NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY "POLITEHNICA"

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Doctoral School of Chemical Engineering and Biotechnology

PhD thesis

Composite polymeric biomaterials with different architectures for tissue engineering applications

– ***Summary*** –

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Keywords: composite biomaterials, chitosan, graphene oxide, electrospinning, 3D-printing, tissue engineering.

The first chapter of the PhD thesis presents a critical study of literature data about insights in tissue engineering and skin tissue engineering. The newest composite polymeric biomaterials with applications in wound dressings are also presented, including polysaccharides (chitosan, alginate etc.), proteins (collagen, gelatin) and graphene oxide-based composite biomaterials. The methods employed in the preparation of scaffolds are also briefly described, such as casting, freeze-drying, electrospinning, and 3D-printing.

The second chapter consists of the extensive presentation of the original contributions, starting from the used materials and characterization methods, objectives and originality of the PhD thesis, then the research studies that form the basis of this thesis are presented.

In the context of pressing need to develop new biomaterials against the background of ongoing advancements in biotechnology, **the general objective of the PhD thesis consists of the design and development of advanced chitosan (CS)-based composite biomaterials with various architectural features and proper characteristics to be used as efficient materials in skin TE, particularly as wound dressings.**

The remarkable characteristics of CS, including its biocompatibility, biodegradability, antibacterial activity, and adaptability in both formulation and functionalization, drive its extensive use as polymeric structures in the field of tissue engineering, particularly in the development of wound dressings. In the pursuit of creating innovative biomaterials with unique morphological and biological attributes, CS is employed either as the primary polymeric matrix within the composite scaffolds or as an auxiliary component in the assembly of intricate multi-component scaffolds.

Composite polymeric biomaterials are prepared by incorporating various types of graphene oxide (GO) derivatives functionalized with carboxyl groups or amine moieties (GO-COOH or rGO-TEPA), along with synthetic polymers (polyethylene oxide - PEO) and various natural polymers (such as SA polysaccharide, GM protein). This is done to enhance mechanical strength, physical stability, and biocompatibility. Furthermore, bioactive agents such as indomethacin-based prodrug and tetracycline hydrochloride (TCH) are integrated to improve the therapeutic qualities of the engineered scaffolds.

The PhD thesis explores a range of manufacturing techniques, as they significantly impact the final properties of scaffolds. It begins with traditional methods such as casting and freeze-drying, resulting in the formation of porous structures incorporating GO-COOH within CS matrix. The study then delves into electrospinning, a well-established technique, enabling the fabrication of nanofibrous structures constituted by CS/PEO polymeric matrix containing GO-COOH and CA-functionalized CS (CsA)/PEO polymeric matrix containing rGO-TEPA. Finally, the research finishes by combining two cutting-edge techniques, 3D printing and electrospinning, to produce a bicomponent assembly with complex architecture comprising a highly porous layer made of 3D-printed sodium alginate (SA)/gelatin methacryloyl (GM) hydrogel and a nanofibrous layer made of electrospun CS/PEO membrane.

To achieve the previously described general objective, the entire research activity is centered on pursuing four specific objectives. The doctoral thesis comprises four research studies, each organized into four subchapters, each guided by a specific objective labeled as "**S.O.**".

- **S.O. 1:** Development of GO-COOH-containing CS composite scaffolds with highly porous architecture using casting and freeze-drying methods and exploration of the chemical interactions between the two compounds;
- **S.O. 2:** Design and fabrication of GO-COOH-containing CS composite scaffolds with nanofibrous structure using electrospinning technique for potential application in TE and investigation of the impact of GO-COOH concentration on the electrospinning ability, structural, morphological and biological features of the electrospun scaffolds;
- **S.O. 3:** Rational design of rGO-TEPA-containing CsA composite nanofibrous membranes using electrospinning technique as efficient antibacterial materials used in skin TE and investigation of the effect of rGO-TEPA concentration on the structural, rheological, morphological, and biological properties of the electrospun membranes;
- **S.O. 4:** Building of complex bicomponent scaffolds based on CS/PEO nanofibrous membrane loaded with IMC-based prodrug and SA/GM hydrogel loaded with TCH using the combination strategy of electrospinning and 3D-printing technologies, and the evaluation of their *in vitro* performances for use as efficient wound dressings.

