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Pseudo-thermosetting chitosan hydrogels for biomedical application☆

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Dedicated to the 60th anniversary of Prof. H. Junginger.

Abstract

To prepare transparent chitosan/β-glycerophosphate (βGP) pseudo-thermosetting hydrogels, the deacetylation degree (DD) of chitosan has been modified by reacetylation with acetic anhydride. Two methods (I and II) of reacetylation have been compared and have shown that the use of previously filtered chitosan, dilution of acetic anhydride and reduction of temperature in method II improves efficiency and reproducibility. Chitosans with DD ranging from 35.0 to 83.2% have been prepared according to method II under homogeneous and non-homogeneous reacetylation conditions and the turbidity of chitosan/βGP hydrogels containing homogeneously or non-homogeneously reacetylated chitosan has been investigated. Turbidity is shown to be modulated by the DD of chitosan and by the homogeneity of the medium during reacetylation, which influences the distribution mode of the chitosan monomers. The preparation of transparent chitosan/βGP hydrogels requires a homogeneously reacetylated chitosan with a DD between 35 and 50%.

Keywords: Chitosan; Hydrogel; Turbidity; Drug delivery system; Reacetylation

1. Introduction

Chitosan is a copolymer of β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose (Fig. 1). This polycationic biopolymer is generally obtained by alkaline deacetylation from chitin (Fig. 1), the main component of the exoskeleton of crustaceans, such as shrimps (Muzzarelli,

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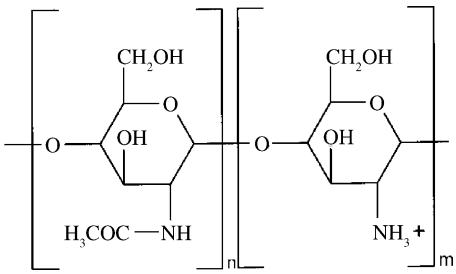

Fig. 1. Structure of chitosan (degree of deacetylation is given by $(m/(m+n))100$) and of chitin ($m \ll n$).

1973). The main parameters influencing its characteristics are molecular weight (MW), crystallinity and morphology. Moreover, the degree of deacetylation (DD), which represents the percentage of deacetylated monomers (Fig. 1), and the distribution mode of the monomers are other essential parameters of chitosan (Domard, 2000) influencing important properties such as solubility (Baumann and Faust, 2001; Sashiwa et al., 2002) and viscosity (Mucha, 1997). Consequently, modification of the DD and of the distribution mode of the chitosan monomers is a common way to modulate the properties of chitosan hydrogels. Ideally, this modification should be easy to perform without the addition of toxic reactants, in order to avoid a decrease of the intrinsic biocompatibility of chitosan. In addition, chitosan should not be degraded and the process should be reproducible and efficient. The DD of commercial chitosan (generally about 80%) can be increased by further deacetylation using concentrated NaOH solutions at temperatures above 100 °C. However this process generally induces degradation that lowers the chitosan MW (Knaul et al., 1998). In order to avoid degradation, deacetylation by enzymatic methods has been suggested (Martinou et al., 1995). More common than deacetylation is the modification of DD by reacetylation, which results in a reduced DD. Reacetylation is generally performed by the addition of acetic anhydride to re-functionalise the amine of chitosan deacetylated monomers with an acetyl group. Consequently, the ratio of the acetylated monomers on the deacetylated monomers is increased, which favours the hydrophobicity of polymeric chains and contributes to the formation of secondary interchain interactions, leading to gelation (Vachoud et al., 2000). As reacetylation is performed in solution, the addition of acid is required to solubilise chitosan. Since the reaction of acetic anhydride with chitosan induces the formation of acetic acid, this latter is generally used (Hirano et al., 1993; Vachoud et al., 2000; Baumann and Faust, 2001). Reacetylation is not an amine specific reaction and the hydroxyl groups of chitosan are generally acetylated at the same time, which decreases the efficiency and reproducibility of the reacetylation process. Therefore, if a large proportion of *O*-acetyl groups is not desired, they have to be removed, for example by treatment with methanolic KOH solution for 6 h (Ogawa and Yui, 1993). However, a simpler method is the use of scavenger groups during the reacetylation reaction in order to minimise *O*-acetylation. For example, an alcohol can be added to the acidic solution of chitosan (Hirano and Yamaguchi, 1976b). In addition to the protective effect, alcohol favours reacetylation by decreasing the dielectric constant of the medium, but on the other hand minimises polymeric chain repulsion and enhances hydrophobic interactions, which increases the viscosity of the solution during reacetylation and often leads to gelation (Aiba, 1994). However, this does not disturb reacetylation, which is a fast reaction step that has been shown to be completed before gelation starts (Domard, 2000). Nevertheless, attention must be paid to the fraction of alcohol added. If this fraction is too high, gelation is favoured and combined with a high proportion of reacetylated monomers this can lead to the formation of an irreversible chitin gel (Vachoud et al., 2000), from which chitosan cannot be precipitated. On the other hand, if the proportion is too low, the efficiency of the reacetylation of amino groups decreases due to the acetylation of hydroxyl groups. The alcohol generally used is methanol (Hirano et al., 1993; Baumann and Faust, 2001). It has been shown that a concentration of 80% (v/v) of methanol allows the best reacetylation efficiency and that a higher concentration only favours gelation (Aiba, 1994). Propanediol (Vachoud et al., 2000) can also be used, but methanol has approximately the same dielectric constant and therefore the same protective effect. In addition, methanol has the advantage of a lower boiling point, which favours final drying of the reacetylated chitosan.

