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SUMMARY OF DOCTORAL THESIS

INTELLIGENT MATERIALS BASED ON STIMULI SENSITIVE POLYMERS FOR BIOMEDICAL APPLICATIONS

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Iași, 2023

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Dedication and perseverance are essential in the development of a doctoral thesis. All these aspects take shape under the careful guidance of the scientific coordinator.

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INTRODUCTION

The response to external stimuli is a fundamental and intrinsic characteristic of living systems, crucial for maintaining normal functions, performing complex activities, and combating diseases.

From unicellular organisms to multicellular species, almost all important physiological processes are regulated by chemical, biological, or physical signals, including cell division, proliferation, differentiation, cell apoptosis, as well as respiration, photosynthesis, and immune regulation. Often, these responses are accompanied by structural transformations of systems at the molecular, cellular, and macroscopic levels. At the molecular level, enzymes, ion channels, and other transport proteins often undergo structural transitions that facilitate binding to small molecules, ions, or other substances. At the cellular level, biomembranes dynamically modulate their architectures through interactions with a variety of molecules, especially macromolecules, such as proteins, biopolymers, etc. These conformational changes play an important role in cellular processes, such as exocytosis and endocytosis.

Inspired by the organizational systems of living organisms and how they react to fluctuating exogenous conditions and processes, researchers have attempted to mimic these natural phenomena and design certain systems, typically polymeric, that can recognize specific changes in external factors and respond by modifying certain physicochemical properties. Polymers possessing these properties have been termed ***"stimuli-responsive polymers"*** or ***"intelligent polymers."***

Stimuli-responsive polymers undergo reversible changes in their physical or chemical properties in response to small variations in external factors. These stimuli induce macroscopic responses in polymeric materials, such as swelling, collapsing, or transitions from solution to gel. Additionally, these macromolecular compounds can simultaneously respond to multiple external stimuli, such as pH, light, temperature, ionic strength, electric field, biochemical substances, etc. From a practical perspective, such capacity to recognize and react to environmental changes can be exploited to create high-performance devices that can enhance activities in various domains: biomedical, food industry, textile industry, cosmetics, bioseparations, and bioprocessing.

For biomedical applications, intelligent polymeric materials can be obtained in the form of hydrogels, films, capsules, micro- and nano-particles, and micelles. These

formulations can be used as controlled drug release systems, diagnostic devices, supports for tissue engineering, optical systems, biosensors, or microelectromechanical systems.

The general objective of the doctoral thesis consisted of the development of intelligent systems for the controlled release of drugs. These pharmaceutical formulations ensure the release of therapeutic substances into the body whenever physiological parameters are outside normal limits, thereby improving drug efficacy by controlling the rate, timing, and site of release.

It is well-known that in the case of conventional formulations, after administration, the concentration of the active ingredient in the blood quickly reaches a peak and then decreases just as rapidly to a value at which dose repetition is necessary. Often, the maximum concentration reaches levels higher than the effective therapeutic level, potentially exceeding the toxicity threshold. Conventional controlled release systems eliminate variations in the concentration of the active ingredient in the blood and ensure a therapeutic level over an extended period. However, there are several clinical situations when such an approach is not sufficient and efficient. These include the release of insulin for patients with diabetes, antiarrhythmics for patients with cardiac rhythm disorders, inhibitors of gastric acid secretion for ulcer control, nitrates for patients with angina pectoris, as well as selective beta-blockers, immunizations, cancer chemotherapy, etc. Therefore, to improve patient compliance with existing therapies, it is crucial to design and implement innovative systems, and smart polymers are the key to overcoming these difficulties, especially surpassing biological barriers, releasing the drug only under certain pathological conditions, and maintaining the action of the active ingredient for an extended period.

The thesis is structured into two parts: the first part includes literature data regarding stimuli-responsive polymers, followed by the classification and description of intelligent macromolecular matrices (micro- and nano-particles, hydrogels, micelles, films). The second part presents the original results obtained during the doctoral period.

Chapter I provides general information about stimuli-responsive polymers, the classification of these macromolecular compounds, as well as the advantages of their use in the biomedical field.

Chapter II includes information regarding the materials used in the thesis, synthesis methods, and characterization of both linear stimuli-responsive polymers and the intelligent macromolecular matrices obtained.

Chapter III presents original results regarding the synthesis and characterization of pH and temperature-sensitive microspheres based on poly(N-isopropylacrylamide-co-vinylimidazole) copolymers (P(NIPAAm-co-VI)) and poly(N-isopropylacrylamide-co-4-vinyl pyridine) copolymers (P(NIPAAm-co-4-VP)).

In subchapter III.1, linear copolymers based on NIPAAm and VI were synthesized through radical copolymerization of the two co-monomers, and the chemical composition was determined for which the copolymer exhibits a lower critical solution temperature (LCST) close to that of the human body under simulated physiological conditions. This copolymer was then used for the synthesis of microspheres sensitive to pH and temperature and loaded with doxorubicin. These microspheres can self-disintegrate into monodisperse nanospheres under conditions similar to those encountered in physiological fluids (pH = 7.4, T = 36 °C). After intravenous administration into the bloodstream, the nanospheres are stable and release a small amount of the drug. However, in environments simulating endosomal and lysosomal conditions (pH = 5.5 and pH = 5.0, respectively), they dissolve and release the entire encapsulated active ingredient. The nanospheres were biologically tested using two cancer cell lines, hepatocellular carcinoma (HepG2) and lung adenocarcinoma A549 cells, and the data showed a higher susceptibility for the HepG2 cell line. Therefore, they can be used as controlled release systems for doxorubicin in hepatic tumors.

Subchapter III.2 presents original results regarding the synthesis and characterization of poly(N-isopropylacrylamide-co-4-vinyl pyridine) microspheres. Firstly, the pH and temperature-sensitive linear polymer was synthesized and characterized using IR and NMR spectroscopy, and the pKa value was determined through potentiometric titration. The critical temperature (LCST) of the copolymer was also determined. The microspheres were obtained from the linear copolymer using an original solvent evaporation method, and dexamethasone was selected as the drug for encapsulation in the microspheres. Following the conducted tests, it was found that these microparticulate systems loaded with dexamethasone are stable under physiological conditions but rapidly dissolve under simulated physiological conditions with pH = 5.0, specific to tumor microenvironments, consequently releasing the entire amount of the drug.

Chapter IV presents original results regarding the development of new double cross-linked hydrogels sensitive to temperature and pH. Initially, a conventional thermosensitive hydrogel was synthesized through radical polymerization of N-isopropylacrylamide with N-hydroxyethyl acrylamide in the presence of N, N'-methylene

bisacrylamide, used as a cross-linking agent. Subsequently, the pH-sensitive double cross-linked structures were obtained from the previously prepared conventional hydrogels by immersing them in a solution of poly(methyl vinyl ether-alt-maleic acid) (P(VME/AM)) and subsequent thermal cross-linking. As an innovation, the double cross-linked hydrogels were prepared in the absence of any organic solvent or cross-linking agent, both of which are generally toxic to the body. The new matrices were characterized compositionally through FT-IR spectroscopy, and morphologically through scanning electron microscopy, and their swelling capacity and mechanical properties were studied. Metoclopramide was selected as a model drug to investigate the matrix's ability to control the release rate of the active ingredient. The obtained results showed that in the gastric fluid (pH = 1.2 and T = 36 °C), the hydrogel is in a collapsed state due to the protonation of carboxyl groups, hindering drug diffusion. In the intestinal fluid (pH = 7.4), carboxyl groups are ionized, the hydrogel swells, and the drug is released more rapidly. In conclusion, double cross-linked hydrogels are recommended for the oral administration of metoclopramide or other biologically active similar compounds.

Chapter V presents original results regarding the preparation and characterization of new thermosensitive injectable gels, either based on a physical mixture of Poloxamer 407 and carboxymethyl pullulan (CMP) or by grafting Poloxamer 407 onto carboxymethyl pullulan.

Subchapter V.1 presents the study of miscibility in an aqueous solution between the two polymeric components (Poloxamer and CMP) and the determination of the effect of CMP concentration and degree of substitution on the sol-gel transition temperature, gelation kinetics, and mechanical properties of the thermogels obtained at 37 °C. Rheological studies showed that due to the relatively low relative viscosity, adding CMP to the poloxamer solution does not significantly affect the rheological parameters of the thermogels. A decrease in the gelation temperature and an increase in the hardness of the gels were observed for concentrations of 0.4 - 1% CMP in the mixture. The gels exhibited good recovery of structure even after a 1000% strain, and the gel containing 1% CMP showed a gelation temperature of 26 °C and good hardness. This mixture can be used as a base for an in situ gel formulation, particularly in dermal applications.

In subchapter V.2, a new pH and temperature-sensitive copolymer was synthesized and characterized by grafting Poloxamer 407 onto CMP (Plx-g-CMP). The structure of the grafted copolymer was evaluated using Fourier-transform infrared spectroscopy and nuclear magnetic resonance. The study of the copolymer concentration's

effect on the gelation behavior was conducted through rheological tests. These tests highlighted high elasticity and the excellent ability of the copolymer to recover its initial structure after the applied force or external stimuli were removed. Furthermore, the hydrogel exhibited sustained release of amoxicillin (model drug) for 168 hours. The results indicate that this copolymer can be used as an injectable hydrogel, particularly for the reconstruction of damaged cartilage.

In the thesis's final section, **general conclusions** are presented, along with a bibliography.

OBJECTIVES

- Obtaining temperature and pH-sensitive microparticles loaded with doxorubicin (MS-DXR) from the preformed linear copolymer P(NIPAAm-co-VI) using the solvent evaporation method for drug release at the tumor level induced experimentally by A549 and HepG2 cell lines.
- Preparation of temperature and pH-sensitive microparticles (MS-DEX) using the solvent evaporation method from the preformed copolymer P(NIPAAm-co-4-VP) and dexamethasone used as platforms for the release of the active principle at the tumor microenvironments.
- Synthesis of conventional thermosensitive hydrogels (PNH) through a radical polymerization reaction of NIPAAm and HEAAm in the presence of the redox initiation system KPS/TEMED.
- Obtaining double cross-linked hydrogels sensitive to pH and temperature (DC) from conventional hydrogels (PNH) and P(MVE/MA) of different concentrations used as matrices for the release of metoclopramide.
- Study of the behavior of physical mixtures based on Poloxamer and carboxymethyl pullulan (CMP) of different concentrations and with certain degrees of substitution.
- Synthesis of a pH and temperature-sensitive grafted copolymer through a coupling reaction between the amine groups of monofunctionalized poloxamer and the carboxylic groups present on the pullulan chains used as a system for the controlled release of amoxicillin and as a potential matrix for joint cartilage restoration.

The doctoral thesis entitled "**Intelligent materials based on stimuli sensitive polymers for biomedical applications**" comprises **250** pages divided into **5** chapters,

including **13** Tables, **84** Figures, **12** Schemes, **41** Equations, and **432** bibliographic references.

PERSONAL CONTRIBUTIONS

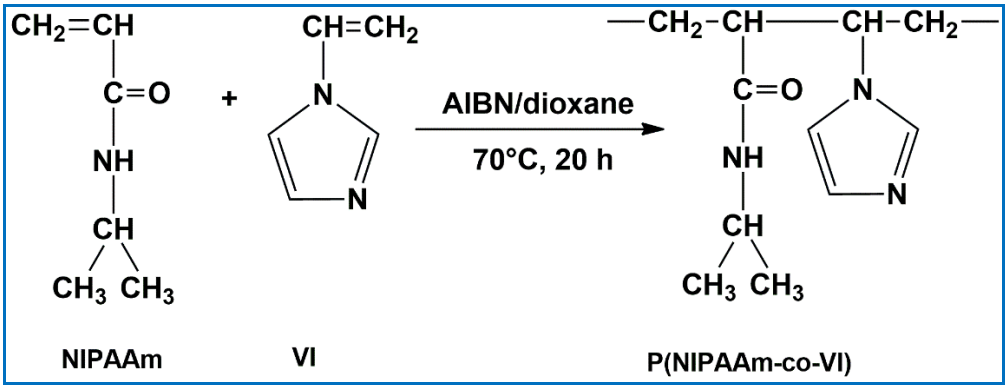
Chapter III. pH/TEMPERATURE SENSITIVE MICRO- AND NANO-PARTICLES

III.1. MICRO- AND NANO-SPHERES FROM POLY(N-ISOPROPYLACRYLAMIDE-co-VINILIMIDAZOLE)

Cancer is among the leading causes of death globally, and the most commonly used methods for its treatment are surgery and chemotherapy, which often come with many undesirable side effects due to their lack of selectivity. A resolution to this issue lies in creating systems capable of directing drugs to a specific anatomical region [1]. Over the past decades, systems have been designed to transport drugs through the bloodstream and then be released due to the influence of external stimuli, such as pH [2], temperature [3], or redox potential [4]. pH sensitive drug delivery systems are commonly used in cancer therapy because they can react to pH changes in tumor tissues [5,6]. Generally, the extracellular pH of normal tissues falls within the range of 7.2 – 7.5, whereas the pH specific to tumors is slightly acidic, ranging from 6.4 – 7.0 [7]. Cancer cells behave similarly to normal cells in the process of particle internalization through endocytosis. Their internalization involves transitioning from endosomes to lysosomes, where the pH drops from 6.0 - 5.5 to 5.0 [8]. Therefore, pH-sensitive polymeric systems are among the most suitable models for designing controlled drug delivery systems.

