ELSEVIER

International Journal of Pharmaceutics 203 (2000) 89–98

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Characterization of thermosensitive chitosan gels for the sustained delivery of drugs

E. Ruel-Gariépy[a], A. Chenite[b], C. Chaput[b], S. Guirguis[a], J.-C. Leroux[a,*]

[a] *Faculty of Pharmacy, University of Montreal, PO Box 6128 succ. Centre-Ville, Montreal, Quebec, Canada H3C 3J7*
[b] *BioSynTech Limited, 475 Armand-Frappier, Laval, Quebec, Canada H7V 4B3*

Received 18 February 2000; received in revised form 21 April 2000; accepted 24 April 2000

Abstract

The aim of this study was to investigate the physical properties of a chitosan/glycerophosphate (GP) thermosensitive solution which gels at 37°C and evaluate the in vitro release profiles of different model compounds. The gelation rate was dependent on the temperature and on the chitosan deacetylation degree. The solution containing 84%-deacetylated chitosan could be stored 3 months at 4°C without apparent change in viscosity. The in vitro release profiles of the model compounds depended on the presence of GP in the chitosan solution, on their molecular weight and on the presence of lysozyme in the release media. They were not affected by the electrostatic charge of the model compound when present at low concentrations. During the first 4 h, the release was accompanied by a substantial loss of the gel weight which was mainly attributed to the leaching of water and excess GP. Scanning electron micrographs revealed that the solutions yield gels with a highly porous structure after 24 h of exposure to a continuous flow of phosphate buffered saline. These results indicate that the chitosan/GP thermosensitive solutions gel rapidly at body temperature, can remain in the sol state at 4°C and can sustain the delivery of macromolecules.

Keywords: Chitosan; Thermosensitivity; Hydrogel; Sustained-delivery; Macromolecule

1. Introduction

In recent years, advances in biotechnology have resulted in a great variety of new pharmaceutically active molecules such as recombinant peptides and proteins. So far, for the patient, the most effective delivery route for the administration of macromolecules is the parenteral one. Most polymeric systems used for the extravascular parenteral delivery of drugs or vaccines are microspheres (Bittner et al., 1998) or implants (Davis, 1974; Bodmer et al., 1992). In those systems, the active compound is generally encapsulated by using organic solvents or by submitting it to relatively high temperatures which can cause a loss of activity. Moreover, the insert of an implant requires surgery which adds to the costs and the

* Corresponding author. Tel.: +1-514-3436455; fax: +1-514-3432102.
E-mail address: leroujea@pharm.umontreal.ca (J.-C. Leroux).

0378-5173/00/$ - see front matter
PII: S0378-5173(00)00428-2

risks of this system. Those problems oriented research towards injectable thermosensitive in situ gelling formulations. Some polymers in aqueous solutions are known to exhibit temperature-dependent reversible sol–gel transitions. The polymeric solutions can be injected while kept above or below their transition temperature and form a gel as they reach body temperature. The most studied thermosensitive polymers are the poloxamers which are poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers (Johnston and Miller, 1989; Johnston et al., 1992; Pec et al., 1992). Those polymers exist as a mobile viscous liquid at reduced temperatures but form a rigid semisolid gel network with an increase in temperature. Unfortunately, poloxamer gels are obtained at high polymer concentrations only (between 20 and 30%) (Desai and Blanchard, 1998) and have been shown to erode rapidly (Bhardwaj and Blanchard, 1996). Also, poloxamers are not biodegradable and can cause hyperlipidemia in rats (Palmer et al., 1998). Copolymers of *N*-isopropylacrylamide also exhibit a phase transition around body temperature (Hoffman et al., 1986; Hoffman, 1987; Okano et al., 1990; Dinarvand and D'Emanuel, 1995) but they are not biodegradable and toxicity data is scarce. Recently, aqueous solutions of block copolymers of poly(ethylene oxide) and poly(lactic acid) have been described (Jeong et al., 1997). They exist in the sol state at temperatures around 45°C and gel upon cooling to body temperature. Even though those copolymers are biocompatible and biodegradable, the need to heat the solution to incorporate the drug and inject the system makes this approach less practical. Polysaccharides also can form thermosensitive systems. For example, partially degraded xyloglucan forms gels at concentrations between 1 and 2% (w/w) when their temperature is raised above 22–27°C (Miyazaki et al., 1998). As well, an aqueous solution of ethyl (hydroxyethyl) cellulose in the presence of an ionic surfactant can form a physically cross-linked network at temperatures around 30–40°C (Nystrom et al., 1995). The xyloglucan system is interesting because of the very low concentration of polymer involved but the low transition temperature prevents an easy handling of the solution at room temperature. The second polysaccharide system has the advantage of having a transition temperature closer to body temperature but it must be kept in mind that ionic surfactants are tolerated in vivo only at a relatively low dose (Casarett and Doull, 1991).

