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INSTITUTE OF MACROMOLECULAR CHEMISTRY "PETRU PONI"
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Hybrid polymeric architectures designed as hydrogel structures

Doctoral thesis abstract

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INTRODUCTION

Tissue engineering is a field that "applies principles of biology along with those of engineering to develop functional replacements for the restoration of injured tissue," according to Joseph P. Vacanti [1], who defined it thirty-one years ago. The term "regenerative medicine" was later proposed by William Haseltine [2] to refer to an ever-evolving interdisciplinary field that combines knowledge from various disciplines, including engineering, cell biology, biomechanics, nanotechnology, and biochemistry. This field involves designing scaffolds to replace or regenerate damaged tissues or organs to restore their physiological function. In recent years [3], the two fields have merged into a complex field called “Tissue Engineering and Regenerative Medicine” (TERM), whose main goal is to design scaffolds with complex 3D structures, based on combinations of cells/biopolymers, which exhibit similar physiological functions to a specific tissue/organ to replace or regenerate the injured tissue/organ.

Hydrogels are complex 3D structures used in tissue engineering. In order to be suitable for tissue engineering, they must possess the following characteristics: mechanical stability, porous structure, biodegradability, and biocompatibility. To mimic the extracellular matrix (ECM), research is being conducted on hybrid polymeric matrices, which are formed by blending natural and synthetic polymers [4-6].

The objective of the PhD thesis titled **"Hybrid polymeric architectures designed as hydrogel structures"** was to develop, acquire, and optimize novel hybrid systems using biopolymers, functionalized or not with polymerizable groups, and synthetic polymers. These systems should possess appropriate structures, physico-chemical characteristics, and specific interactions with the biological environment. They should have potential applications as scaffolds for regenerative medicine of skin or bone tissues. The motivation for the research in the PhD thesis is based on the limited number of studies on hybrid hydrogels/biohybrids obtained through 3D printing techniques. The novelty of the studies lies in the use of functionalized polymers with polymerizable groups to create complex structures using innovative 3D printing techniques. The thesis is divided into two parts and includes nine chapters, along with appendices and references.

The first part of the PhD thesis comprises an introductory information chapter consisting of two subchapters. The first subchapter is dedicated to the literature survey of the designing of hybrid hydrogels and the second one describes the 3D printing techniques used to obtain hybrid hydrogels.

The second part of the PhD thesis is structured into 6 chapters and presents the original contributions about the obtaining, structural and physico-chemical characterization, and *in vitro/in vivo* testing of the hybrid systems.

Chapter II presents the investigation of polymers obtained through analogous reactions designed to modify biopolymers, namely a protein (gelatin) and five polysaccharides (chitosan, sodium alginate, xanthan gum, dextran, and hyaluronic acid) with polymerizable groups. These polysaccharides can form three-dimensional networks through cross-linking processes and have potential biomedical applications.

Chapter III involves the preparation and physico-chemical characterization of hybrid hydrogels as topical delivery systems of active principles for applications in tissue engineering of epithelial tissue, namely: (i) polymer-based hydrogels (chitosan, dextran, xanthan and gelatin) functionalized with methacrylic anhydride and two synthetic polymers, acrylamide and N,N'-methylenebis(acrylamide) with applications in cancer therapy, and (ii) hybrid multicomponent gels based on a synthetic copolymer (poly(ethylene brassylate-co-squaric acid, PEBSA) and hyaluronic acid (HA), which exhibit antioxidant capacity due to the incorporation and controlled release of quercetin.

Chapter IV presents the preparation and characterization of two new hybrid hydrogel systems made by 3D printing technique: (i) based on GelMA (with different degrees of functionalization) and poly(ethylene glycol) diacrylate (PEGDA) and (ii) improving the properties of the hydrogels presented in Chapter III, based on PEBSA and hyaluronic acid, by the addition of GelMA, to acquire rheological properties suitable for 3D printing.

The hybrid gels underwent rheological analysis. The resulting hydrogels were examined for structure and morphology, and tested for in vitro biological activity. Additionally, the release profiles of encapsulated active substances were evaluated to confirm their suitability for use as patches.

Chapter V describes the preparation of hybrid inks with printable properties based on two components, PEBSA copolymacrolactone and GMA-modified hyaluronic acid and the encapsulation of two drugs: erythromycin and ibuprofen. The 3D printed hydrogels with multifunctional properties were characterized structurally, morphologically, in vitro and in vivo, as well as in terms of the release of encapsulated drugs to demonstrate their properties as patches with antibacterial and anti-inflammatory activity.

Chapter VI contains information about the obtaining of composite matrices based on GelMA, two types of chitosan methacrylate (LCsMA, HCsMA), hyaluronic acid, hydroxyapatite (Hap), and a colloidal suspension of magnetic nanoparticles (MNPs). The study investigated the influence of chitosan molecular weight and the amount of HAp on the printability, morphology, physicochemical and biological properties of the scaffolds for bone tissue engineering applications.

Chapter VII presents two studies related to obtaining biohybrid architectures. The first analyzes the influence of the nature and molecular weight of gelatin on the development of biohybrid inks and scaffolds with innovative properties based on mixtures of GelMA (A and B), XGMA, and hyaluronic acid made by 3D printing by the extrusion method. The second objective of the research aimed to realize bioarchitectures with a bistratified structure by joining a cellulose base populated with keratinocytes and a GelMA-based scaffold, AlgMA, previously populated with normal human fibroblasts (NHDF cell line). In addition to the physico-chemical properties, tests about immunological activity were carried out through the Elisa assay. After all these experiments, the bistratified bioarchitectures were applied on the surface of plaques made on an animal model (mouse) to prove their effect on the essential factors for the healing process.

Chapter VIII contains general information about the materials used in the experimental study, as well as the equipment used for the characterization of the obtained hydrogels.

The last chapter of the thesis, Chapter IX, contains the general conclusions regarding the experimental results obtained, as well as the development perspectives in the field of natural/synthetic polymers.

The doctoral thesis entitled "Hybrid architectures designed as hydrogel structures" consists of 266 pages divided into IX chapters, including 39 tables, 182 figures, and 454 bibliographical references.

Original contributions

Chapter II. Functionalization reactions of natural polymers with polymerizable groups

The advantages of biopolymers, polysaccharides, and proteins, such as biocompatibility, biodegradability, biomimeticity, and bioactivity, compared to synthetic polymers, recommend their use for obtaining scaffolds with applications in regenerative medicine [7, 8]. However, there are several drawbacks related to the processing methods of natural polymers, such as low solubility, temperature sensitivity, or low mechanical strength. The solution to these shortcomings is the grafting of novel groups onto the polymeric structure to give them several additional characteristics [9]. Such resulting polymers can be used to obtain different 3D architectures with various applications, depending on their composition, processing method, or crosslinking method [10].

This study aimed to obtain functionalized polymers with methacrylic groups using polymer-analogous transformations with methacrylic anhydride or glycidyl methacrylate, obtaining polymer derivatives capable of forming 3D networks by crosslinking/autoreticulation processes, usable as scaffolds in biomedical applications. For their realization, the following specific objectives were proposed:

Objective 1. Gelatin functionalization by selective methacrylation reactions of biopolymer with methacrylic anhydride.

Objective 2. Chitosan functionalization: creation of a neutral, physiologically soluble chitosan derivative with C=C groups through nucleophilic substitution with methacrylic anhydride.

Objective 3. Functionalization of sodium alginate, xanthan gum and dextran by selective functionalization reactions of polysaccharides with methacrylic anhydride.

Objective 4. Functionalization of hyaluronic acid: obtaining a derivative with C=C groups by functionalization reactions using the epoxide ring opening method of the glycidyl methacrylate monomer.

II.1. Protein functionalization reaction

II.1.1. Gelatin functionalization protocol with methacrylic anhydride

In this study, the polymer chains of two distinct types of gelatin, Gelatin A (of porcine origin, gel viscosity ~175 g Bloom) and Gelatin B (of bovine origin, gel viscosity ~225 g Bloom), were modified using the protocol described by Camci-Unal and his research group [11], with minor modifications of the original method. Two homogeneous yellow solutions of gelatin obtained in PBS (pH 7.2, 0.01M), brought to a temperature of 50^{o}C, 8mL of methacrylic anhydride (MA, 94%) were added at a rate of 0,5 mL/min, under conditions of continuous stirring and constant temperature (300 rpm, 50^{o}C).

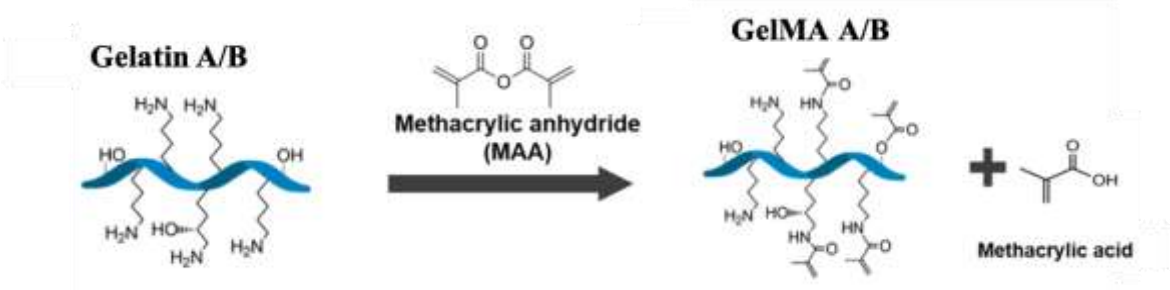

Figure 1. *Modification reaction of the amino group in the gelatin structure with methacrylic anhydride [12,13].*

The influence of reaction time on the degree of functionalization for type A gelatin was studied. Chemical modifications were carried out for different time intervals: 2, 4, 6, and 8

hours. Gelatin B was reacted with methacrylic anhydride for 6 hours. The chemical structure of the modified polymers was confirmed using ^{1}H NMR spectroscopy, and colorimetric analysis was used to determine the degree of substitution of the reaction products.

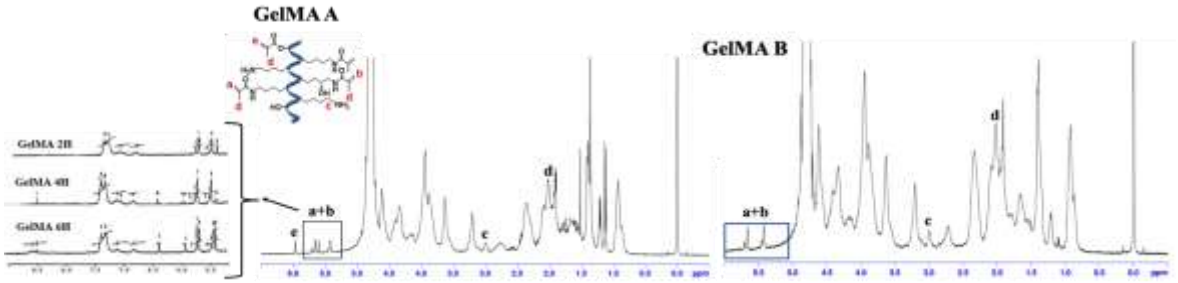

Figure 2. *^{1}H NMR spectra of methacrylated gelatin A/B (as a function of functionalization reaction time for gelatin A), chemical structures adapted from [13].*

Similar protocols were used for the chemical modification of chitosan, dextran, and xanthan gum as the method used for the reaction of gelatin with methacrylic anhydride, but the polymer solvent was modified according to the solubilization properties of each biopolymer.

Hyaluronic acid (HA, molecular weight Mw=1,5-1,8x106Da) was modified using glycidyl methacrylate to obtain HA methacrylate (HAGMA) [14,15]. In the first step, a 0.4% HA solution was made using distilled water as the solvent. To initiate the reaction, the hyaluronic acid solution, under continuous stirring, was heated at 50°C for one hour, with the simultaneous addition of glycidyl methacrylate and a solution of p-toluenesulfonic acid (2%) in N,N-dimethyl formamide (DMF).

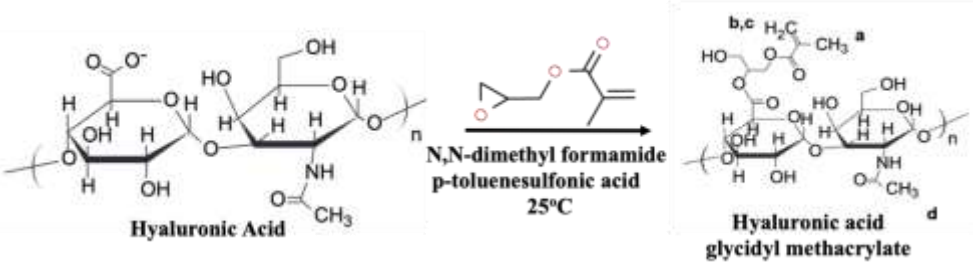

Figure 3. *Functionalization reaction of hyaluronic acid with glycidyl methacrylate [16,17].*

The influence of the polymer/glycidyl derivative ratio on the degree of functionalization was studied by performing 7 different modification reactions. The reactions were carried out for a time interval of 30 hours, constant temperature and stirring (25^{o}C, 300 rpm).

