NANOTECHNOLOGY APPROACHES FOR IMPROVED BASED-DRUG DELIVERY SYSTEMS OF VASOACTIVE INTESTINAL PEPTIDE

Rebecca Klippstein Martín
CABIMER (Andalusian Centre for Molecular Biology and Regenerative Medicine)-University of Seville
Direction and Supervision: Prof. Dr. D. Pozo Perez

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“The scientist is not a person who gives the right answers, he's one who asks the right questions.”  Claude Lévi-Strauss
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I. INTRODUCTION
1. Nanoparticles for biomedical applications:

1.1 Nanomedicine and its actual impact

Nanotechnology is considered by many as a “big revolution”. This technological leap controls materials at a nano-scale level and has driven developments of new applications in modern molecular medicine by using nanodevices, such as nanoparticles (NPs) (West and Halas 2000). NPs are defined as particles with sizes between 1 and 100 nm approximately that show properties not found in bulk samples of the same material (Auffan, Rose et al. 2009). In recent years we have seen the emergence of nanoscience and nanotechnology as cutting-edge research areas that are being rapidly developed. In particular, engineered nanoparticles constitute an extensive field of research due to the translational potential for biomedical applications (West and Halas 2000). The efforts are mainly related to the application of nanoengineering methods and materials to develop new diagnostic platforms and more effective therapies for human diseases. Currently, there is a growing interest in different nanosystems and their biological features as nanocarriers of chemotherapeutic agents and other compounds known as “smart drug delivery” products. Drug delivery nano-systems are being studied to allow more selective and potent treatments as well as to improve the efficacy/toxicity ratio of our current and future therapeutic arsenal (Malam, Lim et al. 2011). The technical designs and the experimental approaches are aimed at formulating therapeutic agents in biocompatible nanocomposites such as noble metals, organic and inorganic nanoparticles, nano-capsules, and a variety of micellar systems (Kumar Khanna 2012).

Moreover, recently developed methodologies related to surface functionalization provide materials with the final properties required for a desired application (Friedman, Claypool et al. 2013). Nowadays, nanotechnology has brought an unprecedented variety of revolutionary approaches for molecular medicine. Some examples of what engineered NPs can offer to modern clinical practice include the detection of biological analytes (reducing sample size, reagent volumes and faster
results) (Rosi and Mirkin 2005), specific targeting to the action site (Drechsler, Erdogan et al. 2004), efficient drug delivery into the target cell (Drechsler, Erdogan et al. 2004), or in vivo real-time monitoring of cellular events (Morawski, Lanza et al. 2005). Such combinatorial NPs may eventually provide the means to achieve “personalised medicine” by tailoring NP functionality to individual responses. In this context, development of bio-functional NPs could dramatically improve already existing NP characteristics and provide a tool to achieve better therapies (Jain 2005).

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Table 1. European Technology Platform Nanomedicine strategic research. The table shows the importance of smart nano-systems for therapeutic treatments, advanced diagnosis and intelligent delivery to overcome different challenges in cancer, diabetes and neurodegenerative, cardiovascular and inflammatory diseases.

An important initiative, led by industry, has been set up together with the European Commission. A group of 53 European stakeholders, composed of industrial and academic experts, has established a European Technology Platform on Nanomedicine (ETPN)\(^1\). The first task of this high level group was to create a vision

\(^1\) White Paper to the Horizon 2020 Framework Programme for Research and Innovation
document for this highly future-oriented area of nanotechnology-based healthcare in which experts describe an extrapolation of needs and possibilities until 2020. As it has been highlighted by the ETPN in the white paper recently disclosed for research and innovation, nanotechnology is one of the six Key Enabling Technologies (KETs) that has a significant impact on many different medical developments in three main areas: therapeutics, diagnostics/imaging and regenerative medicine \(^1\) (Table 1). Over the last decade the strategic research agendas defined by the nanomedicine community were mainly based on technology and clinical demands. The research and development (R&D) projects implemented under these research priorities in FP6 and FP7 have successfully delivered a lot of new nanomedicines but few products on the market. In consequence, the next level of the strategic development of the ETPN is to emphasise the introduction of “innovation” into the Agenda by improving the translation of nanotechnology R&D into medical applications.

Innovation applied to nanomedicine means enabling personalised medicine through stratification of patients by nano-based diagnostic tests or imaging agents, which is a new and different approach to current medical practice. Another example is the combination of a diagnostic test and a therapy within one type of NP thus making some clinical protocols simpler. However, for such innovations to reach the patient, close and well informed interactions between all actors in the nanomedicine research and development chain (academic, industrial, public and private partners) are mandatory, including special structures to actively manage communication and collaboration between all stakeholders, including regulatory bodies to effectively translate and commercialise ideas.

One of the main challenges to be addressed by current nanotechnology research and management is to improve the crosstalk between different academic disciplines, different industries (pharmaceutical, medical devices and diagnostics),
clinical organisations and regulatory agencies to help structure the development of nanomedicine and to provide channels for early stage clinical proof of concept.\(^1\)

Nanomedicines offer the possibility of improving the therapeutic effect of existing drugs at a relatively low cost and risk compared to other areas making it more reliable to invest in the R&D. The global market for nanomaterials products is expected to grow from a global volume of €200 billion in 2009 to €2 trillion by 2015.\(^2\) In addition, vendors have received funding from various governments, for example, the US, the UK, China and Germany have already invested over US$ 67 billion towards nanotechnology funding. Corporate research and various other forms of private funding are expected to invest almost US$ 250 billion in nanotechnology by 2015. With the market already valued at over US$ 81 billion, additional support from government and private funding has provided the opportunity for many vendors to begin new research projects which will help increase the market growth for the upcoming years.\(^3\) Furthermore, Competitiveness and Innovation Framework Programme (CIP), which complements the Research Framework Programme and supports "Entrepreneurship and Innovation" is also running from 2007 to 2013 and has a budget of approximately 3.6 billion Euros.\(^4\)

1.2 Drug delivery systems

NP based drug delivery systems are of great importance because they are able to improve the pharmacokinetics and biodistribution of a drug and provide controlled release kinetics to the target site. Drug targeting to specific organs and tissues has become one of the critical endeavours of the new century. The search for new drug delivery systems and new modes of action involves a multidisciplinary scientific approach.

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\(^1\) White Paper to the Horizon 2020 Framework Programme for Research and Innovation

\(^2\) European Commission, Nanotechnology: http://ec.europa.eu/nanotechnology

\(^3\) Global Nanomedicine Market Report 2012-2016

\(^4\) CORDIS, EU Funding Opportunities
approach to provide major advantages in improving the therapeutic effects and bioavailability of the drug (Baldeschwieler 1997; Beija, Salvayre et al. 2012). An ideal drug carrier should have high drug-loading capacity, low toxicity, favourable drug release kinetics, biocompatibility, and tissue-specific targeting capability.

Targeted drug delivery is the ability to direct the drug-loaded system to the site of interest (Huynh, Roger et al. 2010), whereas passive targeting is a preferential accumulation of the drug in a specific tissue. Active targeting involves the surface functionalization of NPs with ligands that are selectively recognized by molecules on the surface of cells of interest (Kumar Khanna 2012).

Nanotechnology medical developments over the coming years will have a wide variety of uses and could potentially increase life expectancy (Freitas 2009). Nanomedicines are already moving from passive structures to active structures, through more targeted drug therapies or “smart drugs.” These new drug therapies have already been shown to cause fewer side effects and be more effective than traditional therapies. In the future, nanotechnology will also improve the synthesis of molecular systems that may be similar to living systems. These molecular structures could be the basis for the regeneration or replacement of body parts that are currently lost due to infection, accident, or disease (Verma, Domb et al. 2011). The number of products approaching the Food and Drugs Administration (FDA) approval and review processes will likely grow as time moves forward and as new nanotechnology medical applications are developed.

In the present, there is a dramatic increase in the number of reports on the use of NPs in medical applications. However, there is a general need in terms of comprehensive characterization of the published NP systems, which requires an additional effort towards interdisciplinary research at the nanoscience/pharmaceutical interface. While, it is beyond the capability of many research groups to perform detailed physicochemical characterization, tracking analysis, pharmacokinetics and
circulatory half-life studies, the scientific community needs methods of standardization for physicochemical characterization and biological evaluation which is required for clinical translation to the field, not only in clinical testing, but also to reduce the number of conflicting data already published in the scientific literature (Howard and Peer 2013).

Nanomedicine has the potential to impact significantly on key elements related to the diagnosis and treatment of life-threatening diseases. In this sense, drug delivery in the field of oncology has received a successful input after the introduction of several novel nanomedicine products into clinical trials due to the lack of specific anticancer treatments (Liu, Solomon et al. 2013; Rink, Plebanek et al. 2013). Currently, there is a lot of research going on to design novel nanodevices detecting and treating cancer at its earliest stages by tracking its location within the body and delivering anticancer drugs specifically to malignant cells. Regarding conventional anticancer treatments, they are toxic to both tumour and normal cells, thus the efficacy of chemotherapy is often limited by the side effects of the drugs, the need of higher doses and repeated administrations (Juliano 2013).

Therefore, the oncology area has felt the biggest impact from nanomedicine because of the exploitation of the enhanced permeability and retention (EPR) effect in tumour tissues. These newly formed vessels in the tumour are usually abnormal in form and architecture. They consist of poorly-aligned defective endothelial cells with wide fenestrations, lacking a smooth muscle layer, or innervation and with a wider lumen (McDonald and Baluk 2002). The NPs can go through these fenestrations passively and get in contact with the tumour tissue more easily. With the discovery of the EPR effect, passive targeting of chemotherapeutics to solid tumour tissues is an achievable objective given specific particle sizes and chemical characteristics. As its name implies, the EPR effect is the selective accumulation of macromolecules in solid tumour tissue and the retention of those macromolecules within the tissue for a prolonged time due to increased leakage of tumour blood vessels and decreased
effective lymphatic drainage (Maeda, Fang et al. 2003). Figure 1 summarizes the characteristics of normal and tumour tissues, which can affect the passage and retention of NPs.

Figure 1. Enhanced permeation and retention effect (EPR). Upper figure shows the vasculature of normal tissue. This vasculature is not fenestrated and only permits the passage of small molecules. Whereas the lower figure shows the tumour vasculature which is poorly aligned and has wide fenestrations. This permits the leakage of NPs and its accumulation in the tumour site and the following therapeutic effect on the cancer cells.

Besides some pathologically-related situations that facilitate the passive transfer of a limited numbers of NPs, multiple obstacles are encountered on the way to their target which affect their bioavailability and must face primary anatomical barriers such
as the lung epithelium as well as internal barriers including the blood–brain barrier (BBB), the blood–testis barrier (BTB), the blood retina barriers (BRB) and mucosal barriers (Schleh, Semmler-Behnke et al. 2012; Pietroiusti, Campagnolo et al. 2013). The appropriate delivery system for different compounds would be one that protects them from the biological environment, while facilitates their transport through biological barriers. Thus, in order to understand the potential hazard of NPs towards these critical organs, one must understand the nature of biological barriers and how NPs may cross or interact with cellular components of these barriers, leading to detrimental effects. Importantly, each of these NP properties will also likely influence the adsorption of biomolecules onto the surface of NPs, and the bio-corona formed may influence the distribution and toxicity potential of NPs (Pietroiusti, Campagnolo et al. 2013). As a relevant example, over the past few years, many efforts have been made to understand the mechanisms of the NP uptake into the brain. This mechanism appears to be receptor-mediated endocytosis in brain capillary endothelial cells. Modification of the NP surface with covalently attached targeting ligands or by coating with certain surfactants enabling the adsorption of specific plasma proteins are necessary for this receptor-mediated uptake (Wohlfart, Gelperina et al. 2012).

Research in designing and engineering long-circulating NPs, so-called ‘stealth’ NPs, has been attracting increasing interest as a new platform for targeted drug delivery, especially in chemotherapy. In particular, the modification of NPs surfaces with poly(ethylene glycol) (PEG) derivatives has shown a decreased uptake of NPs by mononuclear phagocyte system cells and, hence, an increased circulation time, which is an advantage in cancer biology by allowing passive accumulation in the tumour. In the last few years, the new-generation technique of grafting ligands on the NP surface in order to target and penetrate specific cancer cells has been developed. Since ligand-receptor interactions can be highly selective, active targeting allows a more precise and specific treatment of the site of interest (Emerich and Thanos 2007). Surface modification of drug carriers with bioactive molecules can be adsorbed, coated, conjugated or linked to them interact with cell receptors and have demonstrate a
selective affinity for a specific cell or tissue type and can subsequently enhance drug uptake (Huynh, Roger et al. 2010).

For the utilization of functionalized NPs several considerations have to be taken, as the use of targeting ligands can enhance the distribution to secondary target sites of non-intended tissues (Kaminskas, Boyd et al. 2011). In fact, the disadvantage of using non-antibody ligands is their non-selective expression (Allen 2002). But, on the other hand, immunocojugates pose problems related to immunogenicity and the retention in the reticuloendothelial system (RES). For example, after intravenous injection occurs, NPs are cleared from the plasma within a few minutes due to opsonisation and subsequent phagocytosis by the cells of the RES (Bennewitz and Saltzman 2009). Macrophages in the RES located in the liver and the spleen vividly take up particles bound with serum proteins. In this case, surface modification can reduce the opsonization by minimizing the clearance and leading to improved pharmacokinetic properties. The most common example is PEG, a hydrophilic polymer, which constrains the binding of plasma proteins and prevents aggregation induced by salts and proteins in the serum (Taratula, Garbuzenko et al. 2009). This approach has been used in a variety of NP systems to improve the circulation half-life, and therefore enhancing a key feature related to drug delivery. The surface modification of the NP can also incorporate specific coatings to change the lipophilicity/hydrophobicity profile and prevent the uptake by immune cells to improve cell recognition. This fact prevents opsonisation and recognition from phagocytes and thus avoids undesired immune responses. In addition, PEG can also reduce the access of enzymes and therefore reduce their degradation (Kaminskas, Boyd et al. 2011). In fact, in vivo NPs coated with PEG increase their circulation time from several minutes to many thousand and enhance residence times up to 200-fold in humans (Park, Fong et al. 2009; Gaillard 2010; Habgood and Ek 2010).

Therefore, the two most important aspects of NP drug delivery must be:
1. The **coating** and **functionalization** of the NPs for **specific targeting** to the tissue. The coating of the NPs can modify the circulation time and the pharmacokinetics of the NPs. In addition, functionalization with proteins or antibodies can enhance the delivery of the drug and reduce non-specific associated toxicity.

2. The **time release of the drug** to prevent non-specific toxicity. Here, the drug must not diffuse out of the particle while it is still in the circulatory system, and must remain encapsulated until the NP reaches the target. Once it gets in contact with the target the loaded drug must be released.

In summary, site-specific targeting drug delivery is important in the therapeutic modulation of effective drug dose and disease control. The particle size and surface characteristics (for passive or active targeting) can be selected, the release of the drug can be controlled and different routes of administration can be used according to the treatment (**Figure 2**).

**Figure 2. Smart drugs: NP drug delivery for personalised medicine.** Traditional drugs are spread after administration throughout the body due to the lack of specificity. In contrast, NP
specific drug delivery targets tissues reducing the need of high drug doses and producing as a result less systemic toxicity.

1.2 Types of nanoparticles

Extensive libraries of NPs, composed of an assortment of different sizes, shapes, and materials, and with various chemical and surface properties, have already been constructed (Schutz, Juillerat-Jeanneret et al. 2013). The field of nanotechnology is under constant growth and new additions continue to complete these libraries. There are a wide variety of NPs which can be multi-task by combining different functionalities in a single stable construct, for example incorporate a drug, a contrast agent and a targeting molecule in the same system (Figure 3). Some of their basic properties and current known uses in biotechnology, particularly in nanomedicine will be described. During the last 30 years, there has been a great improvement in the synthesis and characterization of nanomaterials. Basically, NPs are classified in two main groups depending on the type of material, organic and inorganic. Special attention will be paid to metallic NPs and liposomes, due to the experimental work developed during the current PhD project.
Some examples of NPs are showed above, such as micelles, nanotubes, polymeric and metallic NPs. Different molecules, such as therapeutic, directing or contrast agents (for example, drugs, antibodies, peptides or fluorophores) can be used to further modify and confer novel properties for different applications.

NPs are carriers for conventional drugs as well as for peptides, proteins, enzymes or antigens. According to the process used for preparation of NPs, nanospheres or nanocapsules can be obtained (Figure 4). Nanospheres or most commonly called NPs are homogeneous matrix systems in which the drug is dispersed.
throughout the particles, whereas nanocapsules are vesicular systems in which the drug is confined to an internal cavity surrounded by a membrane (Vauthier and Bouchemal 2009) (Figure 4).

**Figure 4. Schematic representation of nanospheres and nanocapsules.** Nanospheres have homogeneous matrix systems in which the drug is dispersed throughout the particle, whereas in nanocapsules the drug is confined into an internal cavity.

Basic understanding of how nanomaterials—the building blocks of nanotechnology—interact with the cells and their biological consequences are beginning to evolve. The constituents of NPs for biomedical applications need to be physiologically compatible (usually the term used is biocompatible) and they need to be biodegradable (it disintegrates in a physiological environment) into harmless components or to have the ability to be excreted via kidney or bile (Naahidi, Jafari et al. 2013).

