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Intelligent Copolymers Based On Poly (N–Isopropilacrylamide). PART II: Grafts polysaccharide to obtain new biomaterials for biomedical and pharmacological applications

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Abstract: Biopolymers such as polysaccharides are compounds that have functional groups and they are very susceptible to be used in chemical modifications and also allows them to synthesizer of new copolymers (used as graft–like chains). Poly (N–Isopropylacrylamide) PNIPAm, is a thermosensitive synthetic polymer widely used in the preparation of intelligent gels for the biomedical field, but have some limitations in use as biodegradable matrix or scaffolds. In this research wered the synthesis and characterization of copolymers their PNIPAm grafted with the polysaccharides: chitosan (CS) or hyaluronic acid (HA), were performed to obtain new biodegradable and biocompatible biomaterials that conserve the intelligent character (thermosensitivity).The PNIPAm was in first chemically modified with 3–butenoic acid in order to generate carboxyl end groups on the graft–polymer chain (PNIPAm–co–COOH) which serve as anchor points and then covalently graft the polysaccharides. For the specific case of grafting with hyaluronic acid, it was necessary to perform a second modification using piperazine (PIP) and obtain the graft–polymers PNIPAm–co–COO–g–PIP. All this modification process was previously reported (Carrero et al, 2018). In this case, the polysaccharides used as grafts–like chains were: (1) chitosan oligomers obtained by acid degradation and (2) hyaluronic acid. The characterization of all copolymers obtained was follow by infrared spectroscopic (FT–IR); the differential scanning calorimetric (DSC) technique was used to determine the lower critical solution transition temperature (LCST), resulting in the range of 29–34 °C. Its morphology was studied using scanning electron microscopy (SEM), but previously was simulate an inject process, for the reversible gel character presented by these novel copolymers; resulting a high porosity and interconnection between pores (scaffold–like micrometric structures). Hemocompatibility assays were performed on agar/blood systems, showing non cytotoxicity. All these results give these graft–copolymers a high potentiality of use as scaffolds in tissue engineering and also for pharmacological applications.

Keywords: Poly (N–Isopropylacrylamide); Chitosan; Hyaluronic acid; Intelligent graft copolymer; LCST temperature; Scaffolds.

Introduction

The science and technology of biomaterials has develop enormously in recent decades. Examples of this development are the intelligent materials that respond reversibly to a change in their environment, such as those exhibited by intelligent hydrogels[1,2]. Materials sensitive to stimuli that are capable of modifying their conformation and properties in response to changes in different physiological variables, receive more attention for the manufacture and design of therapeutic devices for biomedical applications[3,4]. Especially, materials sensitive to temperature and pH are the most studied because these parameters change naturally and can be easily controlled[2], also because pH and temperature are physiological parameters that controlled several organics route in regenerative process.

Thanks to the intelligent thermosensitive character, Poly (N–Isopropylacrylamide) (PNIPAm) has become one of the most studied synthetic polymers in the reported scientific literature[5,6]. Particularly through copolymers synthesis and grafting reactions, because it is possible to obtain a macromolecular chain with one or more monomers attached in the form of blocks, or even side chains or ramifications[7,8]. In this last type of copolymerization, different monomers are covalently bound to the main backbone of the polymer. Depending on the degree of bonding and the incorporated side chain length, the physical/chemistry, morphological and mechanical properties of the grafted copolymer will be defined[9]. This is one of the reason why graft copolymers have attracted increasing attention for applications from materials science to biology[8], medicine[10,11] and pharmacology[12,13]. For example, the design of polysaccharides grafted with a thermosensitive synthetic polymer has been reported[14–17], where the polysaccharide can provide biodegradability character and non–toxicity, while the synthetic polymer provides thermal sensitivity[20].

In the case of PNIPAm, although N–isopropylacrylamide monomer (NIPAm) is cytotoxic, the polymer does not show toxicity[6,18,19]. This led the design of various biomaterials based on this polymer, such as: sensors [21], devices for controlled drug release[22,23], artificial muscles and injectable scaffolds[24,25], between others. However, synthetic biomaterials, in most cases, do not provide biological signals in their molecular chains to facilitate cell–material interaction. Therefore, the modification of synthetic polymers with biopolymers could facilitate the response of the biomaterial with biological systems and improve their biocompatibility. It has been reported in the literature that the incorporation of natural products allows to improve the biocompatibility of synthetic polymers[26,27].

Consider all this aspect, the aim of this work is to obtain an intelligent copolymer based on Poly (N–Isopropylacrylamide) as the bone chain and grafting by side chain incorporation of chitosan oligomers or hyaluronic acid as ramifications. A preliminary evaluation was made on the minimum molar ratio between the main polymer chain and the branch to be grafted. So if a high concentration of the polymer to be grafted is placed it could cause steric impediments, as shown in the proposed model of Figure 1, and is related to the previously reported by Carrero et al (Part I, 2018). This study including the exhaustive characterization, and propose the potential application in these copolymers for biomedical and pharmacological area.