The first study was presented in **subchapter 2.3.** and was focused on the development of CS/GO-COOH composite scaffolds with highly porous architecture and exploration of the chemical interactions between the two compounds. The original contribution of this study consists of a new chemical approach for synthesizing composite CS scaffolds with GO-COOH content using conventional casting and freeze-drying techniques. To achieve complex CS/GO-COOH structures, it is essential to generate a significant quantity of -COOH groups on the GO surface while employing mild chemical conditions. The ultrasonication of GO-COOH sheets within the CS matrix, casting, and freeze-drying as processes, were approached in the synthesis, in order to prepare both a uniform dispersion of GO-COOH and a porous structure of the scaffolds. Spectrophotometric techniques confirmed the molecular-level interaction between the -OH and -NH_2 groups of CS and the -COOH groups of GO-COOH, thereby validating this chemical interaction. ATR-FTIR spectrometry revealed the presence of non-covalent interactions such as hydrogen bonds, in addition to the formation of new amide covalent bonds. Meanwhile, Raman results indicated that the optimal dispersion of GO-COOH occurred within the CS/GO-COOH 0.5% scaffold. Moreover, the effect of GO-COOH concentration on morphology, mechanical attributes, and thermal properties was examined. The μ-CT analysis highlighted a porous and sparse microstructure of the scaffold with the highest concentration of GO-COOH (1%). This observation aligned with the compression test results, which indicated a reduction in mechanical properties attributed to the aggregation of GO-COOH sheets at higher concentrations. Nevertheless, using the TGA technique, an enhancement in the thermal stability of the composite scaffolds became evident. This improvement is indicated by a decrease in the percentage of total weight loss, as the GO-COOH amount increases, from 77% for CS/GO-COOH 0.1% to 67% for CS/GO-COOH 1%.

The second study was presented in **subchapter 2.4.** and contributes to the expansion of scientific field through development of novel composite scaffolds containing CS/PEO matrix and GO-COOH, with a nanofibrous structure. The study aimed to design and fabricate the CS/GO-COOH composite scaffolds by electrospinning technique and examine the impact of GO-COOH concentration on the electrospinning ability, structural, morphological, and *in vitro* biological characteristics of nanofibrous scaffolds. Incorporating GO-COOH within the CS/PEO polymeric matrix offers the advantage of enriching the system with -COOH functionalities. This enrichment can amplify both non-covalent interactions and covalent bonds between the components,

ultimately improving the integrity of the nanofibrous materials. In this complex system of chemical interactions, glutaraldehyde serves as the crosslinking agent for the electrospun scaffolds. It participates in the formation of imine covalent bonds and acetal bridges with the functional groups of CS and GO-COOH, thereby greatly enhancing the stability of the resulting materials. The interactions were verified by FTIR and Raman spectrometry, along with the DLS technique, which enables the assessment of hydrodynamic properties of the system's components. The Raman results highlighted a uniform distribution of GO-COOH sheets within the CS/PG 0.1% and CS/PG 0.2% electrospun composite scaffolds. The nanofibrous microstructure and the uniform dispersion of GO-COOH sheets along the composite nanofibers were observed in SEM micrographs. Additionally, it was found that the nanofiber's average diameter reduced with an increase in GO-COOH concentration, ranging from 141 nm for CS/PG 0.1% to 119 nm for CS/PG 0.5%. Furthermore, the results indicated that these interactions led to a significant improvement of the material elasticity, which was explored at nanoscale using the ultramodern nanoindentation technique. Results from *in vitro* biological tests indicated that, following 72 hours of incubation, CS/PG 0.1% and CS/PG 0.2% promoted the proliferation of fibroblast cells. CS/PG 0.5% presented a cytotoxic effect on the tested cell culture, which may be attributed to the agglomeration of GO-COOH sheets, as evidenced by Raman and SEM analyses. The overall characteristics of CS/PG 0.5% were negatively impacted by the presence of GO-COOH agglomerates.

The third study was presented in **subchapter 2.5.** and its original contribution consists of the incorporation of citric acid-functionalized CS (CsA) and rGO-TEPA into a composite scaffold with nanofibrous structure. The study aimed to design and fabricate CsA/rGO-TEPA composite membranes intended for wound dressings applications, using the electrospinning technique and investigate the influence of rGO-TEPA concentration on structural, rheological, morphological, and biological properties of the electrospun structures. By the covalent functionalization of CS with CA, confirmed via FTIR spectrometry and ^{1}H- and ^{13}C-NMR spectroscopy, numerous -COOH groups were introduced, serving a dual purpose: enhancing the electrospun material's hydrophilicity and promoting the interaction with the $-NH_2$ groups present in the rGO-TEPA structure. Based on Raman results, it was observed that the rGO-TEPA sheets were uniformly dispersed throughout the composite nanofibrous membrane of CsA/PG_T5, indicating the highest degree of exfoliation. An examination of rheological properties and SEM analysis indicated a link between the flow behavior of the precursor systems and the formation of bead-free nanofibers. The

CsA/PG_T5 composite system produced smooth and bead-free nanofibers containing rGO-TEPA sheets that were evenly distributed along the nanofibers. The DSC tests demonstrated that as the concentration of rGO-TEPA increased, there was an improvement in the thermal stability of the composite materials, leading to a higher melting temperature of 68.0°C in CsA/PG_T5 compared to 65.6°C in CsA/PEO. Concerning the *in vitro* biological analyses, the CsA/PG_T2 material presented the highest cell viability, as indicated by the MTT assay, and consequently, the least cytotoxic impact on NCTC fibroblast cells, as demonstrated by the LDH test. Furthermore, all nanofibrous membranes displayed effective anti-biofilm properties against both *P. aeruginosa* and *S. aureus* bacterial strains, with particularly impressive results against Gram-negative bacteria.