NP drug delivery systems have been developed to improve their therapeutic efficacy through principles like controlled release, targeted drug delivery, prolongation of the circulation time or protection from degradation (Kreuter 1994) for the administration of drugs. Besides the intravenous route, colloidal particles have also been administered orally, either for systemic uptake or local activity within the gastrointestinal tract and for local administration on skin and mucosa as for systemic
use by parenteral application or by inhalation (Maincent, Le Verge et al. 1986; Kreuter 1994; Damge, Aprahamian et al. 1996; Chen and Langer 1998).

NPs for pharmaceutical use can be made of lipophilic components or consist of polymeric nature. Other materials for pharmaceutical applications have been studied such as albumin (Elzoghby, Samy et al. 2012), gelatin (Xu, Gattacceca et al. 2013), or calcium alginate (Nesamony, Singh et al. 2012). These NPs can be used to incorporate a drug substance during or after the preparation, depending on the drug properties such as water solubility, molecular weight and aggregation. The active drug can be entrapped inside the NPs, in which case the particles are often referred to as nanocapsules, adsorbed or functionalized to the surface of the NPs. In addition, combinations of these arrangements are also possible. Organic vesicular nanocarriers can easily entrap nucleic acid, drugs or even smaller NPs within their aqueous and lipid cores. Also their surface can be functionalized to improve their pharmacokinetic profile or targeting (Lopez-Davila, Seifalian et al. 2012) (Figure 5).
Figure 5. Schematic representation of different strategies for constructing multifunctional NPs. NPs can include the properties listed above by different modifications in order to obtain numerous benefits, such as toxicity reduction, high levels of drug in the blood or increase the treatment efficacy.

1.2.1 Metallic Nanoparticles

Inorganic NPs are primarily metal-based and have the potential to be synthesized with near monodispersity (Cui, Feng et al. 2009). Noble metal NPs have elicited interest due to their size and shape and unique optoelectronic properties. They have been studied for biomedical applications because of their ease of synthesis, characterization and surface functionalization. In addition, they have been extensively studied for imaging using magnetic resonance and high-resolution superconducting quantum interference devices while their intrinsic properties have been explored for
therapy (Arvizo, Bhattacharyya et al. 2012). Many efforts have been made recently to understand some critical parameters of these NPs for future clinical translation, for example how these nanomaterials interact with cells, their biodistribution, pharmacokinetics and their therapeutic efficacy in different disease models (Arvizo, Bhattacharyya et al. 2012; Liu and Ye 2013).

Metallic NPs and their use may be considered a new truly product since the potential of nanotechnology has been exploited only in recent years, although the synthesis and use of NPs dates back to ancient times. The first evidence of metallic NPs is from 2000 years ago when gold NPs (AuNPs) were part of ancient medicine in India (Arvizo, Bhattacharyya et al. 2012). They used 50 nm AuNPs, called swarnabhasma (gold ash) and mixed them with honey or cow gee. This gold ash was given orally to patients to treat a variety of diseases such as rheumatoid arthritis, asthma, diabetes mellitus and others (Arvizo, Bhattacharyya et al. 2012).

Currently, the most significant metallic NPs are copper, iron oxide, silver, gold and zinc NPs (Faramarzi and Sadighi 2013). They are mainly exploited in applications related to the utilisation of their optical and magnetic properties, conductive inks and catalysts. The red colour of AuNPs in water reflects the surface plasmon (SP) band, a broad absorption band in the visible region. The SP band is due to the collective oscillations of the electron cloud at the surface of NPs that is correlated with the electromagnetic field of the incoming light (Liz-Marzan 2006). In the presence of the oscillating electromagnetic field of light, the free electrons of the NPs undergo an oscillation with respect to the metal lattice (Link and El-Sayed 2003; Jain, Lee et al. 2006). This process is resonant at a particular frequency of the light and is termed the localized surface plasmon resonance (SPR). These SPs enable NPs to absorb light, generate heat, transfer energy, and re-radiate incident photons (Lee and Lee 2010). Due to these unique properties, AuNPs are the subject of substantial research, with enormous applications including biological imaging, electronics, and materials science (Winter, Hager et al. 2011; Liu and Ye 2013). Moreover, metallic surfaces offer unique
opportunities to conjugate ligands such as oligonucleotides, proteins, drugs of interest and antibodies containing functional groups such as thiols, mercaptans, phosphines and amines (Alivisatos, Johnsson et al. 1996) opening a wide range of potential applications in biotechnology (Mody, Siwale et al. 2010).

For silver and gold NPs, proteins and other drugs can bind to the surface of the colloidal particles by one of three possible mechanisms. Two of these mechanisms, ionic and hydrophobic binding are relatively weak interactions that often result in the generation of poor-quality functionalizations. The third method involves the formation of a covalent bond between free sulfhydryl/thiols of the biomolecule and the atoms present on the surface of the particles. Dative bonds are very stable, possessing an energy equivalence of a covalent bond. These functional groups for covalent binding include NH$_2$, COOH and OH, which may be used to link drugs through well described cross linking chemistries such as NHS-based ester formations and EDC-based couplings (Sperling and Parak 2010).

Because of their unique physical properties and capabilities to function at the cell molecular level, metallic NPs have been studied as carriers for targeted drug delivery in therapeutics (Kohler, Sun et al. 2006; Sokolov, Tam et al. 2009). These NPs are promising for therapeutic applications because they can be targeted to a specific tissue through molecule functionalization, increasing the therapeutic index of drugs through site specificity, preventing multidrug resistance and delivering agents efficiently (Song, Zhou et al. 2012). The material properties of each NP system have been developed to enhance delivery to a specific site. Among these, metallic NP probes are emerging as a new class of contrast and tracking agents for different therapies. For example, the synthesis of functionalized SPR gold NPs have found applications in simple but powerful imaging techniques such as dark-field imaging, surface-enhanced Raman spectroscopy (SERS) and optical imaging for the diagnosis of various disease states (Huang, Jain et al. 2007). Moreover, several types of metal NPs are able to convert energy into heat up to 70°C through near-infrared light excitation.
or oscillating magnetic field stimulation (Reynoso, Lee et al. 2013) and also have the
potential to act simultaneously as therapeutic and diagnostic agents, in what is known
as emerging theranostic platforms based NPs (Yoo, Lee et al. 2011).

In the past decade, metal colloids have been the subject of attention as
reflected in a considerable increase in the number of books and reviews. AuNPs are
the focus of intense research for biomedical applications, for example for DNA
detection (Mirkin, Letsinger et al. 1996), to target cancer cells mediated by peptide
functionalization (Kang, Mackey et al. 2010) or deliver drugs like doxorubicin via folate
receptor (Asadishad 2010). Aurimune (CYT-6091) is an example of tumour necrosis
factor (TNF)-alpha bound to PEG-coated gold NPs (~27 nm) developed by CytImmune
Sciences, Inc. for solid tumour therapy (Paciotti, Myer et al. 2004). TNF-alpha is a
potent cytokine with antitumor cytotoxicity which requires incorporation into a
nanocarrier formulation to reduce systemic toxicity. The results show that NP
formulations delayed the tumour growth with local heating using a mammary tumour
xenograft mouse model. Furthermore, El Sayed et al. have established the use of gold
NPs for cancer imaging by selectively transporting AuNPs into the cancer cell nucleus
by conjugating arginine-glycine-aspartic acid peptide (known to target integrin
receptors on the cell surface) and a nuclear localization signal peptide which enables
the translocation to the nucleus to 30 nm AuNPs via PEG (Nakielny and Dreyfuss 1999;
Xue, Atakilit et al. 2001; Gao, Shi et al. 2005). These NPs enable cancer cell specific
targeting and the authors demonstrate the specific targeting to the cytoplasm and
nuclei of cancer cells over those of normal cells (Kang, Mackey et al. 2010).

Gold is one of the most used noble metals in biomedical science, but silver NPs
(AgNPs) have as much attention as gold because of their proved lack of toxicity and
anti-bacterial properties (Fernandez-Montesinos, Castillo et al. 2009). In addition,
AgNPs have unique optical, electrical and biological properties that have attracted
significant attention due to their potential use in many applications, such as catalysis,
biosensing, drug delivery and nanodevice fabrication (Menon, Jadeja et al. 2013). They
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have received considerable attention in biomedical imaging using SERS. In fact, the SP and large effective scattering cross-section of individual AgNPs make them ideal candidates for molecular labelling (Schultz, Smith et al. 2000). As mentioned before, capped AgNPs have many biomedical applications due to its excellent biocompatibility and antibacterial properties. It has been reported that AgNPs interact with virus, bacteria, and the immune system (Lara, Garza-Trevino et al. 2011). Due to their ability to prevent infections, there are several clinical trials in progress with AgNPs. Antibacterial hand gel and catheters made of AgNPs are being tested to compare their antimicrobial effects against conventional ones (Antonelli, De Pascale et al. 2012). In fact, Furno and Coworkers have developed biomaterials by impregnating silicone coated with silver oxide NPs (Furno, Morley et al. 2004). These novel biomaterials were developed with an aim to reduce the antibacterial infections and for the first time the methodology allows silver impregnation as opposed to coating of medical polymers. Even though these particles are not as widely preferred as compared to AuNPs, they have made a tremendous impact on medical science. The interesting property of the noble metals is that they will be continuously used as newer applications and more protocols are being developed.

1.2.1 Liposomes

Liposomes are vesicles containing phospholipid bilayers surrounding an aqueous core, whose diameters can vary from 20 nm to 1 μm. Research on liposome formulations has progressed from conventional vesicles to new generation liposomes. Specifically, the major advantage is that they can encapsulate both hydrophilic and hydrophobic molecules, in their core and membrane respectively (Lopez-Davila, Seifalian et al. 2012). Liposomes are characterized in terms of size, surface charge and number of bilayers. They have been used for more than 30 years as vehicles to improve the delivery of various drugs (Brenner 1989), such as anticancer drugs (doxorubicin), antibiotics (anthracycline, amphotericin B) or vaccines (Baldeschwieler 1997) (Table 1). It exhibits a number of advantages in terms of amphiphilic character,
biocompatibility and surface modification, being a suitable candidate delivery system for biotech drugs (Rawat, Singh et al. 2006). Hydrophobic molecules can be incorporated into the lipid bilayer of liposomes, while hydrophilic compounds can be entrapped in the aqueous core as shown in Figure 4, although it has been described that liposomes tend to be more unstable when loaded with hydrophobic molecules in comparison to hydrophilic ones (Khan, Rezler et al. 2008). In addition, other formulations such as cationic liposomes, temperature sensitive liposomes and virosomes have been developed by modulating the formulation techniques and liposome composition.

The preparation of liposomes are usually simple, rapid and reproducible (Elizondo, Moreno et al. 2011). The lipids are mixed in an organic solvent, which is then evaporated to form a thin lipid film. Hydration and agitation in an aqueous buffer produces a suspension of large multilamellar vesicles (MLV). These vesicles are too large and heterogeneous and their capacity to carry drugs is small to be useful. Therefore, to prepare small unilamellar vesicles (SUV) of sizes ranging from 50 to 200 nm, sonication or filter extrusion is performed. The membrane has two different phases. One, at low temperatures (between 0°C and the lipid transition temperature) considered as a gel phase and above the transition temperature, where the membrane undergoes a fluid phase. The difference between these two phases seems to be the way in which lipids are arranged in the membrane. Above the phase transition liposomes are more malleable, for this reason redispersion in buffer and extrusion should be both made in this phase.

Liposomes have been used as drug delivery systems in various therapeutic applications such as gene therapy, drug-targeting and many others, mainly because of the following advantages (Budai and Szogyi 2001):

1. They are biodegradable and non-toxic.
2. Most molecules can be incorporated into liposomes without modification at a very high concentration.
3. Biologically active molecules are protected by the lipid bilayer of liposomes from damage by chemicals and enzymes after injection into the bloodstream of an organism.

4. Healthy tissues are also protected from the toxicity of the entrapped molecules.

Conventional liposomes are composed of phospholipids and cholesterol. These liposomes are rapidly taken up either by the organs of the RES such as the spleen and liver or by phagocytic cells such as macrophages. Different liposomes have been developed to improve their stability in vivo (Cai, Wang et al. 2012; Sax and Kodama 2013). To prolong liposomes half-life in the body circulation liposomes are coated with PEG which reduces the interaction with plasma proteins or receptors. These liposomes are known as “stealth liposomes”.

Liposome drug loading can be achieved either passively (the drug is encapsulated during liposome formation) or actively (after liposome formation) (Kulkarni, Betageri et al. 1995). Hydrophobic drugs, for example amphotericin B or taxol, can be directly combined into liposomes during vesicle formation. In this case, the amount of uptake depends on drug-lipid interactions. For hydrophilic drugs, the loading depends on the ability of liposomes to trap aqueous buffer containing the dissolved drug during vesicle formation. Trapping effectiveness of hydrophilic drugs is generally less than 30% due to the limited trapped volume. On the other hand, water-soluble drugs that have protonizable amine functions can be actively entrapped by employing pH gradients, which can result in trapping effectiveness close to 100% (Gubernator 2011).

Liposomal formulations form one of the largest groups of clinically approved cancer drug carriers (Table 2) (Chang and Yeh 2012). The best known example is Doxil®, which is the PEG coated liposome formulation of the chemotherapeutic drug called doxorubicin. This drug works by intercalating DNA, with the most serious adverse effect being related to life-threatening heart damage. Doxil® has been
successful tested in clinical trials for several cancer types and has showed significant improvements in pharmacokinetics profiles, tumour accumulation and reduction in toxicity when compared to the free drug (Gabizon 2001). These results have been proved in a phase III clinical trial in patients with breast cancer, where Doxil® exhibited an equivalent efficacy to free drug. In addition, it showed a reduction of the most serious toxicity which is cardiotoxicity and the decrease of neutrophils, but had more skin and mucosal-related side effects (O’Brien, Wigler et al. 2004).

In addition to their capacity for incorporating several agents such as drugs, proteins, siRNA or nanoparticles, they have the ability to conjugate targeting molecules (active targeting). Therefore, these nanocarriers have the potential to be a therapeutic and diagnostic agent (theranostics) (Landen, Chavez-Reyes et al. 2005; Cabral, Nishiyama et al. 2011; Kang, Cho et al. 2011). Another novel approach is triggered drug delivery, where the drug is released after specific stimuli are applied to the target site. Although there are many difficulties encountered in designing a nanocarrier whose triggered release is realistic and effective enough, some of them are already in clinical trials, as is the case of Thermodox®. Thermodox® is a temperature-sensitive doxorubicin liposome that was designed for the treatment of breast and liver cancers and is currently under evaluation in Phase III clinical trials (Lammers, Kiessling et al. 2012).

Furthermore, other anticancer drugs besides doxorubicin have been encapsulated in liposomes such as daunorubicin or paclitaxel. In the case of daunorubicin, which is an anticancer drug used for leukemia and a wide variety of solid tumour treatments, the liposomal formulation is called DaunoXome®. These vesicles are composed of DSPC and cholesterol and are small in size (45nm). They are currently under phase III trial and some results have elucidated higher tumour uptake when measured against free drug (Forssen, Coulter et al. 1992; Gill, Espina et al. 1995; Gill, Wernz et al. 1996). Taxol® (paclitaxel) is a marketed product for the treatment of ovarian, breast, non-small cell lung cancer, and AIDS-related Kaposi’s sarcoma
(Mekhail and Markman 2002). The liposomal formulation of paclitaxel is being developed to potentially reduce toxicities associated with Taxol by eliminating the drug formulation component polyoxyethylated castor oil and it is named LEP-ETU (Zhang, Anyarambhatla et al. 2005).

Liposomal formulations have been used for other applications besides cancer treatments, for example in vaccination protocols. The incorporation of viral membrane proteins or peptide antigens into liposomes has shown to potentiate cell-mediated and humoral immune responses, generating solid and durable immunity against the pathogen. These vaccines are reconstituted viral liposomes, constructed without the genetic information of the virus which makes them unable to replicate and cause infection (Stegmann, Morselt et al. 1987; Gluck, Mischler et al. 1992). Some examples of these vaccines are Epaxal and Inflexal V, hepatitis A virus and influenza virus vaccines respectively (Usonis, Bakasenas et al. 2003; Kanra, Marchisio et al. 2004). In this context, studies have revealed so far that virsomal techniques may not be able to give a superior protective immunity in clinic but play an important role in the prevention of morbidity and lethality associated with vaccines themselves.
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Table 2. Liposomal drug formulations in the market.  
( Based on Hsin-I Chang et al. 2012)

References in Section V.2 (Table References)

To date only passive targeted nanocarriers have been approved although it has been demonstrated widely in vitro that actively targeted nanocarriers show higher internalisation and as a result increased cytotoxicity of cancer cells (Lee, Lee et al. 2008; Li, Ding et al. 2009). Increasing the specificity of cancer treatment leads to a reduction in drug toxicity and its consequent side effects and as a result improves the therapeutic outcomes (Allen, Mumbengegwi et al. 2005). As an example of active targeting nanocarriers, liposomes encapsulating doxorubicin and conjugated to the
F(ab)2 fragment of the human antibody GAH, directed against metastatic stomach cancer is in early phases of clinical trials (Matsumura, Gotoh et al. 2004).

