ELSEVIER

Carbohydrate Polymers 46 (2001) 39–47

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

# Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions

A. Chenite[a,*], M. Buschmann[b], D. Wang[a], C. Chaput[a], N. Kandani[c]

[a]*BIOSYNTECH Limited, 475 Armand Frappier Bd., Laval TechnoPark, Montreal (Laval), PQ, Canada H7V 4B3*
[b]*Department of Chemical Engineering and Institute of Biomedical Engineering, Ecole Polytechnique, Montreal, PQ, Canada H3C 3A7*
[c]*Département de Chimie, Faculté des Sciences Semlalia, Université Kadi Ayad, Marrakech, Maroc*

Received 14 June 2000; revised 25 July 2000; accepted 28 July 2000

**Abstract**

In this study we demonstrate that chitosan solutions can be neutralised up to physiological pH (~7.2) using β-glycerol phosphate without creating immediate gel-like precipitation and furthermore that subsequent heating of these solutions induces hydrogel formation. The addition of the particular basic salt, glycerol phosphate, provides the correct buffering and other physicochemical conditions including control of hydrophobic interactions and hydrogen bonding which are necessary to retain chitosan in solution at neutral pH near 4°C and furthermore to allow gel formation upon heating to 37°C. Rheological investigation evidenced the endothermic gelation of chitosan/β-glycerol phosphate solutions and allowed the establishment of a sol/gel diagram. The gelation process appears to be governed by delicate interplay between the pH and the temperature. The role of β-glycerol phosphate is discussed in the light of relevant literature particularly those indicating the role of glycerol and polyols in the stabilisation of proteins and polysaccharides. 

*Keywords*: Chitosan; Glycerol phosphate; Rheological measurements

## 1. Introduction

Chitosan is an aminopolysaccharide obtained by alkaline deacetylation of chitin, a cellulose-like polymer present in fungal cell walls and exoskeletons of arthropods such as insects, crabs, shrimps, lobsters and other vertebrates (Muzzarelli, 1977). Chitosan is a biodegradable (Struszczyk, Wawro, & Niekraszewicz, 1991), biocompatible (Chandy & Sharma, 1990; Hirano, Seino, Akiyama, & Nonaka, 1990a) and mucoadhesive (He, Davis, & Illum, 1998; Henriksen, Green, Smart, Smistad, & Karlsen, 1996; Lehr, Bouwstra, Schacht, & Junginger, 1992) biopolymer, which is emerging to play a significant role in biomedical applications (Felt, Buri, & Gurny, 1998; Illum, 1998; Madhavan, 1992; Malette, Quigley, Gaines, Johnson, & Rainer, 1983; Sandford, 1988), due to its abundance and wide scope of use. Chitosan has been recommended as an appropriate material for many purposes in pharmaceutical, medical and food industries, where numerous international patents have claimed applications of chitosan in these areas (Nordquist, 1998).

The term chitosan is commonly used to describe a series of chitosan polymers with various weight average molecular weights ($50\,\text{kDa} \leq \bar{M}_w \leq 2000\,\text{kDa}$) and degrees of deacetylation ($40 < \text{DDA} < 98\%$). Chitosan is typically not soluble in water, but chitosan solutions can be obtained in acidic aqueous media which protonate chitosan amino groups, rendering the polymer positively charged and thereby overcoming associative forces between chains. When adding a strong base (i.e. NaOH) to such solutions, chitosan remains in solution up to a pH in the vicinity of 6.2. Further basification, to pH > 6.2, systematically leads to the formation of a hydrated gel-like precipitate. This precipitation, or gel formation, is due to the neutralisation of chitosan amine groups and the consequent removal of repulsive interchain electrostatic forces which subsequently allows for extensive hydrogen bonding and hydrophobic interactions between chains. The inability to maintain chitosan in solution up to a physiological pH in the region of 7.0–7.4, has been the main obstacle to date in the development of certain biomedical applications of chitosan, for example as an encapsulating or delivery system for living cells or for pH-sensitive proteins. In the context of the current study, it

* Corresponding author. Tel: +1-450-686-2437, ext. 232; fax: +1-450-686-8952.
*E-mail address:* chenite@biosyntech.com (A. Chenite).

0144-8617/01/$ - see front matter 
PII: S0144-8617(00)00281-2

is important to note a significant exception to the above general description of the solubility behaviour of chitosan — chitosans with a relatively low DDA, from 40 to 60%, remain in solution up to a pH near 9, and are therefore not the subject of investigation in the current work.

Here we report the preparation and characterisation of thermogelling chitosan solutions formulated at conditions including physiological pH. The endothermically gelling chitosan solution is prepared by supplementing an aqueous solution of chitosan with glycerophosphate salt, an additive which plays three essential roles: (1) to increase the pH into the physiological range of 7.0–7.4; (2) to prevent immediate precipitation or gelation; and (3) to allow for controlled hydrogel formation when an increase in temperature is imposed. Our results suggest that the addition of this particular basic salt provides the correct buffering and other physicochemical conditions including control of hydrophobic interactions and hydrogen bonding which are necessary to retain chitosan in solution at neutral pH and furthermore to allow gel formation upon heating to 37°C. This system is likely to receive considerable attention in the biomedical field, since such liquid polymer solutions can be loaded with therapeutic materials at low but non-freezing temperatures, and then injected into body sites to form degradable gel implants in situ (Chenite et al., 1999). An additional physicochemical characteristic of chitosan bodes well for its use as a scaffold or carrier system in tissue regeneration and repair and local drug and gene delivery. Namely the anionic nature of most human tissues due to the presence of glycosaminoglycans in the extracellular matrix, and the cationic character of chitosan, (at pH $\approx$ 7.2, approximately 17% of amino groups are still protonated), allows for adherence of these thermally gelling solutions to tissue sites. Recently we have also shown that some formulations can be prepared to have physiological pH and osmotic pressure and to thereby offer a suitable micro-environment for living cells to maintain functional characteristics after injection and implant formation (Hoamann, Sun, Binette, McKee, & Buschmann, 2000).