Materials and methods

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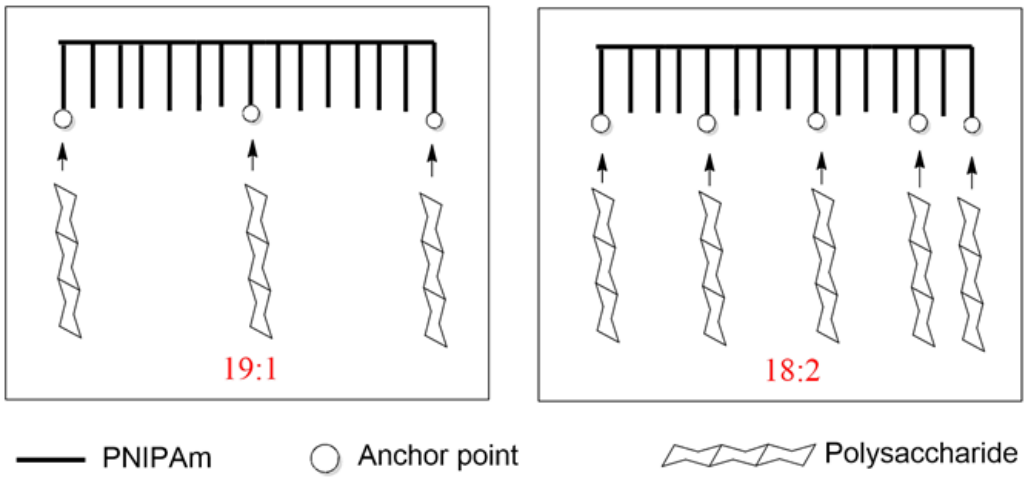

Figure 1– Representative model for the modification of the functionalized PNIPAm chain, which presents anchoring points for grafting reactions with polysaccharides.

Materials

N–Isopropylacrylamide (NIPAm), 3–butenoic acid, piperazine (PIP), 1–ethyl– (3–3–dimethylaminopropyl) carbodiimide (EDC), N–hydroxy–succinimide (NHS) and high molecular weight chitosan (CS) were provided by Sigma–Aldrich. Potassium peroxydisulfate (KPS) Riedel de Haën). Hyaluronic acid (HA), commercial drug of Suprahyal (Laboratory NOLVER). All other chemicals used were analytical grade.

The viscosimetric molecular weights (*Mv*) of grafting polysaccharides were determined using a capillary viscometer, resulting: for HA a *Mv*= $2{,}0x10^6$; and CS oligomers a *Mv* = $2{,}9x10^4$ consider their preparation as oligomers using the acid degradation process reported by Vieira et al[25].

Methods

Synthesis of the precursors PNIPAm–co–COOH y PNIPAm–co–COOH–g–PIP

The precursors were synthesized according to previously described methods reported by Carrero et al[28]. However, in this case the NIPAm monomer was polymerized with 3–butenoic acid at molar ratios of 19: 1 and 18: 2 respectively, thus obtaining the PNIPAm–co–COOH. For the incorporation of HA side chains, the PNIPAm–co–COOH was modified by grafting piperazine (PNIPAm–co–COOH–g–PIP). Figure 1 shows a structural model proposed in this work. In which it reflected how the chemical modification is given with the 3–butenoic acid and/or piperazine, on PNIPAm main chain, and after the incorporation of ramifications of CS(oligomers) or HA.

Synthesis of the graft copolymer PNIPAm–g–CS y PNIPAm–g–HA

A condensation process was carried out for the copolymerization. The carboxylic acid group is coming from PNIPAm–co–COOH, and the amine group is coming from CS, for the synthesis of PNIPAm–g–CS. The process follow with constant stirring for 12 hours, at room temperature. The molar ratio use was PNIPAm–co–COOH: CS 10:1 respectively. During the reaction, EDC was used as the activating agent of the carbonyl groups and NHS as a condensing agent in aqueous medium (EDC/NHS ratio 1:2). Under these conditions the grafting reaction with the amino groups of the chitosan is favored, and proposed in figure 2.

The PNIPAm–g–HA graft is obtained from previously PNIPAm–co–COOH functionalized with piperazine (PNIPAm–co–COOH–g–PIP), which has amino groups available for the reaction with activated carbonyls of HA; due to the presence of the EDC/NHS in the medium, as shown in figure 3. The process follow with constant stirring for 24 h, at room temperature, and consider a PNIPAm–co–COOH–g–PIP:HA ratio 10:0,7.

Obtained each graft copolymer separately, the gels were dialyzed for 48 h, after were freeze–dry by lyophilized, and subsequent characterized.

Characterization

FTIR–ATR spectroscopy of graft copolymers

Samples were analyzed by Fourier Transform infrared spectroscopy FT–IR (coupled to an ATR with ZnSe attenuated total reflectance crystal) Thermo Scientific brand, model Nicolet IS5, performing 32 scans at 4 cm^{-1} resolution, in a range of 4000 and 400 cm^{-1}.

Determination of the LCST of the graft copolymers by using DSC

After lyophilizing the copolymers, each sample was placed in aluminum capsules. The sample weight was approximately 5.00 mg, and 5 µL of distilled water was add, and after capsules were sealed with pressurized hermetic. The tests were perform on a Differential Scanning Calorimetry (DSC) equipment, Perkin–Elmer Model DSC 7. The DSC calorimeter was calibrate with indium (In) in inert nitrogen atmosphere to obtain the corresponding baseline. Only one way of heating was carry out at 20°C/min, in a temperature range from 25 to 50°C for each sample.