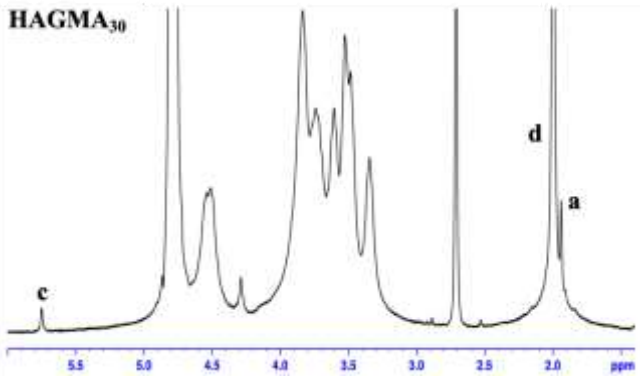

Figure 4. *^{1}H NMR spectrum of glycidyl methacrylate-modified hyaluronic acid (1:30GMA).*

Chapter III. Hybrid hydrogels for the treatment of skin wounds

This study aimed to obtain new hydrogels as topical drug delivery systems for skin cancer therapy. The strategy for the preparation of hydrogels involved cross-linking processes of biopolymers (chitosan, dextran, xanthan, and gelatin) previously modified with methacrylic anhydride with synthetic monomers, namely acrylamide, and N,N'-methylenebis(acrylamide). The chemical modification of the polysaccharides improved their behavior in the crosslinking reaction and did not influence the initial biocompatibility. The obtained hydrogels were used as controlled release systems for doxorubicin, an antitumor agent that prevents DNA replication, and affects topoisomerase II activity and cell function by binding to the cell membrane [34].

III.1. Method of obtaining hybrid hydrogels based on functionalised biopolymers

Obtaining hydrogels based on dextran (a polysaccharide component present in the composition of all synthesized hydrogels), chitosan, gelatin, and xanthan gum, previously modified with methacrylic anhydride, involved several steps.

As shown in Figure 5, in the first step, polymer solutions of 3% (g/v) dextran methacrylate, 3% (g/v) chitosan methacrylate, 3% (g/v) methacrylate gelatin, and 3% (g/v) methacrylate xanthan gum were prepared using a phosphate buffer solution, 0.01 M, pH 7.2, as solvent, under stirring (300 rpm) until a homogeneous mixture was obtained.

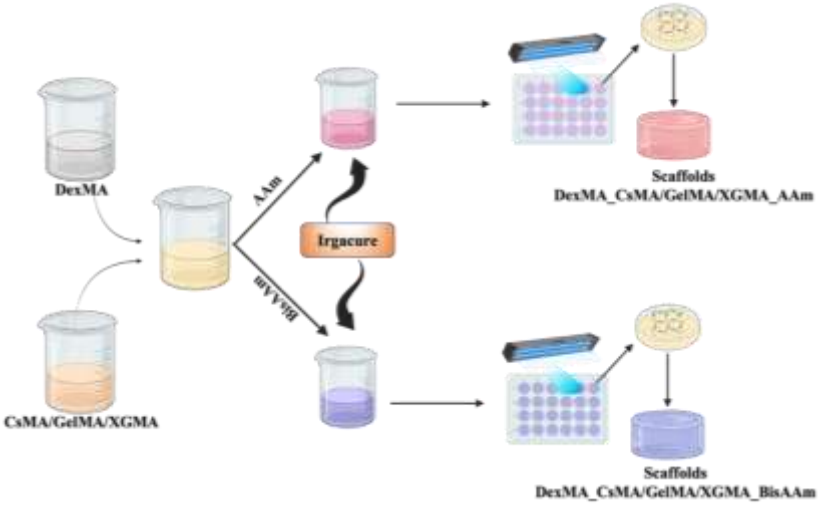

Figure 5. Steps of obtaining DexMA_CsMAAAm/BisAAm, DexMA_GelMA_AAm/BisAAm, DexMA_XGMA_AAm/BisAAm hybrid hydrogels.

After homogenization of the mixtures, the photoinitiator (Irgacure, 2% (w/v)) was added as well as acrylamide (AAm, 10% (w/w, based on the amount of polymers in the mixture)/acrylamide (AAm) and N,N '-methylenebis(acrylamide) (BisAAm): obtained from AAm, 8% (w/w), BisAAm 2% (w/w). The mixing of the systems was continued until complete homogenization and the composition of the resulting gels is detailed in Table 1. The obtained mixtures were poured into 24-well plates (500 μL/well) and exposed to UV light (λ=365nm, 5 min) for 3D network formation.

Table 1. *Composition of hydrogels.*

Polymeric Scaffolds	**DexMA (%)**	**CsMA (%)**	**GelMA (%)**	**XMA (%)**	**AAm (%)**	**BisAAm (%)**
DexMA_CsMA	50	50	-	-	-	-
DexMA_CsMA_AAm	45	45	-	-	10	
DexMA_CsMA_BisAAm	45	45	-	-	8	2
DexMA_GelMA	50	-	50	-	-	-

DexMA_GelMA_AAm	45	-	45	-	10	
DexMA_GelMA_BisAAm	45	-	45	-	8	2
DexMA_XMA	50	-	-	50	-	-
DexMA_XMA_AAm	45	-	-	45	10	
DexMA_XMA_BisAAm	45	-	-	45	8	2

III.1.2. Enrichment of hybrid hydrogels with Doxorubicin

Doxorubicin (Dox) [18] is an antitumoral agent indicated by the FDA for the treatment of several types of cancer: acute lymphoblastic leukemia, osteosarcomas, breast carcinoma, ovarian carcinoma, thyroid carcinoma, etc. Drug loading was performed as follows: the hydrogels were immersed in an alcoholic solution of DOX (0,5 mg/mL in ethyl alcohol) and kept for 24 hours in the dark at 25^{o}C. The solvent was removed by convective drying at 25^{o}C. After drying, the antitumoral drug-encapsulated hydrogels were characterized in terms of DOX release capacity and cytocompatibility with the cellular environment.

III.1.2.1. *In vitro* drug release

The in vitro release of DOX from the hydrogel structure was performed by immersing the hydrogels in PBS (pH = 7.2, 0.01 M) at 37°C. The amount of released drug recorded following the release process was plotted versus time (Figure 6).

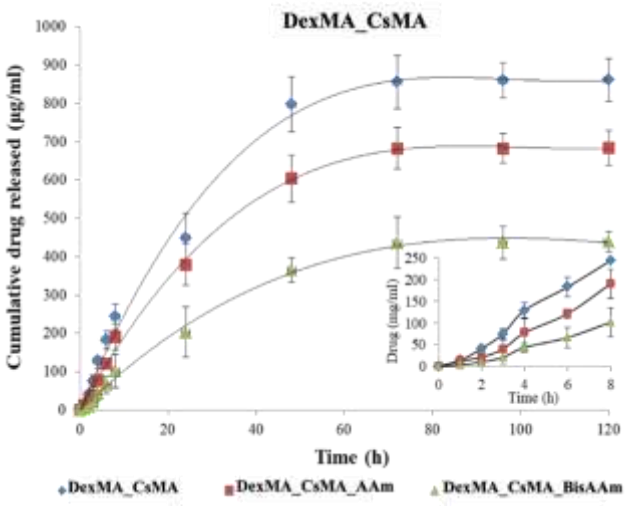

Figure 6. *Release kinetics of Doxorubicin from hydrogels of various compositions.*

The release profiles of the DexMA_CsMA hydrogels showed a steady, controlled release, the maximum concentration of drug released after 50 hours of the experiment. In the case of hydrogels containing AAm and AAm-BisAAm in their composition, the cumulative amount of drug released was lower: 80.56% for DexMA_CsMA, 64.2% for DexMA_CsMA_AAm_BisAAm and 42% for DexMA_CsMA_AAm_BisAAm.

This phenomenon can be explained by the large number of interactions realized between DOX and the AAm/AAm/AAm-BisAAm side groups in the polymer networks. In DexMA_GelMA and DexMA_XGMA hydrogels, this effect is attenuated.

Table 2. Release rate constant (k) and release exponent (n).

Hydrogel	Correlation Coefficient (r2)		Release Rate	Release
	Higuchi	Korsmeyer-Peppas	Constant, k (h^{-n})	Exponent, n
DexMA_CsMA	0.9812	0.9955	0.1403	0.5629
DexMA_CsMA_AAm	0.9799	0.9962	0.1612	0.5719
DexMA_CsMA_BisAAm	0.9843	0.9987	0.1822	0.5685
DexMA_GelMA	0.9892	0.9904	0.1074	0.5270
DexMA_GelMA_AAm	0.9792	0.9973	0.0934	0.5374

DexMA_GelMA_BisAAm	0.9765	0.9965	0.0962	0.5907
DexMA_XGMA	0.9803	0.9944	0.0962	0.5507
DexMA_XGMA_AAm	0.9789	0.9952	0.0943	0.5531
DexMA_XGMA_ BisAAm	0.9806	0.9986	0.1019	0.5496

The obtained results suggest that the mechanism that governs the release kinetics of drug is Korsmeyer-Peppas, because the correlation coefficients values exceeded 0.99 [19]. In addition, other parameters, such as the release rate constant (k) and the release exponent (n), were calculated to determine the mechanism of drug release from the hydrogel structure. The values obtained for the exponent (n) suggested a Fickian-like mechanism (n almost equal to 0.5), which indicated that the drug is released from the hydrogels by diffusion, with the relaxation of the 3D networks having only a small contribution. All these results indicate that the release of doxorubicin from the hydrogel matrix can be controlled by the selection of the underlying natural/synthetic polymers to obtain the hydrogel.

III.1.2.2. Cytotoxicity and drug release studies on cell cultures

After 24 hours of exposure of the cells to the amount of doxorubicin released from the structure of the loaded hydrogels, cell viability was determined and the values obtained ranged between 43% and 66%, as a consequence of drug release (hydrogel-based gelatin having the lowest percentage of viable cells). After 48 hours of the experiment, the trend of decreasing cell viability values was maintained, reaching 26-38%, and after 72 hours of experiment the values were in the range of 18-29%.

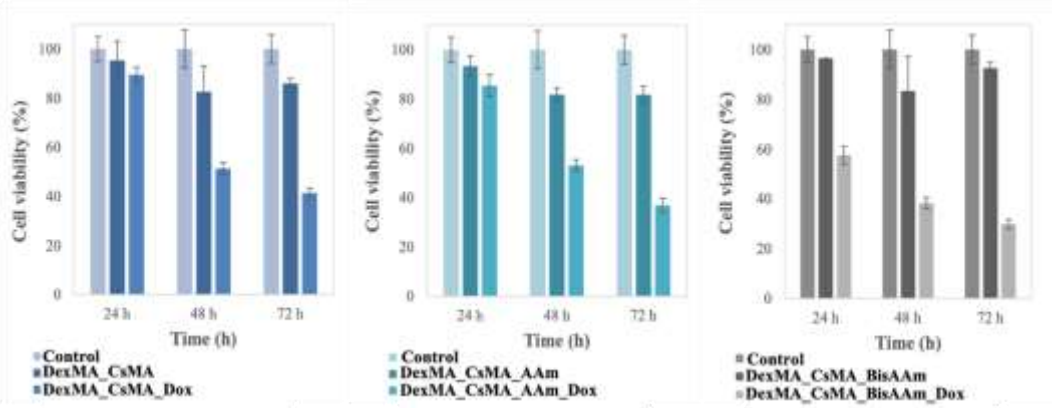

Figure 7. *Cell viability for hydrogels after 24 h, 48 h and 72 h of cell culture.*

III.1.2.3. Cell morphology assay

Differences in cell viability of cells in direct contact with unloaded matrix and drug-loaded hydrogels were analyzed using Calcein AM reagent.

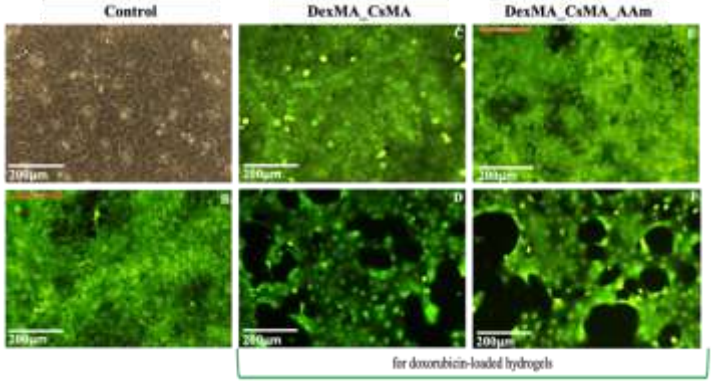

Figure 8. *Cell morphology after 4 days of exposure to direct contact with DexMA_CsMA (C, D) and DexMA_CsMA_AAm (E, F) compared to cells in the control well (A (phase contrast, B*

(stained with calcein AM). Images D , F shows the effect of doxorubicin released from the hydrogel matrix on cell morphology and viability (Calcein AM staining).

Correlation between the MTT results and cell morphology photos is evident, as the morphology images of the MTT samples (Figure 8 C, E) exhibit comparability with the control wells (Figure 8 A,B).

The MTT results of doxorubicin-loaded matrices can be correlated with the appearance of fluorescence microscopy images (Figure 8 D, F), demonstrating the efficacy of the drug on the division capacity of tumor cells by the appearance of areas unpopulated with cells compared to cells not exposed to the drug (Figure 8 C, E), where the well surface is fully populated.

III.2. Hybrid hydrogels based on lactones and hyaluronic acid

The research aimed to obtain hybrid hydrogels using the copolymer, PEBSA, together with hyaluronic acid, and to identify their synergistic properties for their application in the field of epithelial fold regeneration.