2. Vasoactive Intestinal Peptide (VIP)

Among the neuropeptides, the 28-amino acid vasoactive intestinal peptide (VIP) is a molecule that has evolved from being considered a mere neuropeptide/hormone into a novel agent for modifying immune function and, possibly as a cytokine-like molecule (Pozo and Delgado 2004). Originally identified by Said and Mutt in the late 60’s, VIP was originally isolated as a vasodilator and hypotensive peptide (Piper, Said et al. 1970; Said and Mutt 1970). Subsequently, its biochemistry was elucidated and within the first decade its signature features as a neuropeptide/neurotransmitter became consolidated: it is currently known to act as a neuromodulator in many organs and tissues, including heart, lung, thyroid gland, kidney, immune system, urinary tract and genital organs (Henning and Sawmiller 2001). Sustained interest in therapeutic applications of VIP include areas related to neuroprotection (Gozes, Bachar et al. 1998; Gozes and Furman 2003; Gozes 2008), inflammation and autoimmune disorders (Abad, Martinez et al. 2003; Keino, Kezuka et al. 2004; Gonzalez-Rey, Chorny et al. 2007; Hamidi, Prabhakar et al. 2008; Pozo, Anderson et al. 2009), or asthma (Onoue, Yamada et al. 2007; Said, Hamidi et al. 2010).

The amino acid sequence of VIP contains homologies with many gastrointestinal hormones such as pituitary adenylate cyclase-activating peptide (PACAP) and shares some of its receptors (Miyata, Arimura et al. 1989; Miyata, Jiang et al. 1990). In agreement with its widespread distribution, VIP is involved in many physiological and pathophysiological processes related to development, growth, cancers, immune responses, circadian rhythms, control of neuronal and endocrine cells and functions of the digestive, respiratory, reproductive and cardiovascular systems. The VIP peptide is remarkably well conserved across species and is identical in human, cow, pig, rat, dog,
and goat (Mutt 1988). Even across species, amino acid substitutions are conservative and usually do not result in changes in bioactivity (Singh, Jagannathan et al. 2013).

Two heterotrimeric G protein-coupled receptors mediate the actions of VIP, named by VPAC1 and VPAC2, according to the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR), each of them with a unique expression pattern (Harmar, Arimura et al. 1998). VIP and PACAP share receptors VPAC1 and VPAC2, but PAC1 receptor is specific for another peptide called PACAP (Harmar, Fahrenkrug et al. 2012). Each of these receptors is coupled primarily to G proteins, and activate adenylyl cyclase and protein kinase A (PKA), but other pathways are often activated or inhibited in some cells in parallel or downstream of cyclic adenosine monophosphate (cAMP), including pathways involving exchange proteins activated by cAMP (EPACs) (Ster, De Bock et al. 2007), nitric oxide (Murthy, Zhang et al. 1993), phospholipase C (Spengler, Waerber et al. 1993), phosphatidylinositol 3-kinase (Straub and Sharp 1996), MAP kinases (Barrie, Clohessy et al. 1997; Lelievre, Pineau et al. 1998), Jak/STAT, and NFkB (Delgado and Ganea 2000).

The physical sites of interaction between VIP and its VPAC1 receptor remained elusive until the development of extensive photoaffinity experiments showing that the side chains of VIP in position 6, 22, 24 and 28 are in direct contact with different amino acids of the receptor VPAC1 N-ted (Tan, Couvineau et al. 2003; Tan, Couvineau et al. 2004; Ceraudo, Murail et al. 2008). Elucidation of VIP structure by nuclear magnetic resonance (NMR) revealed that most of the 28 amino acid sequence has an α-helical structure (sequence 7–28) with the exception of the N-terminal 1–5 sequence, which has no defined structure in solution when unbound to the receptor (Figure 6). The development of a structural model of the VPAC1 receptor N-ted has made it possible to localize the binding site of VIP. The N-ted structure contains two antiparallel β sheets and is stabilized by three disulphide bonds between residues Cys50 and Cys72, Cys63 and Cys105 and Cys86 (Figure 6). This finding is in good agreement with a
speculative, but largely accepted, mechanism for peptide–ligand interaction with class B GPCRs, which is referred to a ‘two-site’ binding model (Hoare 2005). In this model the central and C-terminal α-helical segments of the ligand are trapped by the N-ted domain of the receptor. In this respect, the integrity of the α-helical conformation seems crucial for the binding to their receptors. Although numerous structure and activity studies have elucidated the molecular interactions between VIP and their receptors, the determination of the structure of VIP receptors is still a challenge and more information will provide and facilitate the emergence of new potent drugs.

Figure 6. VIP molecule interaction with VPAC1 receptor (Couvineau and Laburthe 2012).

The N-ted structure of VPAC1 contains two antiparallel β sheets and is stabilized by three disulphide bonds. The central and C-terminal α-helical segments of VIP are trapped by the N-ted domain of the receptor.

2.2 Functions of VIP in the central nervous system

VIP and its receptors are expressed in a wide variety of brain regions. The widespread distribution of VIP, PACAP and their receptors in both, the central nervous system (CNS) and peripheral nervous system (PNS) suggests an important role in the
nervous system. This fact has led to many hypotheses concerning the physiological functions of these receptors. Although VIP was initially classified as a gut hormone, many studies have demonstrated its activity as a neurotransmitter in the CNS and PNS. Moreover, VIP is expressed throughout different brain regions, with the highest expression levels found in the cerebral cortex, hippocampus, amygdala and hypothalamus (Dickson and Finlayson 2009). It has been shown to enhance glycogen metabolism in the cerebral cortex, regulate embryonic growth and promote neuronal survival (Sorg and Magistretti 1992).

Regarding VIP-receptors, different studies have suggested a role for VPAC1 in learning and memory processes, while VPAC2 and PAC1 seem to be involved in generating normal circadian rhythms and behavioural activities (Harmar, Marston et al. 2002; Aton, Colwell et al. 2005). The recent use of receptor and peptide knockout animals has contributed to important insights into their importance in a number of areas. These including their roles in metabolism, obesity, control of insulin release, various gastrointestinal disorders and in CNS, particularly in regard to roles in regulation of circadian rhythm, neuroprotection and more recently, certain possible human disorders such as schizophrenia and depression (Morell, Souza-Moreira et al. 2012). Due to its potent action on the CNS, VIP and its receptors could be promising therapeutic targets for the treatment of various neurological disorders.

The availability of mutant mice lacking VIP (Colwell, Michel et al. 2003), PACAP (Kawaguchi, Tanaka et al. 2003; Colwell, Michel et al. 2004) and the receptors VPAC1 (Fabricius, Karacay et al. 2011), VPAC2 (Asnicar, Koster et al. 2002) and PAC1 (Hannibal, Jamen et al. 2001; Otto, Martin et al. 2001) has allowed experimental validation of a number of physiological functions for these receptors. For example, VIP and VPAC2 are known to play a role in the control of circadian rhythms in the hypothalamus (Fahrenkrug, Georg et al. 2012; Lucassen, van Diepen et al. 2012). VIP is synthesized in a population of CSN neurones, many of which are thought to receive a direct retinal innervation, and acts on VPAC2 receptors, which are expressed
throughout the CNS. Light entrains the CNS clock through a population of retinal ganglion cells that project to the CNS via the retino-hypothalamic tract and contain both glutamate and PACAP. In addition, mice lacking VIP do not exhibit the daily rise in circulating corticosterone in response to light and have deficiencies in circadian behaviours, such as poor motor rhythmicity in the dark and improper responses to light pulses (Loh, Abad et al. 2008; Dragich, Loh et al. 2010). Moreover, studies of knockout mice lacking the VPAC2 receptor indicate that this receptor is necessary for the generation of normal circadian rhythms of electrical activity, clock gene expression, physiology and behaviour (Harmar, Marston et al. 2002; Cutler, Haraura et al. 2003; Hannibal, Hsiung et al. 2011). VIP-deficient mice and VPAC2 receptor null mice display a severely disrupted circadian phenotype, sharing many common features (Colwell, Michel et al. 2003; Aton, Colwell et al. 2005).

VIP is also thought to play a role in neurodevelopment and in neuroprotection following injury to the CNS. For example, VIP has been shown to be protective against excitotoxin-induced white matter lesions in neonatal mice (Gressens, Marret et al. 1997; Rangon, Goursaud et al. 2005), probably acting through VPAC2 receptors. VPAC2 receptors have also been implicated in the control of astrocyte proliferation (Zupan, Hill et al. 1998). VPAC2 receptors have also been implicated in the VIP-induced expression of the neuroprotective protein activity-dependent neuroprotective protein (ADNP) in astrocytes (Zusev and Gozes 2004) and davunetide, an active fragment of ADNP, is in clinical development for the treatment of neurodegenerative disorders (Gozes 2011). In studies of post-natal hippocampus in vitro, VPAC2 receptor activation was found to expand the pool of neural stem/progenitor cells by preventing either a neuronal or glial fate choice and by supporting their survival, whereas selective VPAC1 receptor activation promoted a neurogenic granule cell fate (Zaben, Sheward et al. 2009). Two recent publications from independent groups have found associations between copy number variation in the gene encoding the VPAC2 receptor and susceptibility to schizophrenia (Levinson, Duan et al. 2011; Piggins 2011). These findings have generated some excitement in the field because they may imply that the
VPAC2 receptor is a potential target for the development of new antipsychotic drugs. VIP has been also described to have a role in other major neurological disorders, as is the case Alzheimer’s disease (AD).

VIP has been implicated in the treatment of this disease due to its neuroprotective properties. The neuronal loss associated with AD results in gross atrophy of affected regions within the temporal and parietal lobes. Functionally, this disease affects primarily the hippocampus and neocortex, causing progressive loss in cognitive ability and memory function, ultimately leading to dementia (Wenk 2003; Delgado, Varela et al. 2008). Regarding this disorder, Delgado et al have shown that VIP could effectively limit harmful Aβ-induced microglia activation and the subsequent release of neurotoxins, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and nitric oxide (NO) (Delgado, Varela et al. 2008). Here, VIP prevented the neuronal cell death that leads to AD pathology in the brain. It has been postulated that the protective effect of VIP occurs upon activation of the VPAC1 receptor by initiating the cAMP/PKA signalling pathway. This activation stimulates neuroprotective glial proteins, such as ADNP and also impairs the nuclear translocation and DNA binding of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) by inhibiting upstream regulatory kinases (Delgado, Varela et al. 2008).

Furthermore, VIP has been shown to protect against neuronal cell death and act as a modulator of the inflammatory immune response. For this reason, it has been implicated as a viable treatment option for Parkinson’s disease (PD). PD is a common neurodegenerative disease of the CNS which primarily impairs an individual’s motor skills and speech. It has been demonstrated by Delgado et al (Delgado and Ganea 2003) that VIP could protect dopaminergic cells from bacterial endotoxin LPS-induced inflammation in mouse embryonic neurons. LPS treatment results in neuronal cell loss and is thought to be mediated by increased microglial activation. Therefore, VIP most likely prevents neurodegeneration by deactivating microglia and the production of proinflammatory mediators via binding to VPAC1. In another study using a mouse model
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of PD, VIP treatment showed a decrease in dopaminergic neuronal loss in the substantia nigra. These experiments demonstrated that the therapeutic abilities of the peptide were performed once again through VPAC1-mediated microglia deactivation and the subsequent production of cytotoxic mediators (Delgado and Ganea 2003).

These findings have shown that VIP is a major neuroprotective factor by inhibiting microglia-derived proinflammatory factors. Therefore, this peptide presents good potential as a therapeutic agent for neurodegenerative disorders of the CNS that affect so many people worldwide.

2.3 Functions of VIP in the immune system

In the last decade, VIP has been clearly identified as a potent anti-inflammatory factor, which acts by regulating the production of both anti- and pro-inflammatory mediators (Gonzalez-Rey, Anderson et al. 2007). From an immunological point of view, VIP has certain characteristics that make it attractive for the control of the mechanisms associated to immune tolerance. In first place, VIP is produced by immune cells, mainly Th2 cells and type 2 CD8 T cells, under the control of inflammatory stress conditions, or following antigenic stimulation in several experimental scenarios (Delgado and Ganea 2001; Delgado, Abad et al. 2002; Delgado, Pozo et al. 2004). Second, VIP exerts its biological actions through various G-protein-coupled receptors (VPAC1, VPAC2 and PAC1), which are expressed on various immune cells and last, VIP signalling involves the activation of the cAMP/protein kinase A (PKA) pathway that is considered a general immunosuppressive signal (Banner and Trevethick 2004). In addition, VIP regulates the expression of co-stimulatory molecules, an action that may be related to the VIP-mediated shift toward Th1 and Th2 differentiation (Delgado, Abad et al. 2002). In this sense, it has recently been reported that VIP prevents the deleterious effects of an experimental model of rheumatoid arthritis, by down-regulating both inflammatory and autoimmune components of the disease.
Numerous evidences demonstrate that VIP is a potent anti-inflammatory agent *in vitro* and *in vivo* that acts at different levels (Delgado, Abad et al. 2002). VIP inhibits phagocytic activity, free radical production, adherence and migration of macrophages (De la Fuente, Delgado et al. 1996) and reduces the production of inflammatory cytokines (TNF-α, IL12, IL6 and IL1b) and various chemokines (Delgado, Pozo et al. 2004). It stimulates the production of anti-inflammatory cytokines such as IL10 and IL1Ra and down-regulates the expression of inducible NO synthase and the subsequent release of NO by macrophages, dendritic cells and microglia (Delgado, Pozo et al. 2004). In addition, VIP inhibits the degranulation of mast cells and reduces the expression of toll-like receptors and associated molecules, for example in human rheumatoid synovial fibroblasts (Gutierrez-Canas, Juarranz et al. 2006) or in inflammatory bowel disease (Gomariz, Arranz et al. 2005).

In relation to key events at the interface between the innate and acquire immune responses, VIP decreases the co-stimulatory activity of s for antigen-specific T cells by down-regulating the expression of the co-stimulatory molecules CD80 and CD86 (Delgado, Reduta et al. 2004). The definitive establishment of VIP as a natural anti-inflammatory factor has been supported by two recent works reporting that mice that lack VIP or the PAC1 receptor show higher systemic inflammatory responses and are more susceptible to die by septic shock (Martinez, Abad et al. 2002; Szema, Hamidi et al. 2006).

Other authors have investigated the influence of VIP not only on inflammatory cells but also on regulatory T cells (Treg cells), known as suppressor T cells which modulate the immune system and maintain tolerance to self-antigens (Schmidt-Weber and Blaser 2004). Szema et al. explored the development of Treg cells in VIP knockout mice, particularly in the thymus and spleen. They observed a tendency towards deficiency of Treg cells in the spleen, which was normalized by VIP treatment and supported the concept that exogenous VIP is able to induce Tregs (Szema, Hamidi et al. 2011). In relation to this study Delgado et al. administered VIP together with a
specific antigen to T cell receptor-transgenic mice and resulted in the expansion of Treg cells, which inhibit T cell proliferation through direct cellular contact (Delgado, Chorny et al. 2005).

VIP restores tolerance in autoimmune disorders by acting at multiple levels. Loss of immune tolerance compromises immune homeostasis and results in the onset of autoimmune disorders (Gonzalez-Rey, Anderson et al. 2007). The capacity of VIP to regulate a wide spectrum of inflammatory factors and to switch the Th1/Th2 balance in favour of Th2 immunity makes it an attractive therapeutic candidate for the treatment of inflammatory disorders and/or Th1-type autoimmune diseases (Gonzalez-Rey, Anderson et al. 2007).

2.4 VIP relevance to cancer

In recent years, there has been a great interest in the research and development of peptide functionalized nanoparticles for diagnostic imaging and cancer therapy (Delehanty, Boeneman et al. 2010). This emergence is attributable to the higher expression of hormone receptors in many tumour cells than in normal tissues (Figure 7), for example GPCRs. (Reubi, Laderach et al. 2000; Reubi 2003). In particular, this phenomenon is of high relevance in the case of VIP receptors. The targeted delivery of therapeutic agents to tumour cells is a challenge because most of the chemotherapeutic agents distribute to the whole body, which results in general toxicity and poor tolerance by patients. Recent advances have opened the way to site-specific targeting and drug delivery by NPs (Brannon-Peppas and Blanchette 2004).

Based on this high occurrence of tumour VIP receptors, a number of potential clinical applications have been evaluated. First, it has been demonstrated that selected tumours, in particular, the VIP receptor-positive cancers, can be visualized in the patient by means of in vitro and in vivo VIP receptor scintigraphy (Virgolini, Raderer et al. 1994; Reubi, Waser et al. 1999). For example, Raderer et al developed scintigraphy
by using $[123I]VIP$ as a radioligand and compared the results with computerized tomography (CT). In this study the VIP scan indicated the presence of disease before CT in four patients (Raderer, Kurtaran et al. 1998). Moreover, several studies have reported an effect of VIP and PACAP analogues on tumour growth in animal tumour models, mediated by specific receptors (Moody, Zia et al. 1993; Maruno, Absood et al. 1998). Therefore, VIP and the related peptide PACAP may be of great potential importance for oncology.

Recently, a high incidence of PAC1 was found in human gliomas, neuroblastomas, and pituitary adenomas (Robberecht, Vertongen et al. 1993; Robberecht, Woussen-Colle et al. 1994; Vertongen, Devalck et al. 1996), whereas VPAC1 was identified in pancreatic, colorectal, prostate and breast cancers (Jiang, Kopras et al. 1997; Reubi, Laderach et al. 2000).