Morphological study of graft copolymers

Because the gel–like character and thermo sensitive response of the copolymers obtained, the process of injectability was simulated according procedure described by Coronado et al[29], Vieira et al[25] and Carrero et al[28]. In this way, the morphology of the copolymers could be closer of the morphology that they can developed in the biomedical applications. The samples studied were those obtained after freeze–drying (xerogel) which were cryogenically fractured in $N_{2(liquid)}$ to ensure the morphological observation of the inside of each sample. Then, they were coated with a thin layer of gold in a Balzers–SCD 030 sputter coater, and a JEOL JSM6390 scanning electron microscope was used for the morphological analysis of sample porosity. The voltage of the SEM equipment was set to 20–25 kV.

Cytotoxicity test by cell hemolysis on blood agar

Blood compatibility was evaluated with hemolysis assay according procedure described by Coronado et al[29], Vieira et al[25] and Carrero et al[28]. The blood was mixed with agar in a totally sterile medium and was gelled for 2 h in an oven at 37 °C. Each lyophilized sample was subjected to 1h of UV irradiation for sterilization. Once the gels were sterile, they were brought into contact with blood/agar system and were transferred in the oven for cell culture at 37 °C and a 5% CO_2 flow. The protocol was perform according to ISO–10993–4 (2002) test assay. The surface around each gel or copolymer sample was observed and photograph several time for a total time of 48 h.

Results and Discussion

Characterization of graft copolymers

After the grafting reaction, the characteristic signals of the precursors PNIPAm–co–COOH and PNIPAm–co–COOH–g–PIP are conserved in the FTIR spectrum. However, the stretch band produced by the presence of hydroxyl groups (–OH) is more pronounced in the FTIR spectrum of the

