PARTIAL REACETYLATION OF CHITOSAN IMPROVES THE THERMOSENSITIVITY OF CHITOSAN/GLYCEROPHOSPHATE SYSTEM.

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Abstract

The influence of randomized distribution of N-acetyl-D-glucosamine on the gelation properties of a chitosan-ß-glycerophosphate (GP) hydrogel system was investigated. Randomization is obtained through partial re-acetylation of the chitosan. Corresponding random chitosan-GP hydrogels were visibly clearer, mechanically stiffer, completely thermo-reversible showing a narrow hysteresis in thermo-reversibility, and solidified faster at lower temperature in comparison to their block chitosan-GP counterparts. These characteristics constitute important improvements for the applicability of chtosan-GP system for potential ocular or topical administration.

Introduction

Chitosan is an aminopolysaccharide obtained by alkaline *N*-deacetylation of chitin, a naturally occurring polysaccharide extracted from crustaceous shells, squid pens or exoskeletons of insects. However, in contrast to chitin, chitosan is soluble in aqueous media under mild acidic conditions. Such solubility makes it an attractive biomaterial for numerous industrial applications particularly in biomedical and pharmaceutical fields.

Natural chitosan occurs as a family of copolymers of D-glucosamine and N-acetyl-D-glucosamine, where the proportion of acetyl residues, called degree of deacetylation (DDA), normally falls between 0 and 50%. Fully deacetylated chitosan (100%) is a homopolymer of D-glucosamine. Chitosan can be modified either through deacetylation or by reacetylating a 100%DDA chitosan homopolymer. The chitosan produced by N-deacetylation under heterogeneous conditions consists of blocks of D-glucosamine and N-acetyl-D-glucosamine sequences, whereas chitosan produced by N-acetylation of 100% DDA chitosans under homogeneous conditions carries a random distribution of N-acetyl-D-glucosamine and D-glucosamine units[1-4].

In addition to the molecular weight (Mw) and the DDA, the distribution of monomer units has been shown to be another factor which affects chitosan properties such as solubility[5,6] and viscosity[7]. The distribution of monomers along with the chain has even been found to affect the digestibility of chitosan by lysozymes[8].

The present work aimed to investigate the influence of random distribution of N-acetyl-D-glucosamine on the gelation properties of the chitosan/ß-glycerophosphate (GP) system. This chitosan-GP system, called BST-Gel™, is a novel family of injectable thermo-setting chitosan-based gels ideally suited for biomedical and pharmaceutical applications. The chitosan-GP system is a neutral liquid solution that can be kept in a liquid state at low temperatures which spontaneously forms a homogeneous gel when heated up to body temperature (37ºC), but without the use of organic solvents, chemical or ionic cross-linkers., Studies of various BST-Gel™ formulations have been shown to be highly biocompatible and appropriate for the encapsulation of living cells, the release of proteins and the design of *in situ* self-forming injectable implants[9-12].

Previous studies also demonstrated that the chitosan DDA is enough to significantly affect the gelation process[10,13] in block chitosan-GP hydrogels.

Materials and Methods

Materials

Raw chitosan material, ($M_w \approx 1.9 \times 10^6$, DDA = 80 %), was purchased from Marinard Ltd. (Qc, Canada). Further deacetylation and purification were performed at BioSyntech Canada Inc. (Qc, Canada). The purified chitosan was characterized and certified as an ultra pure chitosan (Ultrasan™) which meets medical and pharmaceutical standards. The weight average molecular weight and the DDA were determined by size exclusion chromatography (SEC) and ^{1}H-NMR spectroscopy respectively. For SEC, a HP1100 chromatograph equipped with a WAT011545 column connected to WAT011565 guard column in series (Waters Inc., Milford, MA) using an on-line detection obtained with a G1362A differential refractometer, was used. The ^{1}H-NMR spectroscopy experiments were performed at 70°C on a Varian Mercury (400MHz) spectrometer. All chemicals, including acetic anhydride and β-Glycerophosphate disodium salt (GP), were purchased from Sigma-Aldrich Company and used as received.