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>VIP Receptor incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>100%</td>
</tr>
<tr>
<td>Prostate</td>
<td>100%</td>
</tr>
<tr>
<td>Bladder</td>
<td>100%</td>
</tr>
<tr>
<td>Colon</td>
<td>96%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>65%</td>
</tr>
<tr>
<td>Lung</td>
<td>58%</td>
</tr>
<tr>
<td>Stomach</td>
<td>54%</td>
</tr>
<tr>
<td>Liver</td>
<td>49%</td>
</tr>
</tbody>
</table>

Figure 7. Tumour targeting approach by VIP-NPs and the incidence of VIP-receptors in different human tumours.
In particular, human prostate carcinomas have been analysed in order to better understand the differences between human normal and malignant prostate tissue. A study from Collado et al. (Collado, Carmena et al. 2005) showed that the level of expression of VIP receptors was about two times higher in adenocarcinoma samples than in normal tissue, measured by a polymerase chain reaction (PCR) method and by enzyme immune-analysis. In addition, Garcia-Fernandez et al detected VPAC1, VPAC2 and PAC1 proteins in solubilized human normal and tumoral prostate membranes by immunoblot and analyzed by immunohistochemistry the presence of VPAC1, VPAC2 and PAC1 (Garcia-Fernandez, Solano et al. 2003). The receptors were mainly located in the epithelial layer of prostate glands and also the presence of PAC1 was observed in some dispersed cells of the stroma (Garcia-Fernandez, Solano et al. 2003). The functionality of these receptors were also confirmed in both, normal and tumoral tissues by VIP stimulation of adenylyl cyclase activity. Moreover, Reubi performed radio-labelled binding experiments in prostate tumours with 125I-VIP and showed 2.5 times more VIP binding sites during malignant transformation (Reubi 1996). Prostate cancer is the most commonly diagnosed cancer and second most common cause of cancer death 1,2. There is currently no consensus on the optimal management of high-risk prostate cancer because there are different primary modalities available, such as surgery or radiation. In addition there is unstandardized timing of different therapies which makes comparisons of efficacy problematic. Increased understanding into the mechanisms leading to the formation of advanced metastatic disease has increased the development of agents to target these pathways. The early diagnosis of prostate cancer is not reliable in all cases and usually men with localized cancer have few or no symptoms at all. The most common test for prostate cancer screening is Prostate Cancer Antigen blood (PSA) test, which measures abnormal levels of PSA in blood. However, there are additional reasons for having an elevated PSA level, and some men

1 http://health.nih.gov/search_results.aspx?terms=Prostate+Cancer

2 http://www.who.int/gho/ncd/mortality_morbidity/cancer_text/en/
who have prostate cancer do not have elevated PSA. In addition, the potential complications and harmful side effects of treatments for early prostate cancer, such as surgery and radiation therapy include urinary, problems with bowel function, erectile dysfunction and infection. For these reasons other alternatives are being studied and other diagnoses methods are being evaluated.

The studies mentioned above support the notion that this family of receptors are highly expressed in cancerous tissues and that they are maintained in a functional condition during malignant transformation in the human prostate gland. Therefore, they could be considered as an option for effective diagnosis and targeted drug delivery.

2.5 VIP as a therapeutic agent

Small peptides are valuable tools for clinical applications as they offer many distinct advantages over other bioactive molecules like proteins and monoclonal antibodies (Okarvi 2008). Small peptides can be easily synthesized and manipulated to optimize their affinity for a particular receptor and to display a more specific biodistribution pattern. Moreover, peptides have the ability to tolerate the harsh conditions of chemical modifications and/or radiolabeling (Heppeler, Froidevaux et al. 2000). Despite the advantages, the difficulty with peptides is often their short biologic half-life, because of their rapid proteolysis in plasma by endogenous peptidases and proteases. A few years ago, from a therapeutic perspective, VIP was identified as a potential bioactive agent for various diseases as mentioned in sections 2.1, 2.2 and 2.3. However, the effective translation of preclinical studies related to VIP to clinical realities faces several major challenges, most of them are common place for other neuropeptides. Thus, one of the major issues for the use of VIP as a therapeutic agent is that once it has been released into the body it is quickly degraded by enzymes,


leaving the peptide with a very short half-life. In recent years, many studies have been made to elucidate VIP’s beneficial effects, but the native peptide has poor metabolic stability and therefore reduced biological activity (Chapter, White et al. 2010). The VPAC receptors, in particular VPAC1, are very promising targets for the development of therapeutic molecules, but first VIP obstacles have to be solved. While new peptide derivatives specifically targeting VPAC receptor subtypes are now available, however, their short half-life and the inconvenience related to their administration routes make them difficult to use in human therapy.

Protein degradation by endogenous proteases of peptides leads to limited bioavailability. This remains as one of the key issues in drug discovery related programs, without a general solution up to date. To tackle these limitations, two main approaches are under way. A first strategy involves the use of covalent and non-covalent reversible inhibitors of specific proteases. A representative example is the case of inhibitors of dipeptidyl peptidase IV (CD26) that inactivates incretin neuropeptides aimed at the treatment of type 2 diabetes or cancer progress (Karagiannis, Paschos et al. 2012; Nisal, Kela et al. 2012). Unfortunately, although in advanced phase clinical trials, this approach can show safety issues (kidney impairment, immune defects or skin lesions) or limited efficacy which result in contradictory approvals from US or EU regulatory agencies (Drucker, Sherman et al. 2011; Monami, Dicembrini et al. 2011). These precautions regarding long-term treatments with specific proteases inhibitors are reflecting the increasing and complex world of the protease degradome where a given protease is often involved in different key physiological processes increasing the chances of adverse effects (Quesada, Ordonez et al. 2009; Turk, Turk du et al. 2012). The second strategy involves the development of small, non-peptide mimic ligands, but since endogenous ligands are often large molecules (20-40 amino acid residues or more) with large corresponding binding pockets within the receptor and diffuse pharmacophoric domains, the resulting mimic molecules often have reduced affinity and selectivity compared to
their endogenous ligand counterparts (Hoyer and Bartfai 2012). Therefore, neuropeptide applications are still in need of alternative solutions to reduce protein degradation without biological assets impairment. It is to be hoped that recent advances of our knowledge of the structure of the VPAC receptor binding site and more generally of the class B GPCR ligand requirements will lead shortly to the design of non-peptide receptor agonists and/or antagonists. So far, the development of VIP agonists still has to overcome serious limitations, such as poor stability and short duration of action due to kidney clearance, resulting in high dose administration (Gozes, Lilling et al. 1996). For example, BAY 55-9837, a VPAC-2 selective agonist had several limitations with respect to its degradation and low efficacy (Pan, Li et al. 2007). Other alternatives have been studied such as BAY Q9Q28 showing better stability and high affinity to the receptor (Pan, Li et al. 2007). Furthermore, covalent attachment of long chain PEG molecules is another method used to increase the life span of a peptide by increasing its overall stability and solubility. Most importantly, the sustained plasma concentration of PEGylated peptides can reduce the side effects by reducing the levels of the drug. However, success in peptide PEGylation has been limited by the propensity of the large polymers to interfere with peptide function. In this context, nanotechnological approaches could also be another option. VIP functionalization to NPs could increase their half-life, increasing its efficacy and decreasing the dose needed for treatments. Such molecules would be of considerable interest in the therapy of human diseases, in particular inflammatory, neurodegenerative and cancer-related conditions. For the clinical application of VIP, studies aimed at developing an appropriate delivery system of VIP will be a key direction of future research, and the combination of VIP with nanoparticle features might provide novel insight into the therapeutic potential of VIP and its functionalization to nanoparticles.
INTRODUCTION

3. VIP-Engineered Nanoparticles

3.1 Rationale for VIP applications

Many efforts have been made to obtain highly potent VIP analogues (Igarashi, Ito et al. 2005) focusing on the improvement of stability to create drug candidates for the treatment of several diseases including asthma (Bolin, Michalewsky et al. 1995), neurodegenerative diseases (Gozes 2008), impotence (Gozes, Reshef et al. 1994), septic shock (Delgado and Ganea 2001; Lv, Tang et al. 2009), diabetes (Yung, Dela Cruz et al. 2003) or as a tumour imaging agent (Kothari, Prasad et al. 2007). However, none of these analogues has yet reached the clinical stage. Different VIP ligand modifications have been performed in order to affect the binding to the cognate receptors (VPAC1 and VPAC2). Tandem extensions and additional branching methodologies have been carried out on the C-terminal domain, in an attempt to amplify VPAC1 binding. However, these alterations at the C-terminus showed no significant difference in the activation of the VPAC1 receptor to cAMP production when compared to unmodified VIP (Dangoor, Rubinraut et al. 2007). Nonetheless, manipulations at the C-terminal region of VIP have been shown to affect discrimination between VPAC1 and VPAC2 receptors (Caraglia, Carteni et al. 2008). While modifications of the N-terminus have shown increased affinity for the VPAC2 receptor (Langer, Gregoire et al. 2004). Major drawbacks include internal degradation, as well as a difficult balanced equilibrium between potential side effects and low availability of the peptide at the disease site when systemic peptide doses have to be necessarily increased. In this sense, peptide encapsulation and/or functionalization using NPs are important applications that could solve the limitation of the therapeutic use of peptides owing to their short half-lives caused by enzymatic degradation, catalytic antibodies, and spontaneous hydrolysis in biological fluids.

The fact that VIP is so attractive for therapeutic use by itself leads to the study of nano-applications such as a peptide delivery system which may solve the problem of drug break-down by digestive acids and enzymes before they reach their targets.
The half-life of VIP needs to be substantially prolonged in biological fluids to be employed in therapeutics with increased effectiveness, as VIP-based drug design is hampered by the instability of the peptide and has limited bioavailability. Therefore, NP encapsulation protects the peptide contained from being broken down too early and has a slow release mechanism which allows a gradual delivery of its contents.

It can be predicted that the future of drug delivery involves smart systems which maintain the drug at a desired therapeutic level in the body and avoid the need of frequent administration. Additionally, it would be desirable to use a NP that acts only on a unique receptor or biological site of interest with the possibility of being loaded with a drug at different concentrations. For this reason, VIP has been studied as a method of transport to target cells. A variety of primary human tumours such as breast, prostate, urinary bladder, colon, pancreas and lung cancer, among others, express large numbers of high-affinity receptors for VIP (Reubi 1996; Reubi, Laderach et al. 2000) which may represent the molecular basis for NP applications in cancer. One of the main reasons for the use of peptides and peptide receptors in cancer is the possibility of its targeting. VIP could actively target different cancer cells leading to the delivery of drug compounds or imaging agents to cancer cells that overexpress VIP receptors (Reubi 2003). Moreover, the VIP-NPs-mediate high sensitivity and specificity to detect cancer cells offer the potential for early diagnosis and treatment. This is important as the earlier the diagnosis, the less the cost of patient care, and what is the most important, the higher the chances of treatment success that is normally lower in disease at an advanced stage.
Figure 8. Flow-chart depicting the VIP-based approach for targeted cancer. Tumours leaky vasculature is commonly used for NP passive targeting, whereas VIP-NPs can use this approach and in addition take advantage of the effects of active targeting to cells that overexpress VIP receptors and as a result increase the specificity of treatments.

The rationale behind VIP-functionalized NPs is the fact that the active targeting has a higher specificity for the tumour cells, which results as an enhanced therapeutic effect when compared to the passive targeting. Moreover, the NPs reach the tumour by the leaky vasculature as well as the passive targeting due to their size, but have more affinity for the cells.
Receptor scintigraphy using radiolabeled peptides for the localization of tumours and their metastases as well as for radiotherapy is used with a clinical impact at the diagnostic and therapeutic level which emerges as an important treatment option. Therefore, VIP-functionalized NPs can be used as a drug delivery systems to a specific cancer tissues well as for in vivo cell labelling and image acquisition. For example, it has been reported that NPs, such as superparamagnetic iron oxide NPs, could be an option as contrast agents for targeted magnetic resonance imaging, which offers a high potential for diagnosis (Schlorf, Meincke et al. 2010; Liao, Wang et al. 2011).

As a summary, two main applications can be developed with VIP engineered NPs which will be described in more detail in the next section:

1. VIP encapsulated in NPs or attached to its surface in order to act as a drug itself for therapeutic applications.

2. VIP functionalised NPs as a targeting agent to transport drugs to a specific tissue site.

3.2 Types of VIP Engineered Nanoparticles

3.2.1 VIP as a drug for therapeutic applications.

Drug delivery of protein and peptide-based drugs, which represent a growing and important therapeutic class, is hampered by these drugs' very short half-lives. High susceptibility towards enzymatic degradation necessitates frequent drug administration followed by poor adherence to therapy. Furthermore, from a quality control and safety point of view, avoidance of degradation and structural transition during manufacturing and/or long-term storage is also considered necessary for developing peptide/proteins-loaded formulation systems. Drug delivery plays a crucial role in the improvement of therapeutic agents since many drugs have unfavourable drawbacks if applied directly. As well as the chemical and metabolic issues, there is a possible drawback that the systemic administration of VIP or other VIP receptor agonists may cause moderate hypotension and/or other adverse effects depending on
the rate of infusion (Morice, Unwin et al. 1983). Therefore, developing NPs which can work as carriers for a controlled delivery of a peptide is a promising option to improve its availability by reducing its side effects and enhancing its efficacy. Several studies have been developed to establish whether the encapsulation of VIP into NPs enhances its effects or whether its attachment to a surface leads to an appropriate NP transport.

Different NPs have been used to encapsulate VIP, such as liposomes (Gao, Noda et al. 1994; Stark, Andreae et al. 2008), biodegradable protamine oligonucleotide NPs (Wernig, Griesbacher et al. 2008) or poly (ethylene glycol)-poly (lactic acid) NPs (Gao, Wu et al. 2007). Depending on the location where the peptide needs to be delivered, the nature of the NP and/or surface ligands are different. Liposomes are widely used for drug delivery due to their unique properties (Lasic 1998). They are biodegradable, typically made from natural molecules and non-immunogenic, which makes them even more attractive in view of their ability to encapsulate a variety of molecules, including VIP. Stark and co-workers demonstrated that the encapsulation of VIP into liposomes protects the peptide from proteolytic degradation while maintaining its biological activity (Stark, Andreae et al. 2008). Furthermore it has been reported that VIP liposome encapsulation enhances its vasoactive effects on systemic arterial blood pressure (Gao, Noda et al. 1994) while another study showed the delayed release of VIP when administered into liposomes within hyaluronic acid gel for uveitis (Lajavardi, Camelo et al. 2009). However, although liposomes can be highly effective as VIP carriers in some cases, this is not the case of VIP transport to the brain. For this reason, other options have been engineered, such as glucose targeted niosomes-non-ionic surfactant-based liposomes- allowing an efficient delivery of intact VIP to the brain by crossing the bloodbrain barrier following intravenous administration (Dufes, Gaillard et al. 2004) or poly (ethyleneglycol)-poly (lactic acid) nanoparticles modified with wheat germ agglutinin that enhanced VIP transport to the brain by intranasal administration(Gao, Wu et al. 2007). The aim of this study was to investigate the potential of this VIP formulation as a possible delivery system to the brain for the
treatments of neurological diseases. A lipophilic VIP analogue (stearyl-norleucine\textsuperscript{17}) VIP has been proposed for such therapeutic applications (Gozes, Bardea et al. 1996). However, like most endogenic peptides, its potential therapeutic applications are limited by its susceptibility to endopeptidases and poor passage across the blood-brain barrier (McCulloch and Edvinsson 1980; Dogrukol-Ak, Banks et al. 2003).

Other biodegradable NPs have been used as a drug delivery system to enhance protection against proteolytic cleavage as well as cellular uptake; this is the case of protamine-oligonucleotide NPs, which achieve an appropriate binding efficiency of VIP as well as nanoparticle stability (Wernig, Griesbacher et al. 2008). Here, VIP encapsulation occurs during self-assembly of the components and the pharmacological VIP response of encapsulated VIP is investigated using an ex vivo lung arterial model system. The authors achieved high encapsulation efficiency (up to 80%) and a modified VIP response on pulmonary arteries, noting differences in the profile of artery relaxation followed by prolonged vasodilation when compared to aqueous VIP.

In addition, VIP poly(lactide-co-glycolide) (PLGA) nanospheres have been formulated for the treatment of airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease due to its potent bronchodilating and anti-inflammatory effects (Onoue, Matsui et al. 2012). Satomi Onoue et al developed a respirable sustained-release formulation and studied the peptide stability during the formulation process. The rationale for the development of this formulation was to maximize drug concentrations, in this case VIP in the airway systems, while minimize systemic exposure and associated toxicity. Their results show that they could attenuate antigen-evoked inflammatory symptoms and neutrophilia in the respiratory system.

As we have seen, the use of biodegradable NPs for encapsulating VIP offers the advantage of sustained release through its diffusion into the NP matrix and subsequent degradation. This permits a higher or lower release depending on the
composition of the NP with the possibility of designing specific nano-systems according to the treatment and the therapeutic agent. The advantage of VIP encapsulation relies on the facts of peptide protection and sustained release, thus maintaining its activity and increasing its effectiveness for treatments.

Other NPs have been used to enhance the therapeutic/diagnostic potential of VIP with the valuable properties of metal NPs in the field of biosensing and medical imaging owing to their plasmonic properties (Caro 2010). This is the case of silver-protected VIP NPs that retain the functional activity of VIP, assayed by the inhibition of activated microglia under inflammatory conditions (Fernandez-Montesinos, Castillo et al. 2009).

