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Research paper

A novel thermoresponsive hydrogel based on chitosan

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Abstract

Injectable thermosetting chitosan hydrogels are attractive systems for drug delivery and tissue engineering that combine biodegradability, biocompatibility and the ability to form *in situ* gel-like implants. Thermally-induced gelation relies advantageously on biopolymer secondary interactions, avoiding potentially toxic polymerization reactions that may occur with *in situ* polymerizing formulations. In view of a biomedical use, such formulations have to be sterilizable and storable on extended periods without losing their thermosetting properties. These two key features have been studied in the present paper. Chitosans from two different sources were added with several phosphate-free polyols or polyoses as gelling agents. Despite a reduction in chitosan molecular weight following autoclaving, the hydrogels prepared with autoclaved chitosan showed the desired thermosetting properties. Hence, chitosan steam sterilization combined with aseptic preparation of the hydrogel allows a sterile formulation to be obtained. Whereas thermosetting hydrogels were shown to be unstable when refrigerated, freezing was shown to be conceivable as a storage method. When trehalose or mannitol was used as stabilizing agent, the formulation reconstituted from a lyophilizate displayed thermosetting properties and was still injectable, paving the way to the development of a clinically utilizable, novel chitosan thermosetting hydrogel.

Keywords: Hydrogels; Drug delivery; Chitosan; Thermosetting; Thermoresponsive; Lyophilization; *In situ* gelling

1. Introduction

Hydrogels based on both natural and synthetic polymers are currently receiving a great deal of interest, notably due to potential applications in the controlled release of bioactive molecules and tissue engineering. Among biopolymers of interest, chitosan, an aminopolysaccharide obtained by alkaline deacetylation of chitin, stands out by a unique combination of favourable biological properties such as nontoxicity [1], biocompatibility [2,3], biodegradability [4,5], along with mucoadhesive [6], bacteriostatic [7] and wound-healing properties [8]. In addition, this cationic biopolymer has been reported to improve transport across biological barriers [9,10]. Finally, chitosan is very abundant and its production is both environmentally safe and of low cost [11].

Chitosan hydrogels can be divided into two classes: chemical hydrogels, formed by irreversible covalent links, and physical hydrogels, formed by various reversible links [12]. Covalent crosslinking leads to the formation of gels showing enhanced mechanical properties. However, crosslinking agents can interact with incorporated bioactive compounds and are often associated with significant toxicity. For these reasons, physically crosslinked hydrogels have gained increasing attention.

Some hydrogels have the particularity of gelling within the desired tissue or body cavity as a result of polymer interactions. Such *in situ*-forming systems advantageously flow freely as injectable liquids before administration and gel under physiological conditions, in response to stimuli. Temperature-sensitive systems that gel at body temperature are especially attractive. Chenite et al. first described a thermosetting formulation based on chitosan/glycerophosphate

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salt combination [13]. Working with alternative chitosan hydrogels, we observed that gelation is not induced only by temperature but also by time, showing the need for a storage method that would impede or slow down biopolymer interactions. Although storage stability is a major concern in the development of a commercially viable product, this point has not been studied so far for chitosan thermosetting hydrogels. This work aimed at exploring formulations that offer an acceptable shelf life.

Moreover, in view of biomedical application, sterilization is an essential requirement. The sterilization process may affect molecular weight distribution and mechanical properties of polymeric materials, which in turn may induce loss of functionality or biocompatibility, impairing safety and effectiveness of implants [14,15]. Therefore, finding suitable sterilization methods remains a challenge, notably for biodegradable polymers such as aliphatic polyesters, cellulose derivatives, polysaccharides and polypeptides. Chemical sterilization with ethylene oxide is not applicable due to its known toxicity. In addition, interaction of gas residues with functional groups of the polymer surface may alter the polymer's physicochemical and biological properties. In contrast to ethylene oxide sterilization, γ-irradiation does not compromise the biocompatibility of chitosan [16]. Nevertheless, γ-irradiation at sterilizing doses induces main chain scission and some rearrangement of network structure in chitosan fibers and films [17], often dramatically altering thermogelling properties [18]. As for heat treatment, it was shown that dry heat above 80 °C produces chromophores and that the intrinsic viscosity and aqueous solubility decrease with temperatures above 120 °C [3]. Although the autoclaving process was shown to reduce the viscosity of chitosan solutions by 20–50%, it appeared as an appropriate sterilization method [18]. In this study, we selected steam sterilization and evaluated its effect on chitosan thermogelling properties.

