“POLITEHNICA” UNIVERSITY of BUCHAREST

APPLIED CHEMISTRY AND MATERIALS SCIENCE DOCTORAL SCHOOL

Ph.D. Thesis

“New cellulose-based biomaterials for tissue engineering applications”

\- Summary -

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Abstract

Cellulose-based biomaterials may have a multitude of applications in fundamental research and biomedical engineering. Since the existing biomaterial designs are in short supply due to rapid technological advances and several critical applications require cellulose dissolution in aqueous media, in this PhD thesis, new cellulosic hydrogel-based formulations are proposed, where water-based solvent systems and surface functionalization are exploited as strategies for the development of biomaterials suitable for tissue engineering applications.

The strategies proposed herein will preserve cellulose key features and enable the synthesis of cellulose precursors compatible with advanced fabrication techniques. The studies described in Ph.D. Thesis explores polymeric blends of cellulose and naturally derived polymers, which were utilized to aid cellulose in producing efficient biomaterials. This thesis describes the synthesis and characterization of cellulose-alginate films using a co-solvent system, a comprehensive investigation of the printability of cellulose-alginate blends supplemented with additives, the synthesis and characterization of printable pectin-cellulose hydrogels and the synthesis and characterization of printable gelatin methacrylate – cellulose hydrogels. Given the difficulty of designing environmentally sustainable biomaterials based on cellulose, these results demonstrate the polymer's potential to create economically feasible biomaterials with predictable characteristics and structures tailored to the intended use.

This Ph.D. thesis contributes to an expanding body of research in tissue engineering aiding in the development of functional biosynthetic scaffolds. The newly discovered biomaterials are encouraging innovation in the direction of complex manufacturing techniques and processes.

Keywords: Cellulose, Hydrogels, Biomaterials, 3D printing, Tissue Engineering.

Summary of the Ph.D. Thesis

Spectacular advances have been made over the past 20 years in which cellulose-based biomaterials contributed to a multitude of applications in fundamental research and biomedical engineering. Being a complex resource with a wide range of characteristics that may be tuned, cellulose has certain key benefits over conventional materials, making it a compelling platform for biomaterials design, where macroscale effects can be achieved also through different dimensional features of cellulose.

Nonetheless, when working with cellulose, several critical applications, including the biomedical field, require its dissolution in aqueous media. Hydrogels are the leading material class suitable for the design of biosynthetic tissue as the highly hydrated assembly of polymer chains can provides the 3D microenvironment needed for cell survival, adhesion and migration. Typically a difficult task for a variety of reasons, cheaper and environmentally friendly strategies to synthesize cellulose-based hydrogels are now available, where most of them rely on cellulose derivatization. Therefore, it is of great interest to investigate the potential of different strategies that preserve cellulose key features and enable the synthesis of cellulose-based precursors compatible with advanced fabrication techniques.

Up to date, the technological advancements outperform the materials development, so that there is a pressing demand for new biomaterial designs. **Therefore, in this PhD Thesis novel hydrogel-based formulations out of cellulosic materials with applications in tissue engineering are proposed.** With due regard for effective biomaterials for tissue engineering development, two strategies to exploit cellulose were studied: the use of water-based solvent systems and surface functionalization. Moreover, naturally derived polymers were used to assist

cellulose in generating performant biomaterials. Through the comprehensive characterization of the proposed innovative hydrogels, valuable insights on the factors that influence the outcome properties and their suitability for advanced fabrication technique are provided.