III.2.1. Method for obtaining gels based on PEBSA and hyaluronic acid

The hybrid gels were obtained by mixing the synthetic poly (ethylene brassylate-co-squaric acid) copolymer, PEBSA and the biopolymer, hyaluronic acid, in various proportions according to Table 3. The PEBSA copolymer was prepared following the protocol performed by Chiriac et al [16, 17]. The first step involved the production of 5% PEBSA copolymer solutions (EB/SA: 25/75, 50/50 and 75/25), using two types of aprotic solvents with different dipole moments, namely dimethyl sulfoxide (DMSO) and 1,4-dioxane. The second step involved the actual synthesis of the hydrogels by mixing the aqueous solution of HA 1% with PEBSA copolymer solutions in different ratios (as shown in Table 3). The formation of the hydrogels occurred in a short time due to the rapid formation of physical hydrogen bonds between the two polymers. Finally, the prepared gels were characterized, either in a gel state (rheological tests) or after lyophilization (physico-chemical characteristics).

Table 3. *Composition of PEBSA_HA based hydrogels.*

Nr.crt.	Sample	PEBSA type	PEBSA : HA ratio (mL/mL)	Solvent
1	PEBSA_$HA_{1.1}$	25/75	1:1	DMSO
2	PEBSA_$HA_{1.2}$	25/75	1:2.5	DMSO
3	PEBSA_$HA_{1.3}$	25/75	1:2.5	1,4 Dioxan
4	PEBSA_$HA_{1.4}$	25/75	1:5	DMSO
5	PEBSA_$HA_{2.1}$	50/50	1:1	DMSO
6	PEBSA_$HA_{2.2}$	50/50	1:2.5	DMSO
7	PEBSA_$HA_{2.3}$	50/50	1:2.5	1,4 Dioxan
8	PEBSA_$HA_{2.4}$	50/50	1:5	DMSO
9	PEBSA_$HA_{3.1}$	75/25	1:1	DMSO
10	PEBSA_$HA_{3.2}$	75/25	1:2.5	DMSO
11	PEBSA_$HA_{3.3}$	75/25	1:2.5	1,4 Dioxan
12	PEBSA_$HA_{3.4}$	75/25	1:5	DMSO

III.2.1.1. Evaluation of rheological properties

Figure 9 shows the rheological behaviour of the gels under oscillatory shear conditions after the application of shear forces. In the linear range of viscoelasticity, the elastic (G′)

and viscous (G‴) moduli are not influenced by the oscillation frequency (ω). A typical gel structure was observed for most of the analyzed samples, exhibiting: G′ > G′.

Figure 9 (1, 2, 3) shows the dependence of viscoelastic moduli (G′ and G″) and complex viscosity on oscillation frequency, respectively. The analyzed samples exhibited a typical gel behavior (G′ > G′, with values of the parameters G′ and G′ independent of the frequency (ω) values), except for the sample PEBSA_$HA_{2.1}$, which exhibits rheological behaviour specific to liquids. During the experiment, it was observed that the modulus of elasticity, which is correlated with the gel strength, is influenced by the gel composition. Regarding the influence of the ratio between the components, there is a stronger structuring in the case of the PEBSA_$HA_{2.2}$ sample (Figure 9.2) the other 2 gels (PEBSA_$HA_{1.2}$ – Figure 9.1 and PEBSA_$HA_{3.2}$ – Figure 9.3)) having a less gel structure structured.

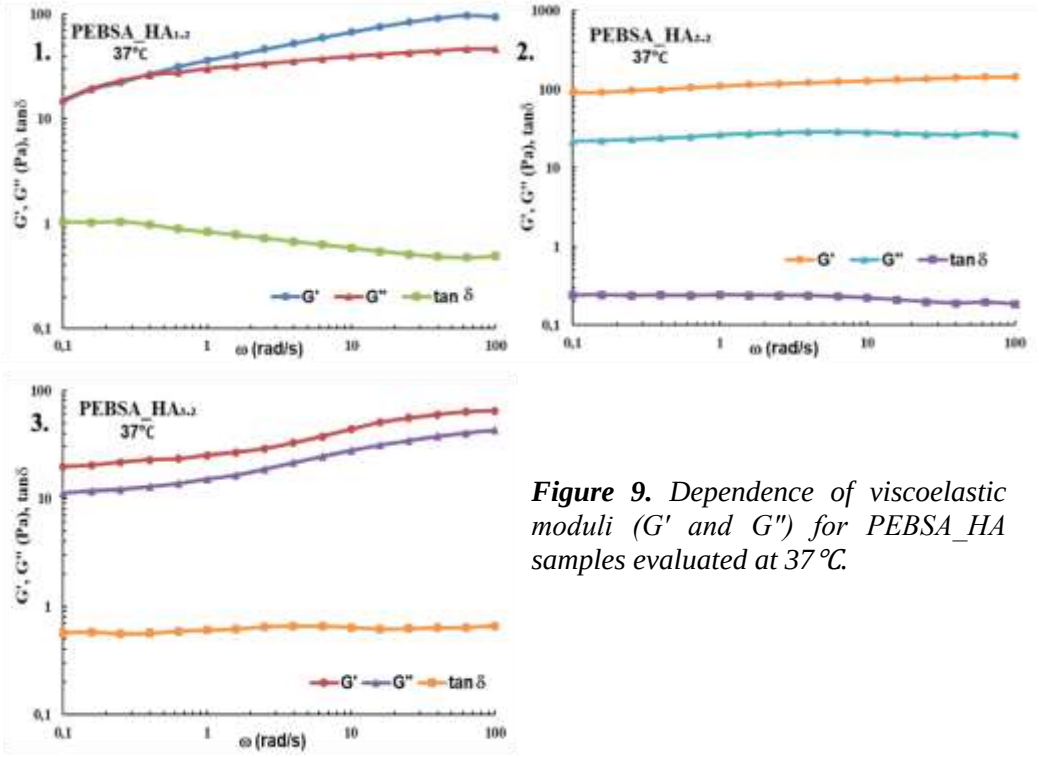

Figure 9. *Dependence of viscoelastic moduli (G' and G") for PEBSA_HA samples evaluated at 37 ℃.*

III.3.1. Obtaining antioxidant bioactive systems

Due to the promising results obtained following the characterization of hybrid hydrogels based on PEBSA and hyaluronic acid, an active substance was incorporated into their structure, namely quercetin. This flavonoid has an effective antioxidant effect, which decreases the secretion of proinflammatory cytokines (IL-1 β, IL-6, TNF-α), chemokines and nitric oxides [22], having a number of beneficial effects, including the reduction of the physiological process of skin aging [23]. Its incorporation was achieved either by its initial complexation with the PEBSA copolymer structure and mixing with an aqueous hyaluronic acid solution, or by its addition into the PEBSA_HA gel composition.

III.3.1.1. Preparation of the copolymer/bioactive substance complex

To obtain the complex between PEBSA and quercetin, two different solutions were prepared, one containing 5% PEBSA and the other containing 2.5% bioactive substance, both of which used DMSO as a solvent due to its high solubilization capacity of 1,4-dioxane. The solutions were mixed at a gravimetric ratio of 2:1 (PEBSA:Q) and kept under magnetic stirring for 24 hours.

III.3.1.2.Preparation of bioactive gels

The preparation of hydrogels based on PEBSA, HA, and quercetin was carried out by three different methods:

1) PEBSA_HA_Q_1: was based on the combination of PEBSA_Q complex with HA. Basically, the PEBSA_Q complex was dispersed in DMSO, and the obtained dispersion was incorporated into 1% HA aqueous solution (as shown in Figure 10);

2) PEBSA_HA_Q_2: was based on the incorporation of the synthetic copolymer PEBSA and the PEBSA_Q complex into the HA solution. Basically, a 5% PEBSA 5% synthetic copolymer solution was made using DMSO as solvent. Then, to this solution, the PEBSA_Q complex and 1% aqueous HA solution were added consecutively (as shown in Figure 10);

3) PEBSA_HA_Q_3: was based on the combination of the 3 compounds: PEBSA, quercetin and HA. Thus, a 5% PEBSA solution was made using DMSO, then the 1% HA solution (in distilled water) was added one at a time, quercetin being incorporated into the gel formed between PEBSA and hyaluronic acid (according to Figure 10);

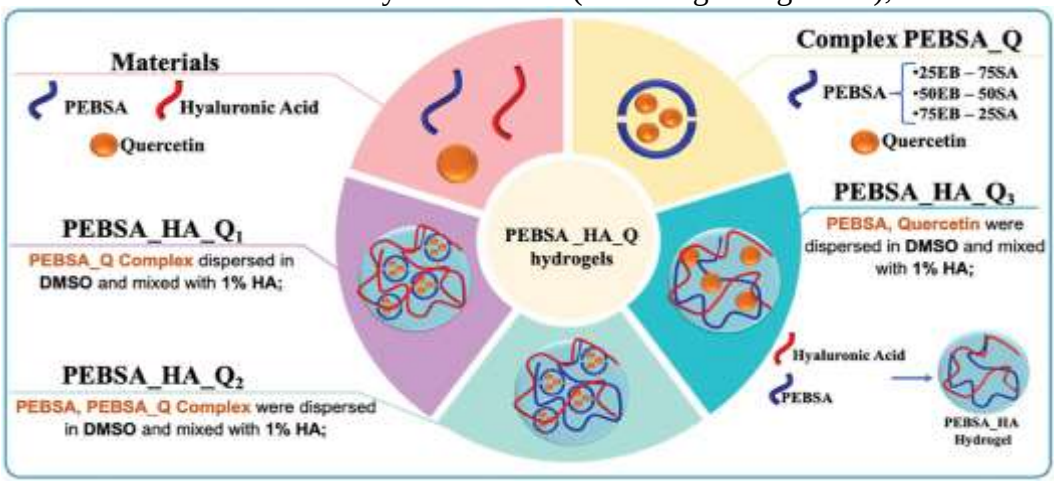

Figura 10. *Preparation scheme for PEBSA _HA and PEBSA _HA_Q hydrogels.*

III.3.1.3. Antioxidant activity

The antioxidant properties of flavonoids are closely related to their chemical structure. Phenolic chemicals can exert their antioxidant effects via two fundamental mechanisms: hydrogen atom transfer and electron donation [34]. In this context and interdependently with the required antioxidant activity, one can opt for the 2nd variant of coupling quercetin within the HA and PEBSA complex in all three types of gels, due to the high and constant antioxidant capacity over the 2 h experiment, exhibited by this variant.

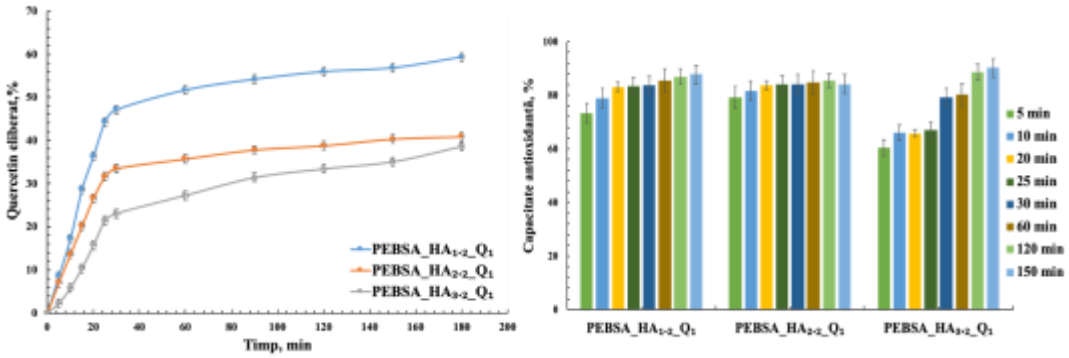

Figure 11. *Evaluation of the release profile of quercetin from the composition of PEBSA_HA_Q (Q_1) hydrogels correlated with antioxidant activity.*

Chapter IV. Hybrid architectures based on methacrylated gelatin and PEGDA realized by 3D printing technique for tissue engineering applications

The study aimed to obtain scaffolds based on GelMA (with different degrees of functionalization) and poly(ethylene glycol) diacrylate (PEGDA), using a 3D printing technique. Throughout the study, the influence of the degree of modification of the methacrylated gelatin on printability, morphology of the matrix, physico-chemical and biological properties were evaluated to determine the applicability of the scaffolds in soft tissue regeneration applications with a focus on wound skin regeneration.

IV.1. Obtaining of GelMA and PEGDA based inks

GelMA A (with different degrees of methacrylation: GelMA A 2h (62%), GelMA A 4h (67%), GelMA A 6h (68%) and GelMA A 8h (72%), a synthetic polymer, PEGDA and a biocompatible photoinitiator, lithium phenyl-2,4,6-trimethyl-benzoyl phosphinated (LAP) were mixed to obtain gels with specific rheological properties for the printing process.

IV.2.Rheological properties of the gels

All hybrid gels showed superior rheological properties after prolonged shear forces (Figure 12). Application of low shear rates (below 0.1 s^{-1}) results in a Newtonian behaviour, the viscosity (η) of the samples being independent of the applied shear rate. When shear rates with values between 0,1 s^{-1} and 100 s^{-1} were applied, all mixtures exhibited pseudoplastic behaviour. According to the literature [24, 25], this type of behaviour favor the extrusion printing process.