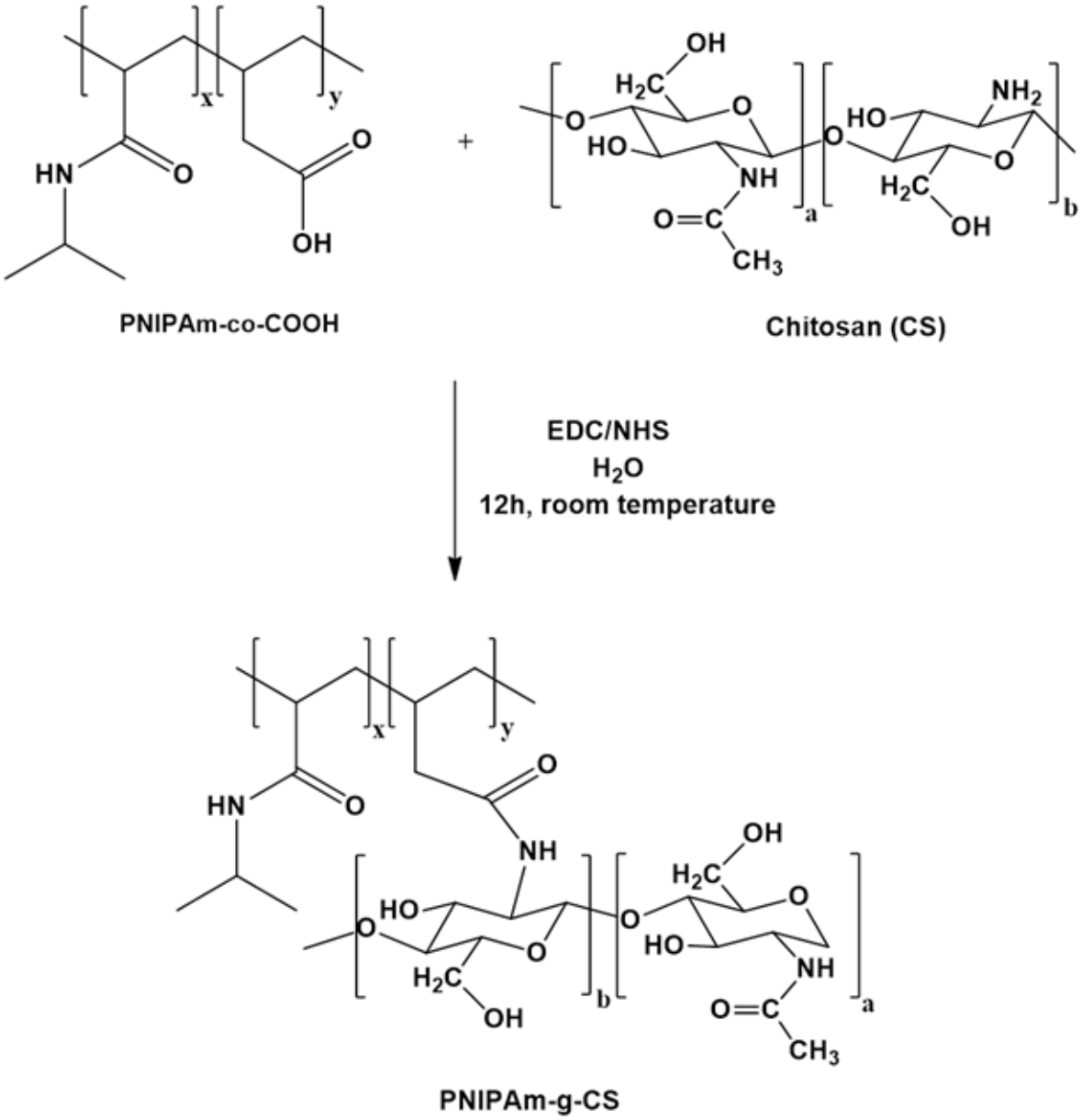

Figure 2 – Copolymerization reaction to obtain the PNIPAm–co–COOH grafts and the chitosan oligos (PNIPAm–g–CS).

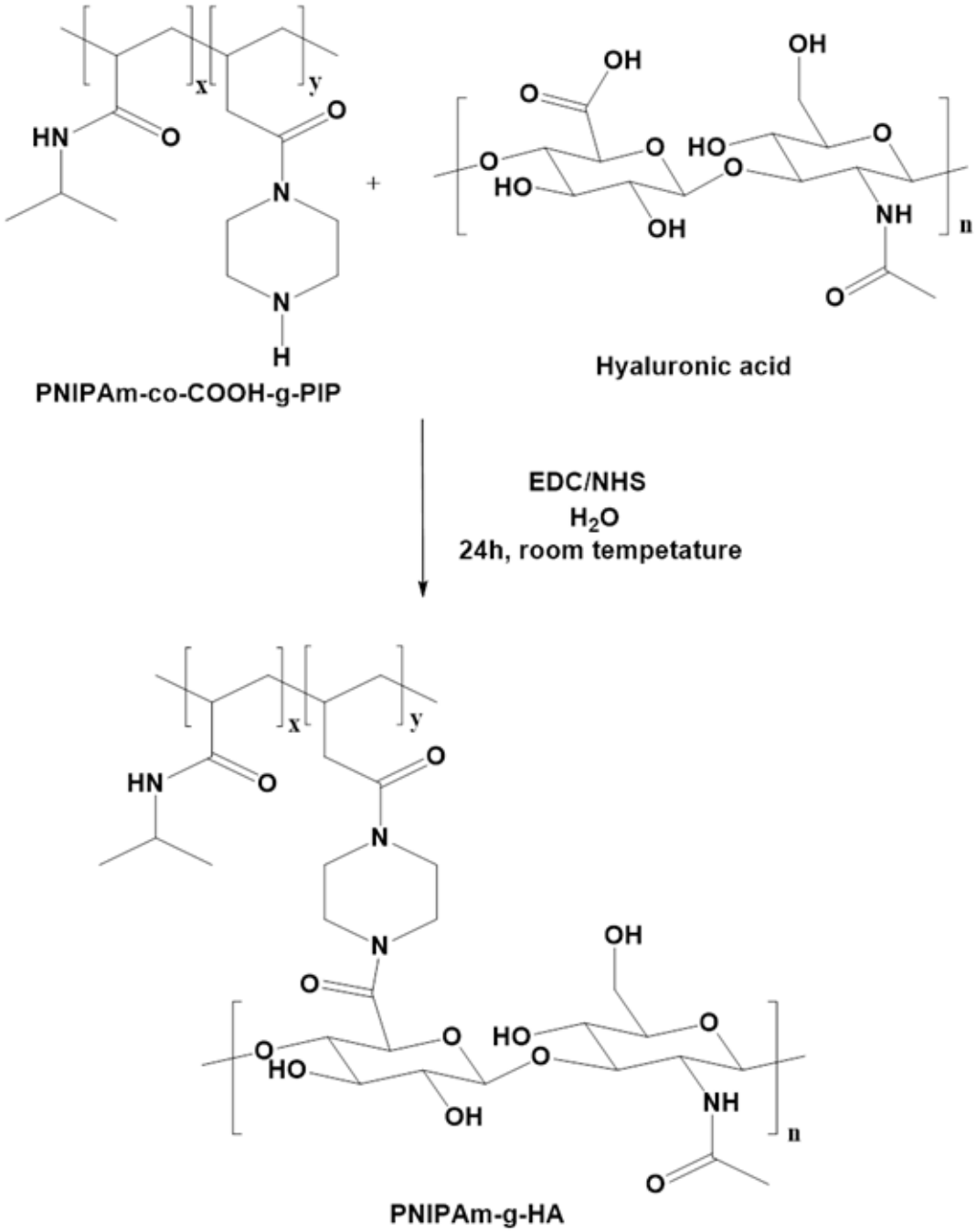

Figure 3 – Copolymerization reaction to obtain the grafts of PNIPAm–co–COOH–g–PIP and hyaluronic acid (PNIPAm–g–HA).

graft copolymers, due to the abundance of these groups in the structure of the incorporated polysaccharide. This signal is present above 3000 cm^{-1}, and can be distinguished in both cases of study (see figure 4A and 5A). In the FTIR figures, the spectrum of the precursors (PNIPAm–co–COOH, and PNIPAm–co–COOH–g–PIP) were present as a control. When CS or HA is incorporate into the copolymer, the structural change is clear and show the characteristic signals of polysaccharides, which have been widely studied and reported in the literature [30–36]. All these structural changes are according to the reactions shown in figures 2 and 3.