In the current state of development, technological advances outperform the material designs, creating an urgent need for novel biomaterial formulations for complex fabrication of scaffolds***. This PhD Thesis's general objective is to develop innovative cellulosic hydrogel-based formulations and to characterize their overall performance in accordance with the advancements and needs in the area of biomedicine***. Following the mission and vision, the research work endeavor:

Objective 1. A viable approach for creating novel biomaterials for TE by combining cellulose with different biopolymers. Natural compounds such as the following were envisaged for this purpose: (i) polysaccharides, such as alginates and pectins, which are highly appealing for developing plant-based biomaterials for specific biomedical applications, notably for drug delivery and tissue regeneration, on account of their renewable nature; (ii) proteins as their usage is critical in the development of cell-interactive biomaterials, gelatin being a valuable tool in the development of biomaterials. When it comes to constructing temporary, biosynthetic scaffolds that allow cell adhesion and three-dimensional tissue growth, cellulose hybrid or composites biomaterials have played a key role.

Objective 2. Exploiting the polymers capability to generate 3D printable formulations and further produce biomaterials with controlled architecture. As many of these technologies are new and, as a result, unexplored, they imply a genuinely interdisciplinary approach to investigation. In light of the new perspectives that 3D printing technologies open up by allowing for ultimate control over the shape, porosity, morphology, and dimensions of a construct, this work aims to explore the possibility of various methods for preserving the essential properties of cellulose and enabling the synthesis of cellulose-based precursors compatible with modern manufacturing processes.

1.2.2. Specific objectives (SO).

Cellulose, either as the primary scaffold material or as an auxiliary component, is aimed to provide biomaterials with particular characteristics. In order to achieve the main goals, strategic and specific objectives were set.

SO1. To design and characterize cellulose-alginate films using a water-based solvent system. For this purpose, the following activities were raised:

1. Use of PEG/NaOH aqueous system as solvent for microcrystalline cellulose
2. Synthesis of biopolymeric films using a dual-crosslinking method
3. Investigate the influence of PEG molecular weight and the effect of the feed ratio between the two natural macromolecules (cellulose and alginate) on the swelling kinetic, spectral, thermal, morphological, and mechanical features.

SO2. To study the additives effect on the printability of cellulose-alginate blends; For this purpose, the following activities were raised:

1. Synthesis and structural characterization of oxidized nanocellulose.
2. Formulation of bi-component inks using one type of cellulose (carboxymethylcellulose or nanocellulose) in combination with sodium alginate, supplemented with additives (glycerol / PEG).
3. Investigation of inks printability and of the additives effect on precursors rheological properties.
4. Optimization of 3D scaffold fabrication and investigation of shape fidelity.

SO3. To design and characterize pectin-cellulose hydrogel-based inks; For this purpose, the following activities were raised:

1. Synthesis and structural characterization of low-methoxylated pectin

2. Formulation of composite inks using oxidized nanocellulose in combination with low-methoxylated pectin.

3. 3D printing using direct extrusion technology.

4. Optimization of the printing process and investigation of the effect of feed ratio between the two natural macromolecules on inks printability

5. Fabrication of 3D scaffolds and evaluation of swelling kinetic, morphological, and mechanical features.

SO4. To design and characterize gelatin methacrylate – cellulose hydrogel-based bioinks. For this purpose, the following activities were raised:

1. Synthesis and structural characterization of gelatin methacrylate of two different types: bovine and fish sourced.

2. Formulation of composite inks using oxidized nanocellulose in combination with gelatin methacrylate.

3. 3D printing using microvalve-based technology

4. Optimization of the printing process and investigation of the effect of feed ratio and type of gelatin on inks printability

5. Fabrication of 3D scaffolds and evaluation of swelling behavior, morphological features, and biocompatibility.

6. Formulation of bioinks and evaluation of cell's viability toward the process of 3D printing.

To achieve these goals, the thesis has been divided into seven chapters.

The structure of ***Chapter 1*** is meant to give a clear insight on the background, the materials, and the design and fabrication of the biosynthetic platforms for tissue engineering. The first section addresses the main principles and developments in tissue engineering, where significant emphasis has been devoted to the use of cellulose in the biomedical field. The following section outlines the materials used to assist cellulose in generating performant biomaterials for tissue engineering and argues the material selection. The biopolymers employed are described here in detail, highlighting their key characteristics of which the end-product may benefit. The third section portrays the fabrication methods used in this PhD Thesis and includes a detailed description of the 3D printing technology. Finally, discussion on the current context and motivation of this study is presented.

