

NOVEL THERMOGELLING HYBRID COPOLYMERS BASED ON CHITOSAN. SYNTHESIS AND PROPERTIES IN AQUEOUS SOLUTION

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Abstract

The aim of this study was the synthesis of a thermally reversible hydrogel based on the controlled grafting on chitosan of a random copolymer of ethylene oxide and propylene oxide (POEP), which is characterized by a Lower Critical Solution Temperature (LCST) between 15 and 50 °C in aqueous acidic condition. The resulting chitosan-POEP derivative gave a solution in acidic water (pH ~ 4), below 40 °C. Upon increasing the temperature above 40°C, it developed hydrophobic interactions, leading to the formation of a physical three-dimensional network. A part of the physico-chemical characterization of aqueous solutions of chitosan-POEP is presented, which highlights the thermoreversibility of hydrogel formation.

Keywords: Chitosan, thermoreversible hydrogels, poly(propylene oxide-co-ethylene oxide), Biomaterials.

1-Introduction

Stimuli responsive polymers are polymers that can change their physical behavior in response to chemical or physical stimuli like variations of temperature, pH, ionic strength and/or electric or magnetic field (1). Over the last fifteen years, these polymers, which can present under different physical forms, have become increasingly important because of their potential applications in the pharmaceutical and biomedical fields. A particularly interesting and important polymeric system is hydrogel forming solutions by a simple phase transition (a sol-gel transition) in aqueous solution induced by a change in temperature (2). This system is of great interest in drug delivery and tissue engineering as injectable hydrogel (3). Indeed, biological substances such as protein or living cells may be simply mixed with the liquid formulation at room temperature, therefore maintaining their activity, and injected into a body environment which induces gelation and thus retention at the site of injection. Additionally, such injectable hydrogels may be more suitable for treating irregularly shaped defects, since rigid chemical hydrogels may be difficult to form into complex configurations. In these systems, the development of physical junctions which is a prerequisite in determining gelation, is generally due to the association of LCST (Low Critical Solution Temperature) polymers. The LCST can be defined as the critical temperature at which a polymer solution undergoes phase transition from a soluble to an insoluble state on heating above the critical temperature. Poly(*N*-isopropylacrylamide) (PNIPAAm) and triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) (PEO-PPO-PEO)- Pluronic®- are the most often used temperature-responsive polymers since they can exhibit a sharp transition near the body temperature (1).

In this context, our objective was to design and synthesize novel thermally reversible hydrogels based on polysaccharides for the development of biocompatible matrices for tissue engineering

and/or drug delivery. One of the most promising polysaccharides in this sense is chitosan. Chitosan has important biological properties but also good film and gel forming characteristics in relation with its molecular structure giving a semi-rigid character but also allowing a cooperative H bond network formation. For some times now, we performed alkylation of chitosan giving interesting thickening properties in aqueous solution. The associative character was related to the length of the grafted alkyl chains as well as to the density of grafting, but also pH, polymer concentration and external salt concentration. Considering these results, the strategy envisaged for the obtention of thermoassociative properties, was to replace hydrophobic alkyl chains by a thermosensitive random copolymer of EO and PO (POEP).

2-Materials and Methods

Materials. Chitosan was kindly provided by FMC BioPolymer AS, Novamatrix (Norway). Its viscosity average molecular weight was determined to be 4×10^5 g/mol (assuming $K=0.076$ mL/g and $a=0.797$) (4). This value was derived from the intrinsic viscosity $[\eta] = 2405$ mL/g (in 0.3 M CH_3COOH / 0.1M CH_3COONa , at 25°C). The POEP derivative (Jeffamine®, $M_n = 2.2 \times 10^3$ g/mol) was obtained from Huntsman (USA). Diacetone galacturonic acid was obtained from CMS Chemicals Limited (Abingdon, UK). All other products and reagents were purchased from Fluka (Buchs, Switzerland).