Chitosan, a polysaccharide derived from crustacean shells by deacetylation of naturally occurring chitin, is currently being investigated for many pharmaceutical applications (Chandy and Sharma, 1990; Illum et al., 1996; Patel and Amiji, 1996). It is a biocompatible and biodegradable copolymer of glucosamine and *N*-acetyl glucosamine (Lee et al., 1995; Miwa et al., 1998; Felt et al., 1999). It has been known to be digestible by lysozyme according to the amount of *N*-acetyl groups and their distribution in the backbone (Machida et al., 1986; Hirano et al., 1989; Aiba 1992; Varum et al., 1996). Chitosan is insoluble at neutral and alkaline pH values. Chitosans with a degree of deacetylation of about 85% have been found to be soluble only up to a pH in the vicinity of 6.2 (Singer and Pittz, 1985). When the pH is brought above this value, the system forms a hydrated gel-like precipitate. We have recently found that when a polyol counterionic monohead salt is used to neutralize the chitosan solution, the latter can remain liquid for long periods of time at or below room temperature. The system can then have a pH value within a physiologically acceptable neutral range (6.8–7.2) and it is only the temperature of the milieu that determines the liquid or gel state; gel formation being observed upon an increase in temperature (Chenite et al., 2000). The aim of this study was to investigate the physical properties of chitosan-based thermosensitive solutions and evaluate the in vitro release profiles of model compounds.

2. Materials and methods

2.1. Materials

The medium molecular weight chitosan was purchased from Aldrich (Milwaukee, WI). The chitosan hydrochlorides Protasan® UP CL213 and UP CL214 were obtained from Pronova Bio-

polymer A.S. (Oslo, Norway). The chitosan hydrochloride CL213 (M_W 421 800) was 84% deacetylated (C84) and the chitosan hydrochloride CL214 (M_W 455 200) was 95% deacetylated (C95). Glycerophosphate containing 50% β-isomer and 50% DL-α-isomer (α-β-GP), β-glycerophosphate (β-GP), calcein, chlorpheniramine maleate, fluoresceine isothiocyanate dextrans (FITC-dextran) and fluoresceine isothiocyanate albumin (FITC-albumin) were from Sigma (St. Louis, MO). Methylene blue and lysozyme were purchased from Fisher (Fairlawn, NJ) and Boehringer Mannheim (Mannheim, Germany), respectively. All other chemicals were reagent grade. All products were used as received. Deionized distilled water from a Milli-Q water system of Millipore (Fisher Scientific Limited, Nepean, On, Canada) was used to prepare all aqueous solutions.

2.2. Preparation of the chitosan/glycerophosphate solution (C/GP solution)

A 1.8% (w/w) chitosan solution was prepared in 0.1 M acetic acid or deionized distilled water with the medium molecular weight chitosan and the chitosan hydrochlorides (C84 and C95), respectively. The chitosan powder was progressively added to the solvent under stirring and mixing was carried on for 1 h. A GP aqueous solution 45% (w/w) was prepared and chilled along with the chitosan solution in an ice bath for 15 min. The cold GP solution was added dropwise to the cold chitosan solution with stirring and the final solution was mixed another 10 min. The final solution contained 7.27% (w/w) GP (Chenite et al., 2000). The α-β-GP was used with the medium molecular weight chitosan and the β-GP was used with the chitosan hydrochlorides because the α-β-GP is incompatible with the chitosan hydrochlorides (Protasan®).

2.3. Rheological measurements

The rheology study was carried out on a Bohlin CVO rheometer (Bohlin Instruments, Cranbury, NJ) equipped with a coaxial-cylinder C-25 geometry in oscillatory mode immediately after preparation of the C/GP solution. The changes in elastic modulus and phase angle were recorded as a function of time at 37 ± 0.1°C. The frequency was fixed at 1.0 Hz during the measurements. The acquisition rate was set up at one point per 13 s. For comparison purposes, the time needed to reach a phase angle of 45° was taken as a measure of the beginning of gelation.

2.4. Stability of C/GP solutions

A C/GP solution using C84 and a C/GP solution using a 1:1 (w/w) mixture of C84 and C95 were prepared. The solutions were stored at either 4°C or room temperature. At different times, the viscosity was measured on a Brookfield RVDV-II + viscosimeter (Brookfield Engineering Laboratories Inc., Stoughton, MA) using a cone-plane CP-52 geometry at 20 ± 0.1°C.

2.5 In vitro release studies

The model compounds were solubilized in deionized distilled water and mixed at different loadings with 3.0 g of chitosan solution with or without GP at room temperature. Samples of 300 mg were placed into continuous flow (10 ml/h) thermostated cells (Merkli et al., 1994) and the release was monitored in phosphate buffered saline (PBS) at 37 ± 0.1°C by spectrophotometry on a Hitachi U-200 spectrophotometer (Hitachi Ltd, Tokyo, Japan) or by spectrofluorimetry on a Aminco-Bowman Series 2 fluorimeter (Spectronics Instruments Inc., Rochester, NY). The area of the gel exposed was 0.8 cm^2.

