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**“POLITEHNICA” UNIVERSITY of BUCHAREST**

**DOCTORAL SCHOOL OF CHEMICAL ENGINEERING AND BIOTECHNOLOGY**

**Ph.D. Thesis**

**“*BIOPOLYMER COMPOSITES FOR MEDICAL APPLICATIONS OBTAINED BY 3D PRINTING*”**

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# Aim and outline of the Thesis

Due to the increasingly frequent bone diseases and the growing appeal for organ transplantation, a multitude of materials and technologies have been widely studied. Among them, gelatin and alginate have gained special interest, because of their versatile and tunable properties, but also because of their fundamental characteristics like solubility in water, biocompatibility, biodegradability, biomimetic and soft mechanical properties and non-immunogenicity. Another important aspect recommending gelatin and alginate biopolymers to be used in tissue engineering is their cost-effectiveness.

Therefore, all the mentioned features propose alginate and gelatin as satisfactory biopolymeric matrices for the synthesis of biopolymer composites with great applicability in regenerative medicine.

The main goal of this PhD Thesis was to synthesize ***Biopolymer composites for medical applications obtained by 3D printing***. In this respect, two biopolymers were selected as raw materials – alginate and gelatin methacrylate which was synthesized from gelatin. In order to achieve effective materials for regenerative medicine, suitable also for the 3D printing process, each biopolymer was modified with different inorganic agents namely, nanoclay and hydroxyapatite. Structural and morphological features of the synthesized materials were thoroughly investigated.

***The general objective of the present PhD Thesis is the design and construction of 3D nanocomposites scaffolds based on two raw materials namely, gelatin methacryloyl and alginate, with potential applications in regenerative medicine, particularly, in bone regeneration.***

To achieve these ambitious goals, the research study had the following objectives:

**Objective 1**

The first objective is represented by the development of new 3D printable materials with applications in bone regeneration, using gelatin methacrylate and alginate as raw materials and nanoclay and hydroxyapatite as reinforcing inorganic nanofillers.

Nanoclays were used to improve the mechanical, rheological, and morphological properties of GelMA and alginate biopolymers, due to their osteoconductive and osteoinductive properties.

Hydroxyapatite doped with different ions was used to improve the mechanical properties of GelMA, due to its osteoconductive and osteoinductive features.

**Objective 2**

The second objective ~~wa~~s the evaluation of the printability properties of the novel nanocomposites, the optimization of the 3D printing parameters and the design and evaluation of the scaffold’s shapes fidelity. In order to investigate the effect of inorganic agents in the polymeric matrix and their effect on osteogenic differentiation, the characterization of the 3D printed nanocomposites scaffolds regarding the biological, morphological, mechanical, nanomechanical, swelling and degradation properties, was thoroughly followed.

## 

## **Specific objectives (SO)**

Using GelMA and alginate as main materials to obtain 3D printable bioinks, the following objectives were settled:

* ***SO1.*** Establishing the 3D printing protocol with optimal characteristics for methacrylate gelatin.In this respect, the following steps were done:
* Synthesis and characterization of gelatin methacrylate (GelMA) with different degrees of methacrylation.
* Preparation of the inks, using three concentrations of GelMA and two concentrations of photoinitiator for each mixture.
* Printability study for each composition obtained using extrusion technology and optimization of the 3D printing parameters.
* Design and manufacture of 3D printed scaffolds based on GelMA.
* Characterization of the 3D printed scaffolds regarding the morphological, nanomechanical, rheological, swelling and degradation properties.

These results were published in: *R.L. Alexa et al. “3D-printed gelatin methacryloyl-based scaffolds with potential application in tissue engineering”, Polymers, 2021, 13(5), 727.*

* ***SO2.*** The improvement of the mechanical, rheological, and biological properties of GelMA. The following actions were made in this regard:
* Synthesis and structural characterization of GelMA.
* Formulation and characterization of the nanocomposite inks based on GelMA and three types of clay.
* Investigation of nanocomposite 3D printability, and observation of the clays effect on the rheological properties of the GelMA hydrogel.
* Optimization of the 3D printing parameters, and evaluation of the scaffold’s shapes fidelity.
* Evaluation of the 3D printed scaffolds regarding the morphological, mechanical, nanomechanical, swelling and degradationproperties in order to investigate the influence of clays when included in GelMA matrix.

