SUMMARY OF PhD THESIS

Design, preparation, and characterization of new composite biomaterials for enhanced efficacy in current and future biomedical applications

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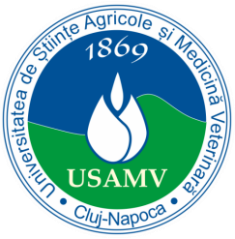

INTRODUCTION

This thesis highlights the significant expansion of research in biomaterials over recent years and their importance in medicine, due to their specific properties and ability to stimulate tissue and organ regeneration. Biomaterials are increasingly used in modern medicine to enhance both human and animal quality of life and longevity.

The introduction covers a wide range of biomaterials, including synthetic and natural polymers, emphasizing their role in tissue engineering and regenerative medicine. It also discusses the advantages and disadvantages of biopolymers and synthetic polymers, with a focus on their potential when combined in composite materials that integrate natural and synthetic components.

The aim of this doctoral thesis is to develop two composite materials with advanced properties, combining at least one organic component with an inorganic one into a unique chemical structure to maximize synergistic effects. Exploring such a strategy is crucial in biotechnology and has the potential to revolutionize approaches to medical challenges such as bacterial infections, epithelial cancer, and wound healing. This approach could lead to the development of more effective and durable treatments to improve public health.

The thesis entitled "Design, Development, and Characterization of New Composite Biomaterials for Enhanced Effectiveness in Current and Future Biomedical Applications" is structured into two distinct parts. The first part, titled "CURRENT STATE OF KNOWLEDGE," comprises two main chapters: **Chapter 1**, "Synthetic Material with Therapeutic Properties - BIOGLASS," and **Chapter 2**, "(Bio)polymers Used in Biomaterial Synthesis (SS, SF, and NaCMC)."

The second part, titled "PERSONAL CONTRIBUTION," includes six chapters: **Chapter 3**, "Motivation and Research Objectives"; **Chapter 4**, "Research Methodology"; **Chapter 5**, "Results and Discussions on the Structure and Cytotoxic Antitumor Effect of Studied Phosphate Glasses"; **Chapter 6**, "Results and Discussions on the Characterization of Composite Biomaterials"; **Chapter 7**, "General Conclusions and Future Perspectives"; and **Chapter 8**, "Originality and Innovative Contributions of the Thesis."

Below, a summary of these chapters will be presented. Additionally, this doctoral thesis includes 15 tables, 63 figures, and 351 bibliographic references.

1. Synthetic material with therapeutic properties - BIOGLASS

In the Chapter 1, the necessity for developing bioactive glasses (BGs) in real-world applications and their evolution in biomedical uses is briefly described. These glasses have been utilized from bone tissue regeneration to promoting and healing skin wounds in tissue engineering. The first BG, known as Bioglass® 45S5, was created by Professor Larry Hench in 1969. His aim was to develop a material capable of bonding with bone tissue without causing adverse reactions from the biological system (Hench, 2006). In the 1980s, it was discovered that bioactive glasses (BGs) can also interact with soft connective tissues (Li et al., 2019). Since then, significant progress has been made in the field of bioactive glasses (BGs), including new production methods, the use of forming/modifying ions, and the creation of 3D scaffolds for various applications in tissue engineering (Kondo et al., 2024; K. Zhang et al., 2022).

A type of bioactive glass that stands out in current research due to its excellent biocompatibility and biodegradability properties is phosphate glasses (PGs) (Monmaturapoj et al., 2022). These glasses are of great interest in the biomedical field for a variety of applications. Moreover, by doping them with various transition metals (MTs), they become true reservoirs of active biological ions that can be released into the target organism upon their degradation (Lapa et al., 2020).

2. (Bio)polymers used in the synthesis of biomaterials (SS, SF, and NaCMC).

Chapter 2 highlights the importance of natural polymers such as silk (SS and SF) and synthetic polymers like sodium carboxymethyl cellulose (NaCMC) in the development of composite materials. Additionally, this chapter explores the latest research in synthesizing new composite materials based on these (bio)polymers in the biomedical field, emphasizing innovation in synthesis methodologies, advanced structural characterization, and evaluation of biological performance of these materials.

