General Abstract

The pharmaceutical industry, while essential for maintaining and improving global health, exerts a considerable environmental and ethical impact throughout the entire lifecycle of its products. This impact originates from the entire lifecycle of pharmaceutical production, energy-intensive processes that contribute substantially to greenhouse gas emissions, the pervasive use of toxic organic solvents, and the generation of large volumes of hazardous wastewater.

In this context, the present research aims at the reduction of the pharmaceutical industry environmental impact by targeting two aspects of the drug development pipeline: the formulation of nanoparticle (NPs) for drug delivery and their preclinical evaluation exploiting organic solvent-free synthesis techniques and advanced *in vitro* models as an alternative to animal experiments.

Two distinct NPs were successfully developed using entirely organic solvent-free fabrication techniques. The first system consisted of chitosan (CS) NPs produced via ionic gelation, a solvent-free method which exploit the electrostatic interaction between the positively charged chitosan polymer and the negatively charged crosslinker sodium tripolyphosphate (TPP). The formulation protocol was optimized to yield NPs with favorable physicochemical proprieties, including small size, low polydispersity index, and high positive zeta potential. To enhance their colloidal stability and stealth capabilities, NPs surface was further functionalized with polyethylene glycol (PEG), which improved their resistance to aggregation and increased the production yield. PEG-CS NPs demonstrated efficient encapsulation of a polyclonal model antibody and exhibited a sustained release profile over time. *In vitro* assays confirmed that the PEG-CS NPs were highly biocompatible in both healthy and melanoma cell lines. Furthermore, confocal microscopy revealed that antibody-loaded PEG-CS NPs were readily internalized by target cells, suggesting their suitability for intracellular drug delivery. Hemocompatibility studies showed no significant platelet activation, supporting their potential for systemic administration *in vivo*.

Concurrently, polyallylamine hydrochloride (PAH) NPs were developed, exploiting the interactions between the cationic polymer and the negatively charged nucleic acids. PAH NPs were assembled through a simple, one-step, water-based self-assembly process, completely avoiding organic solvents exploitment or harsh

synthesis conditions. NPs synthesis was optimized to obtain uniform size distribution and slightly positive surface charge. High encapsulation efficiency (~99%) was achieved and cellular uptake studies on melanoma cell lines demonstrated superior internalization of PAH NPs compared to both free siRNA and a commercially available transfection reagents. Functional validation of the delivery system was conducted by evaluating PAH NPs capability to silence BRAF oncogene. Western blot analysis and cytokines quantification confirmed a significant downregulation of BRAF protein expression following the administration of the treatment, demonstrating effective gene silencing. Moreover, PAH NPs were found to be hemocompatible, further supporting their potential for clinical application.

To support the preclinical evaluation of these systems in a more physiologically relevant, ethical and sustainable manner, an advanced three-dimensional metastatic melanoma model was developed. The model is composed by the dermal compartment, realized with a type I bovine collagen/methacrylated hyaluronic acid hydrogel matrix, which was optimized to support cell viability and proliferation of human dermal fibroblasts. An epidermal layer was developed on top of the dermal matrix by culturing human epidermal keratinocytes at an air-liquid interface, promoting their differentiation into a biomimetic stratified epithelium. The tumor component was established by embedding spheroids of human melanoma cells within the dermal hydrogel, allowing for three-dimensional growth and cell–cell interactions to better resemble tumor physiology. Eventually, the vascular compartment was obtained by seeding human umbilical endothelial cells into the basal compartment of the model, from where they self-assembled into vessel-like structures.

The complete multi-compartmental model, including dermal, epidermal, tumoral, and vascular components could potentially provide a functional, ethical and sustainable platform for melanoma progression mechanisms investigation and innovative drug preclinical validation.

In conclusion, this work successfully achieved the formulation of two innovative, organic solvent-free NPs sets tailored for the systemic delivery of water-soluble therapeutic agents, antibodies and siRNA, with demonstrated *in vitro* efficacy and safety. Moreover, it established a sophisticated, melanoma *in vitro* model that offers an ethical and sustainable and prospectively more reliable alternative to animal-based preclinical testing for their validation. This integrated approach holds significant promise for advancing greener, more predictive, and ethically acceptable practices in pharmaceutical industry.