UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Desenho e produção de novos veículos para entrega de fármacos

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To my parents…

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Abstract

Nanotechnology is a science that conjugates several disciplines from engineering to chemistry, physics and biology at a molecular level. One of the areas of research with particular focus over the last few years is nanomedicine. It is expected that several improvements in medical care, from diagnosis to treatment, will occur, in the near future, due to the application of nanotechnology to medicine. Several biological and chemical compounds are being applied for the production of drug delivery systems at the nanoscale. These biocompatible systems have some interesting properties that make them capable of performing a targeted delivery of drugs and other materials. Lipossomes, solid lipid nanoparticles, dendrimers, nanoemulsions, polymeric nanoparticles and inorganic nanoparticles like carbon nanotubes, magnetic and ceramic nanoparticles are examples of drug delivery systems. Mesoporous silica nanoparticles are ceramic particles with good structural and biological properties that can be easily functionalized, allowing the bond of other compounds, like for instance gold. The objective of the present work was to produce mesoporous silica nanoparticles, to functionalize their surface and also to bond gold nanoparticles to form a core/shell nanosystem. The produced nanoparticles were characterized by Scanning Electron Microscopy, Fourier Transform Infrared spectroscopy and X-Ray Diffraction. Moreover, the loading and release profiles of the nanoparticles were also characterized using ibuprofen as model drug. *In vitro* studies were performed to evaluate the internalization and cytotoxicity of the produced nanocarriers. The results herein presented, suggest that the produce nanosytem are suitable for future drug delivery applications.

Keywords

Core/Shell nanoparticles; Drug Delivery Systems; Nanotechnology; Silica nanoparticles.

Resumo

A nanotecnologia é uma ciência que conjuga diversas áreas científicas tais como engenharia, química, física e a biologia a nível molecular. A nanomedicina é uma das áreas de investigação mais em foco nos últimos anos. Estima-se que diversas melhorias, desde o diagnóstico até ao tratamento venham a surguir num futuro próximo devido à aplicação da nanotecnologia à medicina. Vários compostos químicos e biológicos têm sido aplicados para a produção de sistemas de entrega de drogas à nanoescala. Algumas propriedades que estes sistemas biocompatíveis possuem permitem-lhes efetuar a entrega de fármacos e outros materiais a um tecido alvo específico. Lipossomas, nanopartículas lipídicas sólidas, dendrímeros, nanoemulsões, nanopartículas poliméricas e nanopartículas inorgânicas como nanotubos de carbono e nanopartículas magnéticas e poliméricas, são exemplos de sistemas de entrega de fármacos. As nanopartículas mesoporosas de sílica são nanopartículas cerâmicas com boas propriedades estruturais e biológicas, que podem ser facilmente funcionalizadas à superfície, permitindo a ligação de outros compostos, como por exemplo ouro. O objetivo do presente trabalho foi produzir nanopartículas mesoporosas de sílica, funcionalizar a sua superfície e ligar-lhe nanopartículas de ouro para formar um nanosistema núcleo/concha. As nanopartículas produzidas foram caracterizadas por Microscopia Electrónica de Varrimento, Espectroscopia Infravermelha por Transformada de Fourier e Difracção Raio-X. Os perfis de captação e libertação de fármacos pelas nanopartículas foram também caraterizados. Para avaliar a internalização e a citotoxicidade dos nanoveículos produzidos foram também realizados estudos *in vitro*. Os resultados aqui apresentados sugerem que os nanosistemas produzidos podem futuramente ser aplicados em aplicações de entrega de fármacos.

Palavras-chave

Nanopartículas de Sílica; Nanopartículas núcleo/concha; Nanotecnologia; Sistemas de Entrega de Fármacos.

Resumo alargado

A nanotecnologia pode ser definida como a ciência que estuda os eventos na dimensão dos nanómetros. Diversas áreas científicas tais como engenharia, química, física e a biologia a nível molecular estão enquadradas nela. Todo o processo, desde a síntese até à aplicação de um material ou dispositivo são etapas da nanotecnologia. A nanomedicina é uma das áreas de investigação mais em foco nos últimos anos. O seu desenvolvimento levará a uma melhor compreensão dos processos biológicos que ocorrem à nanoescala. Um dos seus principais objetivos é aproveitar as propriedades e características físicas dos materiais para desenvolver a prática médica em todo o seu processo. Estima-se que diversas melhorias, desde o diagnóstico até ao tratamento venham a surguir num futuro próximo devido à aplicação da nanotecnologia à medicina.

Dentro da nanomedicina encontram-se os sistemas de entrega de drogas. Prevê-se que o seu desenvolvimento venha a revolucionar o panorama farmacêutico e biotecnológico nos próximos anos. Vários compostos químicos e biológicos têm sido aplicados para a produção destes sistemas de entrega de fármacos à nanoescala. Os fármacos podem encontrar-se ligados à superfície do nanotransportador, encapsulados dentro destes ou dissolvidos na sua matriz. Estes sistemas apresentam diversas vantagens sobre os métodos tradicionais de administração de fármacos. Nomeadamente, a protecção de fármacos hidrofóbicos, a deteção do destino dos compostos no organismo, o aumento do tempo de meia-vida de um fármaco na circulação, a entrega intracelular e a possibilidade de entrega de mais do que um composto ao mesmo tempo. Para além disso, estes sistemas são biocompatíveis e possuem a capacidade de entrega de fármacos ou outros materiais a um tecido alvo específico. A capacidade de um sistema entregar especificamente o seu conteúdo, passiva ou ativamente a um alvo selecionado permite que a atuação do fármaco seja mais direccionada e desta forma, actuar ao nível das células afetadas, não interferindo com os tecidos saudáveis circundantes. A aplicação destes sistemas de entrega de fármacos ao corpo humano torna-se por isso muito atrativa. A toxicidade associada a estes sistemas, é uma propriedade fulcral, no campo das aplicações clínicas.

Lipossomas, nanopartículas lipídicas sólidas, dendrímeros, nanoemulsões, nanopartículas poliméricas e nanopartículas inorgânicas como nanotubos de carbono e nanopartículas magnéticas e poliméricas, são exemplos de sistemas de entrega de fármacos. Todos possuem vantagens e desvantagens entre eles dependendo da aplicação a que se destinam. As nanopartículas mesoporosas de sílica são nanopartículas cerâmicas inorgânicas com centenas de canais vazios (mesoporos) arranjados numa estrutura 2D em forma de favos. A aplicação destes nanosistemas em nanomedicina surge recentemente como campo de extensiva pesquisa. Elas possuem boas propriedades estruturais e biológicas que as tornam muito atrativas em termos de entrega de fármacos. Uma das suas propriedades mais relevantes é a facilidade de funcionalização à superfície, permitindo a ligação de outros compostos, como por exemplo ouro. A formação de nanopartículas constituídas por dois ou

mais materiais origina um sistema designado por núcleo/concha. A junção de materiais permite aliar as propriedades de ambos para melhorar a performance do nanosistema.

O objectivo do presente trabalho foi produzir nanopartículas mesoporosas de sílica, funcionalizar a sua superfície e ligar-lhe nanopartículas de ouro para formar um nanosistema núcleo/concha.

As nanopartículas mesoporosas de sílica foram produzidas por uma adaptação do método Stöber e funcionalizadas com aminas para permitir a ligação de nanopartículas de ouro produzidas por uma adaptação do método de Frens. Várias técnicas foram utilizadas para caracterizar os nanoveículos produzidos. Microscopia Electrónica de Varrimento foi realizada para analisar a morfologia e o tamanho das partículas, a Espectroscopia Infravermelha por Transformada de Fourier efetuada permitiu fazer a analise química dos compostos e dos nanoveículos produzidos. Por fim, a Difracção Raio-X foi usada para identificação da estrutura molecular das partículas. Os perfis de captação e libertação de fármacos pelas nanopartículas foram também caracterizados para avaliar a capacidade de aplicação dos sistemas à entrega de fármacos. Para a avaliação da internalização das nanopartículas pelas células procedeu-se à obtenção de imagens de Microscopia Laser de Varrimento Confocal. A citotoxicidade dos nanoveículos produzidos foi avaliada por estudos *in vitro*.

Os resultados aqui apresentados sugerem que os nanosistemas produzidos podem futuramente ser aplicados na entrega direccionada de fármacos.

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List of Acronyms

µm	Micrometers
APTMS	(3-aminopropyl) trimethoxysilane
Au_NPs	Gold Nanoparticles
CLSM	Confocal Laser Scaning Microscopy
cm	Centimeters
CNTs	Carbon Nanotubes
CTAB	Hexadecyltrimethylammonium Bromide
DDS	Drug Delivery System
DNA	Deoxyribonucleic Acid
EPR	Enhanced Permeability
EtOH	Ethanol
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FTIR	Fourier Transform Infrared
HAuCl4.3H2O	Tetrachloroauric (III) Acid
HCl	Hydrochloric Acid
IBP	Ibuprofen
k^+	Positive Control
K^-	Negative Control
MCM	Mobile Crystalline Material
MNPs	Magnetic Nanoparticles
MRI	Magnetic Resonance Imaging
MSNPs	Mesoporous Silica Nanoparticles
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium reagent
MWNTs	Multi-walled Carbon Nanotubes
NaOH	Sodium Hydroxide
NIH	National Institute of Health
nm	Nanometers
PAMAM	Polyamidoamine
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline - Tween 20
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
PNs	Polymeric Nanoparticles

PMS	Phenazine Methosulfate
RES	Reticulo-endothelial System
RME	Receptor Mediated Endocytosis
SBA	Santa Barbara Amorphous
SiAu_NP´s	Silica/gold Nanoparticles
SiAuIBP_NP´s	Silica/gold Ibuprofen Nanoparticles
siRNA	Small Interfering Ribonucleic Acid
SiIBP_NP´s	Silica Ibuprofen Nanoparticles
Si_NP´s	Silica Nanoparticles
SEM	Scaning Electron Microscopy
SWNTs	Single-walled Carbon Nanotubes
TEOS	Tetraethyl Orthosilicate
XRD	X-ray Diffraction

Chapter I - Introduction

1. Introduction

1.1. Nanotechnology

The term nanotechnology, derived from the Greek word for dwarf, "nano", is defined as the science that studies the events in the dimension range between 1 to 100 nanometers (nm) for some authors, while others consider the maximum value of 1000 nm (Sahoo *et al.*, 2003; Koo *et al.*, 2005; Kingsley *et al.*, 2006). Synthesis, characterization and application of a material, device or system production are stages to be fully characterized in the area of nanotechnology (Silva, 2004). In the last decades nanotechnology is being applied for the benefit of the human civilization (Gupta *et al.*, 2012).

This science is not an isolated area, it aggregate a vast number of technological principles of several subjects such as engineering, electronics, chemistry, physics, biology, material science and manufacturing, at a molecular level, to produce new nanodevices (Sahoo *et al.*, 2003). In the area of nanotechnology a huge effort has been done to develop new therapeutics to be used in nanomedicine. Nanomedicine is defined by the National Institute of Health (NIH) as a medical ramification at the molecular scale that search for new techniques of diagnosis, prevention and treatment of diseases (Park, 2007). In fact, nanomedicine is known for their large number of biomedical applications such as gene therapy, imaging and drug delivery systems (DDS) (Sahoo *et al.*, 2003; Koo *et al.*, 2005).

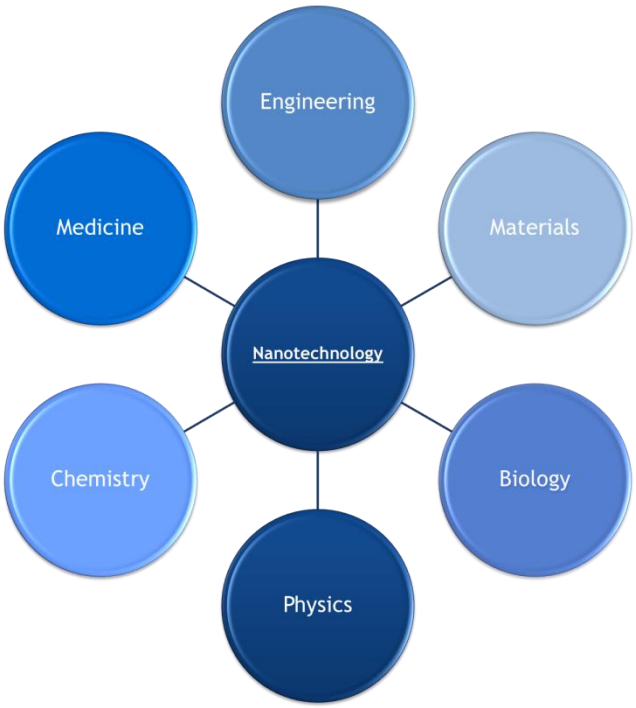

Figure 1 - Nanotechnology applications in biomedical sciences.

1.1.1. Nanomedicine

Nanomedicine has the capacity to improve medical tools in all the stages of medical care, from diagnosis to treatment (Zhang *et al.*, 2012). The metabolic disorders of various diseases take place at the molecular level, enhancing the difficulties for finding effective treatments. The main goal of nanomedicine is to take advantage of the properties and physical characteristics of nanomaterials for medical application, trying to solve these problems (Betty *et al.*, 2010). Therefore, materials can be produced to interact with tissues and cells at a molecular level, having a high degree of specificity (Silva, 2004). Nowadays, scientists are searching and developing new nanomaterials that help transport agents through biological barriers such as ephithelia, endothelia, cell and nuclear membranes, activated monocytes and macrophages of the reticulo-endothelial system (RES). Furthermore, these materials are able to avoid enzymatic degradation and ionic or molecular efflux pumps (Ferrari, 2010). So, it is possible to say that the development of nanomedicine will ultimately lead to a better understanding of the biological processes that take place at the nanoscale level (Salata, 2004). The application of nanotechnology to medicine enhances the availability of new therapeutic weapons and improves the application of several drugs. It also allows a quicker diagnosis (Rosenholm *et al.*, 2011). Nanotechnology in medicine has several levels of action, such as: drug and gene delivery, fluorescent biological labelling, detection of pathogens or proteins, labeling of DNA structure, tissue engineering, tumour destruction (hyperthermia), contrast agents and phagokinetic studies (Salata, 2004). An increase and expansion of this field is expected to happen, making nanomedicine an exciting area that could have a worldwide market of $70-160 billions by 2015 (Shi *et al.*, 2010).

