Development of a Two-Phase Nanofiber Hydrogel System for Sustained Release of Extracellular Vesicles to Enhance Diabetic Wound Healing

Background and Rationale: According to the Centers for Disease Control and Prevention (CDC), as of 2021, an estimated 11.6% of the United States population has diabetes, resulting in a total direct and indirect estimated cost of $413 billion [1]. One of the most prevalent and severe complications contributing to this burden is Diabetic Foot Ulcers (DFUs), a result of hyperglycemia-induced nerve damage. DFUs are particularly challenging to treat due to poor blood circulation, nerve damage, and frequent recurrence [2, 3]. With limited success from current treatments such as surgical debridement, pressure offloading, and skin grafts, DFUs remain challenging to manage, with increased mortality and the risk of chronic, non-healing ulcers [2].

Hydrogels have emerged as a promising approach for managing DFUs due to their ability to retain moisture, promote keratinocyte migration, enhance collagen production and angiogenesis, and reduce scar formation. Hydrogels mimic the extracellular matrix (ECM) and provide structural support for cell growth and tissue regeneration. Hyaluronic acid (HA)-based hydrogels, in particular, have shown enhanced wound healing properties by increasing collagen deposition and blood vessel formation, making them an attractive matrix in improving the healing of chronic wounds like DFUs [4]. HA hydrogels can be further enhanced by loading them with biologically active components such as extracellular vesicles (EVs), cells, or growth factors. EVs, particularly those derived from mesenchymal stem cells (MSCs), have shown great potential in promoting healing, reducing inflammation, and stimulating angiogenesis. However, rapid clearance from the body limits their effectiveness. Incorporating EVs into hydrogels allows for controlled, sustained release at the wound site, enhancing their therapeutic potential [5,6].

Current treatments often focus on one phase of healing, such as inflammation or remodeling, limiting the overall recovery potential [7, 8]. Many hydrogels lack sufficient mechanical stability and volume retention to support gradual tissue healing [8, 9]. Additionally, uncertainty remains about the most effective EV sources for different healing stages, limiting the optimization of EV-based therapies [10]. To address the multifaceted challenges of DFUs, I propose a novel two-phase hydrogel system that delivers both short-term and long-term therapeutic benefits. The first phase of the system will focus on the initial inflammatory response, releasing EVs quickly to control excessive inflammation. The second phase will allow for sustained EV release to support tissue regeneration, angiogenesis, and collagen deposition during the later stages of healing. By granulating and combining these two hydrogel microparticles, we can create a comprehensive, injectable treatment that adapts to the wound healing process. Additionally, the inclusion of polycaprolactone (PCL) nanofibers that are covalently crosslinked to the hydrogels in both phases will enhance mechanical stability and support long-term regeneration, addressing the needs during the early and late stages of the DFU repair process.

Specific Aim 1: Design and validate a two-phase hydrogel system incorporating PCL fibers for short-term and long-term release. This aim focuses on developing and validating a novel two-phase hydrogel system designed for controlled, sustained release of therapeutic agents. One phase will focus on short-term release to address the inflammatory response, while the other will allow for long-term release to support prolonged tissue regeneration. The release profiles will be tuned by varying crosslinking density. The long-term phase will extend EV retention over several weeks by engineering an average pore size of around 1 μm, while short-term phase will be optimized for a 1-week release utilizing larger pore sizes of tens of microns to facilitate host cell infiltration and migration. Both phases will be loaded with EVs from adipose-derived stem cells (ADSCs) and incorporate covalently crosslinked PCL nanofibers to enhance mechanical integrity and volume retention. We will alter the nanofiber concentration to achieve the desired stiffness for each hydrogel phase. These mechanical properties will be measured using rheometry to ensure both phases meet the criteria for effective wound healing. We will validate the system by characterizing the rheological properties, degradation rates, and release kinetics of ADSC-EVs from each phase. We will also explore two approaches to combining the phases: physical mixing or covalent crosslinking, potentially designing a structure with a stiff hydrogel core and a softer outer layer to optimize mechanical stability and controlled release.

