ABSTRACT OF THE THESIS

Myocardial infarction (MI) is one of the leading causes of death worldwide, accounting for nearly 85% of deaths among all cardiovascular diseases (CVDs). In MI, the sudden blockage of coronary artery leads to the death of up to one billion cardiomyocytes (CMs), which in turn triggers a severe inflammatory response and cardiac extracellular matrix (ECM) remodelling. MI maturation phase is characterized by the formation of non-contractile fibrotic scar, mechanically stiffer than healthy cardiac tissue and populated by cardiac fibroblasts (CFs). Currently, the only therapeutic strategy for heart regeneration is heart transplantation, but the limited availability of donors represents a significant issue. To address the fundamental problem of irreversible loss of CMs following MI, different strategies for the regeneration of myocardial tissue have been proposed including the direct reprogramming of fibroblasts into induced cardiomyocytes (iCMs). Direct cardiac reprogramming has been demonstrated by different methods, including the administration of specific microRNAs (miRNAs). Indeed, Paoletti *et al*. demonstrated that a combination of four miRNAs called miRcombo (miR-1, 133, 208, 499), previously identified by Dzau's group in mouse model, was able to trans-differentiate adult human cardiac fibroblasts (AHCFs) into iCMs. This treatment induced gene expression and phenotypical changes in CFs, allowing the progressive formation of *de novo* iCMs. Previously, viral vectors have been mostly used to deliver miRcombo, but their translation to the clinics is limited by different issues, such as immunogenicity and cytotoxicity.

Starting from this background knowledge, the aim of this PhD thesis was the design of a miRcombo delivery system, including: (1) lipoplexes for *in vitro* miRNA delivery; (2) hybrid polymer-lipid miRNA-loaded nanoparticles (miRNAs/NPs) to be use as stable transfection agents *in vitro*; (3) pegylated and ligand-functionalized hybrid NPs for targeted release to fibroblasts populating infarcted myocardium, to promote their direct *in vivo* reprogramming into iCMs and (4) injectable hydrogels to embed miRNAs/NPs for a safer and more efficient targeted treatment of infarcted scar.

Firstly, lipoplexes containing negmiR, as negative control, or miRNA combinations (miRcombo) based on a mixture of cationic lipid [2-(2,3-didodecyloxypropyl)-hydroxyethyl] ammonium bromide (DE) and helper lipid dioleoyl phosphatidylethanolamine (DOPE) were prepared and physicochemically and biologically characterized compared to a commercial lipid transfection agent, DharmaFECT. DE-DOPE/miRNA lipoplexes were prepared via spontaneous electrostatic interactions. The ratio between the positively charged groups on the cationic lipid (N) and the negatively charged groups on miRNA (P) was optimized to get lipoplexes with positive Z-potential, nanometric size and sufficient stability in suspension. DE-DOPE/miRNA lipoplexes at the selected N/P ratio (equal to 3) showed enhanced biocompatibility, ~99 % miRNA loading efficiency and ability to fully deliver miRcombo to AHCFs. Moreover, DE-DOPE/miRNA lipoplexes triggered AHCFs reprogramming into iCMs, resulting in significantly higher expression of cardiac markers as GATA4, MEF2C, TNNT2 and ACTC1 mRNA compared to DharmaFECT based