The PhD Thesis describes novel designs for cellulose-based biomaterials, the main experimental results being presented in *Chapters 2-5.* Given the numerous challenges in shaping material biomechanical properties to emulate the macro and micro structural organization of the natural tissue, cellulose-based materials are envisioned for scaffold design together with different fabrication methods.

Chapter 2 describes the synthesis and characterization of biopolymeric films based on cellulose and alginate, were poly(ethylene glycol) (PEG) /NaOH system was used as a co-solvent. The effect of the feed ratio between the two natural macromolecules as well as the influence of the PEG chain was investigated towards the swelling, kinetic, spectral, thermal, and morphological properties. Out of this chapter the following conclusions can be drown:

1. Biopolymeric films based on cellulose (its microcrystalline form) and alginate were synthetized using a green solvent and a double crosslinking method which led to stable networks in both the dried and water-swollen states.

2. The developed cellulose-alginate films are flexible and have a good thermal stability.

3. The ratio between the two polysaccharides as well as the molecular mass of PEG employed in the synthesis strongly influence the material properties as follows: a higher cellulose content will increase the hydrogel stiffness and also the swelling capacity; higher content of alginate influences the morphological features inducing a higher roughness; while a PEG with a higher molecular weight will increase the compatibility between the two components.

4. Due to the fact that the overall properties can be customized by changing the ratio between cellulose and alginate, these may be promising structural materials, while given the green designing of hydrogel films, it could make a significant contribution to the development of biomedical engineering materials.

In ***Chapter 3***, the plasticizing effect of additives (Glycerol and PEG) on cellulose-based blends used for the fabrication of 3D printed scaffolds for tissue engineering application was studied. A full characterization of the polymer blends and composites in terms of structural, morphological, rheological and printability using direct extrusion technique is presented. Based on the printing results relative to the predesigned structure, it can be concluded that:

1. Bi-component inks can be prepared utilizing carboxymethylcellulose or oxidized nanocellulose nanofibers to modify particular physical characteristics, and by comparison it was observed that nanocellulose based inks exhibit a more pronounced shear-thinning behavior.

2. Glycerol or PEG can be used as wetting agents to improve the printability of the inks and may have antagonist impact on the rheological and printing characteristics of polysaccharide-based polymers.

3. Optimization of the printing process provides a comprehensive description on role and effects that fabrication parameters (pressure, speed) exhibit in direct extrusion process with respect to filament formation and width.

4. Employing glycerol in the ink mixture resulted in the best 3D printing fidelity.

5. Fabricating 3D scaffolds from a cellulosic material coupled with alginate and an appropriate additive not only provides precise geometries but also directs the biological uses of the resulting hybrid hydrogels.

In ***Chapter 4***, novel pectin-nanocellulose printable formulations are developed and scaffolds are fabricated via 3D-printing. The flowing properties and printability of precursor inks are evaluated, while upon crosslinking the performance of the 3D-printed structures are assessed in terms of structural stability, morphological, rehydration and mechanical properties. The selection of most suitable ink formulation is assessed. On the account of investigations carried out, it can be concluded that:

1. 3D printable composite inks prepared by combining oxidized cellulose and low methoxylated pectin can generate self-supporting polysaccharide constructs that can be easily and rapidly crosslinked in cell-friendly conditions.

2. The assessment of flowing behavior showed that pectin addition improves injectability and increase the yield stress while preserving cellulose’s shear-thinning response.

3. Investigation of the printing process considering variation of many fabrication parameters explain the effects of ink properties on printing settings; yet, upon optimization all formulations have exhibited high printability, particularly when higher pectin content was used.

4. Pectin concentrations have both an effect on the viscoelasticity of the hydrogel and an influence on the stiffness of the hydrogel and demonstrated excellent suitability for biomaterials design.