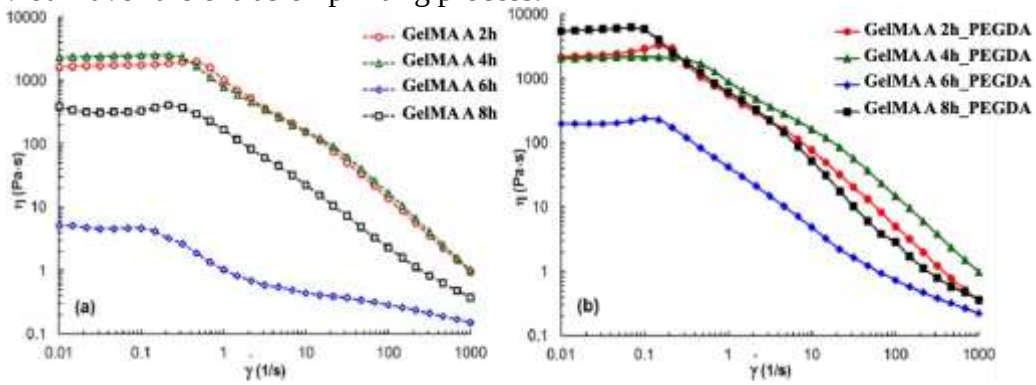

Figure 12. *Apparent viscosity as a function of applied shear rate of GelMA (a) and GelMA_PEGDA (b) based gels at 25°C.*

Thus, in the Nenewtonian region, the apparent viscosity (η) is influenced by the applied shear rate. Thus, gel extrusion through the nozzle can be easily achieved**.**

IV.3. Physico-morphological properties

GelMA and GelMA_PEGDA hydrogels exhibit a porous structure with irregular pore shapes and different pore sizes due to the syneresis phenomenon that takes place during the lyophilization process.

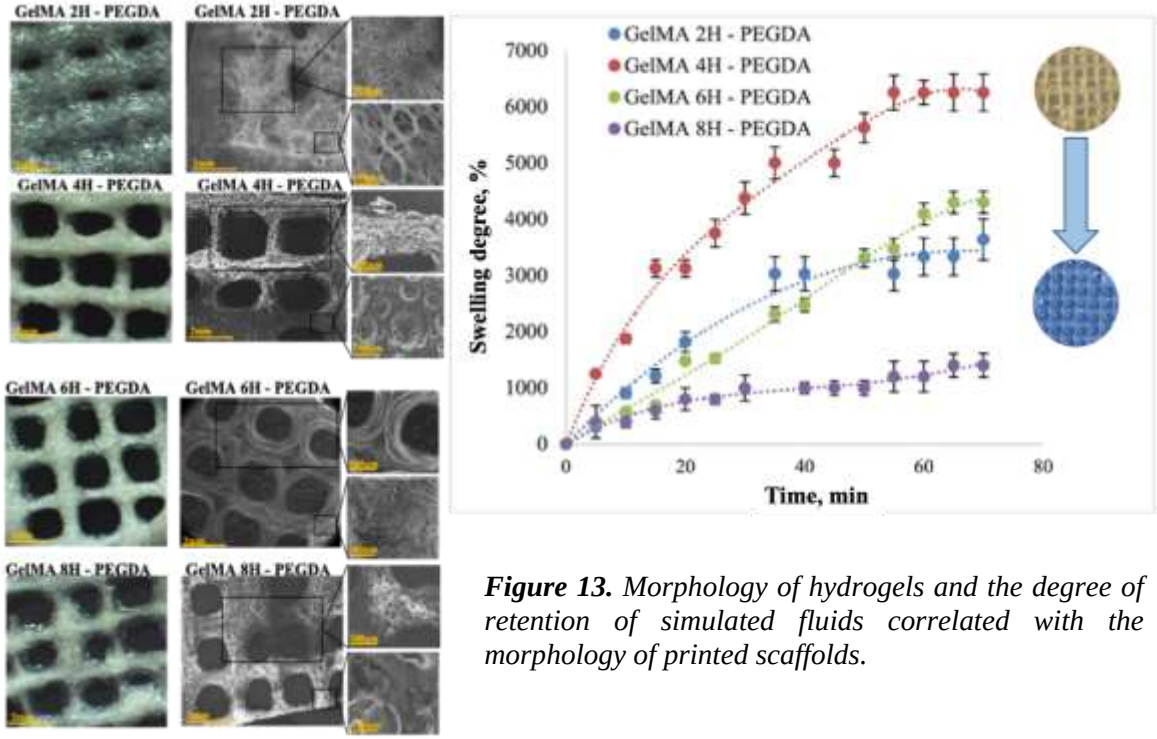

Figure 13. *Morphology of hydrogels and the degree of retention of simulated fluids correlated with the morphology of printed scaffolds.*

The degree of substitution of the GelMA polymer and the position of the methacrylic group on the protein chain influenced the photoreticulation process and contributed to the variation of pore size. The pores are uniformly distributed, easily interconnected, favorable for nutrient diffusion, cell proliferation, cell migration and cell adhesion on the material surface.

The GelMA_PEGDA-based hydrogels retained their shape, revealing values of the swelling degree in the range of 1000 to 6000%. Figure 13 plots the swelling values of 3D-printed scaffolds as a function of time. According to the graphs, it can be seen that all the hydrogels reached a steady state of absorption after 60 minutes of the experiment.

IV.2. Hybrid printed scaffolds based on hyaluronic acid and PEBSA

In order to confer specific shapes and geometries to the structures, multicomponent systems based on PEBSA, HA and GelMA were obtained, which were subsequently improved by adding indomethacin (IND) and profiled by 3D printing. Properties such as IND's release capability and its dependence on component ratios, scaffold composition, and matrix interaction with the biological environment were highlighted.

IV.2.1. Method of obtaining inks

Synthetic materials present high mechanical properties, simplifying the printing process and preserving the shape fidelity of the printed scaffolds, while natural ones present properties such as: low immunogenicity and biocompatibility, biodegradability and bioactivity [28]. Thus, to obtain advanced polymer inks, blends were made between PEBSA copolymer (using three ratios between EB and SA comonomers: 25/75, 50/50 and 75/25) and HA in a 1:2.5 ratio.

The obtained mixtures exhibited low viscosity, which led to collapse of the printed scaffolds during the 3D printing process. To improve the rheological properties of the inks and subsequently, the mechanical properties of the printed matrix, a constant amount of gelatin methacrylate (GelMA A, 68.07% degree of substitution) was added to the initial composition of the inks. GelMA (20% w/v) was dissolved in PBS (pH 7.2, 0.01 M) at 25^{o}C to be incorporated into the initial gel composition of PEBSA copolymer and hyaluronic acid, and LAP (0.025% w/v) was subsequently added. After homogenization, the inks were analyzed in terms of rheological properties and subjected to the 3D printing process to obtain three-dimensional scaffolds.

IV.2.2.Testing of rheological properties

As can be seen from Figure 14, PEBSA_$HA_{2.2}$_GelMA exhibited full recovery of the structure after the application of different types of deforming forces (up to 30,000%), with PEBSA_$HA_{1.2}$_GelMA exhibiting similar behaviour.

When applying strain values between 200% and 20.000%, the PEBSA_$HA_{3.2}$_GelMA specimen can recover about 88 – 89% of the initial strain value. After applying high deformation forces that allow the creation of new interactions between macromolecules (full chain stretching), the strain recovery increases. To prevent deformation and ensure shape fidelity after extrusion, the ink must be resilient to external forces, e.g., the weight of subsequent layers that could cause the collapse and deformation of the matrix structure [29, 30].

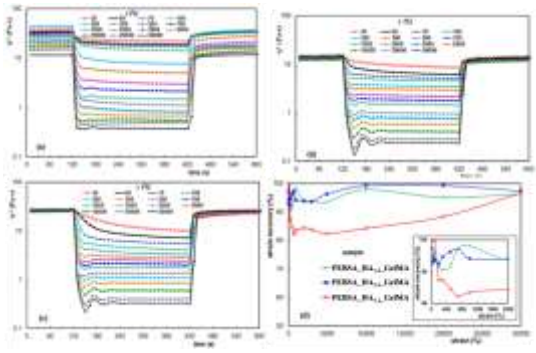

Figure 14. *Self-recovery behavior of the structure after deformation observed in the crosslinked gels (a)PEBSA_$HA_{1.2}$_GelMA; (b)PEBSA_$HA_{2.2}$_GelMA; (c)PEBSA_$HA_{3.2}$_GelMA; (d) Recovery capacity as a function of the force applied to imprint the gels. The inset shows the strain recovery for $\gamma \leq 2000$ %.*

The shear thinning behavior and the self-recovery behavior of the obtained inks (short time required for recovery and high structure recovery capacity of more than 80%) ensure the printability of the inks and the shape fidelity of the printed scaffolds.

IV.2.6. Evaluation of in vitro cytotoxicity

IV.2.6.1. MTT test

The MTT assay was used to evaluate the cytotoxicity of the scaffolds using rabbit abdominal fibroblast cell cultures. Figure 15 shows the cell viability values upon contact with PEBSA_HA_GelMA scaffolds. The MTT results showed an increase in cell viability values to 90% after 72 hours of the experiment (Figure 15).

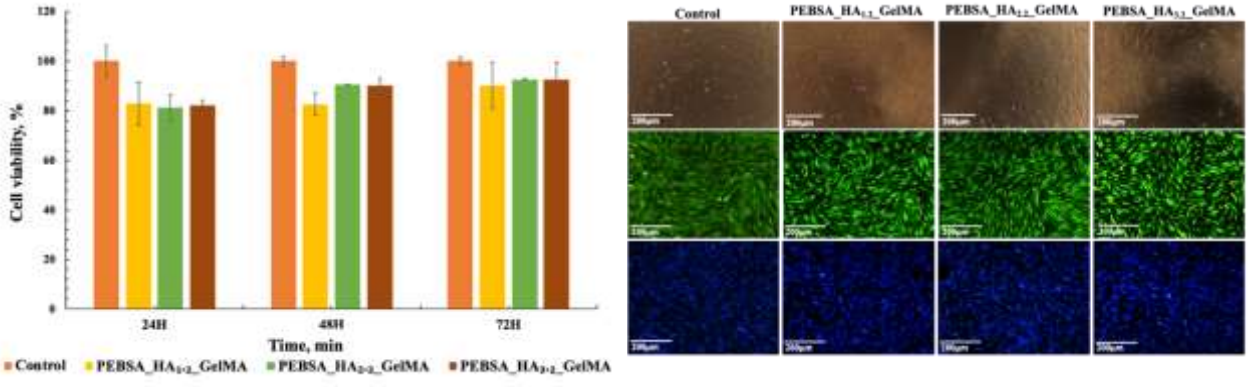

Figure 15. *Cell viability datat and images of viable cells and fixed cells after 72 hours of cell culture with PEBSA_HA_GelMA-based matrix.*

Rabbit abdominal fibroblasts were exposed through direct contact with PEBSA_HA_GelMA hydrogels for 72 hours before morphological examination by staining with Calcein AM and DAPI reagent. The images, taken with an x10 objective, demonstrate the presence of numerous morphologically intact living cells adherent to the substrate, forming a uniform monolayer, and having a normal fibroblast shape.

CHAPTER V. 3D printed hybrid scaffolds based on glycidyl methacrylate-modified hyaluronic acid and a synthetic copolyimacrolactone

The present study aimed to obtain hybrid inks with printable properties based on two polymeric components, namely PEBSA copolymacrolactone and hyaluronic acid. Since the mixing of these two components does not lead to printable inks, as described in Chapter IV, the improvement of the rheological properties of these gels was achieved by modifying the structure of hyaluronic acid through a reaction with glycidyl methacrylate. HAGMA_PEBSA matrices were obtained by 3D printing processes and their properties were studied to demonstrate their application in the regeneration of damaged epithelial tissues. To increase their biofunctionality, in their composition were added two drugs, such as ibuprofen [31] and erythromycin [32], to reduce local inflammation and the risk of wound surface infection, respectively. Properties such as: the release capacity of the two drugs, their synergy, and their dependence on the relative ratios of the components in the composition of the carriers were highlighted..

V.1. Method of obtaining polymeric matrices

Based on the rheological studies presented in Chapter III, where the topic of obtaining PEBSA/HA based gels was discussed, the ratio of 1:2.5 PEBSA and hyaluronic acid methacrylate was selected for this study. According to Table 4, three distinct inks were obtained. The inks were created by combining PEBSA copolymer (using 25/75, 50/50, and

75/25 copolymer variations, the difference being determined by the comonomer ratios, EB/SA) and hyaluronic acid methacrylate in a 1:2.5 ratio. After homogenization, LAP was added, and the inks were extruded to produce the scaffolds.

Table 4. Composition of inks.

Nr.crt.	Scaffold	PEBSA type	HAGMA:PEBSA (mL/mL) ratio	Solvent HAGMA	Solvent PEBSA
1.	HAGMA_PEBSA$_1$	25/75	2.5:1	Water	DMSO
2.	HAGMA_PEBSA$_2$	50/50	2.5:1		
3.	HAGMA_PEBSA$_3$	75/25	2.5:1		

V.1.1.2. Physico-morphologic properties

The morphology of the PEBSA and glycidyl methacrylate-modified hyaluronic acid synthetic copolymer scaffolds was analyzed by scanning electron microscopy, and it was observed that all systems showed the fidelity of the printed shape.