To obtain a temperature and pH-sensitive copolymer, copolymerization of N-isopropylacrylamide (NIPAAm) with vinylimidazole (VI) was employed. VI was chosen to copolymerize with NIPAAm because it contains amine groups that ionize at a pH located at the boundary between physiological and pathological conditions. Moreover, the protonation/deprotonation of VI modulates the thermosensitive properties of P(NIPAAm-co-VI), hence the solubility/insolubility of the copolymer. To achieve this, four copolymer samples were synthesized by radical polymerization in 1,4-dioxane of the two co-monomers in the presence of N,N'-azobisisobutyronitrile (AIBN) as the initiator. The copolymerization reaction took place at 70°C for 20 hours under a nitrogen inert atmosphere (**Scheme 1**). pH and temperature-sensitive copolymers were prepared by varying the molar ratio between the co-monomers NIPAAm:VI (10:0; 10:1; 10:1.5 and

10:2) in the initial reaction mixture. The chemical structure of the copolymer was confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and the spectral analysis allowed for the determination of its chemical composition (molar ratio between co-monomers) (**Figure 1**).



Scheme 1. Synthesis of P(NIPAAm-co-VI) copolymer.

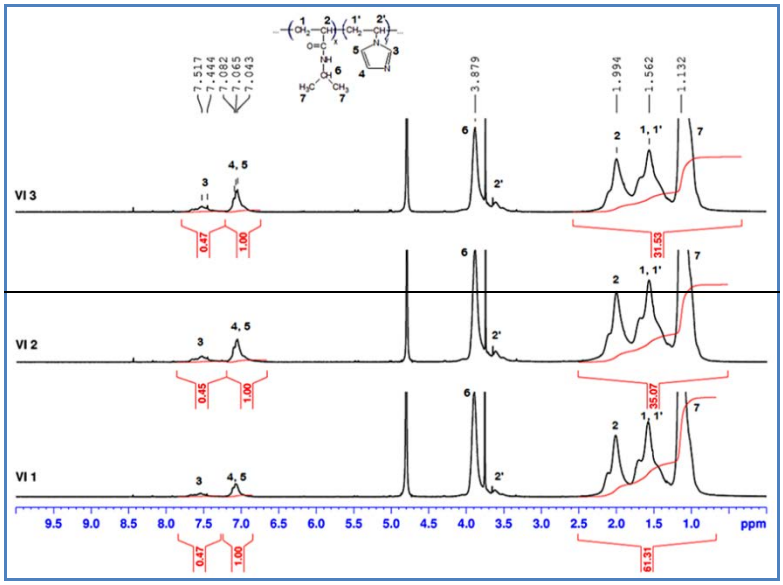


Figure 1. ^{1}H-NMR spectra in D_2O of P(NIPAAm-co-VI) copolymers.

The molar ratio of the co-monomers in P(NIPAAm-co-VI) was calculated from the ^{1}H-NMR spectra (**Figure 1**) using the equations below (**Eq. (1)** and **Eq. (2)**):

$$9x+2y=A1 \qquad \textit{Eq.}\ (1)$$

$$2y=A2 \qquad \textit{Eq.}\ (2)$$

where: x is the mole fraction of NIPAAm and which was calculated as the value of the integral of the methine proton signal (6) and y is the mole fraction of VI.

The copolymer **P(NIPAAm-co-VI)** was used to obtain pH and temperature-sensitive microspheres loaded with doxorubicin (DXR) **(MS-DXR)** through a completely new approach to the solvent evaporation method. The novelty of the technique lies in the fact that the continuous phase is represented by cyclohexane, while the dispersed phase is water. The choice of this binary solvent system was made for the following reasons: (i) the

solvents are immiscible; (ii) water is the best solvent for doxorubicin (DXR); (iii) cyclohexane molecules, which evaporate easily due to its high volatility, entrain less volatile water molecules, significantly reducing the preparation time.

The microspheres were prepared in a slightly acidic solution. The protonation of VI leads to an increase in the critical temperature (LCST) of the copolymer solution, allowing the evaporation of water at a higher temperature without the risk of precipitating the thermosensitive copolymer. Using this method, it was obtained microspheres with a spherical shape in the dry state (**Figure 2A**) and a rough surface (**Figure 2B**). Analysis of SEM images and measurement of at least 200 microspheres led to the conclusion that the microspheres have a relatively wide size distribution with diameters ranging from 500 nm to 8 μm. The microspheres were produced from an aqueous solution using two successive emulsification speeds: an initial speed of 12,000 rpm resulting in the production of nanoscale droplets covered with the surfactant, followed by a speed of 800 rpm resulting in the coagulation of these nanodroplets into solid microspheres of micrometer dimensions. Thus, the microspheres are formed from aggregated nanospheres delimited by the surfactant, which disintegrate upon contact with physiological fluids. Disintegration occurs because soy lecithin is a highly hydrophilic surfactant, and in aqueous media, water penetrates the microspheres, causing their disintegration. The resulting nanoparticles (NS-DXR) are characterized by a hydrodynamic diameter of ~250 nm (**Figure 2C**), a polydispersity index (PDI) of 0.2, and a zeta potential of -9.57 mV.

The percentage of DXR incorporated into the microspheres is 2.0% (w/w), with an encapsulation efficiency of 82.0%. The high values for encapsulation efficiency were expected since the polymer is not soluble in cyclohexane, and drug diffusion is limited only to the interface between the two phases. Under simulated blood flow conditions (phosphate buffer at pH = 7.4), the LCST value of P(NIPAAm-co-VI) is 33.1°C. Therefore, at human body temperature (36 - 37°C), which is above the LCST, the copolymer is completely insoluble, as are the nanospheres made from this copolymer, resulting in very low drug release.

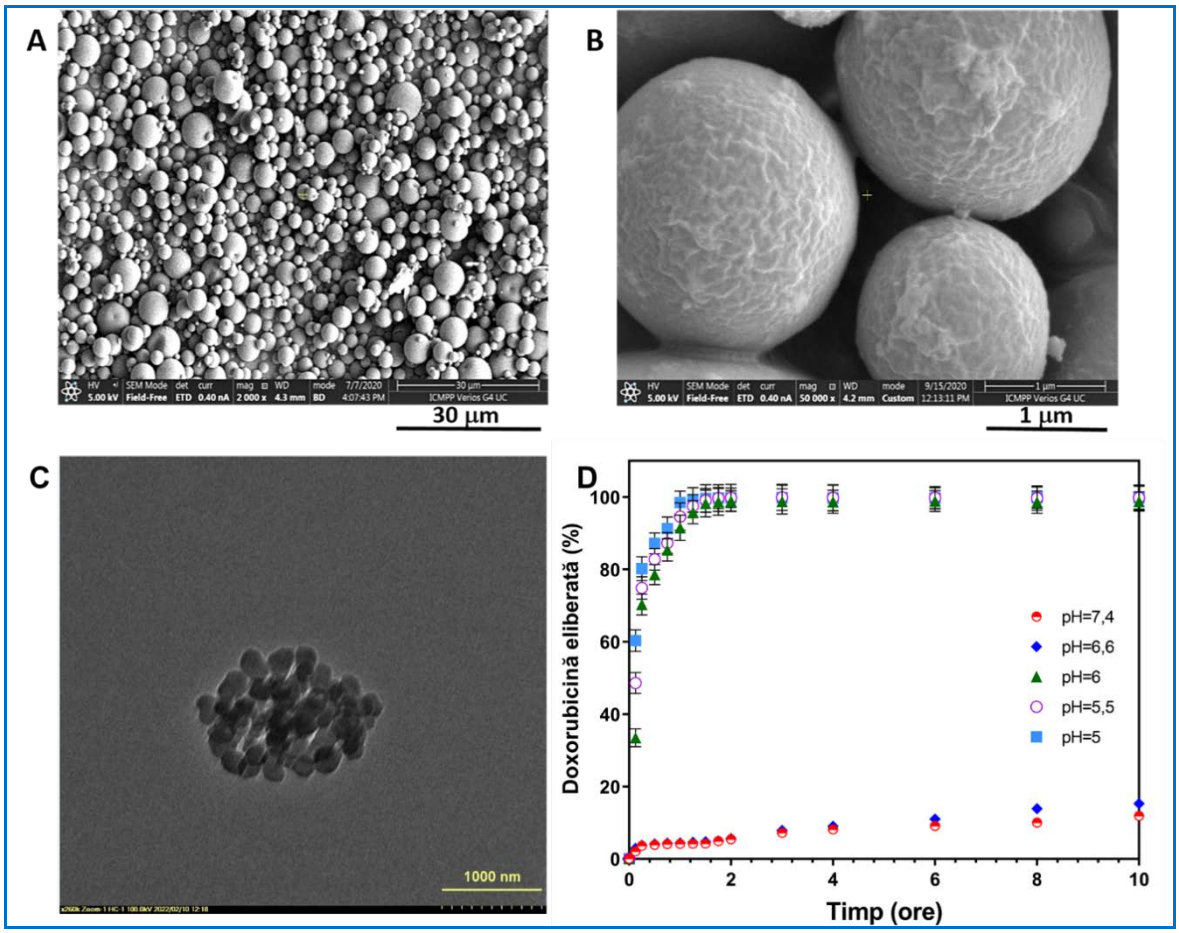


Figure 2. SEM micrographs of DXR-loaded P(NIPAAm-co-VI) microspheres **(A** and **B)**; TEM image of NS-DXR **(C)**; In vitro cumulative release (%) of DXR from NS-DXR under physiological conditions (PB) at different pH values **(D).**

The *in vitro* release studies of DXR (**Figure 2D**) showed that after 2 hours, presumed to be the time nanospheres spend in the bloodstream, only 5.43% of DXR was released, most likely reflecting the fraction of the drug on the nanospheres' surface. In physiological buffer (PB) at pH = 6.6, the LCST value is 34°C, again below human body temperature, and the drug release profile is nearly similar to that at pH = 7.4. Conversely, at pH = 6.0, 5.5, and 5.0, the LCST values are 36.2, 37.7, and 39.7°C, respectively, all critical temperatures being above human body temperature. Therefore, the copolymer and hence the nanospheres dissolve and release the drug in a controlled manner.

Biological tests

Cytocompatibility and cellular internalization tests were conducted at the **Institute of Cell Biology and Pathology "Nicolae Simionescu"** in Bucharest as part of a bilateral collaboration. XTT and ToxiLightTM tests were used to determine the *in vitro* cytotoxicity of nanospheres made of P(NIPAAm-co-VI) loaded with DXR. The cells used for exposure

were HepG2 and A549, and they were exposed to various concentrations of NS, NS-DXR, and free DXR (**Figure 3**). Both methods showed that the viability of HepG2 and A549 cells was not affected by incubation with drug-free nanospheres (NS). This result suggests that NS are cytocompatible with the tested cell lines. According to the results of the XTT test, the nanospheres loaded with doxorubicin and the free drug exhibited higher susceptibility for the HepG2 cell line compared to the A549 cells.

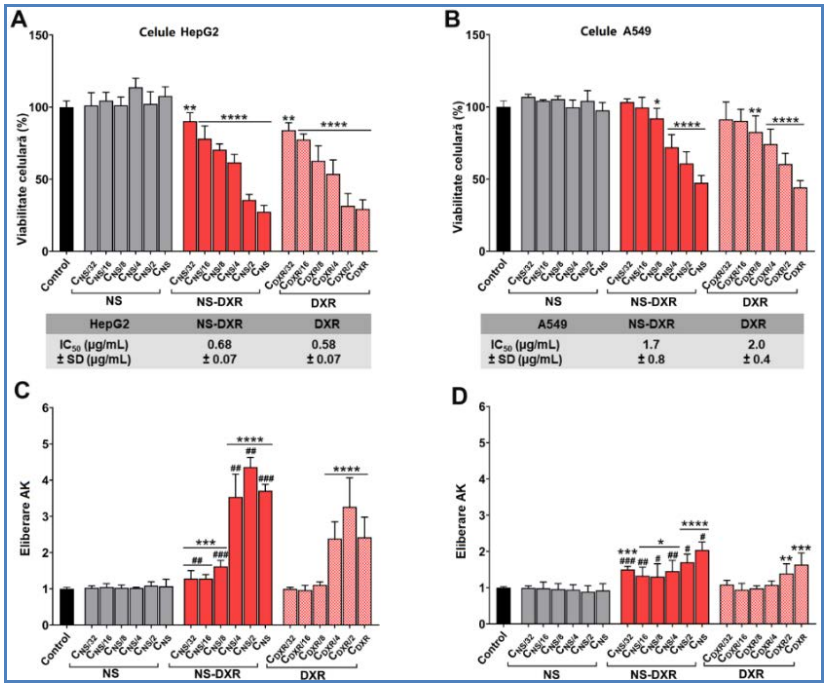


Figure 3. The viability of HepG2 **(A,C)** and A549 **(B,D)** cells after exposure to plain NS, NS-DXR and free DXR determined by XTT **(A,B)** and ToxiLightTM **(C,D)** assays.