Natural silk, produced by the silkworm Bombyx mori L., is a remarkable biopolymer due to its unique structural, mechanical, and chemical properties. The silkworm undergoes a complete life cycle comprising four stages: egg, larva, pupa (chrysalis), and adult moth, with an adult lifespan of about 10 days. During the metamorphosis into the pupal stage, the larvae produce cocoons (nests of natural silk), each consisting of a single long silk filament (Koh et al. 2015). The silk secretion is carried out through the serpiginous apparatus comprising two silk glands: the silk

gland proper and the spinneret gland. The silk filament is composed of two main glycoproteins: sericin and fibroin (Chirila et al., 2013).

Silk sericin (SS) is a water-soluble protein that was previously considered a by-product of the textile industry. Today, SS has gained significant importance in scientific research due to its remarkable mechanical and biological properties. These include moisture regulation capability, UV radiation resistance, high oxygen permeability, excellent biocompatibility, cell growth stimulation effect, and mitogenic effect (Arango et al., 2021). Currently, SS is recognized as a versatile material in various applications, including the synthesis of new biomaterials, tissue engineering, drug delivery, development of cosmetic products, and treatment of acute wounds (Munir et al. 2023). Several techniques are used for extracting SS from silk filament (a process called degumming): chemical treatments, enzymatic methods, and boiling water treatment.

Silk fibroin (SF) represents 60-80% of the composition of silk filament and is responsible for its strength and durability. SF possesses numerous remarkable properties such as biocompatibility, porosity, biodegradability, and mechanical hardness, making it one of the most valued materials in tissue engineering. Furthermore, SF can be processed into various forms including hydrogels, gels, scaffolds, fibers, porous sponges, or films (Ma et al., 2021). Additionally, SF is highly beneficial for simulating the skin microenvironment, scar removal, and alleviating atopic dermatitis. These properties have drawn researchers' interest in tissue engineering for developing dressings for wound treatment (Yang et al., 2017). However, this biopolymer requires a complex process for dissolution and can form various morphologies of regenerated fibroin (RSF) depending on the solvents used. It is insoluble in most solvents, including water, and requires special solutions for manipulation and long-term storage. Processing methods include chemical and enzymatic approaches, as well as advanced treatments to modify the SF structure for diverse applications in tissue engineering

Sodium carboxymethyl cellulose (CMC or NaCMC) is a polysaccharide derivative of cellulose. Its properties, such as solubility, binding strength, and viscosity, are influenced by its degree of purity and substitution. NaCMC can be of food grade, industrial grade, or pharmaceutical grade, each category having specific applications (Rahman et al., 2021). The main characterization methods for NaCMC are also presented. Materials based on NaCMC have extensive applicability due to being biodegradable, non-toxic, biocompatible, and water-retentive, with applications in agriculture, food packaging, textiles, water treatment, tissue engineering, and 3D bioprinting (Tyagi & Thakur 2023). NaCMC also holds enormous potential in tissue engineering as a matrix for cell growth and new tissues (Zennifer et al., 2021). NaCMC is used in the synthesis of new dressings, controlled drug delivery systems, and hydrogels for tissue synthesis de novo (Moreira et al., 2024).

3. Motivation and objectives of this research

Chapter 3 outlines the purpose and objectives of this doctoral thesis. Composite materials science has become crucial in multidisciplinary studies due to the complexity of these materials combining natural and synthetic elements.

The main goal of the research in this doctoral thesis was to design, develop, and characterize two advanced composite materials with special properties geared towards biomedical applications. To achieve this goal, two overarching objectives were defined:

(i) synthesis and characterization of two vitreous oxide systems doped with transitional metal ions (MT).

(ii) development and characterization of two composite materials with potential medical applications.

4. Research methodology

This chapter explains in detail aspects related to: the biological material used in this research; the substances, reagents, and raw materials used in the preparation of glasses and in the synthesis of composite materials; the extraction methods applied; the equipment and techniques used in their characterization; the software used in data processing, as well as a detailed description of the synthesis methodology of the two composite biomaterials.

5. Results and discussion regarding the structure and antitumor cytotoxic effect of the studied phosphate glasses

This chapter presents the results of experiments regarding the structure of phosphate glasses of the two synthesized oxide systems and the *in vitro* evaluation of their biological properties, particularly their anti-tumor characteristics. In brief, the soluble properties of the two phosphate systems were tested based on the added doping concentration. Glass samples in both bulk and powdered forms were used for this purpose. The results indicated that the dissolution rate of phosphate glasses was increased in aqueous medium (DIW) for both systems, except for sample C8 with x = 16 mol% CuO (Oosterbeek et al. 2021; Edathazhe & Shashikala 2018; Zhang & Cresswell 2016). The pH measurements in PBS solution indicated immediate reactivity of the studied glasses, showing a time-dependent decrease in pH value during incubation. This phenomenon resulted from rapid ionic exchange between network-modifying ions attached to NBO (non-bridging oxygen) units and HO in the PBS

solution. Density measurements based on Archimedes' principle were also conducted, highlighting the modifying role of doping ions (V and Cu). Additionally, in the case of the system V_2O_5· (100-x)[0.2CaF_2·0.6P_2O_5·0.2CaO] where 0 ≤ x ≤ 16 mol%, vanadium ions played a dual role as both network formers and modifiers.