The objective of this paper is threefold: first, to evaluate novel phosphate-free (and hence less toxic) gelling agents for chitosan thermosetting hydrogels. Glycerophosphate, as suggested by earlier work [13], presents some drawbacks such as turbidity of the hydrogel and the presence of negatively charged moieties that may interact with numerous bioactive components. Hence, alternative additives were tested. The second objective is to assess the thermogelling properties of formulations prepared from autoclaved reacetylated chitosan. Finally, we tested different storage strategies for phosphate-free thermosetting formulations in order to provide a sterile preparation that can be easily stored, keeping its thermogelling properties and being injectable. Two types of chitosan with different average molecular weights but similar deacetylation degrees were studied.

2. Materials

Chitosan (type I) was an experimental batch obtained from Novamatrix (Oslo, Norway) presenting a deacetylation degree of 59%, measured by nuclear magnetic resonance (NMR). Chitosan (type II) was obtained by reacetylation of a high molecular weight chitosan purchased from Sigma Chemical (St. Louis, MO, USA), according to our procedure described by Berger et al. (method II) [19]. Its deacetylation degree was of 63% measured by NMR. Chitosan concentrations were chosen for each type of chitosan in order to produce formulations that could show gel transition within 3 h, and still being easily injectable. The lower molecular weight type I required a concentration above 1.7% to produce a gel; a concentration of 2% was therefore selected. Type II could gel at 0.9% already; concentrations of 1.1% and 1.3% were used. Deuterium chloride and deuterium oxide were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Hydrochloric acid and sodium hydroxide, both of Pharm. Eur. grade, were purchased from Riedel-de Haën (Seelze, Germany). 1,2-Propanediol (Pharm. Eur. Grade), glycerol (Pharm. Eur. Grade) and trehalose were obtained from Fluka (Buchs, Switzerland).

3. Methods

3.1. Preparation of the chitosan/polyol solutions

Chitosan solution was obtained by dissolving 220–400 mg chitosan in 10 ml HCl 0.1 N under mechanical stirring for 18 h at room temperature. This solution was cooled down to 5 °C using an ice bath and 2 g of gelling agent (at 5 °C) was added. The pH of the gel was then adjusted to 6.8 by dropwise addition of diluted NaOH (at 5 °C). Finally, cold water was added to obtain a total mass of 20 g. The final preparations contained 1.1–2% (w/w) chitosan and 10% (w/w) of a gelling agent. The following polyols or polyoses were evaluated as gelling agents: 1,2-propanediol, glycerol, trehalose, mannitol. When trehalose was used as a gelling agent, it was solubilized in diluted NaOH, cooled down and added to the chitosan solution before pH adjustment.

When specified, the chitosan was first autoclaved in suspension in deionized water (220 or 400 mg chitosan in 10 ml H_2O, 15 min at 121 °C) using a Varioklav® steam sterilizer (H+P Labortechnik, Oberschleissheim, Germany). After cooling down the suspension at room temperature, 83 μl HCl was added and the mixture was kept under stirring for 18 h to allow complete chitosan solubilization. The thermosetting solution was then prepared following the same procedure as described above.

3.2. Hydrogel storage

Several storage conditions were evaluated. The formulation was either refrigerated (4 °C), frozen (−80 °C) or freeze-dried. In the latter case, 10 g of formulation was frozen at −80 °C in a 100-ml penicillin flask before being lyophilized for 24 h with an Edwards Modulyo Freeze dryer (Edwards High Vacuum Ltd, Crowley, UK) (plate

at −50 °C, vacuum 10^{-1} mbar). The lyophilizate obtained was reconstituted by addition of the appropriate amount of cold water under magnetic stirring, at 4 °C.