A variety of techniques are described in the literature for the preparation of peptide encapsulated NPs or peptide functionalized NPs, but few of them make direct reference to VIP. As explained above, VIP can be encapsulated as well as attached to the surface of molecules in order to act like a drug itself or to direct another compound to a specific site. There is an invention that uses these two features of VIP to merge them in a single NP (Paul, Yasuko et al. 1998). The invention describes a method of delivering VIP to the surface and to the intracellular compartment of a target tissue by producing a liposome where VIP is located both on and inside the liposome, i.e. VIP is exposed to the outside solvent and to the luminal space. Other inventions encapsulate VIP but with the aim of a controlled release. This is the case of an invention that describes a water-soluble peptide encapsulation method in order to prepare polymeric microspheres or nanospheres that encapsulate VIP with high efficiency (Ignatious 2001). The author describes a method for reducing the aqueous solubility of the drug, without sacrificing its potency, which is useful in case of high peptide loading and heterogeneous distribution of the drug particles, leading to a non-predictable release profile. This offers an improved encapsulation method when predictable release profiles are needed.
3.2.2 VIP as a targeting agent to a specific tissue

In other applications VIP is used as a surface ligand for targeted delivery. In this case, VIP needs to be efficiently attached to the nanoparticle surface and for this purpose liposomes are commonly used. They offer the possibility of attaching a ligand to the lipid head group or to the distal end of the poly-ethylene-glycerol (PEG) chain on PEG-grafted liposomes and can evade the reticulo-endothelial system as well as have prolonged circulation time in blood. However, these nano-applications that use VIP as surface ligands and not as a cargo (Fig. 1) take advantage of the fact that VIP can be used for targeting specific cells, for example human cancers, and has clinical impact at both diagnostic and therapeutic levels. An example of these nano-systems could be phospholipid nanomicelles grafted with VIP, which have been reported to actively target breast cancer and act as a water-insoluble anticancer drug carriers, (Rubinstein, Soos et al. 2008; Onyuksel, Jeon et al. 2009), for example paclitaxel or an antibiotic derivative named 17-allylamino-17-demethoxy geldanamycin (Onyuksel, Mohanty et al. 2009). In summary, they demonstrated that they could solubilize these drugs at therapeutically relevant concentrations in actively targeted VIP surface-grafted micelles. The cytotoxicity of these micelles to human breast cancer cells was retained implying high affinity to VIP receptors which are overexpressed on these cells and also mediated, in part, their intracellular uptake thereby amplifying drug potency. Pioneer research teams led by Rubinstein and Onyuksel also characterized VIP grafted liposome for targeted breast cancer imaging and could demonstrate that there was significantly more accumulation of VIP liposomes than similar liposomes without VIP, indicating a successful use of VIP receptors for active molecular targeting, rather than passive targeting (Dagar, Krishnadas et al. 2003).


Figure 9. Types of VIP-NPs and their applications. VIP can be encapsulated as well as attached to the surface of particular NPs in order to act like a drug itself or to direct another compound to a specific site. If VIP is encapsulated into a NP it can get to its destination by passive or active cell targeting. By contrast, when VIP is attached to the NP surface it directs the NP to a specific tissue which overexpresses its receptors by active cell targeting. VIP-NPs can be used for a variety of applications such as drug delivery, immunomodulation, diagnosis and cancer therapy.

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Not only targeting to cancer tissues has been studied but also targeting to other cells such as activated T-lymphocytes and macrophages in rheumatoid arthritis (Koo, Rubinstein et al. 2011). May Yue Koo et al. formulated camptothecin sterically stabilized micelles conjugated to VIP to actively bind to VPAC2 overexpressed receptors in the cells mentioned previously. This approach of active targeting long circulating micelles to the effector cells in the arthritic joint enhanced the efficacy of the drug and diminished systemic toxicity. They could observe that a single,
subcutaneous injection of low-dose camptotechin formulation mitigated joint inflammation for at least 32 days after treatment without systemic toxicity in collagen-induced arthritis mice (Koo, Rubinstein et al. 2011).

A major issue for the development of clinical applications based on engineered NPs pertains to toxicology, pharmacokinetic and biodistribution studies (Longmire, Choyke et al. 2008; Malam, Lim et al. 2011). In this sense, there are few data on VIP-engineered NPs, and mainly limited to animal models, which are not a perfect predictor of outcome in humans. Dagar and collaborators have demonstrated in breast tumour-bearing rats that VIP covalently attached to sterically stabilized liposomes encapsulating a radionuclide did not alter the pharmacokinetic parameters of the liposomes, as half-lives data calculated from blood radioactivity indicated (Rubinstein, Patel et al. 1999). Therefore, VIP could be used to decorate labelled, long circulating small liposomes to develop new targeted imaging agents for enhance, and thus, early detection of several cancer types. Although biodistribution studies using liposomes have proved increased brain delivery of VIP (Dufes, Gaillard et al. 2004; Gao, Wu et al. 2007), the authors do not perform comprehensive pharmacokinetic studies, and only indirect data indicate the existence of VIP-engineered NPs clearance by cerebrospinal fluid draining (Dufes, Gaillard et al. 2004). Precise pharmacokinetic analyses of the entire plasma profile, including absorption, distribution and elimination should be included in coming studies, and whenever possible, comparison with VIP engineered NPs intravenous routes of administration should be performed.

3.3 Clinical Potential of VIP

VIP has shown therapeutic and diagnostic potential for a variety of disorders and diseases. The involvement of this peptide in important functions such as the immune system or in other peripheral functions like pain or hypertension, suggests the possibility of its use for potential applications. Based on these functions of the peptide, several clinical trials have been reported using VIP or its analog for asthma (Linden, Hansson et al. 2003) and sarcoidosis (Prasse, Zissel et al. 2010). In the case of
asthma, a double-blind placebo study was performed on 24 patients with moderate stable asthma and they concluded that the inhalation of Ro25-1553 constituted a promising approach for bronchodilatation in patients (Linden, Hansson et al. 2003). In the case of sarcoidosis, a phase II study was conducted in 20 patients with nebulized VIP for four weeks. The inhalation of VIP reduced significantly the production of tumour necrosis factor alpha by bronchoalveolar cells. Here, they concluded that inhalation of VIP could be developed into a new therapeutic principle for chronic lung inflammation (Prasse, Zissel et al. 2010). Moreover, VIP is an important erectile neurotransmitter and has been investigated in combination with phentolamine mesilate (Zhang, Shen et al. 2011). A crossover study with 187 patients was performed and the treatments were effective for erectile dysfunction and recognized benefit of not causing pain (Shah, Dinsmore et al. 2007).

At the diagnostic level VIP has an important clinical impact. This approach takes advantage of the fact that human tumours overexpress VIP receptors. Radiolabeled VIP and its analogs have shown their potential as imaging agents for diagnosing tumours. A biodistribution study was carried out in mice bearing colorectal tumours with 18F-labelled (R8,15,21, L17)-VIP analogue, showing positive results in tumour detection (Cheng, Yin et al. 2007). Furthermore, (123)I-VIP was used to identify primary and metastatic tumor sites in a study comprising 120 carcinoid patients. The results showed high sensitivity for localizing tumour sites and a higher diagnostic yield than conventional imaging techniques (Raderer, Kurtaran et al. 2000).

### 3.4 Current and Future Developments

The majority of the challenges facing the development of VIP-engineered NPs relate to their unique distribution characteristics, physical chemistry, manufacturing processes and drug product characterization. Researchers moving to this field should take into consideration that the slightest changes in particle size can substantially alter the biodistribution, renal excretion and pharmacodynamics of VIP-NPs. Therefore, to ensure consistent VIP-based NP applications into the clinic, factors such as particle
size, surface charge and surface chemistry will require robust characterization with overlapping assays. Although still far from VIP-enabled nanoproducts in the market, the areas of healthcare where VIP nanotechnology can make their greatest contributions are cancer research, inflammatory disorders, neurodegenerative diseases and molecular imaging. Undoubtedly, these carriers provide the hope to treat and diagnose several of the mentioned diseases. However, there are some issues that need to be understood in order to ensure their safety and effectiveness. Nevertheless, in the future, novel VIP-NPs could offer new perspectives for the treatment of diseases and new applications will be developed for personalized molecular medicine.
II. OBJECTIVES
The specific purpose of this project is to study the feasibility as a proof of concept of nanotechnology approaches for VIP improved based-drug delivery systems.

Two main questions have been addressed using VIP engineered NPs:

1. VIP functionalized gold NPs. In this case, VIP could act as a drug/diagnostic molecule for theranostic applications.

2. VIP functionalised liposomes as a targeting agent to transport drugs to a specific tissue site.

The rationale behind the first part of this study is to increase the half-life of VIP as it needs to be substantially prolonged in biological fluids to be employed in therapeutics. VIP-based drug design is hampered by the instability of the peptide and has limited bioavailability. For this reason VIP was functionalized to gold nanoparticles to investigate a possible enzyme protection and the mechanisms involved in this process.

In the second part of this study, VIP was used as a directing agent of a drug-loaded liposome to prostate cancer cells, and was compared with a non-targeted liposome. The aim of this study was to assess the potential of VIP as a ligand for prostate cancer and evaluate the efficacy of the treatment.
Section 1

1. SUMMARY AND RATIONALE
SECTION 1: SUMMARY AND RATIONALE

Lead optimization is one of the most critical steps in drug development in which nanotechnology-based applications offer great potential. Within the molecules in need of lead optimization, ligands for G protein-coupled receptors (GPCRs) are of particular concern, with at least 40% of drugs currently in the market thought to modulate GPCRs. Neuropeptides are a class of ligands for GPCRs. There have been several important improvements in the development of neuropeptide therapeutics with limited success, mainly due to poor bioavailability after protease degradation. Thus, the development of small, non-peptide mimic ligands results in molecules that often have reduced affinity and selectivity compared to their endogenous ligand counterparts. Likewise, the development of inhibitors of specific proteases increases the chances of adverse effects. In this sense, as a paradigm of other peptide-based therapeutics, the neuropeptide VIP is still not available for treating clinical problems despite its potential. For this reason, we looked for an alternative strategy that simultaneously: a) targets the protease substrate (neuropeptide) instead of the protease, and b) makes use of the entire neuropeptide molecule to retain its full biological activity. Remarkably, although it has been hypothesized that surface functionalization of proteins and bioactive peptides on noble metallic nanoclusters might protect from protease degradation, so far there are no formal proofs in this sense. Our aim is to prove that coating gold NPs with the neuropeptide VIP impairs the hydrolytic activity of extracellular proteases, leading to VIP-mediated functional responses after harsh conditions resembling the extracellular circulating proteases milieu. Combining physical and chemical characterization to determine size, dispersion and homogeneity of VIP AuNPs, by AFM and TEM analysis and quantifying the amount of peptide. This is the first study to address the potential protection from protease degradation upon AuNPs functionalization of a given peptide. Besides the implications in the field of neuropeptides, our study places the concept of surface functionalization in the broader perspective of proteins escaping from
extracellular proteases, which could represent a major driven force and an added value to steer the research in the field of engineering NPs.
Section 1

II. MATERIALS AND METHODS
1. Preparation of vasoactive intestinal peptide nanoparticles

1.1 AuNP synthesis

Briefly, a mixture of 1 mg of \((N-(3\text{-dimethylaminopropyl})-N’-\text{ethyl carbodiimide hydrochloride (EDC)}\) and 1.5 mg of \(N\text{-hydroxysuccinimide (NHS)}\) were added to 1 mg of AuNPs in 2 mL of 2-[N-morpholino]ethanesulfonic acid (MES) (50 mM, pH 6.5) and was stirred for 30 min at RT. Gel filtration PD 10 columns were used to eliminate the excess of EDC.

1.2 VIP functionalization.

The filtrated AuNPs were mixed with 75 µg of VIP (1mg/mL) and stirred 20 minutes. Then, 420 mg of amino-PEG was added to the solution and the mix was additionally stirred for 2 h at RT. This solution was loaded into 6 cm segments of seamless cellulose ester dialysis membrane (Sigma MWCO=10000), placed in 4 L beakers of water, and stirred slowly, over the course of 24 hours.

1.3 VIP quantification.

Functionalized VIP was quantified by a peptide quantification assay (LavaPep,Gel Company, CA, USA) according to the manufacturer's recommendations. The method is based on a naturally-occurring fluorescent compound that reversibly binds to lysine, arginine and histidine residues in peptides, and it responds to hydrophobic environments to yield an intensely red fluorescent product. Then, spectrophotometrical absorbances were determined using a fluorescence microplate reader using a 540 nm excitation filter and 630 nm emission filter. There was no interference due to AuNPs. A standard curve of VIP was used to determine the concentration of peptide in the unknown sample.
2. Characterization of VIP-metallic nanoparticles

2.1 AFM imaging

AFM images were obtained with a Molecular Imaging Picoscan 2500 Atomic Force Microscope (Agilent technologies). Tapping mode imaging was performed using standard silicon cantilevers by Nanosensors with a resonance frequency of 45-115 kHz and spring constants of 0.5-0.9 N. Images were obtained with a scan rate of 0.4 Hz and the section analysis of the system was used to measure the diameter and the height of AuNPs and VIP-AuNPs. All the images presented are the original and a flatten command was used to remove the background slope. AFM images were obtained by drying a 20 uL droplet of 0.5 mg/mL of AuNPs deposited on a freshly cleaved mica surface.

2.2 TEM analysis

TEM examinations were obtained after placing a 10 µL single drop of 0.5 mg/mL of the aqueous solution of AuNPs on to a copper grid coated with a carbon film. The grip was left to dry in air for several hours at room temperature. TEM analysis was carried out in a Tecnai T20 (FEI,Netherlands) electron microscope model working at 200 kV.

3. Viability assays

3.1 MTT assay

PC3 cells were previously seeded overnight and treated for 24h in 96-well plates in a final volume of 200 uL. After treatment, the media was removed, and fresh media with 12.5 µl of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent (final concentration 0.5 mg /ml) was added to each well and, after further incubation for 4 h in a humidified atmosphere (37 °C, 5% CO₂), 100 µl of the solubilized solution was added to each well. The plates stood overnight in the incubator in a humidified atmosphere after complete solubilization of the formazan crystals. Then, spectrophotometrical absorbances were determined using a
microplate (ELISA) reader at 595 nm. The studies were performed at least 3 times in duplicates.

4. Enzymatic degradation studies

4.1 Peptide degradation assay

Samples were prepared at 250 μg/mL of AuNPs. The VIP concentration used for peptide degradation assays was 5 μg/mL for both VIP-AuNPs or soluble peptide controls. 500 μL samples of VIP-AuNPs, soluble VIP and AuNPs were incubated with trypsin and α-chymotrypsin (10 U/mg peptide; 0.05 UE/mL) and the enzymatic reaction was allowed to proceed at 37°C for 0, 10 and 30 min. The reaction mixture was stopped by adding HCl to a final concentration of 0.2 M and samples were placed at 4°C.

a) VIP AuNP quantification.

Degraded VIP AuNPs, were loaded into 1 mL cellulose ester dialysis membranes (MWCO 10 kDa), placed in 1 L beakers of MES (pH 4.5) at 4°C, and stirred slowly, recharging with fresh buffer every 12 h over the course of 24 hours. Samples were collected for remaining peptide quantification with the method previously described. In addition, no degraded VIP AuNPs and AuNPs underwent the same treatment to be used as controls.

b) Degraded VIP quantification.

All samples were placed in centrifugal filters and centrifuged at 14000 xg to remove AuNPs from degraded peptidic residues. To determine the concentration of digested VIP, 20 μL of the supernatant was directly injected for HPLC analysis. In addition, soluble VIP was treated in the same way and used as control before and after degradation.
4.2 Evaluation of protective effect by HPLC analytical method

Chromatographic separations were performed on a Waters LC module I plus C18 4.6 mm X 250 mm column. Twenty microliters of sample was injected into the column, and samples were analyzed by a reversed phase HPLC method at room temperature. The mobile phase consisted of 0.1% v/v TFA/water (A) and 0.1% v/v TFA/acetonitrile (B). The gradient conditions were 98-80% A for 10 min, 80-65% A from 10 to 20 min, 65-63% A from 20 to 30 min, 63-80% A from 30 to 35 min, 80-98% from 35 to 37 min, and 98-98% from 37 to 47 min at a flow rate of 1 mL/min. The detection was achieved at a wavelength of 210 nm.

4.3 Intracellular cAMP determinations.

cAMP was quantified using cAMP-Glo Assay according to manufacturer's protocol. Intracellular cAMP was measured in a prostate cancer cell line PC3 after 30 minutes of incubation with VIP-NPs, before and after enzymatic degradation to determine whether there was intact peptide remaining and to evaluate loss of functionality after VIP degradation. Non functionalized AuNPs had no effect on basal cAMP levels as determined after 5, 30, 60 and 180 minutes of incubation.
Section 1

III. RESULTS AND DISCUSSION
1. Characterization of VIP-metallic nanoparticles

1.1 Nanoparticle functionalization

Since AuNPs have proper biocompatibility features in terms of reduced \textit{in vivo} NP destabilization (Bar-Ilan, Albrecht et al. 2009), safety (Kittler, Greulich et al. 2010), and well-tolerant behaviour after systemically delivered in humans, (Davis, Zuckerman et al. 2010) we decided to surface functionalize AuNPs with VIP (Figure 10).

![Synthetic approach to obtain tiopronin-capped gold NPs functionalized with VIP and PEG.](image)

\textbf{Figure 10.} Synthetic approach to obtain tiopronin-capped gold NPs functionalized with VIP and PEG.