1.1.2.1. Drug Delivery Systems

The use of nanotechnology for drug delivery is expected to revolutionize the pharmaceutical and biotechnology panorama in a near future (Farokhzad *et al.*, 2009). There are a large number of biological and chemical compounds that have been used for the production of drug delivery systems at the nanometer scale. These nanocarriers can be synthesised to have drugs absorbed or linked to the particle surface, encapsulated inside the carrier or dissolved within the system matrix (Bamrungsap *et al.*, 2012). Bearing this knowledge in mind, drug targeting has become a challenging but, at the same time promising subject. By improving DDS selectivity for specific cells or sites of action, secondary effects can be diminished and the treatment effectiveness enhanced (Mattheolabakis *et al.*, 2012). DDS presents many advantages over the traditional ways of drug administration. These advantages include:

- stabilization of hydrophobic drugs reducing the use of solvents and surfactants and improving their delivery at the target site;
- capacity of tracking compounds and determine its fate in the human body, i.e.

biodistribution and pharmacokinetcs;

- extended half-life in the circulation;
- reduction of secundary effects due to the specific delivery at the target sites;
- ability to provide transcytosis of drugs across tight epithelial and endothelial barriers;
- possibility of internalization and release of cargo within cells;
- ability to co-deliver two or more drugs into a desired site;
- theragnostic properties, combining therapeutics with diagnostics (Lee *et al.*, 2011; Farokhzad *et al.*, 2009; Bamrungsap *et al.*, 2012; Mattheolabakis *et al.*, 2012).

In order to further improve DDS therapeutic efficacy, researchers must fully understand the interactions of carriers with the biological environment as well as with the target cells and their membrane receptors (Suri *et al.*, 2007).

Some of the most used DDS are summarized below and depicted in figure 2. Food and Drug Administration (FDA) approved DDS are presented in table 1.

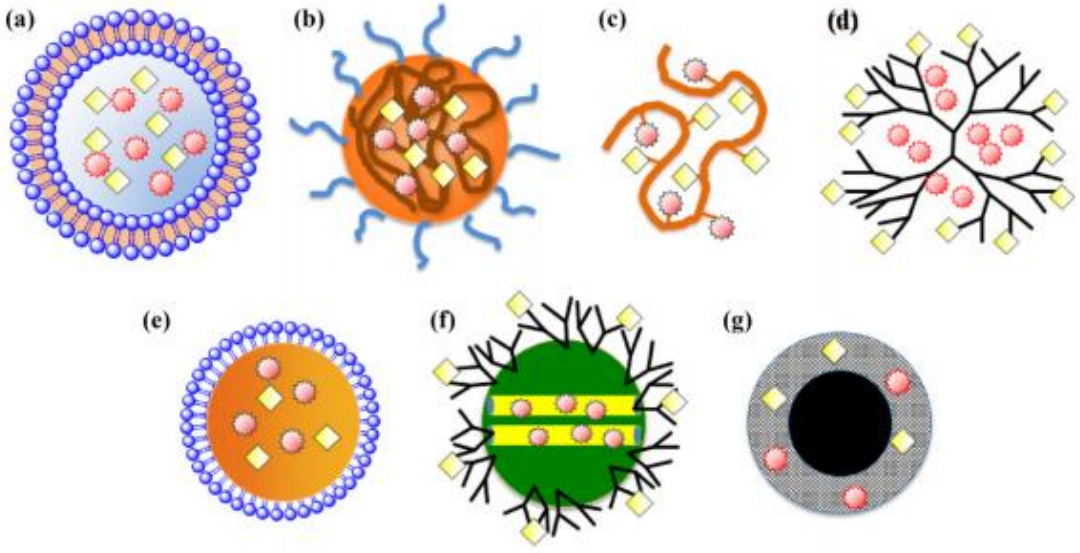

Figure 2 – Schematic representation of some drug delivery systems: (a) Liposome; (b) Polymeric micelle; (c) Polymer-drug conjugate; (d) Dendrimer; (e) Oil nanoemulsion; (F) Mesoporous silica nanoparticle and (g) Iron oxide nanoparticle (Adapted from Hu and Zhang, 2002).

Table 1 - FDA approved DDS available in the market (Adapted from Zhang *et al.*, 2012).

Product name and category	Year of approval	Technology
Liposome & Micelle		
• Doxil	1995	PEGylated liposomal doxorubicin
• Daunoxome	1996	Liposomal daunorubicin
• Ambisome	1997	Liposomal amphotericin
• Estrasorb	1999	Liposomal cytarabine
• Visudyne	2000	Liposomal verteporfin
• Depocyt	2003	Estradiol micellar nanoparticles
• DepoDur	2004	Liposomal morphine sulfate
Polymer-drug conjugate		
• Adagen	1990	PEGylated adenosine deaminase
• Oncaspar	1994	PEGylated L-asparaginase
• PEG-intron	2001	PEGylated interferon alfa-2b
• PEG-ASYS	2002	PEGylated interferon alfa-2a
• Neulasta	2002	PEGylated granulocyte colony-stimulating factor analog
• Somavert	2003	PEGylated recombinant analogue of the human growth hormone
• Macugen	2004	PEGylated anti-VEGF aptamer
• Mircera	2007	PEGylated erythropoetin receptor activators
• Cimzia	2008	PEGylated tumor necrosis factor alpha inhibitor
• Krystexxa	2010	PEGylated urate oxidase
• Omontys	2012	PEGylated peginesatide
Biodegradable materials		
• Zoladex	1989	PLGA/goserelin
• Lupron Depot	1989	PLGA/leuprolide acetate
• Gliadel	1996	Polifeprosan 20/carmustine
• Sandostatin LAR	1998	PLGA-glucose/octreotide acetate
• Atridox	1998	PLA/doxycycline hyclate
• Nutropin depot	1999	PLGA/recombinant human growth hormone
• Trelstar	2000	PLGA/triptorelin pamoatea
• Arestin	2001	PLGA/minocycline
• Eligard	2002	PLGA/leuprolide acetate
• Risperdal Consta	2003	PLGA/risperidone
• Vivitrol	2006	PLGA/naltrexone
• Somatuline	2007	PLGA/dexamethasone
• Ozurdex	2009	PLGA/lanreotide
Protein-based DDS		
• Zevalin	2002	Anti-CD20 monoclonal antibody/yttrium-90
• Bexxar	2003	Anti-CD20 monoclonal antibody/iodine-131
• Abraxane	2005	Albumin/paclitaxel
• Brentuximab Vedotin	2011	Anti-CD30 monoclonal antibody/monomethyl auristatin E

1.2. Targeting

Ultimately for a DDS be applied in the human body, it is necessary to study the interactions between these systems and the biological environment, especially the possibility of intrinsically give to the DDS the capacity to selectively target a specific cell or tissue (Zhang *et al.*, 2012). This ability allows a specific delivery of the DDS to an area of interest, for instance deliver of a drug to cancer cells (Zhang *et al.*, 2012). Ideally, a nanocarrier should be capable of reaching the desired cells with low, to no loss, of its content and also be capable to only deliver it to affected cells, without affecting healthy tissues (Cho *et al.*, 2008).

Researchers generally consider two mechanisms of targeting: passive and active targeting (Koo *et al.*, 2005; Peer *et al.* 2007). Passive targeting is the passage of DDS through targeting sites with leaky microvasculature, namely tumour fenestrations and inflamed tissues, into the tumour interstitium and cells by convection of passive diffusion. High molecular weight compounds only pass across pores, while low molecular weight compounds diffuse rapidly and indiscriminately through the endothelial cell layer of blood capillaries (Koo *et al.*, 2005; Danhier *et al.*, 2010). Taking advantage of the unique pathophysiological characteristic of tumour vessels, the nanocarriers accumulate in this zone by the enhanced permeability and retention (EPR) effect, a characteristic phenomenon of cancer cells that occur due to the extremely disorganization and imperfection of the tumour blood vessels. Such, is caused by an extremely rapid angiogenesis and limited lymphatic drainage (Minko *et al.*, 2004). For effective passive targeting, the nanocarriers have to circulate in the bloodstream for a long period of time, to allow multiple passages by the target site (Koo *et al.*, 2005). This constitutes a problem for the majority of the DDS available, because most of the nanoparticles have a short circulating half-life due to the defence mechanisms of the human body, especially the reticuloendothelial system. One way to overcome this problem is by coating the nanoparticles with hydrophilic polymers such as polyethylene glycol (PEG) (Koo *et al.*, 2005; Torchilin, 2010). An advantage of this type of targeting is that the composition of a DDS does not influence the EPR effect (Mattheolabakis *et al.*, 2012).

Some diseases have, as peculiarity, the expression of epitopes or membrane receptors that can possible be used as active targeting by specific DDS. Thus, ligands that specifically bind to this epitopes or receptors must be attached on the surface of the DDS (Koo *et al.*, 2005) This DDS/target bind and consequently uptake by cells are extremely important for the delivery of drugs that are not easily taken up by cells (Koo *et al.*, 2005). One issue to take into consideration is that the expression of these ligands should be exclusive of the desired cells and not be present on the surface of healthy cells. In addition, an overexpression of these ligands of the target cells is also requirement (Danhier *et al.*, 2010). Targeting ligands can be proteins (antibodies and antibody fragments), nucleic acids (aptamers) and others such as peptides, vitamins and carbohydrates (Peer *et al.*, 2007)

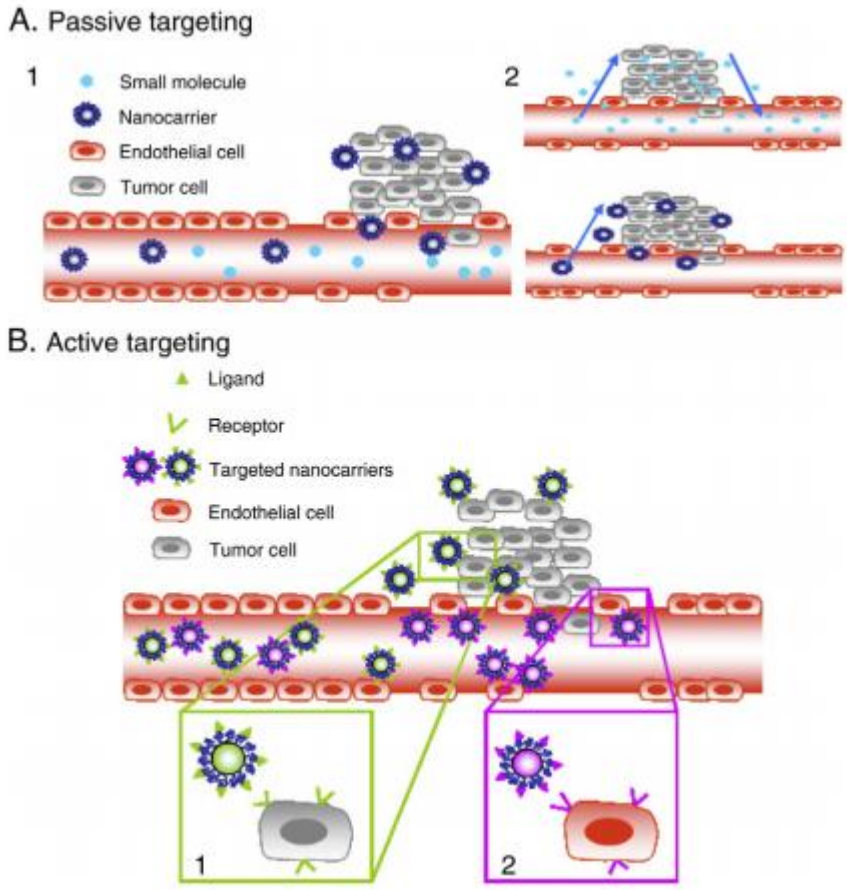

Figure 3 – Types of DDS targeting: (A) Passive targeting of nanocarriers. (1) Nanovehicles arrive to the tumour site through the leaky vasculature. (2) Representation of the influence of size in the retention at tumour site. (B) Active targeting. Nanocarriers bond to cancer cells (1); Nanocarriers bond to angiogenic endothelial cells (2) (Adapted from Danhier *et al.*, 2010).

1.3. Toxicity

One important property of DDS is the potential toxicity associated with the nanoparticles. So, it is important to have into consideration the hazards and safety issues on the use of this type of technology, especially when they are used for drug delivery (De Jong and Born, 2008). Nanoparticles size, chemical composition, surface structure, solubility, shape and aggregation are the main aspects that have to be taken into account in order to evaluate their biological effects in the human body. These properties are intrinsically connected with possible problems in terms of cellular uptake, protein binding, translocation from place of entry to the target site and tissue damage (Jain, 2012). The routes used for nanocarriers administration into the body are also important in order to avoid toxic effects. These routes include the gastrointestinal tract, skin, lung and systemic administration (Jain, 2012). DDS interaction with the surrounding environment, namely cells, body fluids and proteins is the key parameter to assure a successful deliver of the desired content. However, this interaction is also crucial in terms of particles toxicity (Jain, 2012). For the same therapeutic effect, the delivery of a drug within a nanoparticle requires a lower dosage,

when compared with the delivery of free drug. Nevertheless, the particles toxicity itself must be taken into consideration as well (De Jong and Born, 2008). Another point in terms of DDS toxicity is the fate of the particles in the human body. Ideally, the non adsorbed nanoparticles should be excreted trough renal clearance or caught by the liver hepatocytes and then transport to the bile. However, the elimination route of the absorbed nanoparticles is not fully characterized and it is possible that not all nanoparticles are eliminated from the human body. These nanoparticles could be accumulated in several sites of the body. The accumulation of low concentrations of nanoparticles could not represent a high risk, but the accumulation of high doses of DDS can induce toxic secondary effects. Both chemical composition and size might contribute for the absence of nanoparticle elimination (Hagens *et al.,* 2007).

1.4. Lipid Based Nanoparticles

1.4.1. Liposomes

Lipossomes are the most studied nanosystem to be applied in drug delivery (Bamrungsap *et al.*, 2012). They are artificial self-assembled vesicles obtained from amphiphilic phospholipids. Such nanodevices are constituted by one or more aqueous domains surrounded by one or more spherical bilayers of amphipathic lipid molecules with sizes ranging between 50 nm to several micrometers (μm) (Bamrungsap *et al.*, 2012; Mattheolabakis *et al.*, 2012). Having this characteristic structure, liposomes can incorporate both hydrophilic and hydrophobic drugs in the inner aqueous compartment and within the lipid bilayer, respectively (Mattheolabakis *et al.*, 2012). Moreover, liposomes also have noble biological properties such as biocompatibility and biodegradability (Liu and Boyd, 2013). Their size, surface charge and functionality can be easily changed by the addition of agents to the membrane or by modifying their surface chemistry (Bamrungsap *et al.*, 2012). There are four possible processes to load a drug into this DDS: liposome formation in an aqueous solution saturated with soluble drug; the use of organic solvents and solvent exchange mechanisms; the use of lipophilic drugs; or pH gradient methods (Malam *et al.*, 2009). This type of nanodevices is classified accordingly to the number of bilayers presented. Therefore, liposomes can be divided into unilamellar and multilamellar vesicles (Mattheolabakis *et al.*, 2012). Unilamellar vesicles have a mean diameter ranging between 50 to 250 nm and are liposomes with a single lipid bilayer and a large aqueous core, mostly applied to encapsulate hydrophilic drugs. On the other hand, multilamellar vesicles can be defined has liposomes with several concentric lipid bilayers in an onion-ring arrangement which allows the entrapment of lipophilic drugs due to their high lipidic content. This type of liposomes presents a mean diameter ranging from 1 to 5 μm (Mattheolabakis *et al.*, 2012).