NMR spectroscopy. ^1H NMR experiments were performed using a Bruker DRX400 spectrometer operating at 400 MHz. ^{13}C NMR spectra were recorded with a Varian Unity 400 spectrometer operating at 100 MHz. Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm) and calibration was performed using the signal of the residual protons of the solvent as a secondary reference. Deuterium oxide and deuterated chloroform were obtained from SDS (Vitry, France). Details concerning experimental conditions are given in the figure captions. Chitosan-POEP was dissolved in $\text{D}_2\text{O}+\text{DCl}$ at a concentration of 6 g/L during one night.

Rheological experiments. Oscillatory experiments were performed with a cone-plate rheometer (ARES-RFS from TA Instruments). All the dynamic rheological data were checked as a function of strain amplitude to ensure that the measurements were performed in the linear viscoelastic region. For low viscosity systems, the mobile used was a cone-plate with a diameter of 5 cm and an angle of 0.04 rad, and for gel-like systems, the mobile used was a plate-plate system with a diameter of 2 cm. The gap between the plates was 7 mm which lead to a normal force of 50 N (measuring temperature of 25 °C). Experiments were carried with a film of silicone to avoid solvent evaporation. Chitosan-POEP was dissolved in 0.3 M CH_3COOH / 0.1 M CH_3COONa . Salt was added in order to screen the long range electrostatic repulsions between positively charged chains. The dissolution time was at least 12 h at room temperature and allowed at rest for at least 1 h at room temperature. We checked that the rheological properties of the samples did not change with time (from 1 to 24 h)

Differential Scanning Calorimetry (DSC). The DSC studies were recorded by a Micro-DSC III calorimeter (Setaram, France) using the following parameters: 0.4 °C/min between 10 and 80° C. The reference cell was filled with the solvent and the sample compartment with the polymeric solution. The products were dissolved at a concentration of 10 g/L in 0.3 M CH_3COOH with different concentrations of salt ($[\text{CH}_3\text{COONa}] = 0.2$ M, 0.1 M, 0.05 M, 0.025 M)

Synthesis

POEP galacturonamide: Diacetone galacturonic acid (0.373 mg, 1.36 mmol) was dissolved in anhydrous dimethylformamide (35 mL) under nitrogen atmosphere. To this solution, *N*-hydroxybenzotriazole (0.184 g, 1.36 mmol), diisopropylethylamine (0.32 mL, 1.86 mmol), 1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide (0.356 g, 1.86 mmol), and POEP (2 g, 0.91 mmol) were

successively added. After stirring at room temperature for 20 h, the reaction mixture was concentrated under reduced pressure and the residual oil was dissolved in CH₂Cl₂. The organic phase was washed several times with distilled water, and then dried on sodium sulfate, filtered and concentrated under reduced pressure, affording POEP-galacturonamide (1.8 g, 0.75 mmol).

RMN ¹H (CDCl₃, 400 MHz) δ (ppm): 8 (1H, NH), 5.6 (4H, CH galacturonamide), 4.25-4.4 (OH protons of galacturonamide), 3.85-3.3 (CH₂ and CH of the PO and EO units and of galacturonamide), 1.15 (CH₃ of the PO unit).

