediately after infiltration. A protracted period of latency might ensue during which further malignant evolution of the disseminated cell population, of their microenvironment or of both must occur for colonization to proceed to invasive carcinoma.

As an end-stage malignant disease, metastatic relapse is often associated with resistance to therapy. Relapse following systemic treatments might be due to cell intrinsic mechanisms such as genetic alterations that confer drug resistance following a period of therapeutic response. Lung adenocarcinomas with epidermal growth factor receptor (EGFR) mutations respond to EGFR kinase inhibitors but frequently relapse owing to secondary EGFR mutations that confer resistance. Certain mechanisms of drug resistance might simultaneously render the tumour more competent for metastasis.

The most frequent location of metastasis for many types of cancers is the first capillary bed encountered by circulating cells. However, the distribution of metastases varies widely, depending upon the histologic type and anatomic location of the primary tumour. For example 80% of cancers that spread to bone originate from the breast, prostate, lung, thyroid gland, and kidney. Others examples of typical sites of metastasis of different solid cancer are

described in Figure 18. Thus, distant organ infiltration and colonization (separated by a variable period of intervening latency) are general steps that primary tumour cells must accomplish to metastasize.

Tumor type (solid)	Typical sites of metastasis
Breast	Bone, lungs, liver and brain
Lung adenocarcinoma	Brain, bones, adrenal gland and liver
Skin melanoma	Lungs, brain, skin and liver
Colorectal	Liver and lungs
Pancreatic	Liver and lungs
Prostate	Bones
Sarcoma	Lungs

Figure 18: Typical site of metastasis for solid tumours (Nguyen et al., 2009)

2.1.4. Anticancer therapy

The instances of diagnosed cancer are expected to continue to rise as a result of increased life expectancy, aging populations and technological improvements, which are leading to more sophisticated screening techniques and earlier detection of cancer.

The three primary methods of treating cancer are surgery, radiation therapy, and pharmaceuticals, each of which can be used alone or in combination, depending on the type of cancer being treated. The choice of therapy depends upon the location and grade of the tumour and the stage of the disease, as well as the general state of the patient (performance status).

Although complete removal of solid tumours from the body is the goal of treatment, which can sometimes be accomplished through surgery, the tendency for cancers to spread into the surrounding tissue often makes this difficult. There is no single curative method for all cancers, but treatment will generally strive to reduce the spread of malignant cell growth.

A. Surgery

In theory, non-hematological cancers can be cured if entirely removed by surgery, but this is not always possible. Surgery is the oldest modality of cancer therapy and still forms the mainstay of treatment in solid tumours. Surgery is increasing combined with other treatment modalities.When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. In the Halstedian model of cancer progression, tumours grow locally, and then spread to the lymph nodes, then to the rest of the body. This has given rise to the popularity of local-only treatments such as surgery for small cancers. Even small localized tumours are increasingly recognized as possessing metastatic potential. The goal of the surgery can be either the removal of only the tumour, or the entire organ.

Surgical procedures currently involve numerous approaches. Many types of surgical methods for treating cancer and precancerous conditions exist, and investigators continue to research new methods. Some common types of cancer surgery include:

Cryosurgery: During this type of surgery, very cold material is used, such as liquid nitrogen spray or a cold probe, to freeze and destroy cancer cells or cells that may become cancerous, such as irregular cells in a woman's cervix that could become cervical cancer.

Electrosurgery: By applying high-frequency electrical currents, cancer cells are killed, for example, in the buccal cavity or on the skin.

Laser surgery: Laser surgery, used to treat many types of cancer, uses beams of high-intensity light to shrink or vaporize cancer cells. In some cases, the heat of the laser accomplishes this. In other cases, the laser is used to activate a previously administered chemical that cancer cells absorb. When stimulated by light, the chemical kills the cancer cells.

Mohs' surgery: Useful for removing cancer from sensitive areas of the skin, such as near the eye, and for assessing how deep a cancer goes, this method of surgery involves carefully removing cancer layer by layer with a scalpel.

Laparoscopic surgery: A laparoscope is used to see inside the body without making large incisions. Instead, several small incisions are made and a tiny camera and surgical tools are inserted into the body. The surgeon watches a monitor that projects what the camera sees inside your body. The smaller incisions mean faster recovery and a reduced risk of complications. Laparoscopic surgery is used in cancer diagnosis, staging, treatment and symptom relief.

Robotic surgery: In robotic surgery, the surgeon sits away from the operating table and watches a screen that projects a 3D image of the area being operated on. The surgeon uses hand controls that tell a robot how to maneuver surgical tools to perform the operation. Robotic surgery helps the surgeon operate in hard-to-reach areas. But robotic surgical systems are expensive and require specialized training, so robotic surgery is usually available only in specialized medical centers.

Natural orifice surgery: Natural orifice surgery is currently being studied as a way to operate on organs in the abdomen without cutting through the skin. Instead, surgeons pass surgical tools through a natural orifice, such as your mouth, rectum or vagina. For instance, a small incision is made in the wall of the stomach and surgical tools pass into the abdominal cavity in order to take a sample of liver tissue or remove the gallbladder. Natural orifice surgery is experimental, and few operations have been performed this way. It is expected to reduce the risk of infection, pain and other complications of surgery.

Cancer surgery continues to evolve. Researchers are investigating other surgical techniques with a goal of less invasive procedures.

Both, surgery and radiotherapy are essentially local treatments directed at the primary tumour and any loco-regional disease.

B. Pharmaceuticals

Chemotherapy

Chemotherapy is a systemic treatment that can destroy cancer cells and treat distant metastases. Chemotherapy has vastly improved the prognosis of many malignant conditions but it is only curative in a minority of cancers, for example, lymphomas, leukaemias and testicular cancers. Methotrexate, fluorouracil, doxorubicin, tamoxifen, taxol and gemcitabine (Figure 19) are the drugs to most indicate to treat cancer.

Decade	Example
1940	Methotrexate
1950	Fluorouracil
1960	Doxorubicin
1970	Tamoxifen
1980	Taxol
1990	Gemcitabine

Figure 19: History of cancer drug availability

In current usage, the term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells in general, in contrast with targeted therapy. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy. Because some drugs work better together than alone, two or more drugs are often given at the same time, this is called combination chemotherapy. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body.

Actively proliferating cells are the most vulnerable to chemotherapy. Non-dividing cells are killed less effectively by anti-cancer drugs. The cycle (Figure 20) is divided into four main phases. G_1 is the protein synthetic phase; in the S phase, DNA is copied and synthesised prior to cell division, which occurs following chromosome condensation and segregation in G_2 and M phases. The so-called resting phase, G_0, is important. Such cells are not undergoing cell division and may eventually suffer programmed cell death (apoptosis). However, following treatment with chemotherapy or radiotherapy some G_0 cells may be recruited back into cycle. The lethal effects of chemotherapeutic agents vary between different phases of the cell cycle. Cisplatin carboplatin, one of the most utilized drugs for cancer, and the alkylating agents are lethal throughout the cell cycle, but are particularly active in G_2 and at the G_1/S boundary. Antimetabolites such as 5-fluorouracil and methotrexate are most active against

cells in S phase. Etoposide, vinca alkaloids and taxanes are most effective against cells in the G_2/M phases of the cell cycle.

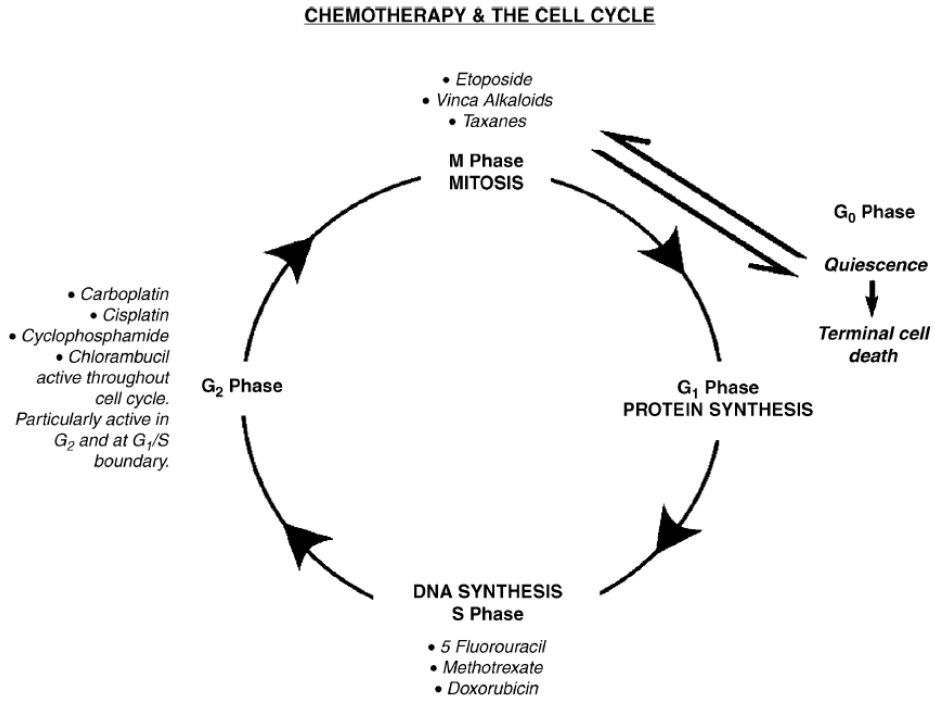


Figure 20: Chemotherapy and the cell cycle (Symonds et al., 2006)

Radiation sensitizers

Chemotherapeutic agents that are highly responsive to ionizing radiation and enhance the effectiveness of radiation treatment are termed radiation sensitizers. Radiation sensitizers act in a number of ways to make cancer cells more susceptible to death by radiation than surrounding normal cells. An ideal radiation sensitizer would reach the tumour in adequate concentrations and act selectively in the tumour compared with normal tissue. It would have predictable pharmacokinetics (pK) for timing with radiation treatment and could be administered with every radiation treatment. The ideal radiation sensitizer would have minimal toxicity itself and minimal or manageable enhancement of radiation toxicity. The ideal radiation sensitizer does not exist today.

Targeted therapies

Targeted therapy, which first became available in the late 1990s, has had a significant impact on the treatment of several types of cancer. This constitutes the use of agents specific

for the deregulated proteins of cancer cells. Small molecule targeted therapy drugs are generally inhibitors of enzymatic domains on mutated, overexpressed, or otherwise critical proteins within the cancer cell. Monoclonal antibody therapy is another strategy in which the therapeutic agent is an antibody which specifically binds to a protein on the surface of the cancer cells. Targeted therapy can also involve small peptides as "homing devices" which can bind to cell surface receptors or affected ECM surrounding the tumour. The trend in the pharmaceutical industry is to provide more and more drugs or therapeutic tools with a personalize approach. This is mainly because of the lack of efficacy of a generalized approach where a population can be seen as one patient with a similar biological profile.

Hormone therapy

It consists to add, block, or remove hormones. To slow or stop the growth of certain cancers (such as prostate and breast cancer), synthetic hormones or other drugs may be given to block the body's natural hormones. Sometimes surgery is needed to remove the gland that makes a certain hormone. For example, many premenopausal breast cancer patients have their ovaries removed to eliminate oestrogen from their body. Oestrogen is thought to cause some types of breast cancer to grow more quickly. These same patients who have tumours classified as oestrogen-receptor positive can be treated with tamoxifen as an accepted alternative to ovarian exeresis. Antioestrogen therapy increases survival for some breast cancers.

C. Radiotherapy

Radiotherapy is the use of ionizing radiation to kill cancer cells. Radiation therapy injures or destroys cells in the target tissue by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. The effects of radiation therapy are localised and confined to the region being treated.

The lethality of radiotherapy is related to its effects on DNA, with the introduction of single-stranded DNA breaks and, to a lesser extent, double-stranded DNA breaks. DNA damage arises because absorption of radiation in tissues leads to the immediate production of ionized atoms, which are raised into excited states. These in turn lead to the formation of

unstable, short-lived free radicals, which interact with cellular constituents. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions. Prescribed treatments typically consist of 25 to 35 fractions, and are administered over periods ranging from a few days to several weeks. Such fractions are intended to deliver a cumulative dose of radiation sufficient to kill cancer cells, while allowing healthy tissue to recover sufficiently between treatments.

Radiation therapy may be used to treat almost every type of solid tumour, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, soft tissue sarcomas, skin, stomach or uterus. Radiation dose to each site depends on a number of factors, including the radiosensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by radiation. As with every form of cancer treatment, radiation therapy is not without its side effects.

For some types of cancer, radiation may be given to areas that do not have evidence of cancer. This is done to prevent cancer cells from growing in the area receiving the radiation. This technique is called prophylactic radiation therapy.

Radiation therapy also can be given to help reduce symptoms such as pain from cancer that has spread to the bones or other parts of the body. This is called palliative radiation therapy**.**

Recent advances in radiation therapy technologies have focused on further improving the ability to target the radiation dose more precisely and to increase the radiation dose in cancer cells, while minimizing the exposure of healthy tissue.

There are two main types of radiation therapy:

- External radiation is delivered by a machine from outside the body
- Internal radioactive materials are placed in the body near the cancer cells (also called implant radiation or brachytherapy)

External beam radiation therapy (EBRT)

EBRT creates a radiation beam and aims it at the tumour.

Electromagnetic radiation

The X-ray radiation adequately covers the tumour but minimizes the dose to the non-tumour normal tissues. Radiation is given in fractions rather than as a single dose.

Conventional fractionation is 1.8 to 2 Gray (Gy) per day, administered five days a week for five to seven weeks, depending on the particular clinical situation. While this schedule is strictly for the convenience of physicians trying to maintain a normal workweek, the relatively long intervals between doses of radiation may allow cancer cells (as well as normal cells) to recover and re-grow.

A number of different radiotherapy schedules have been suggested to overcome this problem. These include hyperfractionation, in which the time between fractions is reduced from 24 hours to 6 to 8 hours to enhance the toxic effects on tumour cells while still preserving an adequate time interval for the recovery of normal cells. Continuous hyperfractionated accelerated radiation therapy (CHART) is an intense schedule of treatment, in which multiple daily fractions are administered within a short period of time. Clinical studies have shown benefits of altered fractionation over conventional treatment for several cancers, including head and neck cancer and non-operable lung cancer.

Three-dimensional conformal radiation therapy (3D-CRT)

3D-CRT is a technique that uses imaging computers to precisely map the volume and location of a tumour. The patient is fitted with a plastic mold or cast to keep the body part still so that the radiation can be aimed more accurately from several directions. By aiming the radiation more precisely at the tumour, it is possible to reduce radiation damage to normal tissues surrounding the tumour by up to 50 percent.

Intensity modulated radiation therapy (IMRT)

IMRT, involves varying, or modulating, the radiation beam intensity across the treatment area. This technique attempts to conform the high dose region of the radiation beam more closely with the shape of the tumour, enabling the delivery of higher doses of radiation to tumours with a reduced impact on surrounding healthy tissue. Using IMRT, medical professionals can design a more individualized treatment plan for each patient. This may result in a higher cancer-control rate and a lower rate of side effects. Adaptive radiation therapy involves adjusting a patient’s radiation therapy plan between fractions to account for changes in the patient’s anatomy, the amount and location of the radiation received by the patient, and the size, shape and location of the tumour. It has been characterized to include as little as an adjustment to the physical position of the patient relative to the radiation source

prior to treatment, as occurs during image guided radiation therapy (IGRT), rather than adjustment to the treatment plan. IGRT consists of an improved type of IMRT device that combines the functions of diagnostic imaging with a computer guided beam control device to accurately direct radiation beams to the tumour area and correct error arising due to movement of patient's internal organs and tissues. By combining imaging with radiation treatment, clinicians can adjust the patient's position relative to the radiation source prior to each treatment to target the tumour more precisely. Compared to traditional IMRT without image guidance, accurate image guidance enables clinicians to improve patient outcomes by concentrating higher doses of radiation at tumours and further reducing the exposure of healthy tissues to radiation.

Stereotactic body radiation therapy (SBRT)

SBRT is a standard form of treatment for primary and metastatic brain cancer. It is delivered using a machine called a gamma knife, which uses converging beams of gamma radiation that meet at a central point within the tumour, where they add up to a very high, precisely focused dose of radiation in a single fraction. Due to this precision, the cancer can be located in an area of the brain or spinal cord that might normally be considered inoperable.

CyberKnife®

CyberKnife® is a non-invasive, precise radiation technique that can deliver concentrated and accurate beams of radiation to any site in the body. This system combines robotics and advanced image guidance cameras to locate the tumour's position in the body and deliver highly focused beams of radiation that converge at the tumour, avoiding normal tissue. It is a successful method used to treat spinal tumours or tumours at other critical locations that are not amenable to open surgery or radiation, as well as to treat medically inoperable patients. It can also be used to treat benign tumours and lesions in a previously irradiated site, or to boost standard radiotherapy.

Particulated radiation

Proton beam radiation therapy

This is one of the most precise and sophisticated forms of EBRT available. The advantage of proton radiation therapy over X-rays is its ability to deliver higher doses of shaped beams of radiation directly into the tumour while minimizing the dose to normal tissues. This leads to reduced side effects and improved survival rates. There are approximately 19 proton treatment centers worldwide.

Internal radioactive materials

Brachytherapy

Brachytherapy or internal radiation therapy uses radiation that is placed very close to or inside the tumour. The radiation source is usually sealed in a small holder called an implant. Implants may be in the form of thin wires, catheters, ribbons, capsules, or seeds. The implant is put directly into the body (NCI).

Radioimmunotherapy (RIT)

Radioimmunotherapy, one of the newest developments in the treatment of non-Hodgkin's lymphoma, has achieved a high tumour response rate (up to 80 percent) in several clinical trials. Radioimmunotherapy uses drugs called monoclonal antibodies (mAb), which have a radioactive isotope attached to them. This is targeted to the surface of a cancer cell, destroying it. Radioimmunotherapy can be used (in a targeted fashion) to treat single cells that have spread around the body. Because the radiation does not concentrate in any one area of the body, radioimmunotherapy does not cause side effects commonly seen with EBRT. The most significant side effect associated with radioimmunotherapy may be a temporary drop in white blood cell or platelet count.

Current trends of radiotherapy combinations

Radiation therapy may be used alone or in combination with other cancer treatments, such as chemotherapy or surgery.

Radiotherapy and Surgery

Malignant tumours of the brain, head and neck, lung, large parts of the gastro-intestinal or genito-urinary tracts, and bone or soft tissues are among the most well known indications for radiosurgical approaches. Improved local and regional control can often be achieved using radiosurgery. Once the gross disease is resected, radiotherapy can be used to kill residual tumour cells. Preoperative radiotherapy can make an unresectable tumour amenable to surgery.

Radiotherapy and chemotherapy

Throughout the past two decades, combinations of radiotherapy with chemotherapy have yielded encouraging results in patients with locally advanced diseases and for whom the prognosis remains dismal in terms of local control and distant metastasis. Patients' benefits have been observed for malignant epithelial tumours such as head and neck, lung and gastrointestinal tumours.

D. Other biological anticancer approaches

Immunotherapy-biotherapy

Immnunotherapy is the development of methods to augment and enhance the body's natural tendency to defend itself against malignant tumours without damaging healthy tissue. Immunological and biotherapy treatments which target therapy are a fraction of total cancer treatment revenues at the current time. Novel treatments such as mAb and vaccines that can develop resistance to cancer in populations are currently developed. Most therapeutic vaccines are in phase III trials at this time. To a more limited extent, interleukins (IL) and interferons are utilized and their use is indicated in tumours such as renal cancer. Other biotherapies include treatments such as antisense oligonucleotides, protein and polypeptide growth factors, methylation modifiers, p53 tumour suppressor proteins, regulatory enzymes and other therapies.

Gene therapy

Gene therapy can defined as the transfer of genetic material into a cell to transiently or permanently alter the cellular phenotype. Introduction of a nucleic acid or target gene (transgene) directly into cells is referred to as transfection. Cancer gene therapy is an

emerging field that was received as one such opportunity to take advantage of the genetic differences between normal and transformed cells. Gene therapy has dampened interest in developing approaches to inactivate oncogenes or replace non-functioning tumour suppressor genes. It designs gene delivery systems (adenoviruses, herpes and non viral), therapeutics gene, selective gene expression or virus oncolysis (Advani et al., 2006).

Stem cell transplantation

It is a method of replacing immature blood-forming cells that were destroyed by cancer treatment. The stem cells are given to the person after treatment to help the bone marrow recover and continue producing healthy blood cells.

E. Other physics-based anticancer approaches

Photodynamic therapy (PDT)

PDT is based on the concept that certain photosensitizers can be localized in neoplastic tissue, and subsequently, these photosensitizers can be activated with the appropriate wavelength of light to generate active molecular species such as free radicals and singlet oxygen ($^1O_2$) that are toxic to tumours. Limitations of light penetration make this therapy most appropriate for small or superficial lesions, such as bladder, oral mucosa, cervical cancer, Barret's oesophagus.

Hyperthermia (HT)

HT means elevation of temperature to a supra-physiological level. It is known to cause direct cytotoxicity and also acts as a radiation and chemosensitizer. HT improves tumour oxygenation and delivery of novel drug carriers, such as liposomal agents. Recent developments in the field of gene therapy may also establish a role of HT as a strategy for targeted, localized induction of gene therapy using the heat shock promoter.

2.1.5. Energy sources for physics-based anticancer approaches

A. Ionizing radiation sources

Radiotherapy equipments and facilities

The US NCI estimates that, despite of advances in systemic therapy, nearly 50% of cancer patients in the US are treated using radiation therapy. Radiation therapy is indicated in approximately 90% of patients with solid tumours, and in the routine medical practice 50 - 60 % of cancer patients are treated with this modality during the disease course. In the US, over 15 million radiation therapy treatments are performed annually in 1.870 radiotherapy centers. Europe counts 911 radiotherapy centers. Globally more than 5.000.000 patients are treated with radiotherapy annually. This makes, apart from surgery, radiotherapy as the major method applied with curative intent for cancer patients (Statistics after J. Sisterson, Particles, no. 17; 1996 and no. 31; 2003). Approximately 90% of patients treated with radiation therapy in the US and other developed countries receive external beam radiation generated by a device called a linear accelerator. Linear accelerators have been widely used for radiation therapy for over 30 years. While radiation therapy is widely available in the US and Western Europe, many developing countries currently do not have a sufficient number of linear accelerators to adequately treat their domestic cancer patient populations.

Photons

- X-rays : LINAC (50 KeV → 200 keV ; MeV)

A linear accelerator produces X-rays by slamming a beam of high energy particles (usually electrons) into a metal target. This gives off X-ray photons in a process called bremsstrahlung, literally “braking radiation”, that occurs when a charged particle undergoes acceleration (or deceleration in this case). The energy of the photons can be as high as the energy of the incoming particles, but the most probable energy is some fraction of that. Most of the photons are given off in the direction of the incident beam, so this results in a beam of photons with a spectrum of energies somewhat less than the incident beam energy.

The simplest device for creating X-rays is the X-ray tube. An X-ray tube is simply a vacuum tube that has a filament on one end that emits electrons and an anode on the other end to collect them. A high voltage is placed between the filament or cathode and the anode to

accelerate the electrons. The energy of the electrons is measured in electron volts. One electron volt is the energy an electron gets when it is accelerated by one volt. If you were to place a voltage of 1 kilovolt across an X-ray tube, the electrons would have an energy of 1 kilo electron volt (or 1 keV). When the electrons hit the anode, bremsstrahlung photons are given off. These photons can have energy up to the energy of the incident particles. Therefore, if 100 kilovolts were placed across the tube, the energy of the electrons would be 100 keV and the photons could have energy of up to 100 keV. Usually, though, the peak of the spectrum is at energy of about 1/3 of the incident energy.

Systems like X-ray tubes work well for energies in the kilovolt range, but become large and cumbersome if used to generate photons in the megavolt range. Since photons with a higher energy can penetrate further into the patient to treat a deep seated tumour, other methods of generating radiation that could reach megavolt energies were developed. There are several types of accelerators that can generate photons with that high of energy, but the one that is most often used is the linear accelerator. This Figure 21 shows a block diagram of a typical linear accelerator or linac.

The linear accelerators in use today use microwaves to accelerate electrons. The waves are generated by a device called a magnetron (you have a smaller version of one in your microwave oven) or by a microwave amplifier called a klystron. The microwaves then travel through a waveguide into an accelerator tube. When electrons are injected into the accelerator tube from an electron gun, they can pick up energy from the waves and be accelerated. The accelerator tube can be manipulated to give different amounts of energy to the electrons and therefore different final energies for the beam. Very large accelerators like the Stanford Linear Accelerator Center can accelerate particles to energies of Giga electron volts. Medical accelerators are more compact and generate electron beams with energies in the Mega electron volt (MeV) range. A bending magnet then steers the beam into a metal target to generate X-rays. The bremsstrahlung process is very inefficient. Only about one tenth of a percent of the electron beam energy is converted into usable X-rays. Most of the rest is dissipated in heat in the target. Therefore, the target must be made of something that is very heat resistant like tungsten and is usually water cooled. Once the X-rays are generated, the beam is collimated and shaped in the treatment head.

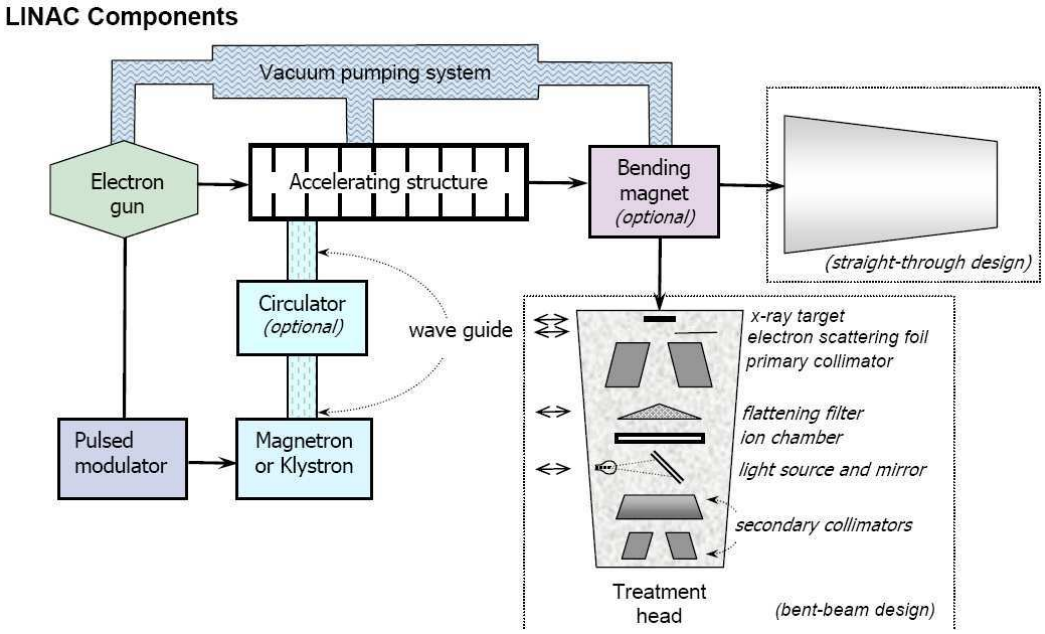


Figure 21 : Linear accelerator model, (BC Cancer Agency, Vancouver)

- Gamma-Rays (Cobalt, Cesium)

Gamma rays are produced when a neutron transforms to a proton and a beta particle. The additional proton changes the atom to barium-137. The nucleus ejects the beta particle. However, the nucleus still has too much energy and ejects a gamma photon (gamma radiation) to become more stable.

Cesium 137 and Cobalt 60 provides an example of radioactive decay by gamma radiation. Cobalt 60 is used in many common industrial applications. Large sources of Cobalt 60 are increasingly used for sterilization food or material. The powerful gamma- rays kill bacteria and other pathogens, without damaging the product.

Electrons: LINAC

Electrons have advantages over photons for some types of treatments. Electrons have a much shorter range and are therefore more desirable for treatments that are very close to the skin. It is important to remember though that by removing the target you get rid of the inefficiency of the bremsstrahlung process. Therefore the electron beam current will have to be lowered by a factor of 1000 (since the X-ray production of the target is 0.1% efficient) to have roughly the same dose as an X-ray beam. Electron beams are described in units of MeV,

as in 6 MeV. Figure 22 shows typical linear energy transfer values for different sort of radiation sources.

Radiation	Linear energy transfer, KeV/μm
Cobalt-60 γ-rays	0.2
250-kV x-rays	2.0
10-MeV protons	4.7
150-MeV protons	0.5
2.5-MeV α-particles	166
2-GeV Fe ions	1,000

Figure 22: Typical linear energy transfer values

B. Laser light source

The term "**laser**" stands for Light Amplification by Stimulated Emission of Radiation. Ordinary light, such as that from a light bulb, has many wavelengths and spreads in all directions. Laser light, on the other hand, has a specific wavelength. It is focused in a narrow beam and creates a very high-intensity light. Laser therapy is often given through a flexible endoscope (a thin, lighted tube used to look at tissues inside the body). The endoscope is fitted with optical fibbers. It is inserted through an opening in the body, such as the mouth, nose, anus or vagina. Laser light is then precisely aimed to cut or destroy a tumour.

- For heat generation: HT therapy

Laser-induced interstitial thermotherapy (LITT) (or interstitial laser photocoagulation) also uses lasers to treat some cancers. LITT is similar to a cancer treatment called HT, which uses heat to shrink tumours by damaging or killing cancer cells.

During LITT, an optical fibber is inserted into a tumour. Laser light at the tip of the fibber raises the temperature of the tumour cells and damages or destroys them.

- For free radical generation: PDT

PDT is another type of cancer treatment that uses lasers. In PDT, certain drugs, called photosensitizers, are injected into a patient and absorbed by cells all over the patient's body. After a couple of days, the agent is found mostly in cancer cells. Laser light is then used to activate the agent and destroy cancer cells.

C. Ultrasound

- For heat generation: HT

New high intensity focused ultrasound (HIFU) treatment has been implemented for the treatment of prostate cancer. The procedure uses ultrasound to destroy deep-seated tissue without affecting the surrounding healthy tissue.

The Sonablate 500 system has approval in Europe for the treatment of prostate diseases. There are currently 35 units being used in medical institutions around the world.

The delivery of this energy to the tumour small area results in an increase in temperature to a point where the lipids (fats) in the cell membrane melt and the proteins denature. A reproducible but small volume of tissue destruction occurs. The distribution of these target lesions is under the control of the clinician.

- For drug release: drug delivery therapy

Using a combination of polymers that respond to temperature, researchers developed multifunctional nanoparticle that can image tumours using ultrasound and simultaneously deliver cell-damaging energy and anticancer drugs to tumours. For instance, ThermoDox® combines doxorubicin with liposomal technology, which provides heat activated drug delivery. Celsion's CEO which developed this product say "It's a liposomal platform that is unique in that, in a very narrow range of temperatures, the liposome decomposes so it allows us to be able to target cancers by adding heat at the local site, causing the doxorubicin to be concentrated at the tumour".

For example, nanoparticles serve as sensitive and specific targeted contrast and drug delivery vehicles by binding to specific cell surface markers. Application of acoustic energy at diagnostic power levels could promote nanoparticle-associated drug delivery by stimulating

increased interaction between the nanoparticle's lipid layer and the targeted cell's plasma membrane (Crowder et al., 2005).

D. Magnetic field

Magnetic field is low-energy radiation that comes from the interaction of electric and magnetic fields. Sources include power lines, electric appliances, radio waves, microwaves, and others.

- For heat generation: HT

New treatment method is based on a defined energy transmission to biocompatible nanoparticles in a magnetic field. The resulting high heat production is determined by the particle type, the frequency of the radiated alternating magnetic field and the magnetic field intensity.

Therapy is more precisely based on injecting aminosilane-coated iron oxide superparamagnetic nanoparticles into a tumour. These nanoparticles are then subjected to a high-frequency alternating magnetic field, causing them to vibrate and produce heat which then damages or destroys the tumour cells. Depending on the temperature attained within the tumour, the method may be used either as HT therapy in support of conventional forms of treatment or by itself as thermoablation for the direct destruction of tumour cells. This approach, now in clinical trials but not yet commercially available, may be used for many different types of solid tumours, as fundamentally all tumour cells may be damaged or destroyed at a certain temperature (Magforce technology).

2.2. Narrowness of the therapeutic index: main concern of anticancer treatments

2.2.1. Limitations of anticancer treatments

Anticancer agents that target the cell cycle and the DNA such as cytotoxics or X-rays are among the most effective in clinical use and have produced significant increase in the survival of patients with cancer when used alone or in combination with drugs that have different mechanisms of actions. They are also extremely toxic and show a narrow therapeutic window. For these reasons, much effort has been put into modifying current treatments in terms of technology innovation, as well as understanding the signal-transduction pathways

that mediate these responses. There is considerable excitement in the cancer field to modify the therapeutical ratio, aiming at efficacy and safety improvements. Concerning the radiation therapy, despite of advances in radiation techniques, most commercially available radiation therapy systems still present significant limitations that restrict clinicians' ability to provide the most effective treatment possible. Many patients with cancer either do not respond, or develop resistance to them.

A. Building the therapeutical window

To get a high probability of cancer disease cure with relatively little risk to healthy tissues and optimal biodistribution (systemic and loco-regional) of the therapeutical agent constitutes the main objective when defining anticancer approaches. The success of treatments in eradicating a tumour depends markedly on the total dose given. Moreover, genetic variations and co-morbidities determine the individual differences in toxicity reactions and bioavailability of agents, which ultimately would be a crucial factor for tumour cell death and incidence of acute and chronic adverse events.

Cancer disease control

The main obstacle to effective treatment is the failure of initial cancer therapy to eradicate a sufficient number of tumour cells to prevent disease recurrence, which significantly affects long-term survival. This population of surviving cells following therapy is called minimal residual disease (MRD), and these cells can go on to find refuge in protective microenvironments. For example, the presence of bone marrow micrometastasis in around 30% of patients with breast cancer at the time of diagnosis is a strong predictor of relapse, despite aggressive treatment, and 15-20% of patients still have disseminated tumour cells in the bone marrow following treatment.

Resistance to antitumour therapy can be subdivided into two broad categories: de novo and acquired. Acquired resistance develops over time as a result of sequential genetic changes that ultimately culminate in complex therapy-resistant phenotypes. Conversely, one form of de novo drug resistance is environment-mediated drug resistance (EMDR), in which tumour cells are transiently protected from apoptosis induced by chemotherapy, radiotherapy or receptor-mediated cell death. This form of drug resistance is rapidly induced by signalling events that are initiated by factors present in the tumour microenvironment and can be

subdivided into two categories: soluble factor mediated drug resistance (SFM-DR), which is induced by cytokines, chemokines and growth factors secreted by fibroblast-like tumour stroma; and cell adhesion-mediated drug resistance (CAM-DR), which is mediated by the adhesion of tumour cell integrins to stromal fibroblasts or to components of the ECM, such as fibronectin, laminin and collagen. As a continuation of this theme, Cordes et al. have coined the analogous term CAM-RR to refer to cell adhesion mediated resistance to radiotherapy.

The selective pressure of therapy eventually leads to the development of acquired resistance in these surviving cells and the outgrowth of MRD, causing disease relapse. EMDR contributes substantially to MRD and to the development of acquired resistance by protecting tumour cells from therapy until they evolve acquired-resistance phenotypes. Data suggests that treatment strategies could more efficiently target the less complex CAM-DR phenotype at earlier stages of disease, before the development of acquired resistance (Meads et al., 2008).

Therapeutic strategies are limited by the tolerance of normal body tissues and this feature frequently determines the delivery of insufficient dose targeting the tumour. Thus, initial treatment would create the supporting conditions for acquired resistance.

Pharmacokinetics (pK)

pK study is measuring compound concentrations in all major tissues after drug administration over a period of time until the elimination phase. It is necessary to monitor the product concentration long enough to fully describe the behaviour *in vivo* (usually 3X half-life; $t_{1/2}$). The pK profile in the blood can be fitted using various programs to obtain key pK parameters that quantitatively describe how the body handles the drug. Important parameters include maximum concentration (C_{max}), $t_{1/2}$, clearance, area under curve (AUC), and mean resident time (MRT), average time that a molecule of a drug stays in the body. When a drug formulation shows prolonged blood circulation, an increased $t_{1/2}$, a reduced clearance, an increased AUC, and an increased MRT are usually observed. pK data are often used in deciding the dose and dose regimen for maintaining a desirable blood concentration for improved therapeutics with minimal side effects. The blood concentration of drugs is highly correlated with their efficacy and toxicity in most cases, especially for free drugs. However, to gain insight into how the body handles the product formulation and how the formulation may affect the efficacy and adverse effects, it is essential to obtain the tissue distribution information for the agent.

In fact, high level of accumulation of a product in the target tissue often results in an enhanced therapeutic effect, and oppositely, a large amount of product distributed to non target organs may cause unwanted toxicity. pK and tissue distribution studies that allow investigators to screen formulations for a given drug are extremely important during drug development. By optimizing the drug formulation, investigators can improve the drug delivery to the target tissue and reduce drug distribution to the non target tissues to obtain increased therapeutic activity with minimal side effects.

When a hydrophilic drug is intravenously injected into the body, without much protein binding, the drug is often quickly eliminated from the blood by renal filtration into the urine. In the case of a hydrophobic drug, the renal clearance is significantly reduced compared to that of the hydrophilic drugs due to an increased level of serum protein binding. The hydrophobic drugs are often transformed into hydrophilic metabolites in the liver and excreted into the bile or eliminated into the urine.

Figure 23 shows a) The supply of a drug to the tissue will depend on its dose and pK. b) After leaving the vasculature, flux through tissue can occur through extracellular or trans-cellular pathways, depending on solubility in water and lipids. Diffusion through water will vary with size or molecular weight (MW). c) Tissue penetration will be determined by the balance between delivery and consumption. Cellular metabolism will reduce drug penetration and build-up within the tissue, and binding and sequestration can increase tissue of a drug but limit its penetration.