To perform this functionalization, in a first step, tiopronin capped AuNPs were obtained by reduction of HAuCl\textsubscript{4} in the presence of tiopronin. After this, VIP was
covalently coupled through its amino terminal end via carbodiimide-mediated amide-forming reactions with the free carboxyl groups of the tiopronin monolayer after their activation with EDC/NHS. Finally, to prevent nonspecific adsorption of proteins, the remaining activated carboxylated groups, not used to anchor VIP molecules, were masked by reaction with an amino-polyethylene glycol (PEG) derivative of 750 daltons. This functionalization process finally rendered VIP-functionalized AuNPs where VIP C-terminus remains free. VIP has a diffuse pharmacophoric profile, and recent evidences indicate a major role of the C-terminal and central parts of VIP (Couvineau, Ceraudo et al. 2012; Harmar, Fahrenkrug et al. 2012). In this sense, our VIP AuNPs could be suitable to functional coupling to VPAC1 receptors. The method for the synthesis of VIP AuNPs described herein produced NPs with final VIP concentrations ≈1.5 uM, depending on the batch, with an average ratio of 2 VIP molecules/AuNP. This concentration range is ideal for VIP biological applications, as the $K_d$ and EC$_{50}$ values for VIP receptors are in the nanomolar range.

1.2 Size and surface characterisation: AFM, TEM and DLS

The resulting AuNPs were analyzed by transmission electron microscopy (TEM). A representative TEM image of these gold NPs is shown in Figure 11A and the statistics shown in Figure 11B is indicative of a monodisperse distribution of particle diameter, with a mean value equal to 3 nm, whereas the minimum and maximum values are 0.5 nm and 5 nm, respectively.
Figure 11. Characterization of AuNPs by TEM. (A) TEM image of AuNPs, scale bar; 50 nm. The inset shows a higher magnification image, scale bar; 5 nm. (B) Statistical analysis of colloid diameter, as evaluated from TEM image.

A representative TM-AFM image of AuNPs and VIP AuNPs deposited on a freshly cleaved mica surface is shown in Figure 12 A and B respectively. Single colloids appear stably bound to the mica, and display homogeneous lateral and vertical dimension in the case of AuNPs nanoparticles and show an increased size in the case of VIP AuNPs, due to the surface functionalization. An estimate of particle diameter can be provided by the vertical size of the colloid above the substrate. Particle diameter is approximately 2.5 nm for AuNPs and 3.5 nm in the case of VIP NPs, in close agreement with values obtained from our TEM characterization. Also, the lateral size is monodisperse but subject to significantly broadening because of the well known tip convolution effect (Howald, Haefke et al. 1994). Taken together, we have developed a reliable method to synthesize homogeneous, stable, and properly characterised VIP AuNPs in order to test our working hypothesis, i.e, the protection from protease degradation upon nanoparticle functionalization.
Figure 12. A) Characterization of AuNPs by AFM. Up-left: Tapping mode topography; Up-right: measurement of NPs height (indicated in the topographic image); Down-left: 3D AFM image; Down-right: statistical analysis of colloids height evaluated from AFM image.
Figure 12. B) Characterization of Au-PEG/VIP NPs by AFM. Up-left: Tapping mode topography; Up-Right: measurement of NPs height (indicated in the topographic image); Down-left: 3D AFM image; Down-right: statistical analysis of colloids height evaluated from AFM image.
1.3 Cytotoxicity studies

In order to evaluate if VIP-AuNPs could be used for the treatment of several diseases, cytotoxicity studies were performed. Figure 13 shows the cell viability after 24 h of incubation with AuNPs and AuNPs-PEG/VIP. Over 90% cell viability was still obtained after 24 h with $10^{-7}$ M VIP, in the case of AuNPs-PEG/VIP and its equivalent amount of NPs in the case of AuNPs (16,6 ug). These results showed that AuNPs and AuNPs-PEG/VIP have low toxicity and could be potentially used due to its good biocompatibility as a carrier or platform for VIP therapeutic purposes.

![MTT assay graph](image)

**Figure 13.** Evaluation of AuNPs and AuNPs-PEG/VIP cytotoxicity by MTT assay after 24h.

2. In vitro assays

2.1 Enzymatic degradation studies

To evidence the relevance of the functionalization strategy on the VIP AuNP and protease interaction, we carried out a HPLC analysis of VIP degradation products.
Figure 14. Functionalized AuNPs impairs hydrolytic protease activity. Experimental approaches. VIP AuNPs were characterized by AFM in order to determine size, dispersion and homogeneity. In the same characterization step VIP functionalization was quantified to determine the initial peptide content. VIP AuNPs were exposed to proteases at different time points and the remaining functionalized peptide was quantified. Furthermore, the resulting functionalized nanoparticles were incubated with cells to study the functional response by cAMP assay. Additional steps consisted in the detection by HPLC of peptides and amino acid residues that have been degraded by proteases and did not remain attached to AuNPs. Finally, these data help to verify the results of the previous step, as they are complementary. That is, the higher the content of free VIP degradation products, the lower the VIP biological activity, and viceversa.

As described in details in the Material and Methods section, treatment of equal amounts of soluble VIP with a mix of the proteases trypsin and α-chymotrypsin resulted in different degradation patterns compared to VIP AuNPs (Figure 14). HPLC
chromatograms of non-degraded soluble VIP and its degradation products after 10 and 30 minutes of treatment with proteases are depicted in Figure 15A, whereas representative chromatograms of VIP AuNPs degradation products after 10 and 30 min of treatment with proteases are shown in Figure 15B. The number of arrows is a figurative representation of the extension of degraded peptides detected by HPLC in the appropriate retention times (Figure 15; central and lower panels). Our strategy to evaluate VIP protection involves the assessment of free, degraded VIP molecules (see Material and Methods), and that is the reason of the absence of VIP signal in Figure 15B (upper panel), as all the neuropeptide remains surface functionalized to AuNPs in the absence of proteases. On the contrary, a strong signal corresponding to soluble, intact VIP is identified around 25 minutes retention time (Figure 15A).

After protease treatment, remarkable differences are observed between VIP and VIP AuNPs degradation patterns, showing higher number of degraded peptides in the case VIP compared to VIP AuNPs (Figure 15; central and lower panels). A main degradation product of soluble VIP is observed after 10 min of protease treatment whereas after 30 min the number of degradation products increase, but none of them predominate (Figure 15A; central and lower panels). In addition, after 10 min of exposure to proteases, the peak corresponding to intact VIP disappears (Figure 15A; central panel). On the other hand, VIP AuNPs show very similar degradation patterns after 10 and 30 min of protease treatment, probably due to the existence of non-detectable residues by HPLC (Figure 15A; central and lower panels). This is consistent with a situation in which NP functionalization restraints molecular interactions in context-specific manners between proteases and their substrates, reducing the number of random events. Of particular relevance is the striking reduction in the number of free peptide degradation products, even at the longest exposure with protease (30 min) shown by VIP AuNPs compared to soluble, non-functionalized VIP (Figure 15B; central and lower panels). Overall, the previous set of experiments demonstrated a notable degree of VIP protection from protease activity,
as the hydrolytic reactions were performed under ideal enzyme reaction conditions used for in vitro evaluation of the protective effects towards other alternative strategies (Smoum, Rubinstein et al. 2006; Sun, Scharff-Poulsen et al. 2008). These observations strongly support the notion that functionalization of the VIP neuropeptide to gold nanoparticles impairs protease activity.

Figure 15. HPLC analysis of Au-PEG/VIP degradation products before and after 10 and 30 min exposure to proteases. (A) Chromatograms of VIP after 0, 10 and 30 min of degradation. (B) Chromatograms of VIP AuNPs degradation products after 0, 10 and 30 min. All samples
were passed through a filter in order to separate functionalized NPs from degraded peptides. The results shown are representative of two independent experiments performed in duplicates.

2.2 Peptide functionalization quantification and cell response to protected VIP AuNPs.

To validate the biological implications of the diminished protease activity upon gold nanoparticle functionalization, we first quantified the VIP neuropeptide in order to determine the content that remained functionalized to AuNPs before and after the exposure to proteases. For that purpose, we used a highly sensitive fluorescence-based peptide quantification procedure at different time points (0, 10 and 30 minutes) after enzymatic degradation. The percentage of VIP decreased gradually to 59% (p=0.017) and 43% (p=0.047) in relation to the initial functionalized peptide content after 10 and 30 minutes of incubation with proteases, respectively (Figure 16). However, the most conclusive question was to determine whether there were functional -biologically relevant- intact neuropeptide molecules attached to the NPs. Thus, we needed to elucidate whether the degradation, even if largely impaired due to NP functionalization, affects the functionalized VIP molecules to some degree leading to a non-functional VIP response by the receptor, or by contrast, an appropriate number of neuropeptide molecules remain intact and give rise to an established, usual cell response mediated by the VIP receptor-effector system.

VIP receptors belong to the B class or family 2 of the GPCRs that characteristically activate the adenylyl cyclase (AC) signaling (Couvineau, Ceraudo et al. 2012; Harmar, Fahrenkrug et al. 2012), therefore increasing the intracellular levels of cyclic adenosine 3′,5′-monophosphate (cAMP), and transducing in this way VIP/VIP receptor interactions to functional responses mediated by the second messenger molecule cAMP. For that purpose, we determined the intracellular cAMP production in the human PC-3 cell line, a well-established cell line for the characterization of VIP-mediated signaling (Harmar, Fahrenkrug et al. 2012) (Figure 16). PC-3 cell incubations
SECTION 1: RESULTS AND DISCUSSION

with non-degraded and degraded VIP AuNPs after 10 and 30 min protease exposure indicate a 74% (p=0.014) and a 44% (p=0.012) of cAMP level compared to the initial cAMP production (see Material and Methods sections for details).

Figure 16. Au-PEG/VIP peptide quantification and cAMP intracellular levels before and after 10 and 30 min exposure to proteases. Peptide quantification at 0, 10 and 30 minutes after enzymatic degradation (black bars) and intracellular cAMP levels in human PC-3 cells treated with intact and degraded VIP AuNPs (hatched bars). Values are mean ± S.E.M. * P<0.05, ** P<0.01, *** P<0.005. The results shown are representative of three independent experiments in the case of VIP quantification and two in the case of cAMP functional assays, all of them performed in duplicates.

These values suggest that a high percentage of VIP molecules remain intact after the exposure to proteases, and therefore retain their ability to interact with cells and to signal a functional response. Moreover, in the context of cell signaling, the short-term responses immediately after ligand-receptor engagement are critical, as they are largely amplified downstream in signaling pathways. Therefore, the fact that we observe a protective effect and a sustained biological response during early
steps in VIP signaling is of great interest as this is an indispensable condition for improving neuropeptide-mediated drugability.

Fig 17. HPLC analysis of Au-VIP degradation products before and after 10 and 30 min exposure to proteases. (A) Chromatograms of VIP-AuNPs degradation products after 10 min and B) 30 min. All samples were passed through a filter in order to separate functionalized NPs from degraded peptides. To confirm that the signals detected by HPLC corresponded to the peptide residues, the same samples were subjected to MALDI-TOF analysis (highlighted graphs). The results shown are representative of two independent experiments performed in duplicates.
Figure 18. Au-VIP peptide quantification and cAMP intracellular levels before and after 10 and 30 min exposure to proteases. Peptide quantification at 0, 10 and 30 minutes after enzymatic degradation (black bars) and intracellular cAMP levels in human PC-3 cells treated with intact and degraded Au-VIP NPs (hatched bars). Values are mean ± S.E.M. * P<0.05, ** P<0.01, *** P<0.005. The results shown are representative of three independent experiments in the case of VIP quantification and two in the case of cAMP functional assays, all of them performed in duplicates.

Although the molecular mechanisms behind this protection are unknown, we might presume it is due to the lack of accessibility of the proteases to the functionalized N-terminal end of the peptide. In addition, protease access to the whole neuropeptide molecule could be hindered by the particular microenvironment resulting from the high density of PEG moieties attached to the gold nanoparticles acting as fences towards the VIP molecules. Notably, the marked effect observed in our case might not be ascribed to substrate crowding, as it has been hypothesized for the case of galactosidases in lactose-functionalized gold nanoclusters (Barrientos, de la Fuente et al. 2009). Taking together, we have combined methods for nanoparticle...
characterization, HPLC measurements and functional readout determinations to demonstrate that VIP functionalization of gold nanoparticles protect from protease degradation. The experimental approaches used in the present work (Figure 4) are the first to formally prove that an additional feature of gold nanoparticles which is of high interest for its biological implications is the capability to impair hydrolytic protease activity.
Conclusions

- We have developed a reliable method to synthesize homogeneous, stable, and properly characterised VIP AuNPs.
- VIP functionalized gold NPs impairs the hydrolitic activity of extracellular proteases.
- VIP-mediated functional responses after harsh conditions resembling the extracellular circulating proteases milieu.
- This the first study to address the potential protection from protease degradation upon AuNPs functionalization of a given protein.
- These findings are pertinent for different fields of research and technological applications:
  a) For the field of neuropeptides and other peptide ligands of GPCRs, where endogenous protein degradation leads to limited bioavailability.
  b) In the case of protease inhibitors development, a modification of the substrate (i.e., NPs functionalization) results in higher degrees of specificity, and it might avoid the safety concerns currently associated with inhibitors.
Figure 19. NP functionalization protects from enzymatic degradation. VIP protection due to NP conjugation produces a functional response on VIP receptor expressing cells.
Section 2

I. SUMMARY AND RATIONALE
Prostate cancer (PCa) is one of the most frequently diagnosed solid tumours in men and is one of the leading causes of mortality in developed countries and with increasing rates in the developing world (Leonetti, Biroccio et al. 2007; Baade, Youlden et al. 2009). The majority of men with newly diagnosed localized prostate cancer may be eligible for active surveillance, surgery or radiation therapy either alone or in combination with androgen deprivation therapy (Stangelberger, Waldert et al. 2008). Moreover, chemotherapy may also be an option for cancers that don't respond to hormone therapy. The decision whether or not to treat localized PCa with curative intent is a patient trade-off between the expected beneficial and harmful effects in terms of patient survival and quality of life (Bangma, Roemeling et al. 2007). Conventional chemotherapy is the most applied treatment for many cancers but has low specificity and limited effectiveness due to its severe side effects. Doxorubicin (Adriamycin) is a broad-spectrum antitumor antibiotic that has been widely used for treatment of several cancers, including breast, ovarian, and prostate cancers (Singal, Li et al. 2000). The effectiveness of doxorubicin is limited due to its high toxicity and side effects, including myelosuppression, alopecia, acute nausea, vomiting, stomatitis, cumulative cardiotoxicity (Rivera 2003), and strong multidrug resistance response in tumour cells after repeated administration (Shen, Chu et al. 2008). Therefore research aims to doxorubicin targeted delivery to PCa cells could be part of the proof of concept studies to reduce repeated administrations and secondary effects as the therapeutic efficacy increases with specific treatments (Sharifi and Steinman 2002; Perrino, Schiattarella et al. 2012). The specific delivery of anticancer drugs to PCa cells has important implications for diagnosis and therapy. Biomarkers that differentiate cancerous tissues from normal tissues can be used as targets for this purpose and one of these attractive molecular targets is VIP receptors which are overexpressed in human PCa compared to normal prostate tissue (Reubi 1995; Reubi 1996) as mentioned in the introduction (section 2.3) in more detail.
For this reason VIP liposomes have been synthesized to exploit VIP-R to actively target carriers to PCa and therefore improve its therapy. Particulate carriers such as liposomes with mean size of about 100 nm are passively targeted by predominantly accumulating at certain sites such as tumours due to the presence of leaky vasculature and liposomal extravasation (section 1.2, Figure 1) (Malam, Loizidou et al. 2009). The use of liposomes is recognized as a promising strategy for improving the delivery of anticancer drugs to tumours, leading to a reduction in drug toxicity and improving the therapeutic outcomes (Gosselin and Lee 2002; Allen, Mumbengegwi et al. 2005). Furthermore, VIP phospholipid liposomes were used to encapsulate doxorubicin in order to deliver it to PCa cells. The aim of this study was to assess the potential of VIP as a ligand for prostate cancer targeting by liposomal nanocarriers. Moreover, we wanted to evaluate the effect of a peptide coupling method on the cellular uptake, cytotoxicity and apoptosis of doxorubicin liposomal formulations. We also addressed in vivo experiments in a preclinical setting in order to evaluate the VIP active driven targeting of the prostate cancer cells by liposomes.
Section 2

II. MATERIALS AND METHODS
1. Cell lines

HFF-1 (ATCC), DU145 (ATCC), PC3 and LnCAP (ICLC) human prostate cancer cells were kindly provided by Dr. M. Japon (Department of endocrine tumorigenesis and hormonal regulation of cancer, Biomedicine institute of Seville, IBIS, CSIC-University of Seville, Spain). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamin, 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C.

1.1 VPAC1 gene expression study by PCR

The expression level of VPAC1 and the Cyclophilin B housekeeping gene was determined in HFF-1, DU145, PC3 and LnCAP by qPCR and agarose gel-electrophoresis. RNA isolation was performed using TriPure isolation reagent (Roche, Switzerland). To quantify VPAC1 expression levels, equal amounts of cDNA were synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). 100 nanograms of cDNA were amplified using 12.5 μL of SYBR qPCR Master Mix (Takara), 200 nM of each primer and H2O up to 25 μL. The PCR amplification scheme was: 10 min at 95 °C followed by 40 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The oligonucleotides were as follows: VPAC1 forward 5-ATGTGCAGATGATCGAGGTG-3, reverse 5-TGTAGCCGGTCTTCACAGAA-3, which yield a PCR product of 324 bp. Cyclophilin B forward 5-CTTCCCCGATGAGAACTTCA-3, reverse 5-TCTTGGTGCTCTCCACCTTC-3, which yield a PCR product of 193 bp. The oligonucleotides were designed using the following sequences: for VPAC1 NCBI Reference Sequence, NM_004624.3 and for Cyclophilin B, NM_000942.4. SYBR-Green detection was followed by the generation of melting curves and visualization of the products to confirm specificity. Quantitative PCR results were obtained using the comparative Ct method. The threshold crossing value was noted for each transcript and normalized to the internal control. Experiments were performed using an ABI Prism 7900 System (Applied Biosystems), and data processing was performed using ABI SDS v2.1 software (Applied Biosystems). PCR products were
SECTION 2: MATERIALS AND METHODS

electrophoresed on a 1.5% agarose gel stained with 0.5 g/ml ethidium bromide and visualized under UV light, followed by (700 Imaging Densitometer, Bio-Rad Laboratories, CA).