## 2. Materials

Medium molecular weight chitosan ($\bar{M}_w \approx 3.5 \times 10^5$) with a high degree of deacetylation (DDA $\sim$ 91%), was generously provided by Maypro (Purchase, NY, USA). The *weight* average molecular weight and the DDA of chitosan were determined by using size exclusion chromatography (SEC) and $^{13}$C-NMR spectroscopy, respectively. For SEC we used a HP1100 chromatograph equipped with WAT011545 column connected to WAT011565 guard column in series (both from Waters Inc., Milford, MA), with an on-line detection obtained with G1362A differential refractometer. $^{13}$C-NMR was performed using a CMX-300 NMR spectrometer operating at 75.4 MHz and room temperature. The DDA was determined following the previously described procedure (Pelletier, Lemire, Sygusch, Chornet, & Overend, 1990).

Hydrated β-glycerophosphate disodium salt (β-GP), ($C_3H_7O_3PO_3Na_25H_2O$; $M_W = 306$), was purchased from Sigma-Aldrich Cie, USA.

### 2.1. Preparation of thermogelling solutions

Clear solutions of chitosan were obtained by dissolving 200 and 400 mg of chitosan in 18 ml of aqueous hydrochloride solutions, 0.05 and 0.1 M, respectively. A series of β-GP solutions were prepared by dissolving from 0 to 1.6 g of β-GP in $\sim$1 ml deionised water and the final volume made up to 2 ml with deionised water. The chitosan solutions were cooled down to $\sim$4°C and continuously stirred while adding drop by drop 2 ml of the β-GP solution. Thus the final 20 ml solutions contained 1 or 2% (w/v) of chitosan corresponding to 0.055 or 0.110 M of amine groups, taking into account the DDA of 91%, and a concentration of β-GP ranging from 0 to 0.262 M.

### 2.2. Turbidity measurements

Turbidity of chitosan and chitosan/β-GP samples was monitored using an LP2000 turbidity Meter (Hanna Instruments), covering a 0–1000 FTU range (FTU, Formazide Turbidity Unit). Aliquots (10 ml) of chitosan and chitosan/β-GP solutions were poured into cuvets and incubated at 37°C. The turbidimeter uses an infrared beam with a wavelength peaking at 890 nm and performs according to the ISO 7027 International Standard. It was calibrated by using two AMCO AEPA-1 standard solutions having 0 and 10 FTU.

### 2.3. Rheological analysis

Rheological measurements were performed on a Bohlin CVO rheometer (Bohlin Instruments, Inc. Grandbury, NJ) using C25 concentric cylinders. Solution aliquots of 12 ml were introduced between the concentric cylinders and then covered with mineral oil in order to prevent evaporation during the measurements. The values of the strain amplitude were verified in order to ensure that all measurements were performed within the linear viscoelastic region, such that the storage modulus ($G'$) and loss modulus ($G''$) were independent of the strain amplitude. Frequency dependent $G'$ and $G''$ of solutions and gels were measured in the frequency range between 0.05 and 100 Hz, at controlled constant temperatures of 10°C (solution) and 37°C (gel). To determine gelation temperature, oscillatory measurements were performed at 1 Hz, while the temperature was increased at the rate of 1°C/min between 4 and 70°C. The gelation temperature was determined as the temperature at which both $G'$ and $G''$ followed a power law ($G' \propto \omega^n$ and $G'' \propto \omega^n$) with the same exponent $n$. The value of $n$ was found to be $\sim$0.48, in accordance with the analysis of Chambon and Winter (1987) and Winter and Chambon (1986). To

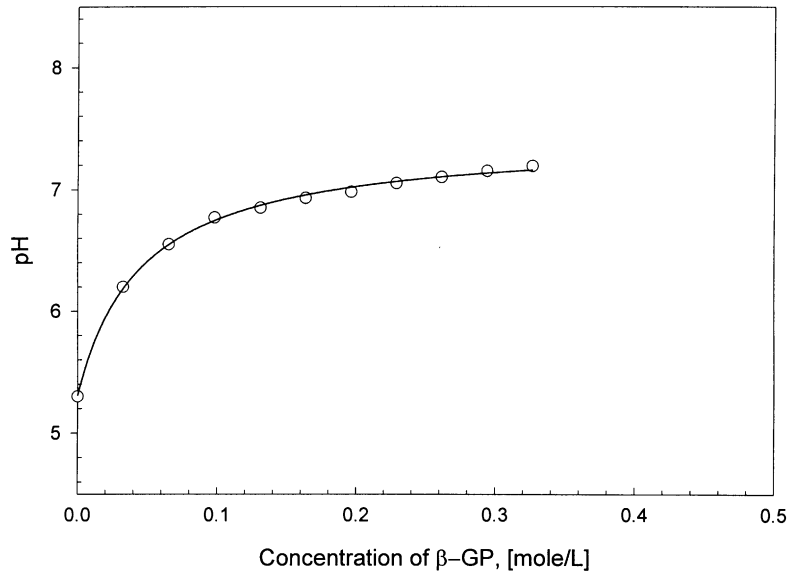


Fig. 1. pH Variation of chitosan solution (2%) as function of β-GP concentration, at room temperature.

determine gelation time, oscillatory measurements at 1 Hz were started just after introducing cold solutions, between 4 and 10°C, into the rheometer chamber which was pre-equilibrated at the desired temperature in the range 30–50°C. The temporal evolution of $G'$ and $G''$ was thereby measured at constant temperature. Gelation time was also determined as the time when both $G'$ and $G''$ followed a power law ($G' \propto \omega^n$ and $G'' \propto \omega^n$) with the same exponent $n$.