These results were published in: *R.L. Alexa et al. “Assessment of naturally sourced mineral clays for the 3Dprinting of biopolymer-based nanocomposite inks”, Nanomaterials, 2021, 11(3), 703.*

* ***SO3.*** Construction and investigation of 3D printed nanocomposites based on GelMA-modified with hydroxyapatite doped with different ions in osteogenic differentiation. In this regard, the subsequent actions were followed:
* Synthesis and structural characterization of GelMA.
* Synthesis and structural characterization of hydroxyapatite dopped with Ce/Zn/Mg ions (HC, HZ, HM).
* Formulation and characterization of nanocomposite materials based on 20% GelMA and different concentrations of hydroxyapatite dopped with Ce/Zn/Mg (GelMA-HC1, GelMA-HC3, GelMA-HC5, GelMA- HZ1, GelMA- HZ5, GelMA- HZ10, GelMA- HM2, GelMA- HM5, GelMA- HM10).
* Based on the biological and morphological results, the selection of the appropriate concentration of HC, HZ, HM, was carried out to be further used in the obtaining of 3D printing inks.
* 3D printing of the materials using extrusion technology and optimization of the printing parameters.
* Investigation of the 3D printed scaffolds shapes fidelity.
* Evaluation of the 3D printed scaffolds' morphological, nanomechanical, swelling, and degrading characteristics as well as how they affect osteogenic differentiation.

These results were published in: *R.L. Alexa et al. “3D Printable Composite Biomaterials Based on GelMA and Hydroxyapatite Powders Doped with Cerium Ions for Bone Tissue Regeneration”, International Journal of Molecular Sciences, 2022, 23(3), 1841.*

*R.L. Alexa et al. “3D Printed Composite Scaffolds of GelMA and Hydroxyapatite Nanopowders Doped with Mg/Zn Ions to Evaluate the Expression of Genes and Proteins of Osteogenic Markers”, Nanomaterials, 2022, 12(19), 3420.*

* ***SO4.***Studying the effect of different types of clay on the biological and osteogenic performances of 3D printed scaffolds. In this respect, the following steps were done:
* Formulation and structural characterization of nanocomposites inks based on alginate (5% w/v) and natural clay, using two concentrations of Cloisite Na (18% w/w, 20% w/w).
* 3D printability study using extrusion technology and evaluation of the scaffold’s shapes fidelity.
* Evaluation of morphological, mechanical, and swelling properties induced by different concentrations of clay in the polymeric matrix.
* Formulation and characterization (structural and rheological) of nanocomposites inks based on alginate and different types of nanoclays (Cloisite Na, Closite 93A, Cloisite 30B, Cloisite 20A, and Cloisite 15A)
* Printability study of the inks and observation of the clays effect induced on the rheological properties of the alginate matrix.
* Optimization of the 3D printing parameters and evaluation of the scaffold’s shapes fidelity using the analysis of the roundness open pores.
* Characterization of the morphological, mechanical, swelling and degradation properties induced by the nanoclays and their effect on osteogenic differentiation.

These results were published in: *R.L. Alexa et al. “3D printing of super concentrated alginate clay ink with potential application in regenerative medicine”, UPB Scientific Bulletin, Series B: Chemistry and Materials, 2021.*

*R.L. Alexa et al. “3D Printing of Alginate-Natural Clay Hydrogel-Based Nanocomposites”, Gels,2021, 7(4), 211.*

To achieve these goals, the thesis entitled ***Biopolymer comp\osites for medical applications obtained by 3D printing,*** was divided in five chapters.

The aim of ***Chapter 1*** is a brief presentation of both the selected materials used in obtaining 3D printable inks, as well as the technology used in the design and 3D printing of 3D constructs employed in tissue engineering (TE).

In the ***first section*** a summary introduction of the general topic area is presented specifically, the features of the biopolymers frequently used in TE, as well as the 3D manufacturing technology.

In the ***second section***, **GelMA** structure and properties, crosslinking methods and applications in 3D printing, are presented.

In the ***third section***, the extraction, the structure and properties, the crosslinking methods and applications in 3D printing are described for the second biopolymeric matrix particularly, the **alginate**.