1.2 VPAC1 protein expression study by WB

HFF-1, DU145, PC3 and LnCAP cells were cultured on 6 well plates (Thermo Scientific, Nunclon Delta Surface) until 80% confluency, washed once with ice-cold PBS, and lysed in ice-cold lysis buffer (RIPA buffer: 50 mM HEPES pH. 7.4, 150 mM Sodium chloride, 1 mM EDTA 1% Nonidet P 40, 0.25% sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 10 mM sodium fluoride, 50 mM sodium orthovanadate) containing Complete, EDTA free Protease inhibitor cocktail Tablets (Roche, Switzerland) for 30 minutes at 4 °C. After centrifugation of the lysates at 21,000 g at 4 °C for 20 minutes, the supernatants were collected and stored at −80 °C until use. The protein concentrations of these extracts were determined with the bicinchoninic acid (BCA) protein assay (Bio-rad Laboratories, California).

Western blot analysis was conducted by employing the corresponding antibody. In brief, equal amounts of lysate proteins (40 μg) were loaded onto 15% SDS-PAGE gel (the loading buffer containing 0.1 M DTT) and subjected to electrophoresis at 200 V. All samples were electrophoretically transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Germany). After blocking with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) overnight at 4 °C and washing for 5 minutes four times with TBST, the membrane was incubated with rabbit anti-human VPAC1 (1:500, Themo Scientific, USA) for 1 hour at room temperature. The membrane was washed as described before with TBST and incubated with an anti-rabbit IgG HPR conjugate secondary antibody (1:20,000, Promega, Madison, Wi, USA) for 1 h at room temperature. The antibody-bound proteins were detected with the ECL chemiluminescence reagent (Millipore, USA) and the signal revealed by autoradiography on Amersham Hyperfim ECL (GE
Healthcare, Buckinghamshire, UK). Quantification of Western Blot by performed by densitometry with ImageQuant TL software after scanning the film on ImageScanner II (GE Healthcare). The relative expression of proteins was normalized to that of loading controls. The values are expressed as percentages of the expression levels in control cells that were arbitrarily set to 100%.

2. Preparation of doxorubicin encapsulated VIP-liposomes

2.1 Liposome formulation

A mixture of phospholipids DPPC, DSPC (molar ratio 6:4), DSPE-PEG2000 (5% of total phospholipids) and DSPE-PEG-Mal (5% of total phospholipids) were dissolved in chloroform:methanol (4:1 v/v) mixture. Multilamellar vesicles were prepared by reverse phase evaporation method. The organic solvent was evaporated in a 25 mL round-bottom flask using a rotavapor (Heidolph, Germany), under vacuum for 30 min at 40°C. After removal of the solvent the dried lipid film was dissolved in 3 mL of di-ethyl-ether and 3 mL of chloroform. To this solution 1.5 mL ammonium sulphate (250 mM pH 5) were added. The resulting two-phase system is sonicated (Ultrasonic cleaner, VWR) for 5 to 10 min until the mixture becomes homogeneous and does not separate for at least 30 min after sonication. The mixture is then placed on the rotary evaporator and the organic solvent is removed until the sample becomes an aqueous suspension. Moreover, the sample is left under vacuum for 30 minutes to ensure the solvent removal and achieve a final lipid concentration of 4mM. Small unilamellar vesicles (SUV) were prepared by further bath sonication at 60°C for 15 minutes. Liposomes were passed down a sephadex G-50 column equilibrated with HEPES buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4) to exchange the external buffer. The eluted liposomes had a transmembrane pH gradient, pH 5.0 inside and pH 7.4 outside, necessary for DOX encapsulation.
2.2 Doxorubicin encapsulation

DOX was encapsulated into the liposomes using the pH gradient technique as previously described (Mayer, Bally et al. 1986). Briefly, after generating a pH transmembrane gradient, liposomes (4 mM total lipid concentration) were incubated with 90 uL of doxorubicin (5 mg/mL). The mixture of liposome and doxorubicin was incubated at 60°C (above both lipids phase transitions) in a water bath for 90 min. After loading, un-trapped doxorubicin was removed by Sephadex G-50 gel filtration in Hepes Buffer pH 7.4. Moreover, the drug encapsulation efficiency was quantified by measuring its fluorescence following the lysis of liposomes with 1% Triton X-100, pH 7.4. The amount of doxorubicin trapped inside the liposomes was determined by a fluorometer (Varioskan Flash, Thermo Electron Corporation, Finland), excitation and emission 485 nm. The size after drug loading was measured by dynamic laser scattering (Zetasizer Nano ZS, MA, US) and the encapsulation efficiency (EE %) was calculated using the below formula:

Encapsulation efficiency (EE %) = (It/Ii) x 100, where It is the intensity of the total amount of drug in the liposome after purification and Ii is the intensity of the total quantity of drug added initially during the preparation. In order to release the drug from inside the liposome, 10 uL of 10% Triton X-100 was added to 100 uL of sample.

2.3 VIP-functionalization

VIP with an additional cysteine at its NH₂ extreme was attached to the outer lipid monolayer of performed doxorubicin liposomes via DSPE-PEG-Mal. The liposomes were flushed with N₂ to deoxygenate the sample and VIP-cys was added in order to conjugate via its cysteine to maleimide-modified termini of PEG chains located on the external surface of the liposomes. An excess of 2-mercaptoethanol (2 mmol/L final concentration) was added to inactivate all unreacted maleimide groups. Unconjugated VIP and any released free small-molecule drugs were separated from the resulting liposomes by dialysis for 24 h.
3. Characterization of VIP-functionalised liposomes

3.1 Quantification of VIP functionalized liposomes

VIP was quantified using a peptide quantification kit (LavaPep, Gel Company, USA). It depends on a small, naturally-occurring fluorescent compound that reversibly binds to lysine, arginine, and histidine residues in peptides to yield an intensely fluorescent product.

The quantification of the peptide was performed after the dialysis of the liposome for 24 h to ensure the quantification of the coupled VIP-liposome. A standard curve (Figure 22) of VIP was used to determine the concentration of peptide in the unknown sample and a serial dilution of the peptide was prepared in water by 4-fold serial dilutions ranging from 0.3 mg/ml to 300 ng/mL. The spectrophotometrical absorbances were determined using a fluorescence microplate reader using a 540 nm excitation filter and 630 nm emission filter. The standard curve was plotted as fluorescence over peptide quantity (log10 fluorescence vs log10 peptide quantity) and the background due to doxorubicin was subtracted by using doxorubicin liposomes samples. Therefore the amount of VIP conjugated to VIP doxorubicin liposomes samples was calculated using the equation of the standard curve.

3.2 Cryo-transmission electron microscopy

In order to visualize the liposomes in a state as close as in their hydrated state cryo-transmission electron microscopy (cryo-TEM) was performed. Samples were vitrified using plunge freezing. Briefly, a 3 µl droplet of the solution containing the sample was suspended in a quantifoil grid and plunged into ethane liquid cooled down to temperatures close to its solidification temperature using FEI Vitrobot Mark IV. This process avoided the presence of ice crystals and the dehydration as well, produced in more standard techniques as negative staining that fully distort the structure of the liposomes. In order to avoid recrystallization of the amorphous ice,
samples were transferred to the cryo-TEM holder (Gatan 626) under liquid nitrogen and later to the microscope avoiding that the temperature of the sample raised -170 °C. The analyses of the samples were performed in a FEI Tecnai T20 equipped with Gatan cryoblades which prevents from contamination and is used low dose mode. This visualization mode allowed focusing close to the area of interest via a beam.

3.3. Liposomes stability study: doxorubicin release studies in Hepes buffer saline and in serum

For in vitro DOX release, 2mL of 4 mM (lipid) Dox–liposome suspension in Hepes Buffer Saline (HBS) pH 7.4 was added to pre-heated 1mL of HBS buffer or 1mL of fetal bovine serum and incubated at different time points at 37 °C in a thermal-shaker at 300 rpm. After incubation at different time points, 100 μL of the samples were collected and diluted in HBS at 1:10 (v/v) and measured by spectrofluorometry. After the last time point, 10 μl of 10% Triton X-100 was added to the sample to obtain maximum doxorubicin release value which was used as positive control.

Doxorubicin release was determined as (%) = (Int_{t=t1} – Int_{t=0})/(Int_{triton} - Int_{t=0}) x 100, where Int_{t=t1} is the intensity measured at each time point, Int_{t=0} corresponds to the intensity of the liposome before the incubation at 37°C and Int_{triton} is the intensity after the addition of Triton X-100 to obtain a complete release of the drug.

4. Cellular uptake studies

HFF-1, DU145, PC3 and LnCAP cells were seeded for 24 hours on poly-D-lysine pre-treated 15 mm diameter cover slips and afterwards, cells were incubated for 2 and 6 hours with 100 μL of 1 mM liposome samples and 900 μL of medium. The liposomes samples were: doxorubicin encapsulated liposomes (DOX-LIP), VIP-doxorubicin encapsulated liposomes (VIP-LIP-DOX) and the equivalent amount of doxorubicin hydrochloride (10 µg/mL) (DOX). Furthermore, cells were washed 2 times with PBS 1X and fixed with 4% PFA for 10 minutes at RT. Afterwards they were
washed again 2 times with PBS 1X and they were incubated with DAPI for 5 minutes at a concentration of 1:5000 (v:v). The coverslips were mounted on Vecta Shield media and sealed for fluorescence microscopy imaging using the 63x objective lens.

5. Cell viability studies

5.1 Optical Microscopy Imaging

DU145, PC3 and LNCAP cells were seeded overnight in 6-well plates in a final volume of 2 mL and treated with 200 μL of 1 mM liposome samples: liposomes (LIP), VIP-functionalized liposomes (VIP-LIP), doxorubicin encapsulated liposomes (LIP-DOX), doxorubicin encapsulated VIP-liposomes (VIP-LIP-DOX) and the equivalent concentration of DOX (10 μg/mL), for 24 and 48 h. The cells were imaged after these time points using brightfield imaging mode and the 40x objective lens.

5.2 MTT and LDH studies

DU145, PC3 and LnCAP cells were seeded overnight in 12-well plates in a final volume of 1mL and treated with 100 μL of 1 mM liposome samples: liposomes (LIP), VIP-functionalized liposomes (VIP-LIP), doxorubicin encapsulated liposomes (LIP-DOX), doxorubicin encapsulated VIP-liposomes (VIP-LIP-DOX) and the equivalent concentration of DOX (10 μg/mL), for 24 and 48 h. After treatment, the media was removed, and fresh media with 62.5 μl of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent (Roche Diagnostics GmbH, Mannheim, Germany) at a final concentration of 0.5 mg/ml was added to each well. After further incubation for 4 h in a humidified atmosphere (37 °C, 5% CO₂), 625 μl of the solubilized solution was added to each well. The plates stood overnight in the incubator in a humidified atmosphere after complete solubilization of the formazan crystals. Then, spectrophotometrical absorbances were determined using a microplate (ELISA) reader at 595 nm. The studies were performed at least 3 times in duplicates.
For LDH determinations cells were previously seeded overnight and treated for 24 and 48 h in 12-well plates in a final volume of 1 mL. Cell cytotoxicity was determined by lactate dehydrogenase (LDH) assay using a kit according to manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany). Experiments were performed at 24 and 48 h at least 3 times in duplicates and 2% of Triton X-100 was used as a positive control.

6. Cell cycle analysis by flow cytometry

The cell cycle of PCa cells was analyzed, whereas apoptosis was quantified by measuring sub-G1 peaks by flow cytometry after 24 and 48 hour cell treatment. Cells were fixed with ethanol and stained with propidium iodide (Sigma, MO, USA) as previously described (Gibson et al., 2002). Briefly, $5 \times 10^5$ treated cells were centrifuged at 3500 rpm for 5 minutes and cells were washed with 5 mL of PBS 1X. Moreover, cells were resuspended in 100 uL of PBS 1X and 900 uL of 70% cold ethanol. Cells were vortexed and incubated for 5 minutes at 4°C. After, cells were centrifuged again and washed twice with 2 mL of PBS 1X. Furthermore, cells were resuspended in a mix of 250 uL of PBS 1X and 250 uL of DNA extraction solution (190 ml Na$_2$HPO$_4$ 0.2 M, 8 ml citric acid 0.1 M, pH7.8) and incubated 10 min at 37 °C. Cells were centrifuged and resuspended in 250 uL of RNase 1X and 40 ug/ml Propidium Iodide) and incubated for 30 min at 37°C in the dark. Finally, cells were analyzed by flow cytometry.

7. In vivo biodistribution studies
7.1 Prostate cancer xenograft tumour model

5 to 6 week old SCID mice (~20 grams) were bred and maintained aseptically in our animal facility. The two tumour cell lines (DU145 and PC3) were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified CO$_2$ atmosphere, and $2.5 \times 10^6$ cells were injected subcutaneously in the right flank of each mouse. Palpable tumours were typically observed within 3 weeks following the injection of cells and
were allowed to progress until at least 500 mm\(^3\) for these studies. The volume of the tumours were measured by a manual digital caliper. For manual caliper measurements of subcutaneous xenografts the two longest perpendicular axes in the x/y plane of each tumour were measured every 2 days. The depth was assumed to be equivalent to the shortest of the perpendicular axes, defined as y. The measurements were calculated according to the following equation as is standard practice (Tomayko and Reynolds 1989):

\[
\text{Xenograft volume} = \frac{xy^2}{2}
\]

7.2 Preparation of fluorescent liposomes

Alexa Fluor 750 succinimidy ester (AF-750) was purchased from Invitrogen (Carlsbad, CA). An aqueous solution of AF-750 (2.5 mM) was created by dissolving AF-750 directly into a 1x PBS. Prior to use, the aqueous solution of AF-750 was heated at 50°C for 30 minutes and incubated overnight at room temperature to ensure complete hydrolysis of the succinimidy ester. For the in vivo studies, the AF-750 probe encapsulated within the particles was significantly quenched when loaded at a concentration above 1 mM while providing sufficient signal for optical imaging and therefore the internal concentration used in these studies was 2.5 mM. Using a partially quenched fluorophore provides an instantaneous indication of dye release due to the detectable increase in fluorescence with de-quenching and facilitates tracking of the circulating particles as well.

Liposomes were prepared by the lipid film hydration and extrusion method. Briefly, lipids dissolved in chloroform were mixed at the molar ratios DPPC:DSPC 6:4, dried into a thin film under vacuum. The dried lipid film was rehydrated in the desired fluorophore solution (2.5 mM) to create a lipid concentration of 50 mg/ml and warmed at 60°C for 50 minutes with gentle vortexing every 10 minutes. The resultant multilamellar lipid vesicles were extruded through a 100 nm polycarbonate filter at 60°C, which is above the liposomal phase transition temperature. The
unencapsulated fluorophore (calcein or AF-750) was then separated from the liposome vesicles by dialysis and collected to a final lipid concentration of 3 mg/ml. The mean diameter of the liposomes (100 ± 20nm) was verified using a Nanosizer.

### 7.3 In vivo imaging

Live animal fluorescence optical imaging monitored the in vivo biodistribution of fluorescently-loaded liposomes using the IVIS Caliper system (Caliper Life Sciences Corp., Alameda, CA). Images were acquired and analyzed with Living Image software 2.5 (Caliper Life Sciences Corp.) with identical illumination settings for all images. The system settings were: binning = 4, exposure time = 5 seconds, field-of-View = 15, f-Stop = 2 and filters with an excitation of 705–780 nm and emission of 810–885 nm. Data are displayed in the unit value of efficiency and represent the ratio of light emitted to light incident. Mice were sedated via inhalation of isoflurane/oxygen and injected with a volume of 150 μL of liposome solutions, such as Alexa 750 encapsulated liposomes (LIP-Alexa750) and VIP functionalized ones, (VIP-LIP-Alexa750) via the tail vein. In vivo images were acquired over 12 hours in four positions (ventral decubitus, right lateral decubitus, left lateral decubitus and dorsal decubitus) and fluorescence of major organs was differentiated.

### 7.4 Ex vivo tumour imaging

Animals were sacrificed immediately following fluorescence imaging and xenograft tumours were excised. Only one untreated tumour was used for this group as a background reference in order to use the other two mice for following experiments. Images were normalized with common minimum and maximum values. The Region of Interest (ROI) were drawn on the DU145 tumours and the average signal intensity within the ROI was used for subsequent quantitative analysis. The fluorescence intensity of DU145 tumour based on in vivo NIRF imaging was presented as mean ± SD (n=3).
Section 2

III. RESULTS AND DISCUSSION
1. Prostate cancer cell lines characterization

1.1 VPAC1 gene expression study by qPCR

The VPAC1 mRNA expression study by qPCR revealed that all of the PCa cell lines expressed VPAC1 and no mRNA was detected for HFF-1. However, the abundance of mRNA varied considerably between the different cell lines. LnCAP showed the highest VPAC1 mRNA expression, almost 100 times more than DU145 expression levels and 1000 times more than PC3 (Figure 20). DU 145 showed an intermediate expression level between LnCAP and PC3 (which showed the lowest expression levels for VPAC1).
Figure 20. VPAC1 gene expression. A) Relative Quantification of VPAC1 versus Cyclophilin B reference gene in 4 cell lines, HFF-1, LnCAP, DU145 and PC3. B) Agarose-gel showing the DNA amplified bands for Cyclophilin B and VPAC1 genes. From left to right, a water control to discard any contamination, M corresponds to the DNA markers, HFF-1, DU145, PC3 and LnCAP cyPB and VPAC1 DNA amplification bands respectively. PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were of the expected sizes of 324 bp (VPAC1) and 193 bp (CyPB).