3.3. Rheological evaluation

Viscoelastic properties of hydrogels were measured shortly after preparation, thawing at 4 °C or reconstitution of the hydrogels, using a Rheostress 1 (Haake, Karlsruhe, Germany) with a cone/plate device (diameter 60 mm, angle 4°). Temperature was controlled with a thermostatic bath Haake DC 30 and a cooling device Haake K10 (Haake, Karlsruhe, Germany) coupled with the rheometer. Hydrogels were placed between the cone and plate (cooled down at 4 °C) and measured after 10 min. All measurements were performed in the linear viscoelastic range and G′ (storage modulus) and G″ (loss modulus) were determined under a constant deformation ($\gamma = 0.05$) at 1.00 Hz for 180 min. The temperature was increased from 4 to 37 °C at 6.6 °C/min over the first 5 min, and maintained at 37 °C over the following 175 min. Evaporation of water leading to drying of hydrogels was minimized by use of a cover surrounding the cone/plate device. The onset of incipient formation of the gel network, which defines the gelation time, is given by the time of crossover of G′ and G″. The measurement of the gelation time showed a reasonably good reproducibility (6% standard deviation when repeated over 3 samples), and the presence or absence of gel transition was a highly reproducible feature.

3.4. Assessment of the injectability

The injectability of the formulations was evaluated with a device composed of a vertical support for a 1.0 ml Luer lock syringe filled with the hydrogel at 5 °C and a pan resting on the piston of the syringe as shown in Fig. 1. A $27G^{1/2} \times 0.5$ inches needle was fixed on the syringe which was positioned in the support. A kilogram mass was placed on this pan and the time necessary for the formulation to be expelled from the syringe was measured.

3.5. Chitosan molecular weight determination

An average molecular weight was measured by asymmetrical flow field-flow fractionation (AFFF) coupled to multiangle light scattering (MLS). Fractionation of the chitosan solution (2 mg/ml in acetate buffer pH 4.5) was performed in a trapezoidal channel, 26.5 cm in length and 350 μm in height, connected to an Eclipse F system (Wyatt Technology Europe, Dernbach, Germany). The bottom of the channel was lined with a regenerated cellulose membrane with a 10 kDa cut-off (Microdyn-Nadir GmbH, Wiesbaden, Germany). The elution medium consisted of acetate buffer pH 4.5. The channel flow was set to 1 ml/min and the injection flow to 0.2 ml/min. The separation started with a focus flow of 1 ml/min for 3 min and was followed by a cross flow of 0.4 ml/min for 25 min. A

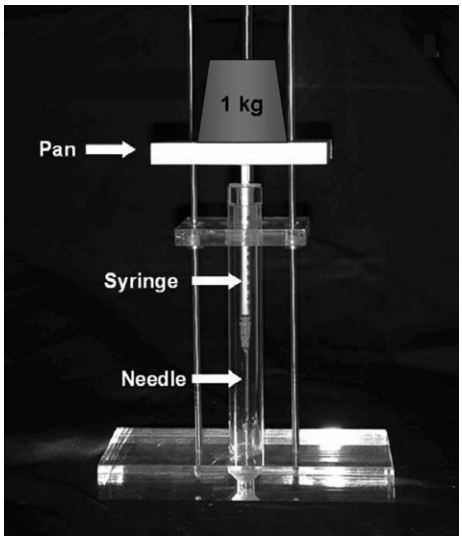

Fig. 1. Device for the measurement of the formulations injectability.

Dawn EOS multi-angle light scattering detector and an Optilab rEx refractive index (RI) detector operating at 25 °C (Wyatt Technology, Santa Barbara, USA) were coupled online with the FFF channel. The light scattering detector was equipped with a GaAs laser (wavelength: 690 nm) and 18 detectors. Scattered light was collected at angles comprised between 14° and 163°. Data were collected and analysed with the Astra version 4.90.08 software, using a refractive index increment (dn/dc) of 0.153 ml/g for chitosan type I and 0.141 for chitosan type II.