However, liposomes also present some limitations such as low encapsulation efficiency, fast burst release of drugs, poor storage stability and lack of tunable triggers (Bamrungsap *et al.*, 2012). One of the main objectives regarding the use of liposomes as DDS, is the improvement of their circulation time in the bloodstream. This has been somehow achieved by the incorporation of PEG into the phospholipids present in the liposomes, forming, therefore, a protective coating. The addition of PEG sterically stabilizes the liposome membrane against opsonisation or uptake by the reticuloendothelial system (Mattheolabakis *et al.*, 2012). Another important goal of the research concerning this type of DDS is the development of targeted liposomes, which can be achieved through the binding of peptides or antibodies to the surface of liposomes (Mattheolabakis *et al.*, 2012). Moreover, modified liposomes have also shown good pharmacokinetic profiles for the delivery of Deoxyribonucleic acid (DNA), antisense oligonucleotide, small interfering ribonucleic acid (siRNA), proteins and chemotherapeutic agents (Bamrungsap *et al.*, 2012). One way used to improve the efficacy of liposomes is the use of pH-sensitive lipids. For low pH environments (usually lower than 7.4), lipossomes vesicles are destabilized and its content released to the exterior (Mattheolabakis *et al.*, 2012).

Liposomes can deliver many types of drugs like anti-cancer, anti-parasite and anti-bacterial, hormones, enzymes and immunoactivators, among others. A few anti-cancer drug formulations are already available in the market, being Doxil® the one most successful to date (Liu and Boyd, 2013). The interaction between liposomes and cells can be achieved through adsorption, fusion, endocytosis or lipid transfer (Wilczewska *et al.*, 2012).

1.4.2. Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are constituted by a lipid matrix (solid at room and body temperatures), which can be made of highly purified triglycerides, complex glyceride mixtures or waxes, and are stabilised by one or more surfactants (Wissing *et al.*, 2003). They have a mean diameter raging from 50 to 1000nm (Müller *et al.*, 2000). There are several methods to produce SLNs such as high pressure homogenization, which is the more commonly used; microemulsion techniques; dispersion of a lipid in a surfactant and precipitation (Müller *et al.*, 2000). This type of nanoparticles has several good properties that make them suitable for drug delivery. Among them, there are the excellent physical stability, protection of incorporated labile drugs from degradation, control of drug release profile, low toxicity, good site-specific targeting and large scale production (Wissing *et al.*, 2003; Mehnert *et al.*, 2012). Notwithstanding, they also have some disadvantages such as limited loading capacity due to solubility in the lipid melt, structure and polymorphic state of the lipid matrix, drug expulsion after polymorphic transition and high water content (Wissing *et al.*, 2003; Mattheolabakis *et al.*, 2012).

SLNs can be administered by oral, parenteral and transdermal administration routes. SLNs can improve the immune response. Adding SLNs to a vaccine formulation allows a slower

release rate, ensuring a prolonged exposure time to the immune system (Mattheolabakis *et al.*, 2012).

1.5. Dendrimers

Dendrimers are synthetic, highly branched globular macromolecules with a three dimensional structure (Gillies and Fréchet, 2005; Restani *et al.*, 2012). The name has origin in the Greek word *dendron*, meaning "tree" and *meros*, meaning "part" (Nanjwade *et al.*, 2009). Dendrimers consist in three different domains: a central core, constituted by a single atom or an atomic group with, at least, two similar chemical functions; several branches with origin in the core that are repeated with at least one branch junction, whose repetition is geometrically organized resulting in a series of concentric layers, called generations; and many terminal functional groups, normally located in the exterior of the macromolecule (Mattheolabakis *et al.*, 2012). Their chemical composition and molecular weight can be precisely controlled providing excellent biocompatibility and pharmacokinetics when within a human body (Bamrungsap *et al.*, 2012). Dendrimers constitution along with their globular structures and internal cavities, allows the encapsulation of drugs in their interior, providing also a controlled release of the drug from the inner core (Bamrungsap *et al.*, 2012). Dendrimers can be synthesized mostly by two methods: the divergent approach and the convergent one. In the divergent technique, monomeric modules are assembled in a radial branch-upon-branch motif to the core site. On the other hand, the convergent approach consists in the reaction of several dendrons (dendrimer sections with multiple terminal groups and a single reactive function at the focal point) with a multifunctional core (Nanjwade *et al.*, 2009). Compared to other types of polymer architecture, dendrimers present many advantages like low polydispersity, high functionality and a nanometer size range, between 1 to 10nm, which facilitates the passage through some biological barriers (Nanjwade *et al.*, 2009; Bamrungsap *et al.*, 2012). Recently, dendrimers have been described as a great improvement in many areas from gene delivery to magnetic resonance imaging. They have also been used for the development of vaccines, antivirals, antibacterials and anticancer therapeutics (Gillies and Fréchet, 2005).

Polyamidoamine (PAMAM) is one of dendrimers already in commercialization and one of the most used in biomedical applications (Gillies and Fréchet, 2005). However, PAMAM-type dendrimers have been demonstrating some drawbacks regarding their cytotoxicity (Restani *et al.*, 2012). To overcome this limitation, Restani and co-workers have synthesised a family of water soluble blue photoluminescence biocompatible and biodegradable PURE-type dendrimers (Restani *et al.*, 2012). Such type of dendrimers, produced using supercritical carbon dioxide ($scCO_2$) conditions have been shown as promising tools for highly sensitive biosensing, cell imaging and disease diagnosis (Restani *et al.*, 2012).

1.6. Nanoemulsions

Nanoemulsions are thermodynamically or kinetically stable dispersions of two immiscible liquids, belonging to the family of colloidal dispersions. In their constitution they present an oil phase and a water phase, with the addition of an interfacial film of surfactant molecules for stabilization (Devarajan and Ravichandran, 2011; Mattheolabakis *et al.,* 2012). The dispersed phase is normally composed by small particles or droplets, with sizes ranging from 5 to 200nm and with a very low oil/water interfacial tension. Moreover, nanoemulsions are transparent, due to the small size of the droplets (Devarajan and Ravichandran, 2011). Depending on their composition, three types of nanoemulsions can be formed: oil in water nanoemulsions, which consist in a continuous aqueous phase with dispersed oil droplets; water in oil nanoemulsions, that contains water droplets dispersed in a continuous oil phase; and bi-continuous nanoemulsions, composed by interdispersed microdomains of oil and water within the system (Lovelyn and Attama, 2011; Devarajan and Ravichandran, 2011). The preparation of nanoemulsions can be achieved by high-pressure homogenization for small size particles involving a drop creation, deformation, and disruption followed by surfactant adsorption, microfluidization or phase invertion temperature technique (Shah *et al*., 2010; Anton *et al*., 2008). Nanoemulsions are applied in different areas, such as biotechnology, diagnostics, cosmetics and drug delivery. A few nanoemulsions are already commercialized (Shah *et al*. 2010).

1.7. Polymeric nanoparticles

Polymeric nanoparticles (PNs) are spherical structures with a maximum size of 1000 nm prepared with natural and/or synthetic polymers (Mattheolabakis *et al*., 2012; Pinto Reis *et al*., 2006). PNs can be categorized in nanospheres and nanocapsules. Nanospheres have a matrix type of structure in which drugs can be absorbed at the sphere or encapsulated within the particle. In the other hand, nanocapsules are vesicular systems with the drug constrained to a cavity constituted by an inner liquid core surrounded by a polymeric membrane. Drugs are usually dissolved in the inner core but may also be adsorbed to the capsule surface (Pinto Reis *et al.,* 2006). Generally, PNs are produced by a two steps method. The first one corresponds to the preparation of an emulsified system (constituted by emulsions, miniemulsions, nanoemulsions or microemulsions). The second one is related to the formation of the nanoparticles by precipitation or gelation of a polymer or by polymerazation of monomers (Vauthier and Bouchemal, 2008). There are other less used methods that do not require the prior preparation of the emulsified system. These methods are based on the precipitation of a polymer in certain conditions to spontaneously form PN´s or self assembly of macromolecules to form nanogels from a polymer solution (Vauthier and Bouchemal, 2008). The most common PNs are made of poly-d,l-lactide-co-glycolide, polylactic acid, poly-ε-caprolactone, poly-alkyl-cyanoacrylates, chitosan and gelatine (Kumari *et al*., 2010). The

specific role of a particular nanoparticle is directly associated with its morphological characteristics, surface chemistry and molecular weight. Surface modification gives the nanoparticles anti-adhesive properties, reducing their clearance by the circulating macrophages. This increases the nanoparticles life time in blood circulation and enhance the number of passages of the nanoparticles through the target sites (Kumari *et al.*, 2010). Morevover, the surface modifications may impair a more controlled release of the drugs encapsulated within the nanoparticles (Kumari *et al.*, 2010). Their exceptional potential in accurate drug and gene delivery makes biodegradable PNs one of the most studied types of nanodevices (Soppimath *et al.*, 2001).

1.8. Inorganic nanoparticles

1.8.1. Carbon nanotubes

Carbon nanotubes (CNTs) are allotropes of carbon with cylindrical nanostructure that belong to the family of fullerenes (Lin *et al.*, 2004; Lacerda *et al.*, 2006). They are constituted exclusively by carbon atoms, in a series of condensed benzene rings arrangement, rolled-up perfectly into a tubular structure (Madani *et al.,* 2012). They were described for the first time in the late 1950s, but their drug delivery properties were only been discovered recently (Madani *et al.,* 2012). CNTs have several advantageous physicochemical properties such as ordered structure, low weight, high mechanical strength, high electrical and thermal conductivity, metallic or semi-metallic behaviour and high surface area (Lacerda *et al.,* 2006). CNTs are usually produced by chemical vapour deposition. This process involves the passage of a hydrocarbon vapour through a tubular reactor with a catalyst material attached. The process is performed at temperatures between 600 and 1200º C to allow de decomposition of the hydrocarbon. After cooling, the CNTs that grow on the catalyst reactor are collected (Paradise and Goswami, 2006; Kumar and Ando, 2010). Other less used methods for CNTs synthesis involve extremely high temperature techniques such as arc discharge and laser ablation (Prasek *et al.*, 2011; Peretz and Regev, 2012). Depending on the number of graphene layers, CNTs can be divided into single-walled carbon nanotubes (SWNTs) or multi-walled carbon nanotubes (MWNTs) (Peretz and Regev, 2012). SWTs are one-dimensional CNTs with diameters of 1-2 nm and lengths ranging from 50 nm to 1 centimetre (cm), with a different behaviour from the nanoparticles in biological environments (Kamalha *et al.,* 2012). They normally appear in bundles due to the strong van der Waals interactions and can exhibit semi-conducting or metallic properties (Klumpp *et al.*, 2006). Due to their flexible properties, it is possible to bend the nanotube to provide multiple binding sites of a functionalized nanotube to a specific cell. Moreover, CNTs also exhibit high surface area, which results from the great number of atoms exposed on the CNTs surface. Such fact results in an efficient loading of multiple molecules in all the nanotube sidewall (Liu *et al.*, 2008). Conversely, MWNTs, are constituted by multiple layers of graphene with a much larger diameter, ranging

from 10 to 100nm, with 0.34 nm between each layer (Kamalha *et al.*, 2012). They are mainly monodispersed and have only semi-conducting properties (Klumpp *et al.,* 2006). Their larger size enables them to deliver large biomolecules like DNA plasmids into cells, but their optical properties are worse than that of SWNTs (Liu *et al.*, 2008).

In order to functionalize CNTs, two methods are commonly used to perform the necessary modification: oxidation by strong acids leading to a reduction of their length and consequent appearance of carboxylic groups in the same reaction, increasing their dispersibility in aqueous solutions and the binding of a large variety of active molecules like peptides, proteins, nucleic acids and other therapeutic agents (Bianco *et al.*, 2005).

CNTs are suitable for several biomedical applications. Such applications are strongly dependent on their size, shape, structure and unique physical properties (Liu *et al.*, 2008). Lately, CNTs have also been described as good vaccine delivery systems and protein transporters (Bianco *et al.*, 2005).

1.8.2. Magnetic nanoparticles

Magnetic nanoparticles (MNPs) have some exciting features making them suitable to be applied in the medical field, especially in nanomedicine. MNPs can truly provide a wide range of improvements in areas like magnetic resonance imaging (MRI) (being applied as dynamic contrast agents), hyperthemic treatment for malignant cells, drug delivery under influence of an external magnetic force and manipulation of cell membranes trough mechanical stress (Berry and Curtis, 2003). The size of these particles can vary from few to tens of nanometres. This makes possible the loading of several agents with a wide range of sizes. They can also be coated with the desired biological molecules to enhance the biding potential of the particle to specific cells and tissues (Pankurst *et al.*, 2003). MNPs are physiological well tolerated, since they present a low toxicity (Berry and Curtis, 2003).

A very interesting property of MNPs is their ability to obey to the Coulomb's law. Such feature enables the application of an external magnetic gradient to manipulate them at distance, directing particles to a specific site, maintaining them until needed and then promote their remotion. Finally, MNPs can be produced to respond to a time-varying magnetic field, with the advantage of the transfer of energy from the exciting field to the nanoparticle whenever needed (Berry and Curtis, 2003; Pankurst *et al.*, 2003). However, for biological applications, a biocompatle coating is needed (Tartaj *et al.*, 2003). The fabrication method is the key to produce a valuable magnetic particle and will determine the particle size, distribution, shape, surface chemistry and magnetical properties (Tartaj *et al.*, 2003).

One of the most researched types of magnetic nanoparticles are the iron oxide ones, which can be either paramagnetic or superparamagnetic (Gupta and Gupta, 2005). One characteristic of these nanodevices is their ability to lose their magnetism as soon as the magnetic field is removed (Berry and Curtis, 2003). Examples of such nanoparticles are magnetite or its oxidized form (maghemita), which are the most common metals in

biomedical applications. Cobalt and nickel, for instance are normally not tested in nanomedicine due to their associated toxicity (Tartaj *et al.*, 2003).

