Chapter 7

General Discussion and Future Perspective
Nanomedicine has been widely developed in the past few decades, with numerous nano-sized materials proposed as delivery platforms for drugs, genes and other agents to treat and diagnose various diseases [1,2]. Thanks to their unique physicochemical properties, and the possibility of being engineered and designed in many different ways, compared to conventional drugs or diagnostic reagents, nanomaterial-based delivery systems possess multiple advantages: for instance, they allow to prolong drug plasma residence time, they can protect drugs from enzymatic degradation and they can be used to deliver poorly soluble drugs, they can be engineered to achieve controlled and stimuli responsive drug release, and they can promote passive and active targeting of tumors [2,3]. However, nanomedicines still remain hard to be translated into clinical formulations and benefit patients. Though thousands of papers on nanomedicine are published each year, relatively few nano-formulations have been approved for marketing [4]. Many factors contribute to the slow clinical translation of nanomedicines, and one of the major obstacles is the still limited understanding of nanomaterial interactions with biological systems [5]. In this context, using liposomes - one of the most clinically established nan-formulation - as nanomedicine models, the aim of this Thesis is to gain a better understanding of nanomaterial behaviors in complex biological environments at a cellular level, and explore new strategies to guide the design of more effective nano-formulations.

The interactions of nanomaterials with complex biological systems involve several key steps and barriers (Fig. 1), which need to be studied one by one. First of all, once introduced in a biological environment, nanomaterials interact immediately with surrounding biological fluids and absorb numerous proteins and biomolecules on their surface, forming a layer known as “protein corona” (Fig. 1, i) corona formation) [6,7]. This corona affects charge, size, and surface properties of nanomaterials and confers them a new biological identity, which influences the following nanomaterial performance, such as cellular uptake, toxicity, and final fate [8,9]. It is known that the synthetic properties of nanomaterials are some of the critical factors which determine corona composition [10]. However, predicting corona formation simply based on nanomaterial properties remains challenging. Different strategies have been explored to modify nanomaterial properties in order to reduce or to avoid protein corona formation, for instance by introduction of poly(ethylene glycol) (PEG) on the
nanomaterial surface [8]. However, these strategies cannot completely suppress protein adsorption and can introduce other problems [8,11,12]. At the same time, growing evidence has indicated that the corona layer can be recognized by specific cell receptors, opening up interesting possibilities to tune corona composition in order to achieve targeted interactions of nanomaterials with cells [13,14].

**Figure 1.** Schematic illustration of the liposome interactions with biological systems at a cellular level. These interactions mainly include 3 steps: i) liposome interaction with biological fluids and corona formation, ii) interactions of liposome-corona complex with cell membranes and their entry into cells, iii) liposome interaction with intracellular components and cargo release. Fig. 1 is reproduced from Chapter 1.

Within this framework, in **Chapter 2**, a liposomes series of tailored surface properties has been prepared and optimized by mixing zwitterionic DOPC and negative DOPG lipids in different ratio, and, in this way, to tune the resulting coronas forming in human serum and determine the effects on liposome interactions with cells (Fig. 2A). The different liposomal formulations showed very different uptake efficiency and kinetics, and by increasing the negative DOPG lipid, formulations with increased uptake levels in the first few hours of exposure were obtained (Fig. 2B). Thus, liposome-corona complexes were isolated from human serum and characterized. The different formulations showed strong differences in the amount and variety of absorbed proteins (Fig. 2C-D). By connecting the abundance of
specific proteins in the corona forming on the different liposomes with the uptake kinetics in cells, candidate corona proteins associated with reduced or increased uptake by cells were identified.

**Figure 2.** Tuning liposome composition to modulate the corona forming in human serum and uptake by cells. (A) Schematic illustration of the work presented in Chapter 2, where liposome composition was tuned in order to modulate corona formation in human serum and the uptake in cells. (B) Uptake kinetics of DOPC, DOPC/G and DOPG liposomes exposed to HeLa cells in cell culture medium supplemented with 4 mg/ml human serum (hsMEM). The results showed that the different liposomes had different cell uptake behavior. (C) Venn diagram of the corona proteins identified on DOPC, DOPC/G and DOPG liposomes. (D) Z-score heat map of the most statistically different proteins in the corona forming on the 3 liposomes (P value ≤ 0.01). By changing liposome composition very different coronas were obtained and very different uptake behavior.