2.6. Mass loss

C/GP solutions were placed into continuous flow (10 ml/h) thermostated cells under the same conditions used in the release studies. At predetermined time intervals, the gels were taken out from the cells, gently blotted and weighed. Then, they were dried in an oven at 80°C for 48 h and weighed again.

2.7. Scanning electron microscopy (SEM)

A C/GP solution using C84 or a 1:1 (w/w) mixture of C84 and C95 was prepared. Samples (300 mg) were placed into continuous flow (10 ml/h) thermostated cells under the same conditions used for the release studies. At predetermined time intervals, the gels were removed, gently rinsed with distilled water, frozen in liquid nitrogen and freeze-dried for 48 h (Freezone, Labconco, Kansas City, MO). The samples were coated with gold/paladium and the surfaces were examined using a Jeol JSM840 electron microscope (Jeol, Boston, MA).

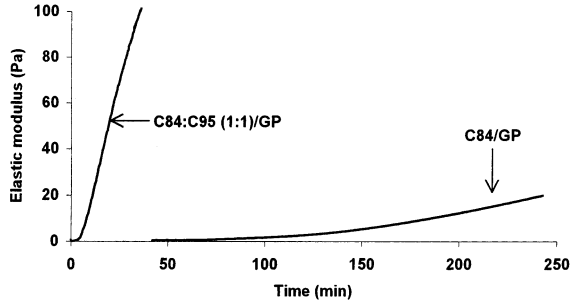

Fig. 1. Elastic modulus as a function of time at 37 ± 0.1°C for two different C/GP solutions. The frequency of oscillation is 1 Hz and the acquisition rate is 1 point every 13 s.

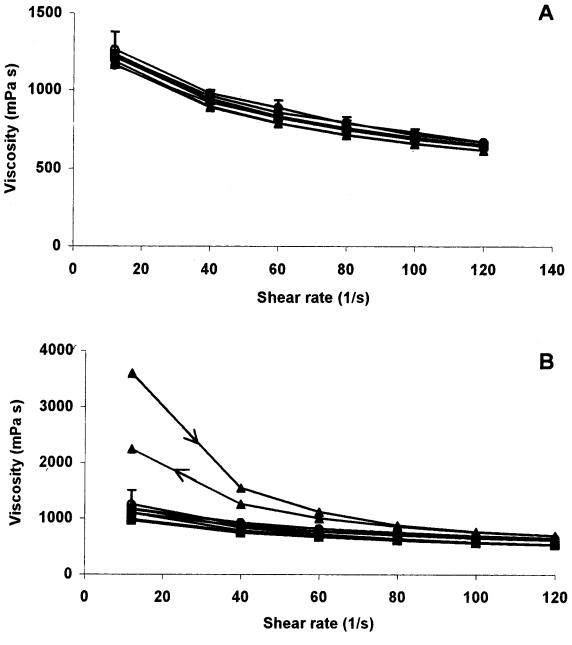

Fig. 2. Viscosity at 20 ± 0.1°C as a function of shear rate for a C84/GP solution depending on the time of storage at 4°C (A) or room temperature (B). After 1 day (●), 7 days (○), 30 days (■), 60 days (□) and 90 days (▲). Each point represents the mean value ± SEM from three measurements.

2.8. Statistical analysis

Data are reported as mean ± SEM. Statistical significance ($P < 0.05$) was determined using one-way ANOVA and Tukey Pairwise Comparison or Student's t-test.

3. Results

3.1. Rheological measurements

Fig. 1 shows the rheological behavior of two C/GP solutions at 37 ± 0.1°C. An increase of the elastic modulus clearly indicates that the liquid solution is turning into a solid-like gel. This transformation from solution to gel took approximately 5 min for the C/GP solution made of a 1:1 (w/w) mixture of C84 and C95 and approximately 140 min for the C/GP solution made of C84. The elastic modulus reached higher values in the case of the C84:C95/GP solution.

3.2. Stability of C/GP solutions

At all times, the C84-based solution exhibited a pseudoplastic behavior i.e. a decrease of viscosity with increasing shear rate. Its viscosity remained unchanged for three months when the solution was kept at 4°C (Fig. 2A) showing that the C84-based system kept at 4°C can remain in the sol state for at least three months. At room temperature, the viscosity was stable for two months but increased at the third month (Fig. 2B). The appearance of a yield stress (Table 1) and thixotropic behavior accompanied this change.