## 3. Results and discussion

Chitosan solutions can be neutralised to pH values between 6.5 and 7.3 via β-GP addition, without inducing immediate precipitation or gelation, provided the temperature is maintained between 4 and 15°C. The pH of these chitosan solutions approached the physiological region when the molar concentration of β-GP exceeded the molar concentration of the amine groups of chitosan (0.110 M in Fig. 1). This ability to maintain chitosan in solution and prevent chain aggregation at neutral or nearly neutral pH is in part due to the mild alkalinity of β-GP ($pK_{a2} = 6.34$) and possibly also due to the presence of the glycerol moiety in β-GP molecules, potentially coating the chitosan polymers and inhibiting chain to chain aggregation. Most importantly, chitosan/β-GP solutions were found to be

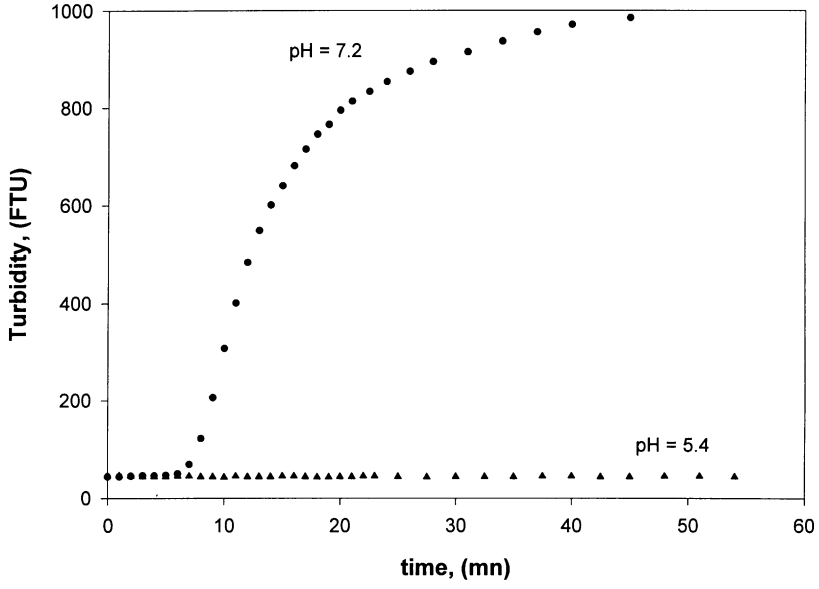


Fig. 2. Turbidity changes with the time of chitosan solutions (2%) incubated at 37°C: (a) in the absence of β-GP at pH ~5.4; and (b) in the presence of β-GP (0.262 M) at pH ~7.2.