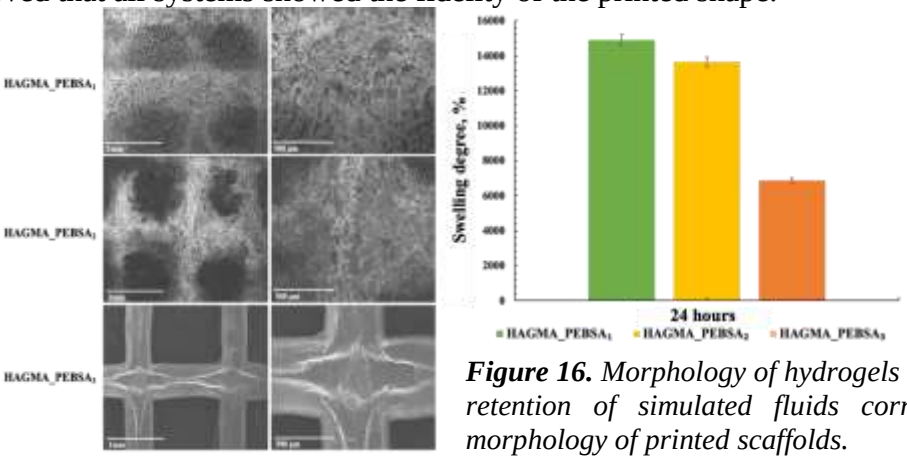

Figure 16. *Morphology of hydrogels and the degree of retention of simulated fluids correlated with the morphology of printed scaffolds.*

V.1.3. Loading and drug releasing profile

Three variations of the mixes were prepared to investigate the controlled drug release characteristics of hyaluronic acid methacrylate (HAGMA) and synthetic copolymacrolactone (PEBSA) based carriers. Each of these mixes had an equal quantity of two active compounds, namely ibuprofen and erythromycin, each reported to be 5% of the relevant polymer concentration in the solution.

The drugs were added to the polymeric solutions after their complete dissolution in a specific solvent (distilled water for HAGMA and dimethyl sulfoxide for PEBSA copolymer variants). After homogenization, 0.01% LAP solution was added to the mixtures as a photoinitiator.

The release of the embedded drugs, ibuprofen and erythromycin, was studied by immersing the loaded hydrogels in a phosphate buffer solution of pH 7.2, 0.01M, to simulate the wounded skin environment, with the experiment carried out at 37°C. The results of the *release tests demonstrate that the release profile of the drugs is influenced by the composition of the hydrogel.*

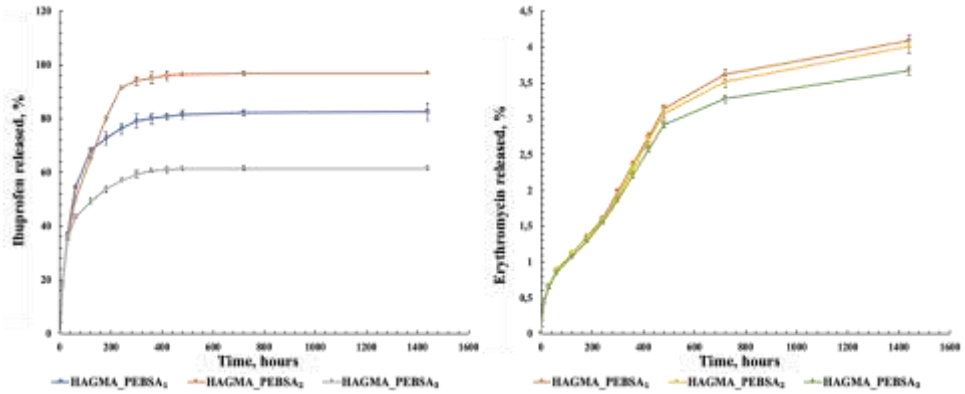

Figure 17. *Release kinetics of the two active principles, ibuprofen-erythromycin from the structure of HAGMA_PEBSA$_1$, HAGMA_PEBSA$_2$, HAGMA_PEBSA$_3$ hydrogels.*

In vitro and in vivo biocompatibility tests

The response of cell cultures of normal human dermal fibroblast cell cultures to the contact with ibuprofen [33] and erythromycin [34] loaded hydrogels was tested. The graph in Figure 19 shows that the viabilities of normal human dermal fibroblasts remained high and proved the non-cytotoxic character of the materials (values of 85-95% after 72 hours of the experiment).

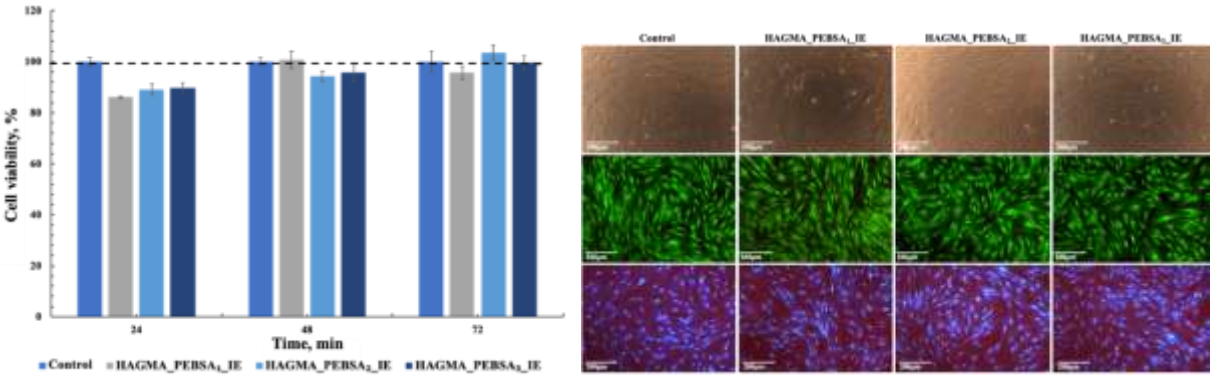

Figure 18. *Cell viability (%) for the drug coated printed scaffolds, after 24, 48 and 72 hours of experiment, measured by MTT technique.*

Following the application of simple HAGMA_PEBSA or HAGMA_PEBSA scaffolds were added active principles, and drug carriers were was tested on animals. The animals' immune defense capacity was assesed by evaluating typical characteristics such as serum opsonic and bactericidal capacity. According to the graphs in Figure 19, the matrix did not affect these parameters, with their values comparable to those of the control animals, demonstrating that the application of the matrices to the wound area was not associated with disturbances in the mouse's immune defense capacity, and the materials did not cause allergic or inflammatory reactions.

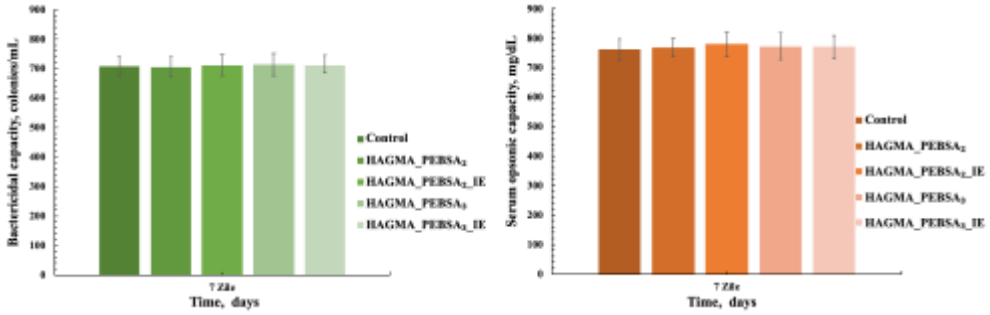

Figure 19. *Influence of printed scaffolds on CO and CB in the mouse. Values are expressed as arithmetic mean ± s.d. of the mean for 5 animals/group.*

Chapter VI. 3D-printed gelatin and chitosan methacrylate, hyaluronic acid, magnetic nanoparticle-based scaffolds for tissue engineering of bone tissue

The present study presents the methodology to obtain 3D printable composite inks based on GelMA, two types of chitosan methacrylate (LCsMA, HCsMA), hyaluronic acid (HA), hydroxyapatite (Hap) and a colloidal suspension of magnetic nanoparticles (MNP). The influence of the molecular weight of chitosan and the amount of HAp on the printability, morphology, physicochemical and biological properties of the matrix for BTE applications were investigated.

VI.1. Preparation of composite inks and their characterization

The preparation of the composite inks involved creating solutions of functionalized polymers (GelMA A with a substitution degree of 71.84%, HCsMA and LCsMA with substitution degrees of 47.50% and 41.60%, respectively), a biopolymer (hyaluronic acid), a colloidal suspension of chitosan-coated magnetite nanoparticles (MNPs) with a hydrodynamic diameter ranging from 140 to 400 nm, as described in article [35], and an inorganic material, hydroxyapatite (HAp).

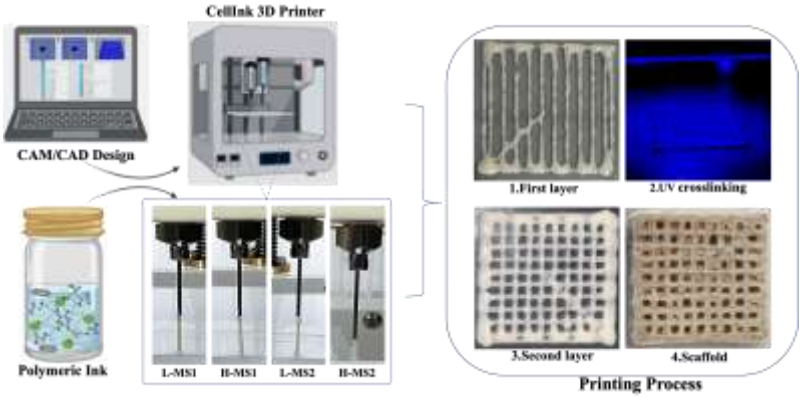

Figure 20. *Fabrication of a photo-crosslinked L/H-MS1 and L/H-MS2 matrix: 1) first layer, 2) crosslinking process, 3) second layer, 4) fully printed matrix.*

VI.1.1. Rheological characterization of composite inks

The shear thinning behavior favors the extrusion of the ink through the nozzle during the printing process, reducing the viscosity of the ink at high shear rates. A high tension threshold value is necessary to maintain the printed shape until the substrate cross-links. Additionally, the value of G′ should be sufficiently high to ensure the prevention of further collapse of the printed structure [36, 37].

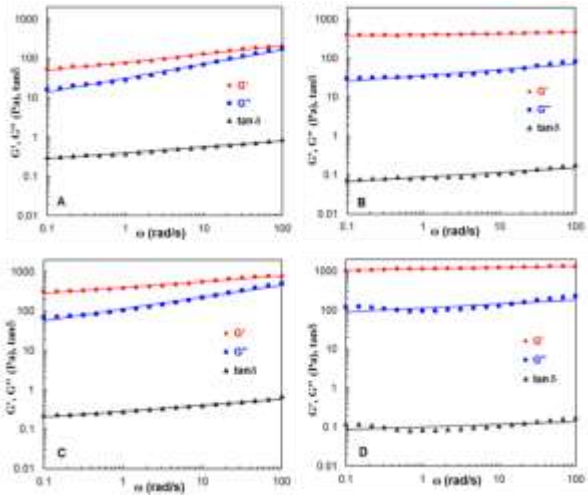

Figure 21. *Viscoelastic parameters of composite inks plotted as a function of oscillation frequency for* γ = 1% *(A) L-MS1; (B) H-MS1; (C) L-MS2; (D) H-MS2.*

The linear viscoelastic region (LVR) was determined by measuring the viscoelastic moduli, G′ and G″, as a function of strain amplitude. In all cases, the limit of the linear viscoelastic deformation linear viscoelastic range (LRV, γ_L) above 10% was reached, thus, frequency sweep tests were performed for γ = 1%. The plots in Fig. 21 A-D) show the variation of viscoelastic moduli and tan δ as a function of oscillation frequency for γ = 1% (in the linear viscoelastic region) at 25°C.

VI.3. Morphological properties of the scaffolds

The obtained matrix exhibits a 3D structure with interconnected pores, on the surface of which either HAp or MNP crystals can be observed, which are distributed throughout the polymer network.

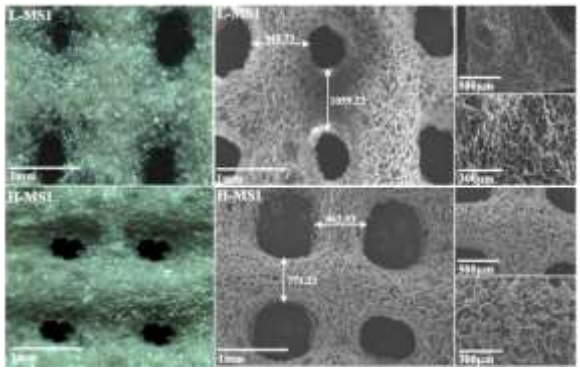

Figure 22. *Stereomicroscopy images and scanning electron microscopy images of the printed substrates, H-MS.*

The scaffolds used in tissue engineering applications must exhibit certain properties related to their composition (adequate rheological characteristics, such as flow resistance) as well as pore size (sizes between 50 and 150 μm determine fibrovascular growth and those between 150 and 500 μm contribute to bone tissue mineralization*).*

VI.4. Degree of swelling

The values of the degree of fluid retention (PBS, pH 7.2, 0.01M) in the 3D scaffold structure are shown in Figure 24. It can be observed that the values are influenced by the molecular weight of the functionalized chitosan, especially for the matrices containing 50%

HAp; the swelling degree values for the L-MS2 scaffold were higher compared to those of the H-MS2 scaffold, the phenomenon being constant since the beginning of the experiment, which can be explained by the formation of a reduced number of cross-linkings in this case.