In general, it is possible to observe with intensity the band in 1044 cm^{-1} attributed to the stretching C–O–C, as well as the stretching signal of carbonyl group C=O around 1600 cm^{-1} and the deformation band of –NH between 1350–1450 cm^{-1}; and the signal in 3000 cm^{-1} is due to stretching –CH.

The amine signal present in the precursor PNIPAm–co–COOH–g–PIP, around 1215 cm^{-1}, disappears due to the formation of an amide bond with hyaluronic acid. A more pronounced signal appeared at 1250 cm^{-1} product of the C–N tension of the new bond between piperazine and HA (see Figure 5B). Similarly, for the PNIPAm–g–CS graft it is possible to observe the appearance of a band in 1250 cm^{-1}, as shown in Figure 4B, which can be attributed to the formation of the amide bond between the precursor PNIPAm–co–COOH and the CS oligomers.

For the copolymer PNIPAm–g–CS, it was verify that the carbonyl band returns to 1624 cm^{-1} indicating the formation of a new amide bond betwe–en the PNIPAm–co–COOH and the chitosan oligomers (see Figure 4B).

Additionally, in figure 5B, the signals between 1500 and 1700 cm^{-1} are better defined in the grafts, than in the precursor and HA. The amide mode–I signal, product of the vibration tension of the carbonyl group, is intensified above 1600 cm^{-1}, as is the amide mode–II signal due to the flexion vibration of the N–H bond and vibration tension of the C–N links is around to 1550 cm^{-1}, confirming the formation of the covalent amide bond between the chain of PNIPAm–co–COOH–g–PIP and HA[37]. This behavior is seen for both PNIPAm–g–HA grafts (ratio 19:1 and 18:2).

Determination of the LCST of the graft copolymers

In Figure 6, it can be observed as all synthesized graft copolymers

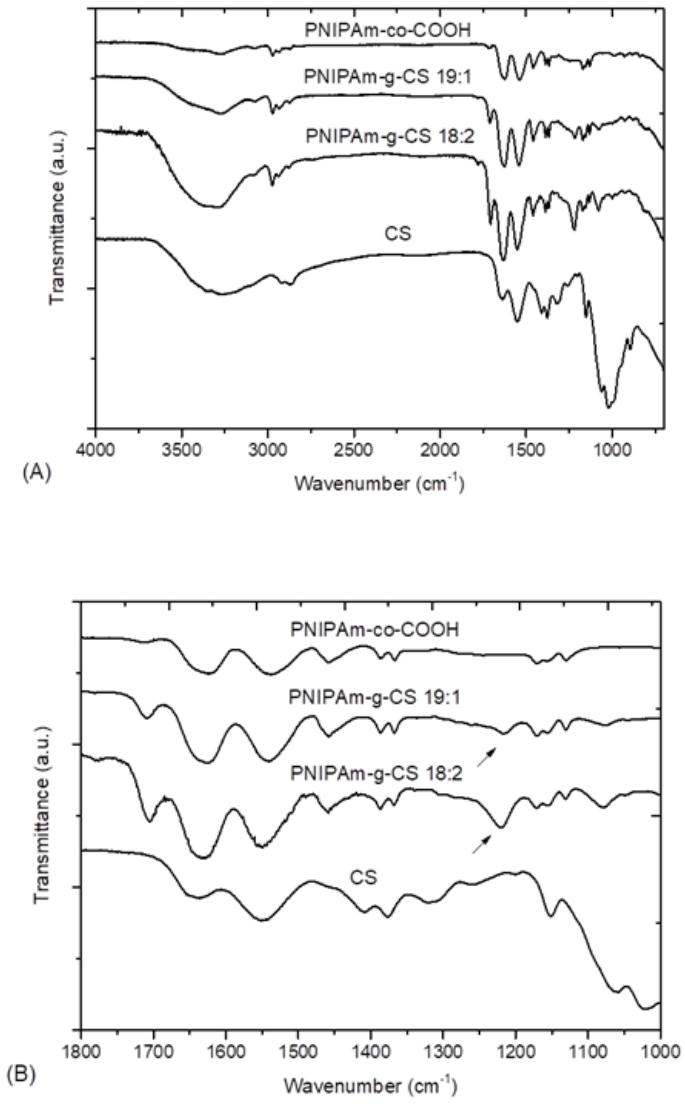

Figure 4 – FTIR spectrum of the precursor PNIPAm–co–COOH, CS and PNIPAm–g–CS grafts in formulations 19:1 and 18:2 in the region (A) 4000–500cm–1 and (B) 1800–1000cm–1.