3.6. Chitosan deacetylation degree measurement

The degree of deacetylation of chitosan (DDA) was determined by a validated ^{1}H NMR method described by Lavertu et al. [20]. ^{1}H NMR spectra were acquired on a Broadband Varian Gemini 300 MHz spectrometer. NMR tubes filled with 5 mg/ml chitosan solution in D_2O with 20% v/v DCl were heated at 80 °C. The ^{1}H NMR experiment was a single pulse sequence with presaturation of the solvent. A 90° pulse corresponding to a pulse width of 11 μs was used. The delay before the application of the pulse was 6 s and the acquisition time was 2 s. The DDA was calculated by using the peaks of protons ^{1}H of both deacetylated and acetylated monomers (equation 3, ref [20]).

4. Results and discussion

4.1. Gelling agents

Besides β-glycerophosphate [13,21], several molecules may accelerate the thermogelation of chitosan solutions: 1,3-propanediol [22], 1,2-propanediol (Fig. 2) as well as glycerol (Fig. 4), mannitol (data not shown) or polyoses such as trehalose (Fig. 6). Although the exact mechanism

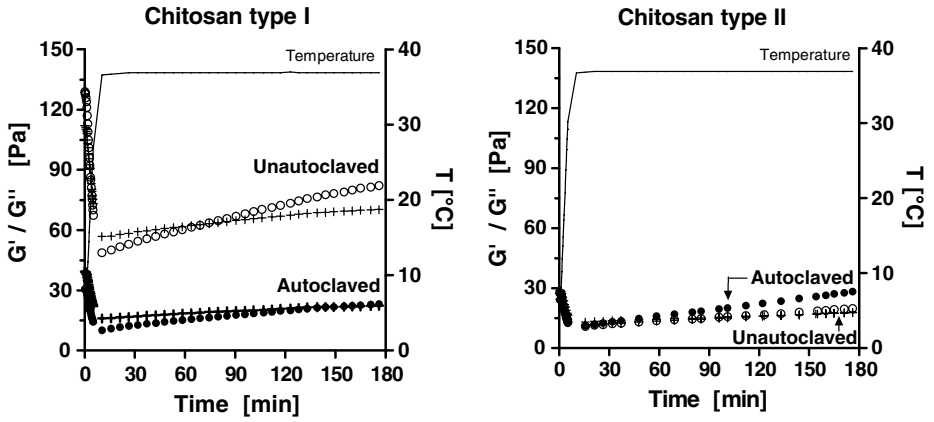

Fig. 2. Viscoelastic behaviour before and after autoclaving of formulations containing 10% 1,2-propanediol and 2% of type I or 1.3% of type II chitosan. The round circles represent the elastic or storage modulus (G′) and the crosses represent the viscous or loss modulus (G″) of the transparent chitosan hydrogels as a function of time, when temperature increases from 4 to 37 °C.

is not yet known, the gelation may be attributed to secondary hydrophobic or hydrogen bonding interactions between polysaccharide chains. Hydrophobic interactions can indeed be favoured by the presence of water-structuring molecules such as polyols [23]. Such an interactive biopolymer system advantageously avoids the potential side effects of an *in situ* polymerization associated to monomer or initiator toxicity.

4.2. Effect of steam sterilization on chitosan solutions

The thermogelling properties of hydrogels prepared with autoclaved chitosan from two different sources are shown in Fig. 2. Both formulations displayed a thermally-induced gelation (defined as the crossover of G′ and G″) that occurred within one to two hours. Hence, steam sterilization of the chitosan suspension is appropriate for the preparation of such thermogelling formulations. One can notice that whereas the autoclaving treatment did not affect the viscoelastic properties of the hydrogel obtained with chitosan type II, the formulation prepared with autoclaved chitosan type I showed significantly reduced elastic and viscous moduli compared to the unautoclaved one. These results are in agreement with the study of Jarry et al. showing that steam sterilization of a chitosan solution having a deacetylation degree of 84% did not impair the ability of a chitosan-glycerophosphate system to form a gel at 37 °C [18].