Chitosan/β-glycerophosphate (βGP) pseudo-thermosetting hydrogels were first described by Chenite et al. (2001). Although chitosan with high DD generally precipitates above pH 6.2, neutralisation at low temperature of such chitosan solutions by addition of βGP prevents precipitation (Chenite et al.,

2001). When temperature is increased, the solution turns into a viscoelastic gel that has been shown to be suitable as a drug delivery system (Chenite et al., 2000). A particular interest in the development of chitosan/βGP hydrogels is their transparency. This paper deals with the modification of the DD and of the distribution mode of the chitosan monomers by reacetylation with acetic anhydride with regard to the turbidity of chitosan/βGP hydrogels. In the first stage, the reproducibility and efficiency of two reacetylation methods were evaluated by measuring the obtained DD. In the second stage, the turbidity of chitosan/βGP hydrogels was determined and its modulation by the DD of chitosan and the homogeneity of the reacetylation reaction was investigated.

2. Materials and methods

2.1. Materials

Chitosan of pharmaceutical grade and high MW was purchased from Aldrich Chemical (Milwaukee, USA) with the following characteristics: DD of 83.2% measured by UV (Muzzarelli and Rocchetti, 1986) and MW of 1136 kDa as determined by a size exclusion chromatographic method (Felt et al., 1999). This chitosan is further called "commercial chitosan". Acetic anhydride was obtained from Carlo Erba (Rodano, Italy) and acetic acid from Merck (Darmstadt, Germany), both of analytical grade. *N*-acetyl-D-glucosamine of HPLC quality was purchased from Fluka Chemie (Buchs, Switzerland) and βGP disodium salt containing less than 0.1% of L-α-isomer from Sigma Chemie (Steinheim, Germany). All other reactants were of analytical grade.