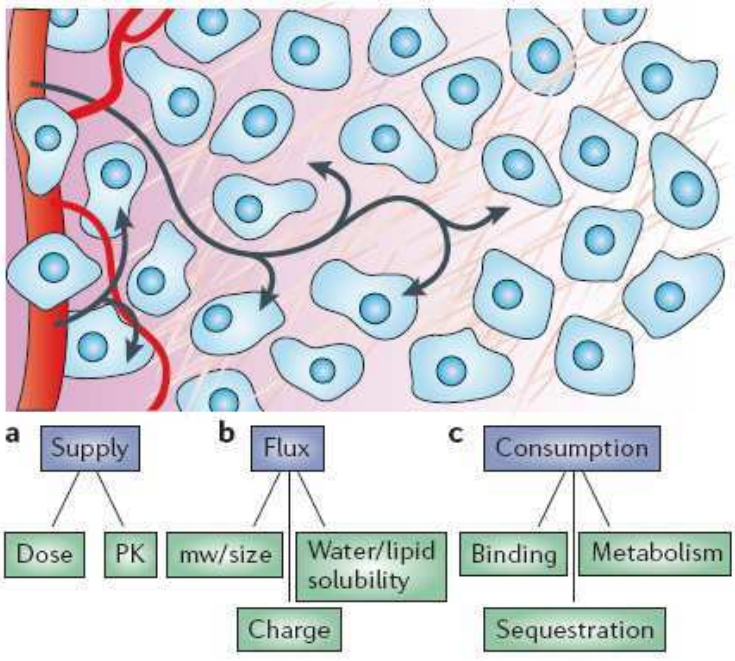


Figure 23: Drug distribution in tissue (Minchinton et al., 2006)

Toxicology

Although the acute toxicity or side effects of cancer therapy which can cause symptoms such as nausea, myelosuppression and alopecia is well defined, the late complications of treatment, including the development of dysplastic or fibrous tissue, continue to evolve because patients are now surviving longer. However, one of the most severe side effects following successful cancer therapy is the diagnosis of a second primary cancer.

Second primary cancers reflect not only the late effects of cancer therapy, but also the influence of environmental factors that were shared with the initial cancer, such as tobacco and alcohol use, diet, immune function, hormonal status and environmental exposures.
Despite this, a lack of molecular genetic markers that are specific for therapy-induced cancer makes it almost impossible to say with absolute confidence whether a second cancer in any given individual is the result of previous therapy. One possible exception to this is genomic microsatellite instability (Hamelin et al., 2008), which has been reported with high frequency, in therapy-related myeloid leukaemia (Allan et al., 2005) and is rare in sporadic myeloid leukaemia. Increased risks of developing a second cancer have been reported after treatment with radiotherapy and with structurally and functionally diverse chemotherapy agents, including alkylating agents, topoisomerase inhibitors and anti-metabolites.

Limited therapeutic window

At present, radiotherapy practice is still essentially pragmatic and based on experience of what happens to the average variant of a particular type of tumour. Even after 100 years of clinical radiotherapy, it is still not clear what the optimal fractionating regime should be. The incidence of severe reactions is dependent on the total radiation dose, the dose per fraction, the overall treatment time, beam type and energy and the surface area of the skin that is exposed to radiation. Over time, the exposure of healthy tissue to radiation energy can result in accumulated damage to healthy tissue in the patient’s body and limit the patient’s future radiation therapy possibilities. It is well recognized that the addition of chemotherapy to radiotherapy (chemoradiotherapy) increases the acute side-effect profile of the treatment, particularly when combined with altered fractionation regimens.

Figure 24 shows the limited therapeutic window with respect to cumulative dose, through which radiotherapy operates: the more radiosensitive the tumour, the wider the

therapeutic window; the more radiosensitive the normal tissue; the greater the risk of permanent damage. Damage to organs such as the spinal cord is of profound importance owing to the lack of repair of normal tissue and potentially severe sequelae. Limited tolerance of the small bowel reduces the amount of radiation that can be given to the pancreas and kidney. In contrast, the cervical mucosa can tolerate very high doses of radiation, allowing for high doses of radiation to be given locally to enable cure. Other late effects of radiotherapy include carcinogenicity, mutagenicity and teratogenicity. All can be ascribed to the effect of ionizing radiation on nuclear DNA, with consequent permanent damage to nuclear material.

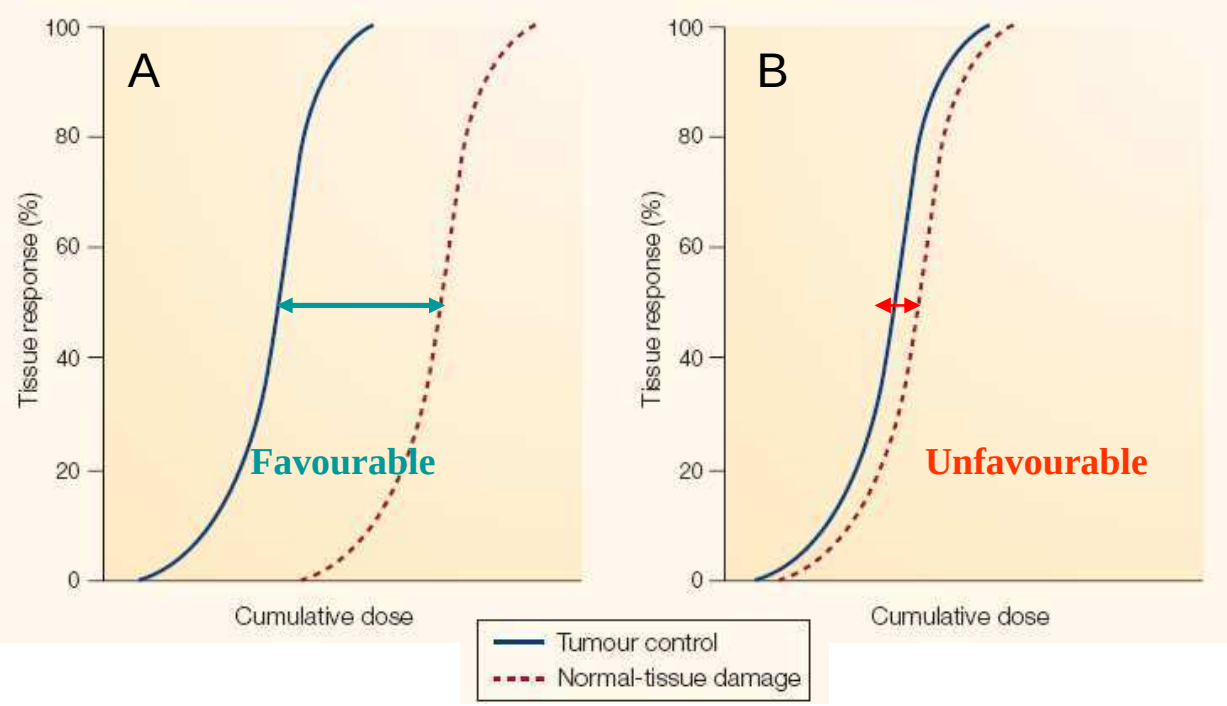


Figure 24: Therapeutic window: increase the therapeutic index (Bernier et al., 2004)

B. How to reconciliate the tolerated dose and the curative dose

NanoXray™ platform is based on a technology that is designed to allow destruction of cancer cells by inert particles. It thus offers a new treatment weapon that could be used alone, or in concert with existing anticancer protocols: chemotherapy, surgery, targeted molecules, and immunotherapy. NanoXray™ particles are intended to be activated from outside the body with a conventional X-ray, after injection. And, efficacy is expected to be proportional to the duration of activation and the number of radiotherapy sessions.

Most solid tumours possess unique pathophysiological characteristics that are not observed in normal tissues or organs, such as extensive angiogenesis and hence multiple neovessels with defective vascular architecture, impaired lymphatic drainage/recovery system, and greatly increased production of a number of permeability mediators. The phenomenon known as the enhanced permeability and retention (EPR) effect for lipid and macromolecular agents has been observed to be universal in solid tumours. Enhanced vascular permeability will enable accumulation of nanoXray^{TM} nanoparticles in the solid tumour tissue.

After nanoXray^{TM} nanoparticles accumulate in the target tissues, a standard X-ray is applied that is intended to generate a local therapeutic effect, designed to destroy only the targeted tumour cells. This mechanism suggests total control of the intended therapeutic effect. In short, usage of nanoXray^{TM} products is intended to resolve radiation therapy's biggest drawback: destruction of healthy tissue and its subsequent deleterious side effects whit high dose X-ray.

NanoPDT platform is designed initially considering traditional routes of medication administration such as intravenous (i.v), oral, or intramuscular. These routes use traditional medication administration systems: needles, syringes, fluted paper cups, i.v bags, and catheters. It must be noted that these medication administration systems may not always optimize rapid delivery of the appropriate concentration of compound to the appropriate site, nor do they necessarily minimize local or systemic toxicity. Thus nanoPDT platform allows a medication delivery system that concentrates photosensitizer where needed and could reduce the destruction of surrounding tissues while minimizing side effects.
The method by which the photosensitizer agent is delivered can have a significant effect on its efficacy. Some drugs have an optimum concentration range within which maximum benefit is derived, and concentrations above or below this range can be toxic or produce no therapeutic benefit at all.

To deliver drugs to specific organs, a range of organic systems (e.g., micelles, liposomes, and polymeric nanoparticles) have been designed. They suffer from limitations, including poor thermal and chemical stability, and rapid elimination by the immune system. In contrast, silica particles offer a biocompatible, stable, and "stealthy" alternative. Pp IX molecules can be easily encapsulated within silica particles which are administered as i.v

injection. Enhanced vascular permeability will enable accumulation of Pp IX silica nanoparticles in the tumour structure. Of the most interest, other challenging issues of PDT can be addressed including surface segregation, and tumour cell uptake with its subcellular organelle localization, which ultimately determines tumour destruction.

PART 2: ACTIVATED NANOPARTICLES TO TREAT CANCER, A WAY TO ENLARGE THE THERAPEUTIC WINDOW OF ANTICANCER APPROACHES

INTRODUCTION

1. Nanobiotix commitment: an innovation-based nanomaterial company for cancer treatment

> Use of nanomaterial as the “active product” to bring meaningful clinical benefit to cancer patients

Nanobiotix is an emerging nanomedicine company combining dramatic advances in nanotechnology and molecular biology to develop nanomaterials that are expected to be turned “on” and “off” *outside the body* to *selectively and safely treat* a variety of cancers. Nanobiotix wants to play the seminal role in making the cancer treatments more effective, less deadly to healthy tissues.

As a general trend, clinical applications exploit nanotechnology to improve the quality and sensitivity of a wide variety of different technologies in order to create new approaches for drug delivery and imaging, with novel targeted agents too. So far, most applications of nanotechnology in medicine have an **enabling function** in many different areas.
Unlike, Nanobiotix has explored other fields, enabling the use of nanomaterials as the “active product” with therapeutic purposes. The company has successfully integrated two worlds, the promising nanotechnology industry and medicine.

To create and develop nanomaterial as being the “active product” for cancer therapy, Nanobiotix has built on a technology founded on two major axes: the key understanding of the biological mechanisms and the ability to elaborate complex structures at the nanometer scale.

Based on the existing technology and preclinical results, Nanobiotix is developing different nanomedicine programs that will sustain its pipeline and will expand the uses of nanoparticles in medicine.

- **nanoXray^{TM} platform** which is based on crystalline nanoparticles to be activated by X-ray
- **nanoPDT platform** which is based on photosensitizer nanocarriers particles for treatment of cancer
- **nanoMag platform** which is based on magnetic particles for treatment and diagnostic of cancer

- **activated drug delivery platform** which uses nanomaterials based on different modalities already in use in hospitals such as drug-delivery systems (DDS) that can be externally triggered

NanoXray™ platform lead product NBTXR3 nanoparticle is a non-drug agent able on its own to kill tumour cells. NBTXR3 nanoparticles can be injected into cancer patients at the tumour site and taken up by cancer cells. Then patients would undergo a standard X-ray procedure that would "switch on" the destructive capability of the NBTXR3 nanoparticles, causing the cancer cells death. NanoXray™, in short, allows for the controlled generation of physical reactions in targeted cells triggered by the application of an external energy source – a standard X-ray.

NBTXR3 clearly is intended to fight the most deadly cancers (Figure 25).

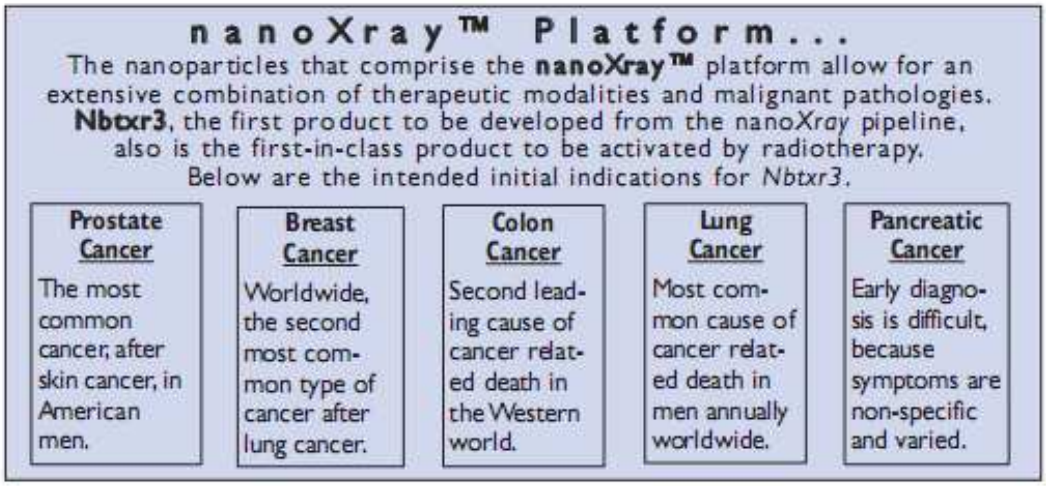


Figure 25 : NBTXR3 intended initial indications

NanoPDT platform offers the possibility of impacting the pK of photosensitizers such as Pp IX and others, but as important as this improvement, nanocarriers introduce the possibility of controlling sub-cellular availability. Efficient PDT may increase the choice of anticancer modalities, opening the possibility of a "new line of local treatment", so far highly limited by the marked changes induced by the radiotherapy in the irradiated tissues. To improve the tumour bioavailability, to reduce photosensitizer accumulation in the skin or to render it "cryptic", to differentially deliver the nanocarriers to cell organelles constitute the major effects of nanoPDT products.

The design and mode of action of NBTXR3 – but also of other nanomaterials currently in Nanobiotix pipeline – is based on a physical effect and not on a biological effect. This design undoubtedly offers a rupture toward current therapies which focus their efforts on developing targeted therapeutics agents such as mAb, issued from the characterisation of molecular features of cancer cells. This is an exciting field, source of new candidates for anticancer treatment which needs a profound knowledge of any cancer type, tissue origin, model, stage and genetic background.

Nanobiotix technology is thought very differently as its nanomaterials present a high level of uncoupling between the therapeutic core of the nanoparticles, which is responsible for the therapeutic effect, and the coating which confers to the material its specificity for action. Such approach allows for a much broader range of application in oncology regardless the disturbed pathways causing uncontrolled growth, proliferation and lack of determined differentiation and programmed death.

Indeed, the development of nanomedicine products which act by physical mechanism of action is a new way of thinking medicine. The rational for design comes from the physic and chemistry, allowing a global approach of cancer therapy. It is not intended to treat diseases through a specific signaling pathway which act on a single disease target. In contrast it is intended to deliver the therapeutic effect through a non specific pathway which works against cancers of a given type or even cancers of many types, a physically-induced pathway level. As opposed to the development of new drugs for therapy, this very unique concept, translated to the clinic, is a new weapon in the fight against cancer.

Today, numerous cancers are difficult to treat due to heavy secondary effects arising when trying to efficiently treat the diseases. Two axes are achievable to solve this issue: either to reduce the toxicity of the treatment, or to increase the tumour control or its destruction.
Nanobiotix aims at playing on both axes and to reach an unprecedented benefit over risk ratio combining major changes in efficacy and allowing possibility of anticancer treatment to patient populations currently excluded from therapies due to their co-morbidities.
Its intention is clearly to break the classical correlation which exists between therapeutic efficacy and corresponding toxicity. Moreover, the principle of occupancy intervention at the sub-cellular level to challenge the narrowness of the therapeutic index is a new paradigm.

2. Nanomaterial and energy interaction: concept and rational for design of nanomaterial

When thinking about devices, currently used in the clinic for diagnosis and therapy, which produce external energy sources, one can easily list the following: radiation emitting devices, MRI, laser and ultrasound. Those external energy sources interact with matter when passing through, generating a significant change in the surrounding media which triggers the visualization and/or the treatment of the area of interest.

A step further would be to ask: how does it work? To answer the question, one should consider the interaction between the energy delivered and the biological media it traverses.

Ionizing down to infrared radiation: the electromagnetic spectrum (Figure 26**)**

Ionizing radiations are capable of breaking the DNA molecule of the cell, thereby preventing cells from growing and dividing. This effect is mainly due to damages created by electrons and/or high energy photons (energy higher than 2 KeV) emitted after ionization.
The term “Ionizing radiations” refers to highly-energetic particles or waves that can detach (ionize) at least one electron from an atom or molecule. Ionizing ability depends on the energy of individual particles or waves, and not on their number. A large flood of particles or waves will not, in the most-common situations, cause ionization if the individual particles or waves are insufficiently energetic. The ability of light waves (photons) to ionize an atom or molecule varies across the electromagnetic spectrum. X-rays and gamma-rays will ionize almost any molecule or atom; far ultraviolet (UV) light will ionize many atoms and molecules; near UV and visible light are ionizing very few molecules; infra-red (IR), microwaves and radio waves are non-ionizing radiations. However, IR is able to excite molecules or atoms.

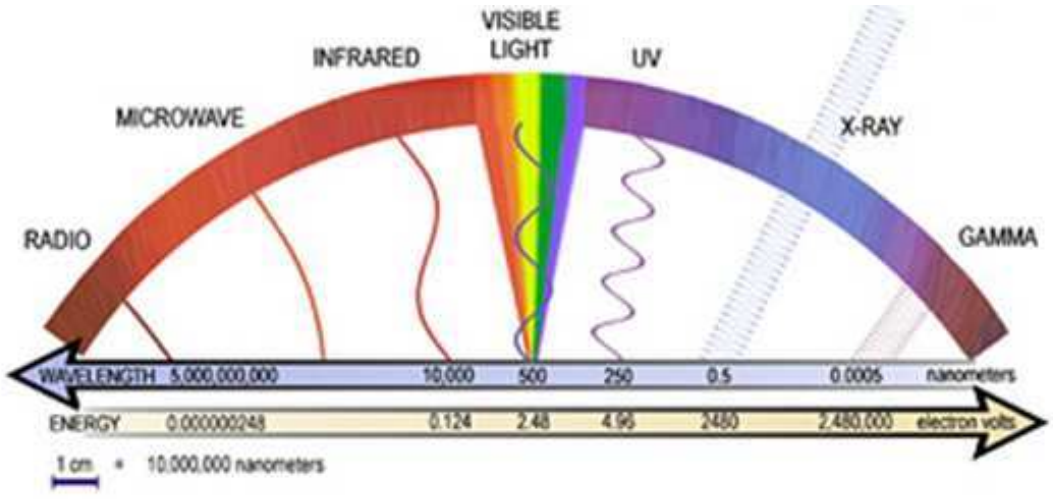


Figure 26: The electromagnetic spectrum

Magnetic field

Magnetic field, generated by MRI devices, interacts with the nucleus of hydrogen atom. Hydrogen is the most abundant element in biological systems and proton in the hydrogen nucleus acts as a tiny magnet with a non zero magnet moment. When placed in a large magnetic field, the randomly oriented magnetic vectors of each proton tend to line up either with (parallel) or against (antiparallel) the direction of the magnetic field. With time, more protons in any given tissue will align parallel to the magnetic field and a single vector of magnetization can be described for the sum or ensemble of protons. Each distinct tissue type has a characteristic rate for its protons to achieve such a magnetization. This magnetization reflects the ability of each tissue's protons to exchange energy with the molecular environment. By adding energy in the form of a radio frequency (RF) pulse, energy which corresponds to the energy difference between the parallel and antiparallel state, the overall magnetic vector is abolished as the protons suddenly begin to switch between the low energy (parallel) and high energy (antiparallel) state in unison. As they all drop together from the high energy state to the low one, the combined energy they give up is released in the form of an RF signal, which can be detected for imaging (Brant-Zawadzki et al., 1985).

Ultrasound

Sound is a physical phenomenon which transfers energy from one point to another. In this respect, it is similar to radiation. It differs from radiation, however, in that sound can pass only through matter and not through a vacuum as radiation can. This is because sound waves are actually vibrations passing through a material. One of the most significant characteristics of sound is its frequency, which is the rate at which the sound source and the material vibrate. The human ear cannot hear or respond to all sound frequencies. The range of frequencies that can be heard by a normal young adult is from approximately 20 Hz to 20.000 Hz (20 kHz). **Ultrasound** has a frequency above this range. Frequencies in the range of 2 MHz (million cycles per second) to 20 MHz are used in diagnostic ultrasound (Figure 27). When a beam of ultrasound pulses is passed into a body, several things happen. Most of the ultrasound energy is absorbed and the beam is attenuated. This is undesirable and does not contribute to the formation of an image like in X-ray imaging. Some of the pulses will be reflected by internal body structures and send **echoes back** to the surface where they are collected and used to form the image. Echoes are produced by surfaces or boundaries between two different types of tissues. Therefore, the general ultrasound image is a display of structures or reflecting surfaces in the body that produce echoes.

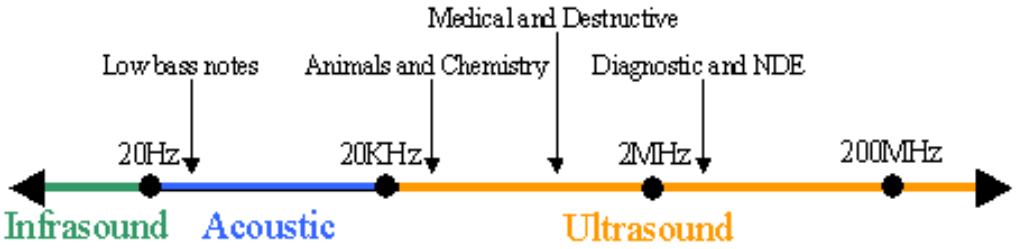


Figure 27: The ultrasound spectrum

Could it be then possible, using nanomaterials in combination with those external energy sources, to improve either the diagnostic or the treatment of a disease, by increasing either the sensitivity and/or the efficacy of the current effect?

Coming back to nanotechnology, an approach is to consider which type of nanomaterial could be used to interact with the delivered energy source to potentially improve the way its energy is exploited. The nanomaterial should first achieve specific characteristics which may allow its interaction with the energy source. Subsequently the nanomaterial-

energy interaction should trigger a biological response in the desired way. Some nanoparticles with market approval or in clinical phase already present such properties. Contrast agents developed for MRI, are typically superparamagnetic nanoparticles which enhance locally MRI signal, hence the diagnostic, providing an efficient biodistribution of the nanoparticles [Resovist®, Schering DE; Feridex®, Advanced Magnetic Industries, Inc. US]. Laser light sources, used to activate metallic nanoparticles such as gold nanoparticles, cause absorption of energy by the nanoparticles and a subsequent generation of heat which locally induce cells and tissues destruction (Maltzahn et al., 2009; O'Neal et al., 2004).

Hence nanotechnology is able to design nanomaterials that are interacting with external energy sources to tackle unmet needs in the clinic. A key step in the development of those nanomaterials will be their safe administration into the human body at the right place, at the right concentration and at the right time.

3. Nanomaterials and biological media interactions: a necessary understanding to bring the concept of nanomaterial as “active product” to the clinic

3.1. The “nano-bio” interface: importance of the protein-corona concept

Knowledge of the nanomaterial-biological interactions is the foundation to understand how engineered nanomaterials and biological entities will communicate and ultimately how nanomaterials and biology, will respond to each other.

At the frontier between two scientific worlds – the molecular and cellular biology and the physic and chemistry – scientists stand at the very beginning of an appreciation of nanomaterials-biological entities interactions. The outcome will support the design of optimized nanosystems which need to reach an exquisite level of interaction with their biological environment for efficient and safe use of nanomaterials in biology.

When nanoparticles enter a biological fluid, they are coated with proteins that may undergo conformational changes, leading to exposure of new epitopes, altered function and/or avidity effects. Further, the interactive nanoparticle surface might be pre-bound to chemical substances that reflect its prior history and could influence its protein adsorption kinetics. While within the biomaterials field the role of adsorbed molecules in cellular responses is acknowledged, there are several new issues at stake where nanoparticles are concerned. The highly selective protein adsorption, added to the fact that particles can reach subcellular locations, results in significant new potential impacts for nanoparticles on protein interactions and cellular behavior.

It is a universal rule of materials in biology that a material is always covered by proteins immediately upon contact with a physiological environment, and it is believed that this phenomenon will also be key to understanding much of the bionanoscience world. Lynch et al, 2007 have recently argued that the effective unit of interest in the cell-nanomaterial interaction is not the nanoparticle per se, but the particle and its ‘corona’ of more or less strongly associated proteins from serum or other body fluids (Lynch et al., 2007 ; Cerdervall et al., 2007). It is important to understand, though, that it is not just the composition and organization of this protein layer, but the exchange times of the proteins on the nanoparticles that are ‘read’ by living cells. In essence, Lynch et al., 2007 expect a huge range of

equilibrium constants (one for each protein) representing the quite different (and competitive) binding mechanisms present. This means that they see the proteins associated with a particle as a 'corona', rather than a solid fixed layer (Figure 28).

The composition of the protein corona at any given time will be determined by the concentrations of the over 3700 proteins in plasma (Muthusamy et al., 2005), and the kinetic on and off rates (or equilibrium binding constants) of each protein for the particular nanoparticle. This corona may not immediately reach equilibrium when exposed to a biological fluid. Proteins with high concentrations and high association rate constants will initially occupy the nanoparticle surface, but may also dissociate quickly to be replaced by proteins of lower concentration, slower exchange, and higher affinity (Cedervall et al., 2007). **Thus the protein corona is the biological identity of a nanoparticle**, as it is what the cell 'sees' and interacts with. The exchange processes may also be important when particles redistribute from one compartment or organ to another, such as upon uptake into cells from the bloodstream, or upon transport from the cytosol to the nucleus.

Because the protein corona could shape the cellular interactions with nanomaterials, a key question is whether such protein interactions depend on particle composition. The nature of the particle surface (for example its hydrophobicity, size, radius of curvature, charge, coatings that exert steric or electrostatic effects) will control which biomolecules interact with the particles – and hence mediate their access to cells. Several proteins are known to form transient complexes with nanoparticles. Complement and immunoglobulin binding leads to particles opsonisation; that is, it promotes receptor-mediated phagocytosis. It is generally perceived that plasma protein binding is important in determining the *in vivo* organ distribution and clearance of carrier particles from the circulation. Proteins and organic substances increase the dissolution rate of particles of ZnO, CdSe, iron oxides, aluminium oxides and hydroxides. Studying the reverse effect of particles on protein is important for understanding potential biological injury due to such changes as protein fibrillation, exposure of new antigenic epitopes and loss of function such as enzymatic activity.

Probing these various interfaces allows the development of predictive relationship between structure and activity. This knowledge is important from the perspective **of a safe and efficient use of nanomaterials** (Nel et al., 2009).

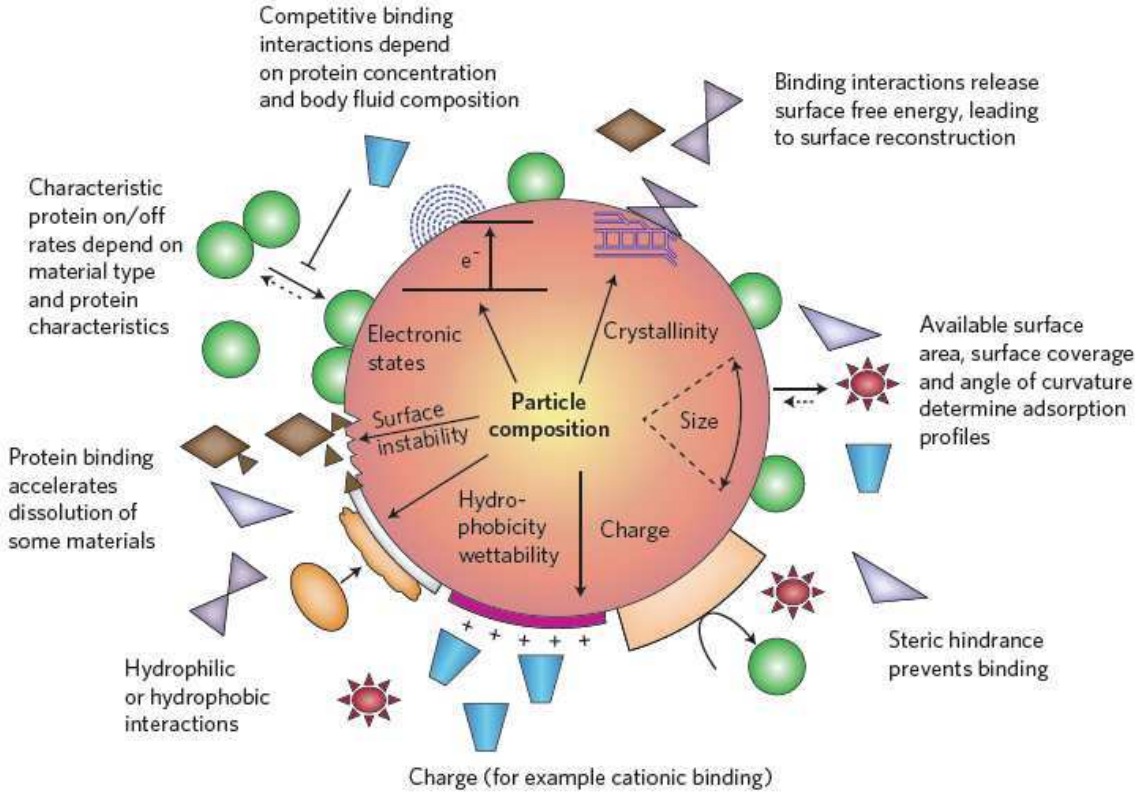


Figure 28: The corona constitutes a primary nano-bio interface that determines the fate of the nanoparticle and can cause deleterious effects on the interactive proteins. Pre-existing or initial material characteristics contribute to the formation of the corona in a biological environment. Characteristic protein attachment/detachment rates, competitive binding interactions, steric hindrance by detergents and adsorbed polymers, and the protein profile of the body fluid lead to dynamic changes in the corona. The corona can change when particles move from one biological compartment to another (Nel et al., 2009).

3.2. The nanomaterial delivery at the right time, at the right place and at the right concentration

Due to the growing importance of nano-biotechnology and its enormous potential to improve drug delivery, the main focus in next years should be on nanomedicine and in particular on nanomaterial delivery. Nano-scale drug carriers play an important role in the safe and efficient delivery of active compounds to their intended site of action, but there are still challenges that need to be solved, like the targeting of specific cell tissues or organs and to overcome biological barriers such as the skin, intestinal, respiratory mucosa or blood-brain-barrier, but also the cells and at the subcellular level, the organelles.

Safe nanoparticles administration, with minimum side effects, and effective bioavailability are the main concerns of current medical cares. Bioavailability refers to the

presence of nanoparticles at the right concentration and the right anatomical area. The ability to alter the pK and pharmacodynamic of a product in the body determines its efficacy and safety. Ultimately, the aim is to deliver nanoparticles at the right time, the right place and right concentration.

3.2.1. Major obstacles to reach the target: tissues, cells and organelles

A. Tissues

In general, biological compartments act as barriers to the passage of nanosized materials, and there are several barriers to consider. Firstly, the epithelium acts as a general barrier to prevent ingress of materials into the body. The next barrier to nanoparticle distribution is the vascular endothelium. Typically most endothelium is continuous with tight junctions between the endothelial cells and the underlying basement membrane. The presence of tight junctions means that the gap between endothelial cells is 2 nm, thus preventing most nanoparticles from exiting the circulation by passage between the cells. In some specialized tissue like liver, the endothelium is fenestrated, thus allowing materials up to 100 nm to pass from the endothelium to the underlying parenchymal cells. In other tissues like spleen, the endothelium is discontinuous, having larger fenestrations, and also lacks basement membrane, allowing exit of very large particles. In contrast, in the brain the endothelial junctions are particularly tight and effective, reducing still further the ability of materials to pass through. This allows the passage of macromolecules through the endothelium, and is thought to be size dependent, so larger macromolecules pass through less easily than smaller molecules. Also, under certain disease conditions, e.g. inflammation, the endothelium can become leaky, allowing a greater exit of particulate materials (Shrivastava et al., 2008).

In fact, hepatic filtration, tissue extravasation, tissue diffusion, kidney filtration play a crucial role on particles diffusion. Endothelia composing the blood vessels have been classified as continuous, fenestrated or discontinuous depending on their morphological features. The continuous endothelium appears in arteries, vessels and the lungs. Fenestrated endothelium appears in glands, digestive mucosa and kidney. Fenestrations have pores of approximately 60 nm. Discontinuous endothelium is a characteristic of the liver (50-100 nm) and bone marrow (Alexis et al., 2008).

B. Cells

Every cell is locked into a membrane, an envelope defender of 8-12 nm, which bounds the cellular compartment and separates it from the surrounding environment. The membrane plays at the same time the role of a filter and a way of transport. On one hand, it controls the entry of the nutritive substances and the exit of the cellular wastes and, on the other hand, it creates an internal environment different from the external environment. It has another important function which is to create and maintain intracellular concentration of specific ions, which are atoms or group of atoms carrying an electric load. The cellular membrane acts also as sensor of external signals, giving to the cells the possibility to answer to the various stimuli they received.

According to the fluid mosaic model of Singer and Nicolson, the biological membranes can be considered as a two-dimensional liquid where all lipids and protein molecules diffuse more or less freely (Singer et al., 1972). This picture may be valid in the space scale of 10 nm. However, the plasma membrane contains different structures or domains that can be classified as (a) protein-protein complexes; (b) lipid rafts, (c) pickets and fences formed by the actin-based cytoskeleton; and (d) large stable structures, such as synapses or desmosomes.

The permeability of membranes is the ease of molecules to pass through. Permeability depends mainly on the electric charge of the molecule and to a lesser extent the molar mass of the molecule. Electrically-neutral and small molecules pass the membrane easier than charged and large ones.

The inability of charged molecules to pass through the cell membrane results in pH parturition of substances throughout the fluid compartment of the body. pH parturition is the tendency for acid molecules to accumulate in basic fluid compartments, and basic molecules to accumulate in acidic compartments. The reason is that acid molecules become positively charged in basic fluids, since they donate a proton. On the other hand, basic molecules become negatively charged in acid fluids, since they receive a proton. Since electric charge decreases the membrane permeability of substances, once an acid molecule enters a basic fluid and becomes electrically charged, it cannot easily escape that compartment and therefore accumulates. Same effect is observed with basic molecules.

C. Organelles

The physicochemical properties of drugs (for example, MW, shape, charge and aqueous solubility) determine their localization into the cell.

1- The **mitochondrion** has been shown to be a critical target in PDT. Lipophilic porphyrins have demonstrated intimate intracellular association with mitochondrial membranes, whilst cationic compounds such as rhodamines and cyanines may accumulate in these organelles due to mitochondrial membrane potential. The inter-membrane space of mitochondria contains a number of soluble molecules whose release from the organelle to the cytosol or the nucleus induces cell death. Thus, molecules that directly trigger mitochondria membrane permeabilisation are efficient cytotoxic drugs. Zhao and his colleagues (2009) have synthesized 25-30 nm silicon phthalocyanine 4 (Pc4), a photosensitizer for PDT encapsulated in silica nanoparticles. Cell viability measurement demonstrated that Pc4 nanoparticles was more phototoxic on non-pigmented human melanoma A375 or pigmented mouse melanoma B16F10 melanoma cells than free Pc4. These nanoparticles were localized both in the mitochondria and lysosomes compartments.

2- The **nucleus** is a membrane-bound organelle and is surrounded by a double membrane. It communicates with the surrounding cytosol via numerous nuclear pores.

The nuclear envelope (NE) is a double membrane, each constituted by lipid bilayer, which encloses the genetic material in eukaryotic cells. The NE also serves as the physical barrier, separating the contents of the nucleus (DNA in particular) from the cytosol (cytoplasm). The outer membrane is continuous with the rough endoplasmic reticulum (ER) while the inner nuclear membrane is the primary residence of several inner nuclear membrane proteins. The outer and inner nuclear membranes are fused at the site of nuclear pore complex insertion. Many nuclear pores are inserted in the nuclear envelope, which facilitate and regulate the exchange of materials (proteins such as transcription factors, and RNA) between the nucleus and the cytoplasm. At present, it is admitted that nanoparticles can not cross the nuclear membrane, but the presence of certain type of nanoparticles (less than 4 nm) has been described into the nucleus (Tsoli et al., 2005; Gu et al., 2009).

Futhermore, others groups have reported the Tat peptide-mediated import of different cargos into cells nucleus, including dye-labeled streptavidin protein, 43 and 90 nm fluorescent beads, as well as ~20 nm quantum dot (QD) for kinetic measurements. Surprisingly, Tat

peptide was able to import 90 nm beads into the nuclei of digitonin-permeabilized cells, suggesting that their interaction with the NE follows a mechanism different from that of NLS (Nuclear Localization Signal). The import kinetics was quantified using Tat peptide-conjugated QD, yielding a kinetic constant of 0.0085 s^{-1}. The results suggest that, compared with NLS, Tat peptide-mediated nuclear import is faster, follows a different pathway, and is capable of importing large nanoparticles. These results have significant implications for the development of new approaches for delivery of cargo into the nuclei of living cells (De Lafuente et al., 2005; Nitin et al., 2009)

3- The **Golgi** apparatus is a membrane-bound structure with a single membrane. It is actually a stack of membrane-bound vesicles that are important in packaging macromolecules for transport elsewhere in the cell. The stack of larger vesicles is surrounded by numerous smaller vesicles containing those packaged macromolecules. The enzymatic or hormonal contents of lysosomes, peroxisomes and secretory vesicles are packaged in membrane-bound vesicles at the periphery of the Golgi apparatus.