The viscosity of the solution made of a 1:1 (w/w) mixture of C84:C95 increased more rapidly. The samples kept at 4°C were stable for 3 days

Table 1
Yield stress (dyne/cm^2) of a C84/GP solution as a function of time of storage at 4 ± 0.1°C or room temperature[a]

No. of days	4°C	Room temperature
1	9.17 ± 0.25	9.11 ± 0.36
7	12.60 ± 2.70	25.77 ± 16.44
30	11.83 ± 0.42	7.63 ± 0.20
60	11.63 ± 0.67	13.23 ± 0.96
90	14.87 ± 5.23	227.60 ± 37.40

[a] Each value is a mean $\pm$ SEM of three measurements.

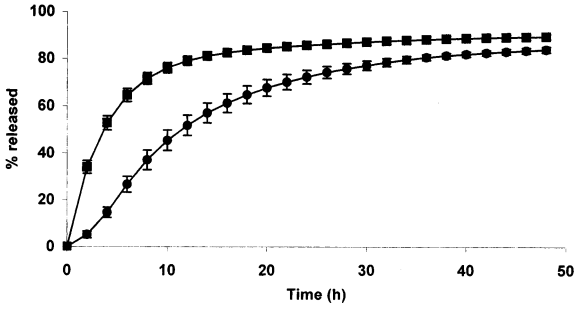

Fig. 3. Chlorpheniramine maleate release profile from a medium molecular weight chitosan solution with (●) or without (■) GP in PBS at pH 7.4 and 37 ± 0.1°C. In each experiment, the loading was 3%. Each point represents the mean value $\pm$ SEM ($n = 5$).

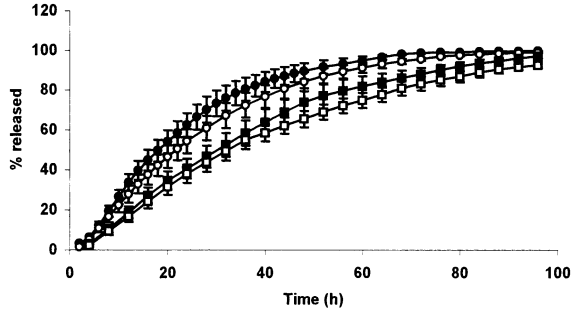

Fig. 4. Release profiles of FITC-dextran of different molecular weights from a C84/GP solution in PBS at pH 7.4 and 37 ± 0.1°C. 12 000 (●), 19 500 (○), 38 260 (■), 148 000 (□). In each experiment, the loading was 3%. Each point represents the mean value $\pm$ SEM ($n = 5$).

but gelled within 7 days (data not shown). The samples kept at room temperature turned into gels in less than 24 h making viscosity measurements impracticable (data not shown).

3.3. In vitro release studies

The incorporation of GP in the medium molecular weight chitosan solution substantially reduced the initial burst release of chlorpheniramine maleate, especially during the first 6 h (Fig. 3). From 0 to 48 h, the release from the chitosan solution with GP was significantly slower than the release from the chitosan solution. Fifty three percent of the chlorpheniramine maleate was released from the chitosan solution within 4 h whereas only 15% was released in the presence of GP over the same period of time. This study showed that GP enables a fast organization of the polymeric network to retain the model compound.

The differences between the release profiles of dextrans with increasing molecular weights from C84/GP solutions were minor but an overall trend is apparent (Fig. 4). As the molecular weight of the model compound increased, the release rate decreased. The release of 90% of the model compound took 52, 60, 76, and 88 h for the dextran with a molecular weight of 12 000, 19 500, 38 260 and 148 000, respectively. The statistical analysis showed a significant difference ($P < 0.05$) only between the dextran with a molecular weight of 12 000 and the dextran with a molecular weight of 148 000. The gelation rate did not influence the release of dextran since the release kinetics between the C84/GP and C84:95/GP gels were similar (data not shown).

In the C84/GP solutions, each mole of NH_3^+ (chitosan) is neutralized by 2.6 moles of PO_4^{2-} (glycerophosphate). To evaluate the influence of excess negative charges on the release of model compounds, a positively charged (methylene blue) and a negatively charged (calcein) molecule were incorporated in the C84/GP solution. The release of methylene blue and calcein from the gel was completed within 48 h (Fig. 5). The release rate was independent of the drug electrostatic charge suggesting that the methylene blue or calcein in small concentration (0.06 and 0.18%, respectively) are not bound by ionic attractions to the polymeric network.

At a pH of about 7.0, which is the pH of the C/GP solution, albumin has a net negative charge.

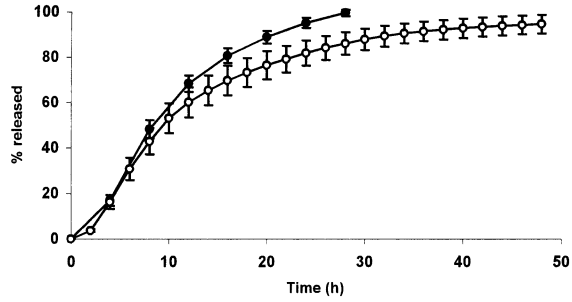

Fig. 5. Methylene blue (●) and calcein (○) release profiles from a C84/GP solution in PBS at pH 7.4 and 37 ± 0.1°C. Each point represents the mean value ± SEM ($n = 5$).

