Chapter 1

General Introduction and Scope of the Thesis
1. Nanomedicine

Nanomedicine, the application of nanotechnology for medical purpose, has attracted a great attention in the past few decades. Thanks to their unique physiochemical properties such as nanoscale size, large surface area to mass ratio and high reactivity, various nano-sized materials including liposomes, micelles, polymeric nanoparticles and inorganic nanoparticles have been proposed as novel tools to deliver drugs, genes, and other agents for therapy and/or diagnosis [1–4]. Compared to conventional drugs that might diffuse nonspecifically after administration, nanoscale materials - benefiting from their size - can passively accumulate through the well-known enhanced permeability and retention (EPR) effect in tumors, taking advantage of the leaky blood vessels and poor lymphatic drainage that characterize many solid tumors (Fig. 1) [5–7]. Alternatively, nanomaterials can be used for active targeting by functionalizing their surface with ligands that bind to specific receptors expressed on the cell surface at the disease site (Fig. 1) [8–10]. In addition to their application for active and passive targeting strategies, nano-sized drug delivery systems also allow improving the solubility of poorly-soluble drugs, carrying large drug payload, prolonging blood circulation half-life, bypassing chemoresistance, and they can be designed to release drugs in a sustained and controlled way [2–4]. These aspects altogether enhance the pharmacokinetics of therapeutic agents and reduce potential side effects [3,11].

Among various nanomaterials, liposomes are considered as one of the most clinically established drug delivery systems [3,12]. Since the introduction on the market in 1995 of the first liposomal formulation, named Doxil, to treat AIDS-related Kaposi’s sarcoma, the application of liposomes in the field of nanomedicine has attracted a lot of attention, and several liposomal formulations have been approved for clinical use [13–16]. Liposomes are vesicles consisting of one or more phospholipid bilayers enclosing discrete aqueous volumes. This unique structure endows liposomal delivery systems the ability to entrap a diverse range of drugs, including hydrophobic molecules inserted into the lipid bilayer and hydrophilic molecules entrapped in their aqueous core (Fig. 2), thus protecting the loaded drugs from the external environment and from degradation [16,17]. Owing to their self-assemble structure and controllable synthetic identity, liposomes with a wide range
of physicochemical properties can be easily formulated (Fig. 2) [13,18]. For instance, liposomes with different surface charge can be obtained by simply changing their lipid composition using neutral, positively charged and negatively charged lipids [19]; long circulating liposomes can be obtained by addition of hydrophilic polymers such as polyethylene glycol (PEG) on their surface [20], and actively targeted formulations can be designed by functionalizing the liposome surface with multiple ligands [18]. Overall, combined with their biocompatibility and biodegradability, all these features make liposomes a versatile tool to deliver efficiently various agents for diagnosis and/or therapy [16,17].

**Figure 1.** Schematic representation of nano-sized materials accumulating in diseased tissue (tumor) by passive targeting and active targeting. Passive tissue targeting can be achieved by extravasation of nanoparticles via the so-called EPR effect, and active cellular targeting can be realized by functionalizing nanoparticles surface with ligands to promote cell-specific recognition and binding. Fig. 1 is adapted with permission from Peer et al. [1].
2. Biological challenges of nanomedicines for their clinical translation

Despite the great advantages of nanomaterial-based drug delivery systems and thousands of papers on nanomedicine published every year, nanomedicines remain hard to be translated into clinical formulations to benefit patients, and currently relatively few nano-formulations have been approved for marketing [21–24]. In 2012, for instance, 5 nano-carriers received great attention and media coverage when they entered into clinical trial, while all of them were terminated by 2016 because of lack of efficiency [21,25]. Additionally, in 2016, after surveying the literature from the previous decade, Wilhelm et al. reported that on average only 0.7% of the administrated nanoparticle dose is delivered to solid tumors [26], which led to an important debate on the success of this technology so far. Overall, there are various reasons that contribute to the slow translation of nano-formulations from bench to beside and limit their efficacy. One of the major obstacles is the so far still limited understanding of the basic mechanisms of interactions of nanomaterials with biological systems [8,18,22,23,27].