1.8.2.1. Gold Nanoparticles

Gold was one of the first metals discovered by humans (Dykman and Khlebtsov, 2011). The first description of colloidal gold nanoparticles (Au_NPs) was made by Michael Faraday in 1857, when he synthesized multicoloured solutions by reacting gold chloride with sodium citrate producing, without knowing, AuNPs with sizes ranging from 12 to 60 nm in diameter (Paciotti *et al.*, 2006; Boisselier and Astruc, 2009). Since then, Au_NPs have been used in varied biological applications. In 1950s the capacity to bond proteins to the particles, without changing their activity allowed their use in immunodiagnostics and histopathology (Paciotti *et al.*, 2006). Recently, Au_NPs have been applied in other biological applications such as, diagnostics and biosensors (Paciotti *et al.*, 2006). The normal oxidation states of gold are +1 (Au [I] or aurous compounds) and +3 (Au [III] or auric compounds) (Jain *et al.*, 2012). There are several ways of producing Au_NPs. Nevertheless, the two main methods that researchers normally use are the citrate reduction of Au [III] derivatives such as aurochloric acid ($HAuCl_4$) in water to Au (0) and the Brust–Schiffrin method, which uses two-phase synthesis and stabilisation by thiols (Boisselier and Astruc, 2009; Jain *et al.*, 2012). The size of AuNPs normally varies between 1 to more than 120 nm (Boisselier and Astruc, 2009). Au_NPs have some characteristic physicochemical properties that make them very exciting and interesting for biomedical applications. Such features include the surface plasmon ressoance (SPR), used for measuring the refractive index of very thin layers of material adsorbed on a metal, the abilility to bind thiol and amine groups, biocompatibility and the ability to control size and optical properties (Pattnaik, 2005; Kim and Jon, 2012; Jain *et al.*, 2012). AuNPs also serve as practical platforms for therapeutic agents, which are related to their high surface area allowing for dense presentation of multifunctional moieties (Yeh *et al.*, 2012). The functionalization of these nanoparticles is one of the subjects that have been widely studied by scientists for the development of biocompatible and multifunctional particles for either diagnostic or therapeutics (theragnostic) (Rana *et al.*, 2011; Jain *et al.*, 2012). They have been used in areas ranging from genomics, biosensors, immunoassays, clinical chemistry, detection and photothermolysis of microorganisms and cancer cells, targeted delivery of drugs, peptides, DNA, and antigens, to optical bioimaging and monitoring of cells and tissues (Dykman and Khlebtsov, 2011).

Is not yet clear the mechanism through which Au_NPs enter into cells, being the non-specific receptor mediated endocytosis (RME) the most accepted to explain their entrance into cells (Jain *et al.*, 2012).

1.8.3. Ceramic nanoparticles

1.8.3.1. Mesoporous Silica nanoparticles

Mesoporous silica nanoparticles (MSNPs) are solid inorganic materials with hundreds of empty channels, called mesoporos, arranged in a 2D network of honeycomb-like porous structure (Slowing *et al.*, 2008; Vivero-Escoto *et al.*, 2010). The most important types of MSNPs are Mobile Crystalline Material-41 (MCM-41), MCM-48 and Santa Barbara Amorphous-15 (SBA-15) (Yang at al., 2011). The synthesis of surfactant-templated mesoporous silica was first performed in 1992 (Douromis *et al.*, 2012). However, the application in controlled release and delivery was held for the first time only in the beginning of the century (Slowing *et al.*, 2008). In recent years, MSNPs have been broadly studied for possible applications in the fields of biotechnology and nanomedicine (Vivero-Escoto *et al.*, 2010). MSNPs have interesting structural properties that make them suitable for application as DDS (Vivero-Escoto *et al.*, 2010; Popat *et al.*, 2011, Yang at al., 2011). The stable and rigid mesostructure gives MSNPs resistance to heat, pH, mechanical stress and hydrolysis (Slowing *et al.*, 2008). Another property of MSNPs is that both the external surface and the internal surface (constituted by the cylindrical pores) can be functionalized (Slowing *et al.*, 2008). Some authors consider the silica framework as a third surface domain (Wu *et al.*, 2011). The capacity of being easily functionalized make MSNPs suitable to be connected with other inorganic particles, like gold, as well as supramolecules and proteins that can possible work as caps forming a core/shell system (Lee *et al.*, 2011; Yang *et al.*, 2011). Furthermore, silica has been used to enhance the biocompatibility of several DDS, like magnetic nanoparticles, polymers and micelles (Slowing *et al.*, 2008). Moreover, the large pore, the large and uniform size of the nanoparticles and the ordered structure, besides the high surface area allow a high loading of drugs or biomolecules within these DDS (Slowing *et al.*, 2008; Popat *et al.*, 2011). They also enhance the therapeutic efficacy of drugs with limited clinical applicability because of the protection against the surrounding environment provided (Rosenholm *et al.*, 2011). The well-controled size provides a good control of the particle-cell interaction allowing the deliver of a precise cargo dose (Wu *et al.*, 2013). Another important property is their high biocompatibility, property that is fundamental for their application in the human body (Popat *et al.*, 2011; Wu *et al.*, 2011)

MSNPs offer a wide range of biomedical applications. These applications include drug delivery, catalyst supports, adsorption and purification of proteins, cell imaging and labelling, enzyme adsorption and immobilisation (Popat *et al.*, 2011). The possibility of being internalized by cells makes MSNPs suitable to be used as carriers of contrast agents. Also, they prolong the imaging time-frame trough the enhancement of their half-lives, reducing their toxicity as well (Rosenholm *et al.*, 2011). Silica has also been used in artificial implants due to its osteogenic properties (Slowing *et al.*, 2008). Figure 4 shows a schematic representation of the loading and release of a mesoporous silica nanoparticle.

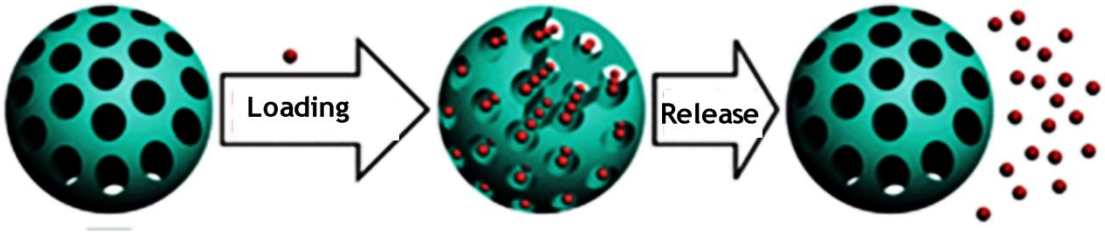

Figure 4 - Representation of the loading and release profile of a mesoporous silica nanoparticle (Adapted from He and Shi, 2011).

1.9. Core/Shell nanoparticles

Nanoparticles constituted by two or more different materials are designated by core/shell nanoparticles. They are composed by an inner material (core) and an outer layer that forms a shell around the inner core (Chaudhuri and Paria, 2011). A broad range of material combinations can be applied for the production of nanocarriers. These combinations are divided in inorganic/inorganic, inorganic/organic, organic/inorganic and organic/organic, depending on the used materials. The application of the nanosystem defines the choice of the materials to be used (Chaudhuri and Paria, 2011). Core/shell systems are highly functional nanoparticles that can be easily modified. The addition of a shell to a nanoparticle provides the nanocarrier enhanced properties, such as better stability, decrease of reactivity and dispersibility, increased functionality and controlled release of materials (Chaudhuri and Paria, 2011). A great attention has been given to these core/shell nanosystems since they are suitable for applications in several fields of science, including biomedical applications. Bioimaging, controlled drug release, targeted drug delivery, cell labelling and tissue engineering are examples of these applications. The inorganic/inorganic class of core/shell nanosystems is the most important one for the production of DDS (Levin *et al.*, 2009; Chaudhuri and Paria, 2011). The growth of a gold shell around a functional nanoparticle, like MCM-41 silica nanoparticle, improves the properties of the nanosystem. The gold shell provides a chemically inert surface layer suitable of functionalization. This shell enhances biocompatibility and preserves the properties of the core material (Levin *et al.*, 2009).

1.10. Objectives

In the present study different nanoparticles were produced in order to obtain a valuable nanosytem to be applied as drug delivery system. The present work plan has the following aims:

- Design and production of several drug delivery systems;
- Characterization of the properties of the different nanoparticles by Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FTIR) and X-Ray Diffraction (XRD);
- Characterization of the loading and release profiles of the nanovehicles;
- Evaluation of the *in vitro* transfection efficiency of the nanoparticles;
- Determination of the cytotoxic profile of the nanoparticles.

Chapter II – Materials and Methods

2. Materials and Methods

2.1. Materials

A549 non-small lung carcinoma cell line was purchased from ATCC (Middlesex, UK). Sodium azide was purchased from Amresco (Solon, USA). Fetal bovine serum (FBS) was acquired from Biochrom AG (Berlin, Germany). Hexane was purchased from JMS Ltda. (Odivelas, Portugal). Tris Base and Sodium Hydroxide (Na OH) were obtained from Fisher Chemical (Loughborough, UK). Poly-d-lysine coated glass bottom dishes (35mm dishes, 10mm glass, 0mm thickness) were from by MatTek Corporation (USA). 96-well plates were purchased from Orange Scientific (Braine-L'Alleud, Belgium). Hydrochloric Acid (HCl) was acquired from Panreac (Barcelona, Spain). (3-aminopropyl) trimethoxysilane (APTMS) and Tetrachloroauric (III) acid (HAuCl4.3H2O) were bought from Alfa Aesar (Karlsruhe, Germany). Ibuprofen and Hexadecyltrimethylammonium Bromide (CTAB) were from Tokyo Chemical Industry (Tokyo, Japan). Tetraethyl orthosilicate (TEOS) was purchased from Acros Organics (Geel, Belgium). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent MTS and Phenazine Methosulfate (PMS) was obtained from Promega (Madison, USA). Amphotericin B, Dialysis tubing cellulose membrane, Ethanol (EtOH), Ham's Nutrient Mixture-F12, Penicillin G, Phosphate-buffered saline (PBS), Streptomycin, Trypsin, Triethylamine and Trisodium Citrate were from Sigma Aldrich (Sintra, Portugal).

2.2. Methods

2.2.1. Synthesis of gold nanoparticles

The gold nanoparticles (Au_NP's) were produced through an adaptation of the Frens method was used (Frens, 1973). The process start with the addition of 0.01g of 0.01% (w/v) tetra-chloroauric[III] acid trihydrate (HAuCl4.3H2O) to 100mL of deionized water heated to boiling, under reflux conditions. After reaching boiling, 1mL of trisodium citrate hydrate 1% (w/v) was added drop by drop to the boiling solution under constant stirring. The previously yellow solution starts to turn blue after a few seconds of reaction, in a process called nucleation. Subsequently, the blue colour solution change into dark red, characteristic of the monodisperse spherical particles formation. The solution was kept under boiling conditions for 10 minutes (min) to complete the gold chloride reduction. After cooling at room temperature, 0.25g of 0.05% (w/v) sodium azide was added in order to preserve the gold nanoparticles. Finally, the resultant solution was stored at 4ºC (Frens, 1973).

2.2.2. Synthesis of silica nanoparticles

For the production of the MCM-41 silica nanoparticles (Si_NP's), a derivation of the Stöber method was applied (Jia *et al.*, 2012). In a round bottom flask 100 mL of Mili-Q water, 720 µL of NaOH (2M) and 0.2 g of CTAB were mixed and kept at 80ºC, under vigorous stirring. After the stabilization of the temperature, 1 mL of TEOS was added dropwise to the mixture and the reaction was left to occur. Two hours later, the reaction was stopped and cooled to room temperature, followed by centrifugation (13.500 rpm, 20 min). The produced particles were dried at room temperature overnight. To remove the surfactants, 120 mL of EtOH and 15 mL of HCl (37%) were added to each gram of particles in a round bottom flask and left at 80º C under refluxing conditions and vigorous stirring for 24 h. Finally the solution was centrifuged (13.500 rpm, 20 min) and washed with deionised water and ethanol several times to completely remove all the surfactants. After drying at room temperature, a Si_NP's powder was obtained.

2.2.3. Functionalization of silica nanoparticles and preparation of silica core/ gold shell nanoparticles

To obtain the silica core/ gold shell nanosystem, from now on designated by SiAu_NP´s, the previously produced Si_NP´s were amino functionalized. To do so, 100 mL of a solution of Si_NP´s in ethanol was placed in a round bottom flask and 1 mL of APTMS was added dropwise and then the mixture was left overnight, under vigorous stirring. After, the mixture was heated to 80º C for one hour, to complete the reaction. Then, the solution was centrifuged (13.500 rpm, 20 min) and washed with ethanol, 5 times, to remove the excess of APTMS. To remove the remaining ethanol, the powder was left to dry at room temperature until evaporation of the solvent. For the synthesis of the SiAu_NP´s a solution of amino functionalized Si_NP´s with ethanol was prepared and added to the Au_NP´s solution, in a ratio of 1:1, by simple contact at room temperature for several hours. After, the solution was centrifuged (13.500 rpm, 20 min) and washed with ethanol several times. A powder of SiAu_NP´s was obtained.

2.2.4. Scanning electron microscopy analysis

In order to characterize the morphology of all the nanoparticles produced, scanning electron microscopy (SEM) analysis was performed. For all the produced nanoparticles analysis, one drop of the solution was added to a 15mm cover glass and left air-dried overnight and then mounted on an aluminium board using a double-side adhesive tape and covered with gold using an Emitech K550 (London, England) sputter coater. Samples were analysed by using a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope operated at an accelerating voltage of 20 kV, at several amplifications (Gaspar *et al.*, 2011; Ribeiro *et al.*,

2009).

2.2.5. Fourier Transform Infrared spectroscopy

The produced nanoparticles were also analysed by FT-IR spectroscopy. In this technique an interference wave, produced by an interferometer, interacts with the sample and the resulting spectrum is analyzed (Faix, 1992). The spectrum obtained gives information about the chemical substances of the tested sample (Almeida *et al.*, 2002). All the spectra were acquired in a Fourier transform infrared spectrophotometer Nicoletis 20 (64 scans, at a range of 4000 to 400cm^{-1}) from Thermo Scientific (Waltham, MA, USA) equipped with a Smart iTR auxiliary module.

2.2.6. X-Ray Diffraction

X-Ray Diffraction (XRD) is a powerful technique used to identify the crystalline phase, the structure refinement and the molecular structures of the samples as well as the grain orientation and strain of the materials (Harris *et al.*, 2001; Tamura *et al.*, 2002; Morgan and Gilman, 2002). To perform the XRD analysis, samples of Au_NP's were first lyophilized and then mounted in silica supports using a double side adhesive tape. The Si_NP's the powder was directly placed in the supports. All the experiments were performed over the range from 5º to 90º 2θ with continuous scans at a rate of 1º 2θ min^{-1}, using a Rigaku Geigger Flex D-max III/c diffractometer (Rigaku Americas Corporation, USA) with a copper ray tube operated at 30kV and 20mA (Gaspar *et al.*, 2011).

2.2.7. Characterization of the loading profile of the vehicles

For ibuprofen be loaded into SiNP's or SiAuNP´s, 20 mL of hexane and 40 mg of ibuprofen were mixed at room temperature under vigorous stirring, in a darkroom. After 30 min of homogenization, 40 mg of SiNP's or SiAuNP´s were added to the solution. The mixtures were collected for 5, 10, 24, 48 and 72 h and then centrifuged (13.500 rpm, 10 min) in order to recover the ibuprofen loaded particles. The supernatants were stored for later quantify the amount of drug loaded in nanodevices using an UV-1700 PharmaSpec spectrophotometer from Shimadzu (Kyoto, Japan) at 264 nm and analyzed with an UVProbe Shimadzu 2.0 software (Zhang *et al.*, 2011).