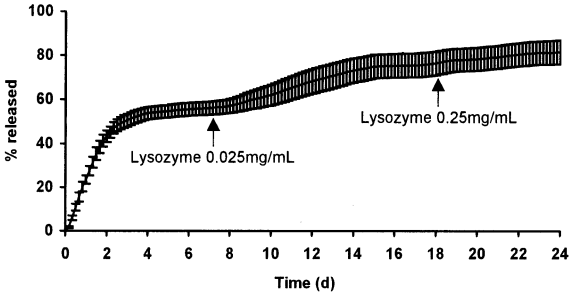

Fig. 6. FITC-albumin release profile from a C84/GP solution in PBS ± lysozyme at pH 7.4 and 37 ± 0.1°C. Each point represents the mean value ± SEM ($n = 5$).

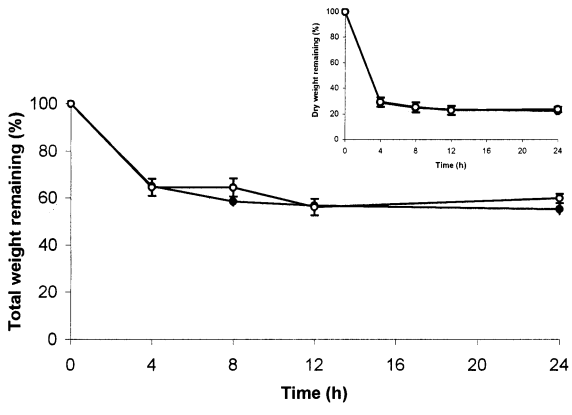

Fig. 7. Total mass of the gelled C/GP solution as a function of time: C84/GP (●) and C84:C95/GP (○). Insert: dry weight of the gelled C/GP solution as a function of time: C84/GP (●) and C84:95/GP (○). Each point represents the mean value ± SEM ($n = 3$).

When albumin was mixed with the C84/GP solution at a high concentration (3%), about 50% was released in 3 days and, afterwards, a plateau was reached (Fig. 6). The remaining albumin was trapped in the gel. In order to release it, lysozyme was added to the release media. After addition of 0.025 mg/ml of lysozyme, another 25% of albumin were released. A subsequent increase in lysozyme concentration (0.25 mg/ml) did not significantly influence the release of albumin.

3.4. Mass loss

At the end of each release study, the gels were taken out of the cells and weighed (data not shown). Approximately 40% of the total weight and 70% of the dry weight was lost at the end of the release study. Fig. 7 reveals that most of the total mass is lost during the first 4 h. During the next 20 h, only 5–10% was lost. Even if the C84:95/GP solution gels more rapidly the mass loss profile is similar to the one of the C84/GP solution. The dried mass lost follows the same kinetics as the total mass. The loss of solid mass during the first 4 h is high, reaching 71% of the initial solid mass.

3.5. Scanning electron microscopy

The micrographs revealed that the gels are porous (Fig. 8). After 4 h of exposure to the release medium (PBS) at 37 ± 0.1°C, the C84/GP solution was solid and pores between 5 and 10 μm, were present. Large pores were expected because of the low polymeric load of the system. With time, there was no substantial increase in pore size. The gel made of C84:95/GP had pores in the same size range.

4. Discussion

In C/GP systems, three types of interactions may be involved during the gelation process: (1) electrostatic attraction between the ammonium group of the chitosan and the phosphate group of the glycerophosphate; (2) hydrogen bonding be-

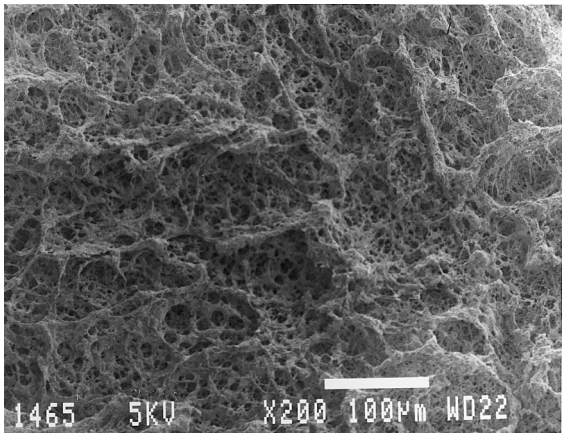

Fig. 8. Scanning electron micrograph of a C84/GP solution after 24 h of exposition to the release medium (PBS) at 37 ± 0.1°C.