The fourth study was presented in **subchapter 2.5.** and significantly contributes to the advancement of wound dressing field by development of a bicomponent scaffold using a combination of diverse biopolymers (including polysaccharides and chemically modified protein), that were processed by electrospinning and 3D-printing technologies, with the integration of various bioactive compounds to facilitate and enhance the wound healing process. This research purposed to create complex bicomponent BiFp@Ht scaffolds consisting of an outer CS/PEO nanofibrous membrane loaded with a pIMC prodrug (Fp) and an inner SA/GM hydrogel loaded with TCH (Ht), using the strategy of combination of electrospinning and 3D-printing technologies, and the assessment of their overall *in vitro* performances to determine their applicability as effective wound dressings. The therapeutic compounds were loaded into the aforementioned polymeric matrices using different methods to facilitate their release, depending on the component that interacts with the wound, owing to their distinct mechanisms of action. On one hand, the 3D-printed hydrogel surface was loaded by physical adsorption with TCH, a potent antibacterial drug recognized for its ability to locally inhibit the bacterial protein synthesis. On the other hand, IMC as IMC-PEG-IMC prodrug, was incorporated within the CS/PEO polymeric blend before the electrospinning process. IMC, an anti-inflammatory drug, was transformed into the IMC-PEG-IMC prodrug through chemical bonding with H_2N-PEG-NH_2, to improve its bioavailability and therapeutic efficacy. This design aimed to accomplish two goals: firstly, a more rapidly release of the antibacterial drug upon near contact with the wound, preventing infection, which is crucial for facilitating efficient regeneration; and secondly, to ensure a gradual and sustained release of IMC for effective immunomodulatory activity throughout the system. The pivotal aspect in designing an effective wound dressing scaffold is the architecture of both layers. Consequently, SEM images

were highly effective in demonstrating the maintained structural characteristics of the bicomponent BiFp@Ht scaffold, highlighting the nanofibrous design of the electrospun Fp membrane and the porous structure of the 3D-printed Ht hydrogel. Based on the results of *in vitro* biological experiments, the BiFp@Ht demonstrated exceptional biocompatibility. The Fp membrane displayed the highest cell viability and growth rate according to the MTT assay, while the Ht hydrogel showed the greatest level of cytotoxicity in the LDH assay, after 72 hours of incubation. The Live/Dead qualitative test results supported the outcomes observed in the MTT and LDH assays. Similarly, the BiFp@Ht scaffold exhibited impressive anti-inflammatory and proangiogenic properties, along with remarkable efficacy against *E. coli* and *S. aureus* bacterial strains. The evaluation of cellular responses highlighted the scaffold's capacity to boost HeLa cell adhesion and growth, while also facilitating the formation of new blood vessels to promote the healing of damaged tissue.

The first work was entitled "*Carboxylated graphene oxide integrated chitosan composite scaffolds as encouraging materials for tissue engineering*", written by the authors: E. Cojocaru, A. M. Onaș, and H. Iovu, and published in the U.P.B. Scientific Bulletin, Series B, volume 82, issue 4, 2020, ISSN 1454-2331.

The second work was entitled "*Synthesis and characterization of electrospun composite scaffolds based on chitosan-carboxylated graphene oxide with potential biomedical applications*", written by the authors: E. Cojocaru, J. Ghitman, E. I. Biru, G. G. Pircalabioru, E. Vasile, and H. Iovu, and published in the Materials MDPI, volume 14, issue 10, 2021, 2535.

The third study was entitled "*Electrospun nanofibrous membranes based on citric acid-functionalized chitosan containing rGO-TEPA with potential application in wound dressings*", written by the authors: E. Cojocaru, J. Ghitman, G. G. Pircalabioru, C. Stavarache, A. Serafim, E. Vasile, and H. Iovu, and published in the Polymers MDPI, volume 14, issue 2, 2022, 294.

The fourth research was entitled "*Electrospun/3D-printed bicomponent scaffold co-loaded with a prodrug and a drug with antibacterial and immunomodulatory properties*", written by the authors: E. Cojocaru, J. Ghitman, G. G. Pircalabioru, A. Zaharia, H. Iovu, and A. Sarbu, and published in the Polymers MDPI, volume 15, issue 13, 2023, 2854.

The third chapter presents the general conclusions of the thesis, and the dissemination of the results was achieved by publications in international journals and oral presentations at international conferences.