The images recorded using fluorescence microscopy revealed that both NS-DXR and free DXR were internalized by cells via endocytosis in a dose- and time-dependent manner. DXR was primarily detected in the cell nuclei, consistent with previous studies involving other DXR delivery systems.

As a preliminary step in further preclinical investigation of the therapeutic potential of doxorubicin-loaded nanospheres after intravenous administration, the *hemolytic behavior* of the plain nanospheres (NS), those loaded with doxorubicin (NS-DXR), and the free drug (DXR) was evaluated. Therefore, the effect of different formulations on the integrity of erythrocyte membranes was assessed by quantifying the released hemoglobin from erythrocytes isolated from C57BL6 mouse strain after incubation with NS, NS-DXR, and free DXR. The results showed that the percentage of hemolysis did not exceed 3.5%, emphasizing that erythrocytes were not affected by incubation with NS, NS-DXR, and DXR. A hemolysis threshold of 5% induced by biomaterials is considered acceptable according to the International Organization for Standardization (ISO) 10993-4:2017.

Localization of NS-DXR and free DXR in various organs after retro-orbital injection in C57BL6 mouse strain was investigated through fluorescence optical imaging based on the fluorescent properties of doxorubicin (**Figure 4**).

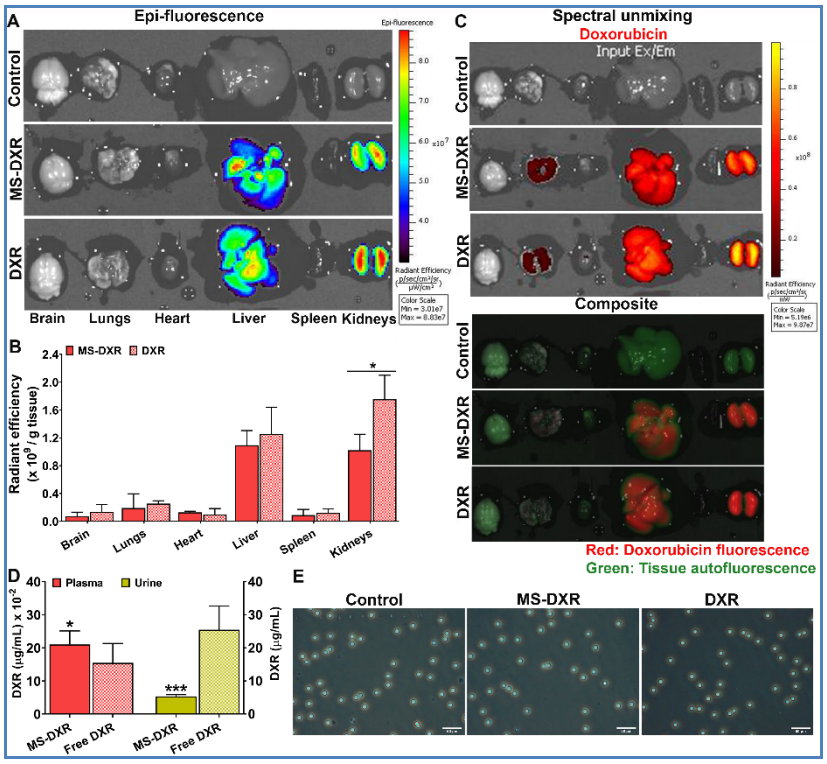


Figure 4. (A) Localization of NS-DXR and free DXR in organs harvested from C57BL/6 mice. **(B)** Quantification of total radiant efficiency in organs. **(C)** Spectral unmixing analysis. **(D)** Quantification of DXR in plasma and urine of mice, 1 h after administration of 3 mg/kg of free DXR or NS-DXR **(E)** Evaluation of *in vivo* hemocompatibility by following the erythrocyte aggregation after administration of NS-DXR and free DXR. Scale bar: 50 µm.

The fluorescence emission data indicate that at 1 hour after administration, both free DXR and drug-loaded nanoparticles accumulate in the liver and kidneys. Additionally, the administration of NS-DXR and free DXR did not cause aggregation of red blood cells, and their appearance was similar to the control group (administration of phosphate buffer) (**Figure 4**).

Therefore, under simulated conditions of blood flow, the synthesized microspheres (**Figure 5A**) disintegrate into stable monodisperse nanoparticles (**Figure 5B**). After interacting with a tumor cell, the nanoparticles are taken up through various types of endocytosis (**Figure 5C**), followed by internalization as they pass from early endosomes to lysosomes, where the nanoparticles self-disintegrate at an acidic pH, releasing the loaded doxorubicin.

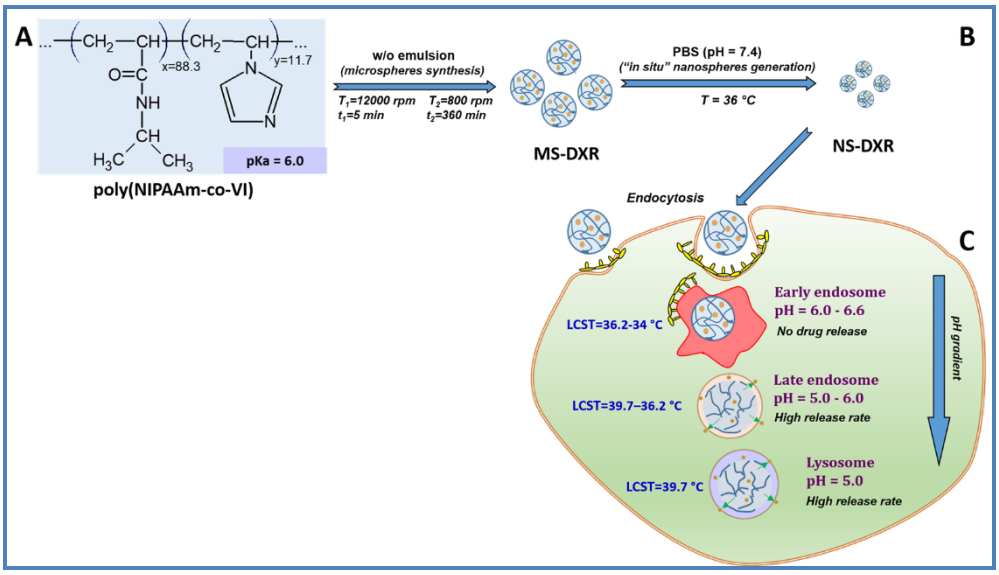


Figure 5. Schematic representation of the potential behavior of DXR-loaded microspheres (MS-DXR) after suspension in physiological buffers and interaction with a tumor cell.

III.2. MICROSPHERES FROM POLY(N-ISOPROPYLACRYLAMIDE-co-4-VINYL PYRIDINE)

A specific characteristic of neoplasms is the relatively low pH and increased temperature compared to physiological values. Poly(4-vinyl pyridine) (P(4-VP)) is a pH-sensitive polymer with a pKa = 5.46 [9], a value often encountered in the tumor microenvironment. Regarding temperature, it can range from 37.5 to 40.5 °C [10]. Therefore, a pH and temperature-sensitive copolymer, P(NIPAAm-co-4-VP), is the ideal candidate for the development of pharmaceutical devices that exploit these small variations in physiological parameters in cancer therapy [11].

The copolymerization of NIPAAm with the hydrophilic monomer 4-VP was carried out through a radical polymerization reaction in an aqueous solution using a redox system consisting of KPS and TEMED as the initiator. The polymerization reaction took place in an inert nitrogen environment at 20 °C for 24 hours. To solubilize 4-VP, it was necessary to acidify the reaction medium with 1N HCl [12]. Three copolymer samples were synthesized by varying the molar ratio between the two comonomers in the initial reaction mixture within the range of 10:1 to 10:2 (**Table 1**). Sample **VP2**, with a 13.70% 4-VP content, was chosen for subsequent experiments as it exhibited an LCST of 22.1 °C under simulated physiological conditions (PB at pH = 7.4), which means that at a temperature of 36 °C, this copolymer is completely insoluble. In a PB solution with a pH of 5.0, **VP2** has an LCST of 36.8 °C, indicating that it becomes soluble at human body temperature.

Table 1. Dependence of LCST on the co-monomer ratio in the initial mixture and in the copolymer (concentration of copolymer solution was 1%, w/v).

Sample code	Co-monomer composition				LCST (°C)				
	In the feed 10^{-3} M (% mol ratio)		In copolymer (% mol ratio)		H_2O	pH=7.4	pH=6.0	pH=5.5	pH=5.0
	NIPPAm	4-VP	NIPAAm	4-VP					
VP_0	10 (100)	0 (0)	100	0	32.6 ± 0.2	28.8 ± 0.3	30.2 ± 0.2	29.2 ± 0.2	31.2 ± 0.3
VP_1[a]	10 (90.9)	1 (9.1)	90.69	9.31	30.1 ± 0.3	26.8 ± 0.2	27.9 ± 0.3	28.6 ± 0.3	33.8 ± 0.1
VP_2[b]	10 (86.96)	1.5 (13.04)	86.30	13.70	27.8 ± 0.3	22.1 ± 0.3	23.5 ± 0.4	27.7 ± 0.3	36.8 ± 0.3
VP_3[c]	10 (83.33)	2 (16.67)	83.0	17.0	23.1 ± 0.2	12.4 ± 0.5	21.1 ± 0.3	24.2 ± 0.4	37.3 ± 0.4

[a]Mn = 10460 g/mol, Mw = 17600 g/mol, IP = 1.68; [b]Mn = 9047 g/mol, Mw = 14.480 g/mol, IP = 1.60; [c]Mn = 12.670 g/mol, Mw = 18.240 g/mol, IP = 1.44 (determined by GPC).

The microspheres of **P(NIPAAm-co-4-VP)** containing dexamethasone (DEX) **(MS-DEX)** were synthesized using the solvent evaporation method (methanol/cyclohexane) (**Figure 6**) since both the polymer and the drug are soluble in methanol (dispersed phase), and methanol is not miscible with cyclohexane (continuous phase). Through this method, the progressive evaporation of methanol from the dispersed polymer solution in the form of liquid droplets allowed the formation of small solid particles based on poly(NIPAAm-co-4-VP) loaded with the drug.

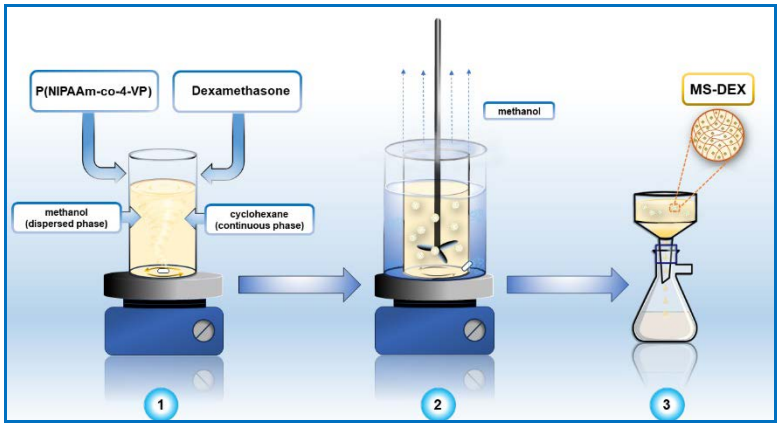


Figure 6. Schematic illustration of the preparation method for MS-DEX microspheres.

SEM image analysis revealed the perfectly round shape of the microspheres in the dry state, their dimensional homogeneity (**Figure 7A**) and their smooth surface (**Figure 7B**). The poly(NIPAAm-co-4VP) microspheres with DEX showed a relatively narrow size distribution with a diameter ranging from 0.5 to 20 μm. However, most of the microspheres were between 4 and 10 μm in size.

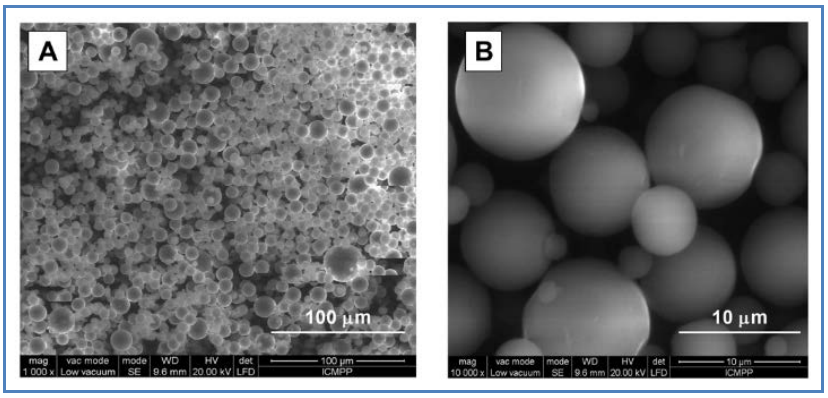


Figura 7. SEM micrographs of DEX-loaded P(NIPAAm-co-4-VP) microspheres: general view **(A)** and surface details **(B)**.