4- The Golgi complex sorts the lysosomal enzyme in the Trans region. **Lysosomal morphology** varies with the state of the cell and its degree of degradative activity. **Lysosomes** carry hydrolases that degrade nucleotides, proteins, lipids, phospholipids, and also remove carbohydrate, sulfate, or phosphate groups from molecules. The hydrolases are active at an acid pH which is fortunate because if they leak out of the lysosome, they are not likely to produce damage (at pH 7.2) unless the cell has become acidic. They degrade the products of ingestion, such as the bacteria that has been taken in by phagocytosis. Lysosomes also degrade worn out organelles such as mitochondria. A third function for lysosomes is to handle the products of receptor-mediated endocytosis such as the receptor, ligand and associated membrane. In this case, the early coalescence of vesicles, bringing in the receptor and ligand, produces an endosome. Then, the introduction of lysosomal enzymes and the lower pH cause release and degradation of the contents. This can be used for recycling of the receptor and other membrane components.

5- There are 2 regions of the ER that differ in both structure and function. One region is called **rough ER** because it has ribosomes attached to the cytoplasmic side of the membrane. The other region is called **smooth ER** because it lacks attached ribosomes. Typically, the smooth ER is a **tubule network** and the rough ER is a series of flattened sacs.

The smooth ER has a wide range of functions including carbohydrate and lipid synthesis. It serves as a transitional area for vesicles that transport ER products to various destinations. The space inside of the ER is called the lumen. The ER is very extensive and is continuous with the nuclear envelope. Since the ER is connected with the nuclear envelope, the lumen of the ER and the space inside the NE are part of the same compartment. Chang and his colleagues (2008) showed that 13 nm gold nanoparticles were co-localized with ER and Golgi apparatus in B16F10 melanoma cells following 18 hours of incubation with cells.

6- The **cytoskeleton** is a dynamic 3D filamentous structure within the cytoplasm. Cellular cytoplasm is dominated by the viscoelastic network of the cytoskeletal lattice**,** comprising microfilaments (actin filaments and contractile actomyosin filaments), microtubules and intermediate filaments. The cytoskeletal lattice is directly responsible for determining cell shape, generating mechanical forces, resisting externally imposed forces, and transducing extracellular biochemical and mechanical stimuli to the cytoplasm. Cytoskeletal dynamics enable the remodeling that is necessary for cell migration and chemotaxis which underlies tissue development, the inflammatory response, and tumour invasion and metastasis. Microfilaments are 3-6 nm in diameter, and are composed mostly of the contractile protein actin, the most abundant cellular protein. Microfilaments are responsible for the cellular movements of gliding, contraction, and cytokinesis (division of the cytoplasm). For instance, Zhang et al., 2009 tested quantum dots (QD) nanoparticles (CdSe/ZnS core/shell structure). They showed that QD nanoparticles with a carboxylic acid surface coating were recognized by lipid rafts but not by clathrin or caveolae in human epidermal keratinocytes (HEKs). QD nanoparticles were internalized into early endosomes and then transferred to late endosomes or lysosomes. QD nanoparticles induced more actin filaments formation in the cytoplasm.

3.2.2. Nanomaterial design for optimized biodistribution and intracellular trafficking: the importance of the nanomaterial size, shape and surface coating

Numerous studies demonstrated the influence of nanoparticles size, shape and surface coating on their biodistribution. Moreover the interactions of nanoparticles with cells are known to be strongly influenced by these parameters which modulated cellular internalization and intracellular trafficking.

A. Nanomaterial size

Particles size plays always a role in how the body responds to, distributes and eliminates materials. By analysis of pegylated nanoparticles uptake by murine macrophages and blood clearance kinetics, blood clearance of the smaller nanoparticles was twice as slow as that of larger nanoparticles (Alexis et al., 2008). Also, biological interactions with nanoparticles could decrease or cancel particles efficacy.

Particle size can also affect the mode of endocytic pathway, cellular uptake and the efficiency of particle processing in the endocytic pathway (Lanone et al., 2006). *In vitro* studies on murine melanoma cell lines B16F10 have demonstrated, with latex nanospheres, a slower uptake for particles size superior to 200 nm relative to the small ones (50 and 100 nm) (Rejman et al., 2004). Decreasing the size also leads to an exponential increase of surface area relative to volume, thus making nanomaterials surface more reactive itself and to surrounding environment. The size of the nanoparticles was shown to have a substantial effect on the proteins adsorption.

Increase nanoparticles uptake into certain tissues may lead accumulation, where they may interfere with the critical functions (Lanone et al., 2006; Kreyling et al., 2006). As partitioning across membranes is not possible for macromolecules, entry into cells is largely governed by biological mechanisms of endocytosis (Shrivastava et al., 2008). These include the uptake of large particles by phagocytosis, performed by specialized cells such as macrophages and neutrophils, and a variety of other endocytic processes at a smaller scale. The best known of these, clathrin-mediated endocytosis, involves a vesicle of a defined size of 100 nm, and can promote uptake through a number of different routes through the cell. A second mechanism, more recently described, is potocytosis involving caveolae, flask-shaped vesicles of 70 nm diameters (Shrivastava et al., 2008). These routes of uptake, which include macropinocytosis, can potentially allow capture of materials up to 300 nm in diameter. All these endocytic routes of uptake involve delivery of material into a subcellular compartment, the endosome, which is still separated from the cell cytoplasm by a membrane.

More recently, uptake of small hydrophobic particles directly into the cytoplasm of cells has been reported in the literature**,** and called patocytosis (Garnett et al., 2006). Patocytosis is a unique macrophage endocytosis pathway in which aggregated low density lipoproteins (LDL) and microcrystalline cholesterol induce and enter a labyrinth of membrane-bound compartments that remain connected to the cell surface. This process has been characterized as size dependent, not occurring for particles larger than 500 nm and

associated with the process of capping, in which the particles become trapped in membrane folds which remain open to the intracellular space.

B. Nanomaterial shape

Particle **shape** varies from perfect spheres, such as droplets, to fibers and more recently nanotubes. Particle shape is known to affect the aerodynamic, as well as the diffusive behaviour of the particles. Particles shape also affects phagocytosis by macrophages. Alveolar macrophages with diameters in the size range of 10-15 µm are not able to completely engulf and clear fiber nanoparticles from the alveolar epithelium.

Regarding **inorganic** metallic or oxide nanoparticles, spherical shape are commonly admitted as having the best conformation for cell uptake. In particular, Chithrani et al., 2006, have shown that spherical gold nanoparticles present the highest cell uptake when compared with rod-shaped gold nanoparticles.

Unlike, Gratton et al., 2007 reported on the internalization of specially designed, monodisperse hydrogel particles into HeLa cells as a function of size, shape, and surface charge. They employed a top-down particle fabrication technique called PRINT that is able to generate uniform population of **organic** micro- and nanoparticles with complete control of size, shape, and surface chemistry. Their findings suggested that HeLa cells readily internalize non spherical particles with dimensions as large as 3 µm by using several different mechanisms of endocytosis. Moreover, it was found that rod-like particles enjoy an appreciable advantage when they come to internalization rates, reminiscent of the advantage that many rod-like bacteria have for internalization in nonphagocytic cells.

C. Nanomaterials surface coating: rational to bring a charged, a steric, or a specific targeted coating

Many nanomaterials are functionalized on their surface to increase blood circulation, make them more biocompatible, and for targeted therapy. While surface functionalization has shown promising applications, functional groups added to the surface can potentially interact with biological components, alter biological functions, and allow passage of nanomaterials that would not normally be taken up by certain cells.

- **Charged surface treating agent: the interaction with cells membrane**

The effect of the surface chemistry of biomaterials on the protein adsorption process has been a topic of great interest for many years, and much is known in this field. Protein

adsorption to various materials has been widely studied and it has been found that factors such as electrostatic interactions, hydrophobic interactions, and specific chemical interactions between the protein and the adsorbent play important roles.

Selective adsorption of proteins on various synthetic adsorbents has been examined under different conditions (such as solution pH and protein concentration) and for many proteins the mechanism of selective adsorption has been attributed to electrostatic interactions.

Figure 29 provides a general recent overview of the major proteins, identified in the literature, bound to an assortment of nanoparticles of different size, chemical make-up, and surface properties which compose the nanoparticle protein-corona.

Though some proteins are specific to certain types of nanoparticles, seemingly dependent on the nanoparticle structure, the proteins that were identified on virtually all nanoparticles are principally albumin, immunoglobulin G (IgG), fibrinogen, and apolipoproteins. What is unknown, however, is the affinity of the proteins binding the nanoparticles. These proteins may dominate the surface of the nanoparticle initially, but then be displaced by proteins of lower abundance, higher affinity, and slower kinetics. This may lead to the presence of other nanoparticle-bound proteins at later times, such as other immunoglobulins, apolipoproteins, and components of the complement system. These proteins, along with albumin and fibrinogen, are important in the clearance process of the body.

Nanoparticles	Identified proteins
Polystyrene with poloxamer 184, 188, 407	Factor B, transferrin, albumin, fibrinogen, IgG, apolipoproteins
Liposomes	Albumin, fibrinogen, apolipoproteins, IgG, α1-antitrypsin, α2-macroglobulin, IgM
Single-walled carbon nanotubes	Albumin
Solid lipid nanoparticles with Tween 80	Fibrinogen, IgG, IgM, apolipoproteins, transthyretin
Solid lipid nanoparticles with poloxamer 188	Fibrinogen, IgG, IgM, apolipoproteins, transthyretin, albumin
Poly(lactic acid) nanoparticles with PEG	Albumin, fibrinogen, IgG, apolipoproteins
Polyhexadecylcyanoacrylate NP	Albumin, fibrinogen, IgG, transferrin
Poly(ε-caprolacton) NP	IgG, apolipoproteins
Polycyanoacrylate NP	Albumin; IgG, IgM, fibrinogen, apolipoproteins
Iron oxide NP	Albumin, IgG, IgM, fibrinogen, C3b, apolipoprotein A-1
Various polymer/copolymer composition NP	Albumin, IgG, fibrinogen, apolipoproteins
Poly($_D$,l-lactic acid) NP	Albumin, IgG, fibrinogen, IgM, apolipoproteins, antithrombin III
Polystyrene with Rhodamine B	Albumin, IgG, fibrinogen, apolipoproteins, PLS:6
Various polymer/copolymer composition NP	Albumin, IgG, fibrinogen, IgM, apolipoproteins, PLS:6, U2
Poly(isobutylcyanoacrylate) with Dextran	Albumin, IgG, fibrinogen, apolipoproteins, serotransferrine, transthyretine
Single-and double-walled carbon nanotubes	Albumin, fibrinogen, apolipoproteins, C1q
Polybutylcyanoacrylate NP with polysorbate 80	Albumin, IgG, fibrinogen, IgM, apolipoproteins
Solid lipid NP with poloxamer or poloxamine coating	Albumin, fibrinogen, apolipoproteins

Figure 29: Summary of various nanoparticles and protein binding (Aggarwal et al., 2009)

Particle adhesion to a cell-surface and engulfment at the adhesion site require specific and non specific binding interactions (Figure 30).

Among the most effective specific binding interactions are those of surface ligands which allow the nanoparticle to interact with complementary molecules or receptors on the cell membrane. These interactions result in receptor-mediated endocytosis. Ligands do not need to be necessary of biological origin – see for instance gold nanoparticles protein-corona inducing specific nanoparticles cell uptake (Chithrani et al., 2006). In contrast, they can comprise chemical moieties, metallic sites, polymers or surface functionalities that promote binding affinity.

Non specific attractive forces that promote cellular contact and particle uptake result from intrinsic nanoparticles characteristics such as surface charge, hydrophobicity and roughness. Among them, surface charge plays an important part in particles interactions with charged phospholipid head groups or protein domains on cell surfaces. For instance, anionic magnetic nanoparticles are described to first adsorb electrostatically to the tumour cell membranes before being internalized within endosomes (Wilhelm et al., 2008). Further, Bose et al., 2004 have reported the binding between apoptotic leukemic T cells and cationic liposomes (100-115 nm). Results showed that the binding of liposomes is mediated through an electrostatic interaction between the positively charged liposome and the translocated anionic phosphatidylserine in the plasma membrane.

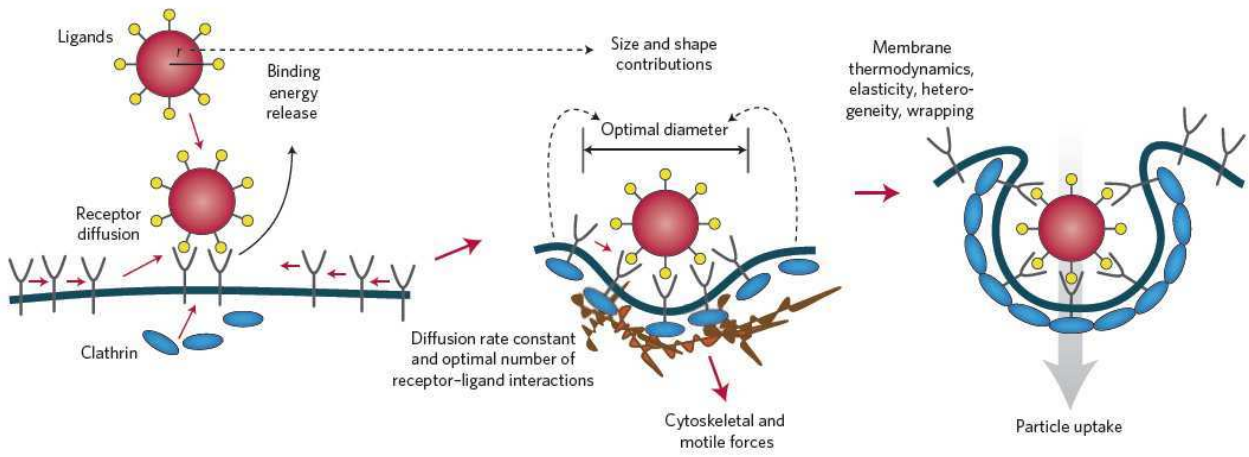


Figure 30: For particle uptake to occur, specific (ligand–receptor) and nonspecific (for example hydrophobic, coulombic). Binding interactions must decrease the free energy at the contact site to overcome resistive forces. The blue ellipses represent clathrin, an example of an endocytic component that engages in energy-dependent uptake of particles. The red spheres represent nanoparticles with attached ligands (yellow dots). These ligands bind to the Y-shaped membrane receptors.

- **Targeting the nanoparticles surface: which option?**

  - **The passive surface targeting: EPR effect**

First demonstrated by Maeda et al., in 1989, the EPR phenomenon is based on two factors:

- The capillary endothelium in malignant tissues is more disorderly and thus more permeable towards macromolecules than the capillary endothelium in normal tissues. Tumours grown subcutaneously have shown to exhibit a characteristic pore cutoff size ranging from 200 nm to 1.2 µm, the majority ranging between 380 and 780 nm (Hobbs et al., 1998). This allows extravasation of circulating polymeric nanoparticles within the tumour interstitium. The increased permeability of the blood vessels in tumours is characteristic of rapid and defective angiogenesis (formation of new blood vessels from existing ones). The hyperpermeable nature of the tumour microcirculation is well described. Numerous morphological studies have demonstrated the existence of interendothelial junctions, transendothelial channels, fenestrations, and vesicular vacuolar organelles in tumour vessels.

- The lack of tumour lymphatic drainage in the tumour bed results in drug accumulation.

Most polymer nanoparticles display the EPR effect. If a chemotherapeutic agent is coupled to a suitable polymer or other molecular carrier via a degradable linker, then such carriers have the potential of increasing the concentration of the chemotherapeutic agent within the tumour tissue. As a result of these characteristics, concentration of polymer-drug conjugates in tumour tissue can reach levels 10 to 100 times higher than that resulting from the administration of the free drug (Maeda et al., 2009).

Most importantly, EPR effect can be observed in almost all human cancers with the exception of hypovascular tumours such as prostate cancer or pancreatic cancer. As clinical examples, Doxil, a liposomal formulation of doxorubicin injected via the hepatic artery, accumulated selectively in hepatocellular carcinoma. Mechanisms and factors involved in the EPR effect, as well as the uniqueness of nanoscale drugs for tumour targeting through EPR effect, are discussed in detail in many reviews (Greish et al., 2007; Maeda et al., 2001 and 2009). Meada et al., 2009 described the factors facilitating EPR effect such as bradykinin, nitric oxide (NO), VEGF, prostaglandins (PG) and matrix metalloproteinases (MMP). EPR

effect can be further augmented by elevating the systemic blood pressure artificially by infusing angiotensin-II, and also by prostaglandin (PG) I2 agonist. Consequently, the delivery of macromolecular drugs is facilitated significantly even to metastatic tumour which has low vascular density (Maeda et al., 2009).

The most commonly used strategy, to achieve "sterically stabilized" nanoparticles is to conjugate polyethylene glycol (PEG) polymers onto the surface of the nanoparticles. A great deal of work has been devoted to developing the so-called "stealth®" particles which are invisible to macrophages, hence increasing nanoparticles blood circulation which is a pre-requisite for EPR effect. PEG is a relatively inert hydrophilic polymer which provides good steric hindrance for preventing proteins binding. Li et al., 2008 have demonstrated that PEGylation reduces the rate of mononuclear phagocyte system (MPS) uptake and increases circulation half time for various types of nanoparticles, including liposomes, polymer-based nanoparticles and hybrid nanoparticles. The area under curve (AUC) of the blood pK profile of the drug irinotecan hydrochloride (CPT-11) formulated in the PEGylated liposome was 6-folds higher than that of non-PEGylated formulation and 36-folds higher than that of the free drug. Akiyama and his colleagues grafted various amounts of PEG onto the surface of gold nanorods and investigated the effects of grafting amount and injection dose on the biodistribution in the tumour-bearing mice (Colon-26) after i.v injection. Higher PEG grafting amount were advantageous for reticuloendothelial system (RES) avoidance and for suppression of aggregation of the gold nanorods in the circulation. A PEG, Au molar ratio of 1.5 was sufficient to confer prolonged circulation of gold nanorods in the blood and to show an EPR effect (Akiyama et al., 2009).

- **Active surface targeting**

To target cell or tissue structures, two general approaches are developed which offer improvement of agent-biological interactions.

Healthy and tumour tissues present differential genotypes with dynamic phenotypes and these differences can be exploited for targeting key biological features present in one system and absent in the corresponding one. Growth factor or vitamin interactions with cancer cells represent a commonly used targeting strategy, as cancer cells often overexpress the receptors for nutrition to maintain their fast-growing metabolism. EGF has been shown to block and reduce tumour expression of the EGF receptor, which is overexpressed in a variety

of tumour cells such as breast and tongue cancers. Additionally, based on the same idea, the vitamin folic acid (folate) has also been used for cancer targeting because folate receptors are frequently overexpressed in a range of tumour cells including ovarian, endometrial and kidney cancers. Transferrin interacts with transferrin receptors, which are overexpressed on a variety of tumour cells (including pancreatic, colon, lung, and bladder cancers) owing to increased metabolic rates.

The second modality is determined by the intention to target particular molecular features of specific cancers and disease stage. There is clinical evidence that trastuzumab, a monoclonal antibody targeting the human EGFR HER, a two tyrosine kinase receptor, is an important component of first-line treatment of patients with HER2-positive metastatic breast cancer (Nielsen et al., 2009).

Antibodies were the first macromolecular ligands used for targeted delivery. The use of mAb became widespread after the discovery of hybridoma technology. Due to their inherent immunogenicity, murine mAb were not suitable for clinical applications. Engineering antibody technologies led to the development of chimeric humanized and fully humanized antibodies. More recently, methods have been developed to produce human immunoglobulins from transgenic mice. Combinatorial phage display libraries have also emerged as a powerful tool to select novel protein ligands. Research using antibodies for targeting applications has led to a better understanding of critical factors affecting targeting. Peptides and antibody fragments have been developed to overcome some of the shortcomings of antibodies, and several examples of these ligands are now under clinical development (Alexis et al., 2008).

It is now well accepted that the binding affinity, stability, and the size of the ligand play a critical role for successful targeting.

Peptides are small, synthetic molecules that can be manufactured in large quantities with excellent quality control. Peptides are more stable than antibodies and unlikely to be immunogenic.

Nucleic acid aptamers are single stranded DNA, RNA or unnatural oligonucleotides which fold into unique structures capable of binding to specific targets with high affinity and specificity (Alexis et al., 2008).

Small molecules such as multitargeted tyrosine kinase inhibitors represent simultaneous interruption of tyrosine kinases and serine/threonine kinases pathways. They serve to block angiogenesis and tumour cell proliferation.

A targeting ligand conjugated to the surface of nanoparticles can recognize and bind receptors, membrane proteins, surface antigens expressed on the target cell surface, which later triggers endocytosis, resulting in an increased level of intracellular delivery. This approach is particularly useful for delivering molecules with low membrane permeability, such as “druggable” DNA, oligonucleotide, and siRNA. Several active targeting strategies (antibodies, carbohydrates, peptides, and small molecule ligands) have been developed and showed promising results (Li et al., 2008).

- **Both passive and active targeting could be the best compromise for efficient nanoparticles bioavailability**

As a summary, passive tissue targeting may be achieved by extravasation of nanoparticles through increasing permeability of the tumour vasculature and ineffective lymphatic drainage, using proper nanoparticle surface functionalization, conferring the so called “stealth®” effect. Further, active cellular targeting may be achieved by functionalizing the nanoparticles surface with ligands that promote cell-specific recognition and binding. Figure 31 summarizes the two approaches, which may be viewed as complementary approaches: targeted nanoparticles concentrate in the tumour interstitium through the EPR effect, exactly as do nanoparticles designed for passive tissue targeting, but once there, nanoparticles are actively taken up by cancer cells after binding to their target antigens on the surface of the cancer cells (Alexis et al., 2008).

Interestingly, Couvreur et al., 2006 have shown, using liposomes, that decorating the surface of the nanocarriers with antibodies directed against tumour-associated antigens needs to be balanced between a sufficient number of antibody molecules per nanocarriers surface to achieve efficient binding and recognition on one hand, and not too many antibodies to avoid complement activation and to keep the ability of the immunovesicles to escape from the recognition by the MPS on the other hand. An optimal coating of 10-30 antibody molecules per nanocarriers seems to allow the combination of an efficient delivery with a limited uptake by the MPS.

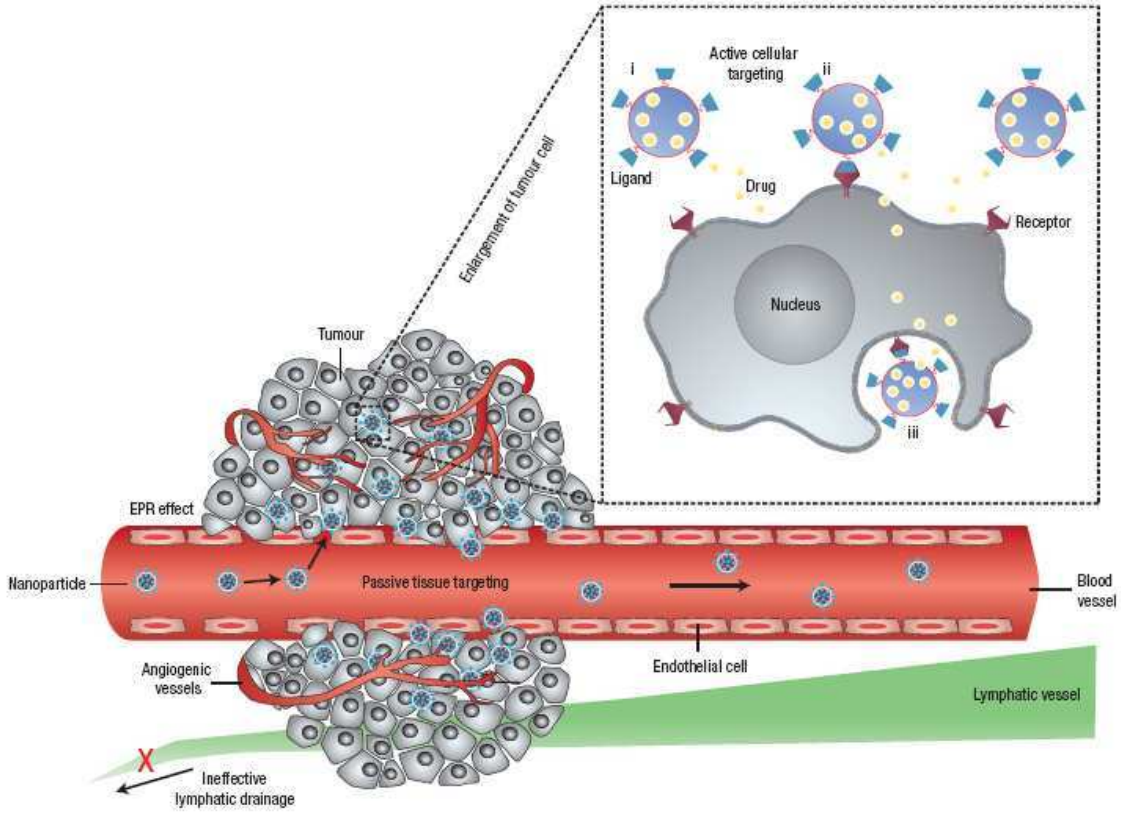


Figure 31: Active and passive targeting of nanoparticles (Peer et al., 2007)

3.3. Toxicological issues: probably the most important concern to address when designing a nanomaterial

An understanding of the hazardous properties of nanomaterials is essential if they are to come into contact with humans. The nanoparticle/biological interface which arises when nanoparticles come into contact with human, embraces particle protein corona formation, particle membrane wrapping, particle trafficking at the subcellular level, which are of most concern when addressing the nanomaterial safety issue.

Nanoparticle protein corona may induce changes in protein structure and function and can lead to potential mechanism of injury that could contribute to disease pathogenesis. Figure 32 illustrates the potential effects on protein structure and function following interaction with nanoparticles. Particles composition at the nanoscale level confers to the nanoparticles their performance characteristics (electronic, magnetic properties among others). However, such characteristics may be responsible for protein structural or functional changes. It is possible that electron confinement or the formation of electron hole pairs at the material surface could lead to cleavage of structural bonds or covalent cross-linking of protein

SH domains. The interaction between human adult hemoglobin (Hb) and bare cadmium sulfide (CdS) quantum dots (QD) has been investigated by fluorescence, synchronous fluorescence, circular dichroism (CD) and Raman spectroscopic techniques under physiological pH 7.4. CdS QD dramatically alter the conformation of Hb, quenching the intrinsic fluorescence of Hb and decreasing the α-helix content of the secondary structure from 72.5% to 60.8%. Raman spectra results indicate that the sulfur atoms of the cysteine residues form direct chemical bonds on the surface of the CdS QD (Shen et al., 2007). Along similar line, when a protein containing cryptic epitopes is denatured on a particle surface, the exposure of new antigenic sites may initiate an immune response, which, if launched against self-protein, could promote autoimmune disease. Here, more than the nanoparticle size or nanoparticle surface area, the nanoparticle reactive surface area constitutes a more accurate measure of particle potential toxicity. While, in biological environment, the nanoparticles agglomeration behaviour takes a role in defining the reactive surface area. Indeed, nanoparticle surface functionalization may influence nanoparticles agglomeration behaviour in biological media – by promoting particles dispersion or, on the contrary, leading to nanoparticles agglomeration – or directly inflicts the reactive surface area.

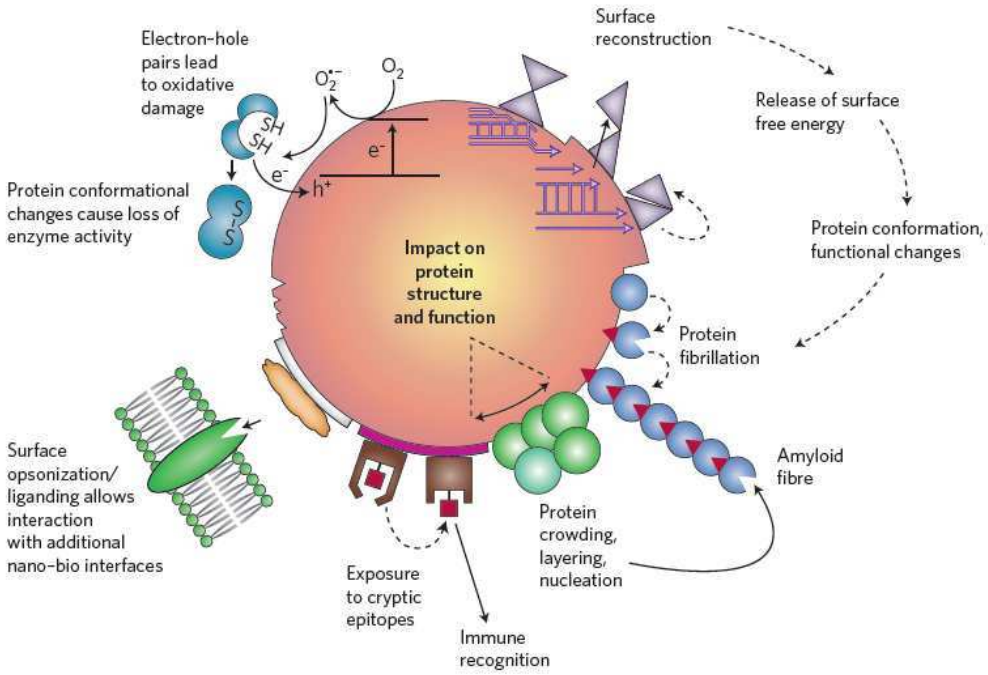


Figure 32: The corona constitutes a primary nano–bio interface that determines the fate of the nanoparticle and can cause deleterious effects on the interactive proteins. Potential changes in protein structure and function as a result of interacting with the nanoparticle surface can lead to potential molecular mechanisms of injury that could contribute to disease pathogenesis. The coloured symbols represent various types of proteins, including charged, lipophilic, conformationally flexible proteins, catalytic enzymes with sensitive thiol groups, and proteins that crowd together or interact to form fibrils (Nel et al., 2009).

Particle adhesion to a cell-surface bilayer and particle engulfment at the adhesion site is the result of promoting forces (such as specific binding induced by ligand-receptor interaction, non specific binding due to particles surface characteristics, optimal particle size and shape,...) and resistive forces (such as stretching and elasticity of cell membrane, receptor diffusion to adhesive front,...). Nanoparticles surface charge plays an important part in particles interactions with charged phospholipid head groups or protein domains on cell surfaces. Nanoparticles with cationic surface charge exert generally stronger effect than their anionic counterparts. However, if the nanoparticles cationic density is not controlled, the interactions may compromise the cell membrane integrity. Nanoparticles shape is also an important factor for nanoparticles cell uptake; studies of pulmonary exposure of pure single-walled carbon nanotubes (SWCNT) have shown that SWCNT are poorly phagocytized by alveolar macrophages; instead, they migrate to the interstitial space in the alveolar wall, where, after close contact with fibroblasts, they directly stimulate cellular proliferation and collagen production.

Nanoparticle trafficking at the subcellular level may exert important effects on organelles. Particularly noteworthy is the impact on lysosomal function. The lysosomal proton pump, which is responsible for acidification, is key to understanding the proton sponge hypothesis, which posits that unsaturated amines on nanoparticles surface are capable of sequestering protons, keeping the pump going and leading to the retention of one Cl^- anion and one water molecule for each proton that enters the lysosome. Ultimately, this process causes lysosomal swelling and rupture. Spillage of the lysosomal content may lead to toxicity. Lysosomes are also involved in nanoparticles dissolution, as illustrated by the toxicity of ZnO or CdSe.

Hence, when designing nanomaterials, the optimal design process appears one of multiple compromises. Above all, potentially competing principles (aggregation, dispersal, transport, bioavailability) must be analysed in detail to formulate the best design strategy for safe but efficient use of the nanoparticles in their intended purpose. Interestingly, surface coating is perceived as an attractive approach to improve nanoparticles safety by playing different roles such as preventing bioreactivity and dissolution. Nanoparticles surface functionalization may also modulate nanoparticles biodistribution and/or nanoparticles cellular trafficking and may be considered for a safe use of nanoparticles in biological media. However, many coatings are recognized as being environmentally labile or degradable, hence an initially non-toxic material may become hazardous after shedding its coat.

4. Summary of PhD study: participation to the development of two types of nanoparticles intended to treat cancer with different rational for design

The present PhD study focuses on the 2 major advanced researches developed by Nanobiotix: the nanoXray™ technology platform and the nanoPDT technology platform. Products issued from those platforms aim to create and develop effective nanomaterials to treat cancer, using external energy sources to carry the "on" / "off" activation.

However, the energy sources used to "activate" the products are notably different (Figure 33). Furthermore, the route of administration is also different; meaning that the rational for design of each nanoparticle is fully different. Further, the platforms are dedicated to radically different therapeutic approaches which share the endpoint of delivering loco-regional control of diseases.

NanoXray™ platform creates and develops nanomaterials which are designed to interact with X-ray radiations to subsequently enhance locally the effect of radiotherapy. NanoXray™ products are inert and electron-dense particles which generate electrons with kinetic energy that will be released into the medium and will generate free radicals, following X-ray absorption. The nanoparticles have an electron density much higher than water, so they have a far more powerful cancer killing effect than standard radiotherapy, and have fewer safety issues than currently available cancer treatments. NBTXR3 is the lead product of nanoXray™ platform, synthesized to enter within the tumour cell by direct injection, without any specific target or interaction. NBTXR3 is a suspension of inert crystalline nanoparticles (±70 nm) of coated hafnium oxide (HfO_2), in water for injection. The NBTXR3 oxide nanoparticles have a simple composition. The hafnium oxide core represents the active component, but only when its electrons are excited by the application of an external beam of X-ray. This core is coated by a layer which ensures stability in fluids and improves biocompatibility.

Moving to longer wavelength throughout the electromagnetic spectrum, the secondary electromagnetic energy source used by Nanobiotix lies in the near-infrared region. Such wavelength of light is able to penetrate biological tissues and may be exploited for clinical purpose. NanoPDT platform has developed nanoparticles that are designed to interact with

laser sources. Existing organic photosensitizers have currently demonstrated interesting efficacy and some of them have received market approval. But their difficulty to perform breakthrough in the clinic relies on their poor pK and their lack of selectivity toward targeted tissues. NanoPDT product (or silica-based nanoparticle) is intended to be administered by intra-venous injection. NanoPDT product consists in a silica-based nanoparticle which successfully encapsulates the photosensitizer to readily improve its pK while preserving its ability to generate reactive oxygen species (ROS) to the surrounding media upon laser light excitation.

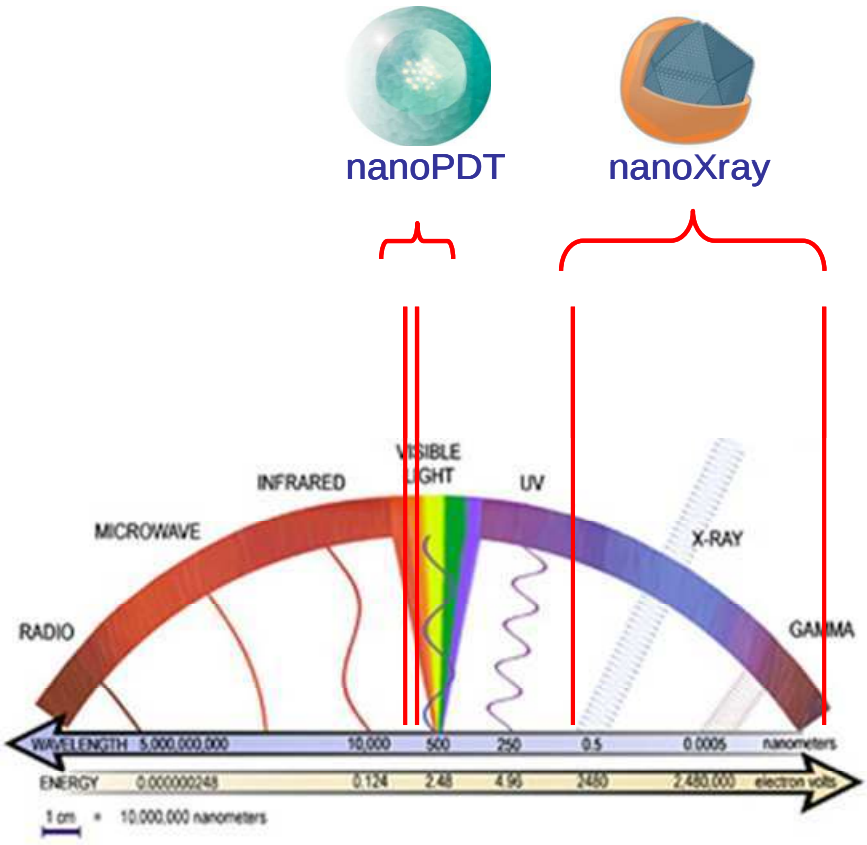


Figure 33 : NanoPDT and nanoXray™ spectrum of activation

NanoXray™ oxide nanoparticles activated with typical radiotherapy treatments or nanoPDT nanocarriers for PDT have clearly for ambition to enlarge the therapeutic window. PhD studies take part to 3 interconnected research axes which are key when establishing the rational for development of the therapeutic products.

In vitro efficacy and toxicity studies

In vitro approach is of particular relevance to assess the nanoparticles toxicity, the nanoparticles efficacy, the nanoparticles subcellular localization effect on efficacy, the type of cellular death, but also to screen different tumour cell types as well as to study the effect of nanoparticles on normal cells. Indeed, many questions may be underpinned in *in vitro* studies when considering the nanoparticle as the 'active product' to enlarge the therapeutic window:

- Are the nanoparticles toxics without activation - for both tumour and healthy cell lines?
- Do nanoparticles enhance tumour cells destruction upon activation by external energy sources - according to schedules and protocols?
- Which sort of cell death is induced by the nanoparticles?
- What are the effects of nanoparticles subcellular localization on efficacy?
- Do nanoparticles induce the same efficacy / toxicity according to tumour biologic specificities: their origin (epithelium and mesenchymal origin, different compartmental structure), human healthy versus malignant tumour cells, radiosensitivity characteristics (radio-sensitive or radio-resistant cancer types)?