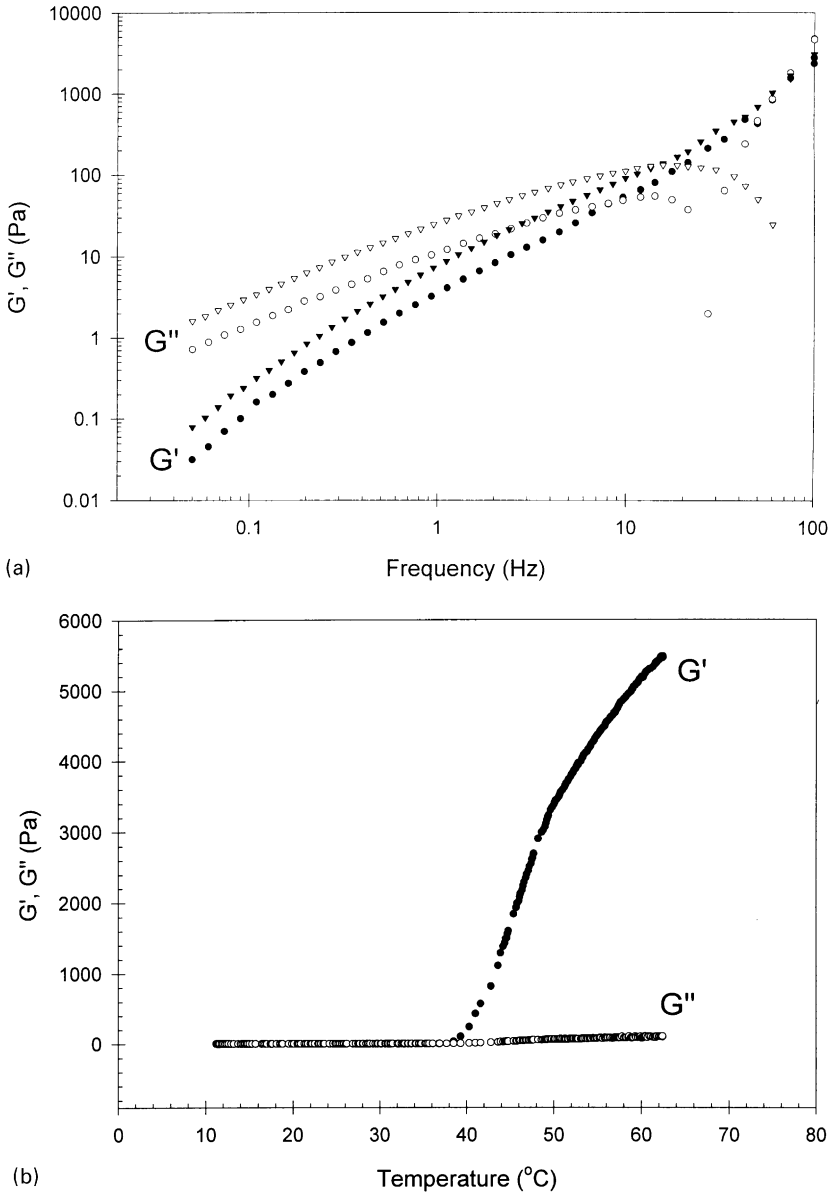


Fig. 3. (a) Frequency-dependence of elastic ($G'$) and viscous ($G''$) modulus of chitosan/β-GP solution (chitosan, 2%; β-GP, 0.262 M; pH ~7.2) (filled and open circles) and of chitosan solution (chitosan, 2%; pH ~5.4) (filled and open triangles), both measured at a low temperature of 10°C. (b) Temperature-dependence of elastic ($G'$) and viscous ($G''$) modulus for chitosan/β-GP solution [chitosan, 2%; β-GP, 0.262 M; pH ~7.2], upon heating from 5 to 70°C. (c) Frequency dependence, at constant temperature (37°C), of the elastic ($G'$) and viscous ($G''$) modulus for chitosan/β-GP solution (chitosan, 2%; β-GP, 0.262 M) previously gelled by heating in (b).

thermosensitive since heating from 4 to 37°C and above induced gelation, visualised by an increase in turbidity which was only present when β-GP was added (Fig. 2). Additionally, the chitosan used in this study was of a high degree of deacetylation (91%) which has been found previously to precipitate below a pH of 6 (Skaugrud, Hagen, Borgersen, & Dornish, 1996).

### *3.1. Rheological characterisation of the temperature-dependent gelation process*

Chitosan solutions with or without added β-GP displayed typical semi-dilute (Nyström, Walderhaug, Hansen, & Lindman, 1995) solution rheological behaviour when measured at low temperature, ~10°C (Fig. 3a). Notably, rheological properties measured at low temperature (~10°C), following the addition of β-GP and attainment of a pH of ~7.15 reduced both $G'$ and $G''$ compared to chitosan alone, potentially due to charge neutralisation and resulting increased flexibility of chitosan polymers, or due to β-GP impeding chitosan/chitosan chain to chain interactions (Fig. 3a). Upon heating, however, from 5 to 70°C, a rapid increase of $G'$ indicated a temperature of incipient gelation near 37°C (Fig. 3b). After incubation at

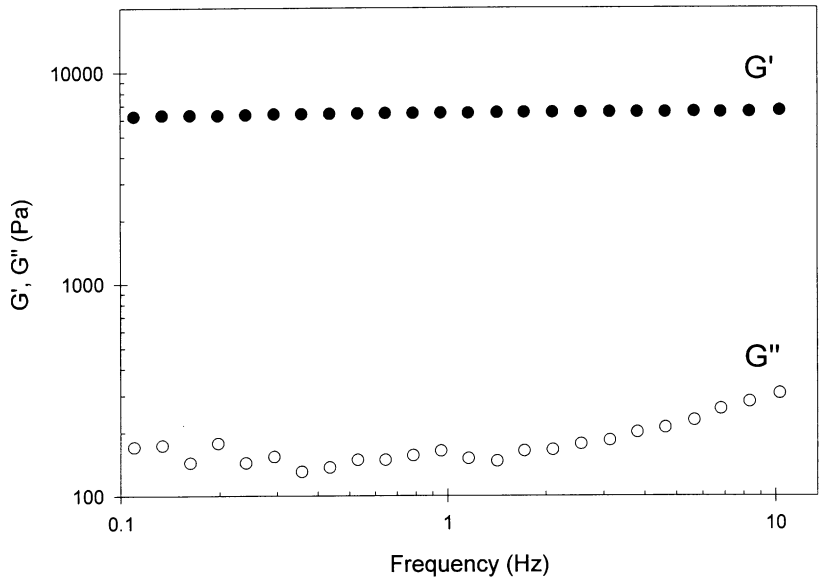


Fig. 3. (*continued*)

37°C for at least 60 min, rheological measurements indicated a nearly frequency independent $G'$, while $G''$ increased slightly with the frequency as is characteristic for hydrogel materials (Clark, Richardson, Ross-Murphy, & Stubbs, 1983; Nishinari, 1997; Nyström et al., 1995) (Fig. 3c). The strength of the gel can be appreciated by the magnitude of $G'$ in the range of several kPa, and by the great difference between $G'$ and $G''$, also indicating a strong gel in the present case with $G' \gg G''$.