2.2.8. Characterization of the release profile of the vehicles

After ibuprofen be loaded into the nanoparticles herein produced, the release studies were performed in order to evaluate the rate of drug release. First, 500 mL of PBS was added to a goblet and placed in a darkroom, at 37º C, pH=7.4 and under constant stirring (200 rpm). The nanoparticles solution was dialysed with a cellulose membrane containing PBS. For the

evaluation of the release profile of drug from the nanoparticles, 2 mL of the samples were periodically taken and substituted by equal amount of PBS. Spectra of the recovered samples were acquired using a UV-1700 PharmaSpec spectrophotometer from Shimadzu (Kyoto, Japan) at 264 nm and analyzed with an UVProbe Shimadzu 2.0 software (Zhang *et al.*, 2011).

2.2.9. Proliferation of A549 non-small lung carcinoma cells in the presence of the various nanoparticles produced

A549 non-small lung carcinoma cells were cultured in Ham‘s F12K medium supplemented with heat-inactivated FBS (10% v/v) and antimycotic (penincilin G (100 U/mL)), spectromycin G (100µg/mL) in an incubator at 37ºC, with a 5% CO_2 humidified atmosphere. First cells were seeded in 25cm^2 T-flasks until reaching confluence. For cell detachment, they were incubated in 0.18% trypsin (1:250) with 5mM EDTA, for 5 min (Gaspar *et al.*, 2011). Then, cells were centrifuged, resuspended in culture medium and reseeded in 75cm^2 T-flasks. After this procedure, cells were kept in culture at 37ºC, with a 5% CO_2 humidified atmosphere (Ribeiro *et al.*, 2009). For the evaluation of cell behaviour in contact with the several nanoparticles produced, A549 cells were seeded with the particles in a 96 well plate, at a density of 12x10^3 cells/well, for 24h. Before seeding, all the plates and materials were sterilized by UV for 30min (Ribeiro *et al.*, 2009). The cell growth was monitored using an Olympus CX14 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

2.2.10. *In vitro* transfection of cells with the nanoparticles

To start the *in vitro* transfection, cells were seeded in glass bottom dishes at a density of 12 x10^3 cells per well, with 1mL of Ham‘s F12K medium supplemented with FBS (10% v/v) but without antibiotic, in order to allow cell transfection (Cunningham *et al.*, 2007). After 24 hours of cell growth, the transfection with the produced nanoparticles was carried out and 4 hours later the process was stopped by changing the antibiotic free medium with fresh complete Ham‘s F12K medium. The nanoparticles used for tranfection were previously loaded with fluorescein isothiocyanate (FITC) at the time of synthesis.

2.2.11. Qualitative Evaluation of *in vitro* transfection

Confocal Laser Scanning Microscopy (CLSM) was used to evaluate the capacity of the various nanoparticles to enter into cells. After transfection, cells were fixed in 4% paraformaldehyde (PFA) for 20 min and then rinsed 10 times with PBS-Tween 20 (PBS-T) solution. To stain the nucleus of the cells a Hoechst 33342® molecular probe was added and incubated for 15 min, followed by 10 washing steps with PBS-T. The glass bottom dishes with transfected cells were visualized using a Zeiss LSM 710 laser scanning confocal microscope

(Carl Zeiss SMT Inc., New York, USA) equipped with a plane-apocromat 63x/DIC objective. Data analysis of CLSM images was performed with Zeiss software (Axio Vs40 V4.5).

2.2.12. Evaluation of the cytotoxic profile of the produced nanoparticles

Nanoparticles toxicity was evaluated through an MTS assay, following the manufacturer instructions. A549 cells, at a density of 12 x 10^3 cells per well, were seeded into 96-well culture plates with 200 µL of Ham's F12K medium supplemented with FBS (10% v/v) without antibiotic. Twenty four hours later, the medium was removed and 200 µL of fresh medium without antibiotic containing the previously sterilised different nanoparticles formulations was added, being changed to complete medium 4h later. After, cells were kept in an incubator at 37ºC, with a 5% CO_2 humidified atmosphere for 24, 72 and 120 h. After incubation, the medium was removed and replaced by 100 µL of fresh culture medium and 20 µL of MTS/PMS reagent solution. This allows the assessment of the mitochondrial redox activity of viable cells, by the reduction of the MTS into a water-soluble purple formazan product. Then, the cells were incubated for 4h at 37ºC, under a 5% CO_2 atmosphere. The absorbance was measured through a microplate reader (Sanofi, Diagnostics Pauster) at 492 nm. Wells with cells in culture medium without nanoparticles were used as negative control and EtOH 96% added to wells with cells as a positive control. Five replicas of each sample were used (Ribeiro *et al.*, 2009).

2.2.13. Statistical analysis

Statistical analysis of the cell viability results were performed using one-way analysis of variance (ANOVA) with the Dunnet's post hoc test. A value of $p< 0.05$ was considered statistically significant (Ribeiro *et al.*, 2009). A MYSTAT 12 statistical package (Systat Software, a subsidiary of Cranes Software International Ltd.) was used for computations.

Chapter III – Results and Discussion

3. Results and Discussion

3.1. Morphological characterization of the produced nanoparticles

SEM images were acquired to characterize the shape and surface morphology of the nanoparticles produced. In addition it also gives the possibility to determine the mean diameter of the nanoparticles.

The gold nanoparticles produced by the previously described Frens method are presented in figure 5A. It is possible to observe an Au_NP with a size of 51 nm which is in line with the data described in the literature (Frens, 1973). These nanoparticles present a not very well defined spherical shape; nevertheless, due to the magnification that must be used to reach such small sizes, it is not possible to accurately describe the form and surface of the nanoparticle.

Figure 5B presents a MCM-41 SEM image. Several Si_NP´s, synthesised by an adaptation of the Stöber method (Jia *et al.*, 2012), with sizes between 424 and 856 nm are shown. The variation of the size range is approximately 400 nm, which is caused by uncontrollable factors during the synthesis process. Although, the nanoparticles generally have sizes near the maximum or the minimum size, having the smallest particles a spherical form and the biggest ones an ovoid shape. The surface of the majority of the Si_NP´s seems to be smooth and it is not possible to visualize the pores of the nanoparticles due to their tiny size.

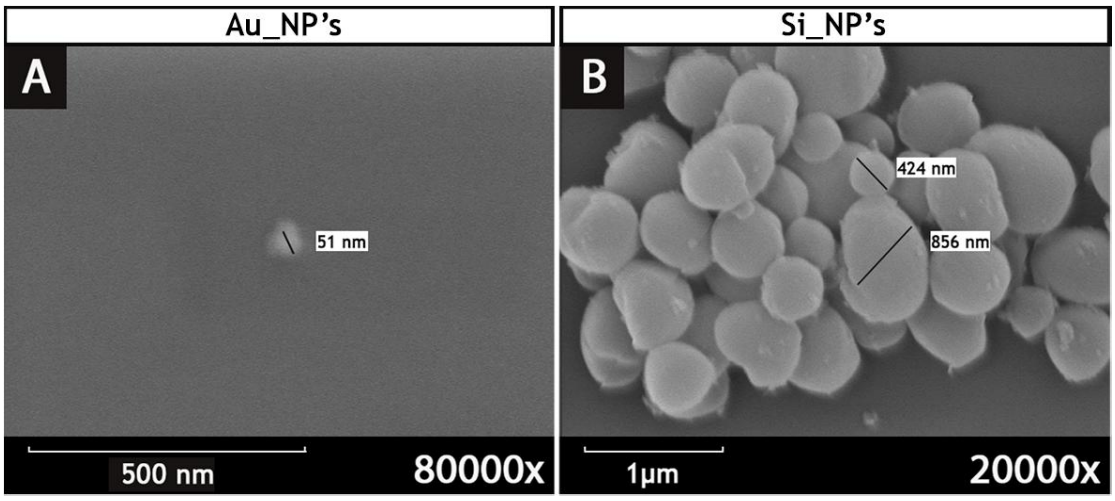

Figure 5 - SEM images of Au_NP´s, magnification 80000x (A) and Si_NP´s, magnification 20000x (B).

The nanosystem produced, constituted by the Si_NP´s mesoporous core with the Au_NP´s shell, is shown in figure 6, and was designated by SiAu_NP´s. The bond of the

Au_NP´s to the Si_NP´s produces no significant changes in the size of the nanoparticles that have sizes between 497 and 773 nm. Again, the smallest nanoparticles present an almost spherical shape, while the nanoparticles with sizes near 800 nm have an ovoid one. It is possible to visualize irregularities in the surface of the nanoparticles, suggesting the positive bond of the Au_NP´s to the amino functionalized Si_NP´s. Another aspect corroborating the connection of gold to the silica is the browning colour of the nanoparticles. This is related with the fact that gold nanoparticles form a shell around the mesopouros silica core.

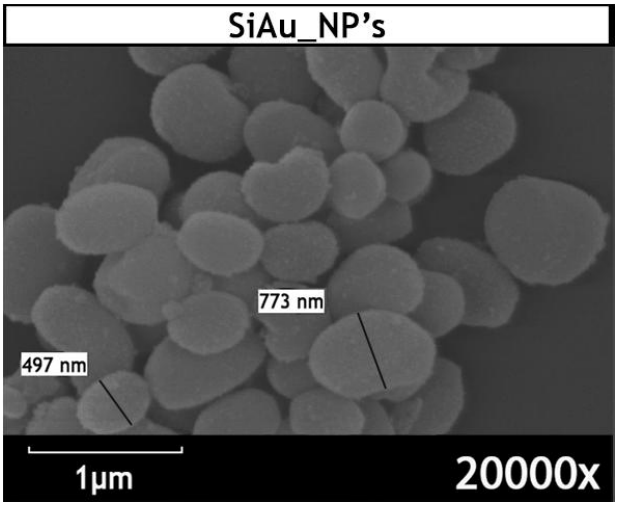

Figure 6 – SEM image of SiAu_NP´s, magnification 20000x.

Figure 7A shows the Si_NP´s loaded with ibuprofen (designated as SiIBP_NP´s). Comparing these nanoparticles with the ones on figure 5B, no visible modifications can be observed in terms of both size and surface, indicating that ibuprofen does not change the particles morphology. The small crystals that appear in the image are aggregates of ibuprofen not encapsulated within the nanoparticles.

In figure 7B, SiAu_NP´s loaded with ibuprofen can be seen. They were named SiAuIBP_NP´s. Once more, no sign of changes on the morphology can be visualized in comparison to SiAu_NP´s of figure 6 and the crystals visualized belong to aggregates that did not enter into the nanocarriers.

The morphology of the nanoparticles produced herein is in accordance with the described in the literature (Manzano *et al*., 2008; Li *et al*., 2011). All the silica formulations present a well defined, almost spherical shape.

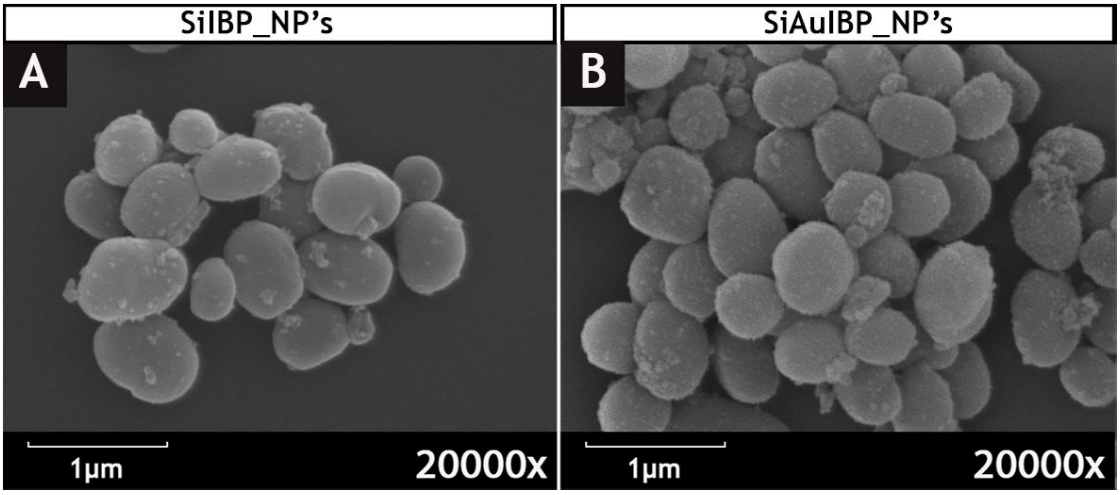

Figure 7 - SEM images of SiIBP_NP´s (A) and SiAuIBP_NP´s (B), magnication 20000x.

3.2. Fourier Transform Infrared Spectroscopy Analysis of the produced nanoparticles

FTIR analysis was performed to evaluate the molecular structure of the different nanoparticles and the compounds used for their production as well as the chemical interactions between them. With this analysis it is possible to observe the characteristic peaks of the compounds and the changes that occur when a new bond is formed, with the addition of a new compound. It is important to demonstrate the presence of the gold and ibuprofen in the newly produced nanoparticles.

The spectrum of the synthesized Au_NP´s is presented in figure 8a. It is possible to see two characteristic peaks of vibrational groups, one in the frequency ranges of 1370-1380 cm^{-1} and 1440-1465 cm^{-1}, that represent the C-H_3 deformations characteristic of the sodium citrate used for particles synthesis (Zhao et Kang, 2011) The other band in the region between 2850-2949 cm^{-1}, corresponds to C-H stretches with four representing peaks within this wavelength range (Mei *et al.*, 2009; Liu *et al.*, 2011; Barboza-Filho *et al.*, 2012).

The produced MCM-41 Si_NP´s spectrum is represented in figure 8b. The most prominent peak observed between 1000-1100 cm^{-1} correspond to the silica characteristic Si-O stretching bridges cross-linking the silicate in the Si-O-Si network structure. The medium peak at 950-970 cm^{-1} is representative of the Si-OH bond of the silicate compounds while the peak at around 790 cm^{-1} is originated by the Si-O vibration. The broad band in the range of 3100-3600 cm^{-1} caused by stretching vibrating absorption peaks of the O-H band in the adsorbed water. Additionally, the small peak between 1620-1640 cm^{-1} corresponds to the H-O-H scissor bending vibration in water (Tourné-Péteilh, 2003; Oliveira *et al.*, 2007; Deshmukh *et al.*, 2007; Parida and Rath, 2009; Ballesteros *et al.*, 2009; Pouretedal and Ahmadi, 2012).

In relation to the SiAu_NP´s (figure 8c) a similar spectrum to that of the SiNP´s was obtained. It is possible to visualize the presence of the three characteristic silica peaks at 1000-1100 cm^{-1} and 790 cm^{-1} as well as of the water O-H vibrations at 3100-3600 cm^{-1} and 1620-1640 cm^{-1} (Oliveira *et al.*, 2007; Parida and Rath, 2009; Ballesteros *et al.*, 2009). The difference between this spectrum and the one from Si_NP´s is related with the intensity of these specific peaks which are significantly lower in the case of the SiAu_NP´s. This lowered absorbance indicates the presence of the gold shell capping the silica core.