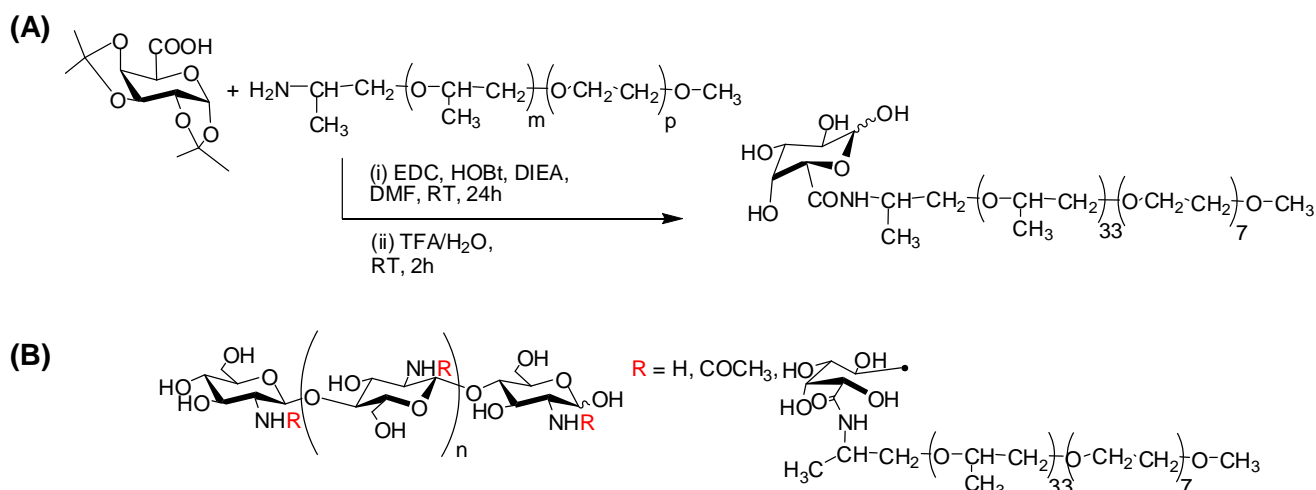
Chitosan-POEP : POEP-galacturonamide (0.4 g, 0.17 mmol) was treated by 3.5 mL of a TFA/water (v/v, 4/1) mixture. After stirring at room temperature for 2 h, the reaction mixture was concentrated under reduced pressure. The residue was then dissolved in diethyl ether and washed with distilled water until to reach a pH value of 5.6. The organic phase was then dried on sodium sulfate, filtered and concentrated to give deprotected POEP-galacturonamide (0.360 g, 0.15 mmol). The resulting product was dissolved in ethanol (10 mL) and added to a solution of chitosan (0.4 g, 2.38 mmol) in a 0.2 M CH₃COOH/EtOH mixture (94mL/40mL). The pH of the reaction mixture was adjusted to 5.1 by addition of a 0.5 M sodium hydroxide aqueous solution, and a solution of sodium cyanoborohydride (0.213 g, 3.3 mmol) in water (2 mL) was added. After stirring at room temperature for 48 h, ethanol was evaporated and chitosan- POEP was precipitated by addition of a 0.5 M sodium hydroxide aqueous solution to reach a pH value of 8. The precipitate was successively washed with H₂O/EtOH mixtures, with different v/v proportions (3/2, 7/3, 4/1, 9/1) and dried to give chitosan-POEP (0.516 g, 98 %).

3-Results and Discussion

3.1 Synthesis of chitosan-POEP and structural characterization

One of the advantages of chitosan is the possibility to perform specific chemical reactions at the C-2 position thanks to the existence of the free amine group. In our previous work (5), alkylation of chitosan was performed by a reductive amination reaction from aldehydic aliphatic chains under homogeneous conditions. Different derivatives were prepared, having different alkyl chain lengths and degrees of substitution (DS), as different solution properties of these derivatives depending on the balance between the electrostatic repulsion and hydrophobic attractive interactions were expected. In aqueous solution, some derivatives with C-12 chains were shown to exhibit remarkable associating properties, giving very viscous solutions. However, this associative character was not shown to be sensitive to temperature. Therefore, in order to obtain reversible associating properties as a function of temperature, we replaced the alkyl chains by thermosensitive chains of a random copolymer of EO and PO (POEP).

The grafting of POEP on chitosan by a reductive amination reaction first involved its functionalization by a reducing sugar. This was based on an amine-acid coupling reaction between a protected galacturonic acid derivative and POEP, followed by deprotection of the sugar moiety (Figure 1). The coupling reaction between the hemiacetal group of the POEP derivative and the amine function of chitosan was performed under conditions described previously (5).



Scheme 1. Chemical modification of chitosan: (A) synthesis of POEP-galacturonamide; (B) obtention of chitosan-POEP by a reductive amination reaction between POEP-galacturonamide and chitosan.