Mechanism of action: nanoparticles trafficking at cellular level

Nanoparticle trafficking at cellular level is a key understanding to yield foundations to establish the schedule of nanoparticles administration in *in vivo* models and the modality of energy source delivery. *In vitro* studies of nanoparticles uptake, localization and clearance are of particular relevance to precise nanoparticles trafficking at the cellular level. Many research axes need to be explored:

- What is the nanoparticles uptake mechanism?
- What is the kinetic of nanoparticles uptake?
- Where are the nanoparticles localized within cells?
- How many nanoparticles are internalized per cells?
- Are nanoparticles exocytosed or are they trapped within organelles and ultimately degraded within the cells?

***In vivo* performance and safety**

In vivo studies encompass the nanoparticles biodistribution, the nanoparticles tumour accumulation and dispersion, the nanoparticle toxicity. Tumours are specific "organs", different from the organ/tissue from which they arose. These 2 genetic entities coexist, and have molecular structure, metabolic and growth behaviors particular to the patient and closely related to the tissue type and cancer disease stage. Exploration of biologic specificity of different cell/tissue/organ is needed. *In vivo* studies must be sustained by *in vitro* studies to bring safe and efficient products in the clinic. Research axes are multiple and some of them are highlighted in the following questions:

- What is the ideal nanoparticle (according to its size, composition, shape and surface coating) in order to optimize biodistribution and/or cellular uptake and/or localization at the subcellular level?
- What is the ideal nanoparticle (according to its size, composition, shape and surface coating) for optimized biocompatibility?
- Are nanoparticles stable in biological media?

Ultimately, the aim of all those studies is to show the benefice of nanoparticles over the current therapeutic approaches. For nanoXray^{TM} applications, the reference control is the radiotherapy alone whereas for nanoPDT applications, the reference control is the free drug.

■ Presentation of the PhD work objectives: **study of interactions between nanoparticles and cancer cells**

Nanobiotix is working on oncology field and therapeutic window enlargement is the main concern in the clinic. Nanobiotix has explored two different pathways: inert therapeutic nanoparticles activated by radiotherapy for local radiation enhancement and silica-based nanoparticles for improved drug delivery for PDT. Specifically, study of interactions between nanoparticles and cancer cells was explored.

The following Figure 34 summarizes the work undertaken during this PhD project.

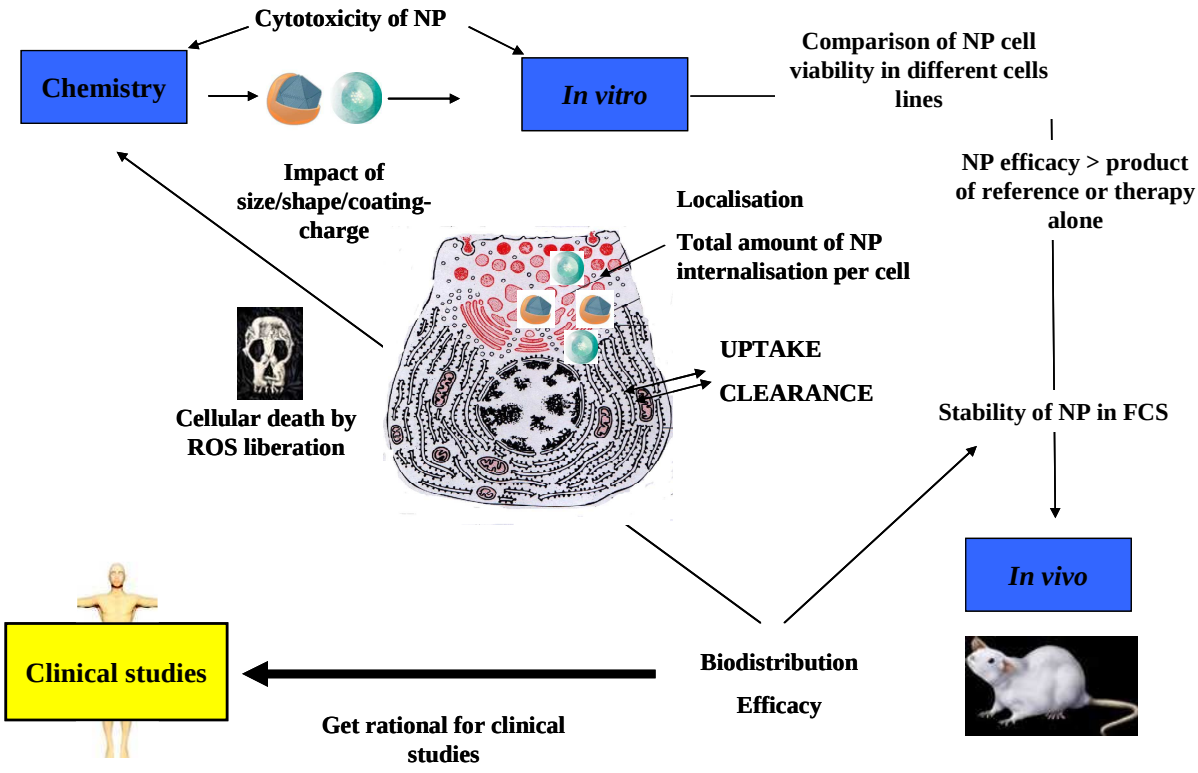


Figure 34: Study of interactions between nanoparticles and cancer cells

NBTXR3 nanoparticles interaction with human tumour cells – binding to membranes efficiency, intracellular uptake, nanoparticles localization and change over time – the effect of nanoparticles localization on efficacy, the nanoparticles dose effect on efficacy, were all the aspects covered by the present study, with the ultimate goal to yield foundation for schedule of NBTXR3 nanoparticles administration in *in vivo* models and the modality of ionizing radiation delivery.

NanoPDT interaction with human tumour cells – intracellular uptake, amount of nanoparticles per cell and kinetic of clearance – were studied to define the optimum schedule for nanoparticles activation. NanoPDT ROS generation quantification and ROS subcellular localization were also covered by the present study. Comparison of nanoPDT biodistribution in different *in vivo* models was underpinned to better understand the role of cell type, engraftment site and stroma contribution.

NanoXray™ therapeutic products allow dramatic increase of X-ray dose on tumour without changing dose applied to healthy tissues. NanoXray™ increases local destruction of the tumour without modifying healthy tissues

1. Nanoparticles activated by X-ray: NBTXR3 mechanism of action

As it is well known, ionizing radiation interacts with atoms or molecules in the cells, particularly with water, to produce free radicals which are responsible for non specific cellular damages, leading to subsequent cell death. As a result of the interaction with a photon, the water molecule will ionize, producing a fast e^- , a photon of reduced energy, and an ionized water molecule: $H_2O \rightarrow H_2O^+ + e^-$. The ionized water molecule will de-excite either by producing Auger e^- or X-ray photon and free radicals. Fast electrons that will be released into the medium will generate free radicals or heat. These electrons with kinetic energy are responsible for the largest effect in radiotherapy.

Hafnium oxide core of nanoXray^{TM} products will interact with a photon or an electron in exactly the same way. However, the probability of absorption (of a photon or an electron) is proportional to the density and the atomic number (Z) of the absorbing material. Water has d = 1, Z = 8, whereas nanoparticles have d > 8, Z > 60. Hence, hafnium oxide nanoparticles will generate the same type of effect as water molecules, but by several orders of magnitude higher.

Primary mode of action of NBTXR3 product

The mechanism of action of nanoparticles activated by X-rays is based on a physical and energetic effect and not on pharmacological, immunological or metabolic effect. The physical and energetic mechanism of action of such a product is based on electron ejection from HfO_2, which generates free radicals after X-ray energy exposure.

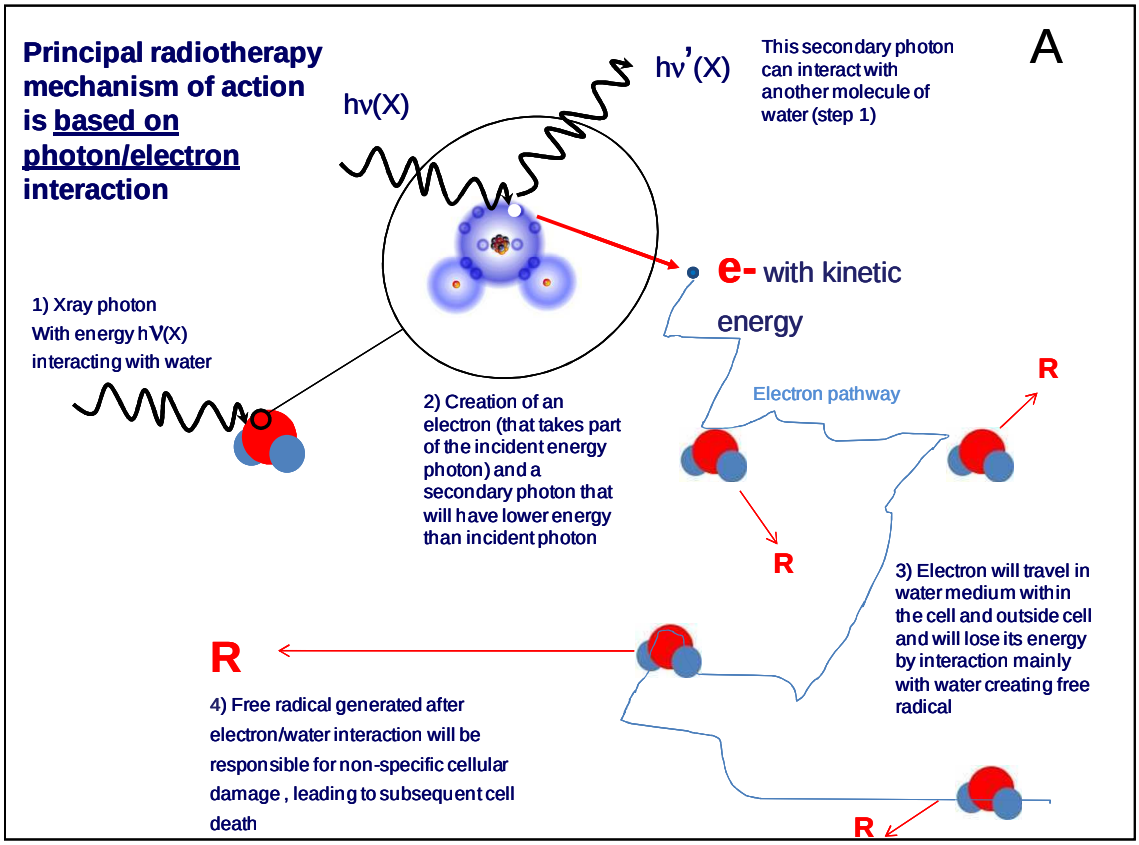
The mode of action of NBTXR3 is described as follows:

Step 1- On/off activation: Energy absorption, electrons emission, and free radicals generation

NBTXR3 works according to an “on-off” activity status:

- When the nanoparticles are not activated, they do not have any effect because they are inert.

- Under external beam X-ray activation as customarily used in radiation oncology,
    - X-rays are absorbed by HfO_2 (NBTXR3) exactly as ionizing radiations are absorbed by water molecules. Meaning an X-ray photon will interact losing a part of its energy with an electron from the atom and will create i) a hole , ii) an electron with kinetic energy and iii) the incident photon will have lost a part of its energy and could participate to subsequent interactions (Figure 35).
    - The electron created will travel in the cellular medium and will lose its energy by interacting several time with water molecules creating free radicals (mainly responsible for cellular damages). See step 2 for cellular damages induced by such free radicals.
    - The hole created in the atom will come back to its initial state by taking an electron from the water molecule in the medium.



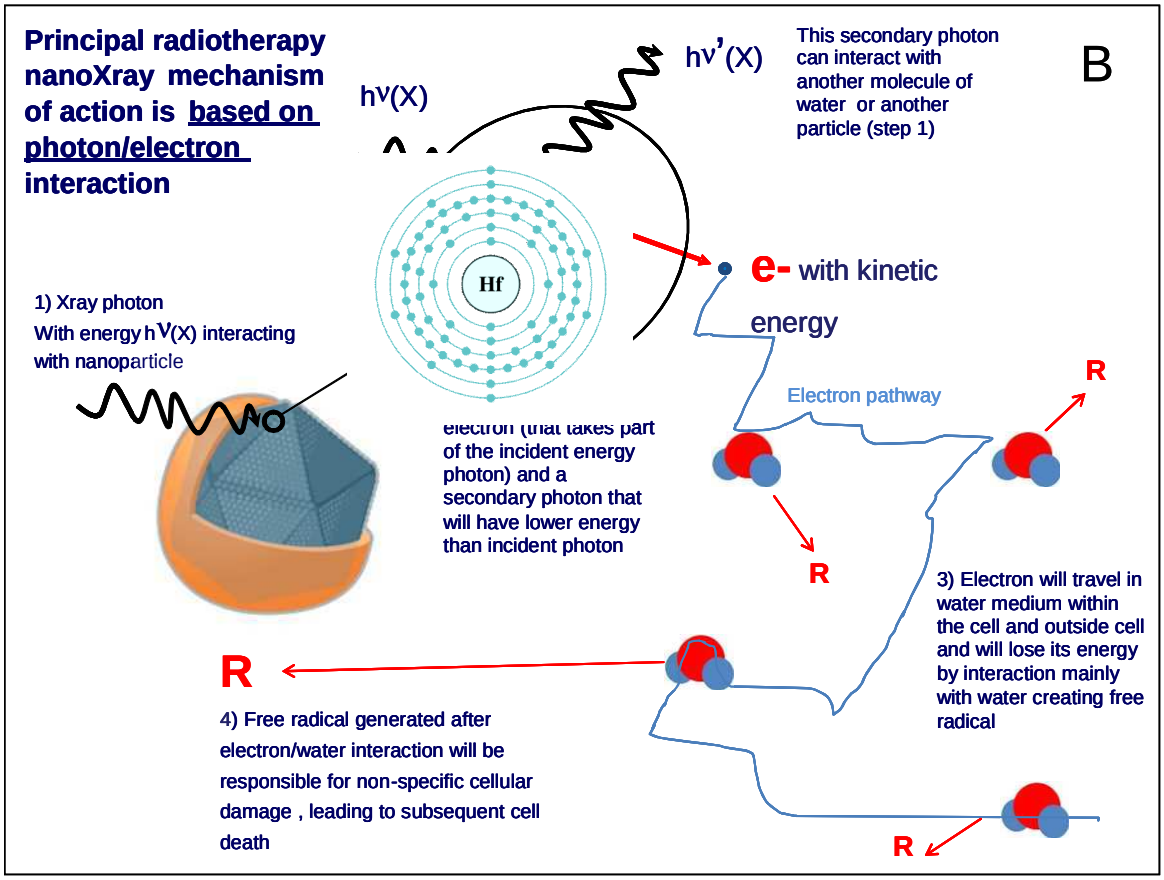


Figure 35: Principal radiotherapy mechanism of action of A/H_2O ; B/NBTXR3

The nanoparticles do not react directly with any biological recipient cell and tissue.

The X-ray irradiation can be applied several times on the same nanoparticle. When the X-ray irradiation is off, NBTXR3 returns to its inactive state.

Step 2- Cellular damage

The free oxygen radicals generated by electrons ejected from the HfO_2 (NBTXR3) and water are quite reactive as they are attempting to form covalent bonds with another species. They can lead to non-specific damages to subcellular organelles and biomolecules around the location of nanoparticles or along the electron pathway that will create free radicals.

Step 3- Subsequent actions on cells

The mode of action of NBTXR3 is mainly explained by the effect of free oxygen radicals leading to cytotoxic effects through non-specific damages to tumour cells. Resultant cellular

destruction is induced by the usual effect of free radicals as for radiotherapy, but enhanced because of the nanoparticles.

All these physical mechanisms mentioned above, can explain the effect of NBTXR3 on tumour cells and support the use of such a therapy in cancer treatment. This mode of action actually mimics the ionizing radiation mode of action on biological systems (Perez et al., 2004; Kufe et al., 2006 and Zhou et al., 2004) (Figure 36).

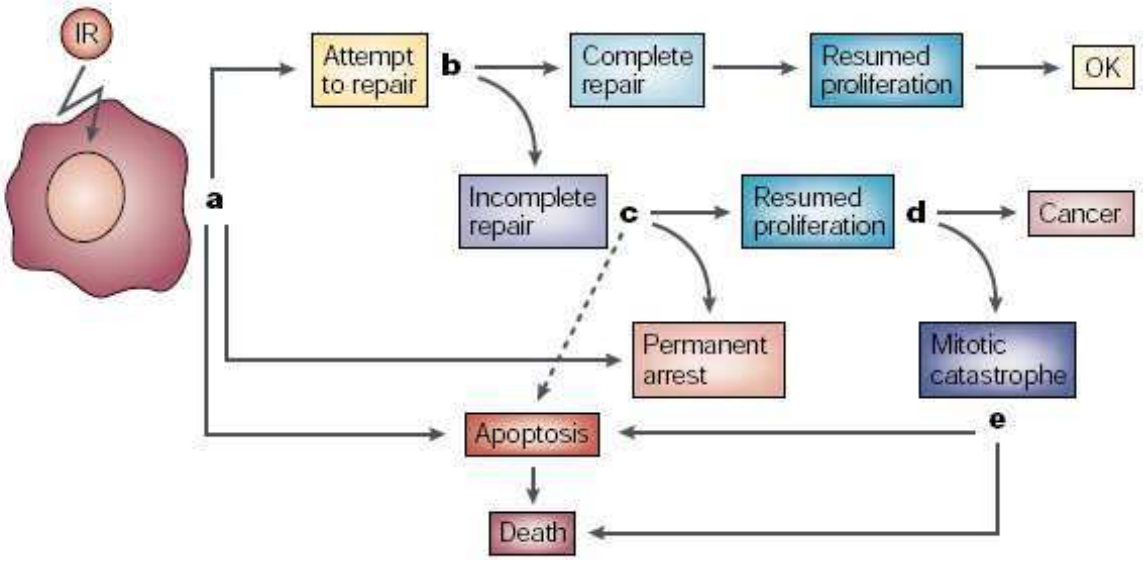


Figure 36: Cell radiation responses (Gudkov et al., 2003)

2. Metallic nanoparticles activated by ionizing radiation to treat cancer

Gold nanoparticles literature survey

The following literature tends to expose the rational for development of gold nanoparticles activated by ionizing radiations to treat cancers. Gold nanoparticles present a high Z (=79) which is expected, providing intra-tumour gold nanoparticles localization, to enhance the dose delivered to a tumour during photon-based radiation therapy, resulting in a differentiate efficacy between tumour and surrounding tissues. Cho et al., 2005 have performed Monte Carlo calculation to estimate the dose enhancement of gold nanoparticles uniformly distributed throughout a tumour, provided some insight into the amount of achievable dose enhancement of gold nanoparticles activated by typical radiation treatment, with a significant higher dose enhancement estimated in the kilovoltage energy range.

Despite the establishment of *in vitro* and *in vivo* efficacy using gold nanoparticles activated by ionizing radiations – supporting the theoretical Monte Carlo calculation –gold nanoparticles safety studies are scarce and disparate and it seems difficult to get a clear view of their potential toxicity in the clinic.

In vitro efficacy and mechanism of action

In their article, Kong et al., 2008 report an *in vitro* performance study of home-made 10.8 nm gold nanoparticles activated by ionizing radiations to treat cancer cells. They put forward (i) the importance of gold nanoparticles localization at the cellular level, (ii) the role of the ionizing radiation energy source and (iii) the differential effect of radiation sensitivity observed in cancer cells versus nonmalignant cells.

The authors have used different surface treating agent, thioglucose (GLU) and 2-Aminoethanethiol (AET) to functionalize gold nanoparticles. Following incubation of functionalized gold nanoparticles with MCF7 breast cancer cells, gold nanoparticles functionalized with thioglucose were mostly found distributed in the cytoplasm of cells. On contrast, gold nanoparticles functionalized with AET were mostly found bound to the cell membranes. The authors have performed *in vitro* MTT assays using the same level of gold nanoparticles per cell for either AET-gold nanoparticles or GLU-gold nanoparticles. Cells either untreated or treated with functionalized gold nanoparticles were irradiated with 200 KVp X-rays with a dose of 10 Gy. GLU-gold nanoparticles and AET-gold nanoparticles were found to induce about 63.5% and 31.7% increase in radiation cytotoxicity, respectively, when compared to irradiation alone. Additional clonogenic survival assays were consistent with previous results. The authors stated that this phenomenon indicates that the location of gold nanoparticles in the cells is an important factor in the increase of radiation cytotoxicity induced by gold nanoparticles. Interestingly, under similar conditions, the authors found that gold nanoparticles increase the radiation sensitivity of MCF7 cancer cells but not that of nonmalignant cells MCF-10A. However, no significant dose enhancement was observed when compared to control, using gold nanoparticles activated by high energy radiation source (^{60}Co, ^{137}Cs or γ-rays).

Rahman et al., 2009 present an *in vitro* performance study of 1.9 nm gold nanoparticles activated by two different energy sources using bovine aortic endothelial cells. The article put forward, the importance of (i) gold nanoparticles concentration at cellular level

and (ii) the role of the energy source, for an enhancement of radiation effect. Interestingly, a dose enhancement effect was observed for cells treated with gold nanoparticles and irradiated using high energy electron beams. Cytotoxicity was observed with gold nanoparticles concentration of 0.25 mM and beyond. Toxicity issue will be discussed specifically in later paragraph, when addressing the safety of gold nanoparticles.

Two energy sources were tested. Studies were done with kilovoltage superficial radiation therapy-type X-rays (80 kV and 150 kV X-rays energy at various dose 0, 1, 2, 3, 4 and 5 Gy) and megavoltage electron beams (6 MeV and 12 MeV). Cells *in vitro* were treated with increased concentration of 1.9 nm gold nanoparticles from Nanoprobes (from 0.25 mM up to 1 mM). After continuous exposure of the cells to the gold nanoparticles for 24 hours, gold nanoparticles, for each concentration tested, were found internalized within the cytoplasm of cells as clusters. Dose enhancement, in cells irradiated with superficial X-rays, was concentration-dependent and energy-dependant. Similar to the superficial X-ray, the results with cells irradiated with electron beams (both 6 MeV and 12 MeV) under identical radiation doses showed that cells containing gold nanoparticles presented a lower survival percentage with increasing concentration of gold nanoparticles, highlighted the ability of gold nanoparticles to enhance the effect of radiation even under high energy electron sources.

***In vivo* efficacy and mechanism of action**

Hainfeld et al., 2004 have established the first proof of tumours eradication, when using gold nanoparticles (1.9 nm from nanoprobes) activated by X-rays following i.v injection.

Syngeneic mouse mammary EMT-6 tumours were grown subcutaneously in the legs of mice. After i.v injection of gold nanoparticles, the tumour region was irradiated with 250 kVp X-rays. A dose of 30 Gy was delivered in a single fraction. Irradiation was performed 2 min following i.v injection. Animal receiving either no radiation or gold without radiation died within 2 weeks. Irradiation alone slowed tumour growth and resulted in 20 % long-term remissions. Animals receiving injection of 1.35 or 2.7 g Au/kg body weight before irradiation showed 50 % and 86 % long term remission. pK, showed an early and rapid rise followed by a slower clearance rate. Gold in tumour peaked at 7.0 ± 1.6 min and fell to one-half of its peak value at 41.2 ± 19.5 min. gold in muscle peaked at 5.3 ± 0.6 min and fell to one-half at 24.2 ± 2.6 min. The gold nanoparticles cleared nearly twice as fast from normal muscle as from

tumour. The preliminary toxicity testing on mice receiving 2.7 g Au/kg lived for more than 1 year without overt clinical signs. Analysis of blood from mice having given 0.8 g Au/kg, 2 weeks after injection showed haematocrits and enzymes within normal range. As perspective from their preliminary results the authors proposed to explore further the potential of gold nanoparticles to enhance the effect of radiotherapy with the following approaches: use of megavoltage sources, direct intratumoural injection, second-generation gold agent with targeting molecule, fractionated radiotherapy. This last approach is of particular relevance as fractionated radiotherapy represents the standard of care of most clinical protocols. In the present study, irradiation is performed 2 min post i.v injection. Obviously, gold nanoparticles, used in the present article, present rapid tumour clearance which appears as a key issue in the context of fractionated radiotherapy. Furthermore, considerable dose (30 Gy) was delivered to the tumours. Hence, those gold nanoparticles may be objected as potential product for development in the clinic.

Chang et al., 2008 present an *in vivo* performance study, using home-made 13 nm gold nanoparticles with sodium citrate acting both as reducing agent and as surface complexing agent, in combination with 6 MeV electron beam to treat tumour. The article highlights the potential of 13 nm gold nanoparticles to significantly retard tumour growth and to prolong survival compared to the radiation alone.

Syngeneic mouse mammary melanoma B16F10 cells were grown subcutaneously in the legs of mice. Gold nanoparticles, 1 g/kg of mice body weight were intravenously injected. Approximately 24 hours post-gold nanoparticles injection, the tumour region was irradiated with 6 MeV electron beam. A single dose of 25 Gy was delivered to the tumour. The results revealed that tumour growth was both retarded in mice receiving either radiation alone or receiving gold nanoparticles followed by radiation. But more importantly, tumour volume in the combination therapy group was significantly smaller compared with that in radiation alone group, whereas administration of gold nanoparticles alone did not exert any antitumour effect on tumour-bearing mice. Biodistribution of gold nanoparticles was performed 24 hours post-i.v injection. A notable accumulation of gold nanoparticles inside the tumour tissues was detected. The tumour-to-tumour surrounding muscle gold ratio was 6.4:1. Nevertheless, higher concentrations of gold nanoparticles were also found in the spleen and the liver, which indicated that the gold nanoparticles were also uptaken by the reticuloendothelial system. Apoptosis in tumours with gold nanoparticles without and with radiotherapy was observed in

TUNEL-stained cryosections and compared to control (no particles) without and with radiotherapy. Noticeably, the number of apoptotic cells detected was significantly higher in the gold nanoparticles and radiation combination group than in the radiation alone group. For a deeper understanding of the mechanism of action, the authors have performed *in vitro* visualization of gold nanoparticles at the cellular level. Eighteen (18) hours post incubation, gold nanoparticles were found inside the B16F10 cells, localized in the ER and Golgi apparatuses. The authors mentioned that continuous ER stress results in apoptotic cell death; therefore, the accumulation of gold nanoparticles in ER and Golgi may also contribute to the increase of the apoptotic potential of cells post irradiation.

The outcome of those two preclinical *in vivo* studies is summarized in the following:

Authors	GNP size	Tumour to normal tissue ratio peak	Clearance (half peak)	Time before irradiation after i.v	Irradiation dose
Hainfeld	1.9 nm	3.5 / 1 at 5 min post i.v	tumour: 41 min normal : 24 min	2 min	30 Gy 250 kVp X-ray
Chang	13 nm	6.4 / 1 at 24 hours post i.v	/	24 hours	25 Gy 6 MeV e^-

Gold nanoparticles with increased nanoparticles size seem more interesting when considering gold nanoparticles retention within tumour, particularly in the perspective of fractionated radiation protocol if multi-nanoparticles injections are to be excluded. However, very high doses are required (or at least presented) in the current studies, which seem quite unrealistic for the clinic, still in the context of fractionated radiotherapy.

Gold nanoparticles trafficking at cellular level.

Chithrani et al. (2006, 2007 and 2009) have deeply explored the effects of gold nanoparticles size, shape and surface coating on intracellular uptake, transport and subcellular nanoparticles localization. HeLa, MCF7, STO (fibroblast) and SNB19 (brain) cell lines were alternatively used for their studies. Spherical gold nanoparticles were synthesized with size of 14, 50 and 74 nm using citric acid as both reducing and complexing agent. Uncoated gold nanoparticles were stabilized in aqueous media by citrate ions, conferring a negative surface

charge to the nanoparticles. Bovine Serum Albumin (BSA) or transferrin proteins were also used as surface treating agent.

Gold nanoparticles group in small vesicles of approximately 500 nm in diameter in the cells cytoplasm, with no nanoparticles found alone in the cytoplasm. Within the vesicles, the nanoparticles appear to be monodisperse.

Authors suggest that uptake of uncoated gold nanoparticles is mediated by non specific adsorption of serum proteins onto the gold surface. This is likely since citric acid stabilizers are weakly bound to the surface of gold nanoparticles and could be desorbed from the metal surface by proteins. These proteins induce the nanoparticles to enter cells via the mechanism of receptor-mediated endocytosis – indeed, *in vitro* nanoparticles cells uptake studies performed at 37°C versus 4°C support their hypothesis. Additionally, their observation of an uptake saturation curve as a function of gold nanoparticles concentration is commonly observed for receptor-mediated endocytosis. Whatever gold nanoparticles size, gold nanoparticles uptake reaches saturation following 6 hours of incubation with cells. Gold nanoparticles, either pre-coated with BSA or transferrin proteins, show similar cells uptake, but to a lesser extend when compared to uncoated gold nanoparticles. The authors state that the surface of the initial uncoated gold nanoparticles (citrate-stabilized nanoparticles) contains a variety of serum proteins and that their diversities may allow entrance into cells via multiple receptors.

Specific studies with gold nanoparticles pre-coated with transferrin also demonstrate cellular uptake with HeLa, SNB19 and STO cells, albeit at different concentrations but with similar trends and further suggest that uptake is likely due to a clathrin mediated process.

Authors put forward the importance of competition between thermodynamic driving force for wrapping (how the membrane encloses a particle which involve factors such as ratio of adhesion and membrane stretching, the membrane's bending energy) and the receptor diffusion kinetics (kinetic of recruitment of receptors to the binding site), for nanoparticle cell uptake. Consistent with previous results, they found that spherical gold nanoparticles with size of 50 nm have the greatest degree in cellular uptake; smaller nanoparticles needed to cluster together to go in and larger nanoparticles having a longer wrapping time due to the slower receptor diffusion kinetic. However, they proposed, as the 50 nm nanoparticle can enter cells as single nanoparticle, that vesicles must fuse later on in the cell after entrance.

Further, detailed TEM studies have shown that nanoparticles are trapped in endosomes before being fused with lysosomes for processing.

Specific mechanisms for nanoparticles cell clearance were conducted with gold nanoparticles precoated with transferrin. Studies were performed with STO, HeLa and SNB19 cell types. Removal of transferrin-coated gold nanoparticles was linearly related to size, which was different than the uptake process. Nanoparticles about to be removed from cells appeared to be localized in late endosomes or lysosomes. Smaller nanoparticles were found to exocytose faster in contrast to larger nanoparticles. Smaller nanoparticles will have less-receptor ligand interactions and the authors speculated that fewer receptor-ligand interactions led to a lower overall binding constant and hence, the transferrin coated nanoparticles could be released more easily.

Despite a huge amount of work performed by Chithrani et al., to deeply understand the effects of gold nanoparticles size, shape and surface coating on intracellular uptake, transport and subcellular nanoparticles localization, no studies have been published, to our knowledge, to evaluate their efficacy when activated by ionizing radiations.

Gold nanoparticles toxicity

It is generally admitted that gold nanoparticles are biocompatible and nontoxic. However, *in vitro* and *in vivo* studies have recently raised the potential toxicity of gold nanoparticles. In the following, a literature survey on spherical gold nanoparticles is presented, excluding gold nanoparticles with size of 1.4 nm which have shown to enter into the nucleus of cells and to interact with DNA inducing significant toxicity even at very low level. (Pan et al., 2007; Tsoli et al., 2005)

In vitro studies

Two papers (Yan et al., 2009 and Connor et al., 2005) present *in vitro* cytotoxicity studies with gold nanoparticles with size of 3.7 nm (HeLa cell line) and ~ 4 nm, 12 nm and 18 nm (K562 leukemia cell line). Nanoparticles were functionalized with different surface treating agents. As a brief summary, the data report that gold nanoparticles are taken up by cells but do not cause acute cytotoxicity. Gold nanoparticles with size of 3.7 nm modified with PEG were found into the nucleus of HeLa cells upon exposure for 24 hours. Following

those results, Pernodet et al., 2006, have focused in greater details on citrate / gold nanoparticles with size of 14 nm and investigated further the effects of gold nanoparticles on cell proliferation, morphological structure, spreading, migration and protein synthesis. In the long run, these factors may be more dangerous as they trigger the growth of defective living tissues. Authors studied these effects on human dermal fibroblast cells and focused on the effects of nanoparticles on individual living cells. The major conclusions of their study is that (1) 14 nm citrate / gold nanoparticles can easily cross cell membranes and accumulate into vacuoles (nanoparticles cells clearance was not discussed). The entry of the nanoparticles appears to be not immediately detrimental to cell function, but rather the formation of an unusually large number of vacuoles probably triggers a serie of secondary events, which eventually hinders other processes; (2) the presence of gold nanoparticles, in a concentration dependant manner, is responsible for abnormal actin filaments (either a depolymerization process or a lack of actin-fiber formation) and ECM constructs in dermal fibroblast. These, in turn, are shown to decrease cell proliferation, adhesion and motility. Further studies are in progress to identify the specific genes expressed and the protein produced. Still, on similar gold nanoparticles, Khan et al., 2007 have performed cytotoxicity assays, uptake studies and gene expression profiling on HeLa cells. Gross changes in gene-expression patterns were not seen after uptake of 18 nm gold nanoparticles into the human cell line HeLa. In spite of their ability to bind to serum proteins non specifically, gold nanoparticles did not induce the splicing of *xbp1* mRNA, which is a marker of unfolded protein response.

Patra et al., 2007 investigated cells specific response to citrate / gold nanoparticles with size of 33 nm. They report that gold nanoparticles-induced death response, in a concentration dependant manner, in human carcinoma lung cell line A549 but not on the 2 others cell lines tested, BHK21 (baby hamster kidney) and HepG2 (human hepatocellular liver carcinoma) which remain unaffected by the presence of gold nanoparticles. Authors confirmed that A549 cytototoxicity is induced by the gold nanoparticles. Gradual increase in gold nanoparticles concentration induces a proportional cleavage of poly(ADP-ribose) polymerase. The programmed nature of the death response is implied, because such cleavage follows activation of caspases.

In vivo studies

Chen et al., 2009 studied *in vivo* toxicity of naked gold nanoparticles with size of 3, 5, 8, 12, 17, 37, 50 and 100 nm. Naked nanoparticles were prepared by seed process in presence

of citrate and $NaBH_4$. To our understanding, citrate is believed to act as complexing agent. Naked gold nanoparticles were injected intraperitoneally into BALB/C mice at dose of 8 mg/kg/week. Gold nanoparticles of 3, 5, 50 and 100 nm did not show harmful effects; whereas gold nanoparticles ranging from 8 to 37 nm induced severe sickness in mice. Pathological examination of the major organs of the mice in the diseased groups indicated an increase of Kupfer cells in the liver, loss of structural integrity in the lungs and diffusion of white pulp in the spleen. The pathological abnormality was associated with the presence of gold nanoparticles at the diseased sites. Modifying the surface of gold nanoparticles by incorporating immunogenic peptides ameliorated their toxicity. This reduction in the toxicity was associated with an increase in the ability to induce antibody response. Gold nanoparticles larger than 50 nm were non toxic to mice, which could be interpreted as a diffusion-restricted region. The non toxic effect of gold nanoparticles smaller than 5 nm could be explained by the increase in antibody response that enhanced the scavenging effect. Further, authors stipulated that urinary excretion may play an important role to remove gold nanoparticles under 5 nm in their model.

Interestingly, Hainfeld et al., 2006 have performed studies with gold nanoparticles of 1.9 nm (gold nanoparticles from Nanoprobes), with the aim to develop the use of such nanoparticles as new X-ray contrast agents. With 10 mg Au/ml initially in the blood, mouse (BALB/C mice bearing EMT-6 subcutaneous mammary tumours) behaviour was unremarkable and neither blood plasma analytes nor organ histology revealed any evidence of toxicity 11 days and 30 days after i.v injection. The highest tissue gold concentration 15 min after injection was in the kidney (10.6 ± 0.2 % of the injected dose per gram of measured tissue – % id/g), followed by tumour (4.2 ± 0.4 % id/g), liver (3.6 ± 0.3 % id/g) and muscle (1.2 ± 0.1 % id/g). However, whole body gold clearance was 77.5 ± 0.4 % of the total injected dose after 5 hours. Muscles and blood were almost gold free 24 hours after injection, while kidney, liver and tumour kept constant gold concentration. Here, the rapid and high level of gold nanoparticle clearance could explain the absence of gold nanoparticle toxicity.

Cho et al., 2009 carried out *in vivo* toxicity study using 13 nm-sized gold nanoparticles coated with PEG, using thiol-terminated PEG (MW 5000). It is worth mentioning that the thiol (SH) function is known to graft strongly to the surface of gold nanoparticles and it is not expected that such surface coating will be displaced by serum proteins. However, serum proteins may interact with the coating. Quite surprisingly, the gold nanoparticles were seen to induce acute inflammation and apoptosis in the liver. The nanoparticles were found to accumulate in the liver and spleen for up to 7 days after i.v injection. In addition, TEM

showed that numerous cytoplasmic vesicles and lysosomes of liver Kupfer cells and spleen macrophages contained PEG-coated gold nanoparticles. Although the transient inflammatory responses were negligible for the toxicity of 13 nm PEG-coated gold nanoparticles, apoptosis was important effects induced by treatment of 13 nm PEG-coated gold nanoparticles.

Despite the establishment of *in vitro* and *in vivo* efficacy using gold nanoparticles in combination with ionizing radiations gold nanoparticles toxicity studies strongly suggest that careful scrutiny of the *in vivo* and *in vitro* toxicities of gold nanoparticles is required even if gold nanoparticles have previously shown to have limited or no toxicity at the cellular level.

3. Enhancement of radiation activity of nanoXray™

3.1. **"Endosomes Bioavailability as Key Parameter for Optimal Efficacy of Radiation Enhancer Nanoparticles on Human Colon Cancer Cells"**

Virginie Simon, Ping Zhang, Laurence Maggiorella, Agnès Pottier, Elsa Borghi, Laurent Levy, Julie Marill ; In Preparation

Medicine is now using physics every day to treat cancer patients. Nanomedicine can help clinicians in delivering safer and more efficacious treatments by shifting the intended effect from the macroscopic to the subcellular level.