![Figure 2. Schematic representation of different types of liposomal formulations as drug delivery systems. (A) Conventional liposome that consists of lipids of various charge and is capable to load both hydrophilic and hydrophobic drugs. (B) PEGylated liposome that is coated with hydrophilic](image-url)
polymers, such as PEG, on its surface acquiring prolonged circulation time \textit{in vivo}. (C) Ligand-targeted liposome that can realize specific targeting thanks to the presence of various targeting ligands, such as peptides and antibodies, on its surface. (D) Theranostic liposome that combines imaging agents and therapeutic components in one system. Fig.2 is adapted with permission from Sercombe et al. [18].

At an organism level, once introduced in the body, nanomaterials have to overcome multiple defense systems such as the reticuloendothelial system (RES) and opsonization, which aim to recognize, neutralize and eliminate foreign substances [18,28]. These defense systems can affect the biodistribution of nanomaterials and promote their clearance, leading to the reduction of their therapeutic efficiency [18,29]. However, achieving a clear understanding of the immune responses to nano-sized materials is still a challenge for scientists and regulatory agencies [30,31]. In addition, disease pathophysiology and heterogeneity can have strong effects on nanomedicine behavior and efficacy [22,23]. For instance, the EPR effect has been widely exploited for passive targeting of tumors, however a recent study performed by Sindhwani et al. showed that in some tumors gaps in tumor vessels allowing nanoparticle extravasation can be rare and on the contrary up to 97% of nanoparticles enter tumors utilizing an energy-dependent pathway through endothelia cells [32]. Additionally, recent reports indicated that many nano-formulations often show negative outcomes in clinical trials because of the poor translation of the results observed in animal models [21,26]. This suggested that differences in the anatomy and physiology between animal species and humans should be taken into account in order to choose better animal models for preclinical studies.

Going down to cellular and intracellular levels where ultimately nanomaterials need to arrive for their action, understanding the extracellular and intracellular nano-bio interactions and controlling the entry and trafficking of nanomaterials into cells remain other major final challenges for the development of successful nanomedicines [33].

Nanomaterial interactions at cellular level include effects due to the modifications they encounter after interaction with biological fluids, the subsequent interactions with the cell membrane, and finally nanomaterial intracellular trafficking and fate.
Before their first interactions with cells, it is known that nanomaterials, once introduced in a biological environment, absorb numerous proteins and other biomolecules on their surface, forming a layer called “protein corona” [34,35]. This corona layer confers nanomaterials a new biological identity by modifying their charge, size and interfacial properties, affecting the subsequent outcomes at cell level [27,36–42]. After these initial interactions with the biological fluids in which they are applied, nanoparticles eventually will reach the membrane of cells and usually enter cells through endocytosis [33]. In order to achieve efficient uptake of nanoparticles in the targeted cells, a better understanding of these first interactions at cell level and nanoparticle internalization mechanism is necessary [43–45]. After their entry, the intracellular fate of nanoparticles also plays a critical role in the success of their application, given that the load they carry (i.e. chemical drugs, proteins or genes etc.) has to be delivered to specific intracellular sites [33,44,45].

Within this context, by using liposomes as a model nanomedicine, the aim of this thesis is to gain a better understanding of the final steps of the nanomaterial journey in biological systems and in particular their interactions at a cellular and subcellular levels. Thus we have studied the modifications liposomes encounter following the interaction with the biological environment in which they are applied, their uptake and intracellular fate, as well as the release of their cargo. A better understanding of these interactions will provide important tools to accurately predict the behavior of nano-formulations at cellular level. In this way, nanocarrier physicochemical properties can be tuned in order to achieve the desired outcomes at cell level, and this ultimately will contribute to accelerate the clinical translation of nanomedicines.