The grafting of the POEP chains on chitosan could be demonstrated by ^1H NMR spectroscopy as can be seen from the intense signal attributed to the methyl groups of the PO units (Figure 2A). This technique also allowed to obtain the DS by digital integration of the proton signals of the methyl groups of the PO units and the H-2 or methyl of the *N*-acetyl glucosamine units. In the present case, the DS was found to be 0.03

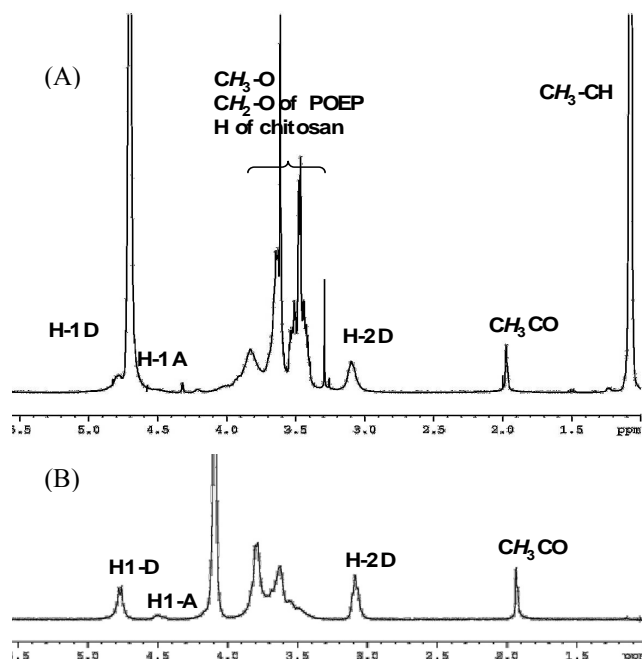


Figure 2. ^1H NMR spectra (400 MHz, 6 mg/mL in $\text{D}_2\text{O}+\text{DCI}$) of (A) POEP-chitosan at 25 °C and (B) native chitosan at 80°C. D : *N*-deacetylated unit; A : *N*-acetylated unit.

3.2 Analysis of the thermosensitive behavior

The temperature dependence of the associative properties of chitosan-POEP was investigated using different complementary techniques. Calorimetry (DSC) allowed to obtain the enthalpy change (ΔH) during the transition of the grafted POEP groups, while dynamic rheology provided information about the elastic and viscous components as a function of the temperature.

Furthermore, aggregation of the POEP groups could be followed at the molecular scale by ^{13}C NMR spectroscopy, by the analysis of the respective intensities of the ^{13}C signals of the free and associated methyl groups of POEP, as shown by Figure 3.

As can be seen on the different spectra of Figure 3, a second peak corresponding to the aggregated methyl group appears upon heating. In addition, its intensity depends on salt concentration as a result of the balance between the polycationic chains and hydrophobic attractive interactions between the POEP groups.