Specific Aim 2: Optimize the bioactive components of the two-phase hydrogel system for enhanced regeneration *in vitro*. This aim focuses on optimizing the bioactive content of hydrogels by comparing EVs derived from ADSCs and bone marrow-derived mesenchymal stem cells (MSCs). EVs will be extracted by subjecting these cells to stress conditions in culture media, followed by purification to ensure high-quality EVs. These will be compared against growth factors, cells, and commercially available treatments like Regranex [11]. To evaluate the regenerative capacity of the hydrogels, several *in vitro* assays will be conducted. Cell viability and infiltration will be assessed by seeding keratinocytes and fibroblasts onto the hydrogels, using live/dead staining (Calcein-AM/EthD-1) to visualize cell penetration and survival in the hydrogel's 3D structure. Angiogenesis potential will be evaluated by seeding endothelial cells and quantifying vessel formation to assess pro-angiogenic effects. Collagen production will be measured by seeding fibroblasts, using picrosirius red staining to quantify collagen deposition as a marker of extracellular matrix regeneration.

Specific Aim 3: Evaluate the performance of the optimized hydrogel system *in vivo* using a diabetic wound healing model. In this aim, we will evaluate the efficacy of the two-phase hydrogel system in promoting wound healing and tissue regeneration in a diabetic rodent model. Full-thickness wounds will be created on diabetic rats, and the animals will be divided into five treatment groups: (1) untreated control group, (2) group treated with a benchmark control, commercially available Regranex, (3) group treated with a single-phase short-term release hydrogel containing EVs, (4) group treated with a single-phase long-term release hydrogel containing EVs, and (5) group treated with the two-phase system containing EVs. Wound closure rates will be monitored at several key time points: days 4, 7, 28, and 56 post-treatment. Histological analysis will assess key markers of healing, including re-epithelialization, collagen deposition, and angiogenesis. Additionally, the inflammatory response will be analyzed by evaluating macrophage infiltration and polarization markers to determine the degree of inflammation modulation. Further analysis will focus on the formation of hair follicles and adipocytes to assess the hydrogel's ability to recover skin appendages, thereby providing a comprehensive evaluation of tissue regeneration.

Intellectual Merit: This project builds on my previous work under Professor Hai-Quan Mao at the Institute of NanoBioTechnology, where I developed nanofiber-containing hydrogels for soft tissue regeneration to prevent radiation fibrosis. By introducing a two-phase hydrogel system with sustained EV release, I aim to address limitations in diabetic wound healing treatments. The innovation stems from the combination of nanofiber hydrogels with EVs for targeted and sustained delivery will enhance tissue regeneration and optimize healing outcomes. This research leverages cutting-edge biomaterials and regenerative methods, providing a solid platform for future wound healing therapies while expanding on my prior expertise.

Broader Impacts: This project has the potential to significantly impact both scientific research and public health. By advancing DFU treatments, which are a major health burden and a leading cause of amputations, this work can improve the quality of life and reduce healthcare costs for millions of patients. The introduction of a two-phase hydrogel system with sustained EV release offers a novel approach to enhance wound healing, addressing current limitations in regenerative medicine. Moreover, with successful results, this technology could be further validated in larger animal models, paving the way for translation into human clinical applications. This step is essential for ensuring the therapeutic potential of this new therapeutic strategy for its widespread clinical adoption. Furthermore, this project will contribute to the broader scientific community through open-access publications and presentations at conferences, ensuring that the knowledge gained is widely disseminated. I am also committed to mentoring undergraduate students and fostering the next generation of scientists. Finally, this research will raise awareness of the importance of innovative wound healing strategies through outreach efforts, potentially inspiring further advancements in the field.

References: 1. CDC, *Diabetes*, 2024; 2. L. Yang et al., *World J Diabetes*, 2022; 3. L. Hu et al., *Skin Res Technol*, 2024; 4. J.R. Bardill et al., *Acta Biomater*, 2022; 5. Y. Ju et al., *Mater Today Bio*, 2023; 6. B. Safari et al., *Eur J Pharm Biopharm*, 2022; 7. H. Peng et al., *Chem Eng J*, 2023; 8. N. Hu et al., *Acta Biomater*, 2023; 9. D. Li et al., *Diabetes Res Clin Pract*, 2022; 10. Y. Xiong et al., *Small*, 2022; 11. U.S. Food and Drug Administration, Regranex (becaplermin) gel, 0.01% prescribing information, 2018.