Conferring to solid state nanoparticles a lipidic coating: design and applications of biomimetic nanoparticles and artificial extracellular vesicles.

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Thesis summary

In recent years, the field of nanomedicine has gained immense attention due to its vast potential. Solid-state nanoparticles, in particular, are considered highly promising for applications in drug delivery, imaging, diagnostics, and even theranostics. In fact, nanoparticles offer significant advantages due to their versatility and ease of customization, which can be adapted to the needs of individual patients, supporting the development of personalized medicine. However, the clinical application of these nanomaterials remains hindered by a series of phenomena, which occur in biological environments, such as aggregation, instability, premature degradation, rapid clearance from the body and nonspecific uptake. Lately, research has focused on the development of lipid-based nanomedicines, such as of liposomes, lipid nanoparticles (as in the case of COVID-19 vaccines) and even on the employment of cell-derived Extracellular Vesicles (EVs), nature's own nano delivery systems. All the above-mentioned structures have in common the presence of lipids, and it is well known that phospholipidic bilayers constitute the natural envelopes of cells and of EVs, making them inherently suited for interacting with biological systems. With this perspective, coating solid-state nanoparticles with lipid layers presents an appealing strategy to enhance their biocompatibility and improve their *in vivo* performances.

The aim of this PhD thesis is to leverage the favourable biological properties of lipid bilayers and explore lipid coatings as a solution to address the challenges associated with pristine solid-state nanoparticles. After providing an in-depth review of the current state of the art regarding lipid-coated nanoparticles for cancer therapy, the thesis progresses to two specific case studies. In the first case, Zinc-Oxide Nanocrystals (ZnO NCs) are utilized as the core of the nanoconstructs, which are tested as therapeutic agents against colorectal cancer (CRC) both in 2D and 3D in vitro models. The innovative approach consists in combining lipid-coated ZnO NCs with acoustic shock waves (SW), a safe external stimulus. This combined treatment aims to enhance the therapeutic efficacy of the nanoparticles while circumventing traditional drug resistance mechanisms, a major hurdle in cancer treatment. The second case study proposes a conceptual shift, from a more "engineering" to a truly biomimetic way of conceiving lipid coatings, drawing inspiration from natural extracellular vesicles. This study was conducted within the framework of the Mimic-KeY project, which aims to develop

fully artificial EVs capable to encapsulate solid-state nanoparticles with therapeutic capabilities, to overcome the limitations that currently hinder the widespread use of natural EVs: high costs and difficulties in isolation and handling procedures and lack of reproducibility and standardization. The dissertation is organized in three chapters.

The first chapter provides a comprehensive overview of solid-state nanoparticles coated with lipidic envelops, specifically for cancer treatment. The considered coatings include liposome-like layers, natural or engineered EVs and also artificial-natural hybrids. The chapter is divided into four main sections that range from traditional 2D monolayer cell cultures to more advanced *in vivo* and 3D models, up to clinical trials, underlying that the body of literature concerning this field becomes sparser with the progressing of the review. What emerged is that the use of lipid membranes as carriers for therapeutic molecules is well-established in both literature and clinical applications, but this is not valid for lipid-coated solid-state nanoparticles, with scarce examples of success in *in vivo* tests and none reaching clinical trials. This field is indeed still in the early stages and currently both the "artificial" and the "natural" ways are equally viable. Artificial, liposome-like coatings have shown promising results *in vitro*, but their inability to naturally target tumors limits their clinical applicability. On the other hand, EVs, often derived from tumor cell lines due to their selective targeting properties, have shown good *in vitro* results but face challenges in extraction, storage, and reproducibility, limiting their use *in vivo* and in clinical trials. Finally, although very promising (since it could in theory overcome the limitations of both liposome- and EV-based coatings), the field of artificial-natural hybrids is almost unexplored, with just a few *in vitro* examples.