For comparison purposes the commercial ibuprofen FTIR spectrum was obtained (figure 9a). It is possible to observe the representative peaks of the drug, being the most characteristic the ones present in the range of 2600-2980 cm^{-1} and at 1700-1720 cm^{-1}. The 2954 and 2923 cm^{-1} highlighted C-H stretching vibrations peaks correspond to the alkyl groups of ibuprofen. The sharp peak at 1719 cm^{-1} belongs to a carboxyl vibration, typical of ibuprofen (Manzano *et al.*, 2008).

The SiIBP_NP´s FTIR representation (figure 9b) have an identical configuration to the Si_NP´s spectrum, being the only difference the appearance of a peak at 2923 cm^{-1}, highlighted in the spectra, that exactly correspond to a C-H stretch characteristic of the ibuprofen. This structural modification indicates the presence of IBP in the nanoparticles (Qu *et al.*, 2006).

The final spectrum, shown in figure 9c, belongs to the SiAuIBP_NP´s. In accordance to the expected results, the spectrum is the same of the SiAu_NP´s with the addition of two peaks at 2954 and 1719 cm^{-1}, corresponding to a C-H and C=O stretch, respectively (highlighted in the figure 7f). These peaks match the ones presented in the commercial IBP spectrum, thus indicating the formation of a hydrogen bond between the carboxylic group of ibuprofen and the silanol group of the SiNP´s and consequently the presence of ibuprofen within SiAuIBP_NP´s (Qu *et al.*, 2006).

The obtainment and analysis of the Au_NP´s, Si_NP´s, IBP, SiAu_NP´s, SiIBP_NP´s and SiAuIBP_NP´s FTIR spectra demonstrated that IBP was loaded within the nanoparticles.

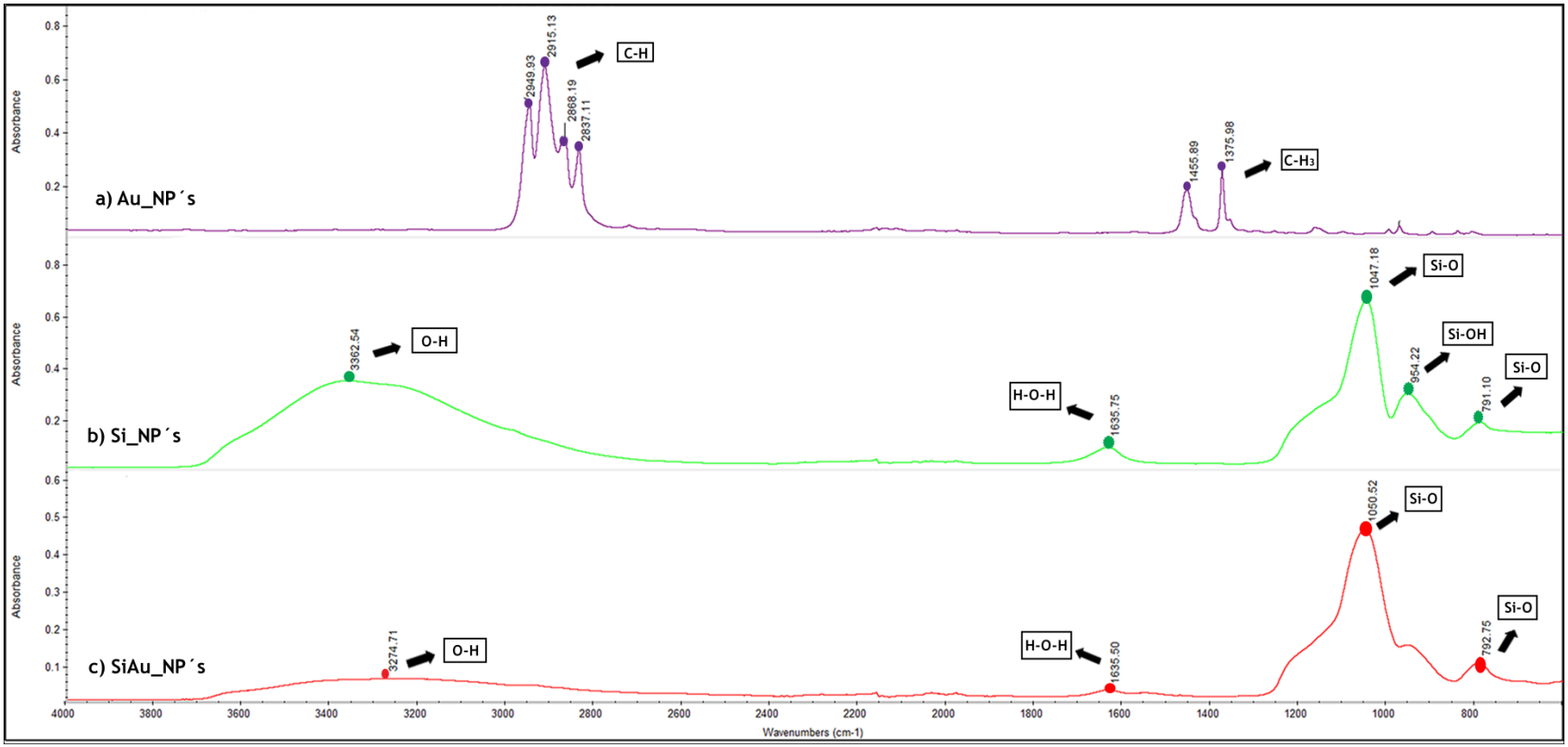

Figure 8 - FTIR spectra of the produced nanoparticles: Au_NP´s (a); Si_NP´s (b) and SiAu_NP´s (c).

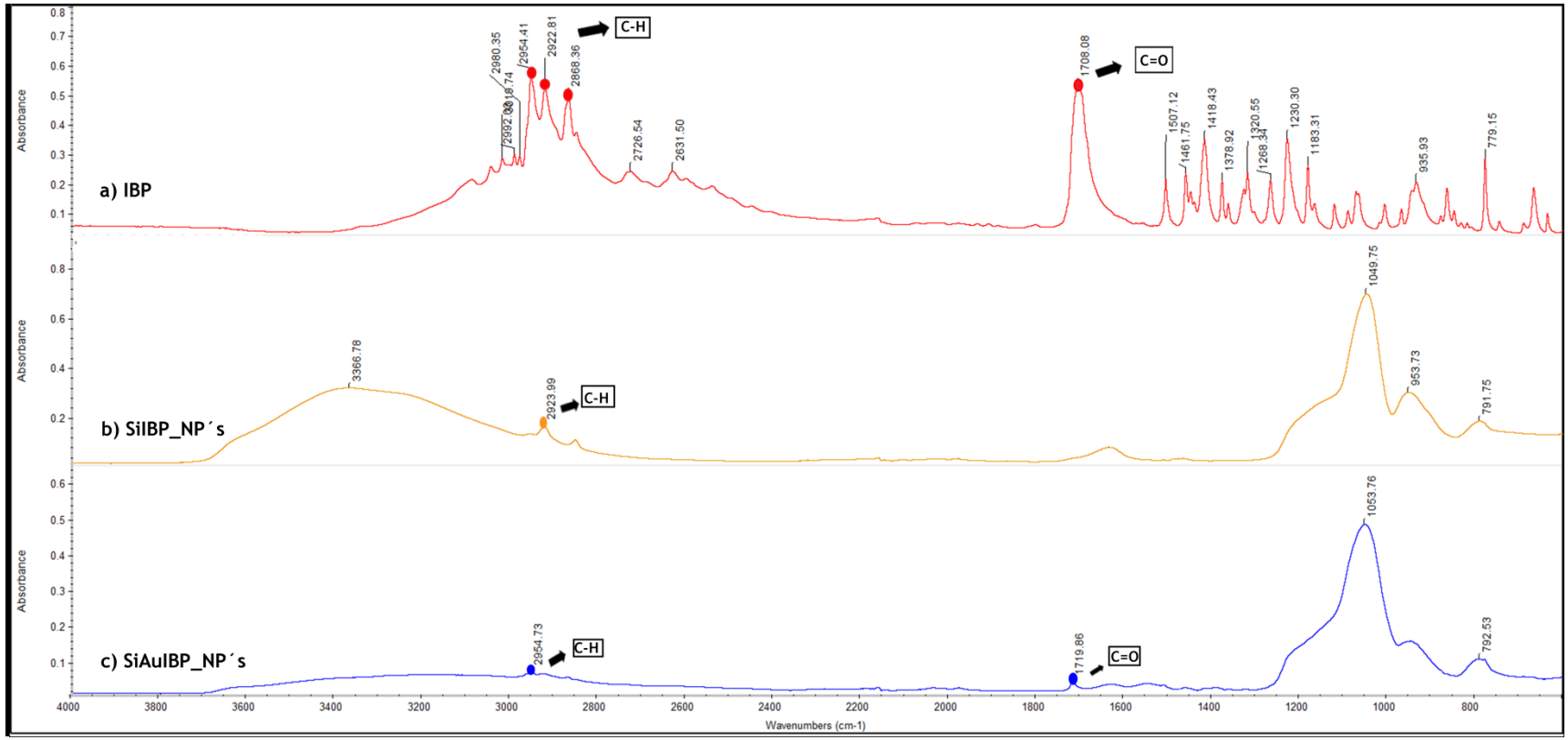

Figure 9 - FTIR spectra of the produced nanoparticles and compounds used: IBP (a); SiIBP_NP´s (b) and SiAuIBP_NP´s (c).

3.3. X-Ray powder diffraction analysis

X-ray powder diffraction analysis was used to determine the physical form (amorphous or crystalline) of the compounds and to demonstrate the formation of the core/shell of silica/gold nanoparticles. Figure 10a, represent the XRD pattern of Au_NP´s. It is possible to observe the gold crystalline structure confirmed by the sharp peaks at 2θ = 111°, 200°, 220° and 311° characteristic of the gold nanoparticles (Huang *et al.*, 2008). The Si_NP´s pattern, figure 10b, present a broad peak at around 2θ = 23°, but no sharp diffraction peaks are shown, confirming the amorphous silica nanoparticles structure (Zhang *et al.*, 2012). The XRD patterns of the produced SiAu_NP´s, shown in figure 10c present both the silica and the gold nanoparticles characteristic peaks. Thus, proving the presence of both compounds in the SiAu_NP´s herein produced.

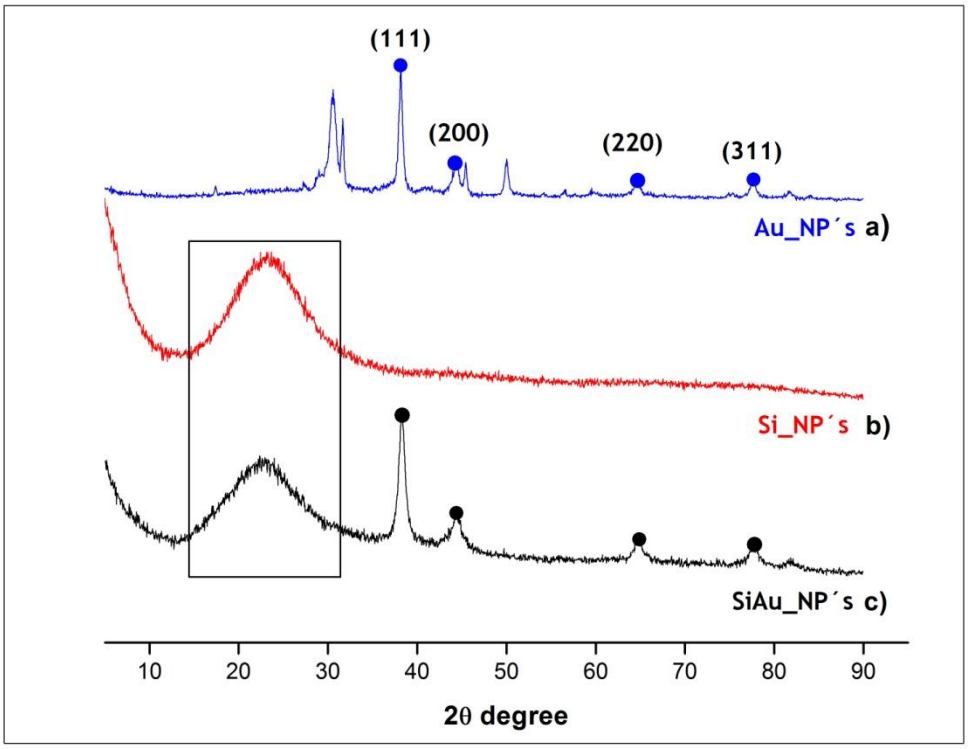

Figure 10 - XRD patterns of Au_NP´s (a), Si_NP´s (b) and SiAu_NP´s (c) at 2θ degree from 5° to 90°.

3.4. Characterization of the loading profile of the vehicles

The loading capacity of the Si_NP´s and SiAu_NP´s produced was characterized by simple immersion loading of IBP. The drug was chosen due to its low cost and easy handling. The particles were added to a solution of ibuprofen in hexane and then collected at 5, 10, 24, 48 and 72 hours for UV-visible absorbance evaluation at 264 nm. From now on, the particles were designated SiIBP_NP´s and SiAuIBP_NP´s. Calibration curves of both the vehicles were

obtained for the determination of the loading profile. The absorbance of several ibuprofen concentrations in hexane was determined at 264 nm to drawn the calibration curves in order to obtain the equations for the calculation of the loading profiles of the vehicles (figures 11A and 12 A).

Figure 11B represents the loading profile of the SiIBP_NP´s and the calibration curve used to calculate the loading percentage of IBP. The nanoparticles do not present a continuous loading profile. It is possible to observe an initial loading burst of ibuprofen during the first hours of contact. The nanoparticles suffered stabilization in the loading of ibuprofen after 5h of immersion. By this time, 15% of the drug was already encapsulated. At around 24 hours of immersion, SiIBP_NP´s resume loading drug. The loading continues during more 24h. The nanoparticles stop then the loading process, reaching a maximum loading after 48h (40% of ibuprofen available in solution was loaded in SiIBP_NP´s). It is possible to state that the results of the SiIBP_NP´s herein produced are satisfactory since, Andersson and co-workers reported that the maximum ibuprofen loading capacity of MCM-41 silica nanoparticles is 41% (Andersson *et al.*, 2008).

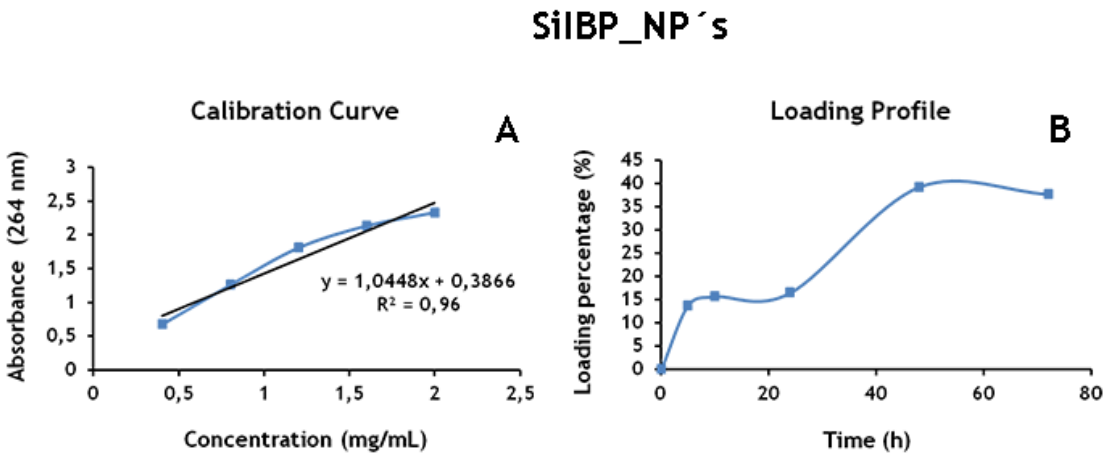

Figure 11 - Loading profile and calibration curve of SiIBP_NP´s.