2.2. Reacetylation

2.2.1. Method I

Method I is an adaptation of the method proposed by Hirano et al. (1976a), where 1000 mg of chitosan are solubilised in 20 ml of 10% acetic acid and diluted with 80–100 ml of methanol, acetylated with acetic anhydride, precipitated, washed with 300 ml of methanol and dried. Typically 1000.0 mg of chitosan were solubilised in 20.0 ml of 10% acetic acid and the volume was completed to 100.0 ml with methanol. The 10% acetic acid/methanol ratio was fixed at 20/80 to combine reaction efficiency with a minimum risk of irreversible gelation (Aiba, 1994). The solution was stirred at room temperature for 3 days to allow complete dissolution of chitosan. Precautions were taken to avoid evaporation of methanol and, if necessary the volume was completed to 100.0 ml with methanol. Reacetylation was performed by the drop by drop addition of various quantities of acetic anhydride (ranging from 0.1 to 0.4 ml), under fast stirring and at room temperature. The solution was stirred for 8 h at room temperature in order to ensure complete reaction. It turned into a gel, which was transferred into Spectra/Por dialysis bags (Spectrum, Rancho Dominguez, USA) for dialysis against 2 l deionised water for 1 week to eliminate the salts produced during reacetylation (Saimoto and Shigemasa, 2000). The water was changed twice a day. At the end of the purification step, the gel had turned into a viscous solution, which was filtered through a 100 μm nylon Mitex filter (Sefar, Heiden, Switzerland) in order to eliminate insoluble particles of chitosan. Chitosan was precipitated by addition of 0.2 M NH_4OH/methanol (50/50) and washed 4 times with 300 ml of methanol. Chitosan was dried for 3 days in the presence of Silicagel, under vacuum, at room temperature and protected from light. The DD was determined according to the method described below.

2.2.2. Method II

Method II is based on method I but includes several modifications. Prior to reacetylation, chitosan was solubilised in 10% acetic acid/methanol (20/80) mixture as described in method I and successively filtered through a 100 μm nylon Mitex filter and through a 5 μm Petex filter (Sefar, Heiden, Switzerland). Chitosan was precipitated, washed and dried as presented in method I. This eliminated insoluble particles of chitosan and allowed one to determine the exact quantity of chitosan subjected to reacetylation. Reacetylation started from this purified chitosan. Typically, 1000.0 mg were solubilised as described in method I, but the volume was only completed to 90.0 ml with methanol. After complete dissolution, the solution was cooled down in an ice-bath. Reacetylation was performed in an ice-bath to decrease the reaction rate and various quantities of cold acetic anhydride (ranging from 0.1 to 0.4 ml) were mixed in 10.0 ml of cold methanol to favour its homogeneous dilution in the chitosan solution (Ogawa and

Yui, 1993; Vachoud et al., 2000; Baumann and Faust, 2001). To reduce the side reaction of alcohol esterification by acetic anhydride (Vachoud et al., 1997), the mixture was immediately added drop by drop to the chitosan solution under fast stirring. The solution was kept under stirring for 1 h in the ice-bath and turned into a gel that was left at room temperature for 7 h, where its temperature rose gently to ensure complete reaction. The gel was transferred into dialysis bags, dialysed, precipitated, washed and dried as described in method I, but no final filtration was performed at the end of the dialysis step. The DD was determined according to the method described below.

2.3. Determination of the deacetylation degree

The DD was determined as described by Muzzarelli and Rocchetti (1986). Briefly, chitosan was solubilised in 1% acetic acid and the DD was determined by measuring its content of *N*-acetyl-glucosamine by UV (Shimadzu UV-1601, Kyoto, Japan) at λ 200, 201, 202, 203 and 204 nm using *N*-acetyl-D-glucosamine solutions as standards.

2.4. Evaluation of the reproducibility and efficiency of reacetylation methods I and II

The correlation between the DD and the amount of acetic anhydride added was studied in order to compare reproducibility and efficiency of the two methods of reacetylation. A linear regression was performed for each method and the squared correlation coefficient (r^2), the standard deviation of the residuals (S) and the Fisher test for significance of the equation (F) were determined. The ratios of the number of moles of reacetylated monomers in 1000 mg of chitosan in relation to the number of moles of acetic anhydride added per 1000 mg of chitosan were determined in order to evaluate the efficiency of the reaction. The number of moles of reacetylated monomers in 1000 mg of chitosan was given by the difference between the number of moles of deacetylated monomers in 1000 mg of the commercial chitosan and in 1000 mg of the reacetylated chitosan (Eq. (1)).