The ***last section*** describes the inorganic fillers used for synthesis of nanocomposite hydrogels, firstly the hydroxyapatite, followed by the next section describing the nanoclay fillers.

**Chapter 2** presents the general and specific objectives of the scientific research**.**

**Chapter 3** was divided in six sections, corresponding to the six articles published during the thesis.

***Section 3.1*** introduces the original contribution referring to the method used to construct 3D scaffolds using as main material gelatin methacryloyl with possible exploitation in regenerative medicine.

In this respect, GelMA with three different methacrylation degrees was synthesized (63%, 64% and 66% w/v.). Then, distinct concentrations of gelatin methacryloyl (10%, 20%, 30% w/v), and specific concentrations of Irgacure 2959 (0.5% and 1% w/v), were explored for each type of GelMA synthesized (GelMA 63%, 64%, 66%w/v).

The aim of ***Section 3.2*** isto describe the method used to develop 3D printable hydrogels based on GelMA and three types of nanoclay (Cloisite Na, Cloisite 30B, Cloisite 15A). Clays were utilized as reinforcing inorganic nanofillers, and GelMA served as the polymeric matrix.

***Section 3.3*** presents the technology and methods used to design and print 3D scaffolds based on gelatin methacryloyl and HA doped with cerium ions with possible utilizations in tissue engineering, respectively in bone regeneration. In the first step, Cerium ions were used to substitute the Ca2+ ions in the of HA structure. Afterwards, three concentrations of HA doped and three concentrations of gelatin methacryloyl, were used to be able to obtain the optimal concentration for the synthesizing of 3D printable hydrogel inks.

The aim of ***Section 3.4*** was to develop bio-inks made from gelatin methacryloyl and HA doped with magnesium and zinc ions, intended for TE application. In this regard, Ca2+ ions that are found in hydroxyapatite composition were substituted with magnesium ions, respectively, zinc ions. Then, a certain concentration of hydroxyapatite doped with magnesium/zinc and three concentrations of GelMA, were explored in the additive manufacturing process of innovative 3D composite platforms.

In ***Section 3.5,*** the developing of3D printable nanocomposite hydrogel inks based on alginate and natural nanoclay is described. Specifically, our study investigated two concentrations (18% and 20% w/v) of Montmorillonite (under the commercially name of Cloisite Na) at a fixed concentration of alginate (5% w/v).

The aim of ***Section 3.6*** wasto obtain biomaterials which exhibit appropriate 3D printability, based on alginate and nanoclays. In this respect, six hydrogels based on alginate and alginate modified with five types of clay, were developed. The clays used for this study, were unmodified clay Cloisite Na, and modified MMT’s, namely Cloisite 30B, Cloisite 93A, Cloisite 20A and Cloisite 15A. All the obtained homogenous mixtures were 3D printed and further the resulted 3D nanocomposite platforms were examined from ~~a~~ structural and morphological perspective.

***Chapter 4*** presents the general conclusions of the concepts and the obtained results, while ***Chapter 5*** provides a brief review of the original contributions of this thesis.

Original contributions:

1. Development of 3D-Printed Gelatin Methacryloyl-Based Scaffolds with Potential Application in Tissue Engineering

For the first time in the field, a systematic study regarding the influence of methacrylation degree, concentration of polymer, and concentration of photoinitiator on 3D printing was performed, using as raw material gelatin methacrylate.

This research work is regarded as being of significant value since it offers a thorough assessment of some parameters that affect the hydrogels based on GelMA's ability to be 3D printed, as well as their mechanical, morphological, and swelling properties.

2. Assessment of Naturally Sourced Mineral Clays for the 3D Printing of Biopolymer-Based Nanocomposite Inks

The development and characterization of the new inks based on GelMA-Cloisite Na, GelMA-Cloisite 30B, GelMA-Cloisite 15A was first reported in this study. The current study is a groundbreaking investigation into the use of natural and altered clay in GelMA-based 3D printing inks.

The effect induced by the mineral clay to the GelMA matrix was studied through rheological and printing tests, showing that clay inclusion in the polymeric matrix led to higher viscosities, consequently, inducing stability during the 3D printing process. Also, 3D printed scaffolds were studied regarding the shape fidelity using a roundness analysis of open pores, and regarding morphological, mechanical, and swelling properties.