NBTXR3 is designed to resolve cancer therapy's biggest drawback: destruction of healthy tissue and its subsequent deleterious side effects when a high dose of X-ray is necessary. Indeed, high dose of X-ray cause burns and necrosis in tissue around reconstructive wires in mandibular cancer patients after radiation treatments (Castillo et al., 1988). The concept of using high Z materials for dose enhancement in cancer radiotherapy was advanced over 20 years ago by Matsudaira et al., 1980, who measured a radioenhancing effect of iodine on cultured cells. Nath et al., 1990 incorporated iodine into cellular DNA with iododeoxyuridine *in vitro*, and found a radiation enhancement of around 3. Regulla et al., 1998 showed a physical dose enhancement factor (DEF) around 100 within a range of 10 µm and a biological enhancement factor of up to 50 for fibroblast monolayers irradiated on a gold foil. Herold et al., 2000 injected 1.5-3 µm gold particles directly into a tumour followed by irradiation and found that excised cells had reduced plating efficiency.

NanoXray^{TM} plateform concept is the design and development of therapeutic metal oxide nanoparticles which core is hard and highly inert and constitutes the matter for nanoparticles – ionizing radiation interactions triggering emission of electrons losing energy and the subsequent creation of free radicals. NBTXR3 nanoparticles have an on-off mode of action. When the beam of ionizing radiation is stopped the nanoparticles return to the basal inert crystalline status.

NBTXR3 nanoparticles are homogenous in size with a mean hydrodynamic diameter of approximately 70 nm. Such range seems relevant for a superior cellular penetration as shown by Chithrani et al., 2006 with gold nanoparticles. Stability in physiological media is achieved by coating the nanoparticles with a surface treating agent, which ensures the stability of the nanoparticles in physiological fluids.

This paper describes for the first time, *in vitro* studies with NBTXR3 oxide metallic nanoparticles. This article shows NBTXR3 nanoparticles trafficking mechanism at cellular level, using complementary and comparative technical methods.

ARTICLE -I-

Endosomes Bioavailability as Key Parameter for Optimal Efficacy of

Radiation Enhancer Nanoparticles on Human Colon Cancer Cells

Virginie Simon, Ping Zhang, Laurence Maggiorella, Agnès Pottier, Elsa Borghi, Laurent Levy, Julie Marill,

(In Preparation)

3.2. Discussion

The goal of this work was to study *in vitro* enhancement of radiation cytotoxicity in HCT 116 colon cancer cells by local activity of NBTXR3 nanoparticles.

Viability assays (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate for WST-1) and clonogenic assays were performed to evaluate NBTXR3 nanoparticles effect on cell survival.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used for NBTXR3 nanoparticles quantification at the cellular level (nanoparticles bound to the cells membrane and/or internalized). Indeed, ICP-MS presents the advantage to quantify NBTXR3 nanoparticles at cellular level, but has the disadvantage to not differentiate between the total amount of nanoparticles internalized or bound to cell membranes.

TEM semi quantitative analysis was investigated for the first time in order to establish nanoparticles trafficking at cellular level, but also to define optimum schedule to achieve significant tumour cells destruction and to visualize the local effect of activated nanoparticles on cells. The aim of visualizing and analyzing ultrathin sections as a prelude to TEM is to reveal the internal structure of HCT 116 cells at a sufficiently high level of resolution. It is also important to remember that the number of images observed and counted on section planes is crucial for the robustness of semi quantitative analysis.

Through all those assays, the ratio between the number of NBTXR3 nanoparticles and cells was kept constant. Hence, we are strongly confident in our results comparison and conclusions between all those assays.

Taking together those complementary approaches, NBTXR3 nanoparticles mechanism of action on cell is proposed. Most appropriate schedules could be defined for nanoparticles use.

We have demonstrated NBTXR3 nanoparticles dose effect on cell viability. Statistical difference on cell viability between radiotherapy control alone and activated NBTXR3 nanoparticles was established for cells incubated with nanoparticles at a concentration of 100 µM and beyond. DEF estimates the increase in radiation cells response triggered by NBTXR3 nanoparticles, in comparison to the irradiation control alone. DEF of 3.45 and 4.88, for NBTXR3 concentration of 400 µM and 4 Gy irradiation, were obtained for colon HT-29 and HCT 116 cells respectively. Local intracellular activity of NBXT3 nanoparticles was proved. Correlation was observed between NBTXR3 nanoparticles cell uptake, cytoplasmic localization and efficacy. Study of interaction between NBTXR3 nanoparticles and HCT 116

cell in term of cell trafficking was performed for the first time. Based on the analysis of TEM and SEM images, we have shown that NBTXR3 nanoparticles penetrated the cells by endocytosis according to physical interaction of NBTXR3 with cell membrane. Nanoparticles assemble as clusters within endosomes in cytoplasmic compartment. Chithrani et al., 2007 compared the exocytosis of spherical gold nanoparticles, with 3 differents sizes, on HeLa cells. The authors showed that after 8 hours of incubation, the fraction of gold nanoparticles with size of 14, 50 and 74 nm exocytosed was equal to 40%, 20% and 10% respectively. In contrast, NBTXR3 nanoparticles were demonstrated to have long residence within the cell cytoplasm.

Although most studies suggest that DNA is damaged indirectly by hydroxyl radicals, electrons can also cause damage to DNA directly, as illustrated in a recent study in which low-energy electrons emitted from metal films were found to cause DNA strand breaks directly (Carter et al., 2007). In our study, local effect of activated NBTXR3 nanoparticles on cells is observed on both cell nucleus and cell organelles. Increase of the number of micronuclei formation was observed for irradiated cells incubated with NBTXR3 nanoparticles when compared to irradiation control alone. Also, significant cells disorganization was observed for cells incubated 24 hours with NBTXR3 nanoparticles prior irradiation.

In vivo efficacy published results, with gold nanoparticles (Hainfeld et al., 2004; Chang et al., 2008), have only been performed with one single and high irradiation dose (25-30 Gy delivered). Interestingly, our *in vitro* results showed that a dose delivered of 2 Gy (using a kilovoltage X-ray beam) was sufficient to induce a significant enhancement of radiation cytotoxicity, for HCT 116 cells incubated with NBTXR3 nanoparticles. Furthermore, preliminary *in vivo* efficacy on mice bearing HCT 116 tumour xenografted was performed with fractionated irradiation and demonstrated very promising tumours eradication. This is an important consideration, because sparing dose to healthy tissue is of primary concern in all radiation therapy procedures.

Next experimental studies should focus on a deeper cell organelles characterization: structures like lysosomes and autolysosomes should be confirmed using specific fluorescence markers. For instance, colocalization of NBTXR3 nanoparticles and lysosomes within live HCT 116 cells should be evaluated using specific alexa-647 lysosomes marker (excitation/emission at 550/568 nm).

Furthermore, HCT 116 cells TEM imaging could be investigated at 4°C to precise the endocytosis pathway.

Finally, studies should be investigated in others tumour cell lines with biologic specificities: their origin (epithelium and mesenchymal origin, different compartmental structure), human healthy versus malignant tumour cells, radiosensitivity characteristics (radiosensitive or radioresistant cancer types).

4. Conclusion

Gold nanoparticles are the single reference in the literature of metallic nanoparticles, activated by ionizing radiations, to treat cancers. Published studies present efficacy and toxicity results, both *in vitro* and *in vivo*, which support foundation for a rational of development of gold nanoparticles in the clinic.

Concerning the *in vivo* toxicity, Chen et al., 2009 showed that naked gold nanoparticles with size ranging from 8 to 37 nm induced severe sickness in mice. Further, Cho et al., 2009 observed that 13 nm-sized gold nanoparticles coated with PEG, using thiol-terminated polyethylene glycol, a strong anchoring surface treating agent, induced acute inflammation and apoptosis in the liver.

Concerning efficacy, only two preclinical studies are reported (Hainfeld et al., 2004; Chang et al., 2008). Of note, very small size of gold nanoparticles was used for those studies (1.9 nm and 13 nm respectively). In Hainfeld study report, nanoparticles are irradiated 2 min following gold nanoparticles i.v. injection. Because of the gold nanoparticles size (1.9 nm), gold nanoparticles are cleared from tumour very rapidly. Gold nanoparticles with increased nanoparticle size around 50-100 nm are believed to be more attractive for clinical applications when considering the nanoparticles cell uptake (Chithrani et al., 2006) and the nanoparticles retention within tumour cells - the smaller nanoparticles are found to exocytose faster and in higher proportion (Chithrani et al. 2007). Furthermore, in these two *in vivo* studies, a very high dose of irradiation was delivered (30 and 25 Gy respectively) in a single fraction, which seems quite unrealistic for clinical application.

Fractionated radiation therapy constitutes the standard of care for cancer treatment. Recent randomized trials have confirmed that hypofractioned whole-breast irradiation (as for other cancer diseases) is equivalent to more conventional whole breast irradiation with respect to local recurrence and cosmetic outcome. For instance, for whole breast irradiation after breast conserving surgery a 1.8 to 2 Gy daily fractions is given 5 times a week to a total dose of 45 to 50 Gy over 5 weeks with the optional addition of a boost to the primary site of 10 to 16 Gy in 5 to 8 daily fractions over 1 to 1.5 weeks. Through empiric observation, it has

become clear that the therapeutic ratio, the balance between tumour cell kill and normal tissue damage, is affected not only by fraction size but also the total dose of radiation and in some instances overall treatment time and the volume of tissue irradiated (Whelan et al., 2008).

NBTXR3 nanoparticles development has included X-ray standard protocols, as routinely used in the clinic for radiotherapy.

Despite of the establishment of *in vitro* and *in vivo* efficacy gold nanoparticles use in the clinic should present some limitations – such as the use of high energy dose delivered in one fraction – which suggest that gold metallic nanoparticles activated by ionizing radiations are delaying for their application to treat cancer. Furthermore, toxicity studies strongly suggest that careful scrutiny of the *in vivo* and *in vitro* toxicities of gold nanoparticles is required even if gold nanoparticles have previously shown to have limited or no toxicity at the cellular level.

In this work, we have demonstrated the NBTXR3 efficacy in clonogenic tests both in HCT 116 and HT-29. No significant clonogenic toxicity of NBTXR3 was observed in these cell lines. Cell viability assays were also performed on HCT 116 following 2 Gy irradiation with significant difference of efficacy when compared to radiotherapy alone.

NBTXR3 are internalized by endocytosis. TEM analysis studies suggest that NBTXR3 nanoparticles stay within the cells cytoplasm. Furthermore, NBTXR3 nanoparticles trafficking at cellular level, evaluated through viability assays and NBTXR3 nanoparticles quantification, support the hypothesis that nanoparticles have long residence within the cells cytoplasm, in late endosomes.

Furthermore, NBTXR3 nanoparticles have demonstrated to bring survival benefit in mice bearing HCT 116 tumour xenografted (data not shown). The time of NBTXR3 nanoparticles residence within tumour was demonstrated to be longer than 15 days.

As opposed to gold nanoparticles trafficking at cellular level reports and currently published *in vivo* efficacy studies, NBTXR3 results demonstrate a real advantage for use with standard radiotherapy protocols.

Pp IX silica nanoparticles create differential biodistribution between tumour and healthy tissues in terms of tumour uptake and subcellular localization: photodynamic therapy therapeutic window enlargement

1. Photodynamic therapy mechanism of action

Three components are essential in the PDT reaction: the photosensitizer, the light with appropriate wavelength and the presence of oxygen. The interaction among these three constituents is the main part of the treatment effect.

A photosensitizer is a chemical compound that can be excited by light of a specific wavelength. This excitation uses visible or near-infrared light. In photodynamic therapy, either a photosensitizer or the metabolic precursor of one is administered to the patient. The tissue to be treated is exposed to light suitable for exciting the photosensitizer. Usually, the photosensitizer is excited from a ground singlet state to an excited singlet state. It then undergoes intersystem crossing to a longer-lived excited triplet state. One of the few chemical species present in tissue with a ground triplet state is molecular oxygen. When the photosensitizer and an oxygen molecule are in proximity, an energy transfer can take place that allows the photosensitizer to relax to its ground singlet state, and to create an excited singlet state oxygen molecule (Josephsen et al., 2008; Figure 37). Singlet oxygen is a very aggressive chemical species and will very rapidly react with any nearby biomolecules. It is important to note that although PDT results in singlet oxygen species, these reactive radicals are short-lived (µs), with a radius of action of only 0.01 µm, and therefore have very low mutagenic potential for DNA damage. The specific targets depend heavily on the photosensitizer chosen, a point detailed in a later paragraph. Ultimately, these destructive reactions will kill cells through apoptosis or necrosis.

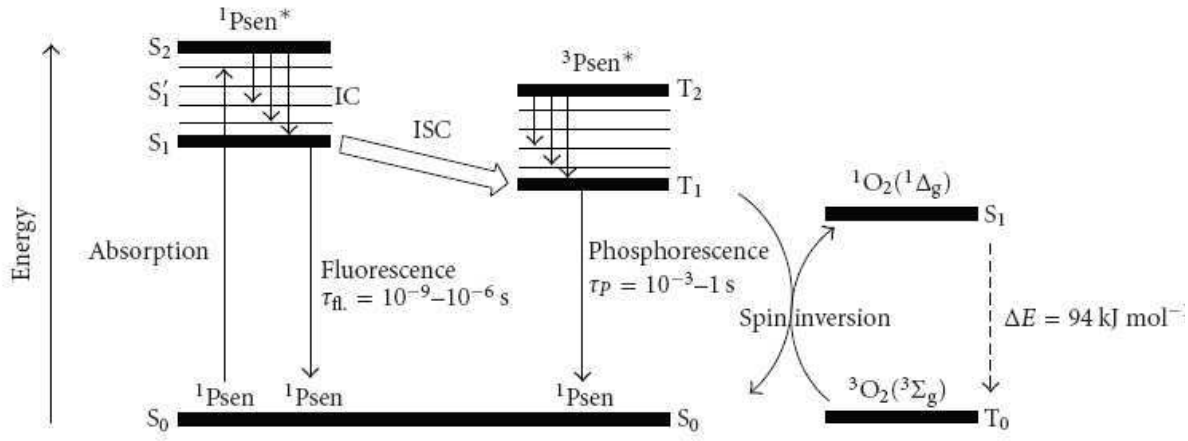


Figure 37: Modified Jablonski energy diagram (Josefsen et al., 2008)

2. Photosensitizer: light excitation

2.1. Optimum for superficial tumours: light penetration in tissues

To carry out PDT successfully *in vivo*, it is necessary to ensure that sufficient light reaches all the diseased **tissues**. This involves understanding how light travels through various tissues and the relative effects of light absorption and scattering. In PDT, it is important to be able to **predict the spatial distribution of light within the target tissue**. Light is either scattered or absorbed when it enters tissues and the extent of both processes depends on the tissue type and light wavelength utilized (Robertson et al., 2009). The light transmission has been experimentally measured for various tissues as a function of the sample thickness and of the wavelength of the incident light. Interestingly, the maximum fluence rates occur below the **surface**. The position of this peak shifts more and more into the tissue with increasing wavelength. Although large variations in the absolute values arise, owing to differences in the biological material, the relative trend of the spectral dependence is quite similar. Most tissues show an increased transparency towards higher wavelengths with a **maximum penetration depth between 700 and 800 nm** (Ochsner et al., 1996). This is also involved because the biological tissue is **inhomogeneous** and the presence of microscopic inhomogeneities (macromolecules, cell organelles, organized cell structure, interstitial layers) makes biological tissues turbid. Multiple scattering within a turbid medium leads to spreading of a light beam and loss of directionality. Absorption is largely due to endogenous tissue chromophores such as hemoglobin, myoglobin and cytochromes. The light source for PDT must exhibit suitable spectral characteristics which coincide with the maximum absorption wavelength range of the photosensitizer applied in order to generate enough ROS to produce a cytotoxic effect. Thus, for efficient photodynamic laser mediated therapy, light excitation between 700 and 800 nm is preferred to allow an **increased penetration depth with minimal light scattering while expecting maximum photosensitizer activation**, with the most tissue destruction occurring within the targeted tissue (Robertson et al., 2009).

Photosensitizer from porphyrin family, and their lead product Photofrin®, are the first generation of photosensitizer developed for PDT. On account of their highly conjugated skeleton, porphyrins have a characteristic ultra-violet visible spectrum which consists of an intense Soret band at approximately 400 nm, followed by four longer weak absorptions (ε > 20 000 $lmol^{-1}cm^{-1}$) Q bands (500, 540, 570 and 630 nm respectively) (Figure 38).

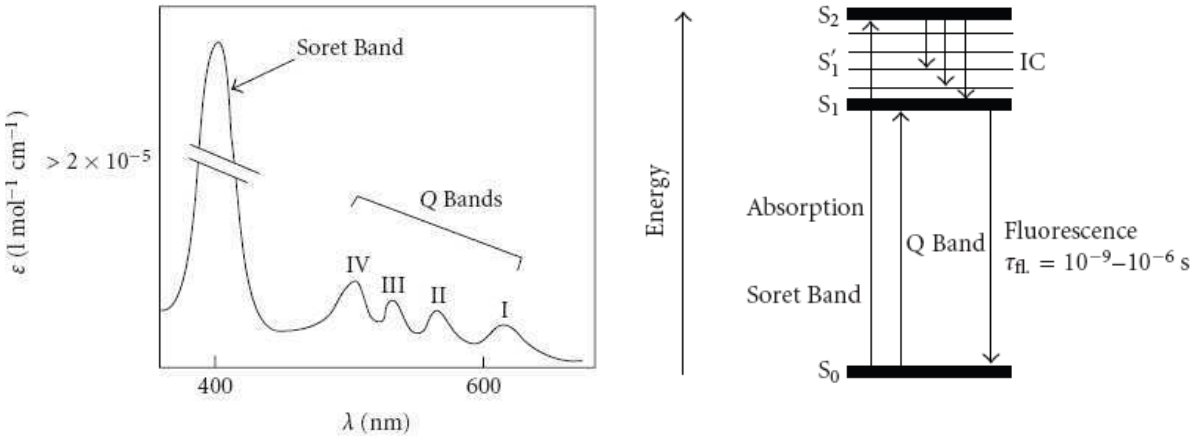


Figure 38: Typical porphyrin absorption spectrum and modification of Jablonski energy diagram (Josefsen et al., 2008)

Despite the weak absorption properties of Photofrin® in the near-infrared region, the 630 nm absorption peak was selected for light excitation in order to penetrate tissues. However, for light in the near infrared region (630 nm), a "rule of thumb" is that about **2-5 mm** of tissue can be treated, depending on the optical properties of the tissue, and the dosimetry used (Wang et al., 1999; Figure 39). That is why the most common application of PDT was intended so far, for superficial tumours treatment.

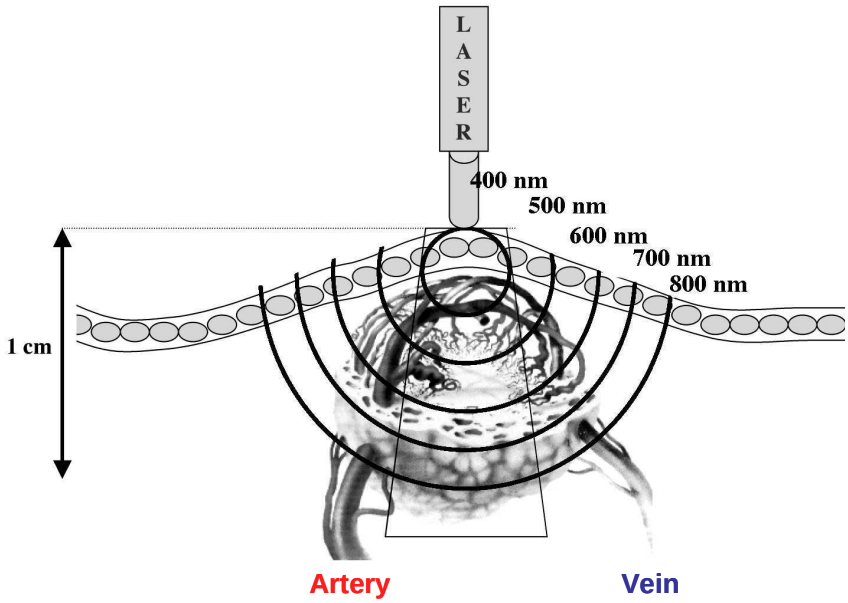


Figure 39 : Light penetration deep in tissues function wavelength: limitation of PDT (Croisy et al., 2005)

2.2. Chemistry improvement: second, third generation of photosensitizer

While the major disadvantages associated with the **first generation photosensitizer hematoporphyrin (HpD) and Photofrin®** have not prevented the treatment of some cancers and other diseases, they have markedly reduced the successful application of these photosensitizers to a wider field of disease.

It is today commonly admitted that a well-designed photosensitizer should exhibit the following properties: long-wave absorption, good singlet oxygen quantum yield, quite simple structure and easy synthesis process route, good *in vivo* stability and reduce adverse effects. This area of research led to the development of a **second generation of photosensitizers**, designed to minimise the drawbacks of the first generation photosensitizer. A number of new sensitizers were therefore developed to overcome these short comings such as 5-Aminolaevulinic acid (5-ALA), Verteprofin, Purlytin, Foscan®, Lutex, ATMPn, Zinc phthalocyanine CGP55847 and Naphthalocyanines (Figure 40).

CLASS OF PHOTOSENSITISER	LONGEST WAVELENGTH ABSORPTION/nm	EXTINCTION COEFFICIENT/ $M^{-1}cm^{-1}$	DRUGS IN CLINICAL TRIAL (Phase I–III)	DRUGS APPROVED FOR PDT (PRECLINICAL AND CLINICAL)
Porphyrins	620–640	3,500	—	Photofrin Levulan Metvix
(Expanded Porphyrins)				
Porphycenes	610–650	50,000		ATMPn
Texaphyrins	730–770	40,000	Lu-Tex Optrin Antrin Xcytrin Benzvix Hexvix	
Chlorins	650–690	40,000	Foscan Puryltin	Visudyne
Bacteriochlorins	730–800	150,000	—	—
Phthalocyanines	680–780	200,000	CGP55847 PC4 Photosense	—
Naphthalocyanines	740–780	250,000	—	—

Figure 40: Summary of a collection of different photosensitiser types and their absorption data (Josefsen et al., 2008)

A number of the second generation photosensitizer described earlier contains a chelated central metal ion. Expanded porphyrins have a larger central binding cavity, increasing the number of potential metals it can accommodate. The metallation of a number of these chromophores has generated a variety of photosensitizers with improved photophysical properties. The effectiveness of these metallo-photosensitizers depends largely (but not definitively) on the nature of the coordinated central metal ion. Chromophores chelated to diamagnetic transition metals and lanthanide ions have shown the greatest potential as photodynamic agents, a consequence of the heavy metal effect enhancing the rate of intersystem crossing. As a result, a number of these metallated tetrapyrrole-based macrocycles are currently photosensitizers of choice, particularly the zinc (II), aluminium (III), and tin (IV) complexes. Ochsner et al (1996) showed that the Zinc(II)-phthalocyanine absorption maximum lies at about 670 nm and its associated extinction coefficient is at least 60 times larger than that of Photofrin® at its treatment wavelength (630 nm). **The greater the extinction coefficient value,** the smaller is the drug dosage required to induce a cytotoxic response and the risk of provoking systemic toxic reactions is clearly diminished. **The longer absorption wavelength** correlates with a higher penetration depth. Since the skin phototoxicity is dominated by the absorption of sunlight, a shift of the main absorption peak to higher wavelengths and a smaller overlap of the absorption spectrum of the sensitizer with the sunlight emission spectrum are expected to reduce the risk of inducing phototoxic reactions.

Despite significant improvement in the design of photosensitizer for a more efficient interaction with laser light and a subsequent enhancement of ROS generation, many drawbacks are pendant which had reduced their development in the clinic. Indeed, most of the developed photosensitizers are hydrophobic and aggregate easily under physiological condition which results in the difficulty in preparing pharmaceutical formulations that enable parenteral administration. Moreover, even with hydrophilic ones, the accumulation selectivity to specific cells or tissues is still usually too low for clinical use.

Works have recently focused on designing a **third generation** of photosensitizer, with specific designed to effect greater selectivity and specificity toward targeted tissues.

Maillard and his colleagues (2007) have screened 17 new potential products for PDT application. *In vitro* photocytotoxicity of hydrophenylporphyrins and chlorines and their glycoconjugated derivated (glucose/galactose/mannose) were tested both in retinoblastoma cell line (Y79) and HT-29 cell line. The goal was to determine whether the covalent coupling

of glycoside residues to the macrocycle would lead to a significant enhancement in PDT retinoblastoma treatment for the derived conjugates relative to the parent free OH compounds. These results showed that the nature and anomeric configuration of the sugar component are important for both *in vitro* cytotoxicity and efficacy. For instance, glycosylated porphyrins in which the galactose component is separated from the *p*-hydroxyphenyl motif by a diethyleneglycol linker display a 10 times greater photocytoxicity for α configuration compared by β one.

At present, the next generation of photosensitizer is the development of **nanocarriers**. Nanocarrier is intended to: (1) protect a drug from degradation, (2) enhance drug absorption by facilitating diffusion through epithelium, (3) modify pK and drug tissue distribution profile, and (4) improve intracellular penetration and distribution (Couvreur et al., 2006). Those nanocarrier systems are developed to induce greater selectivity and specificity when compared with the "free" photosensitizer molecule in order to increase the therapeutic effect.

2.3. Presentation of approved photosensitizers and current research

2.3.1. Porphyrin, texaphyrin and chlorin photosensitizers in clinics

Porphyrins are a heterocyclic aromatic ring made from four pyrrole subunits joined on opposite sides through four methine links. Most current clinical porphyrin photosensitizers derivate are Photofrin®, Levulan®, Metvix® and Visudyne®.

Photofrin® was the first drug for PDT to receive regulatory approval for the treatment of obstructing oesophageal cancer in 1993 in Canada and in 1995 in US. Photofrin® is commercially available from Axcan Pharma, Inc. and has the longest clinical history and patient track record (Figure 41). The photosensitizer consists of about 60 compounds and therefore it is difficult to reproduce its composition. It is actually a proprietary combination of monomers, dimers, and oligomers. It should be noted that the first reports of PDT success in patients using Photofrin® were on **bladder** cancer treatment (Allison et al., 2004).

	Disease	Drug	Country
Pre-cancer	Actinic keratosis	Levulan, Metvix®	EU
	Barett's oesophagus	Photofrin®	EU, US.
	Cervical dysplasia	Photofrin®	Japan
Cancer	Basal-cell carcinoma	Metvix ®	EU
	Cervical cancer	Photofrin®	Japan
	Endobroncheal cancer	Photofrin®	Canada, Denmark, Finland, France, Germany, Ireland, Japan, The Netherlands, UK, US
	Oesophageal cancer	Photofrin®	Canada, Denmark, Finland, France, Ireland, Japan, The Netherlands, UK, US.
	Gastric cancer	Photofrin®	Japan
	Head and neck cancer	Foscan®	EU
	Papillary bladder cancer	Photofrin®	Canada

Figure 41: Type of cancer and approved PDT drugs

Texaphyrins are expanded, porphyrin-like macrocycles which complex large metal cations. The dyes have a high absorbance peak in the near infrared, 730-770 nm. Most current clinical texaphyrin photosensitizer derivate is Antrin®.

Chlorins constitute a group of molecules very similar to porphyrins. Chlorins can also be synthesized from porphyrins. In contrast to porphyrins, chlorins have the strongest absorption peaks in the red part of the spectrum, which give the compounds a green colour. In comparison to the porphyrin structure, the chlorin has at least one double bond missing in the pyrrole rings. Most current clinical chlorin photosensitizers derivate are Foscan®, LS11 and Photochlor (Figure 42).

Platform	Drug	Substance	Manufacturer
Porphyrin	Photofrin®	HpD	Axcan Pharma, Inc.
	Levulan®	ALA	DUSA Pharmaceuticals, Inc.
	Metvix®	M-ALA	PhotoCure ASA
	Visudyne®	Vertiporfin	Novartis Pharmaceuticals
Texaphyrin	Antrin®	Lutexaphyrin	Pharmacylics
Chlorin	Foscan®	Temoporphin	Biolitec Pharma Ltd.
	LS11	Talaporfin	Light Science
	Photochlor	HPPH	RPCI

Figure 42: List of the current clinical photosensitizers and their manufacturers

2.3.2. Others trends

Development of new photosensitizers as well as the optimization of PDT protocols, such as fractionation of light or drugs, well-designed clinical trials which involve selectively localized photosensitizers and convenient light sources will improve the prospects for the use of PDT in cancer.

In addition, current research topics are emerging with the ambition to enlarge the possibility for PDT application, by either increasing the photosensitizer light interaction using already approved photosensitizers (two photon absorption concept), or by bringing additional modalities to current treatments such as combined diagnostic and PDT (theranostic application) or even combined therapy.

▪ **Two photon absorption** (TPA): One of the most relevant remaining limitation of PDT is the limited light penetration within tissues. TPA-induced excitation of photosensitizer is a promising approach for increasing light penetration because it makes it possible to use two photons of lesser energy (higher wavelength) to produce an excitation that would “normally” be produced by the absorption of a single photon of higher energy (lower wavelength). Indeed, appropriate photosensitizers can simultaneously absorb two photons of lower energy, which makes excitation possible in the near IR region, thereby avoiding

wasteful tissue absorption or scattering and allowing a deeper penetration of light into the tissue. Kim et al., 2007 describ the synthesis of organically modified silica nanoparticles in which 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide **(**HPPH) and an excess of 9,10-bis[4'-(4''-aminostyryl)styryl]anthracene (BDSA), a highly two-photon-active molecule acting as an energy donor, were co-encapsulated. HPPH absorption in nanoparticles had significant overlap with the fluorescence of BDSA aggregates, which enabled an efficient energy transfer through fluorescence resonance energy transfer (FRET) mechanisms. After indirect two-photon excitation (850 nm), the authors provided evidence that the energy of the near-IR light was sufficiently upconverted by BDSA aggregates to be able to excite HPPH, leading to formation of $^1O_2$. The intracellular FRET efficiency was estimated as being ~36%. Drastic changes in the morphology of the cells were observed, which were indicative of impending death as a result of photocytotoxicity of HPPH.

▪ **Diagnostic and therapy:** Lai et al., 2008 present a highly uniform Fe_3O_4/SiO_2 core/shell nanoparticles functionalized by phosphorescent iridium (Ir) complexes to form Fe_3O_4/SiO_2 Ir. Authors have strategically designed a hydrophilic Ir complex suited for simultaneous phosphorescence imaging and $^1O_2$ production. MTT assays on HeLa cells showed that almost 100% of the cells were viable without activation, even after 24 hours of incubation with 100 mg/ml of Fe_3O_4/SiO_2 Ir. Cellular uptake was dose responsive and the uptake could be imaged by MRI even at the lowest concentration (detection maximal of 10^4 cells). In this study, Fe_3O_4/SiO_2 Ir composite demonstrated its potential in multiple applications, the magnetic core providing the capability for MRI and the Ir complex greatly enhancing the spin-orbit coupling for phosphorescent labeling, and simultaneous $^1O_2$ generation inducing apoptosis.

▪ **Combined therapy**: Gu et al., 2005 report the synthesis, characterization, and cellular uptake of the conjugate of porphyrin and iron oxide nanoparticles, which may lead to a bimodal anticancer agent which could be used in the combinational treatment of PDT and HT. The HeLa cells were incubated with the Fe_3O_4-porphyrin nanoparticles at 37 °C for 5 hours without showing observable dark toxicity. When cells were exposed to irradiation during 10 min the cells changed their conformation and went in apoptosis cell death. Cells were then observed under a fluorescence microscope. Fe_3O_4-porphyrin nanoparticles are uptaken by the HeLa cells, likely as the result of endocytosis into the cytoplasm. Also HT therapy was not studied, the thermal stability of the nanoparticle was performed. Following

nanoparticles boiling in a water-methanol solution for 30 min, no loss of porphyrin fluorescence spectrum was detected, suggesting a good tolerance of the nanoparticles and a subsequent eligible candidate for HT.

3. Systemic administration of photosensitizer for very localized treatment

3.1. Half life of generated ROS and distance for ROS effect: subcellular localization of the photosensitizer is required

PDT is an anticancer approach, which achieves tumour cell destruction after light excitation of the photosensitizer within a well restricted area. The high selectivity of tumour site cytoxicity is based on the application of the external energy on the cancer anatomical localisation for agents usually administered by systemic route and with broad body distribution.

Also, these sites of photodamage may reflect the localisation of the photosensitiser in the cell, in subcellular organelles and close to biomolecules. A variety of cellular components such as amino acids (particularly cysteine, histidine, tryptophan, tyrosine and methionine), nucleosides (mainly guanine) and unsaturated lipids can react with singlet oxygen. The diffusion distance of singlet oxygen is relatively short (about 0.01 μm), therefore the photosensitiser must associate intimately with the substrate for efficient photosensitisation to occur.

Many factors determine the cellular targets of photosensitizer. The incubation parameters and mode of delivery as well as the chemical nature of the photosensitiser can all influence subcellular localisation, creating a number of potential targets for photodamage. In cell culture studies with porphyrin based photosensitizer, short incubation times (up to 1 hour) prior to illumination leads primarily to membrane damage whereas extended incubation periods followed by light exposure results in damage to cellular organelles and macromolecules.

Hydrophobic (lipophilic) compounds preferentially bind membranes and will target structures such as the plasma membrane, mitochondria, lysosomes, ER and the nucleus. Oxidative degradation of membrane lipids can cause the loss of membrane integrity, resulting in impaired membrane transport mechanisms and increased permeability and rupturing of membranes. Cross-linking of membrane associated polypeptides may result in the inactivation of enzymes, receptors and ion channels. Photosensitizers with anionic substituents, such as

sulphonate or carboxyl groups, have been observed to localize preferentially in the cytoplasm and relocate to the nucleus upon illumination (Patito et al., 2001), whereas lipophilic photosensitizers functionalized with cationic groups are believed to (preferentially) traverse the mitochondrial membrane and accumulate in the mitochondrion (Dummin et al., 1997) - the subcellular organelle widely demonstrated to be a key component in the preferred (apoptotic) cell death pathway. Exactly which physicochemical/structural properties and mechanisms are behind these specific distributions and localizations and how to maximize tumour tissue selectivity over normal tissue accumulation are issues still under investigation.

Figure 43 (from Castano et al., 2005) illustrates some of the cellular and molecular signalling pathways that have been determined to occur in cells treated with PDT *in vitro* (Argarwal et al., 1991; Dahle et al., 1997).

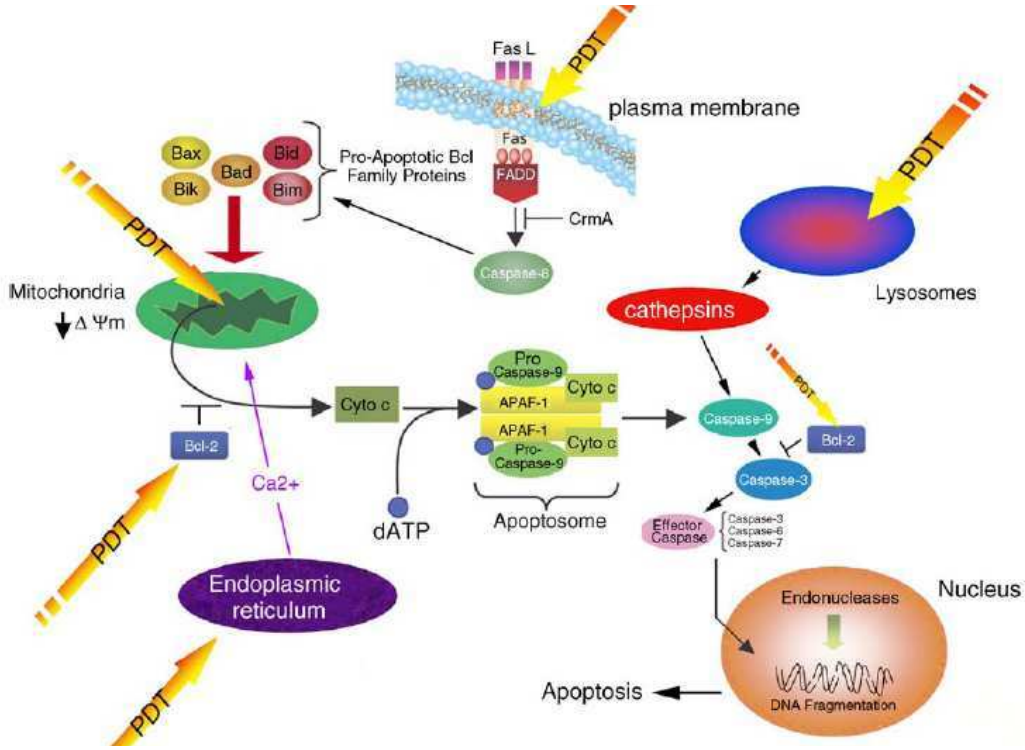


Figure 43: Cellular signalling pathways leading to apoptosis in cells after PDT. ROS generation depending photosensitizers localization (Castano et al., 2005)

3.2. Pharmacokinetics, pharmacodynamic and subsequent adverse effects of photosensitizer: example of Photofrin®

3.2.1 Pharmacokinetic

The pK of porfimer sodium (Photofrin®) has been studied in mice, rats, guinea pigs, dogs, and also humans. The results of pK investigations are basically very similar and are always the same for both genders. In general Photofrin® is infused at 2 mg/kg in an outpatient setting. About 24 to 48 hours later illuminations occur generally by a diffusing fiber or more rarely by a micro lens (which is unidirectional). Depending on the clinical situation light dose of 150 J/cm^2 (lens) or 200-300 J/cm^2 is employed.