The evaluation of the chitosan type I molecular weight by asymmetrical flow field-flow fractionation (AFFF) coupled to multiangle light scattering (MLS) resulted in the following average molecular weights (M_w): 2200 kDa without autoclaving and 410 kDa when autoclaved. A similar reduction of molecular weight was measured by Jarry et al. after autoclaving a chitosan of 537 kDa in the same conditions [24]. A reduced M_w decrease was observed for chitosan type II, whose M_w values were significantly higher: 3620 kDa without autoclaving and 1220 kDa autoclaved.

Still to notice is the fact that the thermogelling properties were also maintained with autoclaved chitosan when trehalose (Fig. 6), glycerol (Fig. 5a) or mannitol (data not shown) was used instead of 1,2-propanediol. Hence, since the chitosan/polyol system keeps the desired thermogelling properties when chitosan is previously autoclaved, this sterilization process may be envisaged for the preparation of sterile hydrogels. Only chitosans exhibiting highly reduced molecular weights when autoclaved might not be adapted to this sterilization method in specific applications where maximal mechanical performance is essential for implant function.

4.3. Storage stability assessment

As previously stated, gelation not only occurs by heating to 37 °C, but is also induced with time at room temperature. Hence, several approaches were evaluated to prevent gelation to occur during storage: refrigeration, freezing and freeze-drying.

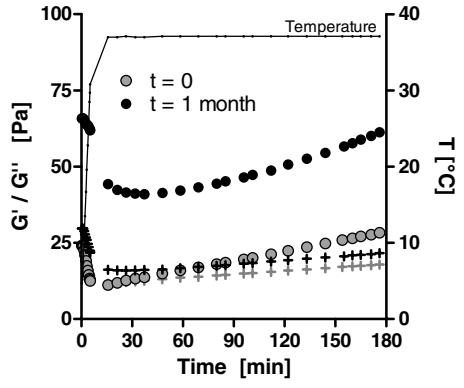

Fig. 3. Viscoelastic behaviour of a formulation made of 1.3% chitosan type II and 10% 1,2-propanediol just after preparation and after 1 month storage at 4 °C. The round circles represent the elastic (or storage) modulus (G′) and the crosses represent the viscous (or loss) modulus (G″) of the transparent chitosan hydrogel as a function of time, when temperature increases from 4 to 37 °C.

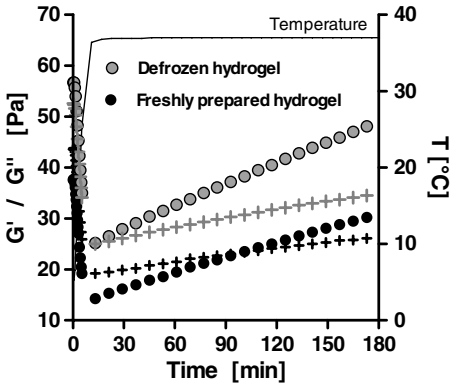

Fig. 4. Viscoelastic behaviour of a formulation made of 2% autoclaved chitosan type I and 10% glycerol. Half of the preparation was frozen at −80 °C in a Petri dish and stored at −20 °C. Its viscoelastic properties were measured after thawing at 4 °C. The round circles represent the elastic (or storage) modulus (G′) and the crosses represent the viscous (or loss) modulus (G″) of the transparent chitosan hydrogel as a function of time, when temperature increases from 4 to 37 °C.

