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# Chitosan–glycerol-phosphate (GP) gels release freely diffusible GP and possess titratable fixed charge

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ABSTRACT

Chitosan–glycerol-phosphate (GP) solutions undergo a sol–gel transition upon heating. A model of heat-induced transfer of protons from chitosan to GP in solution has been proposed to explain this sol–gel transition. This model suggests that reduced inter-chain electrostatic repulsion by a decreased protonation of positively charged amino groups – via proton transfer to $GP^{2-}$ thereby reduced to $GP^{-}$ – would allow chitosan polymer chains to precipitate together and form a solid hydrogel structure. The hypothesis that GP has the single role of acting as a proton acceptor and not as a divalent electrostatic cross-linker of chitosan amine groups suggests that it should freely diffuse out of the gel after the gel formation. We found indeed that GP is free to diffuse and the experimental diffusion profile corresponds to a free diffusion model indicating that it plays no role in electrostatic cross-linking.

Finally since chitosan amine groups in the gel are not bound to GP, we examined whether they are titratable in the gel. We show that chitosan in the hydrogel indeed possesses titratable amine groups with significant fixed charge up to +80 mM and follows the same ionization behavior as chitosan in solution. The results of these studies are significant in light of the current and future biomedical applications of this system, including its recent clinical approval as a biomaterial for cartilage repair.



## 1. Introduction

Chitosan is a biodegradable polyelectrolyte produced by deacetylation of chitin, a polysaccharide found in the shell of crustaceans (Hoppe-Seyler, 1894). Chitosan is composed of glucosamine and N-acetyl-glucosamine monomers linked by a $\beta$-(1→4) glycosidic bonds. The fraction of deacetylated monomers ($f_D$) refers to the fraction of monomers that are glucosamine. A thermosensitive solution composed of a mixture of chitosan and glycerol phosphate (GP) which turns into a solid hydrogel upon heating has been described (Chenite, Buschmann, Wang, Chaput & Kandani, 2001). This system was subsequently developed for cartilage repair (Chevrier, Hoemann, Sun & Buschmann, 2006; Hoemann, Sun, Légaré, McKee & Buschmann, 2005) and has been approved for clinical use in the European Community under the name BST-CarGel (http://bst-cargel.piramal.com/media/index.html). Recently, a mechanistic model for the thermal gelation process of chitosan–GP systems has been proposed (Filion, Lavertu & Buschmann, 2007; Lavertu, Filion & Buschmann, 2008). In this model, the presence of GP allows for a reduction in the charge state of chitosan upon heating via proton transfer from chitosan to GP thereby neutralizing divalent $GP^{2-}$ to the monovalent $GP^{-}$ form. The underlying cause of proton transfer from chitosan amine groups to GP is due to different sensitivities to temperature of their ionization constants ($pK_a$); the chitosan $pK_a$ drops with temperature, as is typical for many titratable groups, while the $pK_a$ of GP is largely temperature independent, thus allowing GP to accept protons that are released from chitosan when temperature is increased. This model implies then that GP is not bound to chitosan as an electrostatic crosslinker or hydrophobicity inducing agent would be, but rather is free to diffuse within the gel since it functions only as a proton acceptor. This proposed model indicates that chitosan transforms from a solution to a gel state due to neutralization of its amine groups after donating protons to GP, since attractive chain-chain interactions (van der Waal's and others) will then predominate after a reduction of interchain electrostatic repulsion and create a solid hydrogel. The primary goal of our current study was to investigate a hypothesis generated by this model, namely that GP should freely diffuse out of the chitosan–GP gel after gelation since its role as a proton acceptor does not require a continued presence of GP as would alternative roles such as electrostatic cross-linking

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or a dehydration effect due to increased GP hydrophobicity with heat. We also investigated the hypothesis that amine groups in chitosan–GP gels remain titratable, since binding to GP is absent, and can therefore provide these gels with a cationic character at pH near or slightly below neutral pH.

Diffusion in gels has been widely studied and numerous different methods for measuring diffusion coefficients (Lauffer, 1961; Muhr & Blanshard, 1982) can be used. In the present study, we equilibrated freshly generated chitosan–GP gels with a bulk solution and measured the increasing concentration of phosphorus in the bulk solution over time. Diffusion of caffeine in chitosan gels has been studied (Payet, Ponton, Agnely, Colinart & Grossiord, 2003). They found diffusion coefficients in the gels to vary from $0.51 \times 10^{-5}$ to $0.55 \times 10^{-5}$ cm$^2$/s (caffeine MW = 194 g/mol).

Since the presence of fixed charge groups from amine groups of chitosan in the gel can alter concentrations of mobile ions like GP via the Donnan effect, and release rates of charged molecules, we also measured the fixed charge density of these chitosan gels using radioactive probes $Na^{22}$ and $Cl^{36}$, as previously performed by Maroudas for other hydrated systems bearing fixed charge such as articular cartilage (Maroudas & Evans, 1972). We then directly tested our hypotheses that GP is freely diffusing and not bound to the gel via quantitative comparison of measured GP concentration in the bulk solution to the prediction of a mathematical model that accounts for the geometry of the gel and the presence of fixed charge groups in the gel.