The neutralisation behaviour of chitosan/β-GP solutions is evidently a central characteristic which determines their solubility and phase transition phenomena. Gelation temperature, for example, determined by rheological measurements, is greatly affected by the pH of a prepared chitosan/β-GP solution (Fig. 4). A solution composed of 2% chitosan with a pH of 7.2 gels at ~37°C, while slight acidification to pH 6.85 increases the gelation temperature to near 50°C. This monotonic decrease of gelation temperature with increasing pH (Fig. 4) suggests that the number of charged ammonium groups on the chitosan chain is an important parameter controlling gelation in this system. A reduction in charge density on the chitosan chain appears to reduce interchain electrostatic repulsion and permit a smaller addition of thermal energy to initiate gelation.

The temporal evolution of $G'$ and $G''$, measured for a chitosan/β-GP solution composed of 2% of chitosan

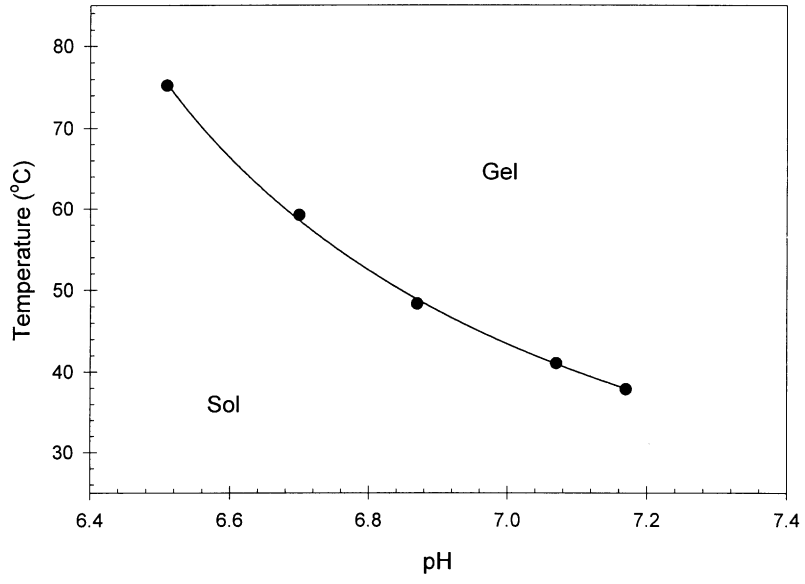


Fig. 4. Gelation temperature as function of the pH of the chitosan solution (chitosan, 2%; β-GP, 0.262 M). The pH differences were generated by altering the concentration of HCl solution used to dissolve a constant amount of chitosan.

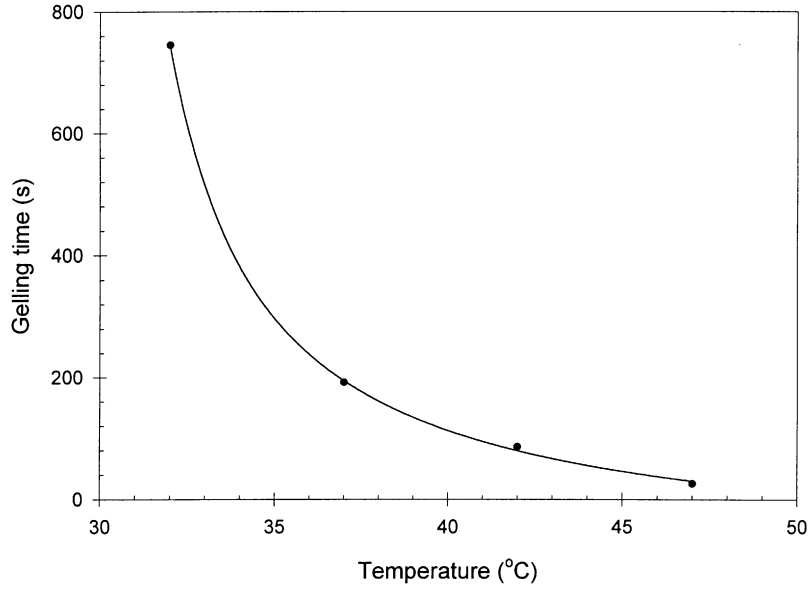


Fig. 5. Temperature-dependence of gelation time of chitosan/β-GP solution (chitosan, 2%; β-GP, 0.262 M; pH ~ 7.2).

(pH = 7.2) at various temperatures, allowed the determination of the dependence of gelling time on temperature (Fig. 5). Gelling time appeared to display an exponential decrease with temperature varying from 13 min at 32°C to 2 min at 42°C for this formulation of chitosan/β-GP solutions. Increasing temperature or addition of heat is therefore a factor that also accelerates the gelation process probably via the acceleration of the formation of junction zones of the polymers. Thus taking together these observations of a dependence of gelation temperature on pH (Fig. 4) and of gelation time on temperature (Fig. 5), the gelation process of chitosan/β-GP solutions appears to be governed by a coupling between pH, temperature and the neutralisation degree of the chitosan chain in the presence of glycerol phosphate.