tween the chitosan chains as a consequence of reduced electrostatic repulsion after neutralization of the chitosan solution with GP; and (3) chitosan-chitosan hydrophobic interactions. When kept at low temperatures C/GP solutions having a pH of about 7 might not immediately turn into a gel (Fig. 2A). Gelation occurs upon an increase in temperature. This observation implies that some repulsive forces between the chitosan chains are stabilized at low temperature and weakened at high temperature. We hypothesized that the polyol part of GP prevents or slows down gelation at low temperature. Polyols are known to stabilize certain compounds in aqueous solutions and to promote the formation of a shield of water around some macromolecules in polyol-water mixtures (Back et al., 1979). Gekko and Koga (1983) and Gekko et al. (1987) showed that the addition of polyols to aqueous solutions of collagen or carrageenan raised the transition and gelation temperature, respectively, meaning that the initial structure was reinforced by polyols so more energy was needed to break it. This group also reported a negative preferential interaction parameter of glycerol to protein for four proteins in glycerol–water mixtures at 20°C, indicating a deficiency of glycerol in the immediate domain of the protein. This means that the proteins were preferentially hydrated; there was a shield of water around the proteins (Gekko and Timasheff, 1981). Na and Timasheff (1981) obtained similar results in their study of tubulin in glycerol solution. With those results in mind, we postulated that the addition of GP to the chitosan solution promoted the protective hydration of the chitosan chains. Therefore, at low temperatures, it prevents their association to form a gel, even at a neutral pH. Raising the temperature increases the chitosan-chitosan hydrophobic interactions which are expected to play a major role in the gelation of C/GP solutions (Chenite et al., 2000). Depending on the deacetylation degree of chitosan, gelation can occur at relatively low temperatures. For instance, C95/GP solutions gel after 8–10 h at 4°C (data not shown) and the gelation rate can be significantly slowed down by the addition of C84. Nevertheless, even solutions of C84/GP start to gel after 3 months of storage at room temperature (Fig. 2B). This means that C/GP solutions can slowly evolve to the gel state even below 37°C. The sol-gel transition does not occur at a determined temperature but starts as soon as the polyol salt is added. This behavior is different from that of poloxamer (Lenaerts et al., 1987) or xyloglucan (Miyazaki et al., 1998) solutions which will change from sol to gel only when the temperature is raised above their phase transition temperature. However, we showed that gelation of C84/GP solutions can be substantially slowed down at 4°C which can ensure the system an adequate shelf-life (Fig. 2A). The increase in gelation rate observed with chitosans of higher deacetylation degree might be attributed to the increase in cross-link density between the phosphate groups of GP and the ammonium groups of chitosan. Another factor that can influence the sol-gel transition is the pH of the final solution. Chenite et al. (2000) demonstrated that the transition temperature of a freshly prepared 91% deacetylated chitosan/GP solution was around 45°C at a pH value of 6.85 and was decreased to 37°C when the pH was brought up to 7.0. One should be aware that the transition temperatures measured in this study may not be true transition temperatures but rather reflect the gelation rate; gelation being faster at higher pH values.

The release studies allowed a better understanding of the gelation kinetics. Fig. 3 shows that the

presence of GP is essential for a fast organization of the polymeric network in order to retain the model compound. In the absence of GP, the chitosan solution eventually solidified as a consequence of the diffusion of the buffer into the system but the process was too slow or the structure formed was not organized enough to avoid burst release. From Fig. 4, it can be seen that the release profiles of compounds having a molecular weight between 12 000 and 148 000 do not differ substantially. There was no important decrease in release rate above a certain molecular weight threshold because the pore size was considerably larger than the size of the released molecule. Despite a difference in the gelation rate, the release kinetics between the C84/GP and C84:C95/GP systems were comparable, probably because the two gels have similar porous structures as revealed by SEM (data not shown). Accordingly, C/GP solutions could be used to deliver relatively large molecules over a period of several hours. Release in this case seems to be more sustained than that observed with other polysaccharide hydrogels such as xyloglucan and dextran gels with similar polymeric loads (Hennink et al., 1996; Miyazaki et al., 1998). However, a more prolonged release would require the pre-encapsulation of drugs into particles (microspheres, liposomes) in the micrometer size range which could then be incorporated in the gel (Bochot et al., 1998). The release of the model compounds is thought to occur mostly by diffusion but could be accelerated by the weight loss of the gels. However, from Fig. 7 it can be seen that the release kinetics do not parallel the weight loss profiles. After 4 h, almost 70% of the gel solid content is lost (35% of total hydrated weight) even though more than 80% of the initial drug load remains in the gel. This suggests that during the first hours there is a considerable leaching of excess GP (and water) which does not contribute to the physical cross-linking of the gel. The three-dimensional network of the gel did not change significantly over time, indicating that there was no substantial erosion of the polymeric matrix. However, excess GP is needed to increase the pH and thus, the gelation rate. Our results are in accordance with currently ongoing studies which revealed that most of the GP rapidly diffuses out of the gel and that the overall charge in the gel is nearly neutral (Buschmann et al., personal communication). The electroneutrality of the gel network was confirmed by the release studies with compounds bearing an electrostatic charge (Fig. 5). When present at low concentrations (less 0.2%), their release profiles are comparable regardless of the electrostatic charge suggesting that no free charges are available to retain the model compound. However, at high concentrations, negatively charged compounds such as albumin may displace GP and bind to chitosan via the ammonium groups. Indeed at 3% loading, the release of albumin reached a plateau after 3 days, suggesting that part of the albumin was linked to the polymeric network. The addition of lysozyme to the release media allowed partial release of the entrapped albumin but 100% recovery was not possible since the gel made of C84 was relatively resistant to the action of the enzyme. After 17 days of exposure to lysozyme, the gels had a final hydrated mass comparable to that of gels not exposed to lysozyme. However, the gels had lost their three-dimensional structure. Our results are consistent with those found in the literature. Tomihata and Ikada (1997) reported that the in vitro degradation of chitosan films in 0.1 M PBS containing 4 mg/ml of lysozyme at pH 7.4 and 37°C occurred less rapidly as their deacetylation degree became higher. Chitosans with degrees of deacetylation higher than 73% were virtually resistant against enzymatic hydrolysis at this lysozyme concentration. In another paper, Hata et al. (1999) reported that as the deacetylation degree of the carboxymethyl chitin increased, the change in viscosity after addition of lysozyme (0.018 mg/ml) became slower.