$$N = \left[\left(\frac{m}{[\mathrm{MW_d} \times \mathrm{DD_c}] + [\mathrm{MW_a} \times (1 - \mathrm{DD_c})]}\right) \times \mathrm{DD_c}\right] - \left[\left(\frac{m}{[\mathrm{MW_d} \times \mathrm{DD_r}] + [\mathrm{MW_a} \times (1 - \mathrm{DD_r})]}\right) \times \mathrm{DD_r}\right] \quad (1)$$

N, number of moles of reacetylated monomers; m, mass of chitosan; $\mathrm{MW_d}$, molecular weight of a deacetylated monomer (162.16 g/mol); $\mathrm{MW_a}$, molecular weight of an acetylated monomer (203.19 g/mol); $\mathrm{DD_c}$, degree of deacetylation of commercial chitosan (83.2%); $\mathrm{DD_r}$, degree of deacetylation of reacetylated chitosan.

For both methods, the average ratio was calculated and their difference was evaluated with a Mann–Whitney test.

2.5. Reacetylation of chitosan under homogeneous or non-homogeneous conditions

Five thousand milligrams of chitosan were reacetylated to various DD according to method II. In order to ensure homogeneous conditions, a stirring propeller (IKA Eurostar-D, Staufen, Germany) was used during the reacetylation. For the preparation under non-homogeneous conditions, stirring was performed with a magnetic stirrer (IKA-combimag, Staufen, Germany).

2.6. Determination of turbidity of chitosan/βGP hydrogels

Chitosan/βGP hydrogels were prepared according to the method described by Chenite et al. (2001). The added chitosan was either the commercial chitosan or a chitosan reacetylated under homogeneous or non-homogeneous conditions. Briefly, 100.0 mg of chitosan were solubilised in 5.0 ml of 0.1 N HCl at room temperature for 24 h. After complete dissolution, this solution was cooled down in an ice-bath. A solution of 1000.0 mg of βGP in 5.0 ml of deionised water was prepared at room temperature and cooled down in an ice-bath. It was added drop-wise under fast stirring to the chitosan solution in an ice-bath to form a viscous solution. This latter was stirred for 10 min and then stored at 4 °C. The hydrogel was formed by heating chitosan/βGP solution in a UV quartz cell (Perkin-Elmer, Wellesley, USA) in a water bath at 37 °C for 2 h. Turbidity was then measured at 620 nm with a UV spectrophotometer (Shimadzu UV-1601, Kyoto, Japan)

using formazin suspensions as standards (Horwitz, 1960). Briefly, hydrazine sulfate was reacted with hexamethylenetetramine to induce formazin precipitation. Standards of known formazin turbidity units (FTU) were prepared by appropriate dilution.

3. Results

Fig. 2a and b represent the obtained DD as a function of the molar ratio of added acetic anhydride and chitosan for methods I and II, respectively. It can be seen that poor reproducibility was obtained with method I (Fig. 2a and Table 1). Indeed, the linear regression has a r^2 value of only 0.41 and the obtained DD are dispersed. On the other hand, the resulting DD have a narrower distribution with method II and a r^2 value of 0.88 and a smaller S value for the linear regression illustrate that a much better reproducibility was obtained (Fig. 2b and Table 1).

In addition to a better reproducibility, method II was also more efficient, as shown by the steeper slope of the linear regression relating the DD obtained and the molar ratio of added acetic anhydride and chitosan (Table 1). In other words, for the same molar ratio (about 0.9) the obtained DD was about 30% with method II, while it was about 50% with method I (Fig. 2). This indicates that a greater proportion of the added acetic anhydride reacted with chitosan in method II. Indeed, the average number of moles of reacetylated monomers in relation to the average number of moles of added acetic anhydride was higher with method II (Table 1).