The IBP loading profile and calibration curve of SiAu_NP´s is presented in figure 12. The nanoparticles show an initial burst continuous loading of ibuprofen until beginning to slow down at around 10 hours of immersion. By this time, 30% of the drug was already encapsulated. The maximum amount of drug loaded (40%) is achieved 48 hours after the beginning of the experiment.

SiAuIBP_NP´s have a maximum loading capacity similar to the SiIBP_NP´s. However, the loading profile is somewhat different. SiAuIBP_NP´s have the capacity to initially load more drug than SiIBP_NP´s, reaching a good load of ibuprofen much earlier. It is also important of note that the loading profile of SiIBP_NP´s is more tortuous than the one of the SiAu_NP´s.

The results demonstrate that both the nanocarriers have a good maximum loading capacity. The fast load of ibuprofen within the nanoparticles is characteristic of the unique

mesoporous structure of silica nanoparticles (Zhang *et al.*, 2011). The addition of the gold shell to the silica core allows a faster loading of drug, maintaining the maximum loading capacity.

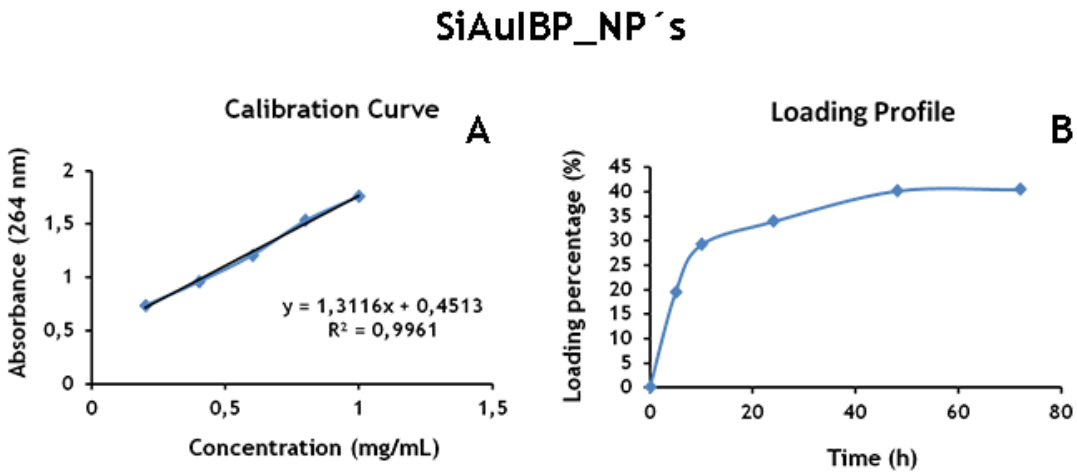

Figure 12 - Loading profile and calibration curve of SiAuIBP_NP´s.

3.5. Characterization of the release profile of the vehicles

The produced silica carriers were maintained in a PBS solution at 37° C, pH=7.4, to simulate the physiological conditions. Samples of the solution were collected with 20 min of interval. Their absorbance was analysed at 264 nm to evaluate the release profile of the vehicles. Calibration curves of both the vehicles were obtained for the determination of the release profile. The absorbance of several ibuprofen concentrations in PBS was determined at 264 nm to perform the calibration curves (figures 13A and 14A). These curves give the equations for the calculation of the release profiles of the nanocarrirers.

Figure 13 shows the release profile and the calibration curve of SiIBP_NP´s. It is possible to observe that the nanoparticles present a continuous release profile. By 140 min after the beginning of the assay almost all the loaded ibuprofen is already released. The unique structure of the SiIBP_NP´s allows a rapid diffusion of the encapsulated ibuprofen through the well-defined pores (Charnay *et al.*, 2004). In the literature it is reported that MCM-41 types of silica nanoparticles have similar releasing profiles to those of the SiIBP_NP´s produced here (Zhang *et al.*, 2011).

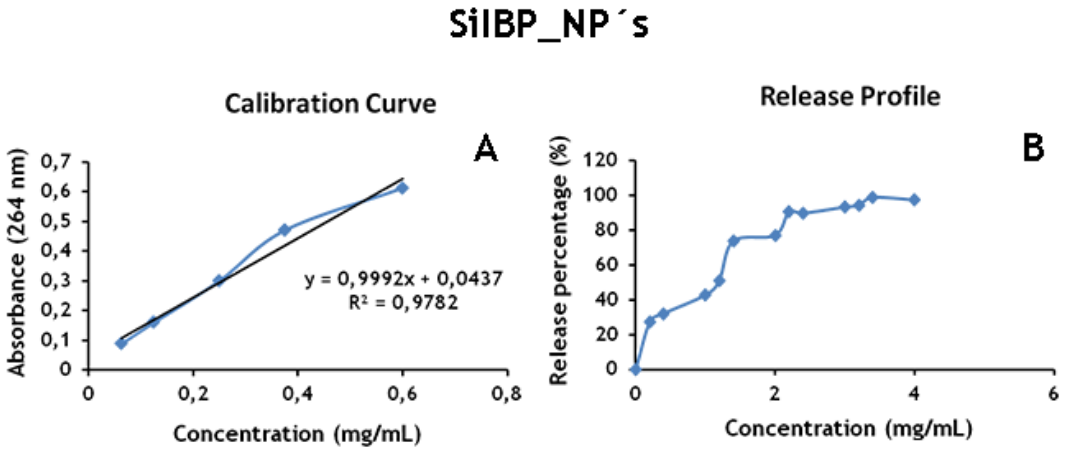

Figure 13 - Release profile and calibration curve of SiIBP_NP´s.

The release profile and the calibration curve of the produced SiAuIBP_NP´s are depicted in figure 14. These produced SiAuIBP_NP´s present a drug release profile similar to the one of the SiIBP_NP´s. However, it is possible to observe that the release rate is slower. The liberation of all the loaded ibuprofen is only achieved after 180 min of the beginning of the experiment. These results suggest that the addition of a gold shell to the mesoporous silica core structure induced the nanovehicle to slow down the release of ibuprofen. This could be very interesting in biomedical applications where a slower release rate is necessary, for instance the release of ibuprofen through time.

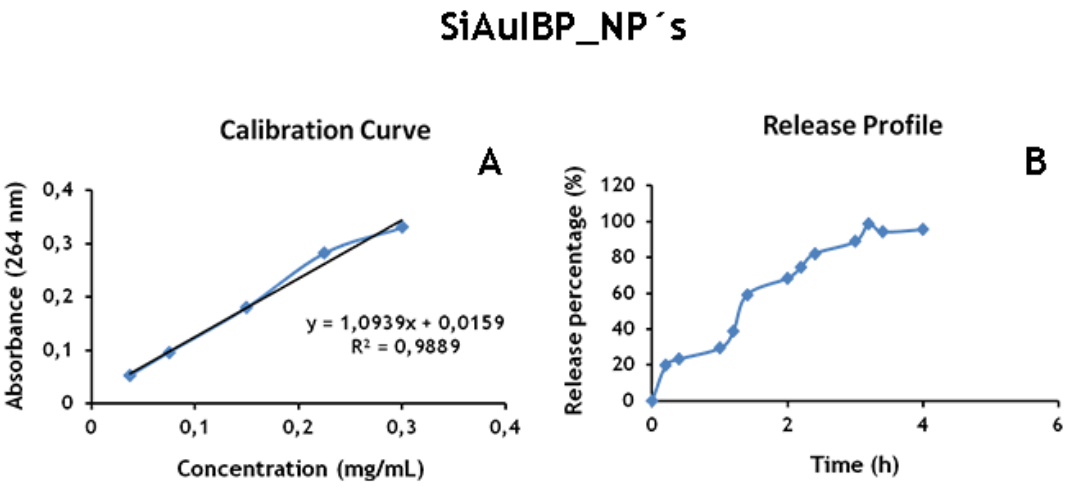

Figure 14 - Release profile and calibration curve of SiAuIBP_NP´s.

3.6. Qualitative evaluation of the in vitro transfection

CLSM is an optical microscopy technique with applications in several sciences, such as biology, chemistry, physics and materials (Van Gough, 2008). This optic technique allows the obtainment of high resolution images. It provides several advantages over other optical microscopy techniques, including enhanced contrast and possibility of 3D analysis. CLSM can be used to collect images of individual slices using the fluorescence or reflection properties of a determined sample in the xy, xz and yz planes (Van Gough, 2008).

The CLSM technique was performed to evaluate the internalization of the produced Si_NP´s and SiAu_NP´s in A549 small lung cancer cells. As depicted in figure 15, Si_NP´s were internalized by a couple of cells, reaching their cytoplasm (green dots in figure). These results show that the produced silica nanoparticles have the ability to surpass the cytoplasmatic membrane of cells.

Figure 16 shows SiAu_NP´s internalized by cells. Once again, nanoparticles reached the cytoplasm of cells. Thus, it is possible to say that the capping of the functionalized Si_NP´s with gold does not affect the capacity of the nanocarriers to enter into cells.

The ability of both types of nanoparticles to reach the cytoplasm of cells allows the delivery of the entrapped drug inside the cell. This capacity gives drugs, like ibuprofen, the possibility to be applied directly within cells. However, it is necessary to give specificity to the nanoparticles to allow them to target cells.

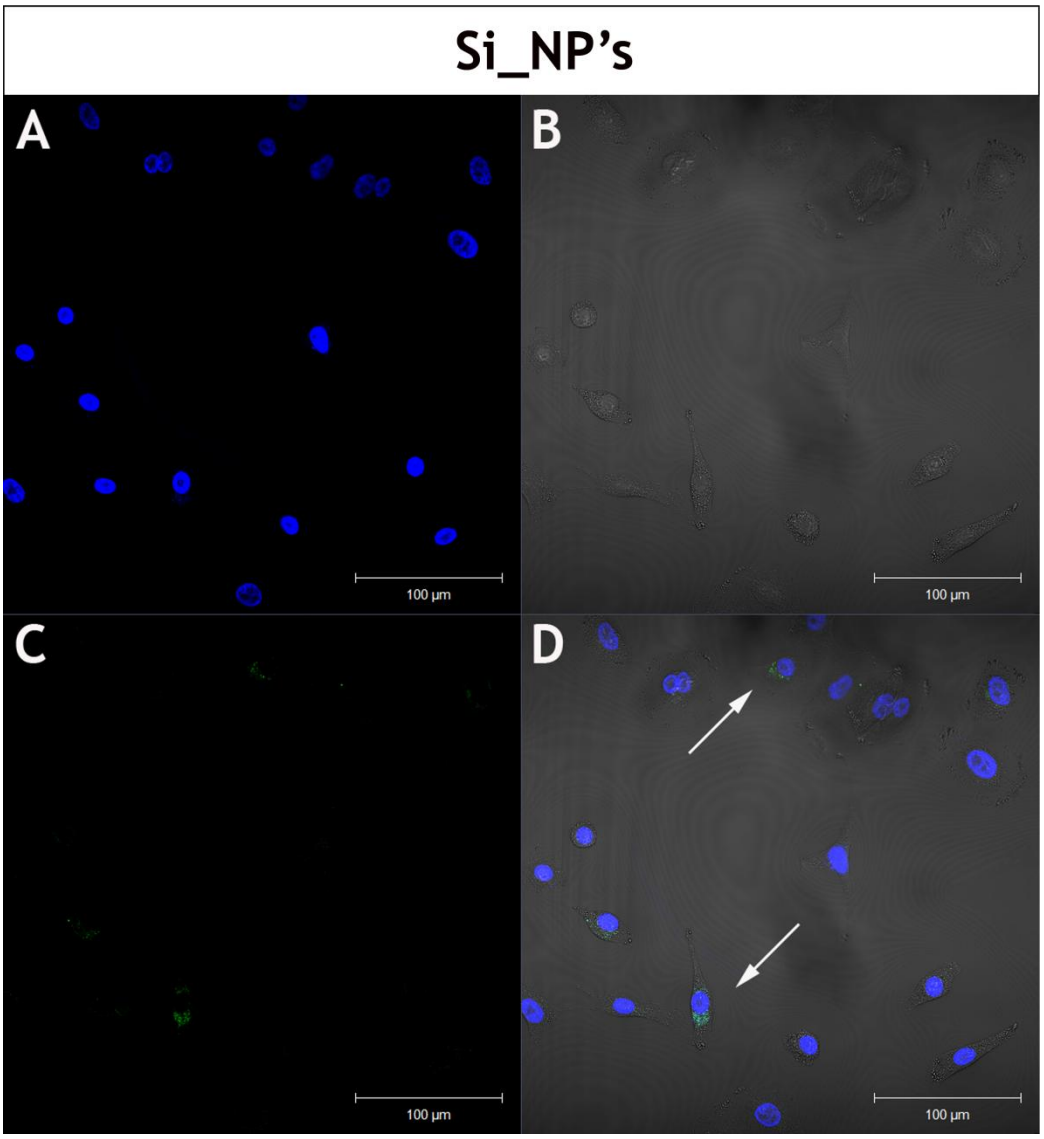

Figure 15 - CLSM images of A549 small lung cancer cells. Nuclear staining with Hoechst® 33342 (blue) (A); Brightfield image (B); FITC labelled Si_NP´s (green) (C); Merged images (D).