1.2 VPAC1 protein expression study by WB

The VPAC1 protein expression study by Western Blot revealed that all of the PCa cell lines expressed VPAC1 protein. LnCAP showed the highest VPAC1 protein expression, in accordance with qPCR results. But PC3 and DU145 showed very similar amounts of VPAC1 (normalized against internal GAPDH controls), showing slightly higher amount in PC3 than in DU145 (Figure 21A). HFF-1 (non-prostatic cell line used as control) showed a weak VPAC1 signal, although it seems to be due to inespecificity, as two bands are observed close to the 55 KDa region (Figure 21B) in the Western Blot.
Figure 21. VPAC1 protein expression in PCa cell lines. A) Normalized values of VPAC1 against internal GAPDH controls for each cell line. B) VPAC1 (62.5KDa) and GAPDH (37 KDa) proteins determined by Western Blot for each cell line.

2. Characterization of VIP-liposomes

2.1 Size and surface characterisation: DLS and cryo-TEM.
In this study VIP functionalised liposomes were prepared consisting of 90 wt% DPPC and DSPC (molar ratio 6:4), 5 wt% DSPE-PEG2000 and 5 wt% DSPE PEG-Mal through the well-known reverse phase evaporation method. Dynamic light scattering (DLS) measurements showed that the size and surface potential of the formed liposomes were 120 nm ± 2.66 (Figure 22). The size was also measured by cryogenic transmission electron microscope (Cryo-TEM) (Figure 23).

Figure 22. Liposome size measurements by DLS. This data shows an average size of 120 nm for three independent measurements, each one as a result of 10 readings.
Figure 23. (A) Scheme of VIP functionalization via DSPE-PEG-Mal and liposome composition. (B) Cryo-TEM of VIP-liposomes (Scale bar 200nm). The image corroborates DLS results, showing an average size of approximately 120 nm.
2.2 Peptide functionalization quantification.

The peptide was bounded to the liposome surface by via its cysteine to the maleimide-modified termini of PEG chains as shown in Figure 23A. The quantification of the peptide was performed after the dialysis of the liposome for 24 h, to ensure the quantification of the coupled VIP-liposome. The result for VIP quantification was calculated using the plotted standard curve (Figure 24) and the amount of functionalized peptide was an average of 15 ug/mL in a 2 mM liposome solution (Figure 25).

\[
y = 0.971x - 3.264 \\
R^2 = 0.990
\]

Figure 24. Standard curve for functionalized VIP determination. The standard curve was plotted as fluorescence over peptide quantity (log10 fluorescence vs log10 peptide quantity).
3. In vitro assays of doxorubicin encapsulated liposomes

3.1 Doxorubicin encapsulation efficiency and stability studies

Liposomes encapsulated an average of 0.42 mg of DOX in a 4 mM lipid concentration. Liposomes with and without VIP and with and without doxorubicin were also prepared as controls for the following experiments. The encapsulation efficiency of the drug was determined an average of 95%.

To evaluate the stability of doxorubicin encapsulated liposomes in HBS and in physical conditions such as 50% serum, liposomes and VIP-liposomes were incubated at 37°C and samples were analyzed every 15 and 30 minutes and at each hour for 5 hours. The release of the drug was measured by a fluoremeter and samples of HBS and 50% serum were used as a reference. Figure 26 depicts the stability of both formulations over time. No difference of stability was observed between the two formulations in HBS, but in serum VIP-liposomes seem more stable, which can be explained by the presence of the

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Figure 25. Quantification of functionalized VIP. Total VIP-lipdox refers to the subtraction of lip-dox to VIP functionalized liposomes due to the interference with doxorubicin’s fluorescence.
peptide on the vesicle that reduces the interaction of the liposome with serum proteins. Rapid release was observed from the non-functionalized liposomes, which released 30% of encapsulated doxorubicin after 30 minutes and 40-60% between 2 and 5 hours. By contrast, VIP-liposomes showed the same release profile as in HBS.

Figure 26. Liposome stability analysis of doxorubicin encapsulated liposomes and VIP functionalized liposomes. A) In HBS and B) Serum.
3.2 In vitro cell uptake

VIP conjugation to liposomes increases visible DOX uptake in VIP-receptor expressing PCa cells. Cells were treated with LIP-DOX, VIP-LIP-DOX and equivalent amount of DOX. PC3, DU145 and LnCAP cells treated with DOX showed red fluorescence mostly in the nucleus (Figure 27). In this case, DAPI staining was not as intense as with the other treatments, due to the previous and quick entrance of DOX into the nucleus, while in the case of liposome treatments, DOX was also observed in the cytoplasm of the cells. Moreover, LIP-DOX and VIP-LIP-DOX had lower intensity in the nucleus and DAPI staining was more visible, but the cells showed higher drug uptake with VIP-LIP-DOX than the untargeted liposomes. This observation is very clear as the red fluorescence is higher in nucleus and cytoplasm in DU145, PC3 and LnCAP cell lines, all of them VPAC1 expressing receptors. This effect is not observed in HFF-1, which doesn’t express VPAC1 or expresses very low levels. In this case, no differences are appreciated between VIP-LIP-DOX and LIP-DOX treatments.
Figure 27. Cellular uptake after 2h incubation by fluorescence microscopy. From A to D, HFF-1, LnCAP, DU-145 and PC3 cells respectively incubated with complete media, lip-dox, VIP-lip-dox and DOX. The blue colour indicates DAPI staining whereas the red colour indicates the uptake of DOX. Purple colour is produced due to the combination of blue and red colour.

3.3 Viability Studies

3.3.1 Optical Microscopy imaging

VIP conjugation to liposomes increases cell death in VIP-receptor expressing PCa cells. After 24 and 48 hour treatments with LIP, LIP-DOX, VIP-LIP, VIP-LIP-DOX and DOX the cells were observed under the optical microscope to evaluate if there was any morphological changes in the cells and if cell death detection was comparable to other cytotoxicity experiments.

After 24 hours, (Figure 28-30) LnCAP cells seemed more sensitive to DOX, LIP-DOX and VIP-LIP-DOX treatments when comparing to the other two cell lines (DU145 and PC3). In the case of LnCAP cells (Figure 28), higher number of dead cells were detected with VIP-LIP-DOX (Figure 28F) treatments than the rest of
DOX formulations (LIP-DOX and DOX). This could be due to a different endocytosis pathway, which in this case could be mediated by receptor. Another interesting observation was detected in Figure 28E and 31E that correspond to LnCAP VIP-LIP treatments after 24 and 48 h. Here, no cell death was observed, as there is no drug in the liposomes, but the morphology of the cells changed, showing long prolongations as much neurite-like processes. This effect was not observed in the case of LIP treatments (Figure 28C and 31C), so this suggests that VIP induced this differentiation in LnCAP cells after 24 and 48 hours of treatment.

In the case of DU145 and PC3, no differences were observed between LIP and VIP-LIP treatments at the two different time points. But DU145 and PC3 also showed higher cell death under VIP-LIP-DOX treatments after 24 (Figure 29 and 30) and 48 h (Figure 32 and 33) in than the other DOX formulations, although not as obvious as observed with LnCAP cells. PC3 cells seemed to be the more resistant than the other two cell lines, as clear differences between treatments weren’t observed until 48 hours (Figure 30 and 33).
Figure 28. LnCAP cells imaging after 24 hour treatment by optical microscopy.
A) Untreated, B) DOX, C) LIP, D) LIP-DOX, E) VIP-LIP, F) VIP-LIP-DOX.
Figure 29. DU145 cells imaging after 24 hour treatment by optical microscopy. A) Untreated, B) Doxorubicin, C) Liposome, D) LIP-DOX, E) VIP-LIP, F) VIP-LIP-DOX.
Figure 30. PC3 cells imaging after 24 hour treatment by optical microscopy.
A) Untreated, B) Doxorubicin, C) Liposome, D) LIP-DOX, E) VIP-LIP, F) VIP-LIP-DOX.
Figure 31. LnCAP cells imaging after 48 hour treatment by optical microscopy.

A) Untreated, B) Doxorubicin, C) Liposome, D) LIP-DOX, E) VIP-LIP, F) VIP-LIP-DOX.
Figure 32. DU145 cells imaging after 48 hour treatment by optical microscopy. A) Untreated, B) Doxorubicin, C) Liposome, D) LIP-DOX, E) VIP-LIP, F) VIP-LIP-DOX.
3.3.2 Citotoxicity and viability determination by LDH and MTT assay.

In vitro viability and cytotoxicity was assessed by MTT and LDH assays (Figure 34 and 35). The cells were exposed to the different formulations for 24 and 48 hours. Each experiment represents the average of at least three different experiments. The cells were exposed to free doxorubicin, doxorubicin encapsulated liposomes, VIP-doxorubicin liposomes and the same liposome
formulations without the drug used as controls. PC3 cell line did not show differences between the two doxorubicin liposome formulations (with and without VIP) and showed very similar cytotoxicity and viability after 24 and 48 h. By contrast, LnCAP shows higher cytotoxicity and lower viability after 24 and 48 h when treated with VIP targeted doxorubicin liposomes.

As shown by PCR it seems that LnCAP express higher levels of VPAC1 receptor than DU145 and PC3. This could be the cause why a higher effect is observed after incubation with VIP-doxorubicin liposomes which enhances the cytotoxicity due to a higher uptake and also an activation of intracellular signalling due to the G-coupled receptors. It has been described that VIP can induce a cellular differentiation n LncAP cells (Gutierrez-Canas, Juarranz et al. 2005), which could sensitize the cells to the doxorubicin treatment.
Figure 34. 24 h cytotoxicity studies by LDH and MTT assay on LnCAP, DU145 and PC3 prostate cancer cell lines. Values are mean ± S.D * P<0.1, ** P<0.05, *** P<0.01
Figure 35. 48 h cytotoxicity studies by LDH and MTT assay on LnCAP, DU145 and PC3 prostate cancer cell lines. Values are mean ± S.D. * P<0.1, ** P<0.05, *** P<0.01
3.4 Apoptosis and cell cycle analysis by flow cytometry

Doxorubicin inhibits cell growth and proliferation primarily through cell cycle arrest and apoptosis inducing mechanism. To further confirm the therapeutic potential of the targeted delivery system, in this case VIP-LIP-DOX, cell cycle analysis was performed using flow cytometry. Figure 35 and Figure 36 show the relative cell cycle blocking activities and the subG1 phase increase due to apoptosis at 24 and 48 hours respectively. SubG1 is used to detect cells that have lost some of their DNA in late stage of apoptosis process following endonucleases activity. Here, the number of hypodiploid cells undergoing this process can be counted in subG1 region of PI histogram. Differences upon 24 and 48 h treatments with free drug and targeted and non-targeted drug liposomes were observed. In the case of 24 h treatments, VIP-LIP-DOX treatment showed the highest cell cycle arrest in SubG1 phase in the case of LnCAP cells but not in the case of PC3 and DU145, which showed these same results but after 48 h. It seems that PC3 and DU145 are more resistant to DOX treatments and that clear differences start to be appreciated after 48 h. This is not the case for LnCAP, where notable differences are observed after 24 h treatments, and after 48 h LIP-DOX and VIP-LIP-DOX reach the same values.

The results observed in the Figure 36 and Figure 37 agree with the cytotoxicity results (Figure 34 and Figure 35), showing a higher cell death effect with targeted liposomes on LNCAP cells than on PC3 and DU145 cells, which show less difference between untargeted and targeted liposomes.
Figure 36. SubG1 analysis by flow cytometry after 24 hours treatment on LnCAP, DU145 and PC3 cells.
Figure 37. SubG1 analysis by flow cytometry after 48 hours treatment on LnCAP, DU145 and PC3 cells.
4. In vivo studies of fluorescent VIP-liposomes

**Animals and Tumours.**

SCID mice were bred and maintained aseptically in our animal facility and tumours were grown subcutaneously in 5–6-week-old mice (~25 grams). The two tumour cell lines (DU145 and PC3) were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified CO₂ atmosphere, and 2.5 x10⁶ cells were injected subcutaneously in the right flank of each mouse.

**3.1 Biodistribution studies in mouse xenograft models**

Biodistribution Near Infrared (NIR) fluorescence studies in DU145 xenograft tumour-bearing mice showed that LIP-Alexa750 and VIP-LIP-Alexa750 have long circulation life-times in the blood. The results revealed a gradual increase in tumour fluorescence, indicating accumulation of the liposomes reaching plateau levels at 12 hours post injection. After 12 hours high fluorescence signal was detected in the upper back blood pool (Figure 38 and 39), specially in the case of LIP-Alexa750. In the case of VIP-LIP-Alexa750 higher fluorescence signals were detected in the tumour site. These results indicate that both formulations have long circulation times, as they could be detected in the blood and in the tumour, but higher tumour accumulation was observed with VIP-LIP-Alexa750 than with LIP-Alexa 750 showing an effective targeting system to the prostate cancer cells.

12 hours postinjection, the mice were anesthetized and sacrificed and the fluorescence intensity was measured in liver, spleen, kidneys, lungs, heart, brain and tumour (Figure 40). Accumulation in the kidneys and spleen was observed in the case of VIP-LIP-Alexa750 treatments, whereas higher intensity was detected in the kidneys in the case of LIP-Alexa750 and almost no signal was detected in the spleen.
SECTION 2: RESULTS AND DISCUSSION

Figure 38. Scheme of areas where NPs with long blood circulation properties can be detected in a ventral decubitus mouse position.

Figure 39. Biodistribution NIR fluorescence images 12 h post. From left to right, two independent experiment images of untreated, LIP-Alexa 750 and VIP-LIP-Alexa 750.
Figure 40. Organ NIR fluorescence biodistribution 12 h post injection. From left to right, brain, liver, kidneys, heart, spleen, lung, skin and tumour.

3.2 Tumour fluorescence quantification

*Ex vivo* imaging studies were performed after 12 hours of intravenous injection in order to quantify the fluorescence signal in the treated tumours and to subtract the background of an untreated one (Figure 41). These results show
that both formulations, LIP-Alexa750 and VIP-LIP-Alexa750 accumulate in the tumour, due to the EPR effect (explained in page X), but higher signals are detected in tumours treated with VIP-LIP-Alexa750 due to tumour targeting and higher cell uptake as occurs \textit{in vitro}. The findings demonstrated the distribution of VIP-LIP-Alexa750 and proved that they experience increased tumour uptake and prolonged circulation half-life.

Figure 41. \textit{Ex vivo} tumour fluorescence imaging and quantification 12 h post injection of VIP-LIP-Alexa750 and LIP-Alexa750 formulation.
Section 2

IV. CONCLUSIONS
Conclusions

- We have developed a reliable method to synthesize homogenous, stable and properly characterized VIP functionalized liposomes.

- VIP-liposomes demonstrated significant cellular binding and uptake by VIP receptor expressing cells (PC3, DU145 and LnCAP) in contrast to unconjugated liposomes.

- VIP-liposomes showed higher therapeutic efficacy in VIP receptor expressing cells than unconjugated liposomes confirmed by cytotoxicity and apoptosis studies.

- *In vivo* biodistribution studies showed VIP-liposome accumulation in liver, kidneys and spleen, whereas unconjugated liposomes showed preference for liver and kidneys after 12 hours of intravenous injection.

- *In vivo* biodistribution studies showed higher VIP-liposome accumulation in the tumour in comparison to unconjugated liposomes after 12 hours of intravenous injection.
V. APPENDICES
## 1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>ADNP</td>
<td>Activity Dependent Neuroprotective Protein</td>
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<tr>
<td>AgNPs</td>
<td>Silver Nanoparticles</td>
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<tr>
<td>AuNPs</td>
<td>Gold Nanoparticles</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>BRB</td>
<td>Blood Retina Barrier</td>
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<td>BTB</td>
<td>Blood Testis Barrier</td>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine Mono-Phosphate</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CT</td>
<td>Computerized Tomography</td>
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<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention</td>
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<tr>
<td>HBS</td>
<td>Hepes Buffer Saline</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>NFkB</td>
<td>Nuclear Factor kappa-light chain- enhancer of activated B cells</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>PACAP</td>
<td>Pituitary AdenylateCyclase-Activating Peptide</td>
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<td>PCa</td>
<td>Prostate Cancer</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PEG</td>
<td>Polyethylene Gycol</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
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<tr>
<td>ROI</td>
<td>Region of Interest</td>
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<tr>
<td>SERS</td>
<td>Surface-enhanced Raman Spectroscopy</td>
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<td>SP</td>
<td>Surface Plasmon</td>
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<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
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<td>VIP</td>
<td>Vasoactive Intestinal Peptide</td>
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<tr>
<td>VPAC1</td>
<td>Vasoactive Intestinal Peptide Receptor 1</td>
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15. (Esposito, Marchisio et al. 2012)
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