The influence of the DD and of the reacetylation conditions on the turbidity of chitosan/βGP hydrogels was investigated with chitosans of DD ranging from

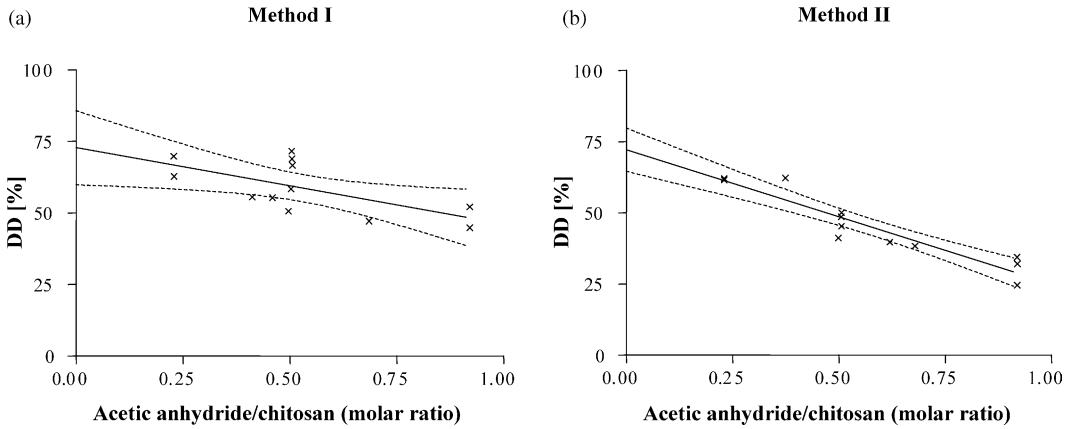

Fig. 2. Degree of deacetylation (DD) obtained as a function of the volume of acetic anhydride added per 1000 mg of chitosan reacetylated according to (a) method I, (b) method II; - - -, linear regressions with 95% confidence intervals ($n = 1$).

Table 1
Reproducibility and efficiency of reacetylation methods I and II: linear regressions and average ratios of the number of reacting moles

Method of reacetylation	r^2[a]	S[b]	F[c]	Average ratios[d]	Equation[e]
I	0.41	7.41	6.9	0.74 ± 0.28	DD = −26.69 × +72.77
II	0.88	4.49	73.6	1.01 ± 0.21	DD = −47.15 × +72.15

[a] Squared correlation coefficient.
[b] Standard deviation of the residuals.
[c] Fisher test for significance of the equation.
[d] Average ratios of the number of moles of reacetylated monomers and the number of moles of added acetic anhydride. As the average ratios are different, the improved method is more efficient than the basic method ($p < 0.02$).
[e] Equation of the obtained degree of deacetylation as a function of the added quantity of acetic anhydride per gram of chitosan.