The results presented here showed that C/GP solutions have the potential to be used as injectable in situ gelling thermosensitive formulations. They can gel at body temperature. The C84/GP solution retains its physical properties for three months and two months when kept at 4°C and room temperature, respectively. C/GP systems can sustain the release of macromolecules over a period of several hours to a few days. Sustained release over more than 1 week will

probably require pre-incorporation of the drug into microparticles or liposomes for those molecules that release is only a function of passive diffusion.

Acknowledgements

This study was supported in part by a grant from The Natural Sciences and Engineering Research Council of Canada.

References

Aiba, S., 1992. Studies on chitosan: 4. Lysozymic hydrolysis of partially N-acetylated chitosans. Int. J. Biol. Macromol. 14, 225–228.

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

Bhardwaj, R., Blanchard, J., 1996. Controlled-release delivery system for the MSH analog melanotan-I using poloxamer 407. J. Pharm. Sci. 85, 915–919.

Bittner, B., Morlock, M., Koll, H., Winter, G., Kissel, T., 1998. Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. Eur. J. Pharm. Biopharm. 45, 295–305.

Bochot, A., Fattal, E., Gulik, A., Couarraze, G., Couvreur, P., 1998. Liposomes dispersed within a thermosensitive gel: a new dosage form for ocular delivery of oligonucleotides. Pharm. Res. 15, 1364–1369.

Bodmer, D., Kissel, T., Traechslin, E., 1992. Factors influencing the release of peptides and proteins from biodegradable parenteral depot systems. J. Controlled Release 21, 129–138.

Casarett, L.J., Doull, J., 1991. Casarett and Doull's Toxicology: The Basic Science of Poisons. Pergamon Press, New York.

Chandy, T., Sharma, C.P., 1990. Chitosan—as a biomaterial. Biomat., Art. Cells, Art. Org. 18, 1–24.

Chenite, A., Chaput, C., Wang, D., Combes, C., Bushmann, M.D., Hoemann, C.H., Leroux, J.C., Atkinson, B.L., Binette, F., Selmani, A., 2000. Novel injectable neutral solutions of chitosan form biodegradible gels in situ for bioactive therapeutic delivery Biomaterial in press.

Davis, B.K., 1974. Diffusion in polymer gel implants. Proc. Nat. Acad. Sci. USA 71, 3120–3123.

Desai, S.D., Blanchard, J., 1998. In vitro evaluation of pluronic F127-based controlled-release ocular delivery systems for pilocarpine. J. Pharm. Sci. 87, 226–230.

Dinarvand, R., D'Emanuel, A., 1995. The use of thermoresponsive hydrogels for on-off release of molecules. J. Controlled Release 36, 221–227.

Felt, O., Furrer, P., Mayer, J.M., Plazonnet, B., Buri, P., Gurny, R., 1999. Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. 180, 185–193.

Gekko, K., Koga, S., 1983. Increased thermal stability of collagen in the presence of sugars and polyols. J. Biochem. 94, 199–205.

Gekko, K., Mugishima, H., Koga, S., 1987. Effects of sugars and polyols on the sol-gel transition of k-carrageenan: calorimetric study. Int. J. Biol. Macromol. 9, 146–152.

Gekko, K., Timasheff, S.N., 1981. Mechanism of protein stabilization by glycerol: Preferential hydratation in glycerol-water mixtures. Biochemistry 20, 4667–4676.