4.3.1. Refrigeration

After prolonged storage at 4 °C, the hydrogel still was temperature-sensitive since it showed an increase of its elastic modulus (G′) upon time at 37 °C. However, this modulus showed significantly higher values and gelation had already occurred before heating, as shown by a G′ greater than G″ already at the beginning of the rheological measurement. Fig. 3 shows the evolution of viscoelastic properties for a type II chitosan with 10% 1,2-propanediol in one month. Similar results were obtained for type I chitosan using either 1,2-propanediol 10% or glycerol 10% as additives. For storage periods longer than one day, initial G′ values higher than G″ were observed with both chitosan types, indicating the occurrence of a sol-gel transition over this period. As a consequence of higher G′ and G″ values upon storage, injectability is decreased: whereas the formulation showed an injection time of 25 s just after the preparation, more than 6 min were needed for the formulation stored for one month at 4 °C to be expelled from the syringe. This period still is acceptable since, on a comparative basis, a commercially available hyaluronic hydrogel (Dermadeep™, Dermatech, Paris, France), designed to be subcutaneously injected, was not injectable with the described device. Therefore, although the formulation is still thermoresponsive and injectable, its lack of stability may lead to poorly reproducible *in vivo* results as a function of the duration of the storage.

4.3.2. Freezing

Freezing was thought to be more promising than refrigeration, since it might congeal the network structure and hence prevent the gelation to occur during storage. As shown in Fig. 4, the gelation time occurred earlier for the thawed formulation compared to the freshly prepared hydrogel, but the thermosetting properties were kept and the viscosity compatible with a clinical use. Similar behaviours were observed when 1,2 propanediol, trehalose or mannitol was used. In the absence of any additive, the control formulation did gel upon thawing. As for the chitosan type I, no additive could prevent gelation upon thawing at the tested concentration. Sol-gel transition was probably favoured by the higher molecular weight of this latter chitosan. Hence, as long as chitosan molecular weight and/or concentration is sufficiently low, chitosan thermogels might be kept frozen for prolonged storage.

4.3.3. Freeze-drying

A lyophilizate that can be easily handled and stored certainly offers advantages over a frozen preparation. The hydrogel is then reconstituted from the lyophilizate by addition of the appropriate amount of water. When glycerol, 1,2-propanediol or 1,3-propanediol was used as gelling agent, the reconstituted formulation showed no gel transition and was not injectable (with a 1 kg mass), whatever being the chitosan source. Fig. 5b shows the viscoelastic

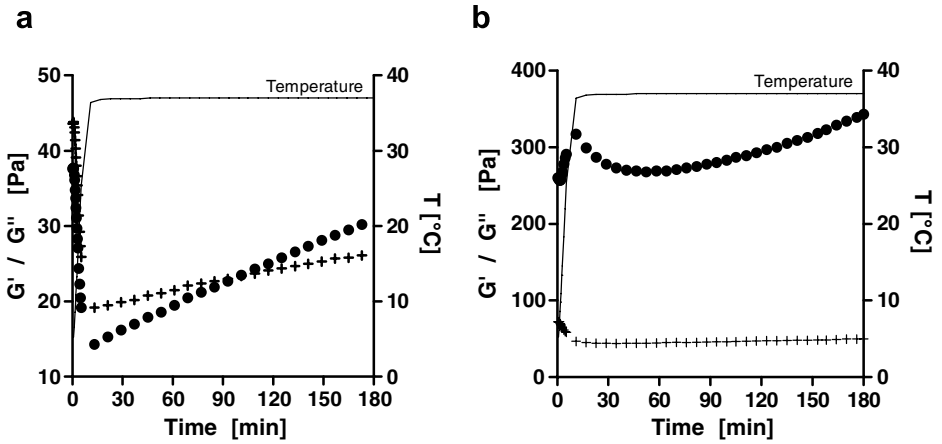

Fig. 5. Viscoelastic behaviour of a formulation made of 2% autoclaved chitosan type I and 10% glycerol. (a) freshly prepared formulation (b) hydrogel reconstituted from the lyophilizate. The round circles represent the elastic (or storage) modulus (G′) and the crosses represent the viscous (or loss) modulus (G″) of the transparent chitosan hydrogel as a function of time, when temperature increases from 4 to 37 °C.