lipoplexes and NegmiR control conditions. Moreover, increased cell percentage positive for cTnT was observed in DE-DOPE/miRcombo transfected AHCFs compared to previously reported reprogramming efficiency (~11 % of cTnT+ cells) using DharmaFECT transfection. Nevertheless, *in vivo* application of DE-DOPE lipoplexes is limited by their instability in physiological conditions, fast release of miRNAs and lack of selective cell-targeting. Therefore, hybrid NPs were formulated based on the previously developed lipoplexes and a synthetic co-polymer (polylactic acid-co-glycolic acid, PLGA). Hybrid NPs were able to synergistically combine the complete miRNA encapsulation ability of lipoplexes and the structural stability of PLGA NPs in a single delivery platform. The new method for the preparation of hybrid NPs was patented (WO 2023/119222 A1). This method was fast and user-friendly, allowed to prepare nanoparticles able to effectively protect miRNAs from degradation and to release them in a sustained and controlled manner over time. Moreover, hybrid NPs were further optimized to achieve long-term storage, by analyzing different storage conditions (4°C; freezing at -80°C and freeze-drying). Hybrid NPs were able to maintain their physicochemical properties, cytocompatibility, sterility and transfection efficiency for up to 14 days storage at -20°C in lyophilized powder form in the presence of trehalose cryoprotectant. Hybrid NPs were herein proposed as new stable transfection agents for microRNA delivery to cardiac cells**.** Then, pegylated and functionalized hybrid NPs (NF-NPs and F-NPs, respectively) were developed for *in vivo* cardiac direct reprogramming. NF-NPs were formulated to expose polyethylene glycol (PEG) and azide reactive groups for ligand conjugation. Surface functionalization of NF-NPs was successfully performed by azide alkyne group click chemistry in the presence of copper catalyst (CuAAC) using a ligand for a selective CFs receptor, the Discoid Domain Receptor-2 (F-NPs). NF-NPs and F-NPs were characterized for their physicochemical properties and biological response, using AHCFs, demonstrating their *in vitro* cytocompatibility and efficient cell internalization for F-NPs. The transfection efficiency of F-NPs loaded with miR-1 was also shown, by evaluating TWF-1 mRNA target downregulation. *In vitro* direct reprogramming was also studied using miRcombo. After 15 days from transfection, AHCFs transfected with F-NPs showed overexpression of TNNT2 cardiac gene and cTnT and α-SAR proteins, as compared to cells treated with NF-NPs, confirming their superior target efficiency. Finally, *in vivo* pre-clinical tests on MI mouse model were performed at BIOEMTECH s.r.l. company in Athens, to evaluate biodistribution and efficacy of F-NPs and NF-NPs. Mice were intramyocardially injected with NF-NPs and F-NPs one-week post-infarction. F-NPs loaded with a fluorescent labelled small interfering RNA (siRNA-Cy5) showed a higher accumulation in heart, the target organ, and permanence until 48 h with a low accumulation in excretion organs, such as liver, kidneys and spleen, differently from NF-NPs loaded with siRNA-Cy5 and siRNA-Cy5 alone, which were poorly retained by the heart tissue. For the efficacy test, different parameters were measured through echocardiography (e.g., dimensions and volumes of left ventricle, global cardiac function and regional cardiac function). A slight improvement in cardiac function was obtained with miRcombo loaded F-NPs treatment compared to NF-NPs and saline, which otherwise maintained or caused a decrease in heart function.

To further enhance the local retention of nanoparticles at the site of injection, increasing treatment efficacy and reducing the off-target effects, an injectable hydrogel was herein designed as a depot for controlled release of nanoparticles *in situ*. Alginate (Alg) was selected for hydrogel preparation, since Alg-based systems are currently being investigated in clinical trials for MI treatment (Algysil-LVR). However, Alg has two main limitations: poor *in vivo* degradability and lack of support for cell adhesion. Therefore, in this work we blended alginate dialdehyde (ADA), an oxidized Alg-form with enhanced degradability properties, and modified carbohydrazide gelatin (GelCDH) with cell adhesive properties, to produce chemically crosslinked injectable hydrogels through Schiff base interaction. Firstly, ADA with four oxidation degree (Dox: 25, 10, 5 and 2.5) were produced through partial oxidation of Alg using sodium metaperiodate. Then, the composition of chemical crosslinked hydrogels was optimized, blending ADA with different Dox, together with Gel-CDH to get hydrogels with high *in vitro* stability, similar mechanical properties to commercial Algisyl LVR and cytocompatibility. ADA5/GelCDH hydrogel was selected as it showed rheological value (G' 1.7 – 2 kPa) similar to Algysl LVR properties similar to Algisyl LVR (G' 0.2-3 kPa), and to cardiac tissue stiffness values (1-6 kPa embryonic; 10-15 kPa adult cardiac tissue). Moreover, ADA5/GelCDH showed the highest *in vitro* stability in PBS among investigated compositions, showing complete degradation/dissolution after 24 days. ADA5/GelCDH hydrogel was characterized by self-healing, shear thinning and injectable properties, fundamental features for the development of drug delivery platform for miRNA-based MI treatment. Then, ADA5/GelCDH hydrogel was loaded with previously developed miRcombo/F-NPs. Gradual and total release (in 14 days) of miRNA/NPs from ADA5/GelCDH hydrogel was observed. Finally, miR-1/NF-NPs and miR-1/F-NPs were loaded into ADA5/GelCDH hydrogel. *In vitro* tests using release media, collected up to 7 days from hydrogel incubation in culture medium, showed TWF-1 mRNA downregulation in AHCF. These preliminary trials are promising and support the next exploration of the safety and efficacy of such drug delivery platform for the *in situ* release of miRNAs-loaded nanoparticles in the treatment of MI.