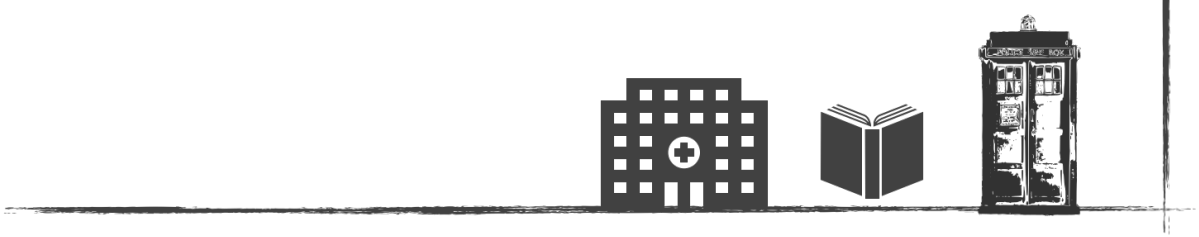
CHAPTER 6:
GENERAL DISCUSSION AND FUTURE PERSPECTIVES



CHAPTER 6: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Nanoparticles as carriers for therapeutic compounds and nutrients are promising platforms from which several areas in medicine may benefit, including treatment and diagnosis of diverse pathologies like cancer and central nervous system disorders. Nevertheless, it is essential to evaluate the cellular response to those nanosized materials in pre-clinical phases to properly translate them to clinical applications. However, many reports are missing important parallels to *in vivo* conditions and often do not describe and discuss the drawbacks or possible alternatives to better understand and improve the nanosystem.

While nanomaterial properties such as size and surface charge have been extensively studied, many other properties are less highlighted. This thesis discusses the role of stiffness in the interaction with polarized endothelial cells mimicking the blood-brain barrier (BBB) in **Chapter 2**. As model particle, we employed poly(N-isopropylmethacrylamide) (p(NIPMAM)) nanogels prepared by precipitation polymerization of which the stiffness was tuned due to the presence of a cross-linker, N,N′-methylenebis(acrylamide), at different densities. Results reveal that even though harder nanogels (NG14, 14 mol% BIS) present higher levels of uptake by the polarized monolayer, the softer nanogels (NG1.5 and NG5, respectively 1.5 and 5 mol% BIS) are favored in the transcytosis through the BBB. No significant variation was detected for particles of the same cross-linking density and a 2-fold difference in size. Interestingly, the internalization as well as the transcytosis level was identical for NG1.5 and NG5, but significantly different from the internalization and transcytosis of NG14, suggesting a rather sharp response to particle stiffness. Based on theoretical models,(1,2) we speculate that the cell membrane bending induced by the softer nanogels is low when compared to the harder nanogel due to the spreading of soft nanogels over the membrane and consequent decreased pressure applied to the membrane. The variation in membrane bending will affect the kinetics of nanogel wrapping and, ultimately, the levels of uptake. Protein corona formation dissimilarities between softer and stiffer nanogels can also contribute to the observed particle interaction with the monolayer. It is noteworthy that protein adhesion on nanogels is low compared to nanoparticles.(3) The opposing effect of nanogel stiffness on their uptake and transcytosis highlights the importance of vesicular trafficking mechanisms and exocytosis in determining transcytosis efficiency, emphasizing that enhanced

internalization does not lead to improved transcytosis. This was also noted when comparing the use of high affinity ligands with low affinity ligands in receptor mediated-transcytosis of ligand-functionalized nanoparticles,(4–6) which brought important insight in the transport mechanism across the BBB. Our finding with non-functionalized particles of different stiffnesses suggests the existence of stiffness-dependent non-ligand-mediated transcytosis. Techniques that can avoid the use of ligands may be beneficial to clinical translation as the efficacy of ligands is subject to inter-patient and intra-patient heterogeneity.

With the applicability of -especially soft- nanogels for crossing the BBB, we next evaluated the role of nanogel stiffness in their interaction with glioma cells and phagocytic cells, specifically nonpolarized macrophages (**Chapter 3**). The uptake of softer nanogels was significantly lower in both cancer and phagocytic cells compared to the uptake of the stiffer nanogel, which correlates with the observed behavior in brain endothelial cells. In macrophages the larger sized nanogels were considerably more internalized than the smaller nanogels. Such variation may be attributed to the augmented contact between the cellular membrane and nanogels of larger sizes. Because the cellular membrane is not completely flat but filled with ruffles, nanoparticles may also interact with those ruffles. Particles with a diameter that is larger than the “flat” regions between ruffles will be able to establish multiple additional points of contact with the membrane (i.e., with ruffles), whereas particles that are smaller than the flat regions maximally connect with the flat region and one ruffle. The optimal particle size for maximal phagocytosis is reached when the particles can connect with a flat region and two ruffles. Below and above this optimal NP size, NP phagocytosis is reduced.(7) The lower uptake of softer nanogels is an important indication of the evasion of those particles from the mononuclear phagocytic system, which increases particles blood half-life. Taking into consideration that soft NG1.5 and NG5 nanogels more efficiently cross the BBB than hard NG14 nanogels, we believe that soft nanogels show great promise for brain targeting.

