Politecnico di Torino

Università degli Studi di Torino

Universitat Politècnica de Catalunya

Ph.D. in Bioengineering and Medical-Surgical Sciences

XXXVI Cycle

Design of a multi-functional, nanoparticles loaded supramolecular hydrogel to treat chronic wounds

Tutors:
Prof. Gianluca Ciardelli
Prof. Clara Mattu
Prof. Tzanov Tzanko
Prof. Monica Boffito

Candidate:
Giulia Crivello

Doctoral Examination Committee:

Prof. Ana Marina Ferreira Duarte, Newcastle University, United Kingdom, *Referee*
Prof. Jochen Salber, Ruhr-University Bochum, Germany, *Referee*
Prof. Chiara Tonda-Turo, Politecnico di Torino
Prof. Chiara Vitale Brovarone, Politecnico di Torino
Prof. Lorenza Draghi, Politecnico di Milano

Abstract of the Thesis

Chronic wounds (CWs) are defined as wounds that have failed to progress through a normal and timely healing process and may arise as a result of different pathologies including infections, tissue hypoxia, trauma, and chronic diseases, such as diabetes and cardiovascular ailments. These wounds represent a significant health concern as they may lead to severe tissue damage and even death. CWs are characterized by elevated levels of proinflammatory cytokines, reactive oxygen species (ROS), and enzymes (e.g., matrix metalloproteases, MMPs, and myeloperoxidase, MPO) which degrade the extracellular matrix (ECM) components and prevent wound closure. Additionally, bacteria in CWs secrete virulence factors that worsen the inflammatory condition. The aggregation of these pathogens into biofilms protects them from antibacterial treatments, preventing their eradication and enhancing the risk of antibiotic resistance. These factors together pose a substantial burden on healthcare systems, with patients necessitating long-term specialized care and repeated hospitalizations. Current treatments for CWs are largely inadequate, with no established gold standard, highlighting the need for innovative solutions that should be easy to handle and capable of combining multiple functions to address the multifactorial nature of CWs.

This PhD project responds to these needs by developing a multi-component dressing solution composed of an injectable hydrogel loaded with hybrid metal/organic nanoparticles (NPs) designed to reduce inflammation and control bacterial proliferation in CWs.

In **Chapter 2,** the development of an injectable, self-healing hydrogel is described. The hydrogel is based on a blend between two custom-made poly(ether urethane)s (PEUs), named CHP407 and SHF68, and α-cyclodextrins (αCDs), resulting in a self-assembled physical system. The two PEUs were synthesized from commercial amphiphilic block copolymers (Poloxamers®), used as macrodiols, and differ by the selection of the chain extender. For CHP407, a cyclic chain extender (1,4-cyclohexanedimethanol) was used, while SHF68 was synthesized with a chain extender containing pending BOC-protected amino groups (n-BOC serinol) and used after BOC-deprotection reaction. The use of custom-made synthetic polymers favors reproducibility and allows better control of the hydrogel properties. The initial tests focused on characterizing CHP407 and SHF68-based hydrogels at different PEUs content (1%, 3%, and 5% w/v) through reological tests and cytocompatibility. The results showed higher rheological performance for CHP407-based hydrogels, with maximum G' and G'' values of 6 kPa and 0.7 kPa respectively at 5% w/v, as compared to 2.5 kPa and 0.2 kPa respectively for SHF68-based hydrogels at the same concentration. However, SHF68-based hydrogels presented higher cytocompatibility with cell viability above 80% for all concentrations tested, while CHP407-based hydrogels presented cell viability above 80% only for the formulation at 1% w/v concentration. Given these premises, two blends (CHP407:SHF68 weight ratio 80:20 and 50:50) at different concentrations (1%, 3%, and 5% w/v) were tested to maximize their mechanical properties and cytocompatibility. These hydrogels were characterized in terms of rheological properties, stability in watery environments, cytocompatibility, and capability to rehydrate and absorb proteins from aqueous media mimicking exudate. The developed hydrogels showed injectability, and self-healing behavior, which allows the gel to comply with the wound shape and to withstand movements. The optimized system (3% w/v, CHP407:SHF68 weight ratio of 80:20) was a fully developed hydrogel, with values of G' and G'' around 10 kPa and 2 kPa, respectively. The optimized gel showed good cytocompatibility, with cell viability > 80% and stability for more than 24 h in conditions of high-medium turnover reproducing highly exudative wounds. The gel absorbed ~ 30% of large proteins from aqueous media and rehydrated in a few seconds. These properties make the hydrogel a suitable dressing in CWs by virtue of its good cytocompatibility, stability, ability to absorb varying quantities of fluids, and protein uptake. Thus, the optimized hydrogel was complemented with additional functionalities, either through combination with nanoparticles or through chemical modification or blending, as described in the following sections.