3. Extracellular and intracellular nano-bio interactions

3.1 Interactions of nanomedicines with biological fluids

Once introduced in a biological environment, nanomaterials, such as liposomes, are first exposed to biological fluids and are immediately covered with plenty of proteins and other biomolecules, forming a layer known as “protein corona” [34,35]. Generally, the corona includes a soft and a hard corona (Fig. 3A). The hard corona
is constituted by the proteins that tightly attach on the nanoparticle surface with high affinity, while the soft corona includes the weakly bound proteins of the outer corona layer, which is very dynamic and exchanges with surrounding biomolecules rapidly [27,46]. On the contrary, due to their high affinity, hard corona proteins have a low exchange rate, thus they have a long residence time on nanomaterials, and because of this they can affect the interactions at cell level, including for instance recognition by cell receptors and the subsequent mechanism of endocytosis (Fig. 3B) [27,36]. Indeed, rather than the bare nanoparticle surface, it is the nanoparticle-corona complex that constitutes the real biological unit that is actually ‘seen’ by cells (Fig. 3B) [27,36,38–41]. For instance, Salvati et al. showed that transferrin-modified nanoparticles lose their targeting abilities because of screening of the targeting ligands after protein corona formation on the particle surface [37], and Digiacoimo et al. demonstrated that after corona formation the internalization pathway of bare liposomes via micropinocytosis was changed to clathrin-dependent endocytosis [47].

Overall, corona formation is a dynamic process, which depends on both the physicochemical properties of the nanomaterials and the properties of the biological environment in which nanomaterials are applied [27].

**Figure 3.** The nanoparticle-corona complex in a biological environment. (A) Proteins absorb strongly to the bare nanoparticle surface (e.g. \( k_1 \)) forming a tightly bound layer of hard corona proteins, while the proteins that exchange rapidly with the environment (e.g. \( k_2 \)) constitute the soft corona layer. (B) The nanoparticle-corona complex constitutes the real unit that interacts with cells. Fig.2 is adapted with permission from Monopoli et al. [36].
3.1.1 Effects of nanomaterial physico-chemical properties on corona formation

It is known that the physicochemical properties of nanomaterials, such as size, charge, topography, curvature, surface chemistry and surface hydrophobicity/hydrophilicity, are some of the critical factors that determine protein corona composition [33,48,49]. Because of the difficulty in predicting nanomaterial behavior after corona formation, different strategies have been developed to “passivate” the nanomaterial surface and reduce protein binding [27,50,51]. Polyethylene glycol, PEG, is currently the most commonly used polymer for reducing protein adsorption thanks to its hydrophilic properties [52]. For example, by modifying gold nanoparticle surfaces with introducing PEG at increasing density, Walkey et al. showed that the total serum protein adsorption was decreased, and this reduced uptake by macrophages [53]. However, in this and other similar studies, it was also shown that protein adsorption cannot be completely suppressed by PEG modification. Additionally, recent works also demonstrated that the stealth properties of PEG are conferred by a specific protein, *i.e.* clusterin, adsorbed in the corona of PEGylated nanomaterials, rather than the reduction of protein adsorption due to PEG [54]. Besides, it has emerged that PEGylation suffers several drawbacks, such as oxidation of PEG in physiological environments and aggregation in high salt solutions, and can lead to the production of antibodies against PEG itself [27,52].

In addition to PEG, several novel anti-fouling polymers have been proposed as potential alternatives, which include polyoxazolines, dendrons, polysaccharides, polypeptoids, and zwitterionic polymers [27]. Besides, pre-absorbing specific proteins on the nanoparticle surface can be also used to suppress serum proteins adsorption [52]. For instance, nanoparticles with a preformed albumin corona showed significant reduction of plasma protein adsorption once applied *in vivo* and this led to reduced cytotoxicity and longer plasma residence time [55]. Similarly, clusterin, which as mentioned above was discovered to play an important role in the stealth effect of PEGylated nanoparticles [54], can be used as another protein candidate to pre-coat the nanoparticle surface for reducing particle clearance. Though this strategy may be reasonably successful for abundant and stable proteins (e.g. albumin), it may remain challenging for low abundant, enzymatically active and unstable
proteins, since in these cases protein purification can be tedious, expensive and may damage their conformation and activity [27].