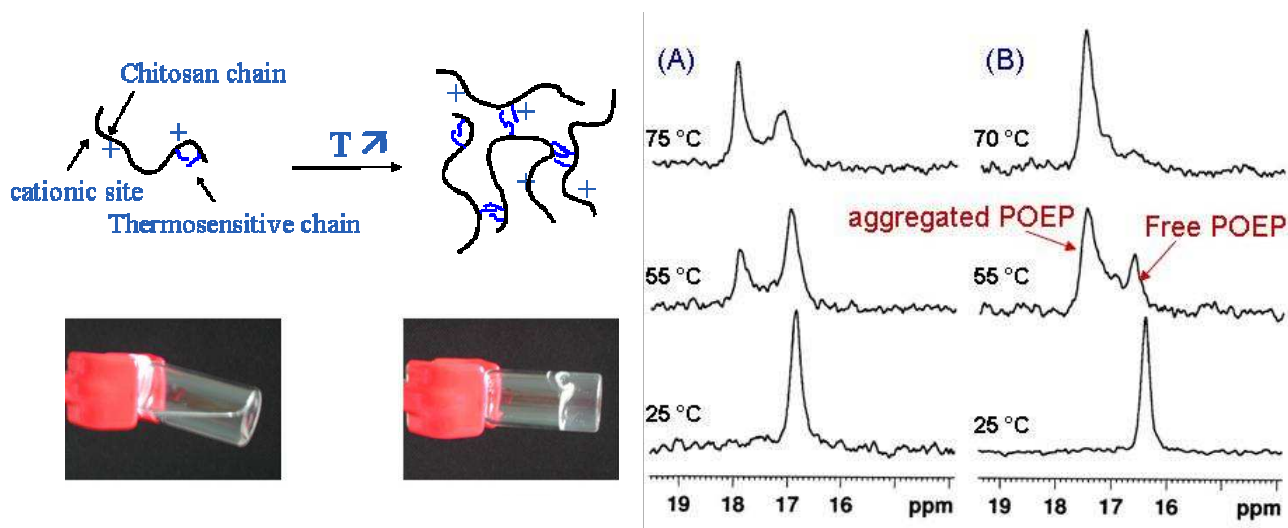


Figure 3. Aggregation of the POEP grafts observed from DEPT45 ^{13}C NMR spectra recorded at different salt concentrations, and illustration by pictures and scheme.

From the calorimetry data given in Table 1, it can be observed that the value of the enthalpy change (ΔH) during the transition of the grafted POEP groups increases whereas the maximum temperature of the ΔH peak tends to decrease with salt concentration, as long as it is lower than or equal to 0.1 M. These results can be attributed to the reinforcement of the aggregation of POEP due to the screening of electrostatic repulsions. At a salt concentration of 0.2 M, an opposite tendency is observed, which could be related to the decrease of the solubility of chitosan-POEP in aqueous solution due to the complete suppression of electrostatic repulsions. Such an assumption had already been evoked from the rheological results obtained under different solvent conditions (6). From these data, the optimal solvent conditions for aggregation of chitosan-POEP appear to be 0.3 M CH_3COOH / 0.1 M CH_3COONa . Indeed, in this solvent the value of ΔH is the highest and the maximum temperature of the ΔH peak, the lowest. Compared to the values obtained for the free POEP, the latter indicate a non-total aggregation of POEP.

Sample	Concentration of POEP (g/L)	[CH ₃ COONa] (M)	Maximum peak temperature (°C)	ΔH (kJ.mol ⁻¹ of PO unit)
POEP	10	0.1	23	6
POEP	3	0.1	23.8	7
Chitosan-POEP	3	0.025	36.2	2.5
Chitosan-POEP	3	0.05	34.3	3.1
Chitosan-POEP	3	0.1	31.6	4
Chitosan-POEP	3	0.2	33.6	3.6

Table 1. Comparison of calorimetry data obtained from solutions of chitosan-POEP under different solvent conditions and free POEP

The formation of a three-dimensional physical network exhibiting a gel-like behavior upon heating the solution of chitosan-POEP could be clearly demonstrated by dynamic oscillary shear measurements.

Figure 5 shows the frequency dependence of the storage modulus (G') and the loss modulus (G'') for a solution of chitosan-POEP at a concentration of about 10 g/L at different temperatures. At 25 °C, the loss modulus, G'' , is higher than the storage modulus, G' , within the whole range of frequencies covered. Those features are characteristic of a viscous behaviour. When the temperature is increased to 45°C, G' becomes higher than G'' within the whole range of frequencies investigated. The solution achieves a gel-like behavior. At 60°C, the G' and G'' curves exhibit almost no dependence with frequency, suggesting the formation of an elastic gel. It should be noted that hydrogels are obtained here at a polymer concentration of 10 g/L, while the lowest concentration of a typical biocompatible thermogelling copolymer (Pluronics[®] F127) that leads to a gel is 200-300g/L.