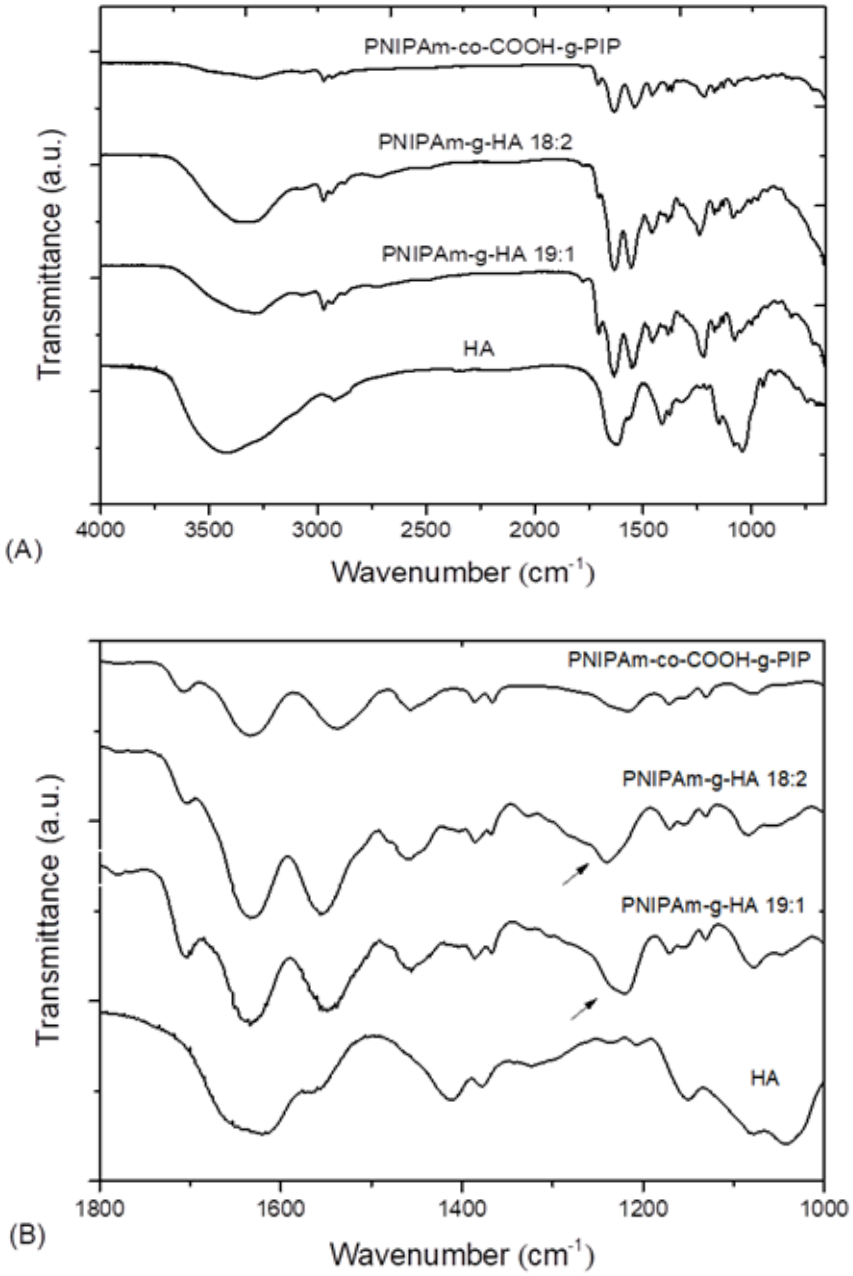

Figure 5 – FTIR spectrum of the precursor PNIPAm–co–COOH–g–PIP, HA and PNIPAm–g–HA grafts in formulations 19:1 and 18:2 in the region (A) 4000–500cm–1 and (B) 1800–1000cm–1.

have a transition temperature (T_{onset} LCST) lower than or equal to the reference homopolymer PNIPAm synthesized (approx. 33°C).

PNIPAm–g–CS grafts, by having shorter chain ramifications (chitosan oligomers), in comparison with their similar formulation of PNIPAm–g–HA, will require less energy to increase polymer/polymer interactions as shown by the ΔH values contemplated, and resume in table 1.

The significant difference in the molecular weight of the polysaccharides used (shown by ***Mv*** of each polysaccharide), can have consequences at the time of making the chemical graft reaction, because in the case of HA, this can increase the viscosity of the system. In consequence, the process of grafting is in disadvantaged and may be less effective, as shown in the yield results reported in Table 1. Also, if grafting occurs with this biopolymer of higher molecular weight, greater molecular entanglements are generated when the system is dissolved (at T < TLCST), but when this system begins to undergo the transition process, these entanglements make it difficult the phase segregation process, requiring a higher energy cost for to induce the thermal transition reflecting in enthalpy results.

The PNIPAm homopolymer presents an $LCST_{onset}$ around 33°C and in the case of the copolymers obtained from the formulation grafted with HA the new products decrease this transition to around 29–30°C, but it seems to have more effective grafting despite requiring greater energy or enthalpy (ΔH). In the case of copolymer grafted with CS oligomers, not change are observed in TLCST, and relate with ΔH, this value is depending of the effective anchor point and the entanglement produce for the oligomers ramifications.