XRD analyses highlighted the vitreous structure of glasses from both systems, while FT-IR and RES analyses underscored significant structural changes. These structural modifications were more pronounced at higher doping concentrations (x ≥ 4 mol%) (Ferraa et al. 2021; Kowalski, Wyrzykowski et al. 2020). EDX mapping showed the homogeneous structure of the glasses, and SEM-EDX measurements revealed numerous irregularly shaped glass fragments with uneven distribution, likely due to sample grinding, confirming the presence of constituent elements as per the preparation recipe.

In vitro studies on the cytotoxic anti-tumor effect of phosphate glasses were conducted using aqueous solutions of glass samples, referred to as glass-based treatments. From each vitreous oxide system, one glass sample alongside the matrix was selected for tumor evaluation: for the vanadium system, samples V7 and M were chosen, and for the copper system, samples C6 and M were selected. Multiple human cell lines were tested, including both cancerous and normal lines: melanoma cell line A-375 [A375] (CRL-1619™) and colon adenocarcinoma cell line Caco-2 [Caco2] (HTB-37™) from ATCC (American Type Culture Collection, Rockville, MD, USA), as well as ovarian carcinoma cell line A2780 and normal human fibroblast cell line Hs-27 from ECACC (European Collection of Authenticated Cell Cultures, Salisbury, UK).

For both vitreous oxide systems, matrix-based treatments (M) exhibited a proliferative effect on the tested cancer cells. In contrast, V7 and C6 treatments (doped with metal ions) demonstrated a strong anti-proliferative effect, dependent on treatment concentration. Considering the results on tumor cell lines, matrix-based treatment (M) was also tested on normal skin cells (Hs-27 fibroblasts), proving to be a non-toxic material capable of supporting attachment and proliferation of these cells due to its nature (Azizi et al., 2021).

6. Results and discussions on the characterization of composite (hybrid) biomaterials

Chapter 6 includes information on the synthesis and characterization results of two composite biomaterials (hydrogel and cream) as follows:

For NaCMC/CA/BG composite hydrogels, their crosslinking was confirmed using FT-IR analysis, which showed two additional distinct peaks around 1716 cm^{-1} and 1743 cm^{-1}, both attributed to carbonyl group stretching (C=O), and another peak observed at ~1217 cm^{-1} associated with the C–O vibration of unreacted carboxylic groups (C–O). SEM-EDX analysis highlighted the homogeneous, porous, and 3D

structure of the hydrogels. These pores are crucial for facilitating water diffusion within the hydrogel network and aiding cell migration (Ngadi et al. 2008). Furthermore, the mechanical properties of the porous medium (texture) are closely linked to the size, shape, type, and distribution of pores, making them vital for tissue engineering applications (Tang et al., 2014).

Rheological studies revealed non-Newtonian, pseudoplastic, and thixotropic behavior for all analyzed hydrogels, indicating excellent injectable performance. These also confirmed that these hydrogels can be smoothly extruded from a syringe without obstruction. This demonstrates their injectability, allowing them to conform closely to skin lesion margins and minimize invasion of surrounding tissues (Cui et al., 2022; Wang et al., 2019).

The antimicrobial activity of the prepared hydrogels was investigated using the Kirby-Bauer disc diffusion method against six bacterial strains, including both Gram-negative and Gram-positive bacteria. The results provided valuable insights into the hydrogels' ability to inhibit the growth and proliferation of these bacterial strains, highlighting their antibacterial nature and potential as biomaterials for various biomedical applications. It is noteworthy that the only bacterium that did not show any inhibition zone was *E. faecalis*.