The second chapter concerns the first case study, introducing at the beginning a proof of concept of the stimuli-responsive treatment, which combines ZnO NCs and shock waves against two CRC cell lines: HT-29 (epithelial phenotype) and Colo 320DM (mesenchymal phenotype). Two forms of ZnO NCs (undoped and iron-doped) were synthesized and compared in terms of physicochemical properties, cell interaction, and therapeutic activity. The results demonstrated significant cytotoxicity when combining either undoped or iron-doped ZnO NCs with shock waves, particularly in the Colo 320DM cell line. In contrast, when applied separately, both treatments had minimal effect. Multiple SW stimulation was tested as well, resulting in improved cytotoxicity, also in HT-29 cells. Cytotoxicity mechanisms for the combined treatment (NCs + SW) were explored, suggesting that the treatment destabilizes cell membranes and induces substantial apoptosis in the Colo 320DM cells. Interestingly, the generation of ROS, particularly hydroxyl radicals, was ruled out as a mechanism through electron paramagnetic resonance measurements.

Successively, the second chapter presents the work made to upgrade the iron-doped ZnO NCs by adding a protective and biomimetic shell made of phospholipids, cholesterol, PEG and a targeting peptide for selective colon cancer targeting. Such nanoconstructs were tested both on a healthy (CCD-18Co) and a CRC (HT-29) cell line, resulting highly biocompatible, hemocompatible and minimally toxic to healthy cells, even at high doses. In contrast, cancerous cells were more sensitive to the nanoparticles, particularly the targeted ones, which were more efficiently internalized by the cancer cells, sparing healthy cells. The combination of nanoparticles and acoustic pressure stimulation was tested in 2D cell cultures, where no significant damage was observed in healthy cells, while CRC cells were subjected to a synergistic cytotoxic effect. Notably, similar synergistic effects were also observed in 3D colorectal cancer spheroid models. At higher nanoparticle doses, combined with

repeated acoustic stimulations, the CRC spheroid mass was completely ablated. Taken together, the findings presented in the second and third chapter validate the potential of the proposed SW-NPs combined treatment and pave the way for further pre-clinical validations.

The third and last chapter relates to the second case study, firstly delving into the principles of EV biomimetics and introducing the "manifesto" of the Mimick-KeY project. This part of the dissertation provides an overview of the emerging approaches to develop and engineer EV-mimicking vesicles, which can constitute an alternative to natural EVs. The bottom-up approach is then identified as the most promising, yet challenging, to achieve a suitable EV-mimicking product and possible procedure to implement it is theorized. The first step would be dedicated to lipidomic studies of naturally occurring EVs, preferably well-known EV subpopulations, with established targeting abilities. Then, a simplification step is needed and requires the identification of the most important lipid components, which can be faced by testing lipidic formulations with increasing complexity. Molecular simulations and other innovative predicting tools can certainly accelerate and optimize this process, assisting the empirical work. Finally, great effort is needed to study natural EVs' protein profile and understand and select the key molecules responsible for their targeting and cargo transfer capabilities. This knowledge will help in decorating artificial EVs with a simplified version of the proteins or peptides that confer these abilities, potentially achieving similar homing and cargo transfer functions as natural EVs.

Afterwards, the chapter gets to the heart of the Mimic-KeY project, presenting the first attempt to implement the principles of the Mimic-KeY project. In this work, "EV mimics" were designed as core-shell hybrid structures, consisting of a degradable organosilica nanocapsule for efficient cargo delivery and controlled release, surrounded by a biomimetic lipid shell that emulates the lipid composition of natural EVs. Three different lipid mixtures based on lipidomic data from the PC3 prostate cancer cell line were conceived, considering the mass percentages of lipid families and fatty acids. These EV-inspired formulations successfully coated the nanocapsules. To optimize the lipid bilayer coating, predictive modelling was utilized, and supramolecular simulations revealed differences in lipid dynamics across the various mimic formulations. The size distribution and Zeta-potential of the different EV mimics were analysed and compared to the ones of natural EVs, finding a high degree of similarity. Additionally, advanced characterization techniques were employed: high-resolution flow cytometry quantified the lipid coating efficiency, and Nile Red-based single-molecule localization microscopy was used to map the polarity of individual EV mimics, revealing notable differences across formulations. Based on the combined results from these techniques, one formulation emerged as the most promising, closely resembling the natural PC3-derived EVs.

The promising results presented in both case studies highlight the potential of lipid-coated nanoparticles and EV-mimicking systems to overcome the current limitations of nanomedicine: this research suggests that these advanced nanostructures could offer safe, controllable, and off-the shelf nanosystems, with the ability to truly give a strong impact to the field of nanomedicine, bringing it to the next level.