At the same time, it is also emerging that specific corona proteins can be recognized by cell receptors, thus they can be used as targeting molecules [56,57]. Lara et al. reported that the corona proteins on the surface of 100 nm silica nanoparticles presented functional epitopes of low-density lipoproteins and immunoglobulin G which can be recognized by specific cell receptors [57]; Caracciolo et al. demonstrated that lipid particles made of DOTAP lipid and DNA can enrich vitronectin after interaction with human plasma and thus promote particle internalization in cancer cells highly expressing the vitronectin αvβ3 integrin receptor [58]; similarly, Zhang et al. developed β-amyloid peptide modified liposomes which can absorb plasma apolipoproteins, and expose receptor-binding domains on their surface to achieve brain targeted delivery [59]. Therefore, instead of trying to avoid corona formation, manipulating corona formation to recruit specific proteins on the nanoparticle surface during their interaction with biological fluids can be used as an alternative strategy to control nanoparticle behavior and promote targeting [27][52].

In order to explore similar strategies to control protein corona formation or to exploit it for novel targeting applications, it is essential to understand how the physicochemical properties of nanoparticles affect their corona composition and how this resulting corona in turn influences nanomaterial outcomes in vivo and at cell level (Fig. 4). Recently, different efforts have been made to connect the synthetic properties of nanoparticles to their biological identities and their biological response. For instance, Walkey et al. established a quantitative model using nanoparticle properties and their serum protein corona composition to predict cell association of a gold nanoparticle library [60]; Bigdeli et al. exploited quantitative structure-activity relationship (QSAR) approaches to correlate the physicochemical properties of liposomes and their protein corona fingerprints to their cellular uptake and viability [61]; and Lazarovits et al. used supervised learning to predict the biological fate of engineered nanoparticles in vivo from the evolution of corona protein patterns on their surface [42].
Overall, a deeper understanding of how nanomaterial physico-chemical properties affect corona formation and – in turn – their cellular outcomes is still required. Within this context, in **Chapter 2** we have used liposomes as a nanomedicine model to prepare a series of nanoparticles with tailored surface properties by mixing zwitterionic and negative charge lipids at different ratio in a systematic way. Their uptake efficiency and kinetics were determined, and the liposome-corona complexes were isolated after exposure to human serum for detailed characterization. In this way, we have obtained liposomes with very different uptake behavior and correlated the abundance of specific corona proteins on the different liposomes with the uptake kinetics in cells. Similar approaches can be used to tune corona composition in a systematic way in order to optimize liposome or other nano-formulations for specific applications.

### 3.1.2 Effects of the biological environment on corona formation

Beside nanoparticle properties, the biological fluids also play an important role in the interaction of nanoparticles with surrounding environment and the resulting
corona. It is known that the corona can be seen as a ‘memory’ of the journey of the nanoparticles through different biological environments [36,62]. When nanoparticles translocate from one biological environment to another, for instance for inhaled nanoparticles from lung fluids through epithelial cells to the bloodstream, their corona will contain not only proteins from the fluid in which they are found (e.g. serum proteins), but also components from the previous environments in which the nanoparticles have transferred (e.g. lung surfactant and membrane components) [36,63]. This is due to the distinctive stability of the hard corona which can be retained at least in part as the nanoparticles transfer to a new environment [36]. At the same time, the corona will also be modified because nanoparticles may be exposed to novel proteins with higher affinity for their surface, capable to displace components of the original corona [36,62].

Indeed, multiple studies have demonstrated that even subtle differences in biological fluids can lead to discrepancies in the corona forming on the same nanoparticles and in the following cell response [36]. These include, for instance, serum heat inactivation [64], serum concentration [65], cell culture media [66], and the species from which the serum is obtained [67], among many others. For instance, Müller et al. showed that the plasma of humans, mice, rabbits and sheeps had very different protein composition (e.g. immunoglobulin), and the stability and corona of nanoparticles was strongly affected by the source of the plasma in which they were dispersed [68]. Notably, they also observed that in many cases the differences in corona composition after dispersion in different plasma were more significant than the differences in the corona forming on nanoparticles with different surface modifications [68]. Similar observations clearly indicate the importance of exact matching of the media for comparative experiments. Similarly, many studies have demonstrated that the nanoparticle-protein corona forming in human serum/plasma is different from that formed in standard cell culture medium supplemented with low amount of fetal bovine serum (FBS) used for in vitro testing, and also from the corona formed in mouse plasma in in vivo studies (Fig. 5). These differences affected also cellular uptake efficiency [67–70]. Overall, these observations suggest that the biological response of nanoparticles obtained in in vitro studies may not accurately predict nanoparticle behavior in vivo. Similarly, outcomes observed in laboratory
animals may not directly translate into humans once nanomedicines reach the clinics [70].