We have also investigated the influence of the concentration of β-GP on gelation temperature for chitosan solutions of two different polymer concentrations (Fig. 6). Gelation temperature was determined by rheology as described for the dependence of gelation temperature on pH. The resulting sol/gel diagram (Fig. 6) indicates a decrease in gelation temperature when the concentration of β-GP is increased for both 1 and 2% (w/v) chitosan solutions. The similarity with the dependence of gelation temperature on pH (Fig. 4) is expected since β-GP addition increases the pH. For both 1 and 2% chitosan, gelation occurs in a region of β-GP

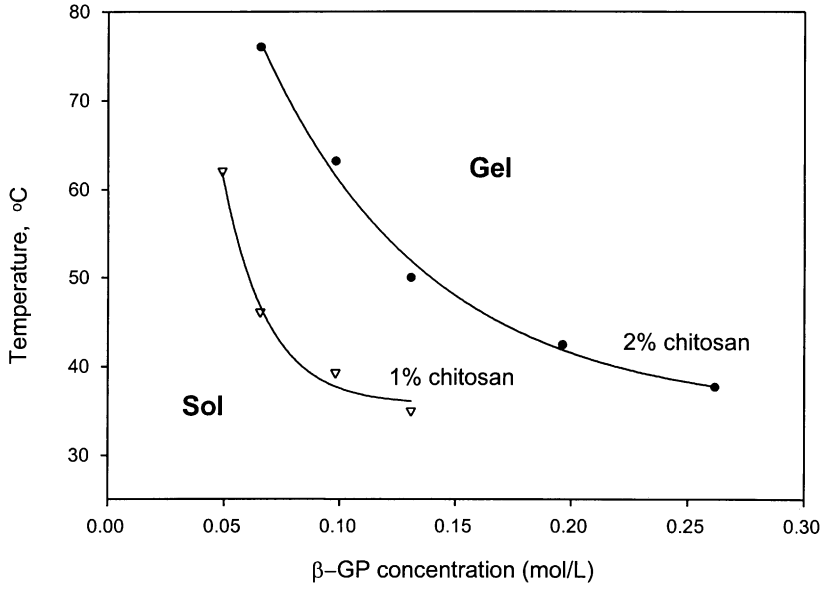


Fig. 6. Sol/gel diagram of chitosan solutions of 1 and 2% w/v concentration, in the presence of varying amounts of β-GP.

concentration corresponding to 1–2 × the molar concentration of amine groups of chitosan (~0.055 M for 1% chitosan and ~0.110 M for 2% chitosan). The profile of gelation temperature versus β-GP concentration is therefore similar for the two polymer concentrations used, with a displacement of approximately the molar concentration difference of polymer amine groups. This is evidently the result of a charge (proton) transfer from chitosan to β-GP upon the addition of the latter, resulting in a decreasing charge density on the polymer chain at higher β-GP concentrations. The amount of β-GP required to achieve a given chain charge density is roughly proportional to the polymer concentration.

It is worthwhile to point out that the results shown here were obtained on highly deacetylated chitosan (DDA of 91%) having an average medium molecular weight of about 450 kD. Since the properties of chitosan solutions depend greatly on these two chemical characteristics, we investigated the influence of these two parameters on the formulation of chitosan/β-GP solutions at physiological pH, as well as their thermal gelation. We found that the temperature of incipient gelation increases as the degree of deacetylation decreases, while the molecular weight showed no significant effect on the temperature of gelation (Chenite et al., 2000). Moreover, for chitosan deacetylated to about 95%, the gelation temperature has been lowered to around 34°C.

### 3.2. Potential mechanisms of gelation

Our current study has revealed several important consequences of β-GP addition to chitosan solutions, in particular the maintenance of chitosan solubility at physiological pH and the temperature-sensitive character of these chitosan/β-GP solutions allowing for rapid hydrogel formation upon heating. We have furthermore demonstrated a clear interdependence of pH, β-GP concentration, and temperature, all of which significantly affect gel formation. This study does not provide sufficient data to allow a complete molecular description of the mechanism of gelation to be proposed, however some indications of important parameters and molecular interactions can be gleaned from our results and from published literature for other thermally sensitive polymer systems. In considering molecular mechanisms of gelation for these systems it is important to keep in mind the broad range of molecular interactions which can occur in aqueous solutions of the cationic polyelectrolyte chitosan and the divalent anionic base glycerol phosphate including: (1) electrostatic repulsion between like-charged chitosan chains; (2) electrostatic attraction between oppositely charged chitosan and the phosphate moiety of β-GP; (3) attractive hydrophobic and hydrogen bonding between chitosan chains; and (4) the hydrophobic or water-structuring character of the glycerol moiety of β-GP. The precipitation of chitosan upon increasing the pH above a critical value (such as ~6.2 when using a strong base) can, for example, be explained by a reduction of charge density along the polymer backbone reducing interchain electrostatic repulsion and allowing the attractive hydrophobic and hydrogen-bonding forces to predominate and precipitate chitosan. Addition of β-GP rather than a strong base maintains solubility of chitosan to a much higher pH (~7.2) probably due to its mild basic character and potentially due to an attraction of phosphate moieties of β-GP to remaining charged amine groups ($NH_3^+$) of chitosan, and thereby exposing the glycerol moiety to separate chitosan chains in solution and maintain its solubility at low temperature. Upon heating of these chitosan/β-GP solutions, physical junction zones of chitosan chain segments throughout the solution occur to form a hydrogel, necessarily by inducing a sudden preponderance of attractive hydrophobic and hydrogen bonding forces over interchain electrostatic repulsion. This thermally induced shift in attractive versus repulsive interchain forces could arise from many sources including: (1) reduced chitosan chain polarity and increased hydrophobicity upon heating; (2) reduced polarity and increased structuring of free water by the glycerol moeity of β-GP thus dehydrating chitosan chains and also causing increased interchain hydrophobic attraction; and (3) a thermally induced transfer of protons from chitosan amine groups to the phosphate moeity of β-GP thereby further reducing both chain charge density and chitosan attraction to β-GP and allowing for preponderance of attractive interchain hydrophobic and hydrogen-bonding forces between chains. Importantly we may exclude the purely ionic cross-linking that is responsible for the gelation of chitosan aqueous solutions with other divalent anions including oxalate (Hirano, Yamaguchi, Fukui, & Iwata, 1990b), molybdate (Draget, Vårum, Moen, Gynnild, & SmidsrØd, 1992), sulphate or phosphate ions, since such ions induce immediate temperature-insensitive precipitation and at a relatively acid pH where the chitosan retains it positive charge. Furthermore, we have also found that β-GP is freely diffusible after gelation and is not retained in the physically cross-linked network (Filion et al., in preparation). Finally, the contribution of hydrogen bonding versus hydrophobic forces could be pH-dependent. Unlike hydrogen bonds, hydrophobic forces are known to be temperature-dependent and were suggested to be a source of the thermoreversibility found previously in chitosan/β-GP gels (Chenite et al., 2000).