Dexamethasone was incorporated into the P(NIPAAm-co-4-VP)-based microspheres during particle synthesis because both the polymer and the drug are soluble in methanol. Furthermore, methanol is a volatile solvent and is one of the few organic solvents immiscible with cyclohexane. The percentage of drug incorporated into the microspheres was 8.89% (w/w) and the encapsulation efficiency was 97.8%. The results were predictable as the polymer and drug are not soluble in cyclohexane and therefore should be recovered in the dried microparticles after solvent evaporation.

In order to obtain information regarding the degree of dispersion of DEX in the polymeric matrix of the microspheres, as well as the interaction between the drug and the polymer, differential scanning calorimetry (DSC) analyses were conducted for both the microspheres with DEX and for DEX alone, and for the microspheres without DEX (**Figure 8**). The dexamethasone thermogram (**Figure 8(a)**) displays a peak around 270°C corresponding to the drug's melting point. In the absence of the drug, the microspheres do not show any peak in the melting point region of DEX (**Figure 8(b)**), and the polymer has a glass transition temperature (Tg) of 128°C. For the drug-loaded microspheres, the thermogram is almost similar to that obtained in the absence of DEX, but the Tg value is slightly higher (136°C) (**Figure 8(c)**). The absence of the dexamethasone melting point at 270°C highlights the molecular dispersion of the drug within the polymeric network [13]. The increase in Tg suggests that the hydrogen bonds between the drug and the polymer are stronger than those between polymer-polymer, and furthermore, DEX acts as a weak cross-linking agent.

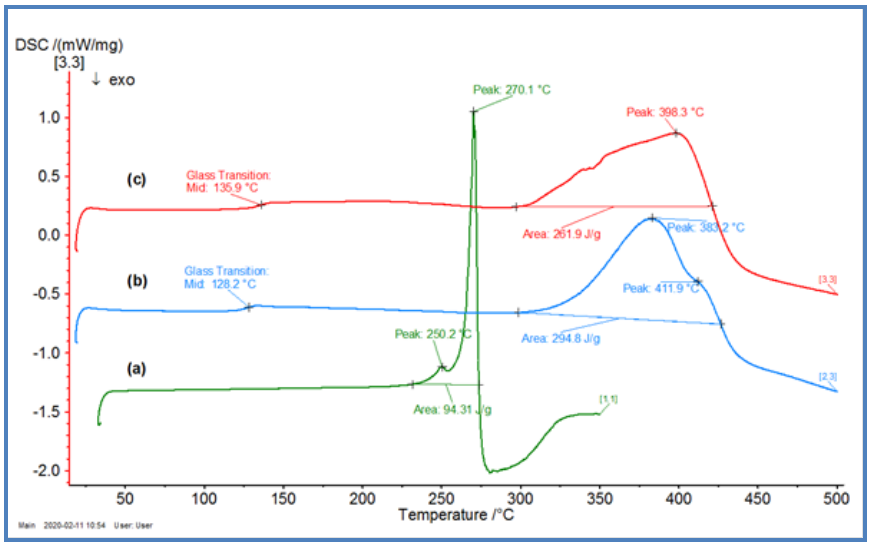


Figure 8. DSC thermograms of pure DEX (**a**), empty P(NIPAAm-co-4-VP) microspheres (**b**) and loaded microspheres with DEX (**c**). Scanning rate: 10°C·min^{-1}.

The DEX release studies were conducted under simulated physiological conditions (phosphate buffer at pH = 7.4) since pH and temperature-sensitive microspheres circulate in the bloodstream for a certain period of time. Additionally, the release kinetics were determined at lower pH values, simulating endosomal (pH = 6.0 – 5.5) and lysosomal (pH = 5.0) conditions after particle internalization (**Figure 9**). At pH = 7.4, the LCST (lower critical solution temperature) of the P(NIPAAm-co-4VP) copolymer is 22.1°C. Therefore, at human body temperature (36°C), which is above the LCST, the copolymer is insoluble, and the drug release rate is very low. After 2 hours, assumed to be the time the microspheres spend in the bloodstream, only 7% (g/g) of DEX was released, likely due to dissolution of the drug fraction from the microspheres' surface. Conversely, at pH = 5.0, the LCST is 36.8°C. Under these conditions, the microspheres undergo solubilization and release the drug. After 2 hours at pH = 5.0, 52% (g/g) of the loaded drug was released, followed by a gradual release, and nearly the entire amount of the active ingredient was released after 24 hours.

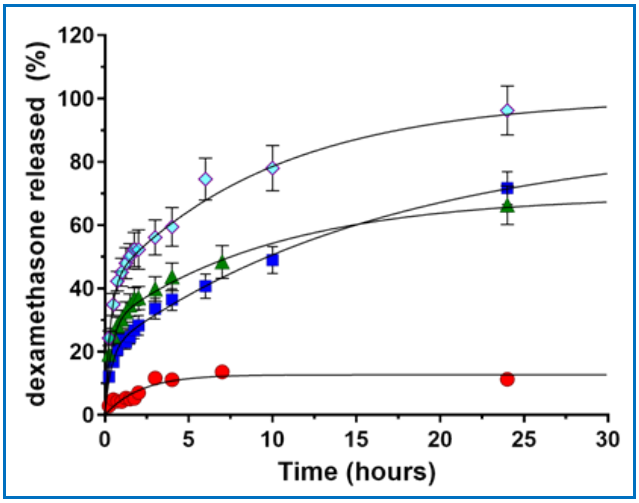
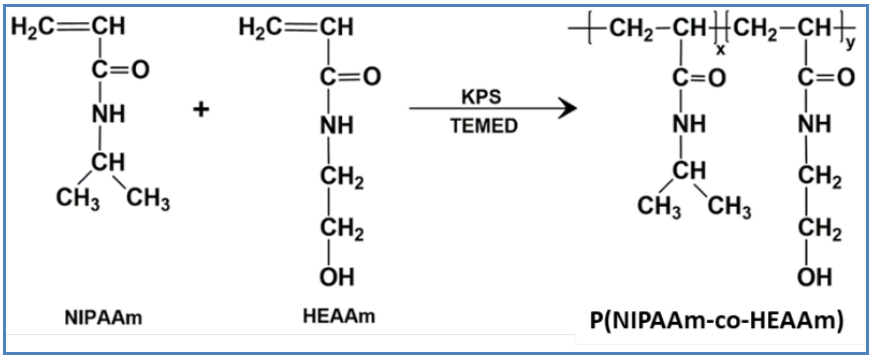


Figure 9. Release profiles of dexamethasone from P(NIPAAm-co-4VP) microspheres under normal physiological conditions (PB) at pH = 7.4 (circles) and under acidic conditions at pH = 6.0 (squares), pH = 5.5 (triangles), and pH = 5.0 (diamonds).

Chapter IV. pH/TEMPERATURE SENSITIVE DOUBLE CROSS-LINKED HYDROGELS

Hydrogels based on smart polymers exhibit sensitivity to small changes in the external environment through shape or volume modifications. This class of materials is commonly used as a support for drug delivery due to their high biocompatibility [14], spatio-temporal control over drug release, protection of the bioactive compound from harsh conditions within the body, and many more [15]. The polymeric matrix of stimuli-responsive hydrogels used as drug transport systems can protect the active principles against hydrolytic or enzymatic degradation in physiological fluids, such as degradation in the low pH of the stomach [16]. The choice of appropriate cross-linker(s) and adjusting their degree of cross-linking is one way to improve the mechanical properties of hydrogels [17] and better optimize drug loading, protection, and release [16,17].

In order to utilize NIPAAm-based copolymers in the biomedical field, copolymerization of NIPAAm with hydrophilic monomers is employed to adjust the phase transition around physiological temperature. Consequently, a thermosensitive copolymer based on NIPAAm and HEAAm was synthesized and characterized, using different molar ratios of the co-monomers in the initial reaction mixture. The radical copolymerization reaction of the two co-monomers occurred in water at room temperature, using the redox pair formed by KPS and TEMED as the initiator (Scheme 2).



Scheme 2. Synthesis of P(NIPAAm-co-HEAAm) copolymer.

In order to determine the chemical composition at which the copolymer exhibits a lower critical solution temperature (LCST) close to that of the human body, a series of copolymer syntheses P(NIPAAm-co-HEAAm) were carried out initially, varying the molar ratio between the 2 co-monomers (1:1, 2:1, and 5:1) in the initial reaction mixture (**Table 2**). Copolymer **H3**, with a NIPAAm content of 82% (mol) and HEAAm content of 18% (mol), has a LCST of 38.3 °C, a value close to the temperature of the human body.

Table 2. Dependence of LCST on the co-monomer ratio in the initial mixture and in the copolymer (concentration of copolymer solution was 1%, w/v).

Sample Code	Comonomer Composition				LCST (°C) in PB, pH=7.4	Yield (%)
	In the Feed × 10^{-3}M (% moles)		In copolymer (% moles)			
	NIPAAm	HEAAm	NIPAAm	HEAAm		
H_1	1 (50)	1 (50)	50.77	49.23	71.1	87.4
H_2	2 (66.66)	1 (33.33)	65.79	34.21	49.1	89.3
H_3	5 (83.33)	1 (16.67)	81.97	18.03	38.3	92.8

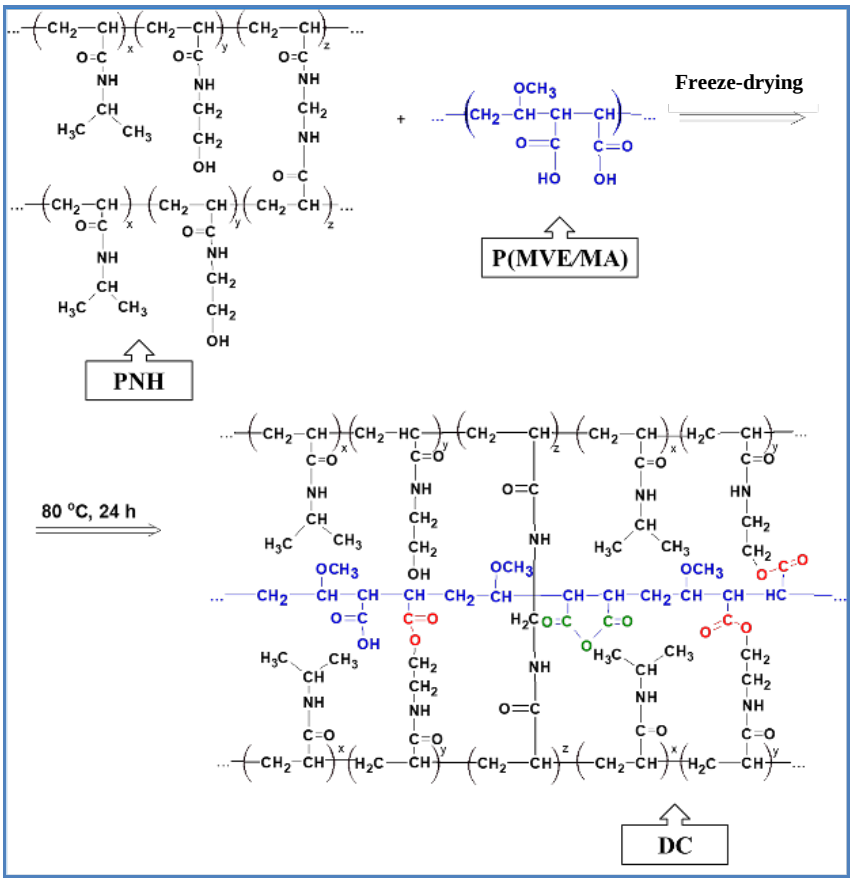
Conventional temperature-sensitive hydrogels (**PNH**) were prepared by radical polymerization of NIPAAm and HEAAm monomers in water, using a molar ratio of 5:1 between the co-monomers and in the presence of a cross-linking agent, N,N-methylenebisacrylamide (BisAAm) (**Scheme 3**).

Double-cross-linked hydrogels (DC) were obtained from the conventional samples by immersing them in a solution of poly(methyl vinyl ether-co-maleic acid) P(MVE/MA) at different concentrations (2 and 5%, w/v) (**Table 3**). The swollen hydrogel samples were quickly frozen with liquid nitrogen and lyophilized for 2 days. The resulting semi-interpenetrating network samples were obtained in a cylindrical shape and consist of a first cross-linked network based on P(NIPAAm-co-HEAAm) and a second non-cross-linked network made of P(MVE/MA) (**Scheme 3**). To cross-link the two polymeric networks, the samples were placed in a vacuum oven at 80 °C for 24 hours. At this temperature, due to condensation reactions, both esterification occurred between the hydroxyl groups of the PNH and the carboxylic groups of P(MVE/MA), as well as the anhydride formation involving the carboxylic groups of P(MVE/MA).