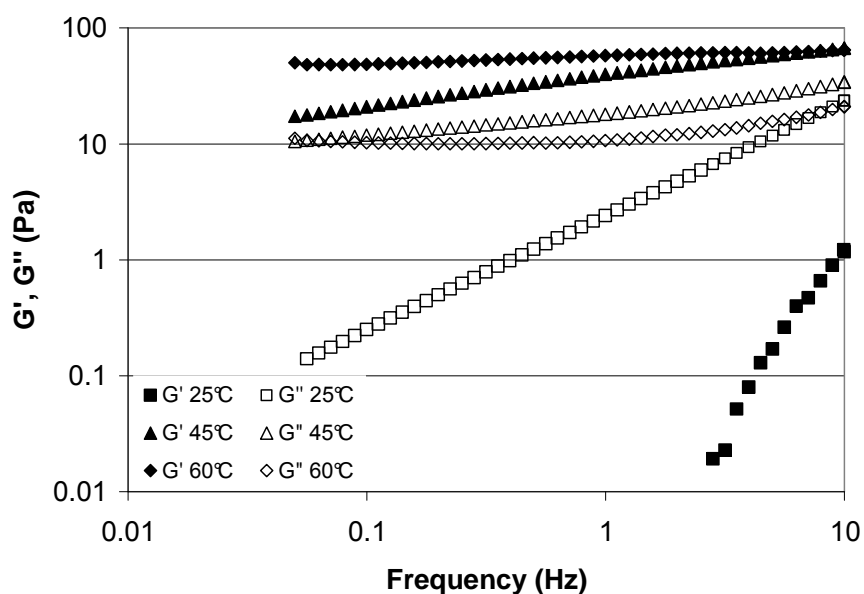


Figure 5 Variation of the dynamic moduli as a function of the frequency for the chitosan-POEP at temperatures of 25, 45 and 60°C.

Figure 6 displays the variation of the dynamic moduli as a function of the temperature for the initial chitosan, chitosan with C10 alkyl chain, and chitosan-POEP at a fixed frequency of 1 Hz. Below 44°C, the solution of POEP-chitosan exhibits a viscous character with the loss modulus higher than the storage modulus. Above 44°C, the storage modulus becomes higher than the loss modulus as a result of the formation of hydrophobic domains of POEP groups.

By comparison, initial chitosan and chitosan with alkyl chains exhibit a viscous behaviour in the entire range of temperature investigated.

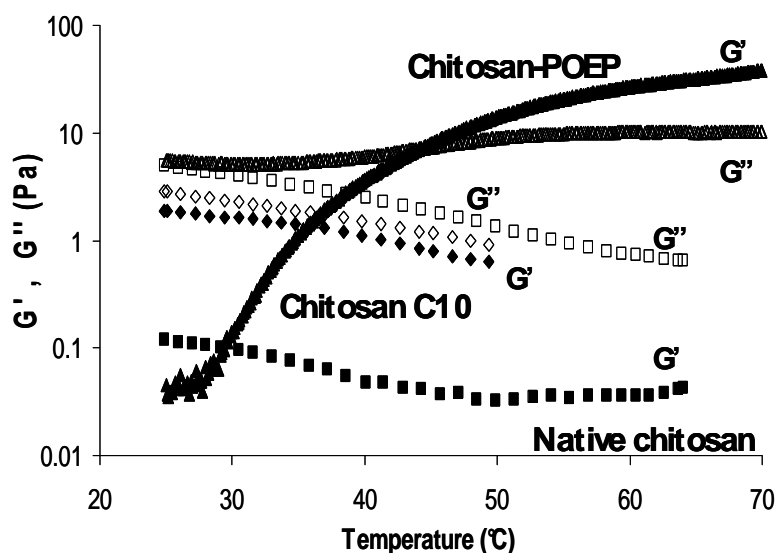


Figure 6. Variation of the dynamic moduli as a function of the temperature for the initial chitosan, chitosan with C10 alkyl chain, and chitosan-POEP at a frequency of 1Hz. The temperature was increased from 25 to 65-70°C at a rate of 1°C/min.

Conclusion

In conclusion, a novel hybrid copolymer of chitosan and a thermosensitive synthetic polyether was synthesized, exhibiting a remarkable thermally reversible hydrogel behavior in aqueous solution. Compared to other thermally reversible hydrogels such as Pluronics[®], this system leads to hydrogels at much lower polymer concentrations while taking advantages of the biological properties of chitosan, such as mucoadhesivity. Such thermally reversible hydrogels may thus find interesting biomedical applications as developed above. For this purpose, further chemical modifications are under investigation to obtain samples which are soluble in aqueous solution at neutral pH, which should be more favourable for such applications.

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