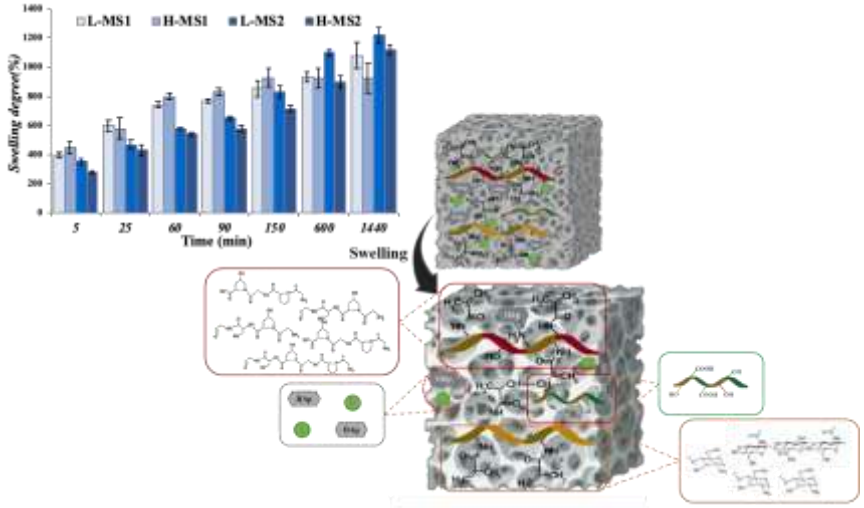

Figure 23. *Swelling behaviour of composite matrices.*

CHAPTER VII. Biohybrid architectures based on functionalized polymers obtained by 3D (bio)printing technique

The purpose of this study was to examine how the nature and molecular weight of gelatin affect the development of biohybrid inks and scaffolds. The study focused on creating blends of GelMA (A and B), XGMA, and hyaluronic acid (HA) for 3D printing using the extrusion method. The properties of the polymeric hydrogels, including the retention of simulated fluids, biodegradability kinetics, adhesion to epithelial tissue surface, and interaction with normal human fibroblasts and normal keratinocytes, were analyzed to demonstrate their versatility. This versatility allows for their use in creating scaffolds through (bio)printing processes for applications in regenerative medicine, particularly in wound healing.

VII.1.Preparation of inks to obtain hybrid scaffolds

Inks based on two types of gelatin (with different origins: porcine type A and bovine type B), XG and HA, were prepared to obtain the printed scaffolds. Gelatins and XG were subjected to polymer-analogous transformations (by polymer-analogous transformations with MA) for 6 hours, to obtain functionalized polymers with degrees of substitution of 71.84% and 63.02% (for gelatins) and 12% in the case of XG, respectively, according to the method and the results of characterization tests presented in Chapter II. The inks were made by dissolving the polymers in a culture medium (DMEM F12 HAM) containing 0,01% LAP, a solution made by keeping the solution at a constant temperature of 60^{o}C for 30 minutes, according to the manufacturer's protocol (Sigma Aldrich, Germany).

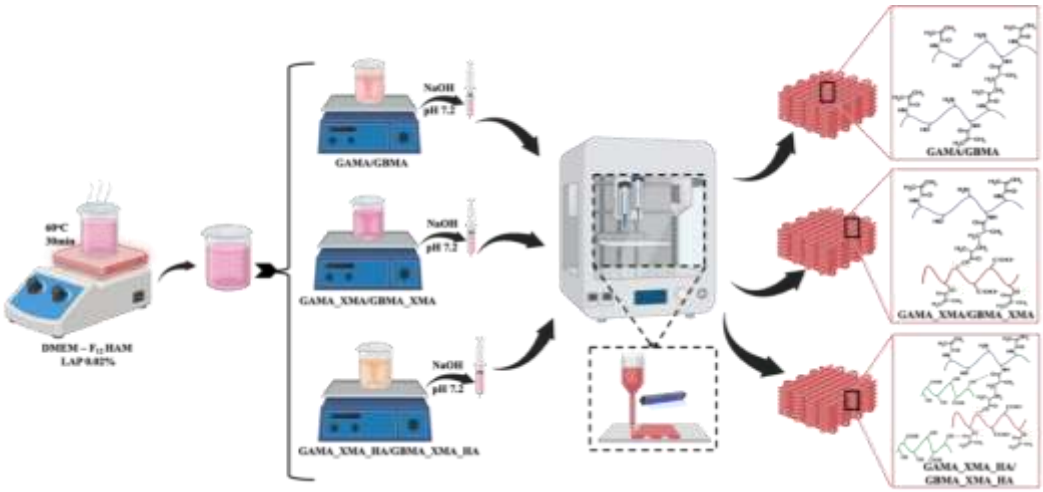

Figure 24. *Steps in the production of GAMA/GBMA_XMA and GAMA/GBMA_XMA_HA hydrogels.*

VII.1.1. Rheological measurements

For all the compositions, the upper limit of the linear viscoelasticity range, LVR (γ_{LVR}), was reached at strain values, γ, above 60 %, with $G' > G''$ and $\tan\delta < 0.2$ for $\gamma < \gamma_{LVR}$, suggesting a behaviour similar to solid materials.

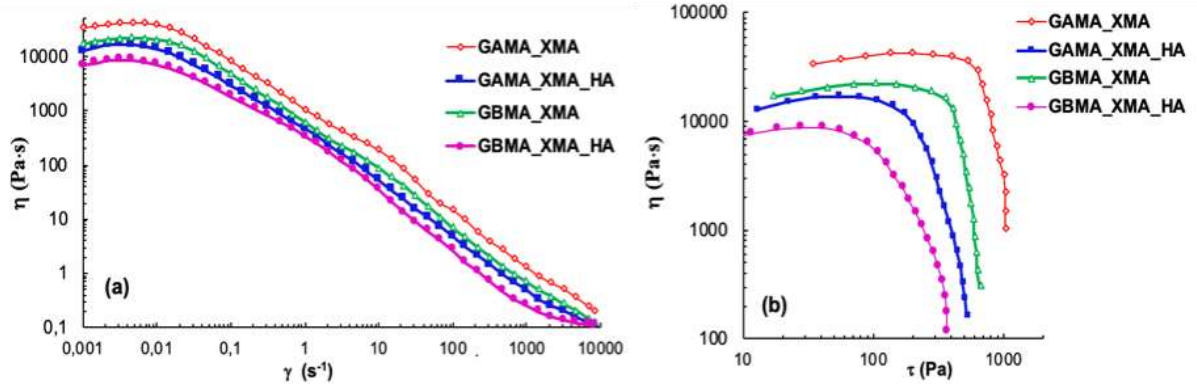

Figure 25. *Variation of viscosity of mixtures as a function of (a) shear rate and (b) shear effort.*

HA-containing gels have a low gel strength, which is further reduced in gels containing GBMA compared to those containing GAMA. A correlation may be established between the gel strength and the values of the yield stress threshold (μo) shown in Figure 25.

VII.1.9.3. Wound healing tests

Wound healing [38] is a complex process involving various cell types, characteristic of the dermis, epidermis and leukocytes. This process is regulated by cytokines and growth factors. Due to the fact that the 3D-printed arrays have wound healing as an application, it was intended to study the effect on cell migration. For this study, cells that play an important role in the regeneration process of epithelial tissue, human dermal fibroblasts (NHDF cell line), which play an important role in the generation of the extracellular matrix.

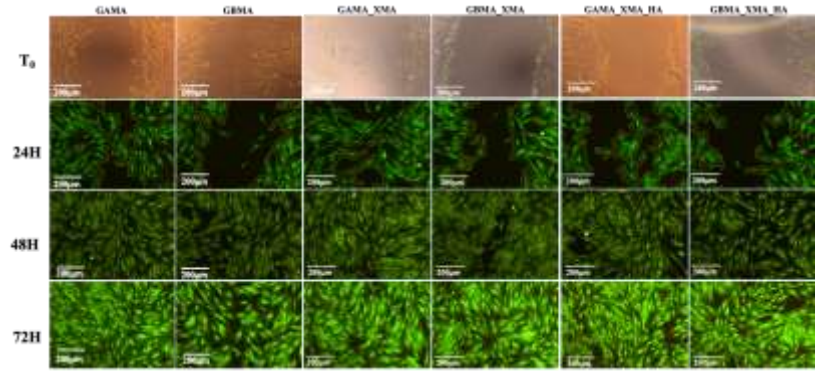

Figure 26. *In vitro evaluation of normal human dermal fibroblast migration in the presence of GAMA/GBMA_XMA and GAMA/GBMA_XMA_HA hydrogels (highlighted with Calcein AM reagent at different time points).*

For quantification of signaling molecules, a culture medium was taken from Petri dishes in which 3D-printed scaffolds were incubated after the population with fibroblasts, after 72 hours of contact. After processing the obtained data, it was observed that the control values (cells that were not in contact with the materials) have low interleukin concentration values compared to the concentration values obtained after testing the incubation medium of the populated materials, which demonstrates that the samples induce the secretion of IL-6, involved in wound healing.

VII.2. 3D printed biohybrid architectures with applications on wound skin regeneration.

The research aimed to realize bioarchitectures with a bistratified structure by joining a cellulose base populated with keratinocytes and a GelMA_AlgMA scaffold populated with normal human fibroblasts. In addition to the physicochemical properties, immunological activity tests were carried out by the Elisa assay, after which the arrays were applied to the surface wound of the animal model (mouse).

VII.3. Obtaining biohybrid scaffolds by 3D printing

3D bioprinting is a biofabrication technique that allows precise control over various parameters, including the deposition of cells, extracellular matrix components, and biochemical factors. This technique enables the creation of structures with well-defined architectures through the layer-by-layer deposition of polymer inks or bioinks [39]. In this context, it was proposed to obtain bioprinted matrix based on GelMA and AlgMA. Following the characterization tests performed previously, the polymer mixture obtained in an equal ratio between the functionalized polymers, GelMA_AlgMA$_2$, was used to obtain bioprinted scaffolds.

VII.3.1 Obtaining bistratified biohybrid architectures

A series of distinct steps were followed to obtain the 3D bistratified printed substrates. The first involved the production, by extrusion printing technique, of GelMA and AlgMA (GelMA_AlgMA) based scaffolds, as well as the preparation of the cellulose membrane and the lyophilization of both components.

In the second step, sterilization of the materials was performed by UV exposure. Preparation of the cellulose membrane for human keratinocyte seeding involved depositing a 0.1% collagen solution on the surface of the cellulose membrane in complete culture medium.

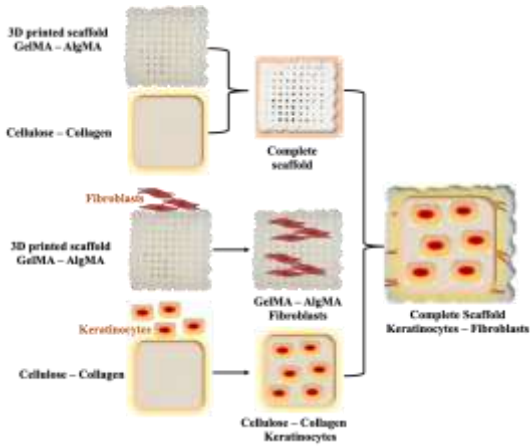

Figure 27. *Stages of obtaining GelMA_AlgMA_cellulose bistratified biohybrid structures (NHDF/HACAT cell lines).*

The printed supports were immersed in culture medium and incubated at 37^{o}C for 3 hours and populated with normal human dermal fibroblasts (NHDF cell line). They were incubated separately for 1 week after which the two types of supports were overlapped and incubated again for a week, the medium being changed every 2 days with a fresh one. After completing these stages, they were tested by interacting with the in vivo environment.

VII.3.3. *In vivo* tests

In order to perform the in vivo experiment, a number of 54 CD1 mice were selected and used in the experiment, 18 mice/group, organized into three groups (group M - control, group C - 3D scaffold (GelMA_AlgMA$_2$) with cells (fibroblasts and keratinocytes) and group FC - 3D scaffold without cells).

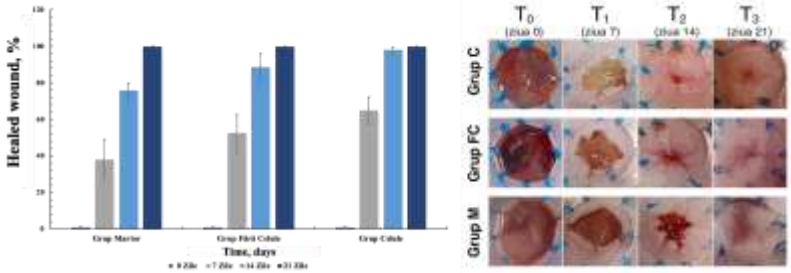

Figure 28*. Healed wound area as a function of time and clinical progression of the healing process.*

At the beginning of the experiment, 108 wounds were created, with two wounds per mouse. Following the euthanization of 6 mice per group, wound size was assessed, and samples were taken for histologic and immunohistochemical analysis at intervals of 7, 14, and 21 days.

VII.3.4: Histologic and immunohistochemical Analyses

Following 21 days of the experiment, groups C and FC showed full epidermal remodeling, whereas group M was still in the process of organizing new tissue. Evaluation of wounds in groups C and FC revealed a normal structure of the dermis and hypodermis, with connective fibers organized in a systematic manner, suggesting full healing. The dermis and

connective fibers in group M exhibited only minor disorganization.