- Pharmacology study in *in vivo* models:

For general conditions in **mice**, no effect was observed in 25 or 50 mg/kg porfimer sodium administered intravenously. At 100 mg/kg and 200 mg/kg there were some disorders observed and beyond at 200 mg/kg all animals died at least within 3 days which is not surprising because this is within the range of the Lethal Dose 50 (LD_{50}). With regard to the central nervous system, porfimer sodium administered intravenously at doses of 50 mg/kg and above resulted in decreased movement, lengthened thiopental-induced sleep, and inhibition of strychnine–induced convulsions. A transient lowering of body temperature at 50 mg/kg in **rats** was observed which returned to normal 3 hours later. Movement of isolated **guinea pig** ileum was inhibited by porfimer sodium at 10 μg/ml, a plasma level which may be equivalent to 5 mg/kg administered intravenously to mice. In heart and circulatory system there was no effect on blood pressure, pulse rate and electrocardiogram when tested in dogs up to the highest dose of 16 mg/kg. There was no influence on lung function.

- Example of pK study in human:

Following a 2 mg/kg dose of porfimer sodium to four male **cancer patients**, the average peak plasma concentration was 15 ± 3 mcg/ml (microgram/ml), the elimination $t_{1/2}$ was 250 ± 285 hours, the steady-state volume of distribution was 0.49 ± 0.28 l/kg, and the total plasma clearance as 0.051 ± 0.035 ml/min/kg. The mean plasma concentration at 48 hours was 2.6 ± 0.4 mcg/ml. The influence of impaired hepatic function on Photofrin® disposition has not been evaluated. *In vitro* studies have shown that Photofrin® was covered

by protein from human serum at a very high level (approximately 90%). The binding was independent of concentration over the concentration range of 20-100 mcg/ml. A course of therapy consisted in one injection of Photofrin® (2 mg/kg administered as a slow i.v injection over 3-5 min) followed by up to two nonthermal applications of 630 nm laser light. Doses of 300 J/cm of tumour length were used in oesophageal cancer. Doses of 200 J/cm were used in endobronchial cancer for both palliation of obstructing cancer and treatment of superficial lesions. The first application of light occurred 40-50 hours after injection. Debridement of residua was performed via endoscopy/bronchoscopy 96-120 hours after injection, after which any residual tumour could be retreated with a second laser light application at the same dose used for the initial treatment. Additional courses of PDT with Photofrin® were allowed after 1 month, up to a maximum of three courses (FDA report, 2000).

- Some specific pK parameters:

A. Absorption-bioavailability

Intravenous (i.v) or intraperitoneal (i.p) single dose (5 mg/kg) pK in mice indicated a long half-life for plasma elimination of residual [^{14}C]-radioactivity associated with the porphyrins in porfimer sodium or with metabolites: triexpontentially decrease with elimination half-lives of about 0.75 (α), 10 (β) and 220 (γ) h, following i.v dosing, and biexponentially after i.p with elimination half-lives of 4 and 220 hours.

B. Metabolism

Biotransformation is difficult to interpret with complex mixtures such as Photofrin®, and metabolism studies per se were not conducted with Photofrin®. However in a biliary excretion study in the rat, the amount of haematoporphyrin (HP) monomer excreted in the bile within 48 hours after dosing was twice the amount of HP injected in the dosing preparation. This indicates that some of ester/ether linkages in the porphyrin excreted dimer/oligomer fraction were hydrolysed *in vivo*.

C. Excretion

There is only one investigation for the elimination profile of the ^{14}C porfimer. Urine and feces of rats were collected each 24 hours for 7 days. The major route (42 %) of elimination of ^{14}C porfimer sodium was via the feces, whereas only 4% of the dose was

excreted in the urine. It is suggested that the fecal route is mainly via bile excretion because 23 % of porfimer were excreted into bile within 48 hours in **rats**. Also this is in accordance with **human** data where excretion data have shown that the major route of elimination is fecal suggesting biliary excretion of 28 % of the i.v dose over 72 hours.

D. Others

In an animal model (**hamster)** bearing a pancreatic tumour it was shown that porfimer had a high affinity to tumour tissue and was retained for a long time in this tumour tissue. In other similar studies in mice it was shown that the amount of labelled hematoporphyrin derivate in tumour tissue was in fact higher than in muscle and skin but lower than in liver, spleen and kidney which were the favoured organs for distribution. The placental transfer of Photofrin® was studied in pregnant **rats** (n=5) following a single 20 mg/kg i.v. dose of Photofrin® given on day 18 of gestation. No porphyrin derivatives were detected in amniotic fluid or foetuses. The transfer of porfimer sodium into breast milk was studied in lactating rats (n=5) following a single 20 mg/kg i.v. dose on day 9 after delivery. Trace concentrations of porphyrins excreted were found in breast milk between 6 and 48 hours after dosing with a maximum concentration of 7.4 µg/ml at 24 hours post injection (EMEA, 2004).

3.2.2. Pharmacodynamic

A. Distribution in *in vivo* models

In **mice and rats**, the results showed that, although most of an i.v. dose was removed from circulation by the **liver, spleen and kidney**, potentially useful concentrations were retained for long periods in other tissues, including implanted human tumours. The protein binding in rat, dog and human was comparable within the species between 80 and 90 % and was not dependent on the concentration of porfimer sodium. It could be shown that porfimer sodium has a strong affinity to lipoprotein, especially to low LDL. In plasma, lipoproteins may be porphyrin carriers to the tumour tissue and its corresponding receptors.

A specific biodistribution study showed the highest content of Photofrin® in liver, kidney and spleen (Figure 44). Porphyrin content in tumour is smaller than in these organs, and the porphyrin concentration does not exceed 10 µg/g wet tissues, while in liver, kidney and spleen it reaches 70, 20.4 and 20.5 µg/g respectively. Quantification of the distribution and the pK of Photofrin® in normal and tumour tissue biopsies of the human bile duct have been investigated. Results demonstrated that Photofrin® accumulated in human bile duct

adenocarcinoma with a tumour to normal tissue fluorescence ratio of about 2:l after 24 and 48 hours of Photofrin® administration. Skin biodistribution was very low compared with others organs (Pahernik et al., 1998).

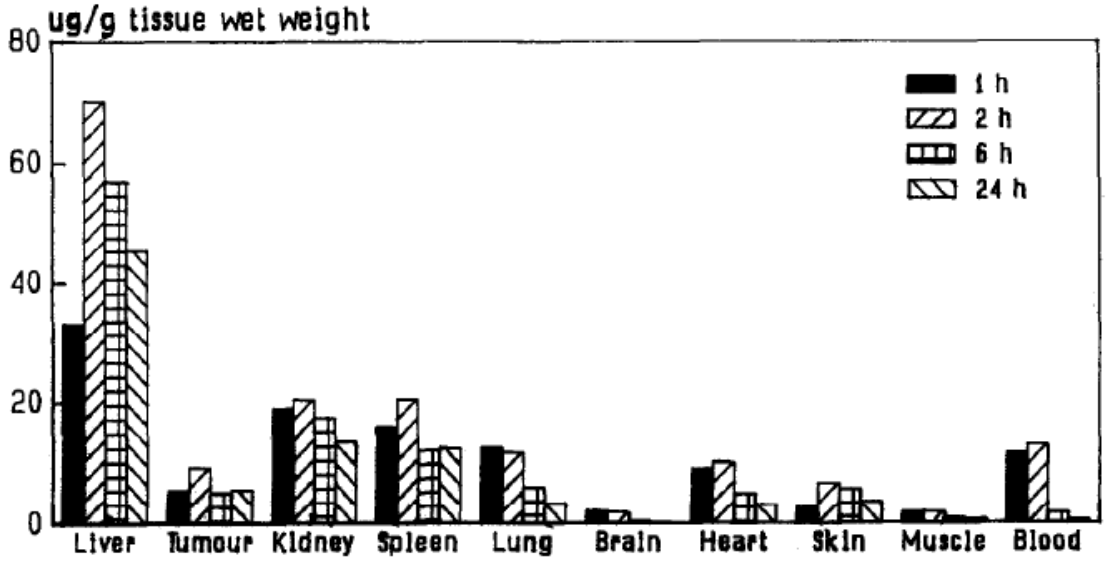


Figure 44 : Example of *in vivo* Photofrin® biodistribution study: single i.v. administration (20 mg/kg) after 1, 2, 6 and 24 hours in nude mice implanted with a small cell lung carcinoma (Tronconi et al., 1995)

3.2.3. Side effects

Photofrin® causes long lasting **cutaneous photosensitivity**, as it is absorbed by the skin. For this reason, patients who have been treated with Photofrin® need to **avoid sunlight for 4-6 weeks** (Dolmans et al., 2003). These side effects are very special and incapacitating.

In fact, **all** patients who receive Photofrin® will be photosensitive and must observe precautions to avoid exposure of **skin and eyes** to direct sunlight or bright indoor light (from examination lamps, including dental lamps, operating room lamps, unshaded light bulbs at close proximity, etc.). The photosensitivity is due to residual drug which will be present in all parts of the skin. Exposure of the skin to ambient indoor light is, however, beneficial because the remaining drug will be inactivated gradually and safely through a photobleaching reaction.

Many changes at the molecular level have been associated with Photofrin®. Diminution of the activity of a number of **enzymes** involved in membrane biosynthesis,

particularly acyltransferases was noted. Mitochondria are a significant intracellular target for Photofrin®, and inhibition of the associated respiratory processes produces cell death. Human bladder transitional carcinoma cells, following sublethal PDT treatment, were found to release arachidonic acid metabolites, mainly thromboxane B2 and PG E2 in a biphasic manner. Photocytotoxicity can be partially mitigated by agents such as **indomethacin** which block the action of PGE, suggesting a relationship between PGE and photodynamic cell damage. PDT treatment of macrophages *in vitro* stimulates the production of tumour necrosis factor (TNF), a cytokine known to induce haemorrhagic necrosis of tumours. The urine of bladder cancer patients following Photofrin® has been shown to contain elevated levels of the **cytokines** interleukin-1 (IL-1), interleukin-2 (IL-2) and TNF. It is not known whether this is a direct or indirect effect of PDT. Mitomycin C, which blocks cells at G2/M, enhances the effects of PDT. In a human colon adenocarcinoma cell line mitomycin C treated cells were shown to take up significantly higher levels of Photofrin® than untreated controls. Etanidazole, misonidazole and trifluoro-misonidazole were all found to be **photoprotective** against Photofrin® when cells were incubated for 24 hours with Photofrin® under aerobic and limited oxygen (0.3%) conditions (EMEA, 2004).

4. Nanocarriers players: a way to enhance selectivity and specificity of photosensitizer to improve their tumour bioavailability

Work has recently focused on designing systems to induce greater selectivity and specificity of photosensitizer in order to enhance tumour cell uptake. The principle of using carriers may increase the tumour concentration and thus, lead to high bioavailability to improve the therapeutic effect.

4.1. Silica-based nanocarriers of photosensitizers: literature survey

Regarding the literature, several ways of nanocarrier's synthesis have been reported. Very complex to very simple synthesis has been addressed and products tested both *in vitro* and *in vi*vo models.

-Nanocarrier: photosensitizer covently linked

Ohulchanskyy et al., 2007 report a new formulation of nanoparticles (organically modified silica (ORMOSIL) nanoparticles) in which the photosensitizer based on iodobenzylpyropheophorbide, derivated of HPPH, was covalently attached to the silica matrix. They found that the covalently incorporated photosensitizer molecules retained their spectroscopic and functional properties and could robustly generate cytotoxic singlet oxygen molecules upon photoirradiation. The advantage offered by this covalently linked nanofabrication (≈20 nm) is that the drug is not released during systemic circulation, which is, according to the authors, often a problem with physical encapsulation. These nanoparticles are also avidly uptaken by tumour cells *in vitro* and demonstrate phototoxic action, thereby highlighting their potential in diagnosis and PDT of cancer.

Brevet et al., 2009 report new class of mesoporous silica nanoparticles based on $Si(OEt)_4$±mannose with size approximately of 100 nm. Particles were elaborated by **covalent** incorporation of a water-soluble photosensitizer and by covering their external surface with mannose residues. Authors have proved that these mannose functionalized mesoporous silica nanoparticles presented a much higher *in vitro* photoefficiency (MTT test) in MDA-MB-231 breast cancer cells than non-functionalized nanoparticles. The higher efficiency of mannose-functionalized mesoporous silica nanoparticles must be due to an active endocytosis via mannose receptors.

Rossi et al., 2008, in a chemical study, present the synthesis of Pp IX silica nanoparticles. The entrapment of Pp IX in silica spheres was achieved by modification of Pp IX molecules with an organosilane reagent. Pp IX was **firmly attached** to the silica matrix. Generation of singlet oxygen was measured in higher proportion than **free photosensitizer**. This means that the immobilization of the Pp IX molecules increased the potential of the photosensitizer to perform PDT.

Tu and colleagues, 2009 report a surface modification process that conjugates a photosensitizer, Pp IX, with mesoporous silica nanoparticles through covalent bonding to obtain Pp IX-modified nanoparticles for PDT study. Mesoporous nanoparticles were hexagonal disk shape with average particle size of 110 nm and thickness of 90 nm. *In vitro* tests performed with HeLa cells revealed high cellular-uptake efficiency and the phototoxicity

was found to be both irradiation time- and dosage-dependent. In order to determine the proper incubation time, HeLa cells were incubated with nanoparticles suspension for 0.5, 1, 2, and 4 hours. Authors found that after 2 hours of incubation, most cells (95%) have uptaken nanoparticles. Cells treated with nanoparticles showed significant intracellular staining due to Pp IX in the cytoplasm, indicating the accumulation of nanoparticles. The cytotoxicity performed after 2 hours treatments were examined using the MTT assay. Results indicated that both necrosis and apoptosis can be induced in HeLa cells after nanoparticles-mediated PDT.

- Nanocarrier: photosensitizer no covently linked

Roy, and colleagues, (2003) report encapsulation of the hydrophobic photosensitizer **HPPH**, which is currently undergoing phase I and II clinical trials for esophageal cancer, by controlled hydrolysis of triethoxyvinylsilane in micellar media. *In vitro* experiments showed significant level of cell death for both HPPH-Tween-80 micelles and HPPH-nanoparticles (30 nm) on HeLa and UCI-107 ovarian carcinoma cell lines.

Yan et al., 2003 compare the spectroscopic properties of free meta-tetra(hydroxyphenyl)-chlorin (*m*-THPC) and of *m*-THPC embedded in **silicon-based nanoparticles**. An interesting result was that $^1O_2$ delivery by the nanoparticles exceeded that of the free *m*-THPC.

Tang et al., 2005 show study of nanoparticles loaded with methylene blue (MB). Encapsulation of MB was performed in three types of sub-200 nm nanoparticles: polyacrylamide, sol-gel silica and organically modified silicate (ORMOSIL). *In vitro* PDT study using the MB-loaded polyacrylamide nanoparticles was conducted on rat C6 glioma tumour cells with positive photodynamic results. The 3 silica-based nanoparticles are worth undergoing additional investigation.

He et al., 2009 have developed bifunctional nanoparticles-based carrier for simultaneous PDT and *in vivo* imaging by encapsulating MB alone in a phosphonate terminated silica matrix. Both *in vitro* and *in vivo* PDT studies were performed on HeLa cells treated with MB-encapsulated silica nanoparticles. *In vitro* cytotoxicity (without activation) tests on HeLa cells were performed. Concentration of the MB-encapsulated nanoparticles with 1 mg/ml was the optimal concentration for efficient phototoxicity with minimum

cytotoxicity without activation. Then, *in vivo* tests were investigated on mice bearing Hela tumours and preliminary results showed that particles can effectively induce cell death. Furthermore, it was possible to visualize *in vivo* in real time tumour obtained with optical imaging system.

4.2. Selection the of simplest process route to design Pp IX silica-based nanocarrier

"One Pot Synthesis of New Hybrid Versatile Nanocarrier Exhibiting Efficient Stability in Biological Environment for Use in Photodynamic Therapy"

Edouard Thiénot, Matthieu Germain, Kelthoum Piejos, Virginie Simon, Audrey Darmon, Julie Marill, Elsa Borghi, Laurent Levy, Jean-François Hochepied, Agnès Pottier ; Submitted

At present, it is commonly admitted that the ideal photosensitizer would be a chemically stable and pure drug with preferential uptake in tumour, rapid clearance, and a strong absorption peak at light wavelengths > 630 nm. Above all, low toxicity and high selectivity are the primary characteristics highlighted by clinicians.

This article describes the chemical possibilities to define the optimal components and the simplest structure of these products for intended use in the clinics. This paper underlines the key synthesis parameters that may tune the size of the silica-based nanocarriers within the nanometer range, the stability studies in mouse serum media, and the interest to add a second biocompatible inorganic coating to tune the flexibility, hence the permeability, of the silica core and to improve the stability of the silica-based protoporphyrin IX (Pp IX) nanocarriers. For additional results, *in vitro* cell viability tests have been engaged in order to confirm the biological activity of nanocarriers in our cellular model.

ARTICLE -II-

One Pot Synthesis of New Hybrid Versatile Nanocarrier Exhibiting Efficient Stability in Biological Environment for Use in Photodynamic Therapy

Edouard Thiénot, Matthieu Germain, Kelthoum Piejos, Virginie Simon, Audrey Darmon, Julie Marill, Elsa Borghi, Laurent Levy, Jean-François Hochepied, Agnès Pottier

(Submitted)

4.3. Discussion

Different nanoparticle chemistries have been developed in the nanoPDT platform to define the optimal components and the simplest structure of these products for intended use in the clinics. Further, most efforts have been focused on selecting one product which low complexity avoids as much as possible metabolic and tissue interactions and fulfills the following conditions. First to keep the simplest synthesis route, avoiding the use of targeting agent but taking advantage of the EPR effect that tumour tissues offer, owing to their “leaky” vasculature. Then, to keep stability such as ability to produce ROS of the encapsulated photosensitizers, at least for the necessary time to accumulate within tumour tissues and to produce its effect upon light activation.

At the first step, synthesis of monolayer Pp IX silica nanoparticles was detailed for different sizes. Nanoparticles are spherical and highly monodisperse with sizes of 10±5 nm, 25±5 nm, 65±20 nm, 90±20 nm and 160±30 nm. In fact, initial micelle formation is affected by various factors including temperature, ionic strength, pH, surfactant species etc. For non-ionic surfactants, the critical micelle concentration (CMC) decreases with an increasing temperature due to an increase in hydrophobicity (Kim et al., 2004). Balance between hydrophobicity and hydrophilicity may change and induce increase of micelle size. For control of the size of the Pp IX silica-based nanocarriers from 10 nm to 200 nm, temperature was increase from 18°C to 37°C.

With the ambition to optimize the *in vivo* performance of the nanoPDT, stability studies of the Pp IX silica-base nanocarrier in biological media were developed. Pp IX has specific absorbance and fluorescence spectra which permitted to simplify the stability studies in mouse serum media. Results showed that Pp IX silica-based nanoparticles present a modification of Pp IX absorption spectrum after t=2 hours aging in 100% mouse serum and a corresponding loss of Pp IX fluorescence emission.

In order to generate silica-based Pp IX nanocarriers with efficient stabilization in biological environments, second generation of photosensitizer vehicle was developed. The hypothesis of adding a second biocompatible coating to tune the flexibility of the silica-based nanocarrier backbone has been successful. Different sort of coatings such as dextran, silane PEG (PEG-Si) or aminopropylsilane (APS) and sodium trimetaphosphate (STMP) were produced and were tested in *in vitro* viability assays (data not shown) .

The cellular stability assay is based on the preservation of activity of Pp IX following Pp IX nanocarriers incubation in 100 % fetal calf serum (FCS).

Dextran is a coating agent commonly used to functionalize oxide nanoparticles, in particular magnetite nanoparticles contrast agent, to increase their blood circulation. Dextran is electrically neutral at neutral pH and the binding at the surface of iron oxide is thought to be labile (physisorption). It is also reported that dextran can be desorbed in biological environment, yielding to a direct contact between magnetite nanoparticles and biological compounds (Auffan et al., 2009). Our results show that dextran molecule, added as second biocompatible coating, did not improve nanoPDT stability in biological media.

Functional silanes develop covalent links with silanol groups present on silica surfaces. However, in aqueous media and particularly under neutral pH conditions, polymerisation of silanes competes with silane-silica surface anchoring (De Monredon et al., 2004). Hence, heterogneous silane deposition on nanoPDT surface is likely. Indeed, we did not observe improved nanoPDT stability in biological media using functional silane as second biocompatible coating agent.

Phosphate compounds, such as sodium trimetaphosphate are considered as complexing agent for most oxide surface. However, silica surface behaves differently. Furthermore, both silica surface and sodium trimetaphosphate are negatively charged at neutral pH. We hypothesized that sodium cations, present in the solution, drive the interaction between phosphate compound and nanoPDT silanol surface groups. Indeed, Pp IX silica-based nanoparticles coated with STMP, the bilayer nanocarriers, showed the best stability in biological media, highlighting the importance of a second specific biocompatible coating.

Roach et al., 2005 report protein adsorption (BSA and fibrinogen) onto model hydrophobic (CH_3) and hydrophilic (OH) surfaces. Authors investigated proteins adsorption using quartz crystal microbalance (QCM) and grazing angle infrared spectroscopy. The data show that albumin undergoes adsorption via a single step whereas fibrinogen adsorption is a more complex, multistage process. Albumin has a stronger affinity toward the CH_3 compared to OH terminated surface. In contrast fibrinogen adheres more rapidly to both surfaces, having a slightly higher affinity toward the hydrophobic surface. Conformational assessment of the adsorbed proteins shows that after initial 1 hour incubation few further time-dependent changes are observed. Both proteins exhibited a less secondary structure upon adsorption onto a hydrophobic surface than onto a hydrophilic surface, with the effect observed greatest for albumin. Data presented in this article suggest that proteins (albumin and fibrinogen) are able to interact with hydrophilic surface with a stronger affinity with hydrophobic surface. Their adsorption may further induce a conformational change in protein structure. In our present study, interaction of silica-based nanocarriers and protein from serum is likely to occur.

Albumin is the most abundant protein in serum and it is suggested that its interaction with hydroxyl group of silica-based nanocarriers readily occurs. However, the 3 dimensional structure of the nanocarrier backbone present some flexibility and hydrophobic vinyl groups, initially constraint within the core of the nanocarrier upon exposure to aqueous environment, may get oriented toward the external surface, triggered by albumin, upon exposure with serum media. Such affinity - vinyl hydrophobic groups / albumin - could be an explanation for the loss of stability of the entrapped Pp IX which may become exposed to the serum media. Interestingly, 50% effective concentration (EC_{50}) value of Pp IX silica-based nanocarriers, the monolayer nanocarrier, incubated in 100% FCS media, decreases with incubation time and reaches the EC_{50} value of free Pp IX in serum media. The role of STMP would then to stiffen the silicone backbone of the silica-based nanocarrier by forcing the silanol groups to interact with the phosphate groups, likely via sodium counter ions. Hence, confining the hydrophobic vinyl groups within the core of the nanocarrier and stabilizing the Pp IX molecules toward external media.

The ability of STMP modified Pp IX silica-based nanocarrier, the bilayer nanocarrier, to kill cells, seems to be preserved for enough time to accumulate within tumour tissues and to produce its effect upon light activation. Indeed, for the highest STMP content, bilayer nanocarriers *in vitro* data, show preservation of Pp IX activity up to 12 hours. The second generation of nanoPDT products, the bilayer nanocarriers, anticipate promises for efficient *in vivo* efficacy, due to their enhance stability – ability of the entrapped photosensitizers to generate ROS upon laser light excitation – in biological environment.

5. Interaction of Pp IX silica nanoparticles with biological systems

5.1. **"Pp IX Silica Nanoparticles Demonstrate Differential Interactions with *In Vitro* Tumour cell Lines and *In Vivo* Mouse Models of Human Cancers"**

Virginie Simon, Corinne Devaux, Audrey Darmon, Thibault Donnet, Edouard Thiénot, Matthieu Germain, Jérôme Honnorat, Alex Duval, Agnès Pottier, Elsa Borghi, Laurent Levy, Julie Marill ; Photochemistry, Photobiology, 2009

In the recent years, PDT has been successfully used in various cancers: skin, lung, uterus, gastrointestinal. Nevertheless, because of important side effects such as long lasting skin phototoxicity, PDT clinical application has been narrow. One of the most potential

revolution in cancer care was the use of nanoparticles. As shown in first paper, Nanobiotix created and developed a new alternative for the therapy photodynamic: the nanocarriers. A drug, Pp IX has been chosen to be encapsulated in a porous silica shell.

This second paper describes the *in vitro* and *in vivo* studies performed with Pp IX silica nanoparticles, the monolayer nanocarrier. Different parameters have been studied to obtain the best nanoparticles efficacy such as incubation time, activation time, particles concentrations, post-illumination time, with the weaker nanoparticles toxicity. Preliminary tests permitted to compare nanoparticles with free Pp IX, the monomer of Photofrin® sensitizer. Furthermore, efficacy was compared on various tumour cells lines, adherent (HCT 116, HT-29, epidermoid cell line A431, MDA-MB 231 and MCF7) and in lymphoblastoid suspension cell line (LLBC37).

In this work, *in vivo* studies were performed in tumour bearing animals –2 human epithelial cancers (HCT 116, A549) and 1 human Glioblastoma Multiforme – which permitted first observations on tumour ⁄ skin ratio accumulation of the product. Concerning time accumulation dynamic, tumour cell type is likely a major determinant but tumour microenvironment could more influence the differential observed.

In this work, Pp IX was successfully encapsulated into a silica nanoparticle that preserves the photodynamic activity of the photosensitizer. Our results demonstrate that the encapsulation in nanoparticles greatly improves the photo-stability and photodynamic efficacy of Pp IX. Furthermore, it opens some fields for exploration regarding the differential biodistribution of the nanoparticles in tumour models with various origins, and xenografted using two different approaches, human tumour cell lines and fragment of tumour from a patient. Stroma, vessels irrigating the tumour, and even growth conditions may trigger cell adaptative functions and cytokine secretion, which support tissue and cell differential biodistribution. Next step of research is to be focused on *in vivo* efficacy of epithelial and non-epithelial models.

These findings suggest that Pp IX and probably other photosensitizers encapsulated could be a promising approach not only for improving PDT efficacy but rather to also explore other route of administration, and thus trigger a more specific based-disease of PDT in the clinical setting.

ARTICLE -III-

Pp IX Silica Nanoparticles Demonstrate Differential Interactions with *In Vitro* Tumour cell Lines and *In Vivo* Mouse Models of Human Cancers

Virginie Simon, Corinne Devaux, Audrey Darmon, Thibault Donnet, Edouard Thiénot, Matthieu Germain, Jérôme Honnorat, Alex Duval, Agnès Pottier, Elsa Borghi, Laurent Levy, Julie Marill

(Photochemistry, Photobiology, 2009)

5.2. Discussion

In vitro tests investigation represent a sophisticated and reproducible system with which basic questions can be answered and which may help to understand what happens *in vivo*. Cell culture experiments allow for comprehensive investigations of particles-cell interactions. These experiments are necessary to study the effects of Pp IX silica nanoparticles on intracellular uptake, transport and subcellular nanoparticles localization.

This article introduces some advanced researches in i*n vitro* and in *in vivo* models with the ambition to improve knowledge on the role of biological factors in the photodamage.

At first toxicity and efficacy of Pp IX silica nanoparticles have been investigated. The WST-1 cell proliferation assay has been chosen. It is a colorimetric assay which is based on the cleavage of a tetrazolium salt, 3-(4,5-diMethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), by mitochondrial dehydrogenases to form formazan in viable cells. WST-1 has many advantages: it can be used to assay either adherent or suspension cell, it is very sensitive, it can detect 500 to 50.000 cells in a single well of a 96-well plate and it is possible to test of lot of conditions (concentrations, different products, controls…) in only 1 experiment. 3 hours of incubation and 20 min of illumination has been defined as the best particles efficacy conditions with an EC_{50} for 6 different cells lines (adherent or suspension cells) between 0.40 µM ± 0.01 for the LLBC37 and 1.13 µM ± 0.15 for the MDA-MB-231. In these conditions, Pp IX silica nanoparticles were always more efficient (more than 1 Log) than free Pp IX.

Pp IX silica nanoparticles are able to enter into all cells lines tested and diffused in all cytoplasm. The mechanism by which nanoparticles penetrate in HCT 116 cells without specific receptors on their outer surface is assumed to involve a passive uptake. In fact, cell viability assays performed at 4°C showed EC_{50}=0.4 µM (same than at 37°C). Others molecules such as glucose or homeoproteins (Holcman et al., 2007) diffuse through cellular membranes by passive penetration. Pp IX nanoparticles passive uptake may be initiated by Van der Waals forces, electrostatic charges, steric interactions, or interfacial tension effect. Others tests should be investigated in order to determine the passive way (for instance spectroscopy correlated with fluorescence) and extended to other cells types. Chithrani et al., 2007 showed gold size effect for nanoparticle uptake (receptor-mediated endocytosis) and clearance (the smaller exocytosed more rapidly). In this *in vitro* study, no size effect (10 to 60 nm) on HCT 116 uptake and cell viability was showed (EC_{50}=0.4 µM). It was no surprising to quantify the same total amount of particles whatever the size considering the role of passive

uptake. In fact, in the passive diffusion process, the membrane behaves as an inert lipid–pore boundary, and nanoparticles traverse this barrier either by diffusion through the lipoprotein region or, alternatively, filtering through aqueous pores (channels) without the cellular expenditure of energy (Mottier at al., 2006). In addition, for the first time, clearance of silica nanoparticles on tumour cells is established. Only 24 hours after the end of incubation all Pp IX silica nanoparticles were cleared. However, silica shells trafficking at cellular level was different than metallic particles such as gold (Chithrani et al., 2007) as no size effect on nanoparticle trafficking is observed (passive way is thought to participate to cellular uptake). Pp IX silica nanoparticles total clearance in HCT 116, 24 hours after medium renewal, suggested that we have to work on the therapeutic window for *in vivo* application in order to optimize schedule for nanoparticles activation.

In vivo studies were performed in tumour bearing animals which permitted first observations of nanoparticles distribution and tumour/skin ratio accumulation. The 3 tumour models behaved differently according to the maximal accumulation time point: 20, 16 and 2 hours for HCT 116, A549 and for Glioblastoma Multiforme respectively. Tumour and liver was the target in terms of nanoparticle-related fluorescence. These results proved that tumour cell types are likely a major determinant in biodistribution. Furthermore, tumour environment such as stroma could more influence this differential time of accumulation dynamic (including nanoparticles internalization and clearance). The ECM could act as a dispersive filter, controlling the composition of extracellular fluid and the rate of molecular trafficking (Muerkoster et al., 2008; Jackson et al., 2008; Minchinton et al., 2006; McKee et al., 2006; Pluen et al., 2001 and Netti et al., 2000).

Finally, deeper comprehension of cell death mechanism was detailed in this article. ROS generation has very short migration distance and was colocalized with nanoparticles after activation. Nanoparticles localization was key of the efficacy. Higher particles concentrations yielded more marked difference and higher ROS amounts caused larger cell photodamage and consequently larger cell death.

We have successfully controled Pp IX silica nanoparticles parameters to optimize the *in vitro* efficacy. First *in vivo* biodistributions were very encouraging because of the specific tumour accumulation in 3 different models, especially with Glioblastoma Multiforme model. Glioblastoma Multiforme is the most aggressive of the primary brain tumours and could be chosen as cancer model for clinical study. *In vivo* efficacy should be investigated and nanoparticles synthesis improved to follow the preclinical steps advancement for medical device development.

6. Generals PDT conclusions and perspectives

PDT has emerged as one of the important therapeutic options in management of cancer (Allison et al., 2008). PDT leads to selective and irreversible destruction of diseased tissues, without damaging adjacent healthy ones. Despite its advantages over current treatments, PDT is yet to gain general clinical acceptance. There are several technical difficulties in the application of PDT to a wide range of diseases. First currently FDA approved PDT photosensitizers, absorb in the visible spectral region below 700 nm, where light penetration into the skin is only a few millimeters, thus clinically limiting PDT to treat surface and relatively superficial lesions. However, this has to be weighed against the availability of advanced fibre-optic scopes which can reach most body cavities. Second is the difficulty in preparing pharmaceutical formulations that enable parenteral administration because most existing photosensitizers are hydrophobic and aggregate easily under physiological condition. Thirdly, the accumulation selectivity to diseased tissues is often not high enough for clinical use. Regarding all PDT limitations, we tried to define the best photosensitizer nanocarriers.

What is the ideal system for the next generation?

Regarding the literature, MacRobert exposed "the **ideal photoproperties for a sensitizer**" (MacRobert et al., 1989):

▪ be chemically pure, at least have a known structure, or consist of a well defined mixture.

▪ have a minimal dark toxicity and only become cytotoxic in presence of light.

▪ be preferentially retained by the target tissue (specificity),

▪ be rapidly excreted from the body (no systemic or skin photosensitizing effects)

▪ have a high photochemical reactivity (high triplet state field, long triplet state lifetimes)

▪ be able to effectively produce singlet oxygen and other reactive oxygen species

▪ have a strong absorbance with a high extinction coefficient for the wavelength range 600-800 nm, where tissue penetration of light is high.

All these criteria were examined and tested on both chemistry and biology research team. Nanoparticles offer solutions to each of the three difficulties. Nanoparticles carrying

photosensitizer by different strategies (in particular photosensitizer encapsulation) offer benefits of hydrophilicity and appropriate size for passive targeting to tumour tissues by the enhanced EPR effect. Selective accumulation can be enhanced by modifying the monocarrier surface using targeting agent such as mAb or specific tumour seeking molecules. Pp IX silica nanoparticles has a lot of advantages: very simple chemistry, better efficacy than free Pp IX for all *in vitro* cells lines tested, good tumour biodistribution. Further, improved stability in biological media is expected with second generation of nanoPDT products, the bilayer nanocarriers.

Efficient PDT may increase the choice of anticancer modalities, opening the possibility of a "new line of local treatment". Pp IX silica nanoparticles hold promises to enhance the current therapeutic window.

What kind of tests for the future?

For the future, it will be interesting to test biodistribution of Pp IX-STMP coated nanoparticles, the bilayer nanocarriers. Nanocarriers with bilayer coating could have different time of tumour maximal accumulation and potential others organs target. For Pp IX silica nanoparticles application it is necessary to decrease side effect by comparison with Photofrin®. As we know, Photofrin® has significant long lasting skin accumulation and it will be necessary to quantify the nanoPDT distribution in this large organ. Deeper exploration of pK characteristics on skin, tumour and liver should be planned.

In all *in vitro* and *in vivo* tests 25 nm Pp IX silica nanoparticles were studied. In HCT 116 viability and quantification tests showed that there are no size effect between 10-60 nm ranges. Approximately 0.7 molecule of Pp IX per particle could be encapsulated in 25 nm silica shell. Hence, 60 nm silica nanoparticles should allow for a much higher photosensitizer encapsulation. If we increase the total amount of Pp IX per particle it will be theoretically possible to increase efficacy. If chemical department research succeeds to encapsulate 7 molecules of Pp IX per particles (ten times higher) it will be interesting to perform biodistribution studies with monolayer and bilayer nanocarriers with size of 60 nm. Then, if particles accumulate into the tumour, further *in vivo* efficacy tests should be run.

Finally, evaluation of subcellular localisation in healthy and human tumour cell lines could be very useful for the complete *in vitro* nanocarrier comprehension.

Such hybrid versatile nanocarrier is expected to reach one of the key challenges of the domain by allowing the delivery of the nanocarriers at the right site and the right dose.

GENERALS CONCLUSIONS AND PERSPECTIVES

The scope of this PhD work is to broaden the current thinking and knowledge in view of recent developments in relation to nanotechnology-based oncology products. Specifically, study of interactions between nanoparticles activated by external electromagnetic energy sources and cancer cells for enhancement of therapeutic window was performed.

Nanotechnology is able to design nanomaterials with new modalities such as the use of active products for breakthrough in cancer care. Nanotechnology may also bring an enabling function such as the use of nanocarriers for impacting the pK of molecules and introducing the possibility of controlling sub-cellular bioavailability. Nanomaterials size allows interactions with biological entities, hence allows pathways to interact with biology. However, communication has to be established and required a deep appreciation of the nanoparticles and biological entities interaction for safe and efficient use of nanoparticles in biology. When nanoparticles meet medicine, new possibilities for cancer treatment are envisaged such as the use of outside body energy source to innovative products activation.

This PhD work participates to the development of two types of nanoparticles, NBTXR3 and nanoPDT, intended with different rational for design. For both nanoparticles, study of interactions between nanoparticles and cancer cells was performed. NBTXR3 activated with typical radiotherapy treatments or nanoPDT nanocarriers developed for PDT have clearly for ambition to enlarge the therapeutic window. Both therapeutic products are intended to make the cancer treatment more effective, less deadly to healthy tissues.

NBTXR3 is the lead product of nanoXray^{TM} platform. We have demonstrated on colon cancer cell lines that NBTXR3 nanoparticles penetrate cells via endocytosis ; that the NBTXR3 nanoparticules are into the cytoplasm into endosomes and then lysosomes; showed the importance of NBTXR3 nanoparticles cell internalization to achieve a significant higher radiosensitization effect; suggested that NBTXR3 nanoparticles appear to have long residence within cells; visualized that endosomes containing NBTXR3 nanoparticles fused into the cytoplasm and that NBTXR3 nanoparticles activated by ionizing radiation increase micronuclei formation. Finally, the highest efficacy appears to be correlated to the maximum amount of nanoparticle per cell.

Results form the basis for the understanding of factors, which are determinant of the significant radiation enhancement demonstrated by irradiated NBTXR3 nanoparticles on

colon cancer line models. Moreover they have yield foundations to establish schedule of NBTXR3 nanoparticles administration in *in vivo* models and the modality of ionizing radiation delivery. Currently, preclinical studies are ongoing. Preliminary *in vivo* efficacy study was conducted in mice bearing HCT 116 tumours grafted on the flank: NBTXR3 nanoparticles in combination with radiotherapy leads to a total regression of tumour on all animals compared to control (treated mice subjected to radiotherapy alone) (data not shown).