Preparation of fully deacetylated chitosan

The raw chitosan ($M_w \approx 1{,}9 \times 10^6$, DDA = 80 %) was treated with 50% sodium hydroxide for 30 minutes at 133°C. Although these treatment conditions could have been sufficient, this step was repeated in order to ensure complete removal of acetyl groups (100% DDA). Subsequently, the chitosan was subjected to an ultra-purification treatment followed by precipitation to form a very fine white powder. Full deacetylation of chitosan (DDA ~ 100%) was confirmed by ^{1}H-NMR.

Homogeneous N-reacetylation of chitosan

The homogeneous N-reacetylation of chitosan (100%) was performed according to a method adapted from that described by Hirano[14]. Briefly, chitosan (1g) was dissolved in 30 ml of aqueous acetic acid solution (10% v/v). After complete dissolution, a highly viscous 3% chitosan solution was obtained. Then, 80 ml of methanol was admixed drop-wise under continuous stirring to obtain a clear homogeneous solution. While maintaining the stirring, a desired amount of acetic anhydride, dissolved beforehand in 5 ml of methanol, was added drop-wise to the resulting solution. The mixture was allowed to react at room temperature for about 24 hours, after which it was filtered and dialyzed against distilled water for one day, using Spectra/Por1 membrane (molecular weight cut-off 6000 Da). Then, re-acetylated chitosan was precipitated in 200 ml of NaOH (0.1M). The precipitate was filtered, washed with distilled water until the pH of the solution was around 7 and dried under vacuum.

Preparation of the gelling system

Chitosan was dissolved in aqueous hydrochloric acid in order to obtain a 2.5% (w/v) clear solution (pH ≈ 6.3). Chitosan solution was cooled to ~ 4°C in an ice bath and a solution of GP in distilled water was added drop-wise under continuous stirring. The final concentrations were 2% (w/v) chitosan and 0.098 M GP.

Rheological analysis

Rheological measurements were performed on a Bohlin CVO rheometer (Bohlin Instruments Inc., Grandbury, NJ) using C14 concentric cylinders. Solution aliquots of 3 ml were introduced between the concentric cylinders and covered with mineral oil in order to prevent evaporation during the measurements. The gelation temperature was determined by oscillatory measurements performed at a frequency of 1 Hz, between 5 and 70ºC at a rate of 1ºC/min. The gelation temperature was determined as that at which the elastic modulus (G') crosses the viscous modulus (G").

Results and Discussion

Characterization of re-acetylated chitosan

The DDA of re-acetylated chitosan samples was established using ^{1}H-NMR spectroscopy. A typical ^{1}H-NMR spectrum recorded for chitosan having a DDA of 84% is shown in Figure 1. The DDA was calculated using integrals of the peak of anomeric proton H1 of glucosamine monomer [H1(GA)] and of the peak of the three proton of acetyl group [H(Ac)] following the equation[15]:

$$DDA(\%) = \frac{3 \cdot H1(GA)}{3 \cdot H1(GA) + H(Ac)} \times 100$$

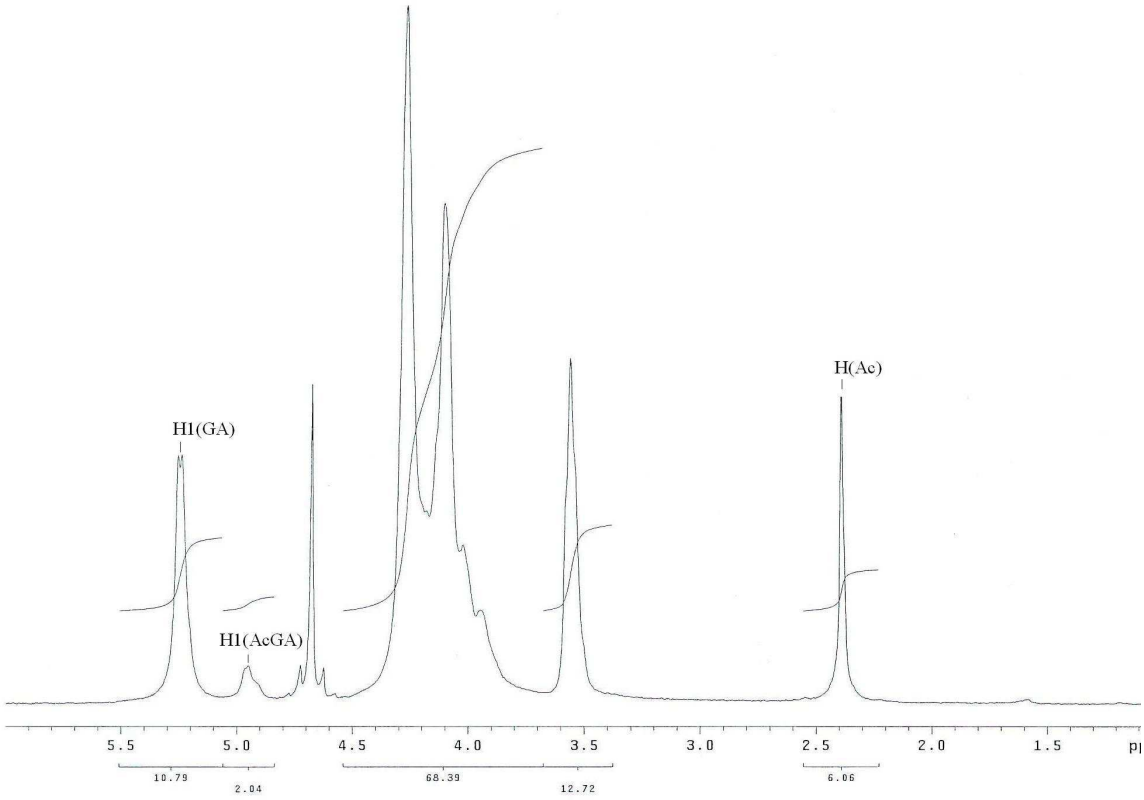

Figure 1 : ^{1}H-NMR spectrum of random chitosan.

Figure 2 displays a quasi-linear relationship between the DDA obtained for the re-acetylated samples and the amount of acetic anhydride used for the reacetylation reaction, within the range of this study.

Equivalent DDA values were also calculated using the peaks of protons H1 of both glucosamine and acetylglucosamine monomers, [H1(GA)] and [H1(AcGA)]. This allowed the confirmation of the absence of O-acetylation during the re-acetylation process. The proton NMR spectroscopy has also been used to confirm the random distribution of the acetyl groups on the polymer chain, using the region of resonance of anomeric protons according to the method described by Vårum *et al*[4].

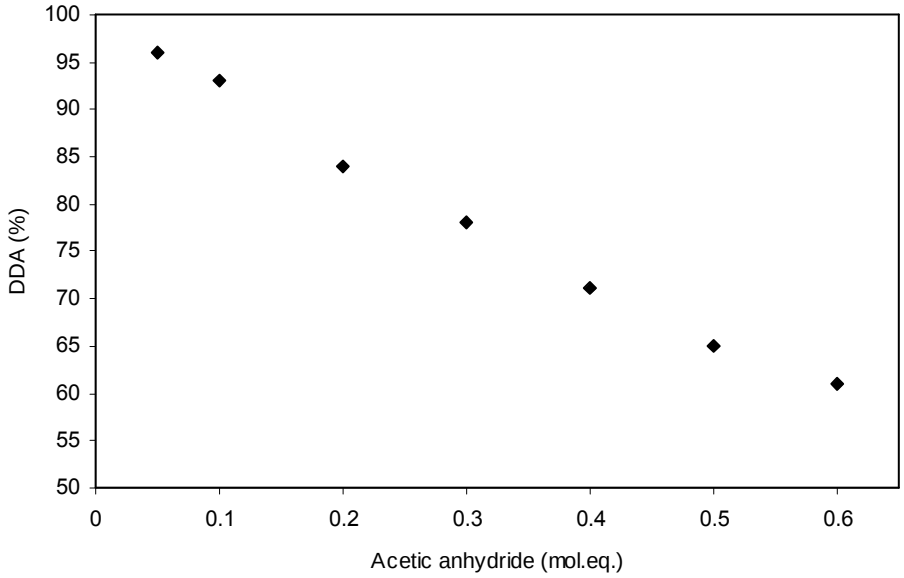

Figure 2 : DDA of the re-acetylated chitosan samples as function of the acetic anhydride ratio.