It has been reported in the literature that polymers with a low LCST, which provide networks undergoing reversible phase transitions, show an enormous potential to develop useful drug delivery systems to control both the site and the release rate[3]. All thanks to its LCST transition that allows gelling in situ, which can also favor the design of injectable scaffolds, because ideally, the polymer solution exists in the liquid state at room temperature and forms a gel at human body temperature. Also, thermal transition induced physical crosslinked hydrogels that have several favorable properties, for example they do not need organic crosslinkers

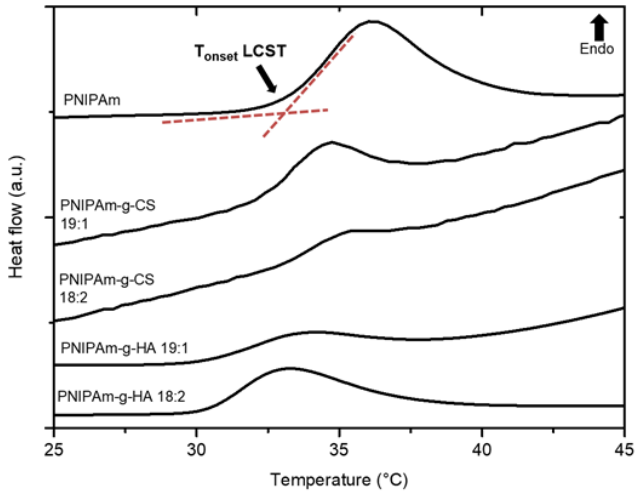

Figure 6 – Determination of the LCST transition by DSC for the PNIPAm and the grafts PNIPAm–g–CS and PNIPAm–g–HA

SAMPLE	Yield (%)	DSC	
		T_{onset} LCST (±0.1) ºC	ΔH_{LCST} (±0.1)J/g
PNIPAm	-	33.1	17.9
PNIPAm-g-HA 19:1	72.5	29.4	19.2
PNIPAm-g-HA 18:2	78.1	30.5	10.5
PNIPAm-g-CS 19:1	84.4	32.6	14.8
PNIPAm-g-CS 18:2	76.6	33.1	4.5

Table 1 – Yield reaction and transition LCST of graft copolymers synthesized.

and have no thermal effect on the surrounding tissues[38].

Study of copolymer morphology observed by SEM

During the development of new materials, the study of porosity is one of the most important aspects to investigate, because it allows to evaluate the potential of a new biomaterial due to its close relationship with processes of fluid exchange, biocompatibility, biodegradability, vascularization, etc[25,39–41]. Consequently, the size of the pore and the interconnection of the network determine how is the interaction process: hydrogel–organism, and are increasingly used in the development of scaffolds with applications in tissue engineering[42,43]. The porosity and interconnectivity of the network are responsible for the response of the tissues where they can be implanted[44]. When the pores are larger, there is a greater amount of surface exposed to the biological environment, so the interaction of the biomaterial with the organism is also favored. Further the pore size, another variable of great importance is the interconnection of the network, since if a 3D network presents interconnected channels, a better flow and greater reach of the aqueous medium (therefore of nutrients) will be obtained in the new material[29].

The injectability process was simulate, to see how the shear stresses could generate a morphological change in the final structure of the gels. In this way, when the gel is going through the needle of an injector, and after the sample pass the limit temperature for the LCST transition, some porosity could be create permanently. It can be observe in figure 7. The formulations 19:1 and 18:2 of the PNIPAm–g–CS graft–copolymers present a homogeneous morphology as a "honeycomb" (due to the shape of their interconnected pores). While the PNIPAm–g–HA micrograph shows a high porosity with less homogeneity and continuity. This evidence can be interpreting taking into account the molecular size (*Mv*) of both grafted polysaccharides. From which it can be inferred that the smaller the size of the grafted chain, there will be minor molecular entanglements with the backbone of the copolymer, which may result in a certain ordering in the solid gel state, which confers a greater size of the pores formed among them. In the case of HA, with large Mv, all the entanglement could be non–favorable for the generation of pores and interconnectivity; or because the LCST transition occur first and can not give time to the molecules to get certain order in the xerogel state.

In general, the synthesized materials show scaffolding structures with interconnected pores, whose homogeneity varies depending on the formulation and the molecular weight of the polysaccharide. The observed morphology is an important requirements to be able to estimate the biomedical or tissue engineering applications that these graft copolymers could have.

Cytotoxic studies

The cytotoxic studies by hemolysis performed in an agar / blood system allowed to evaluate the hemocompatible character of the synthesized materials. Noting that graft copolymers obtained are not toxic, since none of them shows evidence of cell lysis, as shown in Figure 8.