Among the next steps, the use of different types of energy and deeper studies of biologic specificity are important research axis which should be addressed. Indeed, many *in vitro* studies should be completed such as define the type and proportion of cell death which is induced by the nanoparticles for both tumour and healthy cell lines. Then, we should prove that NBTXR3 is the ideal nanoparticle according to its size and surface coating in order to optimize biodistribution and/or cellular uptake and/or localization at the subcellular level. We should then evaluate their efficacy and toxicity *in vivo* in other tumours cells lines than HCT 116.

Regarding these results, NBTXR3 nanoparticles offer a breakthrough approach to create efficient pathways to cancer therapy. NanoXray^{TM} plateform is a new treatment weapon that could be used alone, or in concert with existing anticancer protocols: chemotherapy, surgery, targeted molecules and immunotherapy. Efficacy is expected to be proportional to the duration of activation and the number of radiotherapy sessions.

NanoPDT plateform develops photosensitizer nanocarriers. We have proved that silica-based nanocarriers are efficient carriers for drugs to protect the drug from exposure to aqueous environment; that silica-based nanocarriers present a relevant range of porosity in aqueous solution to efficiently entrap the Pp IX photosensitizer (a physical encapsulation of Pp IX molecule within nanocarriers was performed with the will to simplify as much as possible both the product synthesis route and its final composition); that the addition of a second coating to form a bilayer monocarrier significantly preserves the ability of the Pp IX silica-based nanocarriers to kill cell after aging in 100% FCS media.

In addition, previous preclinical studies have demonstrated *in vitro* the essential non toxicity of this Pp IX silica nanoparticle formulation and interestingly, a very good tolerance in *in vivo* models. We showed that Pp IX silica nanoparticles of 10-60 nm range did behave in

the same way concerning cell uptake and cell viability; that internalization mechanism involves a high proportion of passive internalization with accumulation within the cytoplasm of cells; that Pp IX silica nanoparticle uptake was dependent upon cell type, as shown by the greater uptake into HCT 116 than HT 29 cells; that the experiments supposed a saturation threshold in their intracellular accumulation at this time point without any evidence of toxicity and that for the first time, clearance of silica nanoparticles on tumor cells was reported. For all the 6 cell lines tested, nanoparticles localization was diffuse into the cytoplasmic area. Furthermore, ROS generation was significantly improved in the presence of Pp IX silica nanoparticles in both HCT 116 and HT-29 cells lines: higher concentrations yielded more marked difference. Higher ROS amounts caused larger cell photodamages and consequently better phototoxic effects. For all tumour cells lines tested *in vitro*, Pp IX silica nanoparticles were more efficient than free Pp IX.

In vivo studies were performed in tumour-bearing animals which permitted first observations on tumour ⁄ skin ratio accumulation of the product. Kinetics demonstrated by semi-quantitative analysis showed that tumour models behaved differently according to the maximal tumour accumulation time point. Liver fluorescence intensity remained remarkably constant over the time period investigated and no urine excretion was found.

Recently Nanobiotix has published results of a preclinical study with product issued from the nanoPDT technology platform: the preclinical study has validated the applicability of using nanoPDT to treat glioblastoma multiforme, one of the most prevalent brain tumours.

Many *in vitro* studies should be further addressed such as characterize the nanoparticles toxicity without activation for healthy cell lines; define the type and proportion of cell death which is induced by the nanoparticles for both tumour and healthy cell lines; define the sub-localization of nanoparticles into cells; explore the specific endocytosis players; precise uptake and clearance ratios between tumour cell lines; test the biodistribution and the biocompatibility of different size of Pp IX silica nanoparticles as well as the bilayer nanocarriers and finally test the *in vivo* efficacy of theses products in various tumour cells lines such as HCT 116 and glioblastoma multiform models.

Of course, there is much more work to be done and preclinical findings hold promises for the future development of nanoPDT technology.

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RESUME DETAILLE DE LA THESE

TABLE DES MATIERES

I-RECHERCHE EN NANOMEDECINE

1.1. La Nanomédecine: quand les nanotechnologies rencontrent le domaine de la santé

1.1.1 L'importance d'être à l'échelle nanométrique pour interagir avec le monde biologique

L'année 1959 est essentielle pour l'essor des nanotechnologies et plus particulièrement pour son application au domaine de la santé. En effet, Richard Feynman dans son discours novateur 'There's Plenty of Room at the Bottom, An Invitation to Enter a New Field of Physics", a été visionnaire en avançant l'idée de construire des objets capables de manœuvrer à l'échelle des cellules biologiques.

Depuis, les nanotechnologies ont ouvert la voie vers un 'nanomonde', et génèrent des systèmes simples ou élaborés dont la taille varie de quelques nanomètres à quelques centaines de nanomètres. Ces 'nanosystèmes' ont aujourd'hui l'ambition d'occuper une place fondamentale en médecine. De part leur taille nanométrique, ces 'nanosystèmes' ont la capacité d'interagir avec les entités biologiques telles que les tissus, les cellules, voire d'opérer au sein même des cellules biologiques.

Cependant, si la taille de ces 'nanosystèmes' permet d'agir à l'échelle du monde biologique, il apparaît essentiel aujourd'hui que ces objets interagissent avec les systèmes biologiques pour être utilisés sans danger et de façon effective en médecine.

1.1.2 Définition de la nanomédecine

La nanomédecine est l'application de la nanotechnologie au domaine de la santé. La nanomédecine se réfère à la signification originelle de la nanotechnologie, laquelle se sert des effets physiques qui se produisent à l'échelle nanométrique dans les objets.

La nanomédecine développe aujourd'hui des systèmes dont les champs d'applications couvrent les domaines de la vectorisation des médicaments, l'exploration plus ciblée et moins pénible pour les patients. De plus, un diagnostic plus précoce des maladies peut permettre d'aboutir à une médecine plus préventive et plus personnalisée, c'est-à-dire prenant en compte les spécificités biologiques de chaque individu.

1.1.3 Quels nanomatériaux peuvent jouer un rôle en médecine et pourquoi

Les nanomatériaux développés pour des applications en médecine sont fonctions orientées. Les systèmes de libération de médicaments visent à améliorer le rapport bénéfice sur risque de certaines molécules thérapeutiques. Ces 'nanosystèmes' sont un exemple typique d'utilisation des nanotechnologies pour apporter une fonctionnalité supplémentaire bénéfique à la pratique médicale. Les nanomatériaux peuvent aussi, de part leur propriétés spécifiques, apporter de nouvelles modalités d'usage et permettre de réaliser des avancées sans précédent pour le traitement des patients.

A- Les nanomatériaux organiques : systèmes de libération contrôlée de molécules thérapeutiques

Les nanomatériaux organiques englobent une grande variété de structures. Elles ont comme but commun de transporter et éventuellement de délivrer des molécules au niveau d'un site spécifique par voie contrôlée. Les applications de nanomatériaux les plus développées sont à l'heure actuelle celles des **liposomes,** des **micelles,** des **nanoparticules polymériques** et des **dendrimères.**

B- Les nanomatériaux inorganiques : une nouvelle modalité de traitement

Les nanomatériaux inorganiques sont particulièrement prometteurs pour des applications médicales. En effet, le cœur de certains nanomatériaux possède des propriétés exclusives à l'échelle nanométrique dues à ce que l'on définit comme l'effet quantique. Le ratio surface sur volume augmente quand la taille des matériaux décroît et de nouvelles propriétés, telles que électroniques ou magnétiques, peuvent être observées et exploitées pour des applications médicales spécifiques. Les applications de nanomatériaux les plus développées sont à l'heure actuelle celles des **nanoparticules superparamagnétiques (oxydes de fer),** des **quantum dots** et des **nanoparticules métalliques (or).**

C- L'avenir des nanomatériaux en nanomédecine

Le « National Nanotechnology Initiative » et la « Plateforme Européenne de Nanomédecine » ont travaillé pour anticiper et définir les futures applications des

nanoparticules dans le domaine de la santé. Les priorités de demain ciblent le diagnostic *in vitro* et *in vivo*, le développement de nanotransporteurs à visée thérapeutique, la recherche de nouveaux implants et la régénération des tissus, les systèmes biologiques d'ingénierie ainsi que l'innovation dans le domaine de la médecine instrumentale et des dispositifs médicaux.

1.2. La maladie cancéreuse

1.2.1. Le cancer

A- Epidémiologie

Le cancer demeure l'une des causes majeure de maladie, avec plus de 10 millions de nouveaux cas diagnostiqués chaque année dans le monde. Dans les pays développés, le cancer est l'une des principales causes de mortalité : il a tué plus de 6,7 millions de personnes à travers le monde en 2002. Cette tendance ne va pas être freinée dans les prochaines décennies et les prévisions restent pessimistes avec une estimation de 10,3 millions de personnes tuées et plus de 16 millions de nouveaux cas détectés d'ici 2020 (Organisation Mondiale de la Santé ; OMS, 2007). La figure 1 présente la mortalité à 5 ans pour les cancers les plus répandus :

Cancers	ovaires	pancréas	colorectal	vessie	rein	sein	estomac	foie	prostate	poumon	oesophage	thyroïde
Mortalité à 5 ans	68%	96%	54%	36%	52%	27%	79%	95%	44%	86%	92%	25%

Figure 1. Statistiques du cancer (Simon *et al.*, 2007)

Les progrès de la médecine et de la recherche pharmaceutique sont importants, puisque globalement 50 % des cas sont guéris en France ; néanmoins, le traitement contre le cancer reste plus que jamais un enjeu majeur en terme de santé publique.

B- Caractéristiques moléculaires du cancer

Le cancer ne peut pas être considéré comme une maladie mais comme une multitude de maladies constituée d'une centaine de sous-types. Les cancers peuvent être classés en deux catégories : les hématologiques (malignes du sang) et les tumeurs solides. Quand les cellules du corps deviennent anormales et qu'elles se divisent sans contrôle, la tumeur se forme. Elle peut être de type maligne ou bénigne. La caractéristique unique, commune à ces cancers est la

prolifération anormale des cellules. Par des moyens chimiques et mécaniques, les cellules tumorales vont s'insérer dans l'espace séparant les cellules normales ou à leur niveau, les tuant. Le cancer est une maladie génétique (Holland *et al*., 2006). Les bases génétiques de la tumorigénèse changent énormément d'un cancer à un autre mais les étapes requises pour la formation de métastases sont similaires pour toutes les tumeurs. Par détachement du site primitif, les cellules utilisent d'une façon prédominante, soit les vaisseaux lymphatiques, soit la voie sanguine. Ces étapes impliquent des stimulations lymphangiogéniques et angiogéniques au niveau de la tumeur mais aussi des perturbations au niveau de l'environnement local de la tumeur. L'invasion et les métastases tuent leur hôte par deux processus : l'invasion locale et la colonisation des organes distants et la génération de dommages cellulaires induits.

C- Principaux traitements pour le cancer

Les trois principaux traitements du cancer sont la chirurgie, la radiothérapie et la chimiothérapie. Chacun de ces traitements peut être utilisé seul ou en combinaison, selon le type de tumeur traité. Le choix du traitement va dépendre de la localisation et du type tumoral, de son stade de développement et de l'état général du patient.

La chirurgie

La plupart du temps, les cancers de type non hématologique peuvent être soignés intégralement par la chirurgie. La chirurgie est le plus ancien traitement pour le cancer et reste le traitement d'éradication des cancers solides. Lorsque le cancer est métastasé à d'autres sites de l'organisme, l'utilisation de la chirurgie devient alors controversée.

La chimiothérapie

La chimiothérapie est un traitement systémique permettant de tuer les cellules tumorales de localisation primitive et de traiter les métastases éloignées. La chimiothérapie a énormément amélioré le pronostic de nombreux cancers. Elle reste néanmoins peu curative exceptée pour quelques tumeurs comme les lymphomes, les leucémies et le cancer des testicules. Le terme chimiothérapie réfère souvent à des médicaments de type cytotoxiques qui affectent les divisions cellulaires rapides des cellules anormales. Les médicaments issus

de la chimiothérapie interfèrent à différents stades du cycle de division cellulaire, par exemple au moment de la duplication de l'acide désoxyribonucléique (ADN) ou à celui de la séparation des chromosomes néoformés.

La radiothérapie

La radiothérapie est l'utilisation de radiations ionisantes pour tuer les cellules cancéreuses. Ce traitement va léser ou tuer les cellules tumorales en endommageant le matériel génétique, les rendant ainsi incapables de continuer de croître et de se diviser. Le but de la radiothérapie est de créer un maximum de dommages aux cellules tumorales, tout en affectant le moins possible les cellules saines. Les effets de la radiothérapie sont alors localisés et confinés à la région du traitement.

1.2.2. Etroitesse de la fenêtre thérapeutique: la limitation majeure des traitements anti-cancéreux

A- Les limitations des traitements anti-cancéreux

Actuellement, les agents anti-tumoraux ciblant le cycle cellulaire et l'ADN, comme les agents cytotoxiques ou les rayons X, sont les plus efficaces en clinique. Ils ont permis d'améliorer de manière significative la survie des patients quand ils sont utilisés seuls ou en combinaison avec d'autres médicaments ayant des mécanismes d'action différents.

Néanmoins, des efforts dans le domaine de la recherche se poursuivent pour améliorer les traitements actuels. Il s'avère important par exemple de comprendre les voies de signalisations de transduction qui vont médier les réponses cellulaires. De nombreux espoirs résident dans la modification du rapport thérapeutique bénéfice sur risque. Malgré les avancées techniques, la plupart des équipements de radiothérapie actuellement sur le marché présentent de nombreuses limitations qui restreignent significativement le traitement. Beaucoup de patients atteints d'une tumeur ne répondent pas aux radiations ou développent une résistance à celles-ci.

B- L'élargissement de la fenêtre thérapeutique

L'objectif des approches pour traiter le cancer est d'obtenir des traitements présentant une forte probabilité de **guérison** avec le **minimum de risque** pour les tissus sains et une **biodistribution** optimale (systémique ou loco-régionale) de l'agent thérapeutique.

Le contrôle du cancer

Le principal obstacle pour traiter de façon effective le cancer est l'échec de la thérapie initiale. En effet, cette dernière ne permet pas toujours d'éradiquer un nombre suffisant de cellules tumorales pour éviter la récurrence de la maladie, ceci affectant de façon significative la survie à long terme des patients. La population de cellules qui a survécu au traitement est appelée maladie résiduelle microscopique (MRD) (quelques millions de cellules malignes) et est responsable des rechutes. Ces cellules peuvent trouver refuge dans le microenvironnement, ou le stroma, qui les protègent.

Les stratégies thérapeutiques sont limitées par le degré de tolérance des tissus sains ; en effet des doses très élevées permettraient d'éradiquer la totalité des cellules tumorales, mais entraîneraient également la mort des cellules saines avec des conséquences délétères pour le patient. Ce problème impose très souvent une limitation pour délivrer la dose nécessaire au traitement de la tumeur et permet en conséquence à quelques cellules malignes d'échapper au traitement.

La pharmacocinétique

Le but de la pharmacocinétique est de fournir les connaissances nécessaires à l'adaptation de la posologie pour obtenir les concentrations plasmatiques suffisantes d'un médicament entraînant l'effet optimum, c'est-à-dire la meilleure efficacité avec le minimum d'effets indésirables. En effet, le médicament est inefficace lorsqu'il est administré à de trop faibles concentrations ; et lorsque celles-ci sont trop élevées, les effets indésirables prédominent sur l'efficacité.

En pharmacologie clinique, le paramètre facilement et directement accessible est la concentration plasmatique du médicament. Les différences pharmacocinétiques entre médicaments proviennent essentiellement de la facilité avec laquelle ils traversent les membranes biologiques et leur métabolisation par les enzymes présentes chez le patient. L'acquisition des connaissances pharmacocinétiques est nécessaire à une prescription correcte.

La toxicité

L'un des effets secondaires les plus sévères à une thérapie efficace est le développement d'un second cancer primitif. Celui-ci reflète non seulement les effets tardifs de

la thérapie, mais aussi le rôle des facteurs environnementaux présents lors de l'apparition du premier cancer: le tabac, la consommation d'alcool, l'alimentation, la fonction immunitaire, le statut hormonal et l'exposition environnementale.

Des risques accrus de développement de seconds cancers primitifs sont identifiés après des traitements par radiothérapie et avec des agents thérapeutiques tels que les agents alkylants, les inhibiteurs de la topoisomérase et les anti-métabolites.

La fenêtre thérapeutique restreinte

Le choix du traitement par radiothérapie est empirique et essentiellement basé sur la connaissance du radiothérapeute en fonction de l'organe ciblé. Cent ans de pratique en radiothérapie n'ont toujours pas permis de définir clairement le régime de fractions optimales types à appliquer aux patients. La fréquence d'apparitions d'effets secondaires sévères est dépendante de la dose totale d'irradiation, de la dose par fraction, du temps de traitement total, du type de rayonnements, de l'énergie utilisée et de la surface totale de la peau exposée aux rayons. Au fur et à mesure du temps, l'exposition à des radiations au niveau des tissus sains va entraîner l'accumulation de dommages et limiter la possibilité de futurs traitements. Il est également admis que la combinaison de la radiothérapie avec d'autres thérapies telles que la chimiothérapie (chimioradiothérapie) augmente les effets secondaires sévères. Ce phénomène est particulièrement exacerbé quand cette combinaison se fait avec des protocoles de fractionnements.

La figure 2B montre une fenêtre thérapeutique restreinte. Dans le cas de la radiothérapie, plus la tumeur est radiosensible et plus la fenêtre thérapeutique est large (cas 2A) ; plus les tissus normaux sont radiosensibles et plus le risque est élevé d'engendrer des dommages permanents. D'autres effets secondaires dus à la radiothérapie sont également décrits : carcinogénicité, mutagénicité et tératogénicité. Ces effets sont assimilés comme permanents puisque les radiations ionisantes vont induire des dommages au niveau du génome.

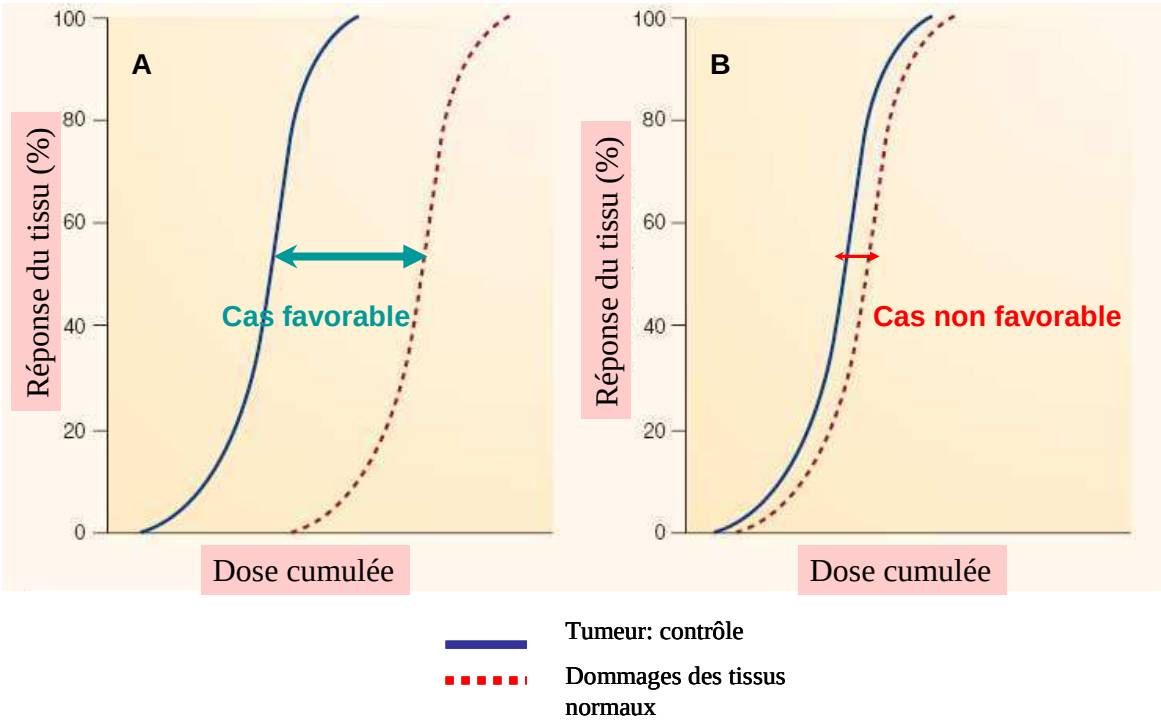

Figure 2: La fenêtre thérapeutique : augmenter l'index thérapeutique (Bernier *et al*., 2004)

C- Comment réconcilier la dose tolérée avec la dose curative

La plateforme nanoXray^{TM} se base sur une technologie ciblant la destruction des cellules tumorales par des nanoparticules inertes. Cette technologie ouvre de nouvelles perspectives pour le traitement du cancer. Les nanoparticules sont conçues pour être activées après injection par une source de rayon X externe. L'efficacité du traitement est proportionnelle à la durée d'activation et au nombre de sessions de la radiothérapie.

La plupart des tumeurs solides possèdent des caractéristiques physiopathologiques uniques qui ne sont pas observées dans les tissus et organes sains : angiogenèse étendue entraînant la formation de néovaisseaux multiples présentant une architecture vasculaire défectueuse et l'absence de drainage lymphatique. Ces caractéristiques sont responsables du phénomène de perméabilité et de rétention de macromolécules (dit "effet EPR", pour Enhanced Permeability and Retention effect) dans les tumeurs solides. Cette perméabilité vasculaire spécifique des tumeurs permet l'accumulation des nanoparticules nanoXray^{TM}.

L'utilisation des produits issus de la plateforme nanoXray^{TM} est destinée à résoudre le problème majeur de la radiothérapie: la destruction des tissus cancéreux en limitant l'énergie délivrée aux tissus sains.

La plateforme nanoPDT a été conçue en considérant les voies d'administration traditionnelles des molécules thérapeutiques : la voie intraveineuse, orale ou intramusculaire. La plateforme nanoPDT permet la libération de molécules photosensibilisantes au site tumoral même pour permettre de réduire la destruction des tissus environnants et ainsi minimiser les effets secondaires.

II-DES NANOPARTICULES ACTIVABLES POUR LE TRAITEMENT DU CANCER : UN MOYEN D'ELARGIR LA FENETRE THERAPEUTIQUE DES APPROCHES ANTI-CANCEREUSES

2.1 Introduction

2.1.1 Nanobiotix développe des nanomatériaux 'activables' pour apporter des solutions cliniques aux patients

Nanobiotix est une entreprise **innovante** qui travaille dans le domaine de la nanotechnologie pour le traitement du cancer. Nanobiotix met au point des nanoparticules permettant la destruction spécifique de cellules cancéreuses par la génération de réactions physiques contrôlées. Ces nanoparticules sont activables par différentes sources d'énergie externes selon une utilisation de type **« on »** et **« off »**.

Pour créer ces nouveaux nanomatériaux 'activables', Nanobiotix a développé une technologie fondée sur deux axes majeurs : la compréhension fine des mécanismes biologiques et la capacité à élaborer des structures complexes à l'échelle nanométrique.

Nanobiotix développe différents programmes de recherches, basés sur le concept de particules activables, pour élargir le champ d'application des nanoparticules en médecine :

▪ La plateforme **nanoXray^{TM}** est basée sur le concept de nanoparticules cristallines activables par rayons X.

▪ La plateforme **nanoPDT** est basée sur le concept de nanotransporteurs encapsulant des agents photosensibilisants pour le traitement du cancer.

▪ La plateforme **nanoMag** est basée sur le concept de particules magnétiques pour le traitement et le diagnostic du cancer.

▪ La plateforme « **libération de médicaments actifs** » est basée sur le concept de systèmes de libération de médicaments par stimuli externes.

La plateforme nanoXray^{TM} développe des nanomatériaux dont les propriétés structurales permettent d'interagir avec les rayons X, permettant d'augmenter localement les effets de la radiothérapie. NBTXR3 représente la première génération de produits issus de cette plateforme. NBTXR3 est destiné à être injecté par voie intratumorale chez les patients, les nanoparticules étant préférentiellement internalisées dans les cellules cancéreuses. Les patients sont ensuite exposés à des rayons X qui vont activer les nanoparticules et permettre la destruction sélective des tissus tumoraux. NBTXR3 a pour ambition de lutter contre les cancers les plus dévastateurs comme celui des poumons, du pancréas, du colon, de la prostate ou du sein.

La plateforme nanoPDT développe des nanotransporteurs qui offrent la possibilité de modifier de façon significative la pharmacocinétique de photosensibilisants comme la protoporphyrine IX (Pp IX). Au-delà, et de façon toute aussi importante, ces nanotransporteurs offrent la possibilité de contrôler la disponibilité subcellulaire. Augmenter la biodisponibilité au niveau de la tumeur, réduire l'accumulation du photosensibilisant au niveau de la peau et distribuer de manière différenciée le nanotransporteur au niveau des organelles des cellules, constituent les effets majeurs apportés par les produits issus de la plateforme nanoPDT.

Les mécanismes d'action des produits développés par Nanobiotix sont basés sur des effets physiques et non biologiques. Cette approche permet une véritable rupture au regard des thérapies usuellement développées. Ces matériaux possèdent en effet un haut degré de découplage entre le cœur de la nanoparticule, qui apporte l'effet thérapeutique recherché et le revêtement de surface de la nanoparticule qui confère à l'ensemble de la structure sa spécificité d'action.

Un des problèmes majeurs aujourd'hui dans le traitement du cancer est de vouloir rendre très effectifs certains traitements et ainsi exacerber leurs effets secondaires. Deux axes peuvent permettre de résoudre ce problème : réduire la toxicité associée au traitement ou améliorer le contrôle ou la destruction de la tumeur. Nanobiotix se propose de jouer sur **les**

deux dimensions à la fois, et d'atteindre un ratio bénéfice sur risque jamais atteint jusqu'à présent. Il s'agit clairement de briser la corrélation classique entre efficacité thérapeutique et toxicité associée pour l'organisme. Au-delà, le principe d'intervention des nanomatériaux, qui se situe au niveau subcellulaire pour améliorer la fenêtre thérapeutique, représente un nouveau paradigme.

2.1.2 Interaction entre les nanomatériaux et le milieu biologique : une compréhension nécessaire pour amener les nanomatériaux en clinique

Les connaissances des interactions entre les nanomatériaux et la biologie sont essentielles pour comprendre comment les nanomatériaux vont communiquer avec les entités biologiques et *in fine* comment les nanomatériaux et la biologie vont interagir ensemble. A la frontière entre deux mondes scientifiques – la biologie moléculaire et cellulaire et la physique et la chimie – les chercheurs sont aux prémices de la compréhension des interactions entre les nanomatériaux et les entités biologiques. Les résultats issus de ces recherches vont constituer les fondements pour élaborer des 'nanosystèmes' plus performants permettant de développer des interactions adaptées avec le milieu biologique pour augmenter l'efficacité et la tolérance de ces nanomatériaux en clinique.

Les interactions entre les nanoparticules et les protéines sont considérées comme essentielles en nanomédecine et en nanotoxicité depuis la mise en place du concept de nanoparticules-protéines « couronnes ». Quand les nanoparticules entrent dans un milieu biologique, les protéines présentes dans le milieu interagissent avec les particules ce qui peut générer une modification de leurs conformations ou altérer leurs fonctions biologiques. Cette interaction peut aussi réduire l'efficacité du traitement et/ou induire des effets secondaires. La nature de la surface de la particule (taille, rayon de courbure, charge, fonctionnalisation) influence le type de protéines interagissant avec les nanoparticules. L'albumine, les immunoglobulines (essentiellement l'IgG et l'IgM) et le fibrinogène sont les protéines qui interagissent le plus souvent avec les nanoparticules.

Afin de pouvoir délivrer les nanomatériaux au bon moment, au bon endroit et à la bonne concentration, plusieurs défis doivent être relevés. Les nanomatériaux doivent s'accumuler préférentiellement au niveau des tissus tumoraux et doivent pour cela être capables de franchir les barrières biologiques comme la peau, les intestins, les muqueuses, mais aussi de s'internaliser dans les cellules et enfin à l'échelle subcellulaire dans les organelles. De nombreuses études ont d'hors et déjà démontré l'influence des caractéristiques physico-chimiques des nanoparticules pour franchir ces barrières.

La taille et la forme des nanoparticules ont un impact sur leur biodistribution et leur pénétration cellulaire. La charge des nanoparticules permet de gérer leurs interactions avec les membranes cellulaires et subcellulaires. Le choix d'un revêtement de surface adapté permet de cibler de façon passive ou active la zone à traiter.

La conception des nanomatériaux, selon le principe d'intervention souhaité, doit cependant toujours être mise en regard des effets potentiellement toxiques qui peuvent apparaître suite à l'interaction de ces nanomatériaux avec le milieu biologique qu'ils rencontrent.

2.1.3 Présentation du travail de thèse : participation au développement de deux types de nanoparticules pour le traitement du cancer selon deux approches différentes

Le travail réalisé au cours de cette thèse s'intéresse à l'étude de deux axes majeurs de recherches développés par Nanobiotix: la plateforme nanoXray^{TM} et la plateforme nanoPDT.

Les produits issus de ces deux plateformes ont pour but de créer et de développer des nanomatériaux activables, utilisant des sources d'énergie externes de type « on » / « off ». Cependant, les sources d'énergies utilisées pour activer les produits sont différentes, de part l'énergie mise en jeu (source rayons X ou laser ; Figure 3). De plus, la voie d'administration des produits est également différente ce qui implique une approche très distincte dans la conception des nanomatériaux issus des plateformes nanoXray^{TM} et nanoPDT. Ces différentes plateformes, basées sur des approches thérapeutiques radicalement différentes, ont comme objectif commun de permettre un contrôle locorégional de libération.

● NBTXR3, issu de la plateforme **nanoXray^{TM}**, est une suspension biocompatible de nanoparticules cristallines et inertes d'oxyde d'hafnium (HfO_2 ; ± 70 nm) dans de l'eau pour préparation injectable (PPI). L'oxyde d'hafnium constitue le cœur thérapeutique de la nanoparticule, seulement lorsque ses électrons sont excités par l'application des rayons X. Le cœur est recouvert par un revêtement assurant la stabilité et la biocompatibilité des nanoparticules. Les applications thérapeutiques de ces particules sont des cancers de type **profonds**.

● Les **nanoPDT** sont des nanoparticules – ou nanotransporteurs – constituées de silice, encapsulant une molécule photosensible. La taille des nanoparticules peut être ajustée sur une large gamme, couvrant le domaine de 10 à 200 nm. Ces nanoparticules sont activables

par une source laser. Les applications thérapeutiques de ces particules sont des cancers de type **superficiels.**

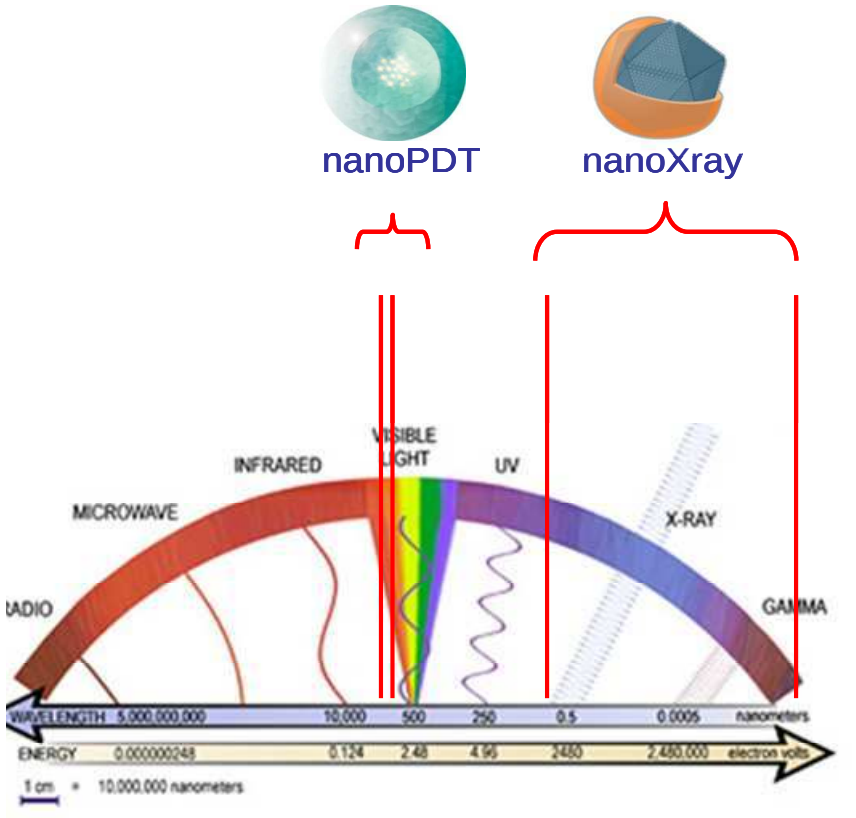

Figure 3. Deux différents types de nanoparticules nanoPDT et nanoXray^{TM} activables par énergie électromagnétique

Les études réalisées au cours de cette thèse participent à trois axes de recherche primordiaux, permettant de poser les bases d'un développement des produits thérapeutiques en clinique.

1) <u>Etudes de l'efficacité et de la toxicité *in vitro*</u>

L'approche *in vitro* est particulièrement pertinente pour tester la toxicité, l'efficacité des nanoparticules, l'effet de la localisation des nanoparticules à l'échelle subcellulaire sur l'efficacité, le type de mort cellulaire induit, mais aussi pour passer au crible différents types de cellules tumorales et normales. Plusieurs questions doivent être approchées pour développer les nanoparticules comme « produits actifs »:

- Est-ce que les nanoparticules sont toxiques sur des lignées tumorales et/ou saines en l'absence d'activation?

- Est-ce que les nanoparticules augmentent la destruction des cellules tumorales après activation par une source externe d'énergie selon les conditions et protocoles établis?

- Quels sont les types de mort cellulaire induits par les nanoparticules ?

- Quel est le rôle de la localisation subcellulaire, et du nombre total de nanoparticules internalisé, sur l'efficacité ?

- Est-ce que les nanoparticules induisent la même toxicité et/ou efficacité selon les spécificités biologiques des tumeurs : leur origine (origine épithéliale ou mésenchymateuse, structure compartimentale différente), cellules humaines saines versus cellules tumorales malignes, caractéristiques de radiosensibilité (lignées radiosensibles ou radiorésistantes)?

2) Mécanisme d'action : trafic intracellulaire des nanoparticules

L'étude mécanistique des nanoparticules *in vitro* est essentielle pour la compréhension et la mise au point des protocoles d'administration des nanoparticules et des conditions d'activation dans des modèles *in vivo*. Les études de pénétration cellulaire, de localisation et de clairance des nanoparticules sont la base de la compréhension du trafic intracellulaire. Plusieurs axes de recherches sont explorés :

- Par quel mécanisme les nanoparticules sont-elles internalisées ?
- Quelle est la cinétique d'entrée des nanoparticules ?
- Où sont localisées les nanoparticules dans les cellules ? Dans quelles organelles ?
- Est-ce que les nanoparticules ressortent des cellules ou bien restent-elles confinées dans des organelles pour être dégradées ?

3) Etudes de la performance et de la tolérance *in vivo*

Les études *in vivo* englobent la biodistribution des nanoparticules, l'accumulation et la dispersion des nanoparticules dans la tumeur et leur toxicité. Les tumeurs peuvent être apparentées à des « organes » spécifiques, différents des organes/tissus dont elles sont issues. Ces deux entités génétiques coexistent, et ont une structure moléculaire, métabolique et un comportement de croissance spécifiques pour chaque patient et sont fortement liées au type tissulaire et au stade de développement du cancer. L'exploration des spécificités biologiques des différents cellules/tissus/organes est nécessaire. Les études *in vivo* doivent être soutenues par les études *in vitro* pour amener un produit efficace et non toxique au stade du

développement clinique. Les axes de recherches sont nombreux et englobent les questions suivantes :

- Quel est la nanoparticule idéale (selon sa taille, composition, forme et fonctionnalisation) afin d'optimiser la biodistribution et/ou la pénétration cellulaire et/ou la localisation au niveau subcellulaire ?
- Quelle est la nanoparticule idéale (selon sa taille, composition, forme et fonctionnalisation) afin d'optimiser la biocompatibilité ?
- Les nanoparticules sont elles stables en milieu biologique ?

Le but de toutes ces approches est de montrer le réel bénéfice de ces nanoparticules par rapport aux approches thérapeutiques actuellement développées. Concernant la plateforme nanoXray^{TM}, la radiothérapie seule constitue la référence. Pour la plateforme nanoPDT, la molécule photosensible - Pp IX libre - est la molécule de référence.

Nanobiotix travaille dans le domaine du traitement du cancer et l'élargissement de la fenêtre thérapeutique est essentiel pour le développement de nouveaux produits en clinique. Deux voies différentes ont été explorées par Nanobiotix : le développement de nanoparticules thérapeutiques inertes en combinaison avec la radiothérapie pour augmenter localement l'effet des radiations et le développement de nanoparticules à base de silice pour améliorer la distribution de médicaments pour la thérapie photodynamique. Plus particulièrement, l'étude des interactions entre les nanoparticules et les cellules tumorales a été explorée.

Les interactions des nanoparticules NBTXR3 avec les cellules tumorales – pénétration cellulaire, localisation subcellulaire et évolution au cours du temps – l'impact de la localisation des nanoparticules sur l'efficacité, l'effet dose des nanoparticules sur l'efficacité sont tous les points abordés dans le cadre de cette étude. Le but final étant de définir des conditions d'administration des nanoparticules NBTXR3 dans les modèles *in vivo* ainsi que les modalités d'activation par rayons X.

Les interactions des particules nanoPDT avec les cellules tumorales – pénétration cellulaire, quantité totale de particules par cellule et cinétique de clairance – ont été étudiées dans le but d'optimiser les conditions d'activation. La quantification des radicaux libres générés et leur localisation ont aussi été étudiées au cours de ce travail de thèse. Une comparaison de la biodistribution des nanoPDT dans différents modèles *in vivo* a été

entreprise afin de mieux comprendre le rôle du type cellulaire, du site de greffage et de la contribution du stroma.