Nanogel stiffness also showed an effect on cellular viability. Glioma cells showed an inverse relationship between ROS induction and NG stiffness, i.e., highest ROS induction with the softest NG. This is a very interesting response, considering that ROS production is one of the main mechanisms by which chemotherapeutics kill cancer cells The ROS levels exhibited by the macrophages were higher than by C6 cells for all NGs, but adaptive mechanisms allow them to survive the increased

stress.(8,9) In direct co-culture, the cytotoxic effect of the soft NG (NG1.5) was reduced and the ROS level of the combined cell populations was considerably lower than the combined levels of the monocultures. Direct co-culture also influenced the internalization of nanogels, causing enhanced uptake by the macrophages. The changes in toxicity and internalization behaviors could be ascribed to changes in phenotyping and function of the macrophages as induced by the concomitant presence of cancer cells. The phagocytic capacity of macrophages is enhanced when they are stimulated,(10–12) which occurs in the presence of tumor-derived factors secreted by the glioma cells. Also, ROS trigger the activity of ROS-scavengers promoting the scavenging of excessive ROS, which will lead to the reduction of the toxic effect by NGs in the glioma cells. The ROS generation by soft (p(NIPMAM)) NGs in cancer cells can be exploited in drug delivery systems to control the release of therapeutic compounds.(13,14) Also, other stimuli-dependent triggers can be incorporated in the system like nanoparticle-mediated hyperthermia to chemosensitize cancer cells, which can in the case of the thermoresponsive p(NIPMAM) NGs can also promote drug release drug due to the collapse of the NGs.(15–17)

Moreover, results from **Chapter 2** and **3** support the need of evaluating nanomaterial potential in conditions more relatable with *in vivo* settings, considering possible biological barriers, e.g. blood-brain barrier and phagocytic cells.

In **Chapter 4**, homotypic adhesion between breast cancer cells was exploited to achieve a more effective nanotherapeutic system. Poly (D, L-lactic-co-glycolic acid) nanocarriers (NCs) were coated with MCF-7 breast cancer cells membrane and their interaction with MCF-7 breast cancer cells, non-tumorigenic breast cells, and lung cancer cells was analyzed and showed an increase in interaction between membrane-coated NCs for all cellular types when compared to non-coated-nanocarriers. The enhanced nanoparticle-cell adhesion for all cellular types relates to the presence of common membrane proteins e.g. epithelial cell adhesion molecule (EpCAM).(18–20)

However, when the membrane-coated NCs were loaded with paclitaxel, a chemotherapeutic, there was a statistically significant reduction in cellular viability only for MCF-7 cells, i.e., the cell line that the membrane was derived from. Engineering nanoparticles with tumor-derived material seems an effective way to improve nanotherapeutics efficacy and targetability. Also, it is a less arduous process to design tailored nanotherapeutics, avoiding the identification and production of specific ligands. Tumor cell membrane extraction can be envisioned as part of a personalized treatment

where patient tumor cells can be proliferated *ex vivo* prior to membrane extraction for custom-made patient-specific nanotherapeutics, while avoiding inefficacy related to intra-patient heterogeneity. Rao et al(21) already brought some important results to this topic employing head and neck squamous cell carcinoma patient-derived tumor cells as coating for nanoparticles and describing a superior effect for treatments and postsurgery treatments that were targeted with cellular coating from the same source as the produced *in vivo* model. In addition, cells can be genetically engineered to produce specific molecules at the cell surface or associated with extracellular vesicles which can be used to coat NPs to target the tumor microenvironment.

To conclude, **Chapter 5** reports the development of light-induced release from liposomes by means of photochemical and thermal isomerization mediated by synthetic molecular motors. Control of light-driven molecular motors shows spatial and temporal precisions. By incorporating the molecular motors into the lipid bilayers of liposomes, calcein was released only with the input of UV irradiation and longer exposure times were shown to increase the calcein release from the liposomes. Gaining precise control over the release of molecules from drug delivery system is essential and the molecular motors showed an outstanding regulation with a simple strategy. These results unlock possibilities for the application of molecular motors in nanomedicine and advances towards precisely controlled-release systems. Valuable advancements would be the use of molecular motors that respond to longer wavelength, allowing for better penetration of tissues, or their use in polymersomes, a versatile system thanks to the ease of polymer synthesis.(22) Delivery systems that can identify and respond precisely to external or internal cues aiming at "*on demand*" release kinetics are of great interest for the delivery of therapeutic compounds. However, to be able to create these systems a deeper understanding of nanomaterial-cell and -tissue interaction is necessary.

This thesis presents novel nanosized delivery systems, specifically nanogels, membrane-coated PLGA particles, and liposomes with molecular motors, that were designed to improve therapeutic efficacy. The described studies expand our knowledge of nanoparticle properties, specifically stiffness, size, and cell affinity, that affect NP interactions with biological systems.

Future perspectives

The study of nanomaterials-cell interaction in this thesis has provided insights into the field of nanotechnology applied to medicine. We demonstrated that transport across the blood-brain barrier and internalization of polymeric nanogels can be regulated by tuning particle stiffness. As a next step, we would be interested in investigating the nanogels intracellular with vesicular trafficking and its impact on the exocytosis of the nanogels. Further, *in vivo* experiments are needed to confirm if the stiffness effect observed in the filter-free blood-brain barrier model agrees with the *in vivo* behavior of soft NPs, and if soft NPs would be advantageous as a drug delivery system to the brain. Likewise, the effect of nanoparticle stiffness on the *in vivo* drug delivery to glioblastoma needs to be evaluated, and compared with the results from *in vitro* co-culture of glioma cells and peripheral macrophages mimicking the tumor microenvironment (TME) in order to assess the predictive value of the co-culture system. In addition, exploring NP behavior in 3D co-culture systems will take the possible influence of the extracellular matrix and tissue structure into account. It would be of our interest to prepare cancer-cell coated nanocarriers using patient-derived tumor cells, and analyse their interaction with primary tumors and circulating tumor cells (CTCs). Finally, studying on demand drug release from molecular motor-containing liposomes in cells is essential to determine the level of control over drug delivery as well as safety in response to the illumination of cells with different light sources. The technology should also be translated to other carrier systems, e.g. polymerosomes and polymeric nanoparticles, and be explored in areas as photodynamic therapy.

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