Chapter 3 describes the combination of the optimized hydrogel with hybrid polymer/inorganic NPs to obtain a dressing with antibacterial and antioxidative properties. To this aim, hybrid organic/inorganic NPs were obtained by combining the antibacterial properties of cobalt, with a lignin-based material (Lig-TA-LTH). Lig-TA-LTH was obtained by functionalizing lignin with the antioxidant tannic acid (TA) and L-tyrosine hydroxamate (LTH), an inhibitor of MMPs. The obtained Lig-TA-LTH is expected to enhance the antioxidant properties of Lig and to impart inhibitory activity against MMPs and MPO, two key enzymes involved in altering healing CWs. The obtained NPs (CoLig NPs) presented an irregular round shape with a size of around 200 nm. The incorporation of Lig-TA-LTH, with its polyphenolic nature, resulted in nearly 80% ROS scavenging activity at 2.5 mg/mL. Additionally, the NPs were able to inhibit more than 80% of the activity of MMPs and MPO. The presence of cobalt resulted in growth inhibition of *S. aureus* and *P. aeruginosa* at concentrations above 0.3 mg/mL and 0.6 mg/mL, respectively. Cytocompatibility tests indicated that cells could be treated with CoLig NPs at concentrations up to 0.6 mg/mL. Therefore, a concentration of CoLig NPs of 0.6 mg/mL was selected as the optimal condition to combine bacteria inhibition with high cytocompatibility and ROS scavenging ability.

CoLig NPs were then integrated into the optimized hydrogel, resulting in enhanced stability (up to 48 h) and improved rheological properties, with a more than 50% increase of G' and G''. The nature of the interactions between the NPs and the hydrogel components was investigated through cryo-scanning electron microscopy (cryoSEM) and Attenuated Total Reflectance–Fourier Transformed Infrared (ATR-FTIR) spectroscopy demonstrating a 40% increase in the crosslinking of the hydrogel network through interactions between NPs and both, αCDs and SHF68. Additionally, the hydrogel allowed the sustained release of the NPs with a nearly zero-order release kinetics. The antibacterial and antioxidant properties of the components released from the hydrogel were also confirmed by observing bacterial membrane disruption and ROS scavenging activity, which was above 40% throughout the hydrogel life. The composite system demonstrated significant potential for CW treatment due to its ease of application and multifunctional therapeutic actions combining antioxidant, anti-inflammatory, and antibacterial properties.

In **Chapter 4**, CoLig NPs were modified to include anti-biofilm capabilities, as previous tests in Chapter 3 highlighted the absence of antibiofilm functionalities albeit a promising antibacterial effect. The presence of biofilms in CWs results in hampered healing and in the development of antibiotic resistance. Therefore, wound dressings with biofilm disruption capacity are highly needed. To achieve this, two strategies were implemented: the first based on CoLig NPs coating with a nitric oxide (NO) donor to obtain NO-CoLig NPs, and the second based on substituting bare cobalt with a complex of cobalt and the photo-excitable dye phthalocyanine, to obtain CoPc-Lig NPs to be used for photothermal biofilm disruption. NO delivery is known to alter the nitroxidative state, therefore inducing destabilization of biofilms, while promoting regeneration. The negatively-charged CoLig NPs were successfully coated with positively charged amino cellulose, which was conjugated with a NO donor (S-nitroso glutathione, GSNO). While the coating significantly improved the cytocompatibility of CoLig NPs, no antibiofilm effects were observed, suggesting scarce NO release from GSNO in the test conditions. The second approach proposed photothermal treatment of biofilms. CoPc-Lig-NPs presented ROS scavenging ability at 0.2 mg/mL and MMPs inhibition at 0.8 mg/mL. Additionally, they were cytocompatible up to 4 mg/mL, significantly improving the cytocompatibility of CoLig NPs. At 4 mg/ml CoPc-lig NPs presented 4.8 log reduction of *S. aureus* growth. They could be internalized into both *S. aureus* and *P. aeruginosa* biofilms and produced a temperature increase of 7 °C upon near-infrared (NIR) light excitation. This temperature gradient led to a decrease in bacteria viability and a loss in biofilm integrity. Additionally, these NPs could be used in photoacoustic imaging responding to the need for monitoring CWs treatments. The photoacoustic signal was stable over time and could be detected in *ex vivo* phantoms. The developed CoPc-Lig NPs, combining antibacterial cobalt, antioxidant polyphenols, and photo-excitable phthalocyanines, deserve further investigation in CWs treatment.