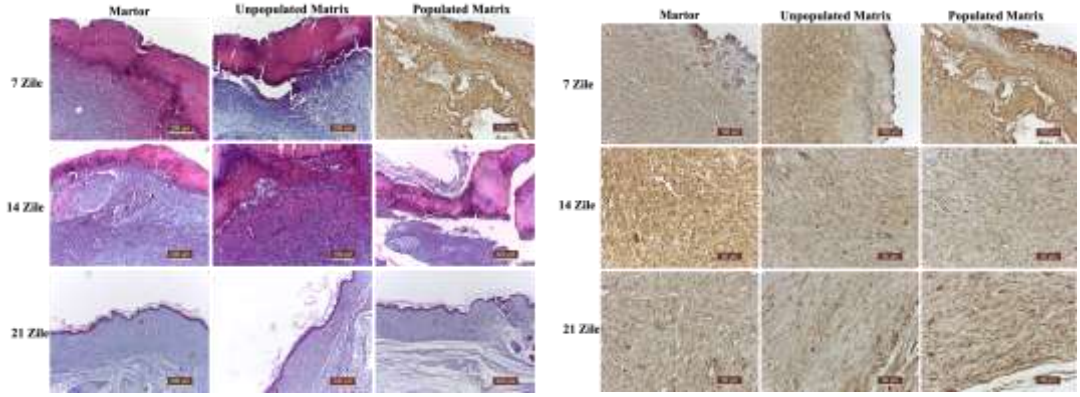

Figure 29. *Results of histological examination by Masson trichrome method and immunohistochemical examination (VEGF activity for lesions in contact with the simple matrix and biohybrid matrix), staining by Hematoxylin-Eosin method.*

VII.3. Obtaining biohybrid scaffolds by 3D bioprinting

Three-dimensional bioprinting is a biofabrication method that allows for precise control of several aspects, including the deposition of cells, extracellular matrix components, and biochemical variables. This process creates objects with well defined topologies by depositing polymer inks or bioinks layer by layer. Furthermore, the 3D bioprinting technology enables the acquisition of intricate structures with biomimetic characteristics, resulting in structures that closely resemble physiological tissues.

. In this context, it was proposed to obtain bioprinted matrix based on GelMA and AlgMA. It was chosen due to superior swelling properties that favored cell adhesion on the surface and inside the polymer matrix.

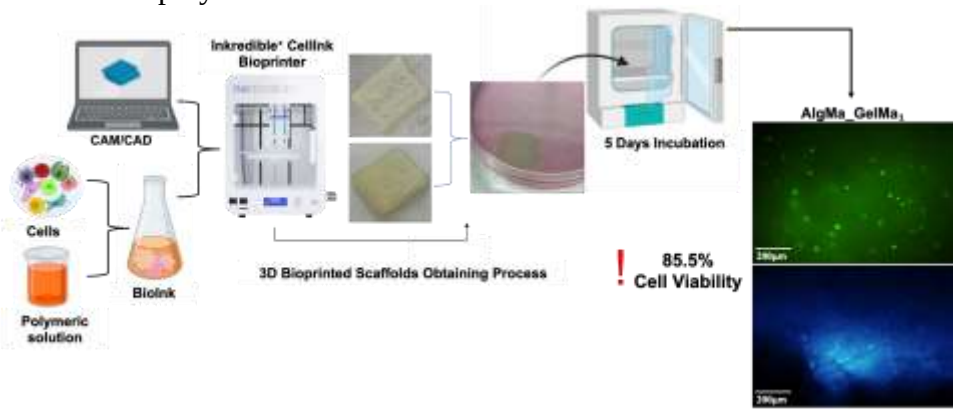

Figure 30. *Steps of 3D bioprinting process.*

Inks based on GelMA and AlgMA, allowed cell bioprinting. Cell viability ranged between 78.2% and 85.3% for the substrates analyzed. The quantification of the number of viable cells after the completion of the 3D printing process of the bioinks and the incubation of the populated matrices, was performed by relating the number of viable cells in the scaffold to the total number of cells in the cell suspension added to the polymer mixture composition that underwent the bioprinting process.

Final Conclusions

The studies can be summarized by the following points:

Functionalization reactions of natural polymers with polymerizable groups

The tests revealed obtaining polymers with different degrees of modification, as follows:

⇒ (i)GelMA with different degrees of modification between 46% and 64% depending on the reaction time with methacrylic anhydride;
(ii)CsMA (42%)
(iii)AlgMA (12%)
(iv)XGMA (10%)
(v)HAGMA, with low degrees of modification depending on the amount of monomer added (values ranging from 32-53%);

⇒ All of them were evaluated through Nuclear Magnetic Resonance and Fourier Transform Infrared Spectroscopy tests, the modification results being comparable to those reported in the literature.

It has been demonstrated the obtaining of functionalized polymers with methacrylic groups, by means of polymer-analog transformations with methacrylic anhydride or glycidyl methacrylate, for the formation of polymeric derivatives capable of forming networks, through crosslinking/self-crosslinking processes, with biomedical applicability.

Hybrid hydrogels for the treatment of skin wounds

The study aimed the development of hybrid hydrogels, with applications in the treatment of skin cancer through the release of localized doxorubin. It was shown that matrices can be created by combining dextran with chitosan/gelatin/xanthan gum (whose chain was changed with methacrylic anhydride). Synthetic polymers (acrylamide and N,N'-methylenebis(acrylamide)) were added to form chemically cross-linked structures, highlighting the antitumoral effect of the matrices obtained by various characterization methods.

⇒ FT-IR spectral analysis demonstrated that the addition of synthetic polymers (acrylamide and N,N'-methylenebis(acrylamide)) led to the formation of a higher number of covalent interactions.

⇒ Immersion in simulated fluid (pH 7.2 phosphate buffer solution) of the polymeric matrices demonstrated that all hydrogels reach a high degree of swelling at equilibrium, the obtained data could be correlated with the morphological structure of the hydrogels (porosity and pore distribution).

⇒ The release profile of doxorubicin from the hydrogel matrices varied between a rapid-release mechanism and a balanced, sustained, controlled release profile, appropriate in terms of the recommendations for topical administration of active ingredients in skin cancer therapy. Aspects related to the release profiles of doxorubicin were demonstrated and supported by the application of the Higuchi and Korsmeyer-Peppas kinetic models.

Doxorubicin-loaded hydrogels are effective on tumor keratinocytes (A431 line), data indicating that the hydrogels exert a cell division-reducing and apoptosis-inducing effect and can be successfully applied in tumoral therapy.

Hybrid hydrogels based on lactones and hyaluronic acid

⇒ This study aimed to synthesize a novel hydrogel for use in tissue engineering applications, specifically wound healing, by using PEBSA copolymacrolactone and hyaluronic acid in varying polymeric ratios. Quercetin was added to the hydrogel to give it antioxidant properties.

⇒ The formation of the gel networks was confirmed by rheological tests, the majority of the systems exhibited values of the specific viscoelastic parameters $G' > G''$ and $\tan \delta < 1$.

⇒ FT-IR spectral analysis demonstrated the formation of hydrogen bonds between the -C=O groups from the PEBSA chain structure and the -OH groups on the hyaluronic acid chain.

⇒ The retention degrees obtained by immersion of the hydrogels in simulated fluids demonstrate that the matrices exhibit properties characteristic of superabsorbent materials, showing high values of the degrees of swelling at equilibrium, exceeding 2000%. The testing of PEBSA_HA based materials, by the MTT method, performed on rabbit abdominal fibroblast cultures, demonstrated that during the 3 days of the experiment, the cells maintained viabilities of more than 90%, proving favorable properties for the use of the obtained materials in biological applications.

⇒ The encapsulation of the active principle, quercetin, in the matrix structure was achieved by three distinct methods. Antioxidant activity was demonstrated using the DPPH reagent assay, the study demonstrating the possibility of modulating the release profile of the active principle, interdependent with the required antioxidant response: for antioxidant activity for a long time, quercetin must be introduced during matrix formation (with the formation of intermolecular physical bonds between quercetin, HA and PEBSA) and for a fast-acting antioxidant activity, the flavonoid must be coupled by a complexation procedure with the PEBSA polymer, followed by the addition of HA.

The obtained materials show interdependencies between the different physicochemical characteristics of the polymers such as rheology, morphological properties but also biological properties such as biocompatibility, properties that make them suitable for biomedical applications, especially in the treatment of skin wounds.

Hybrid architectures based on methacrylated gelatin and PEGDA realized by 3D printing technique with applications in tissue engineering

⇒ The aim was to obtain substrates by rapid prototyping of extruded printable ink-based arrays. The inks were obtained by mixing GelMA polymer (with varying degrees of substitution), photoinitiator and constant amounts of PEGDA. The properties of the substrates were evaluated by various characterization methods to demonstrate that the degree of substitution of the modified polymer influences the printing parameters, the rheological properties of the gels and the physico-chemical properties of the realized scaffolds.

⇒ The results of rheology tests revealed that GelMA_PEGDA gels exhibit viscoelastic properties, low flow stress and shear thinning behaviour, important characteristics for extrusion printing processes.

⇒ Under simulated physiological conditions, the 3D scaffolds show a behaviour specific to superabsorbent hydrogels, exhibiting stability over time, a property acquired by the addition of PEGDA polymer.

⇒ The interaction of the scaffolds with the cell environment was evidenced by evaluating the viability values of two specific epidermal tissue cell lines (fibroblasts and keratinocytes). Both cell types maintained their viability, showing values exceeding 95% after 3 days of experiment, demonstrating that the matrices can be applied in soft tissue regeneration processes as they do not influence the cell proliferation mechanisms.

⇒ The bioadhesivity characteristics are significantly improved when PEGDA polymer is involved in the realization of the 3D network.

In conclusion, the corroboration of all the characterization results showed that the hydrogel structures fabricated by 3D printing exhibit suitable characteristics for soft tissue engineering applications.

HA and PEBSA-based printed hybrid carriers

⇒ The study aimed to obtain hybrid hydrogels based on PEBSA, a synthetic copolimacrolactone, and HA, a polysaccharide with wound-healing properties, followed by the addition of GelMA to the composition to obtain inks with properties applicable in 3D printing processes. For the application of the materials as patches with anti-inflammatory effect, the printed matrices were loaded with indomethacin.

⇒ Rheology tests revealed gels based on PEBSA, HA and GelMA with viscoelastic properties, flow resistance and thinning behavior under shear forces, properties suitable for their use as printable inks.

⇒ FT-IR spectral analysis demonstrated hydrogen bond formation between the C=O bonds of the PEBSA chains and the -OH groups of the HA chains, also showing the bands of the characteristic groups of GelMA.

⇒ Scanning electron microscopy attests to the formation of 3D structures, which retain the printed shape. Moreover, they are porous and capable of creating a biomimetic environment for cell growth and development.

⇒ The printed hydrogels also demonstrate compact structures that enable the controlled incorporation and release of the selected active ingredient, indomethacin.

⇒ Retention tests of simulated fluids reveal that, under physiological conditions, the matrices exhibit high absorption capacity and enhanced stability, attributed to the presence of GelMA in their composition.

⇒ In order to evaluate the cytocompatibility, the polymeric scaffolds were placed in direct contact with albino rabbit abdominal fibroblasts. Throughout the 72-hour experiment, the cells maintained viability values of 80%, providing evidence in support of the usage of the obtained materials for future biological applications.

The non-toxic properties and very good biocompatibility of the matrix, together with their structural, morphological and physicochemical properties, indicate the potential of the carriers to be used in medical applications, such as patches for personalized medicine.

3D printed of hybrid scaffolds based on glycidyl methacrylate-modified hyaluronic acid and a synthetic copolyimacrolactone

⇒ The study confirms the obtaining of hydrogels based on PEBSA, a synthetic copolymacrolactone, and glycidyl methacrylate functionalized hyaluronic acid to obtain stable scaffolds by (bio)3D printing processes using the extrusion techniqueTo utilize the samples as patches with anti-inflammatory and bacteriostatic effects for wound healing, the printed matrices were loaded with two active ingredients, ibuprofen and erythromycin.

⇒ FT-IR spectral analysis demonstrated the formation of hydrogen bonds between the C=O groups of the PEBSA chain and -OH of the HAGMA chain, the creation of 3D networks by chemical cross-linking reaction between the hyaluronic acid methacrylate chains, and the appearance of characteristic HAGMA bands in the spectra.

⇒ The encapsulation of active principles has also been demonstrated by the appearance of drug-specific bands in the FT-IR spectra.

⇒ Scanning electron microscopy attests to the formation of a 3D porous structure, influenced by the composition of the synthetic copolymer PEBSA, which can create a biomimetic environment for cell growth and development. Thus, the imprinted hydrogels exhibit compact structures useful for the controlled incorporation and release of active ingredients.

⇒ To assess cytocompatibility, human cells were placed in direct contact with 3D printed scaffolds, both simple and encapsulated. Throughout the 72-hour experiment, the cells maintained vitality values of more than 80%, indicating that the obtained materials can be used for additional biological applications.

⇒ In addition to the cytocompatibility tests, in vivo tests were performed by applying the hydrogels to a dorsal incision made on white Swiss mice. The obtained results demonstrated that the matrices do not induce significant changes in hematological, biochemical and immunological parameters compared to the use of commercial patches.

The corroboration of all characterization results, such as those related to biocompatibility properties, structural, morphological and physico-chemical properties, as well as the results highlighted by the application of the matrices on animal models (mouse) indicate the potential of the scaffolds to be used in medical applications, such as patches with antibacterial and anti-inflammatory properties applicable in personalized medicine.

3D-printed gelatin/modified chitosan, hyaluronic acid, magnetic nanoparticles based substrates for bone tissue engineering

⇒ Matrices with tunable properties for bone tissue engineering (BTE) applications were designed and fabricated using an extrusion 3D printing technique. These matrices were created using composite inks composed of methacrylated gelatin, low molecular weight chitosan methacrylate, hyaluronic acid, hydroxyapatite, and magnetic nanoparticles. The stability and mechanical strength of the substrate were ensured by crosslinking immediately after the extrusion process, with the methacrylate groups pre-activated by the addition of a non-toxic photoinitiator, LAP.