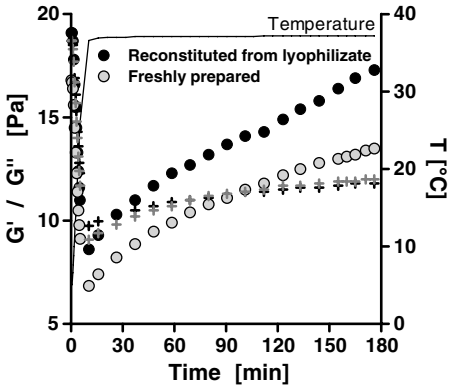

Fig. 6. Viscoelastic behaviour of a formulation made of 1.1% autoclaved chitosan type II and 10% trehalose. The round circles represent the elastic (or storage) modulus (G′) and the crosses represent the viscous (or loss) modulus (G″) of the transparent chitosan hydrogel as a function of time, when temperature increases from 4 to 37 °C.

behaviour of the reconstituted formulation containing 10% of glycerol. The elastic modulus was, before and during heating, more than 3 times higher than the viscous modulus and remained roughly constant, around 300 Pa. Both the elastic and the viscous moduli of the very firm gel obtained were significantly higher than that of the freshly prepared formulation whose injectability was of 28 s. A comparable rheogram was measured with 1,2-propanediol as gelling agent. This behaviour might be attributed to a too low polyol glass transition temperature with respect to lyophilization temperature, thus hindering the preservation of chitosan structure during the lyophilization process.

In contrast, when a polyol such as trehalose or mannitol was used, the rheological behaviour of the reconstituted formulation was comparable to that of the freshly prepared solution, as illustrated in Fig. 6: G′ was smaller than G″ before heating, abruptly decreased when the sample reached 37 °C, and then steadily increased with time, going beyond G″ in less than 30 min. Both types of chitosan preserved thermogelling properties with 8% or 10% trehalose, as well as with 10% mannitol. Some additional experiments showed that grinding of freeze-dried samples facilitates hydration but might affect the rheological behaviour of the reconstituted hydrogel. Further work should be carried out to evaluate the impact of granulometry on the viscoelastic properties of formulations reconstituted from freeze-dried powders.

Therefore, although lyophilization is not appropriate for compounds such as glycerol and propanediols, probably because of their plastifying properties, it certainly offers an attractive alternative for preparations containing solid, high glass transition temperature polyols.

5. Conclusions

Steam sterilization of the biopolymer in suspension was shown to preserve thermogelling properties, despite the decreased molecular weight. Thus, chitosan autoclaving followed by aseptic processing allows a sterile thermosetting preparation to be obtained. A key feature for injectable in-situ forming composition is the stability of the formulation. Whereas the thermosetting hydrogels were shown to be unstable at room temperature as well as during refrigeration, freezing in presence of various gelling agents was proved to be an appropriate storage method. The most convenient and attractive approach certainly consists in a lyophilizate that can be easily handled and stored. This approach was shown to be very promising with stabilizing agents such as trehalose: the reconstituted formulations were injectable and had the desired thermosetting properties. This study represents a significant step towards the development of a thermosetting hydrogel based on reacetylated chitosan and a phosphate-free additive. The latter can be chosen in order to tailor the mechanical properties of the gel, and as a function of the foreseen application.