Sample Code	Conventional hydrogels used to obtain DC hydrogels	P(MVE/MA) (%, g/v) in	
		feed solution	final hydrogel
$DC_{0.4}$–P2	$PNH_{0.4}$	2	25.88 ± 2.4
$DC_{0.4}$ –P5	$PNH_{0.4}$	5	44.57 ± 1.8
$DC_{0.6}$–P2	$PNH_{0.6}$	2	25.55 ± 2.2
$DC_{0.6}$–P5	$PNH_{0.6}$	5	40.67 ± 1.9
$DC_{0.8}$–P2	$PNH_{0.8}$	2	22.90 ± 2.1
$DC_{0.8}$–P5	$PNH_{0.8}$	5	34.58 ± 1.5

Table 3. Composition of the initial reaction mixture and of double cross-linked hydrogels.

The addition of P(MVE/MA) to conventional hydrogels significantly altered the pore morphology (**Figure 10**). Since the **DC** hydrogels are obtained after the formation of **PNH**, it is expected that the pore size remains un-changed, but they are differently filled depending on the concentration of P(MVE/MA) used.



Scheme 3. Schematic illustration of the chemical reactions involved in the synthesis of double-cross-linked hydrogels (**DC**).

Furthermore, a fairly homogeneous interpenetration of P(MVE/MA) into the networks of conventional hydrogels was observed, resulting from both good diffusion of P(MVE/MA) into **PNH** and intermolecular interactions such as hydrogen bonding between P(MVE/MA) and **PNH**, stabilizing the hydrogel network.

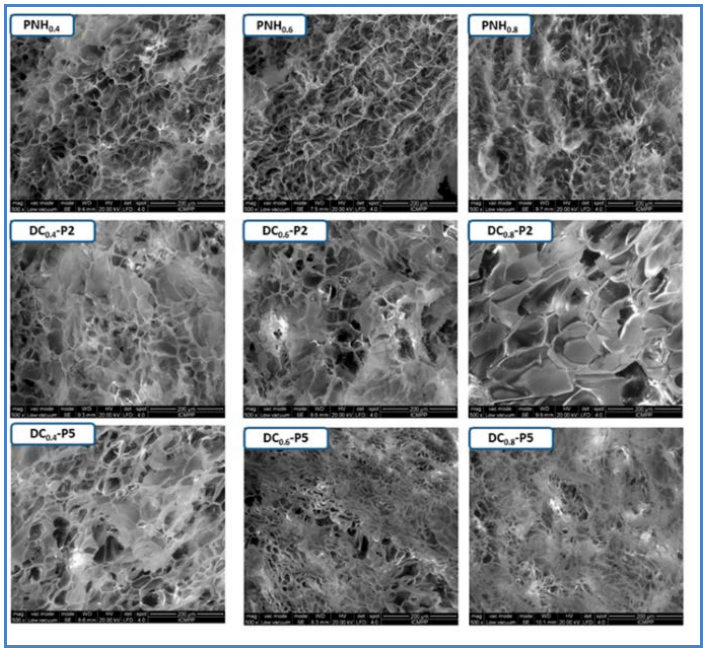


Figure 10. SEM micrographs of conventional (PNH) and double-cross-linked (DC-P) hydrogels. Scale bar corresponds to 200 μm.

The double cross-linked hydrogels (**$DC_{0.6}$-P2** and **$DC_{0.6}$-P5**) obtained from conventional hydrogels PNH cross-linked with 0.6% BisAAm (**$PNH_{0.6}$**) were chosen for further studies because they contain approximately the same amount of cross-linked P(MVE/MA) as $PNH_{0.4}$, but more than $PNH_{0.8}$ (**Table 3**).

The presence of P(MVE/MA) in the double-cross-linked hydrogels significantly modifies the value of the volume phase transition temperature (VPTT) and influences the degrees of swelling and the shape of the swelling curves. In the presence of the drug (**Metoclopramide, MET**) that interacts electrostatically with the new carboxyl groups introduced in the network of the double-cross-linked hydrogels, there is a decrease in the VPTT value in PB to a value close to physiological temperature (**Figure 11**) and a steeper slope of the swelling curves. This behavior is due to the hydrophobic character imparted to the hydrogels by the electrostatic interaction of the drug (hydrophobic) with the carboxyl groups of maleic acid in P(MVE/MA). In an acidic environment, the carboxyl groups are protonated, and the hydrogel is practically collapsed. Therefore, the influence of MET is almost negligible regardless of the concentration used (1 or 5 mg/ml), and the VPTT values of the double-cross-linked hydrogels decrease to values comparable to those of the conventional hydrogels.

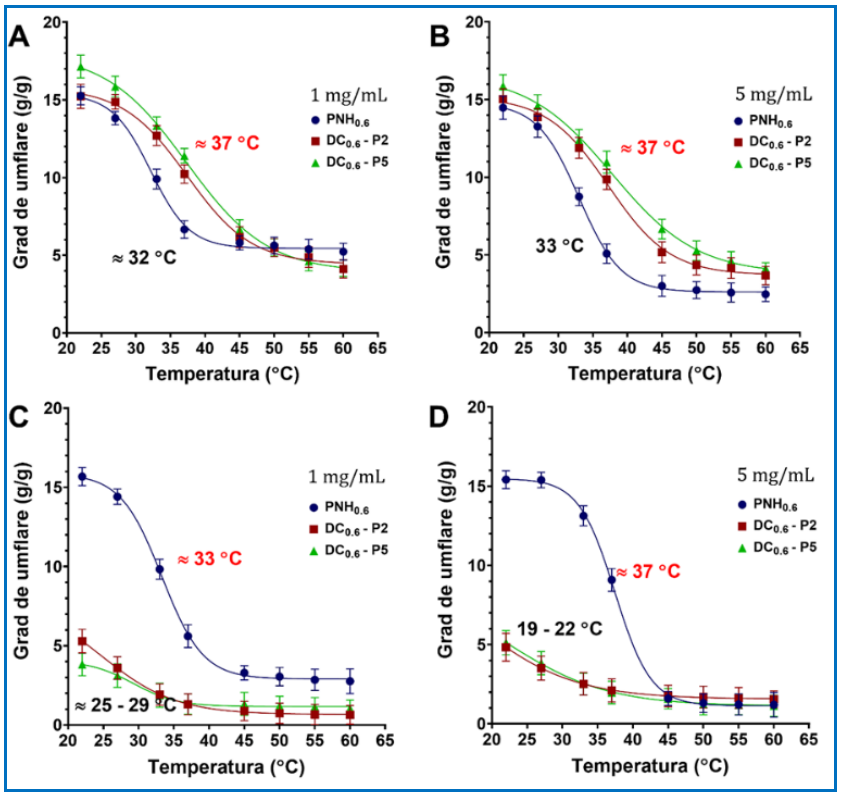


Figure 11. Swelling ratios of conventional ($PNH_{0.6}$) and double cross-linked ($DC_{0.6}$-P2 and $DC_{0.6}$-P5) hydrogels as function of temperature in the presence of drug (MET) at different concentrations in simulated physiological conditions: PB at pH=7.4 (**A,B**) and ABS at pH=1.2 (**C,D**).

To determine the *mechanical resistance* of DC hydrogels to compression, they were swollen at equilibrium in buffer solutions with pH = 7.4 and pH = 1.2, simulating physiological conditions. The values of the elastic modulus increase from 2.78 kPa for the conventional hydrogel sample (PNH0.6) to 6.54 kPa for the double-cross-linked hydrogel sample (DC0.6-P5) (**Figure 12**). The values of the elastic modulus of the DC hydrogels are still low compared to other double-cross-linked hydrogels, making them highly elastic.

However, the highest deformation resistance value was observed for the DC0.6-P5 sample at 21.32 kPa in ABS, significantly higher compared to PNH0.6, which has a value of 4.5 kPa in the same medium. These characteristics recommend these hydrogels as matrices for tissue engineering or other biomedical applications where the supports used need to be soft and flexible yet sufficiently resistant to external forces.

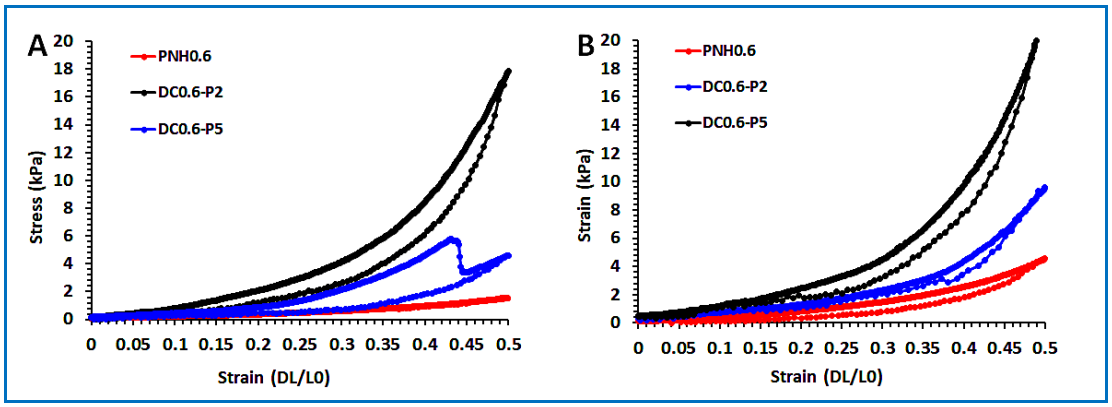


Figure 12. Stress-strain curves for conventional and double cross-linked hydrogels determined in simulated physiological conditions: PB at pH=7.4 (**A**) and ABS at pH=1.2 (**B**).

The release kinetics of metoclopramide from the hydrogels were studied in a buffer solution at pH=7.4 and pH=1.2 at 37°C (**Figure 13**). In phosphate buffer (PB), MET is released more rapidly from the double-cross-linked hydrogel (DC) than from the conventional hydrogel (PNH) due to the higher degree of swelling (ionization of carboxylic groups) (**Figure 13A**). Conversely, at pH=1.2, the double-cross-linked hydrogels are fully protonated, the polymeric network is collapsed, and consequently, the release rate is slower (**Figure 13B**). It's worth noting that the drug is more soluble at pH=1.2 (in the hydrochloride form) than at pH=7.4, and thus, the polymeric matrix significantly contributes to the delayed drug release in gastric fluids.

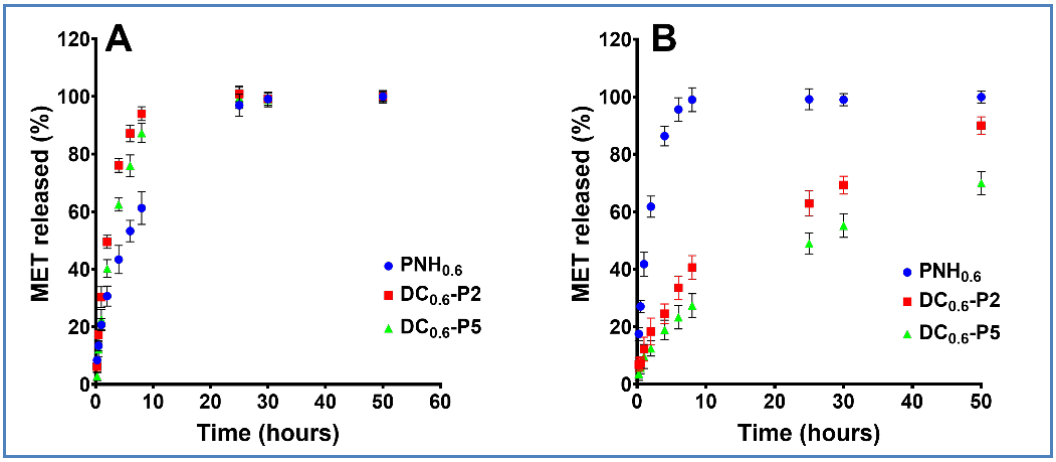


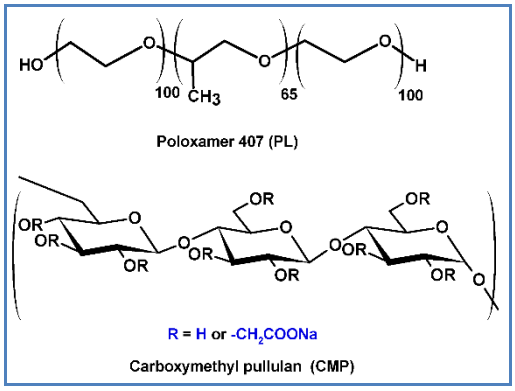
Figure 13. Release kinetics of metoclopramide from $DC_{0.6}$ hydrogels in PB at pH = 7.4 (**A**) and ABS at pH = 1.2 (**B**) buffer solutions.