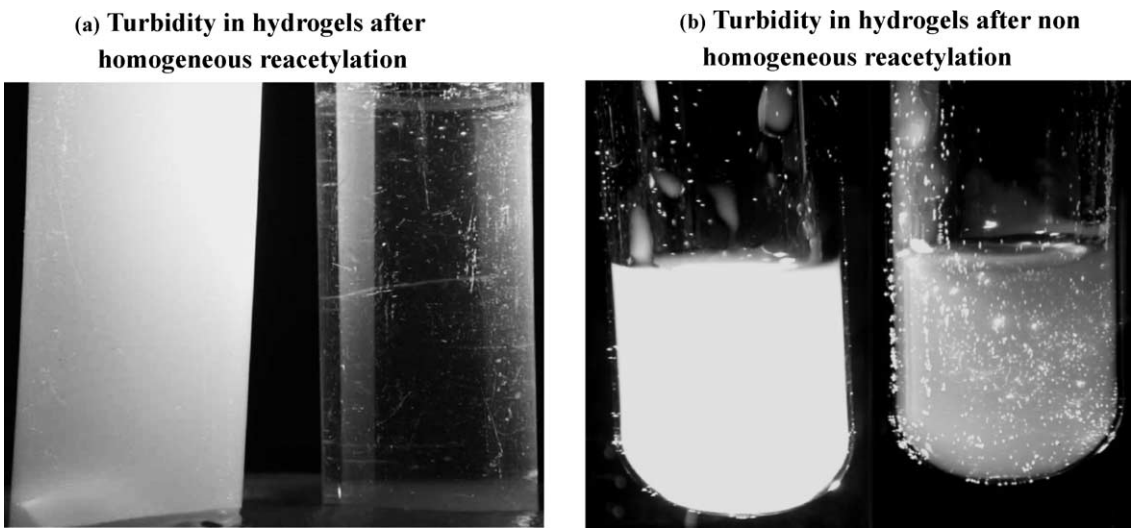

Fig. 3. Optical appearance of chitosan/β-glycerophosphate hydrogels containing; (a) the commercial chitosan with a degree of deacetylation (DD) of 82.3% (left) and a homogeneously reacetylated chitosan with a DD of 50.0% (right); (b) the commercial chitosan with a DD of 82.3% (left) and a non-homogeneously reacetylated chitosan with a DD of 48.3% (right).

35.0 to 83.2%. The turbidity of chitosan/βGP hydrogels was found to be influenced by the DD of chitosan. Indeed, a hydrogel containing the commercial chitosan was turbid (Fig. 3a, on the left), but the turbidity decreased with a decreasing DD (Fig. 4). Turbidity depended not only on the obtained DD, but also on the homogeneity of the reacetylation. If reacetylation was performed under homogeneous conditions, hydrogels were optically transparent with DD of 50% or less, as can be seen in Fig. 3a (on the right). If reacetylation was performed under non-homogeneous conditions, due to inadequate stirring for example, turbidity decreased,

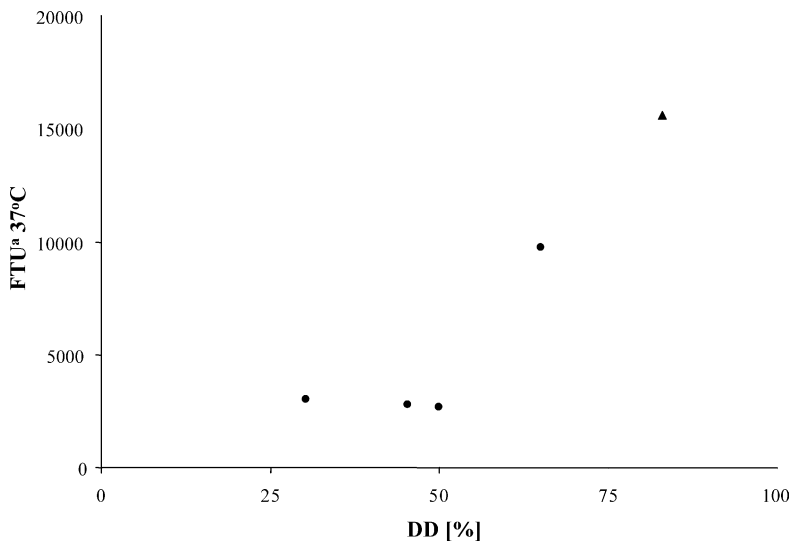

Fig. 4. Turbidity of chitosan/β-glycerophosphate hydrogels after gelation in relation to the degree of deacetylation of the chitosan; (▲) commercial chitosan; (●) homogeneously reacetylated chitosan; Formazin Turbidity Units[a] (FTU[a]) ($n = 1$).

compared to the hydrogel containing the commercial chitosan, but hydrogels looked turbid whatever the DD of chitosan (Fig. 3b).

4. Discussion

The poor reproducibility of method I can be explained by the lack of homogeneity of the chitosan solution. Indeed, large insoluble particles decreasing the homogeneity of the chitosan solution were still visible in the solution after 3 days of stirring. In addition, transparent gel particles were observed, since the chitosan solution gelled immediately in contact with acetic anhydride drops. At room temperature, reacetylation was obviously fast in the vicinity of the acetic anhydride drops, leading to a very low DD and to formation of insoluble chitin gel particles (Vachoud et al., 2000). Consequently, method I allowed only poor diffusion of the acetic anhydride in the solution. Method II allowed a better homogeneity of the solution and consequently a better reproducibility. Indeed, no insoluble particles were observed with method II due to previous filtration. Dilution and cooling of acetic anhydride avoided a fast reacetylation and the formation of chitin gel particles. In addition, the reaction rate was reproducible since reacetylation was always performed at the same temperature in an ice-bath.