Dissolution tests showed that the re-acetylated samples could be dissolved in less acid than the heterogeneously deacetylated chitosan samples, due to the uniform distribution of positive charges along the chitosan chains, which is further evidence of a random acetyl group distribution. Figure 3 depicts the volume of HCl (0.1M) needed for complete dissolution of 0.1g of re-acetylated chitosan and the resulting pH for each solution depending on the DDA of the chitosan. All solutions were completed with water in order to obtain a final concentration of 2% in chitosan.

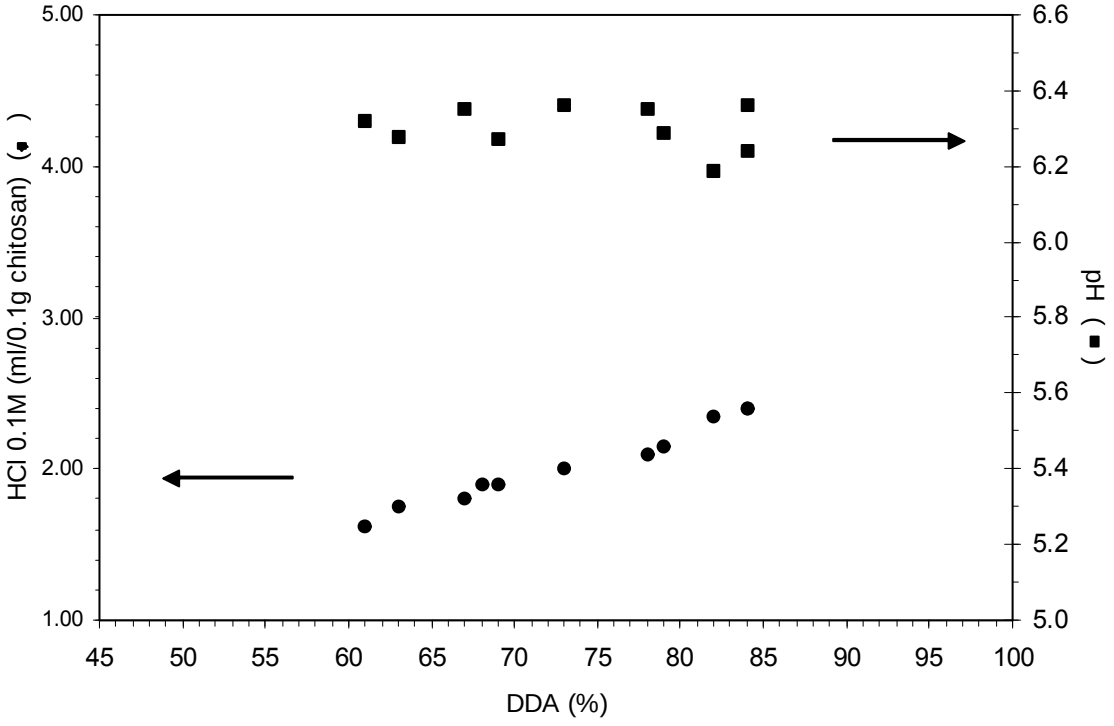

Figure 3 : Volume of HCl needed to dissolve 0.1g of polymer and pH of 2% solution versus the chitosan DDA.

Characterization of chitosan-GP gelling system

The chitosan-GP systems prepared with re-acetylated chitosan (random distribution of the acetyl groups) were characterized and their properties have been compared with those of systems prepared with deacetylated chitosan (block distribution of the acetyl groups)[9,10]. Figure 4 shows chitosan-GP samples in solution and hydrogel state at 37°C. It clearly indicates that a chitosan-GP hydrogel prepared with re-acetylated chitosan (random distribution) is optically transparent, in contrast to that prepared with block chitosan, which is optically translucent or turbid. We found that using random chitosan leads to transparent chitosan-GP hydrogels regardless of chitosan DDA, within the range of 40 to 86%. This result is different from that reported by Berger *et al.*[16], probably because full deacetylation (100% DDA) of their chitosan was likely not attained. It is believed that the optical transparency is due to the high number and the size of micro-domains where junctions formed during the gelation process. Indeed, as the numerous micro-domains are much smaller with random chitosan, the light is only slightly scattered when it passes through the hydrogel, and thus appears transparent[18].