Chapter V. “IN SITU” GELLING POLYMER SYSTEMS

Thermogels are materials that are in liquid state at room temperature and undergo a sol–gel transition upon heating at a temperature close to that of the human body (36°C) [18]. Due to this property, thermogels can be used as injectable matrices for the transport and release of drugs [19,20], as well as in tissue engineering applications due to their similarity to the extracellular matrix (ECM). One approach to obtaining polymer systems that gel "in situ" is using physical mixtures. These systems can be obtained from Poloxamer 407 and pullulan, with these components already being studied and evaluated as potential pharmaceutical devices for intradermal [21] or dermal [22] use. Another approach involves grafting synthetic polymers onto natural polysaccharides, as the resulting copolymers combine the advantages of both types of polymers, especially the biocompatibility required for applications that involve interaction with biological tissues [23]. Compared to physical mixtures, grafted copolymers based on Poloxamer 407 can form reversible gels with high elasticity in aqueous media at low copolymer concentrations.

V.2. THERMOGELS BASED ON PHYSICAL MIXTURES OF POLOXAMER AND CARBOXYMETHYL PULLULAN

In this chapter, the study of polymeric systems based on a physical mixture of Poloxamer 407 (Plx) at a concentration of 17% (w/v) and carboxymethyl pullulan (CMP) with three different degrees of substitution ($CMP_{0.42}$, $CMP_{0.95}$, and $CMP_{1.6}$) is presented as potential injectable matrices for pharmaceutical applications or tissue engineering [22].



Chemical structure of the two polymers used for the synthesis of thermogels is presented in **Figure 14.**

Figure 14. Chemical structure of Poloxamer 407 (Plx) and carboxymethyl pullulan (CMP).

The physical mixtures were obtained by adding CMP to the stock solution of Plx (17%, w/w) (Plx17) under gentle agitation, alternately at 4°C and room temperature (~23°C). Aqueous mixtures of Plx17/CMPx were prepared, where CMP had three different

degrees of substitution (x = 0.42; 0.95; and 1.6), and the concentration of CMP in the mixture varied from 0 to 4% (w/v).

The first stage of the study involved determining the miscibility of the two polymers in aqueous solution using viscometry. All miscibility studies of Plx with CMP were conducted in aqueous solutions at 37°C, considering *that the aim of the study is to obtain a system that gels around human body temperature*. The physical mixtures of Plx/CMP exhibited specific behavior of polyelectrolytes. The values of the parameters ε (deviation from the ideal experimental values of intrinsic viscosity [η]) and B (viscometric interaction between polymer segments) highlighted that the interaction between Plx and CMP chains results in an increase in the hydrodynamic volume of isolated coiled unimers or the existence of mixed unimers of CMP and Plx with increased sizes. This deviation is more pronounced for $CMP_{0.42}$ in the presence of large fractions of Plx. On the other hand, for large fractions of $CMP_{1.6}$, deviations from the ideal hydrodynamic volume are very small, and some interactions occur between the two polymers.

The gelling capacity of the 17% Plx solution in the presence of $CMP_{0.95}$ and $CMP_{1.6}$ was investigated using two different methods. In the first stage, the sol-gel transition temperature was determined using the tube inversion method [24]. To determine the sol-gel transition temperature (Tsol-gel), the Plx17/CMPx mixture solution was heated at a rate of 0.2°C/min from 20 to 40°C, and equilibrated at each temperature for 5 minutes. The sol–gel transition temperature was recorded as the temperature at which the liquid was immobile (no movement of the meniscus over a period of 30 s) (**Figure 15**).

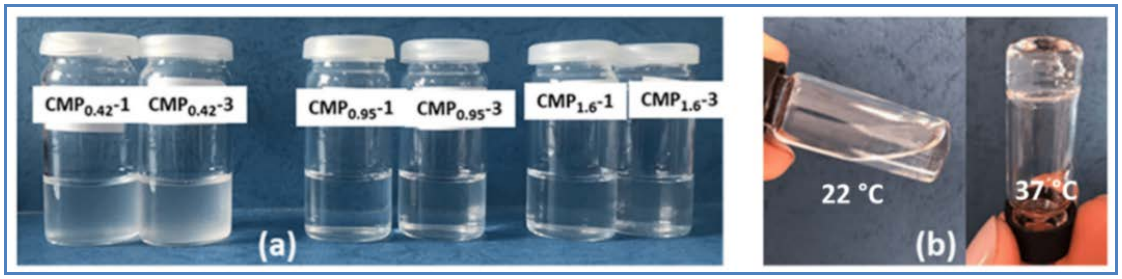


Figure 15. (a) Concentrated poloxamer solution (17%) with 1% and 3% added $CMP_{0.42}$, $CMP_{0.95}$, and $CMP_{1.6}$ (from left to right) at room temperature. (**b**) Tube inversion method for 17% poloxamer with 1% $CMP_{0.95}$ below (left) and above (right) the gelation temperature.

A temperature increase in gelation was observed, reaching a maximum at 29.5 °C in the presence of low polyelectrolyte concentrations (below 0.5%) (**Figure 16**). As expected, further increase in CMP concentration resulted in a decrease in gelation temperature [25]. Moreover, at 5% CMP in the Plx/CMP mixture, a very soft gel was formed that exhibited flow properties, and the gelation temperature could not be measured

using the tube inversion method. This behavior could be explained by the modest viscosity of CMP due to its relatively low molecular weight and high flexibility of its chains compared to other polysaccharides.

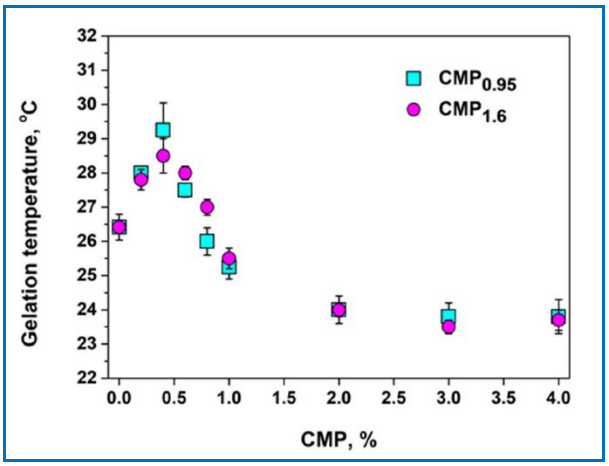


Figure 16. Variation of the gelation temperature (determined by test inversion method) of 17% Plx solution with the addition of CMP in different concentrations.

The addition of CMP in low concentrations (below 0.5%) results in a decrease in the hardness of Plx17/CMPx gels (**Figure 17**).

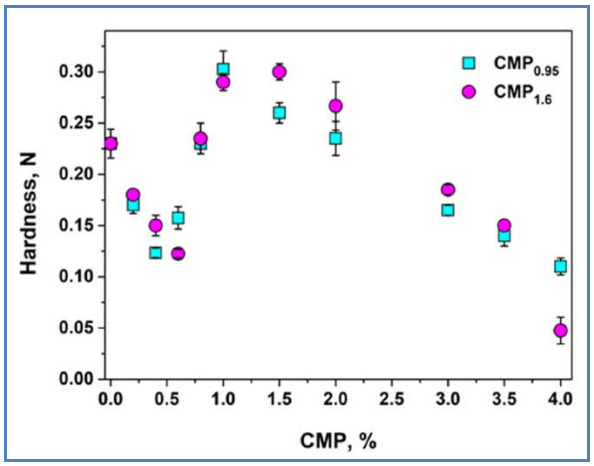


Figure 17. Variation of the gel hardness (texture analysis method) of 17% Plx solution with the addition of CMP in different concentrations.

The increase in CMP concentration in the mixture from 0.5 to 1.5% is accompanied by an increase in the strength of the gels, with a maximum observed at concentrations of 1% $CMP_{0.95}$ and 1.5% $CMP_{1.6}$.

The gels obtained from Plx17% with $CMP_{0.95}$ and $CMP_{1.6}$ behave similarly due to their relatively high charge densities, likely resulting in similar configurations in solution. Consequently, the gelation behavior through rheological tests was studied only for the **Plx17/$CMP_{0.95}$** gels.

Figure 18 illustrates the variation of rheological parameters (G′, G″, and tan δ) with increasing temperature and the determination of the sol-gel transition temperature. The initial solution state of the mixtures is highlighted by the small values of the viscoelastic moduli, $G' < G''$ and $\tan \delta > 1$. Below the temperature T_0, marking the onset of gelation, Plx micelles and unimers coexist in the solution with CMP chains, and the temperature increase has a small influence on the rheological parameters. Above T_0, when

the temperature increases by less than 10 °C, the viscoelastic moduli show a sudden increase, with G′ increasing more rapidly compared to G″. This is due to structural changes induced by temperature, transitioning from micelles to polymicelles and network structure. At the transition point from solution to the gel state, denoted as $T_{sol–gel}$ (which is close to T_0), G′ = G″, and tan δ = 1. Above this temperature, G′ > G″, and around the gelation temperature T_{gel}, the network is formed and equilibrium is achieved. The samples behave differently as the concentration of $CMP_{0.95}$ in the 17% Plx solution increases (**Figure 18(b)**). In this case, the values of the temperatures T_0 and $T_{sol-gel}$ shift to lower values. The addition of a small amount of CMP leads to a decrease in transition temperatures and disrupts the gelation kinetics and the Plx network structure.

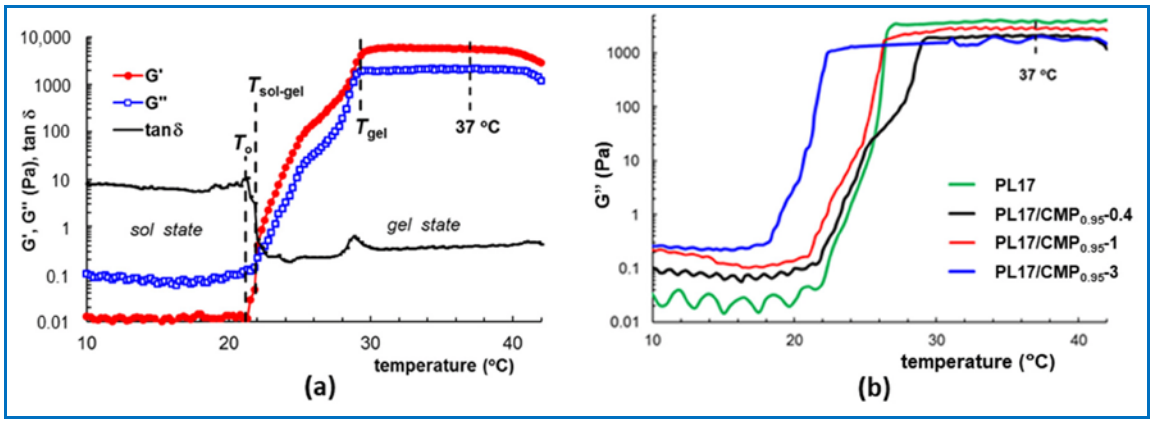


Figure 18. Sol-gel transition illustrated for sample $Plx17/CMP_{0.95}$-0.4 through the dependence of the viscoelastic parameters on temperature (**a**); variation of loss modulus during temperature induced gelation for $Plx17/CMP_{0.95}$ samples (**b**) (heating rate of 0.5 °C/min, ω = 1 rad/s, γ = 1%).

The experimental data shows that as the concentration of CMP increases, $T_{sol-gel}$ decreases, but T_{gel} (which is in good agreement with Tgel measured by the tube inversion method) exhibits a peculiar behavior: it first increases (sample $Plx17/CMP_{0.95}$-0.4) and then decreases.

The addition of CMP has two different effects on the gelation of physical mixtures: (i) it increases the concentration of Plx in the polyelectrolyte-free domains ($T_{sol-gel}$ decreases with increasing CMP concentration), (ii) it disturbs the aggregation of Plx micelles and the gel formation (hence the specific rheological parameters of the gels formed from Plx and CMP are lower compared to the parameters of gels obtained only from Plx). At low concentrations, the polyelectrolyte has an extended conformation of its chains and divides the Plx network into many domains, thus reducing the gel's strength (**Figure 19(b)**). As the CMP concentration increases, the conformation of the polysaccharide becomes more coiled, forcing the aggregation of Plx micelles into

macrodomains. Consequently, the interactions between micelles are much stronger, and the strength of the Plx network increases when up to 1% CMP is added to the system. As the CMP concentration added to the mixture exceeds 1%, the gel's flexibility increases due to the progressive isolation of Plx micelles, and the rheological parameters decrease.