The lower efficiency of method I can be explained by the entrapment of a part of the acetic anhydride inside chitin gel particles that did not swell nor solubilise during the course of the reaction, since chitin is insoluble in diluted acid or organic solvents (Vachoud et al., 2000). Therefore, less reactant was available to react with chitosan. In addition, some of the added reactant could be absorbed by the insoluble particles observed with method I. It should be noted that the average of the ratios of the number of moles of reacetylated chitosan and added acetic anhydride was found to be about 1 for method II. This value seems rather surprising, as we did not expect to observe a stoichiometric reaction between acetic anhydride and the amino groups of chitosan. According to the literature, even if methanol was added as a protecting group for the hydroxyl groups of chitosan, some of these latter should be acetylated (Aiba, 1994) and the observed average of the ratios should be smaller than 1. A moderate *O*-acetylation of chitosan hydroxyl groups may be of interest, as this improves water solubility of the reacetylated chitosan (Sashiwa et al., 2002).

A hypothesis based on the distribution mode of the chitosan monomers has been put forward to explain the differences in turbidity of chitosan/βGP hydrogels containing homogeneously or non homogeneously reacetylated chitosans. Generally, commercially available chitosan is industrially prepared by deacetylation of solid chitin particles (Muzzarelli, 1973). Since deacetylation preferentially occurs in the amorphous zones of chitin and at the surface of the particles, the conditions are non-homogeneous and the monomers have a block-type distribution (Aiba, 1991). If chitosan is reacetylated under homogeneous conditions, the monomers adopt a random distribution, which induces a decrease of the crystallinity of chitosan (Aiba, 1991; Ogawa and Yui, 1993; Milot et al., 1998). On the other hand, if reacetylation is performed under non-homogeneous conditions, a block-type distribution of the monomers is obtained (Baumann and Faust, 2001), as shown by a higher crystallinity of chitosan (Ogawa and Yui, 1993).

During the formation of chitosan/βGP hydrogels, monomers of the same type (either acetylated or deacetylated) interact together (Berger et al., submitted for publication). These interactions lead to the formation of two types of domains, hydrophilic domains containing the deacetylated charged monomers and most of the negatively charged βGP and hydrophobic domains containing the acetylated uncharged monomers. Since the composition of the two types of domains is different, they are likely to have different refractive indexes, leading to light-scattering. Due to the block distribution of monomers in commercial chitosan or chitosan reacetylated under non-homogeneous conditions, the domains formed during gelation are large. Consequently, the light-scattering they induce leads to turbid hydrogels (Fig. 5a). During the formation of hydrogels containing a chitosan reacetylated under homogeneous conditions to a DD of about 50% or less, numerous microdomains are formed, due to the random distribution of monomers. Consequently, light is only slightly scattered when it passes through the hydrogel which therefore looks transparent (Fig. 5b). In addition to the different sizes of the domains formed during thermogelation, the different distribution modes have opposite effects on chitosan solubility, which can also influence

