Abstract of the thesis

Skeletal muscle possesses a remarkable regenerative capacity following minor injuries; however, this ability is severely compromised in conditions characterized by extensive tissue loss or chronic degeneration, such as volumetric muscle loss (VML), traumatic injuries, and several neuromuscular disorders. In these pathological conditions, the endogenous regenerative response is insufficient to restore the highly organized architecture and contractile function of skeletal muscle, leading to permanent functional impairment. Current therapeutic strategies are often unable to restore both the structural organization and functional contractility of damaged tissue, highlighting the need for advanced regenerative approaches capable of recreating the complex biochemical, mechanical, and electromechanical microenvironment of native skeletal muscle. In this context, this PhD thesis focused on the development of multifunctional biomaterial-based platforms integrating structural support, biophysical stimulation, and targeted RNA delivery to promote skeletal muscle regeneration.

The first objective of this work addressed a major challenge in skeletal muscle regenerative medicine: the efficient and selective delivery of RNA therapeutics. Although RNA-based drugs have demonstrated extraordinary therapeutic potential, their clinical translation remains largely limited by the absence of delivery systems capable of efficiently targeting extrahepatic tissues. Building upon a previously developed hybrid polymer-lipid nanoparticle platform, nanoparticles were functionalized with the skeletal muscle-homing peptide M12 to achieve active targeting toward muscle cells. A systematic optimization strategy was performed by investigating different PEG spacer lengths and peptide surface densities in order to maximize ligand accessibility while maintaining favorable physicochemical characteristics. The optimized formulation preserved high oligonucleotide encapsulation efficiency, colloidal stability, and excellent cytocompatibility, including after repeated administrations. Compared with non-functionalized nanoparticles, M12-functionalized nanoparticles significantly enhanced cellular uptake in murine myoblasts, differentiated myotubes, and primary human skeletal muscle myoblasts, while producing only minimal increases in uptake by hepatic cells, indicating improved tissue selectivity. Mechanistic studies further demonstrated that M12 functionalization accelerates the early stages of nanoparticle internalization through an energy-dependent process consistent with receptor-mediated endocytosis. Importantly, the improved intracellular delivery translated into enhanced biological activity, as delivery of miR-1 significantly promoted skeletal muscle differentiation, increasing myosin heavy chain expression, differentiation index, fusion index, and myotube formation in both murine and human skeletal muscle cells. Preliminary *in vivo* biodistribution studies showed limited skeletal muscle accumulation following systemic administration, confirming the intrinsic barriers associated with intravenous muscle targeting, whereas intramuscular administration resulted in prolonged local retention of M12-functionalized nanoparticles compared with non-functionalized controls. These findings demonstrate that rational optimization of ligand presentation represents an effective strategy to improve active targeting and establish hybrid polymer-lipid nanoparticles as promising vehicles for localized RNA delivery to skeletal muscle.

Following the development of the targeted delivery system for RNA delivery, the second objective of this thesis focused on the development of an injectable electroactive hydrogel for minimally invasive treatment of VML. Injectable hydrogels represent an attractive

solution for skeletal muscle regeneration as they can conform to irregular defects while providing a temporary extracellular matrix-like environment that supports cell infiltration and tissue remodeling. However, conventional injectable hydrogels often exhibit limited mechanical stability and lack the ability to provide the biophysical stimuli required to guide muscle regeneration. To address these limitations, an injectable hydrogel based on alginate dialdehyde (ADA) and carbohydrazide-modified gelatin (GelCDH) was developed and systematically optimized by varying the ADA oxidation degree and polymer composition. Hydrazone crosslinking enabled the production of hydrogels exhibiting prolonged stability and mechanical properties suitable for skeletal muscle tissue engineering. Subsequently, fragmented electrospun poly(vinylidene fluoride) (PVDF) fibers were incorporated within the hydrogel matrix to introduce piezoelectric functionality while preserving biocompatibility, homogeneous fiber distribution, and viscoelastic behavior. The resulting electroactive hydrogels successfully converted externally applied ultrasound into localized electrical stimuli capable of enhancing myogenic differentiation. *In vitro* studies demonstrated significantly increased myosin heavy chain expression, differentiation index, and fusion index following ultrasound stimulation compared with non-stimulated controls, highlighting the potential of remotely activated piezoelectric biomaterials as minimally invasive therapeutic platforms for skeletal muscle regeneration.

The third objective of this thesis translated the knowledge acquired from injectable biomaterials into the field of skeletal muscle tissue engineering by developing a multifunctional piezoelectric bioink for extrusion-based three-dimensional bioprinting. While injectable hydrogels are particularly suited for minimally invasive therapies, bioprinting offers the possibility of generating highly organized tissue constructs capable of reproducing the anisotropic architecture of native skeletal muscle. To this end, ADA/Gel hydrogels were optimized by combining ionic and enzymatic external crosslinking strategies to achieve suitable rheological properties, prolonged structural stability, and high printability. Fragmented PVDF fibers were incorporated to generate fibrous and piezoelectric bioinks while maintaining homogeneous fiber dispersion, shear-thinning behavior, and excellent cell viability. The resulting bioinks enabled the fabrication of stable 3D constructs with high shape fidelity. Upon ultrasound stimulation, piezoelectric activation significantly enhanced myogenic differentiation and myotube maturation compared with control hydrogels. Proteomic analyses further provided mechanistic insights into the biological effects of piezoelectric stimulation, revealing the upregulation of proteins associated with skeletal muscle differentiation and maturation, including ACTN3, CAVIN4, MYH3, and MYH8, together with the enrichment of pathways involved in mechanotransduction, cytoskeletal organization, extracellular matrix remodeling, muscle development, and calcium signaling. Furthermore, the intrinsic alignment induced during the bioprinting process, generated anisotropic microenvironments that more closely reproduced the structural organization of native skeletal muscle and promoted enhanced muscle maturation.

In conclusion, this PhD thesis demonstrates that combining biomaterial engineering, electroactive materials, biofabrication, and targeted nanomedicine provides complementary strategies to enhance skeletal muscle regeneration. The developed injectable piezoelectric hydrogels, M12-functionalized RNA-delivery nanoparticles, and multifunctional bioinks promote myogenesis through structural support, electromechanical stimulation, and selective therapeutic miRNA delivery. Together, these platforms represent promising approaches for the treatment of VML and other skeletal muscle disorders.