The tests of cytotoxicity through the use of agar gels enriched with blood, allow the verification of the degradative activity by the action of the cytotoxic components present in a given biomaterial[45]. In case of the possible diffusion of cytotoxic components present in prepared hydrogels or gels, in a certain time, there would be evidence of lysis or decomposition by the rupture of red blood cells (call this process hemolysis)[46]. If, after

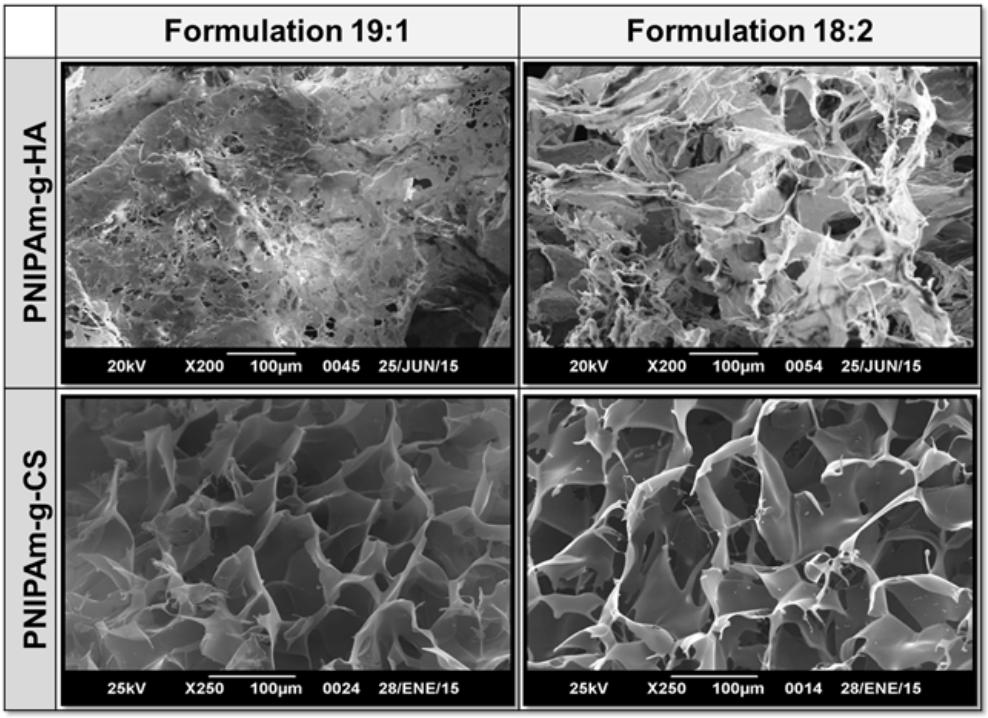

Figure 7 – Micrographs of PNIPAm–g–CS and PNIPAm–g–HA grafts copolymers.

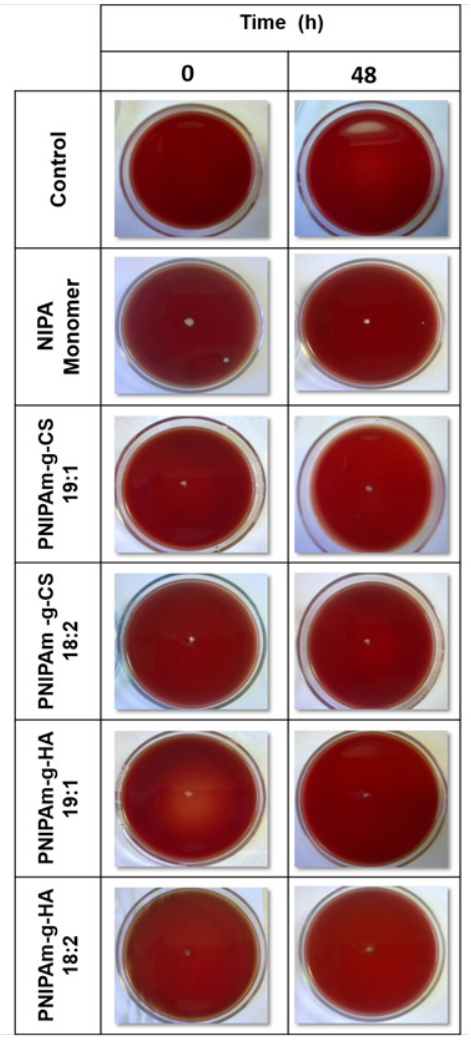

Figure 8 – Non–cytotoxic evidence of the PNIPAm–g–CS and PNIPAm–g–HA grafts in contact with the agar/blood at time 0 and after 48h.

48 h of contact between the agar/blood and the biomaterial, hemolysis does not occur, the biomaterial is consider non−cytotoxic[41]. When in the presence of a cytotoxic material, hemolysis is evidenced by the formation of a whitish halo around the contact area of the material seeded in the agar/blood, an indication that a lysis process has occurred as a cytotoxic response[29,46].

Figure 8, resume the hemo−compatibility test results, and the formation of the white halo around the surface of the copolymers gel is not observe (after 48 h), therefore, the synthesized copolymeric gels can be considered non−cytotoxic compounds. As a control, the NIPA monomer was used, in which it is possible to observe, after 48 h, a slight halo formed at the border of the sample and the agar/blood medium.

Conclusions

The results obtained in this study, such as the thermal transition LCST at temperatures close to the human body of the graft copolymers PNIPAm−−g−HA and PNIPAm−g−CS, as well as their injectable character and the porous morphology observe, are indicative that these new water−soluble biomaterials have a high potential to be used in applications such as: injectable scaffolds for tissue engineering and/or encapsulation/controlled release of drugs. Additionally, the results of non−toxicity on contact with blood give to these copolymers a hemocompatible character.

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