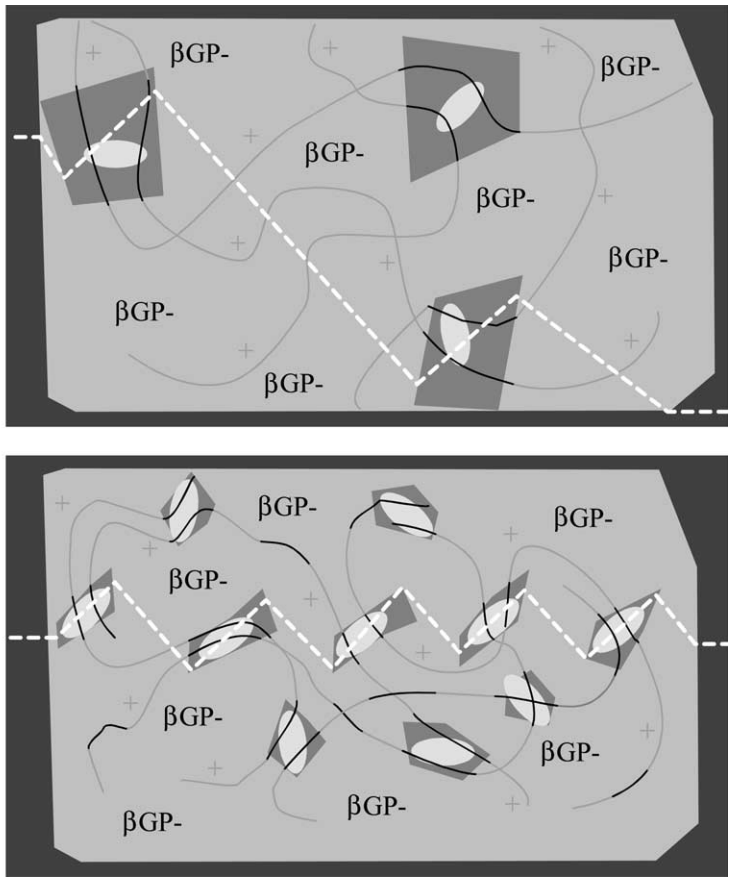

Fig. 5. Schematic representation of the formation of domains in chitosan/β-glycerophosphate (βGP) hydrogels after thermogelation and their influence on light scattering; (a) degree of deacetylation (DD) of chitosan of about 85% with a block distribution of the monomers; (b) DD of chitosan of about 50% with a random distribution of the monomers; (+ ▬) positively charged deacetylated monomers; (▬) uncharged acetylated monomers; ( ) hydrophilic domain; ( ) hydrophobic domain; ( ) hydrophobic interaction; βGP− negatively charged βGP; ( ) light and background.

turbidity. Indeed, reacetylation to a DD of around 50% performed under homogeneous conditions allows enhancement of chitosan solubility due to the random distribution of the positive charges along the polymeric backbone (Kurita et al., 1977) combined with *O*-acetylation of monomers (Sashiwa et al., 2002). This increases the solubilisation of the polymeric chains and favours the transparency of the hydrogel. On the other hand, a block distribution is well known to induce solubility problems (Kurita et al., 1991; Baumann and Faust, 2001). Therefore, micro-precipitations can occur in hydrophobic domains, leading to an increase in turbidity.

5. Conclusions

This study has shown that the efficiency and reproducibility of the reacetylation of chitosan can be improved compared to published methods by previous filtration of chitosan, dilution of acetic anhydride and reduction of the reaction temperature. The modified reacetylation method proposed (method II), allows preparation of chitosan with a desired DD and therefore facilitates the modulation of the properties of chitosan hydrogels. In addition, it has been shown that the properties of a chitosan hydrogel, such as turbidity of chitosan/βGP hydrogels, are not only mod-

ulated by the DD of the used chitosan but also by the distribution mode of the monomers, influenced by reacetylation conditions such as homogeneity of the medium. Moreover, as the distribution mode of the monomers is well known to influence interchain interactions, drug release should be modulated by the reacetylation method. It is likely that a random distribution of monomers would prolong drug release due to additional interchain interactions. Further investigations are required to characterise this potential modulation. When commercialising a reacetylated chitosan, reacetylation conditions or distribution mode of monomers should be mentioned as additional information to the standard data, such as DD and MW. Particular attention should be paid to the homogeneity of the reacetylation reaction if a random distribution is required. This study has shown that stirring was an important parameter determining the homogeneity of the medium. Consequently, adaptations should be considered during scale-up processes. Finally, it has been shown that chitosan/βGP hydrogels are transparent if the chitosan used has been reacetylated under homogeneous conditions and has a DD of 50% or less.

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