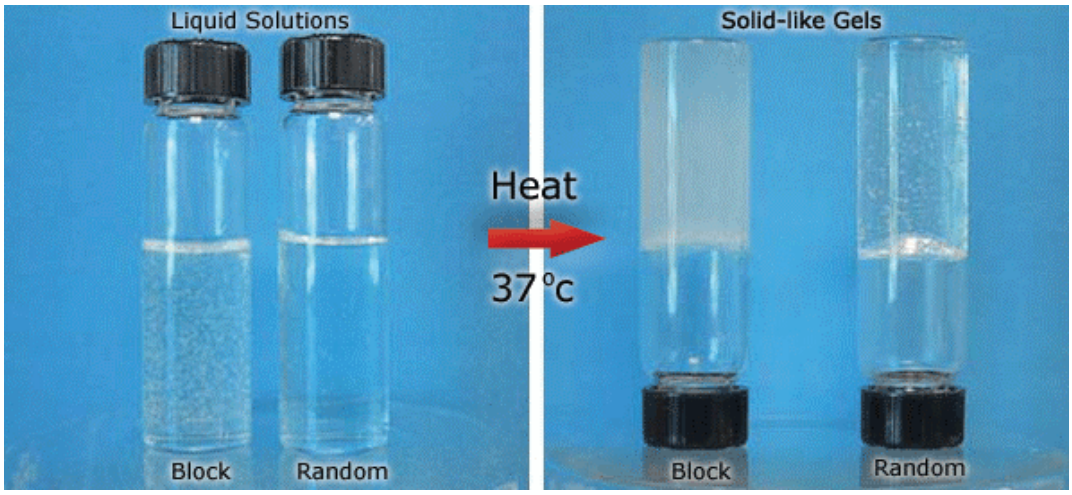

Figure 4 : Optical appearance of chitosan-GP solution and hydrogels prepared with block and random chitosan.

Rheological analysis also demonstrated the differences between chitosan-GP hydrogels prepared with random and block chitosans. Figure 5 shows a representative example of the temperature-dependence of the elastic (G') modulus during the heating-cooling cycle for a chitosan-GP prepared with random chitosan sample (73% DDA). The drastic increase of G' upon heating above 50°C indicates the formation of a hydrogel, which returns to solution upon cooling below room temperature. The hydrogel appears to form around 48°C during the heating, and disappears at 43°C, the temperature below which the hydrogel is completely transformed back to the liquid solution during the cooling. This is manifested as a very narrow hysteresis.