2.2 La thérapie nanoXray™ permet une augmentation significative de la dose de rayons X au niveau de la tumeur sans changer la dose appliquée aux tissus sains

2.2.1 Nanoparticules activables par rayons X : mécanisme d'action de NBTXR3

Les radiations ionisantes interagissent avec les atomes ou les molécules dans les cellules, en particulier avec les molécules d'eau, pour produire des radicaux libres, qui sont responsables de dommages cellulaires non spécifiques conduisant à la mort de la cellule. L'interaction d'un photon X avec une molécule d'eau génère l'ionisation de cette dernière, produisant un électron d'énergie cinétique élevée et un photon d'énergie réduite. Les électrons générés vont perdre leur énergie par interactions multiples avec le milieu environnant, produisant des radicaux libres et de la chaleur. Ces électrons sont majoritairement responsables des effets obtenus en radiothérapie. Les rayons X sont absorbés par l'oxyde d'hafnium, constituant le cœur de la nanoparticule, exactement comme les radiations ionisantes sont absorbées par les molécules d'eau. Cependant, la probabilité d'absorption d'un photon étant proportionnelle au numéro atomique (Z) et à la densité du composé qu'il traverse, la nanoparticule d'oxyde d'hafnium va générer les mêmes types d'effets que les molécules d'eau, mais avec un ordre de grandeur bien supérieur. Les cellules sont ainsi détruites spécifiquement par la production localisée et contrôlée de radicaux libres.

2.2.2 Etat de l'art : des nanoparticules d'or activables par radiation ionisante pour traiter le cancer

La question de l'augmentation de la dose, liée à la présence de matériaux possédant un Z élevé au sein de la zone à traiter par radiothérapie a été abordé, notamment via les calculs Monte Carlo. Ainsi, les nanoparticules d'or ont été perçues comme des systèmes prometteurs pour le traitement de tumeurs, activées par les radiations ionisantes.

A- Trafic intracellulaire des nanoparticules d'or

Chithrani *et al*., 2006, 2007 et 2009, ont largement travaillé l'influence de la taille de nanoparticules d'or sphériques sur l'internalisation cellulaire, le transport et la localisation subcellulaire des nanoparticules. Les nanoparticules d'or (or/citrate) ont été isolées avec des tailles de 14, 30, 50 et 74 nm par réduction du précurseur HAuCl4 en présence d'acide citrique jouant le rôle de réducteur, mais aussi de complexant de la surface des nanoparticules. Les lignées cellulaires HeLa (tumeurs ovariennes), MCF-7 (tumeurs mammaires), STO (fibroblastes) et SNB19 (tumeurs du cerveau) ont alternativement servi de support à leurs études. Les nanoparticules d'or/citrate sont internalisées par voie d'endocytose – récepteur médié - au sein des cellules. Les résultats suggèrent que la surface initiale des nanoparticules d'or/citrate est modifiée par les protéines du sérum, ce qui permet aux nanoparticules d'être internalisées par reconnaissance spécifique de ces protéines avec la membrane cellulaire. Les nanoparticules sont systématiquement observées, sous formes de clusters, dans des vésicules de taille de l'ordre de 500 nm dans le compartiment cytoplasmique des cellules. Le maximum d'internalisation est observé pour des nanoparticules présentant une taille de 50 nm. Ces résultats peuvent être expliqués par un mécanisme compétitif qui met en jeu la notion de « wrapping time », associé aux caractéristiques de la membrane cellulaire et de cinétique de diffusion des récepteurs sur les sites d'internalisation.

Une étude plus spécifique de nanoparticules d'or fonctionnalisées avec de la transferrine a montré que les nanoparticules sont internalisées par un processus chlathrine dépendant. La question de la clairance de ces mêmes nanoparticules d'or fonctionnalisées par de la transferrine a également été abordée sur la lignée cellulaire HeLa (Chithrani *et al*., 2007). Toutes les nanoparticules d'or, indépendamment de leur taille, ressortent des cellules mais avec des cinétiques différentes ; la cinétique de clairance étant plus rapide pour les petites particules. Les nanoparticules apparaissent localisées dans des endosomes tardifs ou lysosomes. Ces vésicules semblent diffuser vers la périphérie de la cellule, fusionner avec la membrane plasmique et enfin relarguer les nanoparticules dans le milieu extérieur. La balance entre la pénétration cellulaire et la clairance (demi-vie d'internalisation) des nanoparticules d'or de 14, 50 et 74 nm a été établie à 0,3, 0,5 et 0,75 heures respectivement.

Bien qu'un travail considérable ait été entrepris par Chithrani *et al*., pour étudier l'influence de la taille et du revêtement de surface des nanoparticules d'or sur l'internalisation cellulaire, le transport et la localisation subcellulaire des nanoparticules,

aucune étude n'a été publiée à ce jour – dans l'état de nos connaissances – pour évaluer l'efficacité de ces nanoparticules par activation avec des radiations ionisantes.

La **localisation** des nanoparticules d'or peut être suivie grâce aux greffages de sondes fluorescentes en surface des nanoparticules ou par visualisation directe en microscopie électronique. La localisation des nanoparticules d'or à l'échelle subcellulaire dépend essentiellement de la fonctionnalisation de la surface de la particule mais aussi de sa taille. Les publications décrivent en majorité les nanoparticules d'or dans le compartiment cytoplasmique, dans des endosomes (Rahman *et al*., 2009 ; Chithrani *et al*., 2006, 2007 et 2009). Chang *et al* (2008) ont cependant observé la localisation de nanoparticules d'or de 13 nm et présentant un revêtement citrate dans le réticulum endothélial et l'appareil de Golgi de cellules tumorales B16F10. Par ailleurs, deux articles décrivent une localisation de nanoparticules d'or dans le noyau de la cellule: des clusters de 1,4 nm pouvant interagir avec l'ADN du noyau (Tsoli *et al*., 2005) et des particules de 3,7 nm fonctionnalisées avec de l'acide 3-mercaptopropionic-polyéthylène glycol (MPA-PEG).

B- Efficacité in vitro et in vivo des nanoparticules d'or

Des tests de viabilité cellulaire ainsi que des tests de clonogénicité ont été réalisées sur la lignée MCF-7 et sur son analogue non malin MCF-10A avec des particules d'or de 10,8 nm fonctionnalisées avec de la cysteamine (AET) ou du thioglucose (Glu). Les cellules, traitées ou non par des nanoparticules d'or – à iso concentrations en nanoparticules d'or au niveau cellulaire – ont reçu une dose de 10 Gray (Gy) avec un générateur de rayons X (200 KVp). Cette étude démontre que les deux types de nanoparticules d'or augmentent la sensibilité de la radiation au niveau des cellules cancéreuses mais qu'aucun effet significatif n'a été observé sur des cellules saines (Kong *et al*., 2008).

Rahman *et al*., 2009 ont étudié les interactions entre des nanoparticules d'or (1,9 nm) et un modèle de cellules endothéliales (BAECs). Les cellules ont été traitées avec des concentrations en nanoparticules d'or croissantes de 0,25 mM à 1 mM. Les études de survie cellulaire, pour différentes doses d'irradiation (0, 1, 2, 3, 4 et 5 Gy) avec un générateur de rayon X (80 KVp et 150 KVp) ont montré un effet dose particules dès 0,25 mM et au-delà. Cependant une étude de viabilité cellulaire, en absence d'activation, a aussi montré une cytotoxicité induite par la seule présence des nanoparticules, fonction de la concentration en nanoparticules (30% de diminution de viabilité cellulaire à été observée pour une concentration en nanoparticule de 1 mM).

Seules deux études précliniques ont été publiées à ce jour sur l'utilisation de nanoparticules d'or activées par des radiations ionisantes pour traiter des tumeurs. Dans une première étude menée par Hainfeld *et al*. (2004), la pousse de tumeurs mammaires de type EMT-6 implantée sur des souris a été suivie. Deux minutes après une injection intraveineuse de particules d'or de 1,9 nm, une dose unique de 30 Gy a été délivrée par une source X (250 kVp) sur la tumeur. 86% de taux de survie à 1 an ont été obtenus pour les animaux ayant reçu le traitement avec les nanoparticules contre seulement 20% pour les animaux exposés à la radiothérapie seule. Dans une deuxième étude, Chang *et al*., 2008, ont étudié l'efficacité de nanoparticules d'or de 13 nm sur des souris porteuses de cellules de mélanomes B16F10. Vingt quatre heures après injection, une dose unique de 25 Gy a été délivrée par une source d'électron de 6 MeV. Une réduction significative de la progression tumorale a été démontrée.

Les données de ces deux études précliniques sont reprises dans la figure 4 :

	Taille des particules d'or	**Ratio du pic tumeur sur tissue normal**	**Clairance**	**Temps d'irradiation après injection i.v**	**Dose d'irradiation**
Hainfeld	1.9 nm	3.5 / 1 à 5 min post i.v	tumeur : 41 min normal : 24 min	2 min	30 Gy 250 kVp X-ray
Chang	13 nm	6.4 / 1 à 24 heures post i.v	/	24 heures	25 Gy 6 MeV e^-

Figure 4. Comparaison des protocoles d'administration et d'activation des nanoparticules d'or

Des nanoparticules d'or présentant des tailles supérieures à celles proposées dans ces deux études précliniques pourraient être intéressantes pour des traitements impliquant des protocoles d'irradiation fractionnée. En effet, la taille des nanoparticules devrait permettre leur rétention au niveau du site tumoral (Chang *et al*., 2008) et favoriser leur accumulation intracellulaire (Chithrani *et al*., 2006, 2007) ce qui réduirait la nécessité d'administrations répétées. Au-delà, les deux études présentées utilisent des doses d'irradiation très élevées. Ces doses ne sont pas adaptées pour des protocoles utilisés en clinique, surtout dans un contexte de radiation fractionnée.

Par ailleurs, les études de toxicité réalisées sur les nanoparticules d'or restent encore peu nombreuses et disparates. Il semble difficile aujourd'hui de juger de la pertinence d'un potentiel développement de ces produits en clinique.

2.2.3. Augmentation de l'effet de la radiothérapie par les nanoparticules nanoXray™

Article 1: « Importance de la biodisponibilité des nanoparticules NBTXR3 dans les endosomes pour optimiser l'efficacité de la radiothérapie sur des cellules tumorales humaines de colon »

Virginie Simon, Ping Zhang, Laurence Maggiorella, Agnès Pottier, Elsa Borghi, Laurent Levy, Julie Marill ; en préparation

Résumé de l'article:

Le concept innovant de concevoir des nanoproduits d'oxyde métallique pour des applications anti-tumorales marque une avancée considérable dans l'utilisation de nanomatériaux comme produits thérapeutiques. NBTXR3 est une suspension aqueuse stérile de nanoparticules, composée d'un cœur d'oxyde d'hafnium et d'une couche superficielle biocompatible. Le cœur dense et inerte est la cible d'interactions avec les radiations ionisantes. L'interaction entre les radiations ionisantes et les nanoparticules NBTXR3 produit des électrons qui vont perdre leur énergie par interactions multiples avec le milieu environnant, produisant des radicaux libres. Les nanoparticules NBTXR3 permettent une augmentation significative de la dose de rayons X dans les lignées tumorales de colon HCT 116 et HT-29 et présentent un effet concentration dépendant. L'interaction des nanoparticules NBTXR3 avec les membranes est indépendante d'un processus spécifique d'internalisation. L'étude de l'internalisation des nanoparticules dans les cellules, leur localisation cytoplasmique et leur efficacité a montré une corrélation entre la localisation intracellulaire des nanoparticules et l'augmentation de la cytotoxicité des radiations sur les cellules tumorales HCT 116. Les effets intracellulaires engendrés par les nanoparticules NBTXR3 activées ont été observés aussi bien au niveau cytoplasmique que nucléaire. Les nanoparticules présentent un temps de résidence long au niveau du cytoplasme et se retrouvent dans les endosomes tardifs. La biodisponibilité spécifique des particules au niveau des endosomes semble être le paramètre clef pour augmenter significativement la destruction des cellules tumorales par les radiations.

2.3 Des Pp IX nanotransporteurs créent une localisation et une biodistribution entre la tumeur et les tissus sains différenciées: élargissement de la fenêtre thérapeutique de la thérapie photodynamique

2.3.1 Mécanisme d'action de la thérapie photodynamique

La thérapie photodynamique est basée sur un mécanisme de photo oxydation de la matière. Ce type de traitement requiert: une **molécule photosensible** qui s'accumule préférentiellement au sein des tumeurs, une **source lumineuse** capable d'exciter cette molécule et un milieu riche en **oxygène**. L'oxygène singulet, l'agent cytotoxique principal produit lors de la thérapie photodynamique, est une forme hautement réactive de l'oxygène produit à partir du dioxygène cellulaire.

Lors de l'exposition à des rayonnements laser, les molécules photosensibles génèrent la formation de radicaux libres: les chromophores activés permettent la transformation de l'oxygène moléculaire environnant et des espèces réactives de l'oxygène en **radicaux libres**, qui sont des espèces hautement réactives provoquant des dommages irréversibles dans les cellules tumorales. L'oxygène singulet réagit **localement et rapidement** : la migration d'un oxygène singulet est inférieure à 0,02 µm et celui-ci possède un temps de demi-vie d'environ 52 µs (Rossi *et al*., 2008). Les effets oxydatifs induits par la thérapie photodynamique sont ainsi confinés à une région restreinte. De plus, les longueurs d'ondes utilisées pour activer la molécule photosensible, ne permettent pas de traverser une grande épaisseur de tissu. NanoPDT est, de ce fait, destiné préférentiellement aux **cancers superficiels** comme celui de la peau, de l'oesophage, de la vessie ou de l'estomac. Lorsque l'activation par le laser cesse, les nanoparticules reviennent à leur état inerte.

2.3.2 Présentation des photosensibilisants avec autorisation de mise sur le marché

La famille des **porphyrines** a généré un grand nombre de produits actuellement sur le marché ; Photofrin®, Levulan®, Metvix® and Visudyne® en sont les dérivés. Le médicament **Photofrin®** est le médicament qui a la plus longue histoire clinique et qui a été utilisé sur le plus grand nombre de patients. Il s'agit d'un mixte complexe d'environ 60 composants qui combine des monomères, des dimères et des oligomères dérivant de l'hématoporphyrine. La famille des **chlorines** a conduit à trois produits utilisés fréquemment en clinique : Foscan®

(composé de temoporphyrine), LS11® (composé de talaporfine) et Photochlor® (composé de 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide : HPPH).

Enfin, la famille de **texaphyrine** a permis de développer le produit commercial Antrin® qui est synthétisé à partir du lutexaphyrine.

Le premier succès du Photofrin® a été établi pour le traitement des cancers de la vessie. La première autorisation de mise sur le marché (AMM) en 1993 par la Food and Drug Administration (FDA) a été donnée au Canada puis en 1995 aux Etats-Unis pour le traitement du cancer de l'œsophage. Depuis le médicament Photofrin® a obtenu des AMM dans de nombreux pays : en Europe et aux Etats-Unis pour le cancer de l'œsophage de type Barett ; au Japon pour des dysplasies cervicales ainsi que pour les cancers gastriques ; au Canada pour le cancer de la vessie papillaire ; au Canada, Danemark, Finlande, France, Irlande, Japon, Hollande, Angleterre et Etats-Unis pour le cancer de l'œsophage et au Canada, Danemark, Finlande, France, Irlande, Japon, Hollande, Angleterre et Etats-Unis et l'Allemagne pour le cancer endobronchéale.

2.3.3 Administration par voie générale des photosensibilisants destinés à un traitement local

A- Principales caractéristiques pharmacocinétiques du Photofrin®

Des études pharmacocinétiques du Photofrin® ont été effectuées chez la souris, le rat, le cochon d'inde, le chien ainsi que chez l'homme et se sont avérées pour tous les modèles relativement similaires. En général, Photofrin® est injecté chez les patients à la concentration de 2 mg/kg. L'activation par laser (λ= 630 nm) a lieu entre 24 et 48 heures après injection avec une dose d'environ 200-300 J/cm.

B- Effets secondaires du Photofrin®

Chez la souris et le rat, des études de biodistribution ont montré une accumulation du Photofrin® au niveau du foie, de la rate et du rein. Une quantité minime a été quantifiée au niveau de la peau (Tronconi *et al*., 1995).

Chez l'homme, par opposition, Photofrin® a montré une forte photosensibilité au niveau de la peau. Ceci est dû à une biodistribution du produit assez importante à ce niveau. Les effets secondaires sont par conséquents lourds pour les patients puisqu'ils doivent éviter tout contact avec la lumière naturelle pendant 4 à 6 semaines.

2.3.4 Les nanotransporteurs

A- Etat de l'art des nanotransporteurs de silice

Au regard de la littérature, il existe deux familles de nanotransporteurs à base de silice pour des applications anti-cancéreuses en thérapie photodynamique : celle où l'agent photosensibilisant est greffé à la particule de silice et celle où l'agent photosensibilisant est encapsulé.

Plusieurs équipes ont testé des nanoparticules de silice ayant un protocole de synthèse chimique complexe. En effet, il s'agit d'établir un lien **covalent** entre le photosensibilisant et la coque de silice. L'équipe d'Ohulchanskyy (2007) et celle de Brevet (2009) développent respectivement des nanoparticules de silice où le dérivé du HPPH est greffé à la nanoparticule de silice. Plus proche de nos applications, Rossi et son équipe (2008) et Tu et son équipe (2009) ont synthétisé des nanoparticules de silice mésoporeuses dans lesquelles le Pp IX a été greffé. Tu *et al* ont testé ces particules (110 nm) *in vitro* sur des cellules HeLa. Les résultats indiquent que les nanoparticules sont efficaces. Une autre voie de synthèse consiste à encapsuler **(absence de lien covalent)** dans une coque de silice les photosensibilisants (Yan *et al*., 2003 ; He *et al*., 2009). L'équipe de Tang (2005) a par exemple encapsulé du bleu de méthylène. Roy (2003) et son équipe ont encapsulé du HPPH et montré une bonne efficacité du produit *in vitro*.

B- Nanobiotix a sélectionné un procédé de synthèse simple pour développer des nanotransporteurs contenant le photosensibilisant Pp IX.

Article 2: « Synthèse d'un nouvel hybride polyvalent (nanotransporteur), montrant une stabilité adaptée dans un environnement biologique pour une utilisation en thérapie photodynamique »

Edouard Thiénot, Matthieu Germain, Kelthoum Piejos, Virginie Simon, Audrey Darmon, Julie Marill, Elsa Borghi, Laurent Levy, Jean-François Hochepied et Agnès Pottier ; soumis

Résumé de l'article:

Un nouveau matériau hybride a été conçu par un procédé de synthèse simple, basé sur l'approche sol-gel, pour encapsuler efficacement un photosensibilisant, le Pp IX et préserver ainsi son activité intacte dans un environnement biologique, pour son utilisation en thérapie photodynamique. Ces matériaux, les nanotransporteurs, ont été obtenus sous formes sphériques avec des tailles pouvant être ajustées entre 10 nm et 200 nm. La capacité du Pp IX encapsulé dans les nanotransporteurs à libérer efficacement les radicaux libres sous irradiation laser a été démontrée. De même, sa capacité à induire la mort de cellules tumorales a été testée avec succès *in vitro*. La stabilité des nanotransporteurs a été suivie par absorbance ultraviolet et par émission de fluorescence à la fois en milieu aqueux et en milieu 100% sérum. Une perte de stabilité – évaluée par la capacité de la molécule Pp IX à générer des espèces réactives – a été observée après 2 heures de mûrissement des nanotransporteurs dans le milieu 100% sérum. La flexibilité des nanotransporteurs a été envisagée comme un paramètre essentiel pour préserver l'activité du Pp IX dans un environnement biologique. Un second traitement de surface réalisé sur la première génération de nanotransporteurs, a permis de former un composé bicouche et d'augmenter de façon significative la stabilité du nanotransporteur dans les milieux biologiques. Cette seconde génération de nanotransporteurs ouvre de nouvelles perspectives pour la thérapie photodynamique.

2.3.5 Interactions entre les nanoparticules de silice encapsulant le Pp IX et des systèmes biologiques

Article 3: « Des nanoparticules de silice encapsulant le photosensibilisant Pp IX présentent des interactions spécifiques avec des lignées cellulaires tumorales *in vitro* et des modèles de souris porteuses de cancers humains *in vivo*"

Virginie Simon, Corinne Devaux, Audrey Darmon, Thibault Donnet, Edouard Thiénot, Matthieu Germain, Jérôme Honnorat, Alex Duval, Agnès Pottier, Elsa Borghi, Laurent Levy et Julie Marill ; accepté dans la revue Photochemistry Photobiology.

Résumé de l'article:

Des nanoparticules de silice (nanotransporteurs) encapsulant le Pp IX, développées pour des applications en thérapie photodynamique, ont été testées à la fois dans des modèles *in vitro* et *in vivo*. Cette étude a pour ambition de mieux appréhender le rôle des différents facteurs biologiques sur la génération de photodommages induits par les nanoparticules. Ces nanoparticules de silice encapsulant le Pp IX, les nanotransporteurs de première génération, ont montré leur efficacité dans des conditions d'activation mettant en jeu des températures extrêmes (4°C), ce qui suggère une plus grande proportion d'internalisation par voie passive que par voie active. Pour la première fois, la clairance cellulaire de ces nanotransporteurs a été démontrée. L'estimation de la viabilité cellulaire a été établie dans six lignées de cellules tumorales. Pour tous les types cellulaires, les nanotransporteurs sont plus efficaces que le Pp IX libre. Un fort signal fluorescent démontre la colocalisation des radicaux libres générés et des nanoparticules, corrélé à 100% de mortalité cellulaire. Des études *in vivo* réalisées sur des souris porteuses des lignées HCT 116 (tumeur de colon ; cellules injectées en sous-cutané), A549 (tumeur des poumons ; cellules injectées en sous-cutané) et de glioblastome multiforme (tumeur du cerveau ; fragment de tumeur injecté en sous-cutané), ont montré une meilleure accumulation des nanoparticules au niveau de la tumeur que dans le groupe contrôle (molécule libre), soulignant leur forte sélectivité pour les tissus tumoraux. Comme observé dans les tests *in vitro*, le type de tumeur s'avère être un facteur déterminant pour l'accumulation des nanoparticules. Cependant l'environnement tumoral peut potentiellement encore plus influencer ces différents temps d'accumulation. Ces résultats renforcent le fait que ces nanotransporteurs peuvent devenir une nouvelle alternative pour des applications locales de la thérapie photodynamique.

III-RESULTATS

Les nanoparticules NBTXR3, issues de la plateforme nanoXray^{TM}, activables par radiothérapie, ainsi que les nanotransporteurs nanoPDT développés pour des applications en thérapie photodynamique, ont pour ambition d'élargir la fenêtre thérapeutique. Ces deux

produits thérapeutiques sont destinés à rendre les traitements des cancers plus efficaces et moins nocifs pour les tissus sains.

-Recherches au sein de la plateforme nanoXray^{TM}

Les nanoparticules NBTXR3 induisent une importante diminution de la survie des cellules HCT 116 après irradiation à la dose de 2 Gy. Une différence significative de viabilité cellulaire a été mise en évidence avec les nanoparticules NBTXR3 sous irradiation aux concentrations de 100, 200, 400 et 800 µM comparativement au contrôle (radiothérapie seule). Un effet concentration dépendant a été observé sur la viabilité cellulaire. Des tests de clonogénicité réalisés à la fois sur la lignée radiosensible HCT 116 et sur la lignée radiorésistante de colon HT-29 ont clairement démontré un effet seuil de la radiodestruction pour une concentration en nanoparticules NBTXR3 de 50 µM et 100 µM respectivement, par rapport à la radiothérapie seule. Aucun signe de toxicité associé aux nanoparticules NBTXR3 n'a été observé sur ces deux types de lignées cellulaires.

Concernant les études réalisées sur une lignée de tissu sain (lignée de fibroblastes transformés), aucun effet différentiel n'a été observé entre les nanoparticules NBTXR3 irradiées et la radiothérapie seule à la dose de 2 Gy (résultats non montrés).

La viabilité cellulaire a été mesurée pour différents temps d'incubation avec les nanoparticules NBTXR3 en présence des cellules HCT 116. L'efficacité dépend du temps d'incubation. Elle est observée dès 2 heures. Une différence significative est observée entre 2 heures et 4, 15 et 24 heures d'incubation.

Les images en microscopie électronique à transmission (MET) des cellules HCT 116 incubées 2 et 24 heures avec les nanoparticules NBTXR3 présentent une internalisation différenciée. Après 2 heures, 20% des cellules ont internalisé les nanoparticules NBTXR3. Après 24 heures, 80% des cellules ont internalisé les nanoparticules NBTXR3. Ces dernières sont localisées dans le compartiment cytoplasmique au sein d'endosomes. Pour un nombre total de nanoparticules NBTXR3 internalisées par cellules (entre 60±7,7 $mg.kg^{-1}$ et 103±3,5 $mg.kg^{-1}$ de nanoparticules NBTXR3 quantifiées pour 100 000 cellules sur la lignée HCT 116), la localisation des particules dans les endosomes apparaît être le paramètre essentiel pour augmenter de façon significative l'effet de la radiothérapie.

La viabilité cellulaire est similaire à celle observée pour la radiothérapie seule pour des cellules traitées 2 heures avec NBTXR3 et irradiées immédiatement après avoir renouvelé le milieu. A l'inverse, la viabilité cellulaire diminue de façon significative, par rapport à la

radiothérapie seule, pour des cellules traitées 2 heures avec NBTXR3 et irradiées 48 heures après avoir renouvelé le milieu. Parallèlement, la quantification des nanoparticules NBTXR3 au niveau cellulaire, montre une même concentration de nanoparticules pour des cellules incubées 2 heures avec NBTXR3, suivie par un changement du milieu et incubées aux temps supplémentaires de 0 et 48 heures. Ces résultats renforcent l'hypothèse de la nécessité d'une localisation intracellulaire des nanoparticules pour produire un effet thérapeutique sous irradiation et suggèrent l'absence du phénomène de clairance à une échelle de temps d'au moins 48 heures.

L'analyse et l'interprétation des images MET des cellules HCT 116 traitées avec NBTXR3 et irradiées suggèrent une mort cellulaire par apoptose et par autophagie. Après irradiation, les cellules traitées par NBTXR3 montrent une proportion significative de micronoyaux par rapport à la radiothérapie seule. Une désorganisation cellulaire est aussi observée ainsi que la fusion d'endosomes contenant les nanoparticules pour former des vésicules de grandes tailles, concentrées dans une zone du cytoplasme. La fusion d'endosomes a aussi été décrite pour des temps prolongés d'incubation.

Les nanoparticules NBTXR3 semblent interagir de façon non spécifique avec les membranes cellulaires des cellules HCT 116 et pénétrer par endocytose. L'observation des cellules incubées 24 heures avec les nanoparticules, 96 heures après irradiation, montre encore la présence des nanoparticules dans des vésicules (endosomes et lysosomes). Les nanoparticules semblent rester dans le cytoplasme.

En parallèle du travail réalisé au cours de cette thèse, les nanoparticules NBTXR3 ont été injectées par voie intratumorale sur des souris porteuses de tumeurs HCT 116 greffées sur le flanc. Une irradiation locale de la tumeur a été effectuée. Une régression, voire une éradication, tumorale a été observée chez tous les animaux irradiés alors que le groupe radiothérapie seule ne présente qu'un retard de croissance tumorale : après 60 jours, 90% des animaux ne portaient plus de tumeurs pour le groupe traité avec les nanoparticules NBTXR3 et irradié. Le groupe avec la radiothérapie seule a montré une reprise de la pousse tumorale entraînant le sacrifice des animaux avant la fin de l'étude. Une étude de tolérance sur des souris nudes porteuses de tumeurs HCT 116 a été effectuée par administrations répétées par voie intraveineuse de nanoparticules NBTXR3. La dose administrée a été bien tolérée chez l'ensemble des animaux.

-Recherches au sein de la plateforme nanoPDT

Les nanoPDT ou nanotransporteurs sont des nanoparticules à base de silice encapsulant la molécule photosensible Pp IX. Ces nanoparticules sont de morphologies sphériques et monodisperses en taille. Un contrôle de leur taille entre 10 et 200 nm a été effectué grâce à l'ajustement de la température de la synthèse entre 18°C et 37°C. L'effet de la taille de ces nanotransporteurs de première génération a été évalué *in vitro* sur la lignée HCT 116. Des tests de viabilité cellulaire ont montré des résultats équivalents quelque soit leur taille en accord avec les résultats de cinétique d'internalisation et du nombre total de particules internalisées.

Afin de comprendre les mécanismes d'internalisation de ces nanotransporteurs dans les cellules HCT 116, des tests de viabilité cellulaire ont été effectués à 4°C et à 37°C. La température de 4°C est connue pour bloquer la voie d'internalisation d'endocytose/pinocytose. Les cellules HCT 116 traitées pendant 3 heures avec des nanoparticules à 4°C et activées par laser juste après montrent une EC_{50} (dose efficace 50%) équivalente à celle obtenue dans les mêmes conditions à 37°C. Cependant, le nombre total de nanoparticules internalisées est plus faible à 4°C qu'à 37°C (résultats non montrés). Ces résultats suggèrent une balance d'internalisation entre la voie passive et active. Les nanoparticules sont internalisées dans le cytoplasme. Le signal fluorescent des nanoPDT est diffus à travers tout l'espace cytoplasmique. Des études complémentaires de colocalisation de nanoparticules permettraient de savoir plus précisément avec quelles organelles les particules interagissent.

La question de la clairance des nanoparticules a été abordée. Les cellules HCT 116 ont été traitées pendant 3 heures à plusieurs concentrations de nanoPDT. Puis le milieu a été retiré et du nouveau milieu sans nanoparticule a été ajouté. Vingt quatre heures après la fin de l'incubation, 100% des nanoparticules sont ressorties des cellules. De plus, la clairance des nanoparticules commence très rapidement (dès 2 heures). Ces résultats montrent que l'efficacité des nanoparticules est corrélée à la localisation intracellulaire des nanoPDT.

Un effet temps d'incubation des nanoparticules sur HCT 116 a aussi été démontré en viabilité cellulaire. Trois heures d'incubation (avec une concentration en particules de 1 µM) sont suffisants pour induire 100% de mort cellulaire. Le meilleur temps d'irradiation a été défini comme étant celui de 20 min. Les conditions optimisées retenues sont: 3 heures d'incubation, 20 min d'irradiation et 48 heures de post incubation.

Aucun test *in vitro* n'a montré un bénéfice de la multi-irradiation en terme de réponse cellulaire (résultats non montrés). Les tests de multi-irradiation pourront cependant être entrepris *in vivo* afin de mettre en évidence un éventuel bénéfice.

Des tests de viabilité cellulaire et de toxicité ont été entrepris dans six lignées différentes: HCT 116, HT-29, A431 (tumeur épidermoïde), LLBC37 (lymphoblastoïde), MCF-7 et MDA-MB-231 (tumeurs mammaires). Pour toutes ces lignées, les nanoparticules nanoPDT ont une efficacité supérieure (différence de plus d'un log) à celle du Pp IX libre. Les valeurs d'EC_{50} des nanoparticules sont comprises entre 0,4 et 1,2 µM alors que les valeurs d'EC_{50} du Pp IX libre sont comprises entre 3,4 et 32,7 µM pour l'ensemble des lignées testées.

Une corrélation a été mise en évidence entre la génération de radicaux libres et les concentrations en nanoPDT dans les cellules HCT 116 et HT-29. Cependant, la quantité de nanoparticules internalisées dans la lignée HT-29 est plus faible que celle quantifiée dans la lignée HCT 116. Un maximum de 600 fmol de nanoparticules pour 1000 cellules (3 heures d'incubation à 5 µM de nanoPDT) a été quantifié dans la lignée HCT 116 alors qu'un maximum de 200 fmol de nanoparticules pour 1000 cellules a été quantifié pour HT-29 dans les mêmes conditions. Ces résultats démontrent un différentiel d'internalisation entre les deux lignées testées qui apparaît corrélé aux valeurs respectives des EC_{50} observées.

Une mort cellulaire par apoptose est majoritairement décrite dans la littérature après injection du Pp IX ou du Photofrin® activés. Le mécanisme de mort cellulaire dans toutes les lignées testées devrait être abordé avec nos nanoparticules.

De plus, des études de biodistribution ont été réalisées sur des souris porteuses des différentes lignées (HCT 116, A549 et glioblastome). Les résultats montrent une très bonne accumulation de ces particules au niveau de la tumeur avec des temps d'accumulation maximaux variables selon les modèles testés.

Dans le but d'optimiser la performance des nanoPDT, des études de stabilité dans le milieu biologique ont été entreprises. Les résultats montrent que ces nanotransporteurs de première génération présentent une perte de leur stabilité dès 2 heures d'incubation dans du sérum de souris. En effet, ces nanoparticules présentent une modification du spectre d'absorption du Pp IX et une perte d'émission de fluorescence. Afin de générer un nanotransporteur stable dans les milieux biologiques, des nanotransporteurs de deuxième génération ont été développés.

La seconde génération de produits nanoPDT, a été conçue en réalisant un second revêtement de surface biocompatible (addition de sodium trimétaphosphate : STMP)

permettant de générer un nanotransporteur bicouche. Ce nanotransporteur a montré une excellente amélioration de la stabilité en milieu biologique – capacité du Pp IX encapsulé à générer des radicaux libres responsables de la destruction cellulaire - jusqu'à 12 heures de mûrissement des nanoparticules dans du sérum.

IV-CONCLUSIONS ET PERSPECTIVES GENERALES

Ce travail a permis d'enrichir les connaissances dans le domaine novateur de la nanomédecine. Spécifiquement, l'étude des interactions entre les nanoparticules activées par différentes sources d'énergie électromagnétiques externes et des cellules cancéreuses pour élargir la fenêtre thérapeutique a été explorée.

La nanotechnologie permet de générer des nanomatériaux présentant de nouvelles fonctions, comme l'utilisation de nanoparticules activables pour traiter le cancer. Elle permet aussi de générer des nanomatériaux comme vecteurs de molécules thérapeutiques pour modifier la pharmacocinétique du principe actif et la biodisponibilité subcellulaire. La taille nanométrique des objets permet d'agir à l'échelle du monde biologique. Cependant il apparaît essentiel aujourd'hui que ces objets interagissent avec les systèmes biologiques pour être utilisés sans danger et de façon effective en médecine. Quand le monde des nanoparticules rencontre celui de la médecine, de nouvelles modalités pour le traitement du cancer sont alors envisagées, comme celle de l'utilisation d'une source énergétique externe à l'organisme pour activer les médicaments.

Ce travail de doctorat a contribué au développement de deux types de nanoparticules, NBTXR3 et nanoPDT, destinées à être utilisées pour une même application, le cancer, par deux approches différentes.

NBTXR3 est le produit phare de plateforme nanoXray^{TM}. Nous avons mis en évidence dans des lignées tumorales que les nanoparticules interagissent de façon non spécifique avec la membrane cellulaire et pénètrent dans les cellules *via* un mécanisme d'endocytose, que la localisation de ces nanoparticules est cytoplasmique *via* leur confinement dans des endosomes

puis dans des lysosomes. Les nanoparticules résident dans les endosomes et dans les lysosomes. De plus, l'importance de l'internalisation de ces nanoparticules au niveau cytoplasmique apparaît nécessaire pour obtenir une augmentation significative de l'effet de la radiothérapie.

L'ensemble des ces résultats participe à l'établissement du rationnel de développement de NBTXR3. L'étude de l'efficacité des nanoparticules activées par différents types d'énergie et selon les spécificités biologiques des tumeurs, constitue des axes de recherches qui doivent être explorés.

Des études précliniques se poursuivent actuellement. Des résultats préliminaires d'efficacité *in vivo* montrent que l'administration de NBTXR3 à des souris porteuses de tumeurs de colon (HCT 116) xénogreffées permet une régression totale de tumeur et ce pour tous les animaux traités. La plateforme nanoXray^{TM} développe un traitement totalement novateur qui pourra être utilisé seul ou en synergie avec les protocoles de traitements déjà existants: la chimiothérapie, la chirurgie, l'immunothérapie, etc.

La plateforme nanoPDT axe sa technologie sur l'utilisation de nanoparticules comme nanotransporteurs. Nous avons prouvé que les nanoparticules de silice encapsulant le Pp IX étaient des transporteurs efficaces pour délivrer des médicaments mais aussi pour les protéger de l'exposition à un milieu aqueux environnant. Ces nanotransporteurs permettent de piéger efficacement le Pp IX et présentent une gamme de porosité appropriée pour libérer les radicaux libres après activation par laser dans le milieu environnant. L'encapsulation physique du Pp IX s'avère être la voie de synthèse la plus simple possible. L'addition d'un second composé (le STMP) à la surface des nanoPDT, pour former un système bicouche, permet leur stabilité dans un milieu avec sérum.

De plus, des études précliniques ont démontré la non toxicité de ces nanotransporteurs que ce soit *in vitro* ou *in vivo*. Nous avons montré que les nanoparticules de 10 à 60 nm avaient le même comportement tant au niveau de l'internalisation que de l'efficacité cellulaire. Une internalisation en majorité par voie passive a été démontrée dans la lignée HCT 116. Pour l'ensemble des six lignées cellulaires testées, la localisation des nanoparticules est la même à savoir diffuse dans tout le compartiment cytoplasmique. Une internalisation optimale des nanoPDT dans les cellules entraîne par conséquent un maximum de libération de radicaux libres générés et ainsi induit une réponse optimale en terme de mort cellulaire. Pour toutes les lignées cellulaires testées, les nanotransporteurs nanoPDT sont plus efficaces que la molécule de référence.

Des études *in vivo* réalisées sur des souris porteuses de tumeur ont permis de réaliser les premières observations de l'accumulation du produit en terme de ratio tumeur/peau. Les résultats montrent une forte accumulation du produit dans la tumeur. De plus, l'analyse semi quantitative réalisée sur trois modèles différents de tumeurs a montré des différences de cinétique d'accumulation des nanoPDT.

Récemment, Nanobiotix a publié des résultats préliminaires d'études précliniques sur les produits issus de la plateforme nanoPDT et a validé leur utilisation dans le traitement du glioblastome, un des cancers les plus mortels et les plus fréquents au niveau du cerveau.

Il reste bien évidemment encore beaucoup d'études à effectuer mais ces premiers résultats précliniques nous rendent confiants quant au développement de cette plateforme technologique prometteuse nanoPDT.

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