Evidence can be found in the literature to support the occurrence of these temperature-induced alterations of molecular interactions in chitosan/β-GP solutions listed above. First, considering the polarity of the chitosan chain, poly(ethylene oxide) (PEO) solutions are known to become less soluble and precipitate at higher temperatures in aqueous solutions, potentially due to a conformational transition to a less-polar form (Saeki, Kuwahara, Nakata, & Kaneko, 1976). Polysaccharides can also be considered as ethylene oxide containing polymers, as well as some cellulose derivatives which have demonstrated reduced

solubility in aqueous media upon heating (Karlström, Carlsson, & Lindman, 1990; Sarkar, 1979). Moreover, Park, Choi, and Park (1983) have suggested that at the precipitating pH of chitosan, a conformational change occurs allowing $NH_2$ groups to form intermolecular hydrogen bonds aiding the formation of a hydrated precipitate. Secondly, concerning the role of the glycerol moiety of glycerol phosphate, it has been previously shown that polyols and sugars can stabilise proteins against denaturation due to their structuring effect on water molecules, thereby strengthening protein–protein hydrophobic interactions (Back, Oakenfull, & Smith, 1979; Gekko & Koga, 1983; Gekko, Mugishima, & Koga, 1987; Gekko & Timasheff, 1981; Na, Butz, Bailey, & Carroll, 1986). Gekko and Koga (1983) and Gekko and Timasheff (1981) have additionally shown that the addition of polyols to aqueous solutions of collagen or carrageenan raise the transition temperature from gel to sol upon heating indicating that the initial gel structure was reinforced by the presence of polyols, requiring more thermal energy to disrupt it. Finally, modulation of electrostatic repulsion between chains by controlling chain charge density is the mechanism by which the solution pH controls the state of chitosan. It is thus probable that one effect of temperature on chitosan/β-GP solutions is also to alter the chitosan chain charge density, potentially by reducing it upon heating via intrinsic temperature dependence of the various $pK_a$s or via conformation-charge coupling (conformational dependence of the polyelectrolyte electrostatic free-energy). The presence of divalent ions in solution adds yet another interesting aspect due to the strong pair correlations between divalent ions (compared to monovalent ions) also reducing interchain electrostatic repulsion (Svensson, Jonsson, & Wodward, 1990). Ongoing studies are underway to ascertain the relative importance of each of these potential gelation mechanisms.

## 4. Conclusions

A thermally gelling chitosan system was prepared by neutralising highly deacetylated chitosan solutions with β-glycerol phosphate to retain chitosan in solution at physiological pH. Upon heating to moderate temperatures, these solutions quickly transformed into a hydrogel structure as demonstrated by rheological measurements. Furthermore, the sol/gel transition temperature was pH-sensitive and gelling time was shown to be temperature-dependent. The molecular mechanism of gelation may involve multiple interactions between chitosan, glycerol phosphate, and water, several of which may be thermally modulated. The ability to prepare these low concentration (1–2% w/v) polymer solutions which gel upon mild heating, for example from 4 to 37°C, and which are biocompatible, biodegradable and adhesive to human tissues, provides for new opportunities in the delivery of sensitive therapeutics. Further studies are now being pursued to elucidate the physicochemical mechanism of gelation as well as to investigate the potential of this system for specific biomedical applications including tissue repair and regeneration, and in the delivery of protein- and gene-based therapeutics.

## References


Back, J. F., Oakenfull, D., & Smith, M. B. (1979). Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry*, *18* (23), 5191–5196.

Chambon, F., & Winter, H. H. (1987). Linear viscoelasticity at the gel point of a crosslinking PDMS with imbalanced stoichiometry. *Journal of Rheology*, *31*, 683–697.

Chandy, T., & Sharma, C. P. (1990). Chitosan as a biomaterial. *Biomaterials Artificial Cells and Artificial Organs*, *18*, 1–24.

Chenite, A., Chaput, C., Combes, C., Jalal, F., & Selmani, A. (1999). *Patent* WO09907416A1.

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C. D., Leroux, J. C., Atkinson, B. L., Binette, F., & Selmani, A. (2000). Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*, *21*, 2155–2161.

Clark, A. H., Richardson, R. K., Ross-Murphy, S. B., & Stubbs, J. M. (1983). Structural and mechanical properties of agar/gelatin co-gels. Small-deformation studies. *Macromolecules*, *16*, 1367–1374.

Draget, K. I., Vårum, K. M., Moen, E., Gynnild, H., & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions: a new gelling system. *Biomaterials*, *13*, 635–638.