All these findings enrich the knowledge regarding the behavior of these inorganic clays in the GelMA matrix and in 3D printing process.

3. The development of 3D Printable Composite Biomaterials Based on GelMA and Hydroxyapatite Powders Doped with Cerium Ions for Bone Tissue Regeneration

The development of new nanocomposites bio-inks based on GelMA- Hydroxyapatite Powders Doped with Cerium Ions (GelMA-HC) with applications in 3D printing, were first published in this research study.

In this respect, to select the right concentration of HAP-C and GelMA that ensures scaffold biocompatibility and cell proliferations, three concentrations of HAP, and three concentrations of GelMA were explored, then the biomaterials were physically, chemically, and biologically characterized.

The obtained results showed that the porosity and osteogenic differentiation increased when a concentration of 30% GelMA and a concentration of 3% HC, were used to obtain the composite ink. Therefore, the newly scaffolds based on GelMA-HC5 that show properties and abilities for osteogenic differentiation, are appealing materials to be used in bone regeneration.

4. The development of 3D printed composite scaffolds of GelMA and hydroxyapatite nanopowders doped with Mg/Zn ions to evaluate the expression of genes and proteins of osteogenic markers

Through this research study, new approaches for the synthesis of printable composite inks with applications in bone regeneration were explored.

For the first time, a systematic study based on new 3D printing inks composed of GelMA and hydroxyapatite powders doped with zinc (HZ) and magnesium ions (HM) was performed.

The objectives of this research were to select the right concentration of HZ, HM and GelMA, which ensure printability properties of the inks, scaffolds biocompatibility, cell proliferations and osteogenic differentiations. Investigation of the morphological properties induced by different concentrations of HZ and HM on GelMA matrix, also the printability properties induced by different concentrations of GelMA-HZ and GelMA-HM, represents a pioneering study, the new information obtained making it a valuable one in the field of biomedicine.

Observing the morphological and biological results of the 3D printed scaffolds that maintained their shape fidelity and provided cell viability and osteogenic differentiation, we can state that the newly developed nanocomposites hydrogels based on 25%GelMA-3%Z3, 30% GelMA-3%Z3, 25%GelMA-3%M3, 30%GelMA-3%M3 are suitable composite materials to be used for bone regeneration purposes.

5. 3D Printing of Super Concentrated Alginate Clay Ink with potential application in Regenerative Medicine

The experimental research that enabled the creation of 3D printable inks with potential tissue engineering applications continues by investigating the printability of alginate and its combination with natural clay.

The innovation of this study refers to the development of new 3D printable ink based on alginate and Cloisite Na that allows the obtaining scaffolds with high viscosity, high shape fidelity and high resistance at temperature variations.

Also, the comparative study between the alginate-based hydrogel and the two printing inks based on alginate clay-18% Cloisite Na and alginate clay-20% Cloisite Na, represents pioneering research, providing important information about the characteristics induced to GelMA matrix by different concentrations of clays.

The newly developed ink based on alginate-20% clay, provided reproducibility of the 3D printing process. The 3D printed scaffolds showed high shape fidelity, high resistance at temperature variations, and high porosity, making alginate-20% clay ink an appropriate material for 3D printing technology, with potential application in tissue engineering.

6. 3D Printing of Alginate-Natural Clay Hydrogel-Based Nanocomposites

The originality of this study is provided by the development of the new 3D printable biomaterials based on alginate-Cloisite Na, alginate-Cloisite 30B, alginate-Cloisite 93A, alginate-Cloisite 20A and alginate-Cloisite 15A. This is the first comprehensive investigation into how various types of clay affect the alginate matrix to produce a hydrogel-based ink with applications in additive manufacturing.

Due to the biological results, the high shape fidelity of the 3D printed scaffolds, the mechanical properties and the reproducibility, the alginate-Cloisite Na and alginate-Cloisite 93A nanocomposites were suitable materials to be used for the design of customized scaffolds in bone regeneration.

All these new studies and formulations developed make significant contributions both in understanding the behavior of certain materials and in the design of new biomaterials adequate for 3D printing process and tissue engineering applications.