**Figure 5.** Serum from different species can lead to different corona formation. In the example shown here the human protein corona (HPC) and mouse protein corona (MPC) of functionalized SiO$_2$ nanocarriers are compared. Fig.6 is adapted with permission from Solorio-Rodríguez et al. [67].

Next to effects due to differences in corona composition, other factors, often overlooked, when comparing nanoparticle outcomes in different biological fluids are those due to the presence of excess free biomolecules in solution. For instance, it is a common observation that nanoparticles dispersed in FBS have much higher cell uptake than when they are dispersed in the same concentration of human serum, and that increasing human serum concentration can further reduce nanoparticle uptake [37,57,65,71]. On the one hand, the different cellular uptake efficiency might result from the different corona forming in different biological fluids, as discussed above. On the other hand, the excess free serum proteins in solution may also contribute to such differences (Fig. 6). In fact, serum proteins can be recognized by cell receptors, for example, the free apolipoprotein B in serum can bind to the low-density lipoprotein receptor (LDL receptor), apolipoproteins A-I, A-II, and A-IV can be recognized by the high-density lipoprotein receptor (HDL receptor) and immunoglobulins can be recognized by the Fc receptor [72–74]. These proteins can compete with the nanoparticles and their corona for the same receptors. Thus, when using serum from different species, because of their different composition, the concentration of these free endogenous ligands will be different, and because of this also their competition for cell receptors. What’s more, it is also likely that free serum proteins in the media will have higher affinity for cells from the same species (e.g. human serum on human cell lines), thus they can compete better for the cell receptors.
than proteins of a different species [65,71,75]. However, currently, a full understanding of how the composition of the biological environment affects nanoparticle-cell interactions is still missing and, more in particular, potential effects related to the presence of excess free proteins in solution on nanoparticle outcomes at cell level have not been considered in detail.

![NP-Corona](image)

**Figure 6.** Competition of free low-density lipoprotein (LDL) or apolipoprotein B-100 in human serum with a nanoparticle-corona complex. The corona formed on 100 nm silica nanoparticles in serum includes LDL, which is recognized by the LDL receptor (LDLR) and triggers uptake by cells. At the same time, free LDL and apolipoprotein B-100 in human serum can also bind to the same receptor, leading to receptor occupancy and nanoparticle uptake competition. Fig. 7 is adapted with permission from Lara et al. [57].

To this aim, **Chapter 3** aimed at gaining a better understanding of nanomaterial-cell interactions when using serum proteins from different species. Using liposomes, once again, as a nanocarrier model, cell uptake and corona composition were characterized for liposomes exposed in medium supplemented with FBS or human serum (HS). Next, corona-coated liposomes were isolated and added to cells in serum-free conditions or after re-exposure to media supplemented with either FBS or HS. This has allowed us to differentiate effects of the protein source on corona composition from those due to the excess free proteins in solution.
Overall, when designing nanoparticles for disease therapy or diagnosis, it is clear that the influence of biological fluids should be carefully considered and a comprehensive understanding of the effects related to both corona formation and the presence of free proteins in solution is required to optimize nano-formulations.

3.2 Cell membrane interactions and internalization of nanoparticles

When nanoparticles are exposed to cells, they will first interact with the cell membrane and in most cases they are then internalized by cells (Fig. 7). Unlike small hydrophobic molecules which can partition in cell compartments according to physicochemical equilibrium principles [76], synthetic nanomaterials, because of their size and surface, use various energy-dependent endocytic pathways to gain access into cells (Fig. 7) [77]. This constitutes one of the great advantages of nanomedicines over many conventional drugs, since nanocarriers can be designed to target specific cell types and specific pathways for the intracellular delivery of drugs and they are processed by cells using endogenous pathways [44]. However, it is clear that almost all the internalized nanoparticles are primarily routed to the degradative organelle of cells, the lysosome [77]. This constitutes a major intracellular barrier to drug delivery. Understanding and controlling the initial interactions on the cell membrane and the internalization mechanism of nanoparticle by cells may help not only to discover most efficient receptors and pathways for targeted uptake, but potentially also to discover strategies for endosomal escape and to control the intracellular fate of nanomaterials.