5. Bio-based cellulose-pectin formulations' rheological and printing behavior are shown to be promising in addition to providing effective crosslinking mechanism, and therefore, may be applied in 3D printing for TE application.

In ***Chapter 5***, bioink-formulations based on cellulose and gelatin methacrylate are described. The bioprinting performance using human adipose stem cells (hASCs) of the newly developed hydrogel inks together with the morphological, swelling and biological properties are studied. High fidelity 3D constructs are printed herein using micro-valve based extrusion and a detailed description of influencing parameters is given. Since the development of appropriate bioprinting materials has become a significant area of research, with one of the primary difficulties being the design of consistent, dependable, and cell-interactive formulations, herein are described for the first time extremely dependable and stable bioinks that were based on methacrylamide-modified gelatin in combination with oxidized cellulose nanofibers. Based on the study carried out, it can be concluded that:

1. Protein-polysaccharide physical interactions are effective in the structural integrity of the materials, cellulose supporting gelatin throughout the fabrication process.

2. Investigation of inks printability using the micro-valve based system provided valuable elucidation on the role of fabrication parameters in printing accuracy and proved that the deposition can be mainly controlled by valve parameters to use minimum pressure.

3. Through printing tests, the thermo-sensitive behavior of developed inks was described, showing that formulation comprising the fish-based gelation derivative exhibit a broader printability window, therefore the printing process becomes more convenient and cost-effective.

4. All formulations delivered 3D structures with well-defined internal structure and a high level of shape accuracy, under optimized conditions.

5. Investigation of acellular 3D scaffolds properties in terms of swelling behavior, morphological features and biocompatibility showed that, regardless of gelatin type, a higher protein content decreases the swelling capacity and the porosity as a consequence of an increased hydrogel stiffness. Nonetheless, high cell viability was observed for all samples, especially for protein-rich samples.

6. Ultimately, the bioprinting results established that newly created biomaterial inks can maintain the viability of embedded cells throughout the process of fabrication and that the resulted 3D constructs offer suitable support for cell loading, dispersion, and growth.

The final conclusions and a general overview are presented in ***Chapter 6,*** emphasizing the applicability of these cellulose-based biomaterials in the field of tissue engineering and ***Chapter 7*** provides a review of the original contributions. Overall, this Ph.D. thesis adds to a growing corpus of research in TE providing novel hydrogel-based formulations out of cellulosic materials with tunable biomechanical properties facilitating the development of functional biosynthetic scaffolds. Considering the challenging design of green biocompatible materials based on cellulose, these findings prove the capability of this natural polymer to produce affordable biomaterials with controlled properties and architectures guided by envisioned application. The current research's general conclusion is that newly developed biomaterials are stimulating innovation toward superior manufacturing methods.

Ph.D. thesis “New biopolymer based composites with targeted application” presents main scientific achievements realized, being constituted on four papers which were presented with the following logic: from most common polymeric components using a conventional and highly accessible fabrication technique (chapter 2) to polymer functionalization and layer by layer fabrication (chapter 3) and more and more complex and unexplored printable formulation (nanocellulose and pectin – chapter 4), up to 3D bioprinting, using cell-embedded formulation (chapter 5). Research results obtained during the doctoral stage were disseminated through the publication of 3 articles in ISI quoted journals and one manuscript is to be submitted, with candidate as first author.

The main original contributions are:

1. Development of cellulose-alginate films using a water-based solvent that is environmentally friendly followed by a suitable dual-crosslinking process.

This concept has been never published to date, this being the only research study exploiting the idea of addition of alginate into dissolved cellulose in PEG/NaOH system. The knowledge in the field of biopolymeric films have been improved and strengthened by the synthesis and characterization of these materials that exhibit tunable properties in terms of swelling, flexibility, mechanical strength and stability in the wet state. The ability to tailor the overall properties of the obtained materials by varying the ratio of cellulose to alginate makes them potentially useful structural materials, and the environmentally sustainable design of hydrogel films suggests that they could make important contributions to the field of biomedical engineering materials.