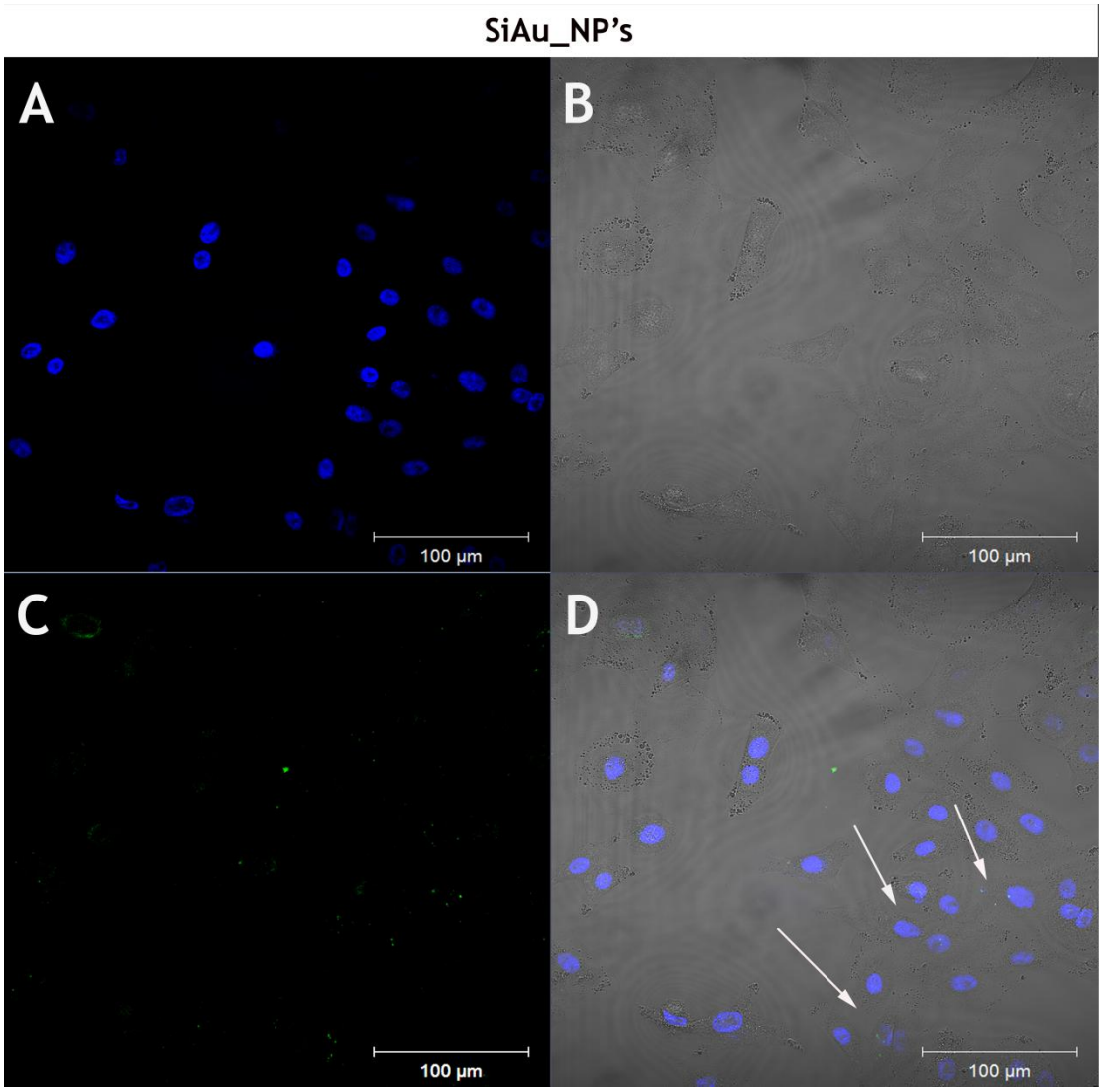

Figure 16 - CLSM images of A549 small lung cancer cells. Nuclear staining with Hoechst® 33342 (blue) (A); Brightfield image (B); FITC labelled SiAu_NP´s (green) (C); Merged images (D).

3.7. Characterization of the cytotoxic profile of the produced nanoparticles

The cytotoxic profile of the nanocarriers was characterized through *in vitro* assays.

This study was performed to address if the synthesized nanoparticles formulations are toxic for cells. Gold itself is reported to have low toxicity. However, the toxicity of gold nanoparticles (due to its low concentration) are not yet been fully studied (Murphy *et al.*, 2008; Alkilany and Murphy, 2010). Size, shape and surface group as well as cell type are the main concerns that have to be taken into account in terms of the evaluation of gold nanoparticles toxicity (Murphy *et al.*, 2008). In other hand, silica nanoparticles are described as having low toxicity as long as they are kept in concentrations lower that 100 µg/mL (De Jong and Borm, 2008). There are evidences in the literature of the toxicity associated with

Si_NP´s (Wu *et al.*, 2011; Bimbo *et al.*, 2012). Nevertheless, relatively limited knowledge about this matter is already published (Bimbo *et al.*, 2012).

As presented in figure 17, the viability of cells was not affected by any of the nanoparticles produced after 24, 72 and 120h. The A549 small lung cancer cells seeded in contact with the nanocarriers, adhered and proliferate in a similar way to that of the negative control (K^-), where cells were seeded only with Ham-F12 medium. The positive control (k^+) show dead cells with their typical spherical shape.

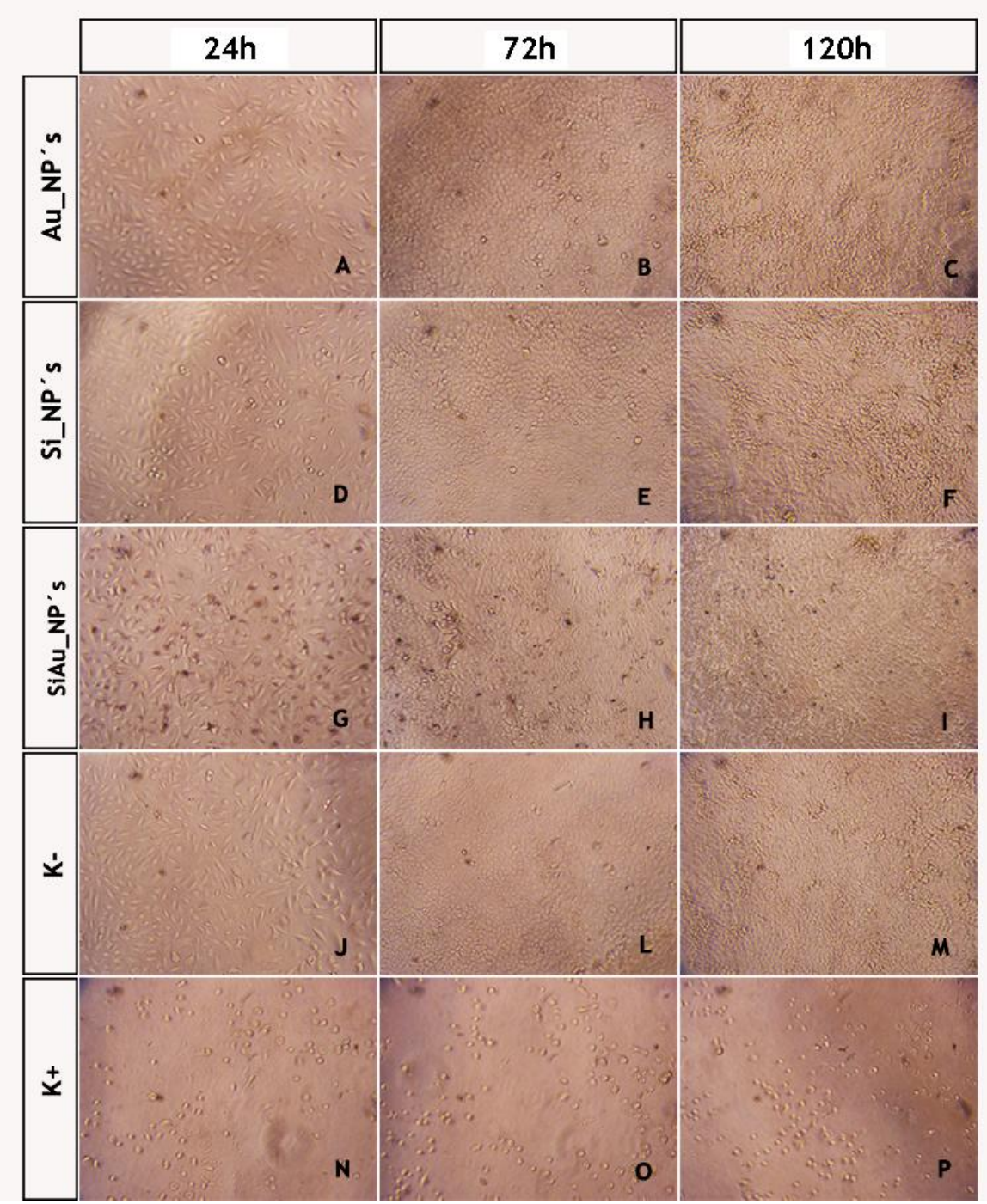

Figure 17 - Inverted Microscope Images of A549 small lung cancer cells 24,72 and 120h after being seeded with Au_NP's, Si_NP´s and SiAu_NP´s. Negative control (K^-), positive control (K^+). Original magnification x100.

The MTS assay (figure 18) was performed to further evaluate the biocompatibility of the produced nanoparticles. In all the used formulations cell viability was similar to that of negative control (K^-) after 24, 72 and 120 h of cell seeding. The positive control (K^+) presents no viable cells. The assay showed a significant difference between the positive control ($p<0,05$) and the negative control and cells in contact with the different nanoparticles after 24, 72 and 120 hours of incubation. Thus, suggesting that the produced nanoparticles formulations did not have an acute cytotoxic effect on cells and are suitable for being applied in different biomedical applications. Silica nanoparticles toxicity is reported to be cell type and nanoparticles concentration associated (Mccarthy *et al.*, 2012). The results obtained herein are similar to those reported by Yu and colleges. They showed that silica nanoparticles do not cause any toxic effect on A549 small lung cancer cells (Yu *et al.*, 2011). Moreover, it is also described that the concentration used in the assays (less than 100 µg/mL) are not toxic to cells (De Jong and Borm, 2008). The addition of gold (SiAu_NP´s) does not change the citotoxic profiles of the nanoparticles. This is in accordance with what was previously published in the literature for gold nanoparticles, which are described as having no toxicity for cells (Alkilany and Murphy, 2010).

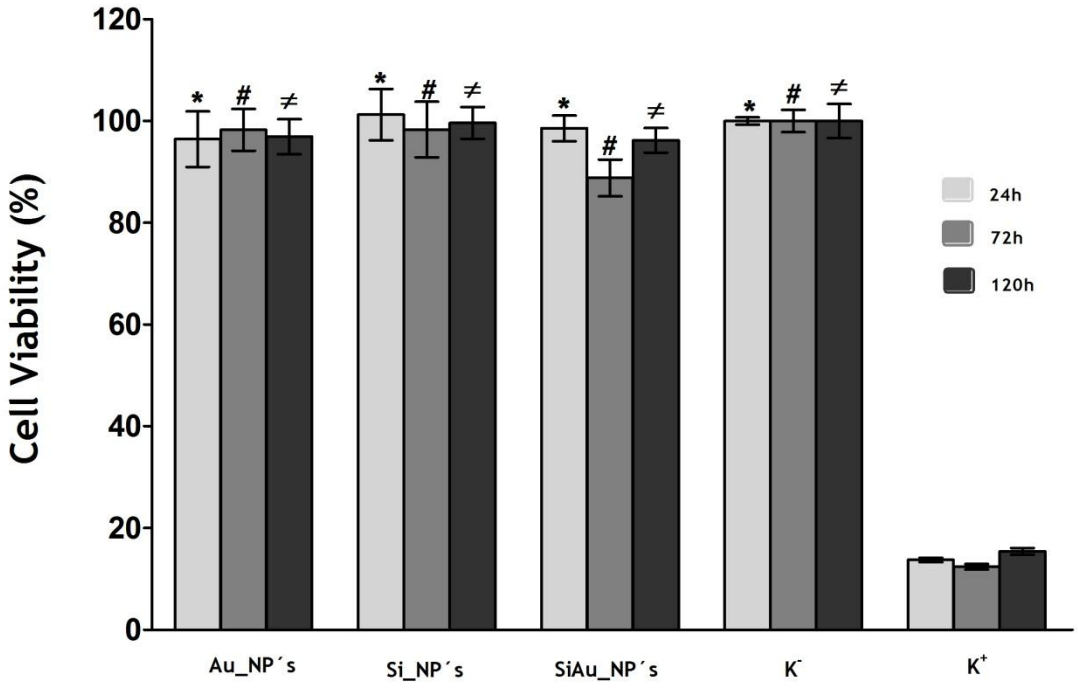

Figure 18 - Evaluation of the cellular viability after exposure to the produced nanoparticles: Au_NP's; Si_NP´s and SiAu_NP´s. Light grey bars represent cell viability at 24h. Medium grey bars represent cell viability at 72 h. Dark grey bars represent cell viability at 120 h. Non-transfected cells were used as negative control (K^-). Cells treated with Ethanol were used as positive control (K+). Each result is mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunet's post hoc test (*$p<0,05$, #$p<0,05$ and ≠$p<0,05$).

Chapter IV – Conclusions and Future Perspectives

4. Conclusions and Future Perspectives

Healthcare is an area of constantly evolution. Thus, the application of nanotechnology to medicine is a promising new trend that is expected to improve medical practice from diagnosis to treatment. Among nanomedicine, the use and development of nanoparticles for the delivery of drugs and other compounds is one of the most studied areas. Mesoporous silica nanoparticles appear as a viable option, since some of their properties, such as the rigid mesostructure, good biocompatibility, high loading capacity and the easy connection to other inorganic particles, like gold, are fundamental for their application of drug delivery.

There are several types of silica nanoparticles. In the present study, MCM-41 Si_NP´s were produced by an adaptationvof the Stöber method. Au_NP´s were produced by an adaptation of the Frens method. To create a stabilization shell that improves some of the nanocarrier properties, the Si_NP´s were bonded to the Au_NP´s creating a new nanosystem, named SiAu_NP´s. To allow the gold binding, the Si_NP´s surface was functionalized with amine groups. Ibuprofen was loaded into Si_NP´s and SiAu_NP´s in order to evaluate the capacity of the nanoparticles to act as DDS.

The morphology of the nanoparticles was characterized by SEM analysis. FTIR and XRD analysis were performed to characterize the chemical composition of the nanoparticles. These analyses confirmed the presence of gold and silica in the nanoparticles and also the presence of ibuprofen within the nanoparticles. Moreover, the loading and release profiles of Si_NP´s and SiAu_NP´s loaded with ibuprofen were characterized. The nanoparticles showed a good loading and release profile. These make them viable options for drug delivery applications.

A549 small cancer lung cells were placed in contact with the produced nanoparticles to perform *in vitro* studies. The internalization of the nanoparticles was evaluated through CLSM. The results obtained showed that Si_NP´s and SiAu_NP´s have the capacity to surpass the cytoplasmatic membrane allowing nanoparticles to deliver drugs inside cancer cells. Furthermore, the cytotoxic assays showed that all the nanoparticles formulations are biocompatible, demonstrating their applicability for being used as DDS in the human body.

The SiAu_NP´s herein produced can be in a near future a good option for drug deliver applications. They combine a mesoporous core with a gold shell. This allows them to have a high ibuprofen loading capacity as well as a characteristic burst release of the drug. Different biomedical application could beneficiate from the nanoparticles produced in this study.

In the future, it would be interesting to load different drugs within the nanoparticles to access if the load and release profiles of SiAu_NP´s are kept. Another newsworthy option will be the load of genes or growth factors, for instance. The produced nanoparticles do not have specificity to any type of cell. Another important point that must be addressed is to functionalize the particles surface in order to confer specificity for cancer cells.

Chapter V – Bibliography

5. Bibliography

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