In **Chapter 5**, SHF68 was functionalized with lig to obtain Lig-SHF68, through the enzymatic conjugation of Lig-TA to amino groups of SHF68, providing ROS-scavenging properties (> 80% at 12.5 mg/mL) and MMPs and MPO inhibitory activity (>50% at 10 mg/mL). The supramolecular hydrogel obtained by combining Lig-SHF68 at 5% w/v and αCDs maintained its antioxidant properties, with ROS scavenging activity above 80% in the first hour of release. Additionally, the weak interactions between lignin, αCDs and other hydrogel components extended the durability of the system. Indeed, at 5% w/v concentration, Lig-SHF68-based hydrogel was stable at 24 h and started to dissolve afterward, whereas SHF68-based hydrogel disassembled after 6 h. Interestingly, the rheological properties of Lig-SHF68 based-gel were lower compared to pristine SHF68 based gel, with G' and G'' values decreasing from 2400 Pa and 200 Pa in Lig-SHF68 hydrogel to 780 Pa and 75 Pa in SHF68-based system (at 5% w/v). To improve stability and rheological properties, Lig-SHF68 was blended with a photosensitive polymer (i.e., poly(ethylene glycol) diacrylate, PEG-DA) to provide additional chemical crosslinking through light irradiation at 365 nm and 10 mW/cm^2 in presence of a catalytic amount of a photoinitiator. The modified hydrogel (5% w/v overall polymer concentration, Lig-SHF68:PEG-DA weight ratio 80:20) presented properties comparable to the hydrogel developed in Chapter 2 by blending CHP407 and SHF68 (3% w/v PEU content, CHP407:SHF68 weight ratio 80:20), showing injectability, self-healing capacity above 70%, and G' and G'' values of 9300 Pa and 2300 Pa, respectively. Additionally, the hydrogel maintained a good antioxidant activity (around 20% throughout its life) and displayed longer durability for over 7 days. The hydrogel was also able to sustain the release of CoPc-Lig NPs and of the antioxidant drug curcumin, with delivery mechanisms dependent on the nature of the encapsulated compound.

Overall, the innovative hydrogel/NP system developed in this PhD project represents a promising tool for CWs treatment. The hydrogel was injectable, granting the ability to adapt to the wound bed, and was able to absorb exudate and maintain moisture. The functionalization with Lig-TA provided MMPs and MPO inhibitory capacity and antioxidant properties. Blending with a photosensitive polymer produced light-mediate chemical crosslinking which improved stability, thus reducing dressing changes. CoLig NPs with broad-spectrum antibacterial activity, antioxidant properties, and capacity to inhibit MMPs and MPO were successfully obtained. CoPc-Lig NPs with photothermal and photoacoustic behavior were also successfully obtained. These particles were cytocompatible, antioxidant, antibacterial, and were able to enter bacteria biofilms. This work presents promising results which deserve further investigation. Future

research should focus on finding the optimal combination between the developed solutions, e.g., combination of CoLig NPs with CoPc-lig NPs to find the optimal balance between broad-spectrum antibacterial activity and antibiofilm capacity. Investigation of the photoacoustic imaging potentialities and a deeper analysis of the tissue regeneration ability of the dressing should also be considered.