Figure 7. Nanomaterials interact with cell membrane and use various endocytic pathways to enter cells. Fig.8 is adapted with permission from Sahay et al. [78].
Within the different pathways cells use for internalizing exogenous materials, mechanisms of endocytosis are usually classified as clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, clathrin/caveolae-independent endocytosis, macropinocytosis and phagocytosis [78]. CME is the main mechanism that cells use to obtain nutrients and internalize membrane components. It involves the formation of invaginations of the cell membrane assisted by clathrin (together with other proteins), and usually is followed by trafficking of the internalized cargo towards the lysosomes [79]. Caveolae-dependent pathways have also been described and are believed to enable transcytosis of internalized cargoes across cells, however this kind of observations is nowadays debated, as also the existence of caveolae-mediated endocytosis [80]. Additionally, several other clathrin/caveolae independent pathways have also been described and are still being investigated [81]. Macropinocytosis is an actin-driven endocytic process, which creates large vesicles by extensions of the cell membrane that engulf larger volumes of extracellular fluids [82]. This pathway is considered important in the uptake of larger nanoparticles [83]. Finally, phagocytosis mainly takes place in professional phagocytes, such as macrophages, for the immune response to pathogens [84].

Overall, a clear understanding on how nanoparticle properties affect the mechanisms cells use for the internalization of nanomaterials is still missing. Thus several studies have tried to investigate how mechanisms of uptake change with nanoparticle properties such as size, charge, material etc. For instance, it is generally observed that charged nanoparticles have higher uptake than neutral and zwitterionic ones [13]. Zwitterionic modifications have received increased attention as a strategy alternative to PEGylation to reduce protein adsorption [27]. However, it is not known yet how zwitterionic modifications affect nanoparticle uptake efficiency, as well as nanoparticle uptake mechanism. In this context, in Chapter 4 we characterized and compared the uptake mechanism of negatively charged and zwitterionic liposomes of same size in HeLa cells. This was done by blocking key components of different endocytic pathways using a panel of common pharmacological inhibitors and RNA interference, and comparing their effects on the uptake of both liposomes. The effects of liposome or other nanocarriers formulation on the mechanisms cells use for their
internalization are other important factors that require a better understanding to allow the optimization of nanomedicines with optimal properties for specific applications.

3.3 Intracellular fate of nanoparticles and drug release

Following uptake, the next crucial step for successful drug delivery is the intracellular sorting and trafficking of nanoparticles, and ultimately the release of the carried drug [33]. The final intracellular destination of nanoparticles can strongly affect their therapeutic/diagnostic efficiency, as well as their potential side toxicity [44]. As mentioned above, it is known that almost all of the endocytosed nanoparticles are primarily routed to the lysosomes [77], which makes lysosomal targeting relatively easy. For instance, Jena et al. developed an optical reporter based on carbon nanotubes to quantitatively map lipid accumulation in the lumen of endolysosomal organelles [85]. However, for any other intracellular target, endosomal escape or cargo release during endolysosomal trafficking are required to avoid lysosome-mediated digestion of the carried drug (Fig. 8) [86]. For example, polyethyleneimine (PEI)-DNA complexes can escape from endosomes via the so-called proton sponge effect to reach the cytosol and accumulate in the nucleus for gene transfection [87].

**Figure. 8** Expected intracellular trafficking of nanoparticles. Nanoparticles can either target to lysosomes or need to escape from endosomes to accumulate in cytosol and/or for subcellular targeting. Fig.9 is adapted with permission from Pearson et al. [86].
In addition, the kinetics of cargo (e.g. genes and drugs) release from nanocarriers after internalization is also crucial for a successful nanomedicine application. For instance, despite their several advantages for clinical use, some studies pointed out that the unloading of the encapsulated cargo from liposomes following endocytosis by the targeted cells is usually a rate-limiting step [88,89]. Seynhaeve et al. have reported that free doxorubicin can kill cells more efficiently than liposomal formulations, such as Doxil, because of the fast diffusion of free drugs through cells and their quicker accumulation in nucleus compared with drugs trapped in liposomes [90]. Similarly, the commercial lipofectamine 2000, commonly used as an efficient gene delivery tool in vitro, is not suitable for therapeutic purposes because its release cannot be controlled, and it shows slow gene transfection [91]. A clear knowledge of when and where the cargo is released from a nanocarrier after its internalization by cells is required for the design of formulations with the required release characteristics.