Hata, H., Onishi, H., Machida, Y., 1999. Evaluation of carboxymethyl chitin as a drug carrier. Effect of the deacetylation degree on the biodegradation and biodisposition characteristics in mice. S.T.P. Pharma Sci. 9, 115–121.

Hennink, W.E., Talsma, H., Borchert, J.C.H., De Smedt, S.C., Demeester, J., 1996. Controlled release of proteins from dextran hydrogels. J. Controlled Release 39, 47–55.

Hirano, S., Tsuchida, H., Nagao, N., 1989. N-acetylation in chitosan and the rate of its enzymic hydrolysis. Biomaterials 10, 574–576.

Hoffman, A.S., 1987. Applications of thermally reversible polymers and hydrogels in therapeutics and diagnotics. J. Controlled Release 6, 297–305.

Hoffman, A.S., Afrassiabi, A., Dong, L.C., 1986. Thermally reversible hydrogels: II. Delivery and selective removal of substances from aqueous solutions. J. Controlled Release 4, 213–222.

Illum, L., Davis, S.S., Pawula, M., Fisher, A.N., Barrett, D.A., Farraj, N.F., Shaw, P.N., 1996. Nasal administration of morphine-6-glucoronide in sheep—A pharmacokinetic study. Biopharm. Drug. Disp. 17, 717–724.

Jeong, B., Bae, Y.H., Lee, D.S., Kim, S.W., 1997. Biodegradable block copolymers as injectable drug-delivery systems. Nature 388, 860–862.

Johnston, T.P., Miller, S.C., 1989. Inulin disposition following intramuscular administration of an inulin/poloxamer gel matrix. J. Parent. Sci. Technol. 43, 279–286.

Johnston, T.P., Punjabi, M.A., Froelich, C.L., 1992. Sustained delivery of interleukine-2 from a poloxamer 407 gel matrix following intraperitoneal injection in mice. Pharm. Res. 9, 425–434.

Lee, K.Y., Ha, W.S., Park, W.H., 1995. Blood compatibility and biodegradability of partially *N*-acetylated chitosan derivatives. Biomaterials 16, 1211–1216.

Lenaerts, V., Triqueneaux, C., Quarton, M., Rieg-Falson, F., Couvreur, P., 1987. Temperature-dependent rheological behavior of pluronic F-127 aqueous solutions. Int. J. Pharm. 39, 121–127.

Machida, Y., Nagai, T., Abe, M., Sannan, T., 1986. Use of chitosan and hydroxypropylchitosan in drug formulations to effect sustained release. Drug Design Deliv. 1, 119–130.

Merkli, A., Heller, J., Tabatabay, C., Gurny, R., 1994. Semisolid hydrophobic bioerodible poly(ortho ester) for potential application in glaucoma filtering surgery. J. Controlled Release 29, 105–112.

Miwa, A., Ishibe, A., Nakano, M., Yamahira, T., Itai, S., Jinno, S., Kawahara, H., 1998. Development of novel chitosan derivatives as micellar carriers of taxol. Pharm. Res. 15, 1844–1850.

Miyazaki, S., Suisha, F., Kawasaki, N., Shirakawa, M., Yamatoya, K., Attwood, D., 1998. Thermally reversible xyloglucan gels as vehicles for rectal drug delivery. J. Controlled Release 56, 75–83.

Na, G.C., Timasheff, S.N., 1981. Interaction of calf brain tubulin with glycerol. J. Mol. Biol. 151, 165–178.

Nystrom, B., Walderhaug, H., Hansen, F.K., 1995. Rheological behavior during thermoreversible gelation of aqueous mixtures of ethyl(hydroxyethyl)cellulose and surfactants. Langmuir 11, 750–757.

Okano, T., Bae, Y.H., Jacobs, H., Kim, S.W., 1990. Thermally on-off switching polymers for drug permeation and release. J. Controlled Release 11, 255–265.

Palmer, W.K., Emeson, E.E., Johnston, T.P., 1998. Poloxamer 407-induced atherogenesis in the C57BL/6 mouse. Atherosclerosis 136, 115–123.

Patel, V.R., Amiji, M.M., 1996. Preparation and characterisation of freeze-dried chitosan-poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharm. Res. 13, 588–593.

Pec, E.A., Wout, Z.G., Johnston, T.P., 1992. Biological activity of urease formulated in poloxamer 407 after intraperitoneal injection in the rat. J. Pharm. Sci. 81, 626–630.

Singer, E.J., Pittz, E.P., 1985. Interaction of surfactants with epidermal tissues: Biochemical and toxicological aspects. In: Rieger, M.M. (Ed.), Surfactants in Cosmetics. Marcel Dekker, New York, pp. 133–194.

Tomihata, K., Ikada, Y., 1997. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials 18, 567–575.

Varum, K.M., Myrh, M.M., Hjerde, R.J.N., Smidsrod, O., 1996. In vitro degradation rates of partially N-acetylated chitosans in human serum. Carbohydrate Res. 299, 99–101.