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References

[1] S.B. Rao, C.P. Sharma, Use of chitosan as a biomaterial: studies on its safety and hemostatic potential, J. Biomed. Mater. Res. 34 (1997) 21–28.
[2] S. Hirano, Y. Noishiki, The blood compatibility of chitosan and *N*-acylchitosans, J. Biomed. Mater. Res. 19 (1985) 413–417.
[3] S. Hirano, H. Seino, Y. Akiyama, et al., A biocompatible material for oral and intravenous administrations, Prog. Biomed. Polym. (1990) 283–290.
[4] R. Chen, H.C. Chen, Chitin enzymology-chitinase, Adv. Chitin Sci. 3 (1998) 16–23.
[5] R.A.A. Muzzarelli, Human enzymatic activities related to the therapeutic administration of chitin derivatives, Cell Mol. Life Sci. 53 (1997) 131–140.
[6] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, et al., *In vitro* evaluation of mucoadhesive properties of chitosan and some other natural polymers, Int. J. Pharm. 78 (1992) 43–48.
[7] X.F. Liu, Y.L. Guan, D.Z. Yang, et al., Antibacterial action of chitosan and carboxymethylated chitosan, J. Appl. Polym. Sci. 79 (2001) 1324–1335.
[8] H. Ueno, T. Mori, T. Fujinaga, Topical formulations and wound healing applications of chitosan, Adv. Drug Deliv. Rev. 52 (2001) 105–115.
[9] V. Dodane, M.A. Khan, J.R. Merwin, Effect of chitosan on epithelial permeability and structure, Int. J. Pharm. 182 (1999) 21–32.
[10] A.F. Kotzé, H.L. Luessen, M. Thanou, et al., Chitosan and chitosan derivatives as absorption enhancers for peptide drugs across mucosal epithelia, in: E. Mathiowitz et al. (Eds.), Bioadhesive Drug Delivery Systems, Marcel Dekker Inc., New York, 1999, pp. 341–385.
[11] M.G. Peter, Applications and environmental aspects of chitin and chitosan, J. Macromol. Sci., Part A: Pure Appl. Chem. A32 (1995) 629–640.
[12] J. Berger, M. Reist, J.M. Mayer, et al., Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, Eur. J. Pharm. Biopharm. 57 (2004) 19–34.

[13] A. Chenite, C. Chaput, D. Wang, et al., Novel injectable neutral solutions of chitosan form biodegradable gels *in situ*, Biomaterials 21 (2000) 2155–2161.
[14] Y.H. An, F.I. Alvi, Q. Kang, et al., Effects of sterilization on implant mechanical property and biocompatibility, Int. J. Artif. Organs 28 (2005) 1126–1137.
[15] S.D. Bruck, E.P. Mueller, Radiation sterilization of polymeric implant materials, J. Biomed. Mater. Res. Appl. Biomater 22 (1988) 133–144.
[16] J. Rosiak, P. Ulanski, M. Kucharska, et al., Radiation sterilization of chitosan sealant for vascular prostheses, J. Radioanal. Nucl. Chem. 1 (1992) 87–96.
[17] L.-Y. Lim, E. Khor, O. Koo, γ-Irradiation of chitosan, J. Biomed. Mater. Res. 43 (1998) 282–290.
[18] C. Jarry, J.-C. Leroux, J. Haeck, et al., Irradiating or autoclaving chitosan/polyol solutions: effect on thermogelling chitosan-β-glycerophosphate systems, Chem. Pharm. Bull. 50 (2002) 1335–1340.
[19] J. Berger, M. Reist, A. Chenite, O. Felt-Baeyens, J.M. Mayer, R. Gurny, Pseudo-thermosetting chitosan hydrogels for biomedical application, Int. J. Pharm. 288 (2) (2005) 197–206.
[20] M. Lavertu, Z. Xia, A.N. Serreqi, et al., A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan, J. Pharm. Biomed. Anal. 32 (2003) 1149–1158.
[21] A. Chenite, M. Buschmann, D. Wang, et al., Rheological characterization of thermogelling chitosan/glycerol-phosphate solutions, Carbohydr. Polym. 46 (2001) 39–47.
[22] J. Berger, Pseudo-thermosetting chitosan hydrogels as potential drug delivery systems, Ph. D thesis, University of Lausanne, 2003.
[23] K. Gekko, H. Ito, Competing solvent effects of polyols and guanidine-hydrochloride on protein stability, J. Biochem. 107 (1987) 572–577.
[24] C. Jarry, C. Chaput, A. Chenite, et al., Effects of steam sterilization on thermogelling chitosan-based gels, J. Biomed. Mater. Res. 58 (2001) 127–135.