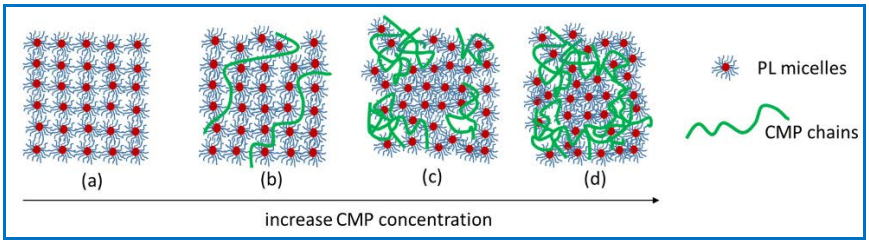
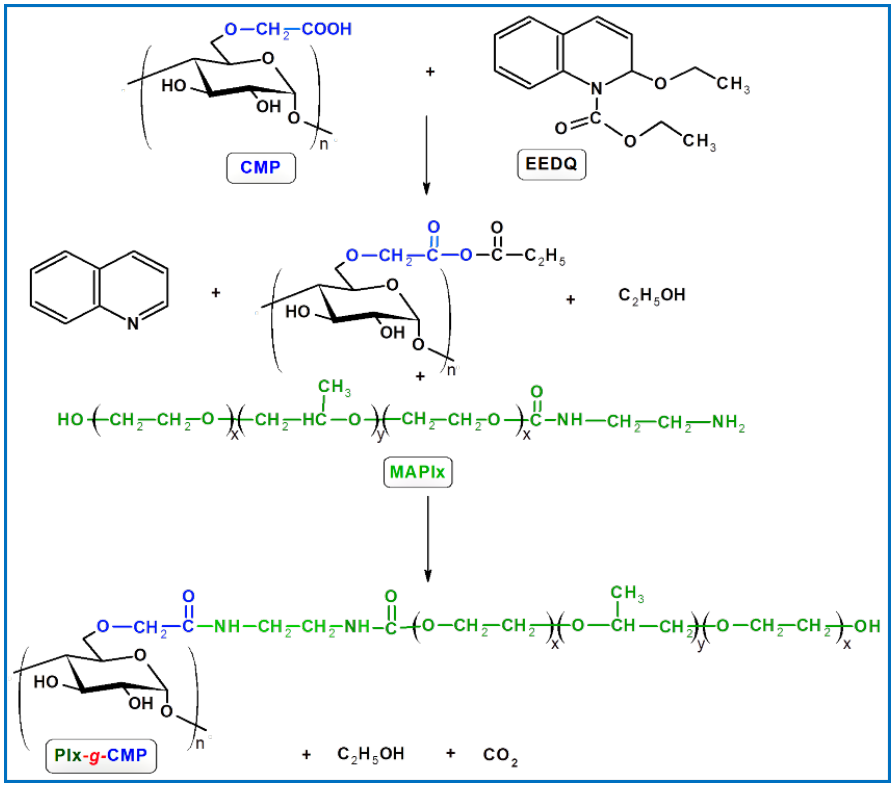


Figure 19. Schematic representation of the influence of CMP concentration on the Plx17/CMP gelation behavior: Plx17 (**a**), Plx17/$CMP_{0.95}$-0.4 (**b**), Plx17/$CMP_{0.95}$-1 (**c**) and Plx17/$CMP_{0.95}$-3 (**d**).

V.3. THERMOGELS BASED ON COPOLYMER POLOXAMER-g-CARBOXYMETHYL PULLULAN

The grafted copolymer poloxamer-g-carboxymethyl pullulan (**Plx-g-CMP**) was prepared through a coupling reaction between the amine groups of a monoamine derivative of Poloxamer 407 and the carboxylic groups present on the carboxymethyl pullulan chains (**Scheme 4**). First, the two derivatives, carboxymethyl pullulan (CMP) and monoamine-modified Poloxamer (MAPlx), were synthesized using methods described in the literature.



Scheme 4. Synthesis route of Plx-g-CMP grafted copolymer.

The temperature at which the polymer undergoes a sol-gel transition was first determined using the tube inversion method, and experiments were performed for both poloxamer and grafted copolymer solutions (**Figure 20**). The gelation temperature decreases with increasing copolymer concentration: from 33°C at a concentration of 11% (w/v) to 20°C at a concentration of 18% (w/v). Furthermore, the sol-gel transition of the grafted copolymer occurs up to a concentration of 11% (w/v), while gelation of poloxamer does not occur at lower concentrations. These observations confirm that Plx-g-CMP forms additional cross-linking points (i.e., hydrogen bonds). Moreover, under simulated physiological conditions at pH = 7.4, the polymer chains are in an extended state, and the environment in which the poly(propylene oxide) (PPO) segments are located, becomes more polar, forcing the PPO segments to aggregate at lower temperatures.

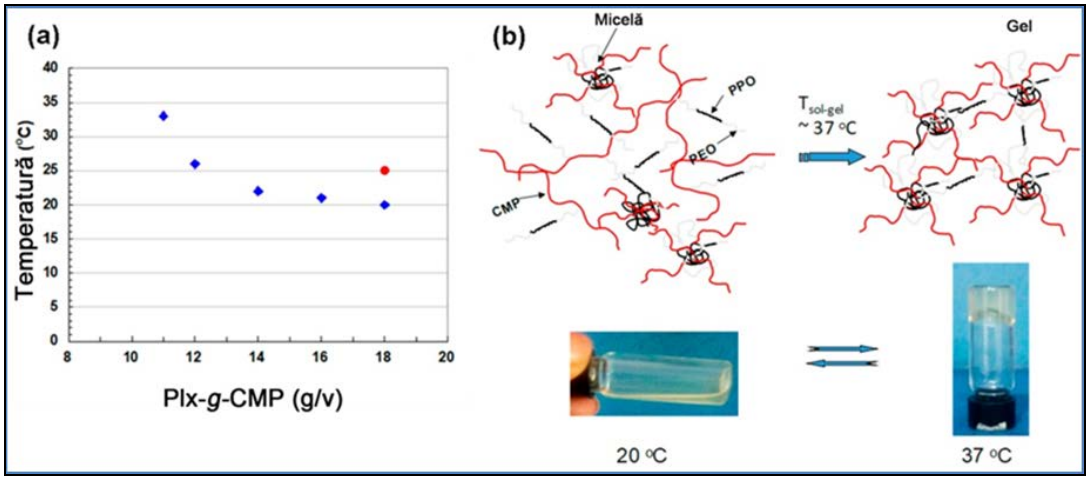


Figure 20. Influence of the copolymer concentration on the gelation temperature of Plx (●) and Plx-g-CMP (◆) in phosphate buffer 0.05 M at pH = 7.4. Schematic representation of sol-gel transition of the Plx-g-CMP solution (**b**).

Rheological studies highlighted that, depending on the temperature at which the copolymer solution was maintained prior to the test, the gelation process of the 13% concentration copolymer sample started either after 990s or after 280s, when a rapid increase in the values of G' and G" was observed (**Figure 21**). Furthermore, it was observed that the change in viscoelastic parameters occurs much more slowly over time as a stable three-dimensional structure forms (tan $\delta < 1$), and this is not influenced by the resting temperature of the samples before the tests or the concentration of the Plx-g-CMP copolymer. The gelation process occurs in two stages (e.g., after 990s and 2500s or 280s and 2000s), probably due to the competition between different types of interactions (hydrogen bonds, hydrophobic interactions) that develop over time.

For higher concentrations (22 and 30%, w/v), the gelation time is on the order of tens of seconds for samples kept at 4 °C, while for samples maintained at 20 °C, gelation occurs almost instantly.

When the sample is subjected to a high oscillatory strain (γ = 100%), G' and G" decrease, indicating the destruction of the network structure (tan δ > 1) and an increase in sample fluidity (**Figure 22**). By changing the strain from a high value of 100% to a low value of 1%, the network structure is recovered within a few seconds. It is also observed that the resting structure is not fully recovered after the first deformation cycle (the value of G' after the first cycle is about 65% of its resting value). The test was repeated five times (maintaining the strain for 200s in each cycle), and it was observed that the recovery process is reversible for the following four deformation cycles; the sample is capable of regaining its 3D network structure after the successive action of external forces (tan δ is approximately 0.14 at rest and increases slowly between 0.2 and 0.4 after applying up to five deformation cycles).

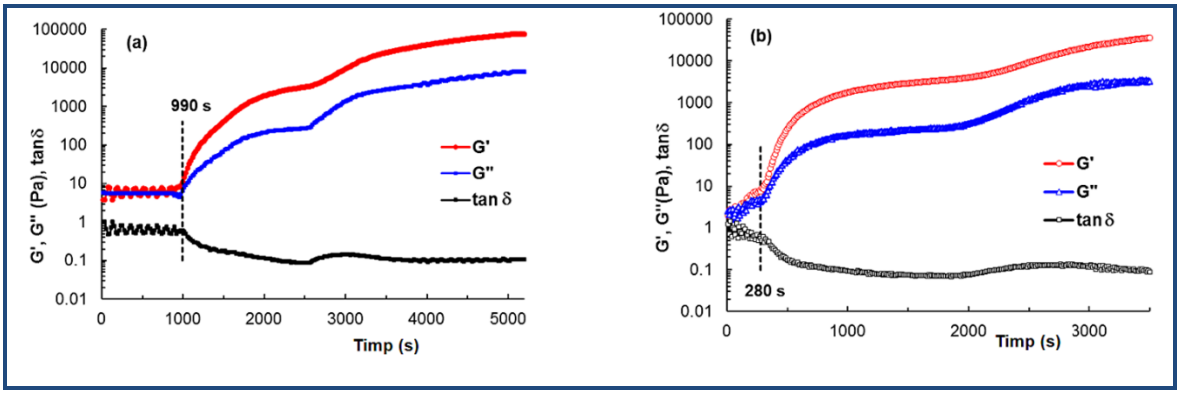
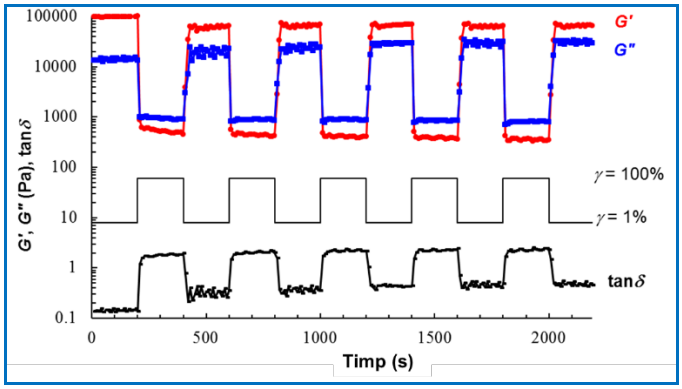


Figure 21. The evolution of the viscoelastic parameters in time for 13% (w/v) Plx-g-CMP after 24 hours of rest at 4 °C (**a**) and 20 °C (**b**) (ω = 1 rad/ s, γ = 1%) raising the sample temperature at 37 °C inside of the rheometer.

Figure 22. The self-healing test for the hydrogel containing 13% (g/v) Plx-g-CMP by following the evolution of the viscoelastic parameters in time, when two successive levels of deformations were applied each for 200 s: 1% and 100% (37 °C and 1 rad/s).



The gel formed by the Plx-g-CMP grafted copolymer at two concentrations and containing amoxicillin (AM) was tested as a potential support matrix in tissue engineering applications.

Release studies of AM from the hydrogel obtained using two copolymer concentrations (13 and 22%, w/v) were conducted under simulated physiological conditions (phosphate buffer (PB) at pH = 7.4) (**Figure 23**). The results of the study show a gradual release of AM from both hydrogels over a period of approximately 168 hours. In the first 6 hours, 41% of the drug is released from the Plx-g-CMP hydrogel obtained at a concentration of 13% (w/v), which is twice the amount released from the hydrogel obtained at a concentration of 22% (w/v). For the next 48 hours, the release curves follow almost the same profile, but the difference is smaller. From this point until the end of the experiment, the release curves follow an approximately parallel trajectory, suggesting that drug release is mainly controlled by its diffusion from the hydrogel matrix. However, the diffusion of AM from the hydrogel into the release fluid should be controlled both by steric interactions with the polymeric network and by possible electrostatic interactions between the opposite charges of the drug and CMP. As long as release studies are conducted in a phosphate buffer with a specific ionic strength (50 mM), electrostatic interactions between AM and the carboxyl groups of CMP are hindered by the presence of competing ions. As a result, the diffusion rate of the drug will be controlled solely by steric interactions between the drug and the polymeric network. Obviously, the denser the network, the higher the probability of intermolecular interactions, and the slower the release rate of amoxicillin.

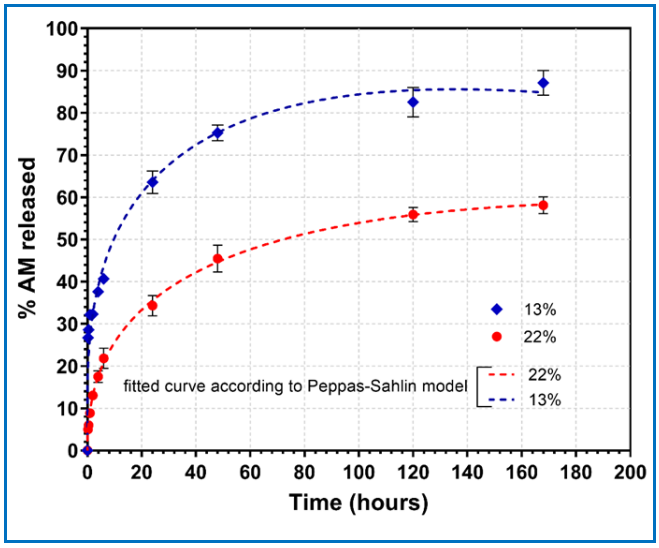


Figure 23. *In vitro* release profiles of amoxicillin from Plx-g-CMP hydrogels obtained at two concentrations (13% and 22%, w/v).