However, nowadays, no standardized methods are available yet for testing the release of drugs from liposomal products or other nanomedicines [92][93]. The commonly used techniques for studying drug release in vitro are based on dialysis and centrifugation: the released drugs are separated from drug-loaded liposomes using either a dialysis membrane or ultracentrifugation and then quantified using methods such as UV/fluorescence spectroscopy or HPLC. Nevertheless, these methods cannot be used to determine cargo release from liposomes in complex biological environments or once inside cells, and the in vitro separation step can affect drug release rate. For instance, when using dialysis, Washington et al. suggested that the drug release rate is affected by the high concentration gradient between the large release medium in the donor compartment and the bulk phase in the receptor compartment [94]. Similarly, centrifugation-based methods can lead to drug release enhancement or even liposome damage when high centrifugal force applied [95]. Additionally, the current methods usually do not take into account the effects of protein binding and corona formation on liposome stability and release properties, as also further effects following their uptake by cells and intracellular trafficking [93].
Within this context, in Chapter 5 we have used sulforhodamine B, a membrane impermeable fluorophore, to mimic hydrophilic drugs entrapped in the inner aqueous volume of liposomes, and by combining flow cytometry analysis and microscopy imaging, we have determined liposome release behavior after internalization by cells. An ideal nanocarrier should not only have efficient drug release in the targeted cells to maximize its therapeutic effects, but also avoid cargo leakage in biological fluids to minimize side effects while it is distributed towards its target. Thus the release properties of liposomes were also studied after exposure to biological fluids and incubation in biological conditions in order to include effects of corona formation and aging of the dispersions. Having observed in the previous Chapters that liposomes with different composition showed very different uptake efficiency and uptake mechanism by cells, in this Chapter the same liposomes were used as a model in order to investigate how the different lipid composition affects liposome stability and intracellular drug release.

4. Liposome targeting with endogenous components

As mentioned above, once introduced in a biological environment, nanomaterials interact with various components, such as biomolecules and proteins from biological fluids, cell membrane receptors and intracellular components, before they arrive to their destination. These interactions are very difficult to predict and it is still difficult to tune nanoparticle properties in order to precisely control their performance in a biological environment using synthetic nanomedicines.

While several studies, such as those in the Chapters presented here, try to understand how to tune nanoparticle properties in order to control their biological outcomes, biomimetic nanotechnology is receiving increasing attention as an alternative strategy to fabricate nanoparticles with defined interactions with biological systems (Fig. 9) [96–98]. Cells are involved in various basic functions. A large portion of these cell-specific functions are governed by their plasma membrane, which regulates - for instance - the interactions with other cells, receives extracellular signals and modulates immunity [96]. Inspired from these basic functions governed by cell membranes, cell-membrane coated nanoparticles have
been recently developed as novel biomimetic nanocarriers. Cell membranes derived from different cell types are used to prepare or to coat nanoparticles, and in this way achieve naturally existing interactions that cells usually have with other cells or biological components, while reducing non-specific or unwanted interactions (Fig. 9) [97,98]. For instance, to benefit of the long circulation, inherent biocompatibility and lack of immunogenicity of erythrocytes, erythrocyte membrane-camouflaged polymeric nanoparticles have been developed. Interestingly, they showed prolonged circulation half time in vivo up to 72 h, in comparison to the same polymeric nanoparticles cores coated with the state-of-the-art synthetic stealth materials [99]. Similarly, taking advantage of the unique ability of leukocytes to accumulate to inflamed sites, leukocyte membrane doped liposomes were used to target inflamed vasculature and selectively deliver dexamethasone to inflamed tissues [100]. Additionally, several studies have suggested that cancer cell membranes can be used to achieve homotypic targeting to the cells from which they are derived, though the mechanism is not clear, and various homotypic cancer cell membrane coated nanoparticles have been developed to try to achieve tumor self-recognition and self-targeting [101–103].
Within this context, in **Chapter 6** we have used cancer cell membrane nanotechnology as a tool to study the interactions of leukemia cells with stromal cells in the bone marrow and in this way investigate new strategies to target acute myeloid leukemia (AML). It is known that acute myeloid leukemia (AML), a cancer of the myeloid line of blood cells, has high treatment failure because of drug resistance, disease relapse and difficulty of targeting due to its various mutations [104–106]. Since in 1978 Schofield proposed the concept of ‘niche’, increasing evidence has suggested that the bone marrow microenvironment (the niche) plays an important role in the development and evolution of this disease, by regulating the leukemic cells or their progenitors via secreted factors, released vesicles and via direct cell-cell interactions [107,108]. Thus, novel strategies to target the interactions of AML with its niche are being investigated. In this context, in this chapter, leukemia and stromal cell models were used to extract cell membranes and prepare cell membrane nanoparticles. Synthetic liposomes such as those used in the other chapters were doped with cell membrane components and characterized. Thus, we have compared cellular uptake levels of liposomes doped with various cell membranes in order to determine their affinities for leukemia and stromal cells. Such approaches can be used to characterize the interactions between stromal and leukemia cells and discover potential novel strategies to target them.