Felt, O., Buri, P., & Gurny, R. (1998). Chitosan: a unique polysaccharide for drug delivery. *Drug Development and Industrial Pharmacy*, *24*, 979–993.

Filion, D., Lavertu, M., Chenite, A., & Buschmann, M., in preparation.

Gekko, K., & Koga, S. (1983). Increased thermal stability of collagen in the presence of sugars and polyols. *Journal of Biochemistry*, *94*, 199–205.

Gekko, K., Mugishima, H., & Koga, S. (1987). Effects of sugars and polyols on the sol–gel transition of κ-carrageenan: calorimetric study. *International Journal of Biological Macromolecules*, *9*, 146–152.

Gekko, K., & Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol–water mixtures. *Biochemistry*, *20*, 4667–4676.

He, P., Davis, S. S., & Illum, L. (1998). In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *International Journal of Pharmaceutics*, *166*, 75–88.

Henriksen, I., Green, K. L., Smart, J. D., Smistad, G., & Karlsen, J. (1996). Bioadhesion of hydrated chitosans: an in vitro study. *International Journal of Pharmaceutics*, *145*, 231–240.

Hirano, S., Seino, H., Akiyama, Y., & Nonaka, I. (1990a). In C. G. Gebelein & R. L. Dunn, *Progress in biomedical polymers* (p. 283). New York: Plenum Press.

Hirano, S., Yamaguchi, R., Fukui, N., & Iwata, M. (1990b). A chitosan oxalate gel: its conversion to an *N*-acetylchitosan gel via a chitosan gel. *Carbohydrate Research*, *201*, 145–149.

Hoamann, C. D., Sun, J., Binette F., McKee, M. D., & Buschmann, M. D., (2000). In *Federation of European Connective Tissue Societies*, 1–5 July, Patras, Greece.

Illum, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharmaceutical Research*, *15*, 1326–1331.

Karlström, G., Carlsson, A., & Lindman, B. (1990). Phase diagrams of nonionic polymer–water systems. Experimental and theoreitical studies of the effects of surfactants and other cosolutes. *Journal of Physical Chemistry*, *94*, 5005–5015.

Lehr, C-M., Bouwstra, J. A., Schacht, E. H., & Junginger, H. E. (1992). In

vitro evaluation of mucoadhesive properties of chitosan and other natural polymers. *International Journal of Pharmaceutics*, *78*, 43–48.

Madhavan, P. (1992). *Chitin, chitosan and their novel applications*, Kochi, India: Central Institute of Fisheries Technology.

Malette, W. G., Quigley, H., Gaines, R. D., Johnson, N. D., & Rainer, G. (1983). Chitosan: a new hemostatic. *Annals of Thoracic Surgery*, *36*, 55–58.

Muzzarelli, R. A. A. (1977). *Chitin*, Oxford: Pergamon Press.

Na, G. C., Butz, L. J., Bailey, D. G., & Carroll, R. J. (1986). In vitro collagen fibril assembly in glycerol solution: evidence for a helical co-operative mechanism involving microfibrils. *Biochemistry*, *25*, 958–966.

Nishinari, K. (1997). Rheological and DSC study of sol–gel transition in aqueous dispersions of industrially important polymers and colloids. *Colloid and Polymer Science*, *275*, 1093–1107.

Nordquist, R. E., et al. (1998). *US Patent* 5,747,475.

Nyström, B., Walderhaug, H., Hansen, F. K., & Lindman, B. (1995). Rheological behavior during thermoreversible gelation of aqueous mixture of ethyl(hydrxyethyl)cellulose and surfactants. *Langmuir*, *11*, 750–757.

Park, J. W., Choi, K-H., & Park, K. K. (1983). Acid–base equilibrium and related properties of chitosan. *Bulletin of the Korean Chemical Society*, *4*, 68–72.

Pelletier, A., Lemire, I., Sygusch, J., Chornet, E., & Overend, R. P. (1990). Chitin/chitosan transformation by thermo-mechano-chemical treatment including characterization by enzymatic depolymerization. *Biotechnology and Bioengineering*, *36*, 310–315.

Saeki, S., Kuwahara, N., Nakata, M., & Kaneko, M. (1976). Upper and lower critical solution temperatures in poly(ethylene glycol) solutions. *Polymer*, *17*, 685–689.

Sandford, P. A. (1988). In G. Skjak, T. Anthonsen & P. Sandford, *Chitin and chitosan: sources, chemistry, biochemistry, physical properties and applications* (p. 51). New York: Elsevier.

Sarkar, N. (1979). Thermal gelation properties of methyl and hydroxypropyl methylcellulose. *Journal of Applied Polymer Science*, *24*, 1073–1087.

Skaugrud, Ø., Hagen, A., Borgersen, B., & Dornish, M., (1996). *International Symposium on Marine and Microbial Polysaccharides*, 7–8 November, Trondheim, Norway.

Struszczyk, H., Wawro, D., & Niekraszewicz (1991). In C. J. Brine, P. A. Sandford & J. P. Zikakis, *Advances in chitin and chitosan* (p. 580). London: Elsevier Applied Science.

Svensson, B., Jonsson, B., & Woodward, C. E. (1990). Monte Carlo simulations of an electric double layer. *Journal of Physical Chemistry*, *94*, 2105–2113.

Winter, H. H., & Chambon, F. (1986). Analysis of linear viscoelasticity of crosslinking polymer at gel point. *Journal of Rheology*, *30*, 367–382.