2. Formulation of bi-component inks using one type of cellulose (carboxymethylcellulose or nanocellulose) in combination with sodium alginate, supplemented with additives (glycerol / PEG).

The obtained oxidized cellulose nanofibers were studied in combination with alginate and promising ink formulations are described, bringing in the foreground the contribution of additives. The study on these ink formulations as well as on the effect of additives provided a comprehensive knowledge on the properties required for 3D printing and the comparative research encouraged the design of new performant ink formulations. Also, this study showed that wetting agents can be used as to improve the printability of the inks, yet a careful selection of the ink components as they can also negatively impact the rheological and printing characteristics of polysaccharide-based polymers. This study stimulates research towards polysaccharide-based materials designs and as a result the disseminated research results had even gained international media interest.

3. Design of new bio-based ink formulations suitable for 3D printing using pectin and oxidized nanocellulose

The employment of this combination of biological materials to produce 3D printable composite inks is reported for the first time and holds tremendous potential in TE as well as for animal-free biofabrication (for instance clean-meat production).

Upon characterization, the outstanding property of pectin to modulate the elasticity, hydration behavior and printability of the materials was proven. After analyzing the flow behavior, it was shown that adding pectin enhances injectability and increases yield stress while also retaining cellulose's shear-thinning response. The impacts of ink qualities on printing settings are explained by an investigation of the printing process that takes numerous fabrication aspects into account.

Investigation of pectin as ink component represents pioneering research making it an attractive choice for biomaterials design. The newly developed formulations showed great printability, especially when pectin concentration was increased and, moreover, hydrogel viscoelasticity and stiffness are both affected by pectin concentrations.

4. Formulation of bioinks based on methacrylamide-modified and oxidized cellulose nanofibers

The research on 3D printable formulations continued exploring the printability of cell-embedded cellulose-gelatin formulations. In this thesis bioinks that exhibit high biocompatibility, reproducibility and very low sensitive upon temperature changes are introduced for the first time. The formulated bioinks based on methacrylamide-modified and oxidized cellulose nanofibers were optimized for micro-valve driven printing process, the study providing in addition a detailed evaluation on the fabrication parameters that influence the printing quality.

The thermosensitive behavior of developed inks was detailed through printing experiments, demonstrating that formulations including the fish-based gelatin derivative showed a wider printability window, making the printing process more convenient and cost-effective. Yet, according to research done on the characteristics of acellular 3D scaffolds, a larger protein concentration reduces swelling capacity and porosity and it was shown to be true independent of gelatin type.

Given the high degree of consistency and high shape fidelity achieved in the 3D printed structures alongside with high cell viability, these formulations are appealing for further development as platforms for TE.

5. Elaboration of printing protocols and parameters-optimization procedures for low viscosity inks using 3D contact printing.

In parallel with the design of biomaterials, to address the limitations of conventional fabrication methods, 3D printing technology was foresighted. In this respect, the technical aspects were studied step by step, from machine operation to virtual 3D modeling, to master the fine tuning of the process so that high fidelity prints will be achieved and further to gain expertise on how to integrate hydrogels as printable formulations, which technology is best suited for specific formulations and ensuring the high standards in bioprinting.

The research studies presented in the thesis were focused on the design and characterization of cellulose-based hydrogels and also on the optimization of the printing protocols and parameters to fabricate well defined 3D structures using direct extrusion and microvalve-based printing. The studies described herein provide comprehensive description of the key fabrication parameters that influence the printing quality, where two different 3D contact printing technologies are employed: direct extrusion and micro-valve based printing.

Overall, this thesis makes valuable contributions to the field of biomedicine through the design and comprehensive characterization of innovative hydrogels and it provides significant insights into the factors that influence the outcome properties of the hydrogels as well as their suitability for use in 3D printing. *The broad implication of the present Ph.D. thesis is that the newly developed biomaterials are driving the innovation toward advanced fabrication techniques.*