CONCLUSIONS

The studies conducted on the preparation and characterization of **temperature- and pH-sensitive microspheres based on P(NIPAAm-co-VI) copolymer** led to the following conclusions:

- A new temperature- and pH-sensitive copolymer was synthesized through a radical polymerization reaction of the co-monomers N-isopropylacrylamide and 1-Vinylimidazole.
- The lower critical solution temperature (LCST) of the copolymer was determined by UV-Vis spectroscopy, and the LCST values for P(NIPAAm-co-VI) increase with the increasing amount of VI in the copolymer. Additionally, for the three samples (VI_1-VI_3), the LCST increases as the pH decreases from 7.4 to 5.0.
- Sample VI_2 was selected for further studies because it is insoluble at pH = 7.4 but soluble at pH = 5.5 and pH = 5.0 at human body temperature, making it an ideal candidate for obtaining controlled drug delivery systems at the tumor level.
- Microparticles loaded with doxorubicin (MS-DXR) were obtained from the linear copolymer P(NIPAAm-co-VI) using the solvent evaporation method.
- MS-DXR have the ability to disintegrate into nanoparticles (NS-DXR) under simulated physiological conditions (pH = 7.4 at 37 °C).
- *In vitro* and *in vivo* studies conducted on NS-DXR show that these polymeric systems are stable once introduced into the bloodstream but solubilize at pH = 6.0 - 5.0 and release the drug (DXR) in a controlled manner into the nuclei of HepG2 and A549 cancer cells.

The following results were obtained from the studies conducted on **pH- and temperature-sensitive microspheres obtained from P(NIPAAm-co-4-VP) copolymer**:

- A novel copolymer with dual sensitivity to temperature and pH was synthesized through a radical polymerization reaction of the co-monomers N-isopropylacrylamide and 4-vinylpyridine.
- The chemical structure of the P(NIPAAm-co-4-VP) copolymer was confirmed, and the compositional ratio was determined by ^{1}H-NMR spectroscopy.
- The lower critical solution temperature (LCST) of the P(NIPAAm-co-4-VP) copolymer was determined using UV-Vis spectroscopy. Under normal physiological conditions (phosphate buffer at pH = 7.4), all three copolymer samples (VP_1-VP_3) exhibited LCST values below human body temperature, and therefore, they are insoluble. Furthermore, in a pH = 7.4 buffer solution, the LCST value decreases with an increase in the amount of 4-VP in the copolymer.
- The VP_2 sample was selected for further study as it is insoluble at pH = 7.4 but soluble at pH = 5.0 at human body temperature, making it suitable for developing drug delivery systems in the tumor microenvironment.

- Microparticles of P(NIPAAm-co-4-VP) loaded with dexamethasone (MS-DEX) were obtained using the solvent evaporation method.
- The degree of dispersion of dexamethasone in the polymeric matrix of the microspheres and the possible interactions between the drug and the polymer were evaluated through differential scanning calorimetry (DSC) analyses. The results highlighted a molecular dispersion of the drug within the polymeric network. Additionally, it was observed that the drug also acted as a plasticizer.
- *In vitro* tests conducted on MS-DEX demonstrated the ability of these polymeric matrices to be used as controlled drug release systems under the acidic conditions of the tumor microenvironment.

The studies conducted on **conventional temperature-sensitive hydrogels obtained from P(NIPAAm-co-HEAAm)** concluded with the following results:

- A linear copolymer based on NIPAAm and HEAAm was obtained using different molar ratios between the two co-monomers in the initial reaction mixture.
- Conventional hydrogels (PNH) were obtained by radical polymerization of NIPAAm and HEAAm monomers in water at a molar ratio of 5:1 and in the presence of the cross-linking agent N,N-methylenebisacrylamide (BisAAm).
- The results of *in vitro* release studies of metoclopramide from conventional hydrogels indicate a rapid release of the drug in PB and ABS buffer solutions.

The studies conducted on **pH/temperature-sensitive double-cross-linked hydrogels** resulted in the following findings:

- Double-cross-linked hydrogels (DC) were obtained by immersing conventional hydrogels in solutions of P(methyl vinyl ether-alt-maleic acid) (P(MVE/MA)) at different concentrations (2 and 5%, w/v). The resulting semi-interpenetrated networks underwent additional cross-linking through a 24-hour heat treatment at 80°C. This method offers the advantage of not requiring the use of solvents or toxic chemical cross-linkers.
- In a pH = 7.4 phosphate buffer solution, it was observed that the volume phase transition temperature (VPTT) in the presence of the drug (MET) approached physiological temperature. This behavior can be attributed to the electrostatic interaction of the hydrophobic drug with the carboxyl groups of maleic acid in P(MVE/MA).
- In a pH = 7.4 buffer solution, MET was released faster from the double-cross-linked hydrogels due to their high degrees of swelling (ionization of carboxyl

groups). Conversely, at pH = 1.2, the DC hydrogels were fully protonated, causing the polymeric network to collapse, resulting in a slower drug release.

From the studies on obtaining and characterizing **thermogels based on physical mixtures of poloxamer and carboxymethyl pullulan**, the following conclusions have emerged:

- The results of miscibility studies through viscometry showed that the carboxymethyl pullulan chains adopt an extended conformation in the presence of Poloxamer, and the interactions between the segments of $CMP_{0.95}$ or $CMP_{1.6}$ chains with Plx are stronger compared to the system formed by $CMP_{0.42}$ and Plx.
- The systems Plx17/$CMP_{0.95}$ and Plx17/$CMP_{1.6}$ are miscible with Poloxamer at a high concentration, while the mixture formed by $CMP_{0.45}$ and Poloxamer exhibits phase separation.
- The addition of CMP has two opposing effects on the gelation of Plx-based mixtures: it increases the concentration of Plx in the regions without polyelectrolytes, reducing $T_{sol\text{-}gel}$, but it also disrupts the aggregation of Plx micelles into an organized network structure, resulting in softer gels.
- Adding 1% CMP to a 17% Plx solution allowed the formation of thermogels with a gelation temperature (T_{gel}) at approximately 26 °C, displaying a moderate hardness.

Following the studies conducted on **thermogels based on poloxamer-g-carboxymethyl pullulan copolymer**, the following conclusions were derived:

- The copolymer was obtained through a coupling reaction between the amine groups of monofunctionalized poloxamer and the carboxyl groups present in carboxymethyl pullulan.
- The degree of substitution (G.S.) of carboxymethyl pullulan was determined using conductometric titration, resulting in a G.S. of 0.87, corresponding to a carboxylic group content of 3.78 mmol/g.
- The gelation capacity of the Plx-g-CMP copolymer was investigated through rheological studies. The copolymer can form a gel under simulated physiological conditions (phosphate buffer at pH = 7.4 and 37 °C). The sol-gel transition temperature of the copolymer is lower (20 °C) compared to native Poloxamer (25 °C) at the same concentration (18%, g/v). Additionally, the sol-gel transition of the grafted copolymer occurs at a concentration as low as 11% (g/v), while the gelation of Poloxamer 407 does not occur at concentrations lower than 18% (w/v).

- Drug release studies using amoxicillin as a model drug from gels obtained using two concentrations of the grafted copolymer (13 and 22%, g/v) in simulated physiological conditions (PB at pH = 7.4) demonstrated controlled release of the drug over a period of approximately 168 hours.
- Based on the physicochemical and rheological properties, it can be concluded that Plx-g-CMP can be effectively used both as a matrix for sustained release of amoxicillin and for cartilage regeneration.

DISSEMINATION OF RESULTS AND OTHER SCIENTIFIC AND PROFESSIONAL TRAINING ACTIVITIES

The results obtained in the doctoral thesis are the subject of five scientific articles and seven oral communications.

Articles published in extenso in specialized journals with international circulation (ISI rated journals):

1. Constantin, M.; Bucatariu, S.; Popescu, I.; **Coşman, B.**; Ascenzi, P.; Fundueanu, G., **2021**. *Intelligent Micro-vehicles For Drug Transport And Controlled Release To Cancer Cells*. Reactive and Functional Polymers 165, pp.104961.
2. Constantin, M.; **Coşman, B.**; Bercea, M.; Ailiesei, G.-L.; Fundueanu, G., **2021**. *Thermosensitive Poloxamer-graft-Carboxymethyl Pullulan: A Potential Injectable Hydrogel for Drug Delivery*. Polymers 13(18), 3025.
3. Fundueanu, G.; Constantin, M.; Turtoi, M.; Bucatariu, S.-M.; **Coşman, B.**; Anghelache, M.; Voicu, G.; Calin, M., **2022**. *Bio-Responsive Carriers for Controlled Delivery of Doxorubicin to Cancer Cells*. Pharmaceutics 14(4), 865.
4. **Coşman, B.-P.**; Bucătariu, S.-M.; Constantin, M.; Fundueanu, G., **2022**. *Temperature/pH-Sensitive Double Cross-Linked Hydrogels as Platform for Controlled Delivery of Metoclopramide*. Gels 8(12), 824.
5. Popescu, I.; Constantin, M.; Bercea, M.; **Coşman, B.-P.**; Suflet, D.M., Fundueanu, G., **2023**. *Poloxamer/Carboxymethyl Pullulan Aqueous Systems—Miscibility and Thermogelation Studies Using Viscometry, Rheology and Dynamic Light Scattering*. Polymers 15(8), 1909.

Communications at scientific events

1. *pH/temperature-sensitive microspheres for drug delivery to the tumors*; Fundueanu G., Constantin M., **Cosman B.**, Bucatariu S.; *International Congress of the „Apollonia" University* FROM Iasi „By promoting excellence, we prepare the future" XXXIth Edition, Iasi, Romania, 01-03.03. 2021
2. *pH/temperature-sensitive interpenetrating polymeric hydrogel*; **Coșman B.**; Bucatariu S.; Constantin M.; Fundueanu G.; *ICMPP - Open Door to the Future Scientific Communications of Young Researchers* MacroYouth 2022, Iași, România,19.11. 2021
3. *pH/thermosensitive copolymer with gelling properties for controlled delivery of drugs*; **Cosman B.**; Constantin M.; Bercea M.; Ailiesei G.-L.; Fundueanu G., *The International Congress of Apollonia University of Iasi*, Iasi, Romania, 28.02.2022 – 01.03.2022
4. *Stimuli-sensitive microspheres for drug delivery to the tumors;* **Coșman B.**; Constantin M.; Bucatariu S.; Fundueanu G., *ICMPP - Open Door to the Future Scientific Communications of Young Researchers* MacroYouth 2022, Iași, România, 18.11.2022
5. *Smart microparticulate systems for the transport and controlled delivery of doxorubicin to tumor cells*; Fundueanu G., **Cosman B.**, Bucatariu S., Constantin M.; *The International Congress of Apollonia University of Iași*, Iași, România, 28.02 – 01.03. 2022
6. *Design of double cross-linked smart hydrogel for biomedical applications*; **Coșman B.**; Bucătariu S.; Fundueanu G.; Constantin M.; *International Congress of the "Apollonia" University of Iasi "By promoting excellence, we prepare the future" XXXIIIth Edition*, Iasi, 2-5.03. 2023
7. *Intelligent polymers for biomedical applications*; Fundueanu-Constantin G., Bucatariu S., **Cosman B.**, Constantin M.; *International Congress of the "Apollonia" University of Iasi "By promoting excellence, we prepare the future"* XXXIIIth Edition, Iasi, 2-5.03. 2023

Articles and related communications on the subject of the doctoral thesis, developed based on collaboration:

1. **Cosman, B.**; Rata, D.M.; Suflet, D.M.; Fundueanu, G.; **2021**. *Chitosan-PVA composite hydrogels: synthesis, characterization and their antibacterial properties*; Proceedings of the Progress in Organic and Macromolecular Compounds Conference, online, pag 127-128.

2. Constantin, M.; **Coşman, B.**; Ascenzi, P.; Simionescu, B.C.; Fundueanu, G., **2022**. *New Chromatographic Insights On Drug:Cyclodextrin Inclusion Complexes And Their Potential Use In Drug Delivery*. Expert Opinion on Drug Delivery 19(12), 1696–1709.

3. *Design of chitosan films via solvent free thermal cross linking method*; Bucatariu S., **Cosman B**., Fundueanu G., Constantin M.; *International Congress of the „Apollonia" University FROM Iasi „By promoting excellence, we prepare the future"* XXXIth Edition, Iasi, Romania, 01-03.03. 2021

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Involvement as a team member in research projects

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2. Ionic polymers based on polysaccharides: correlations between structure, physico-chemical properties and interaction with oppositely charged particle, project **PN-III-P4-ID-PCE-2020-0296**, contract PCE94/2020 (2020-2023).

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