Therefore, tuning the synthetic properties of nanomaterials in a systematic way, as we showed in Chapter 2 with liposomes, provides a novel tool to adjust corona composition and in this way obtain formulations with the desired uptake behavior. Additionally, correlating corona composition and uptake by cells allows to identify corona proteins associated with increased or decreased uptake by cells, which can then be used for targeting strategies or to avoid clearance. Similar approaches can be
Further applied in in vivo studies, and novel insights into effects of corona composition on nanomaterial clearance by the immune system, biodistribution and targeting could be obtained. Clearly, much more work is needed in order to fully understand how nanomaterial properties affect corona formation and how corona formation affects nanomaterial outcomes at cell and organism level. Ideally, with such knowledge, predicting corona formation and biological behavior of nanomaterials based on their physicochemical parameters would allow to guide the design of successful nanomedicines.

Another important factor affecting corona formation is the biological fluid in which nanomaterials are applied [10]. Many studies have demonstrated that subtle differences in biological fluids, such as upon serum heat inactivation, or when using serum originated from different species, and also when serum concentration is varied, can lead to the formation of very different coronas on the same nanomaterials and – as a consequence of this – very different outcomes on cells [9,15–17]. While recently extensive efforts have been focused on effects due to differences in the corona forming on the nanomaterials when different biological fluids are used and the following impact on their interactions with cells, relatively less attention has been paid on additional effects due to the presence of the free biomolecules in these biological fluids.

**Fig. 4** Effects of protein source on liposome-protein corona and cellular uptake. (A) Liposome showed very different uptake in FBS and HS. In order to disentangle the effects due to corona composition and those related to free serum proteins in solution on liposome uptake, FBS and HS corona-coated liposomes were isolated and exposed to HeLa cells in serum free medium as well as
FBS and HS supplemented medium. (B) Uptake kinetics of FBS and HS corona-coated liposomes by HeLa cells in different media. The results showed that FBS and HS corona-coated liposomes have comparable uptake efficiency when exposed to cells in the same medium (serum free or with excess free proteins in solution).

In this context, Chapter 3 aimed to gain a better understanding of nanomaterial interactions with biological systems when using different biological fluids (in this case serum proteins from different source) and to disentangle effects due to differences in corona composition and those related to the presence of free biomolecules in solution. Liposomes were used as nanomedicine models, and they showed very different uptake efficiency when added to cells in medium supplemented with fetal bovine serum (FBS) or human serum (HS). The corona forming in the two sera were very different (Fig. 4A). However, when the excess free proteins were removed and the different corona-coated liposomes were exposed to cells in serum free medium, their uptake was comparable (Fig. 4). Similar results were obtained when the corona-coated liposomes were re-introduced in medium supplemented with either human or bovine serum (Fig. 4). Therefore, in this case, the different uptake efficiency depended primarily on the presence and source of the excess free proteins, rather than the observed differences in corona composition. These results clearly show that the protein source affects nanomaterial behavior on cells not only because of the identity of the absorbed corona proteins, but also via additional effects due to free proteins in solution.

The results from Chapter 3 suggested that the evaluation of nanomaterials and their therapeutic efficiency should carefully take into account similar differences related to the nature of the biological fluids in which they are tested. In particular, also the source of serum proteins used in the medium can strongly affect the outcomes. For instance, in vitro studies should be designed to use matching serum source (or other relevant biological fluid) and cell species, and performing in vitro study using cells and serum source corresponding to the animal models used for in vivo studies may help to narrow the gap between in vitro and in vivo outcomes. Similar considerations should be applied to try to narrow the gap between preclinical studies and clinical trials. Nowadays, many preclinical studies to assess the therapeutic efficiency of nano-drugs are performed in animal models where diseased human
cells/tissues have been implanted (for instance xenograft models) [18,19], which means that the targeted cells/tissues and the serum proteins of the animal model are from different species. Based on the results presented in Chapter 3, one may speculate that in such models nanomedicine targeting and uptake efficiency on the implanted human cells may be higher given that the competition of the excess free proteins in solution is likely to be lower (because of the different animal species). It would be important to test in future studies whether effects similar to those shown in Chapter 3 may take place and may contribute – at least in part - to commonly observed differences in nanomedicine efficacy in the translation from animal models to humans.

Figure 5. Overview of uptake inhibition of DOPC and DOPG liposomes in HeLa cells after treatment with a panel of chemical inhibitors or RNA interference. The symbols are the results obtained in individual experiments (3 to 5 independent replicate experiments) and show the median cell fluorescence intensity averaged over 3 technical replicates, normalized by the results in untreated control cells to indicate the inhibition efficacy. The lines are their average. A black dashed line and a red dashed line are included in each panel as a reference, at 100% and 60% uptake, respectively (with 60% uptake shown as an indicative threshold for inhibition efficacy). The results showed that blocking a series of key components in different endocytic pathways had very different effects on the uptake of zwitterionic DOPC liposomes and negative DOPG liposomes.