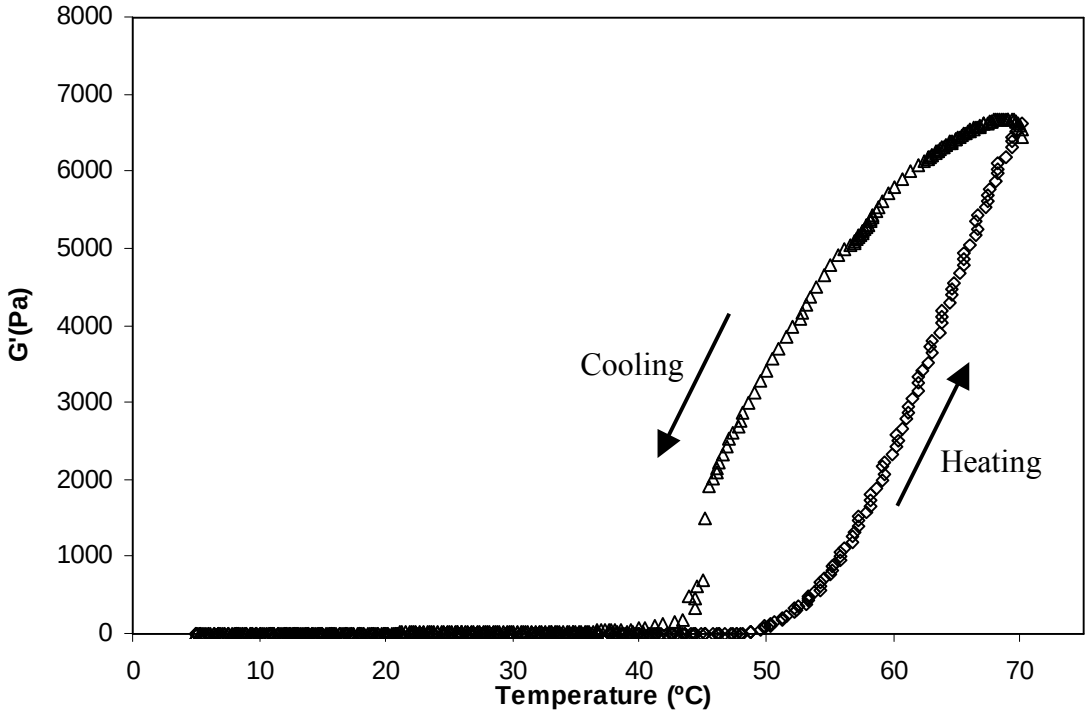

Figure 5 : Temperature dependence of elastic modulus for chitosan-GP (pH ~ 7.0) during heating-cooling cycle between 5 and 70°C.

It is important to note that at this pH value, hydrogels based on block chitosans were only partially thermo-reversible, as previously reported [9]. The complete thermo-reversibility seems to indicate that the micro-domains of junctions are exclusively based on hydrophobic forces, which are well known to strengthen with increased temperatures. However, for a better understanding, it is necessary to probe more extensively into all the forces involved in the formation of chitosan-GP hydrogels.

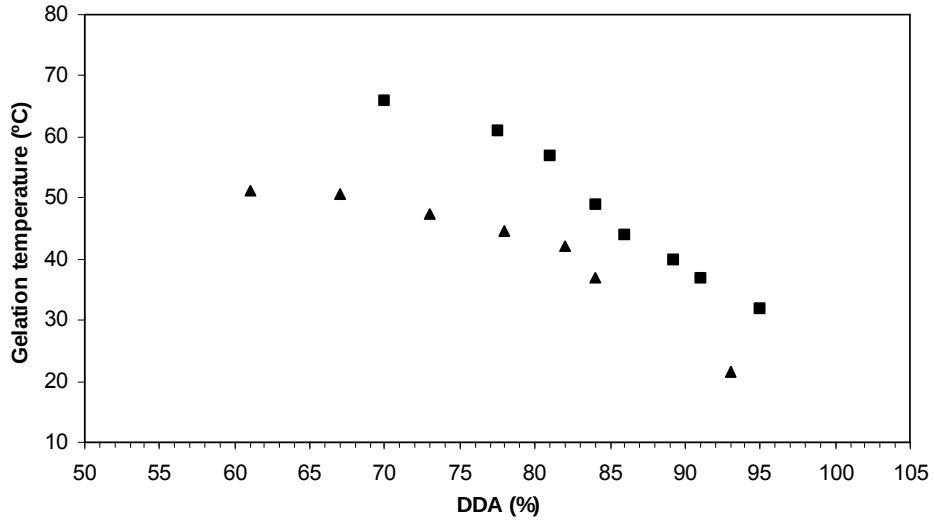

Figure 6 : Gelation temperature of chitosan-GP solution (pH ~ 7.0) as function of DDA of block chitosan (square) and random chitosan (triangle).

Figure 6 shows the dependence of the gelation temperature of chitosan-GP on DDA for both block and random chitosans. It is observed that for a similar DDA, the gelation temperatures of random chitosan hydrogels are always lower than those of hydrogels based on the block chitosan.

Conclusion

The present work demonstrates that the re-acetylation of fully deacetylated chitosan provides chitosan with random acetyl distribution and potentially improved properties. Using this chitosan component within chitosan-GP hydrogels brought several interesting improvements to the gel system, particularly in regards to thermo-sensitivity and optical transparency. The hydrogels are visibly clearer, mechanically stiffer, completely thermo-reversible with a narrow hysteresis and set rapidly at lower temperature in comparison comparison to their block chitosan-GP counterparts. This may render the chitosan-GP hydrogels more attractive for particular applications such as ocular or topical administration.

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