For Gly/BG/SS composite creams, part of the analyses focused on the individual characterization of silk sericin (SS) and bioactive glass (BG). Fourier-transform infrared spectroscopy (FT-IR) studies confirmed the presence of specific functional groups characteristic for each cream type. Additionally, typical absorption bands of functional groups characteristic of silk proteins were observed in the composite cream spectra. However, characteristic bands of BG were not detectable in these FT-IR spectra due to the low concentration of BG used (0.33% w/v), making it undetectable in the analyzed samples. pH measurements revealed the bioactivity of the creams by immersing them in a simulated body fluid similar to blood plasma. In this experiment, the pH decrease was directly proportional to the incubation time and was attributed to degradation processes involving released ions from the sample reacting with ions in the SBF solution. However, the pH changes caused by the composite creams remained within a safe range for the human body. This demonstrates that Gly/BG/SS composite formulations have balanced bioactivity and can be used as topical products.

Rheological studies demonstrated that Gly/BG/SS composite formulations have ideal properties for topical application, providing stable consistency that prevents leakage. These characteristics support the practical application of the creams, ensuring efficient use on the skin(Manian et al., 2022).

Antibacterial tests did not demonstrate antibacterial activity of the composite creams against the two bacterial strains tested (S. aureus and E. coli). However, the results suggest the possibility of a bacteriostatic effect. Further *in vitro* analyses and

tests are required to confirm this hypothesis.

7. General conclusions and future perspectives

7.1. General conclusions

The research conducted in this doctoral thesis has led to the following general conclusions regarding:

I. BIOGLASS SISTEMS

- Two vitreous oxide systems were prepared and studied starting from a phosphate matrix (M) with the system $0.2CaF_2 \cdot 0.6P_2O_5 \cdot 0.2CaO$:

  $\mathbf{S_1} \rightarrow xV_2O_5 \cdot (100 - x)\ [0.2CaF_2 \cdot 0.6P_2O_5 \cdot 0.2CaO]$ where $0 \leq x \leq 16$ mol%;
  $\mathbf{S_2} \rightarrow xCuO \cdot (100 - x)\ [0.2CaF_2 \cdot 0.6P_2O_5 \cdot 0.2CaO]$ where $0 \leq x \leq 16$ mol%.

- The analysis of the two phosphate systems was conducted using a variety of techniques, including X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FT-IR), electron spin resonance (ESR), scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX), pH measurements, density measurements, dissolution tests, and *in vitro* evaluations of their anticancer properties.
- XRD analysis confirmed the presence of vitreous structure for both systems (S1 and S2). The dissolution tests indicated an increased solubility of glass samples in DI water (DIW), except for sample C8 in system S2 (x = 16 mol%).
- SEM measurements confirmed the homogeneous structure of glasses from both systems and showed that they did not undergo significant changes with increasing dopant ion concentrations.
- The phosphate systems (S1 and S2) caused a reduction in the pH value of the PBS solution, indicating their reactivity.
- The density (ρ) of the tested glasses increased, and the molar volume (Vm) decreased with increasing dopant content in the vitreous structure of both systems. The phosphate network structure was significantly affected for both systems.
- Both IR spectra, density measurements, and ESR of the vanadium-doped phosphate system highlighted the dual role of V_2O_5, acting as a network modifier at low concentrations ($x < 1$ mol%) and a network former at high concentrations ($x \geq 1$ mol%).
- For the CuO-doped system, FT-IR spectra revealed the contribution of copper oxide to the glass structure, acting as a network modifier leading to the formation of NBOs. EPR results indicated a high sensitivity of Cu^{2+} ions to the local environment, especially for $x \geq 4$ mol%.
- *In vitro* cytotoxicity studies of V7 glass samples (from system S1) and C6 glass samples (from system S2) demonstrated strong anti-tumor effects on A375, A2780,

and Caco-2 cell lines, respectively A375. In contrast, matrix-based treatment (M, without dopant) showed good biocompatibility, with viability exceeding 70% for all concentrations used in both systems.

II. COMPOSITE MATERIALS

- The two composite materials were prepared using an eco-friendly synthesis method.
- Characterization of the composite materials involved a diverse set of analytical techniques, including Fourier-transform infrared spectroscopy (FT-IR), rheology studies, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX), biodegradation tests, and the Kirby-Bauer disc diffusion method for evaluating antibacterial properties. This comprehensive approach allowed for detailed investigation of the composition, structure, and functional properties of the two composite materials.