**5. Scope and outline of the Thesis**

Nanomedicine has been rapidly developed in the last decades and numerous nanomaterials have been proposed as drug/gene delivery tools to treat various diseases. However, the clinical translation of nano-formulations is slow and relatively few of them have been approved for clinical use so far. One of the main obstacles in the development of nanomedicines is the still limited understanding of nanomaterial interactions with biological systems.
Using liposomes - one of the most clinically established nano-formulations - as a nanomedicine model, this Thesis aims to investigate nanocarrier behavior in complex biological systems, in order to explore new strategies to guide the design of more effective nano-formulations.

**Figure 10.** The liposome journey in a biological environment. This includes three steps: i) the liposome interaction with biological fluids and the rapid formation of protein corona on their surface; ii) the cell recognition of liposome-corona complexes followed by their internalization; iii) liposome interaction with intracellular compartments and the final drug release.

The interactions of liposomes and other nanocarriers with complex biological environments involve several key steps and barriers, and these all need to be studied (Fig. 10). These include, first of all, the interactions with the biological fluids in which the nanocarriers are applied (**Chapter 2 and 3**), secondly, their interactions with the cell membrane and the nanocarrier entry into cells via different mechanisms (**Chapter 4**), and finally, the intracellular trafficking and cargo release, which also affect their final therapeutic efficacy (**Chapter 5**) (Fig. 10). At the same time, cell membrane nanotechnology can be been used to camouflage nanomaterials and develop novel drug delivery tools expressing specific biological signals on their surface, and in this way to help nanocarriers to cross some of these barriers (**Chapter 6**).
Within this context, in this Thesis, we have changed in a systematic way the synthetic properties of liposomes in order to tune the corona they form once applied in serum and modulate cell uptake efficiency and kinetics (Chapter 2). Additionally, we investigated the effect of different biological fluids - in this case serum proteins from different species – on nanomaterial-cell interactions and we have differentiated effects on corona composition from those – often overlooked - due to the presence of excess free proteins in solution (Chapter 3). Thus, we have studied how liposome charge affects the mechanisms cells use for their internalization, focusing in particular on the effects of zwitterionic modifications, given the current interests in zwitterionic particles as an alternative to PEGylation to improve the pharmacokinetics of nanocarriers (Chapter 4). Next to this, we have developed methods to study drug release kinetics of different liposomes once they are in contact with biological fluids, following corona formation and also once they are inside cells after endocytosis (Chapter 5). Finally, we have exploited cell membrane nanotechnology to dope liposomes with cell membranes as a tool to explore and characterize the interactions between leukemia and stromal cells in AML (Chapter 6).

This Thesis contributes to a better understanding of the interactions between nanomaterials and biological systems and in particular of some of the most crucial steps in the journey of nanocarriers at cellular level. A deeper knowledge on how complex biological systems affect nanomaterial behavior will help to tune nanomaterial synthetic properties to achieve the desired biological outcomes at cell and organism levels, thus overall contributing to the design of more efficient nanomedicines.
References