Following corona formation, nanomaterials exposed in a biological environment eventually interact with the cell membrane and in most cases enter cells (Fig. 1, ii) endocytosis). Recently, zwitterionic modifications have received increased attention as a strategy alternative to PEGylation to reduce protein adsorption [8]. The results
of Chapter 2 also showed that addition of the zwitterionic DOPC lipid in liposomes led to lower protein binding capacity, as well as reduced uptake efficiency [20]. However, little is known about the effect of zwitterionic modifications on the mechanism liposomes use to enter cells. Thus, in Chapter 4, the internalization mechanism of liposomes consisting of zwitterionic DOPC lipid or negative DOPG lipid were studied. The results showed that blocking a series of key components involved in various endocytic pathways had very different effects on the uptake of zwitterionic and negative liposomes (Fig. 5). For instance, blocking clathrin-mediated endocytosis strongly reduced the uptake of the negatively charged liposomes, but had almost no effect on the uptake of the zwitterionic ones, while inhibition of macropinocytosis reduced the uptake of both liposomes, but to a different extent. These results indicated that the two liposomes were internalized by cells using different pathways, thus introducing zwitterionic modifications affects not only protein adsorption and uptake efficiency (Chapter 2), but also the mechanisms of liposome uptake by cells (Chapter 4). Clearly, more work is needed to fully understand how zwitterionic groups affect corona formation and how the different corona changes the way cell recognize and process the nanomaterials.

Chapters 2 and 4 showed that zwitterionic and negative liposomes form different corona in biological fluids and enter cells via different endocytic pathways. These differences are likely to affect also their interactions with intracellular components after uptake and the ultimate release of the cargo they carry. These also are crucial steps in the liposome journey in biological systems, which affect the final therapeutic/diagnostic efficiency (Fig. 1, iii) intracellular trafficking and cargo release) [10,21]. Current methods for studying drug release are difficult to use in complex biological environments or to determine cargo release from liposomes once inside cells. Within this context, in Chapter 5 different methods have been used to determine liposome release behavior after internalization by cells and also liposome stability and eventual cargo leakage in biological fluids. Interactions with biological fluids and corona formation can affect bilayer stability and release properties and this can have consequences on release kinetics also after internalization by cells. Thus, the same model liposomes loaded with sulforhodamine B (SRB) to mimic hydrophilic drugs entrapped in the lumen were used, and their release properties in
complex biological environments were compared (Fig. 6). The results showed that DOPG liposomes were able to deliver inside cells very high amounts of SRB in a short time, but the high uptake efficiency and fast release properties inside cells were also accompanied by a substantial loss of the SRB load outside cells upon interaction with serum (possibly connected to the higher protein binding capacity observed in Chapter 2). On the contrary, the uptake efficiency of DOPC liposomes was much lower (as already observed in Chapter 2) and the intracellular cargo release more gradual and sustained over time. This formulation also showed higher stability in biological conditions, when exposed to serum, likely also because of the lower protein adsorption. The methods presented in this Chapter allow to address at least in part some of the limits of simpler \textit{in vitro} release studies, in order to characterize liposome stability in complex biological fluids and release kinetics inside cells. With similar methods and depending on the requirements for specific applications, liposome and other nano-formulations can be tuned to achieve the required balance between stability in serum and drug release kinetics at the target.

\textbf{Fig. 6} Characterization of cargo release kinetics from DOPG and DOPC liposomes inside cells and in complex biological environments, upon corona formation. DOPG liposomes showed high uptake efficiency and fast release properties inside cells, while DOPC liposomes with higher stability in biological environments showed slow internalization and a more gradual cargo release inside cells.
While several studies, such as Chapters 2-5 presented here, try to understand how to tune nanomaterial properties to achieve expected biological outcomes, cell membrane nanotechnology is attracting increasing interests as an alternative strategy to fabricate nanomaterials with defined interactions with biological systems [22,23]. Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells with high treatment failure due to drug resistance and disease relapse[24]. Increasing evidence has suggested that the bone marrow microenvironment (niche), in which leukemia cells can reside, plays an important role in the development and evolution of this disease. A better understanding of the interactions between leukemia cancer cells and their niche can provide novel strategies to target this disease [25]. Within this context, in Chapter 6 cell membrane nanotechnology was exploited to prepare cell membrane nanoparticles from different leukemia and bone marrow stromal cells as a tool to probe the interactions between cancer and stroma.