COMPOSITE HYDROGEL

- An FT-IR analysis confirmed the crosslinking of composite hydrogels with CA, showing the binding of carboxylic groups of CA to the hydroxyl groups of cellulose.
- Rheology study results indicated that all analyzed hydrogels exhibited non-Newtonian, pseudoplastic, and thixotropic behavior, suggesting good injectability performance.
- SEM-EDX analysis demonstrated a porous, homogeneous, and 3D structure of lyophilized composite hydrogels. Crosslinking of hydrogels with CA led to compaction of their structure. EDX analysis confirmed the presence of all elements as per the preparation recipe.
- The antibacterial activity of the hydrogels was tested against both Gram-positive and Gram-negative bacterial strains. The results highlighted the strong antibacterial properties of NaCMC/CA/BG composite hydrogels. Enterococcus faecalis was the only bacterium that showed resistance, including resistance to the antibiotic GEN.

COMPOSITE CREAM

- For composite creams, the synthetic component (BG) was analyzed separately. *In vitro* biocompatibility studies demonstrated that the BG material is non-toxic and capable of supporting the attachment and growth of cells (Hs-27).
- FT-IR analysis showed that there were no significant differences between the extracted silk sericin and the commercial sample. Additionally, FT-IR spectra confirmed the presence of SS in the structure of Gly/BG/SS composite creams.
- *In vitro* biodegradability tests indicated that the pH changes in the SBF

solution caused by the composite creams remained within a safe range for the human body, suggesting that they can be used as a topical product.

- Rheological studies showed that the addition of SS to the glycerin structure (the cream matrix) increased the viscosity and structural stability of the composite creams. SS was the key component in determining the texture of the cream. Moreover, the Gly/BG/SS creams exhibited solid viscoelastic behavior.
- Antibacterial tests did not reveal the antibacterial properties of the composite creams. However, the results suggested the possibility of a bacteriostatic effect. Therefore, further *in vitro* analyses and tests are needed to confirm this hypothesis.

7.2. Future perspectives

Just as any study can further be improved, this is also the case for the current thesis. Based on the obtained results, the research can be easily extended with *in vivo* studies on the two obtained composite materials. Animal testing (rabbits or rats) and even clinical trials would be crucial in defining the final results. Additionally, *in vivo* experiments should be accompanied by histopathology tests, which are highly valuable in evaluating tissue response by identifying inflammation, necrosis, regeneration, and integration of the materials at the cellular level. These tests allow for detailed observation of cellular and tissue interactions, providing essential information on the integration and long-term effectiveness of the studied composite materials.

For vitreous systems, a series of additional tests could reinforce the obtained results. Further *in vitro* studies, such as UV-Vis spectrophotometry, could provide valuable data on the absorption, stability, and state of doped metal ions (vanadium and copper ions). This information would be crucial for understanding and optimizing the behavior and performance of phosphate glasses in the proposed applications. Additionally, antibacterial tests, ranging from the determination of minimum inhibitory concentration (MIC, IC50) to diffusion tests (Kirby-Bauer), could evaluate the antimicrobial potential of these systems. Moreover, thermal treatments applied to phosphate glasses could introduce new properties, opening new directions in research.

Thus, integrating these studies and additional experiments can provide a completer and more detailed picture of both the behavior of the glasses and the effectiveness of applying composite materials. This significantly contributes to advancing knowledge in the fields of biomaterials and biotechnology.

8. The originality and innovative contributions of the thesis

The research activity carried out in this thesis, based on the study of specialized literature, extensive experimental activities, and the use of various physico-chemical techniques for the analysis of the structure of the synthesized products, has led to the following elements of originality:

- Two vitreous systems doped with transition metals (TM) were synthesized, featuring a unique composition on a global scale. The components of the phosphate matrix and their molar ratios were chosen to ensure a high degree of solubilization, while consisting solely of macroelements (Ca, P) and trace elements (F).
- The bioglasses, being highly soluble in aqueous media (≥90%), enabled the creation of a DIW-based BG extract through a proprietary methodology inspired by the literature. Subsequently, these aqueous solutions (extracts) were tested *in vitro* to evaluate biocompatibility and cytotoxicity on cancer cells A275, Caco-2, A2780, and normal cells Hs-27.
- The creation of a glassy matrix (sample M) with excellent adhesion and cellular support properties, which proved to have a proliferative effect on both tumor cell lines and the normal fibroblast line Hs-27.
- The actual realization of the two composite materials (the hydrogel and the composite cream) represents an element of originality. Additionally, obtaining and analyzing these materials based on a set of experimental data regarding their mechanical and biological properties is another element of originality.
- Other elements of originality include: establishing a customized testing program for the obtained materials; developing the products (both synthetic and composite) based on the studied documentation; the entire research conducted to achieve the proposed objectives; and interpreting the results obtained from the tests performed.

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