⇒ Rheological data indicate that low molecular weight functionalized chitosan-based blends outperform high molecular weight chitosan-based inks with comparable hydroxyapatite content and processing conditions.

⇒ SEM images confirmed the formation of 3D-structured scaffolds and interconnected pore networks with sizes ranging from 50-150 μm, which is in agreement with literature data on bone cell size, the parameters being suitable for populating bone cells with cells.
⇒ The behaviour of the materials in contact with simulated biological fluids (PBS and an enzyme mixture) demonstrated that the matrix exhibit properties suitable for implantation in bone tissue.
⇒ Tests performed on *in vitro* cell cultures (MG-63 cell line, osteoblasts, viability and morphology) demonstrated that the 3D scaffolds are cytocompatible.

It has been demonstrated that by adapting the ratio between the polymeric phase and hydroxyapatite, matrices can be developed by 3D printing methods that can have applications in bone regeneration processes and regenerative therapies.

Biohybrid architectures based on functionalized polymers realized by (bio)3D printing technique

⇒ The study focused on developing and characterizing novel GelMA (types A and B), XGMA, and HA-based polymeric scaffolds. These scaffolds were 3D printed using extrusion and have applications in regenerative medicine. In the research, inks based on gelatin, xanthan gum (functionalized with methacrylic anhydride), and hyaluronic acid were developed. Gelatin was chosen for its biocompatibility, biodegradability, and support for cell adhesion. Hyaluronic acid was added to enhance cell adhesion, biocompatibility, and to attain the appropriate ink viscosity for 3D printing.
⇒ The formation of the gel networks was confirmed by rheological tests, the systems exhibiting values of the specific viscoelastic parameters $G' > G''$ and $\tan \delta < 1$.
⇒ GelMA(A and B), XMA and HA hydrogel-based inks were obtained and crosslinked at 365 nm.
⇒ The substrates were tested in terms of morphological structure, demonstrating the achievement of porous structures with pore values of 50-160 μm, values that favor the fibroblast adhesion and growth.
⇒ From the point of view of the interaction with the cellular environment, cell viability values exceeding 90% were evidenced, demonstrating the cytocompatibility of the matrix. Both phase-contrast and fluorescence microscopy images demonstrated that the scaffolds do not have a negative influence on cell shape and development, the cells retaining their characteristic fusiform shape, forming a confluent monolayer on the surface of the wells.

It was demonstrated that the obtained scaffolds can be used in regenerative medicine engineering applications. The research also observed the positive impact of XMA and HA polymers, which significantly improve the printability characteristics and mechanical performance of the printed substrates.

3D printed biohybrid architectures with applications in skin lesion regeneration

⇒ The study focused on the development and characterization of novel GelMA A and AlgMA-based polymeric substrates, 3D printed via extrusion, as well as dual-layered substrates created from two types of materials populated with two interlinked cell lines. These have potential applications in regenerative medicine. Gelatin and sodium alginate based inks (previously functionalized with methacrylic functions) were developed in the research undertaken. Gelatin was chosen due to its properties related to biocompatibility and biodegradability, but also for supporting cell adhesion. The sodium alginate chain being functionalized showed a higher capacity to interact with the cellular environment.

⇒ Cell viability values exceeded 90% demonstrating the cytocompatibility of the scaffolds.

⇒ Both phase-contrast and fluorescence microscopy images demonstrated that the matrix does not have a negative influence on cell shape and development, the cells maintaining their characteristic fusiform shape, forming a confluent monolayer on the surface of the wells.

⇒ Testing of bistratified architectures has shown that the use of a three-dimensional (3D) polymeric cell-supported scaffolds significantly influences the speed of wound healing compared to the natural healing process.

⇒ Histologic and immunohistochemical analyses demonstrated that the 3D cell support not only accelerates wound surface reduction, but also stimulates epidermal regeneration factors essential for the healing process. Thus, the cell-populated 3D polymeric scaffold has demonstrated a superior ability to reduce the time required for wound healing, an effect that can be attributed to the complex interactions between the cells and the 3D matrix, which create a microenvironment conducive to regeneration.

The results highlight the potential of cell-populated 3D assemblies to improve wound treatment, opening new directions for research and clinical application.

Final conclusions

The PhD thesis entitled **"Hybrid polymeric architectures designed as hydrogel structures"** had as its main objective the development of hybrid systems, achieved either through traditional processing methods or by optimizing the rapid prototyping technique using extrusion printing. The novelty of the studies consisted in the use of functionalized polymers with polymerizable groups for the creation of complex structures by innovative 3D printing techniques.

New polymer systems based on biopolymers, polymers functionalized with synthetic and/or polymerizable groups, with appropriate structure, physicochemical properties, and possible interactions with the biological environment, were designed, obtained, and optimized.

Perspectives

As perspectives, the research directions developed in the PhD thesis will be extended and oriented towards:

- **in-depth studies on functionalized polymers with printable properties;**
- **further studies on systems with complex, layered structures;**
- **further studies on printable systems containing cell suspensions in their composition;**
- **the development of new systems for cell culture by optimizing hybrid systems obtained during doctoral studies through the addition of biological molecules involved in cell proliferation and differentiation.**

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Scientific activity

Scientific articles on the subject of doctoral thesis

1. **Nacu I**, Bercea M, Niță L.E, Peptu C.A, Butnaru M, Vereștiuc L, **„3D bioprinted scaffolds based on functionalized gelatin for soft tissue engineering",** *Reactive and Functional Polymers*, Volume 190, **2023. IF 5.1**
2. Luca A, **Nacu I**, Tănăsache S, Peptu C.A, Butnaru M, Vereștiuc L, „**New Methacrylated Biopolymer-Based Hydrogels as Localized Drug Delivery Systems in Skin Cancer Therapy",** *Gels*, Volume 9(5), **2023. IF 4.6**
3. Niță L.E, **Nacu I,** Ghilan A, Rusu A.G, Șerban A.M, Bercea M, Vereștiuc L, Chiriac A.C, „ **Evaluation of hyaluronic acid-polymacrolactone hydrogels with 3D printing capacity",** *International Journal of Biological Macromolecules*, Volume 256, Part 2**, 2024. IF 8.2**
4. **Nacu I,** Ghilan A, Rusu A.G, Bercea M, Niță L.E, Vereștiuc L, Chiriac A.C, **„Hydrogels with Antioxidant Microparticles Systems Based on Hyaluronic Acid for Regenerative Wound Healing",** *Macromolecular Bioscience***, 2024. IF 4.4**
5. Botezatu I, **Nacu I,** Cojocaru F.D, Balan V, Bercea M, Niță L.E, Vereștiuc L, **„3D Printed composite scaffolds based on biopolymers, hydroxyapatite and magnetic nanoparticles for bone tissues defects repair" – in evaluation process**

Scientific articles related to the doctoral thesis

1. Crețu B.E.B, Dodi G, Gardikitios I, Bălan V, **Nacu I,** Stoica I, Stoleru E**,** Rusu A.G, Ghilan A, Niță L.E, Chiriac A.C, **„Bioactive Composite Cryogels Based on Poly (Vinyl Alcohol) and a Polymacrolactone as Tissue Engineering Scaffolds: In Vitro and *In Vivo* Studies",** *Pharmaceutics, 2023.*
2. Onu I, Gherghel R, **Nacu I,** Cojocaru F.D, Vereștiuc L, Matei D.V, Caşcaval D, Şerban I.L, Iordan A.D, Tucaliuc A, Galaction A,**„Can Combining Hyaluronic Acid**

and Physiotherapy in Knee Osteoarthritis Improve the Physicochemical Properties of Synovial Fluid?", *Biomedicines,* **2024.**

3. Şerban A.M, **Nacu I,** Roşca I, Ghilan A, Rusu A.G, Niță L.E, Niță R.D, Chiriac A.C **„Preparation and Characterization of Polymeric Microparticles Based on Poly(ethylene brassylate-co-squaric Acid) Loaded with Norfloxacin",** *Pharmaceutics, **2024.***
4. Platon I.V, Ghiorghita C.A, Lazar M.M, Aprotosoaie A.C, Gradinaru A.C, **Nacu I,** Vereștiuc L, Nicolescu A, Ciocarlan N, Dinu V.M, „**Highly Compressible, Superabsorbent, and Biocompatible Hybrid Cryogel Constructs Comprising Functionalized Chitosan and St. John's Wort Extract",** *Biomacromolecules,* **2024.**
5. Bibire T, Dănilă R, Yilmaz C.N, Vereștiuc L, **Nacu I,** Ursu R.G, Ghiciuc C.M ***„In Vitro* Biological Evaluation of an Alginate-Based Hydrogel Loaded with Rifampicin for Wound Care",** *Pharmaceuticals, **2024.***
6. Afloarea O.T, **Nacu I,** Vereștiuc L, Yilmaz C.N, Panainte A.D, Peptu C.A, Ostafe I.G, Bibire N, ***„In Vitro and Ex Vivo* Evaluation of Novel Methacrylated Chitosan-PNIPAAm-Hyaluronic Acid Hydrogels Loaded with Progesterone for Applications in Vaginal Delivery",** *Polymers,* **2024.**

International presentations

1. **International Conference on e-Health and Bioengineering, Iaşi, 2021:** Donea R, **Nacu I,** Butnaru M, Verestiuc L,"Methacrylated Collagen/Chitosan Based Hydrogels as Scaffolds for Soft Tissue Engineering"
2. **International Conference on e-Health and Bioengineering, Iaşi, 2022: Nacu I,** Baiu T, Niță L.E, Verestiuc L, **"**3D Bioprinted Methacrylated Gelatin-Based Scaffolds", **MENȚIUNE**
3. **International Biomedical Science and Technology Symosium, Ankara, Turcia, 2022: Nacu I,** Nedelcu L, Niță L.E, Verestiuc L,**"**3D Bioprinted hybrid hydrogels based on gelma and functionalised biopolymers for tissue engineering".
4. **International Conference on Bioengineering and Polymer Science, Bucureşti, 2023: Nacu I,** Ilie I, Tunaru A, Niță L.E, Verestiuc L,**"**3D Bioprinted Scaffolds Based on Functionalised Gelatin and Sodium Alginate for Soft Tissue Engineering".
5. ***NanoBioMat Summer Edition, Bucureşti, 2023:*** Botezatu I, **Nacu I,** Cojocaru FD, Bălan V, Verestiuc L „***3D printed scaffolds based on biopolymers-calcium phosphates and magnetic nanoparticles for bone tissue engineering" – Best Paper Award***
6. ***MacroYouth, Iaşi, 2023:*** **Nacu I,** Niță L.E, Verestiuc L, "***Biocompatible Scaffolds based on Functionalised Polymers For Soft Tissue Engineering" – Premiul II***
7. ***NanoBioMat Winter Edition, Bucureşti, 2023:*** **Nacu I,** Ilie I, Tunaru A, Niță L.E, Verestiuc L, ***Biocompatible Hybrid Scaffolds based on Functionalised Gelatin For Soft Tissue Engineering.***
8. ***Conferința Tehnico-științifică a studenților, masteranzilor și doctoranzilor a Universității Tehnice a Moldovei, Chişinău, 2024:*** **Nacu I,** Ilie I, Tunaru A, Niță L.E, Verestiuc, *3D Bioprinted Scaffolds Based on Functionalized Biopolymers for Soft Tissue Engineering.*
9. ***Conferința Tehnico-științifică a studenților, masteranzilor și doctoranzilor a Universității Tehnice a Moldovei, Chişinău, 2024:*** *Ghiață I,* **Nacu I,** Verestiuc L, ***Vascular grafts obtained through 3d printing technologies – Premiul III***
10. ***NanoBioMat Summer Edition, Bucureşti, 2024:*** **Nacu I,** Ilie I, Tunaru A, Niță L.E, Verestiuc L, **Complex 3D printed architectures for skin tissue repair and regeneration. – Best Presentation Award**

International Presentations - Poster Section

1. **Tissue Engineering and Regenerative Medicine International Society, Inc.- European Chapter, 2022 TERMIS-EU Conference, Kracow : Nacu I,** Nedelcu L, Niță L.E, Peptu C.A, Verestiuc L, „**3D Bioprinted scaffolds based on functionalised gelatin/chitosan/xanthan, dextran for soft tissue engineering".**
2. **Tissue Engineering and Regenerative Medicine International Society, Inc.- European Chapter, 2022 TERMIS-EU Conference, Kracow :** Botezatu I, **Nacu I,** Cojocaru FD, Bălan V, Niță L.E ,Verestiuc L, „**3D Printable functionalised gelatin/chitosan, hyaluronic acid, hydroxyapatite and magnetic nanoparticles scaffolds for bone regeneration".**
3. **Appolonia, Iaşi, 2024: Nacu I, Ghilan A, Rusu A.G, Serban A.M, Bercea M , Vereştiuc L, Nita L.E, „Hybrid hydrogel systems based on hyaluronic acid and a copolymacro-lactone structure".**

Research projects

1. New hybrid polymer/peptide hydrogels as innovative platforms designed for cell cultures applications" **PN-III-P2-2.1-PED-2019-2743 (2020 - 2022)**
2. „3D bio-inspired hybrid architectures for deep thickness skin repair and regeneration" **PN-III-P2-2.1-PED-2021-3003 (2022 - 2024)**