Procedures to extract cell membranes were optimized in order to avoid contaminations from other cell compartments and cell membrane nanoparticles were prepared by doping synthetic liposomes, such as those used in the other Chapters, with cell membrane components (Fig. 7A). Cell uptake results showed that all cell membrane-doped liposomes had higher uptake efficiency in all tested cells than the synthetic liposomes (Fig. 7B-D), suggesting that addition of cell membrane components favors interactions with cells (possibly cell receptors) and this leads to increased uptake levels. In addition, liposomes doped with the stromal cell membranes (lipo-ms5) showed the highest uptake (Fig. 7B-D), and this effect was the strongest on the stromal cells themselves (Fig. 7E). These results open up interesting possibilities for future studies. On the one hand, stromal cell membrane nanoparticles can be used as a nanotechnology tool to characterize the interactions between stromal and leukemia cells and identify the proteins involved. On the other hand, stromal cell nanoparticles may be used to improve drug delivery to leukemia cells. On a broader level, identifying the mechanisms behind the higher uptake of cell membrane doped liposomes could allow to discover key players in the interactions between nanomedicines and biological systems to improve their efficacy.
Figure 7. Cell membrane nanoparticles to probe the interactions between stromal and cancer cell in acute myeloid leukemia. (A) Schematic illustration of cell membrane-doped liposome preparation. Fig. 7A is adapted with permission from Molinaro et al. [26]. (B-D) Uptake of synthetic liposomes (lipo) and K562, THP-1 and MS-5 cell membrane doped liposomes (named lipo-k562, lipo-thp1 and lipo-ms5, respectively) in different cell lines. The results showed that in all tested cells, the cell membrane-doped liposomes had higher uptake efficiency than the synthetic liposomes, the effect being the strongest for lipo-ms5 nanoparticles. (E) Comparison of lipo-ms5 uptake efficiency across different cell lines. The results suggested that among the leukemic cell lines uptake of lipo-ms5 in K562 cells was more efficient compared to TF-1 and THP-1, and the effect was the strongest on the stromal cell themselves.

In conclusion, this Thesis aimed to investigate nanomaterial behavior in complex biological systems, in order to define potential new strategies to guide the design of more effective nano-formulations. Using liposomes as a nanomedicine model, we have changed the synthetic properties of nanomaterials in a systematic way to tune the corona they form once introduced in serum and modulate cell uptake efficiency.
and kinetics (Chapter 2). Additionally, the effects of different biological fluids - in this case serum proteins from different species – on nanomaterial-cell interactions were investigated, and we demonstrated that not only the corona composition differed strongly in different biological fluids, as many studies already reported, but also that the presence of excess free proteins in solution from different fluids played an important role in nanomaterial-cell interactions, an aspect which is often overlooked (Chapter 3). As a next step, we studied how nanomaterial-corona complexes enter cells and interact with intracellular components (Chapters 4 and 5). Interestingly, by comparing zwitterionic and negative liposome behavior in biological environments, we observed that, beside different corona formation and uptake efficiency as shown in Chapter 2, nanomaterials with different surface charge entered cells through different endocytic pathways (Chapter 4) and after entering cells, they released their cargo in a very different manner (Chapter 5). What’s more, we have developed methods which can be used to study drug release kinetics from liposomes or other nano-formulations once they are in contact with complex biological fluids, following corona formation, and also once they are inside cells after endocytosis. These methods address at least partially some of the limits of simpler in vitro release methods which are commonly applied (Chapter 5). Finally, we exploited cell membrane nanotechnology to dope liposomes with cell membranes from stromal and leukemia cells in the context of AML, and compare their affinity in different cells. Cellular uptake results suggested that stromal cell membrane-doped liposomes (lipo-ms5) had the potential to be developed as a tool to characterize the interactions between stromal and leukemia cells and in this way to identify novel targets for AML treatment (Chapter 6).

Even though further studies are needed in order to continue to deepen our knowledge on how complex biological systems affect nanomaterial behavior, the results presented in this Thesis have highlighted important aspects in such interactions. Deepening this knowledge will help to tune nanomaterial synthetic properties to achieve the desired biological outcomes at cell and organism levels and ultimately accelerate the development of efficient nanomedicines and their clinical translation.
References