## 2. Materials and methods

### 2.1. Preparation of chitosan–GP gels

#### 2.1.1. Chitosan gel for GP diffusion analysis

Chitosan–GP gels were made by stirring a 2.93%, w/v chitosan chloride salt in 7.5 mL distillated and deionised water – $ddH_2O$ (Protasan UP CL 213, Batch no. 607-783-02, from Pronova Oslo Norway, pH = 5.0 at 21 °C, $f_D$ = 0.84, $L_D$ = 8.6% where $L_D$ refers to the loss on drying and viscosity of 143 cP for a 1%, w/v in $ddH_2O$ solution) on an ice bath to bring the solution to 4 °C. To this solution, we added 2.25 mL of a glucosamine solution (Sigma, G-1514, 2.16%, w/v in 0.1 N NaOH) drop by drop, waiting 15 s between drops. We then added 1 mL of a glycerol phosphate disodium salt solution (Sigma, G-9891, 33.3%, w/v, in $ddH_2O$) also drop by drop. Two milliliters of hydroxyethyl cellulose (Fluka, 54290, 2.5%, w/v in DMEM pH 7.4 solution) were then added to the mixture (Hoemann et al., 2007) in order to obtain a stronger gel to permit manual handling described below. The final pH of this solution was 6.85 at 25 °CA separate aliquot of GP solution was kept for phosphorus content analysis in order to validate its initial concentration.

We used 5 g of the mixture prepared as described in the previous paragraph and poured into Petri dishes of 5.3 cm diameter for GP diffusion analysis. The procedure was performed quickly to prevent solution gelling before settling to have the upper surface parallel to the bottom of the Petri. The remainder of the solutions was poured into 3 cm Petri dishes to analyse initial GP content in the gels. The Petri dishes were placed for 30 min in an incubator at 37 °C and 5% $CO_2$ under 100% relative humidity on a leveled shelf to permit gelation to be completed.

#### 2.1.2. Chitosan gel disks

Chitosan–GP gels were prepared from 5 mL of a 2.0%, w/v chitosan (Aldrich Chemical, Cat. No. 44887-7, $f_D$ = 0.836, $L_D$ = 0.061 and viscosity of 460 cP for a 1 wt.% in 1% acetic acid) solution dissolved in 0.1 M acetic acid (pH 3.98). The solution was autoclaved for 30 min, cooled down and stirred in ice bath. Slowly, 0.5 mL of a 38.8%, w/v disodium glycerol phosphate (Sigma, G-9891) was added to the stirred chitosan. The addition of 1.5 mL hydroxyethyl-cellulose (Fluka, 54290, 16.7%, w/v) solution created a stronger gel capable of being cored and manipulated (Hoemann et al., 2007). This mixture was poured into a 6 cm diameter Petri dish and placed in an incubator at 37 °C for 30 min. We then added 6 mL of DMEM (pH 7.4) on top of the gel for 15 min. A second and third wash were done with 6 mL of fresh DMEM with 15 min interval and the last wash removed after overnight incubation. 4 mm diameter disks were removed with a biopsy punch.

#### 2.1.3. Cartilage disks

Articular cartilage discs were taken from young (1½ year-old adolescent) bovine shoulder joints as described (Dumont et al., 1999) and then frozen in liquid nitrogen until fixed charge measurements. Cartilage discs were used as controls since they are known to bear a negative fixed charge (Maroudas, 1968; Maroudas, Muir & Wingham, 1969).

#### 2.1.4. Agarose disks

A 2.0%, w/v agarose was boiled in TBS and 3 mL of this solution was poured into a 6 cm diameter Petri dish in order to have discs of about 1–1.5 mm thickness when removed with biopsy punches as described for chitosan gel disks. Agarose is composed of d-galactose and 3,6-anhydro-l-galactose which are neutral monomers and was therefore used as a control for an uncharged or neutral gel.

#### 2.1.5. Primary buffer solutions

Saline Hepes buffer and Saline Sodium Carbonate buffer solutions were prepared from Free Acid (Sigma, Cat. No. H3375), $NaHCO_3$ (Sigma, Cat. No. S8875) and NaCl (Fisher Scientific, Cat No. S271-1). The appropriate mass of Hepes or $NaHCO_3$ and NaCl were dissolved in $ddH_2O$ in order to obtain a final concentration of 10 mM (Hepes or Sodium Carbonate) with 50 or 150 mM NaCl. The pH was adjusted using NaOH 1 N (or HCl 1 N) to 6.0, 6.5, 7.0 or 7.5.

#### 2.1.6. Radioactive buffer solutions

Radioactive solutions were made by adding $NaCl^{36}$ aqueous solutions (Amersham, Cat. No. CIS3-100UCI) and $Na^{22}Cl$ (Amersham, Cat. No. SKS1-100UCI) at a 1:20 (5%) volume ratio of the radioactive to primary solutions. To obtain $Na^{22}Cl^{36}$ solutions, $Na^{22}Cl$ and $NaCl^{36}$ aliquots representing 1.0% of the volume of primary solutions were added to separate volumes (1.0 μCi/mL in order to calibrate the scintillation counter). The two volumes were then mixed in 1:1 ratio and final solution was 0.5 μCi/mL.

### 2.2. Measurement of phosphorus content (Kjeldahl digestion)

Gel samples were weighed to estimate their volume and 50 μL aliquots of medium were taken to analyse GP content by phosphorus measurements. Phosphorus content was determined following an established procedure (Liao & Milwaukee, 1994). In this procedure phosphorus from glycerol phosphate and any other phosphorus containing components are transformed to an orthophosphate ion following a Kjeldahl digestion. During the analysis, the *Lachat Quik Chem AE* instrument injects an ammonium molybdate and potassium antimoin tartrate solution under acidic conditions. The complex then formed absorbs light at 880 nm and this measurement is proportional to the phosphorus concentration. The absorption is compared to that obtained from standard phosphorus solutions.

### 2.3. Analysis of GP diffusion

Fig. 1 shows a schematic of the gel and GP concentration profiles calculated with the model presented below. The $x$-axis passes through the center of the Petri dish with $x = 0$ at the bottom surface

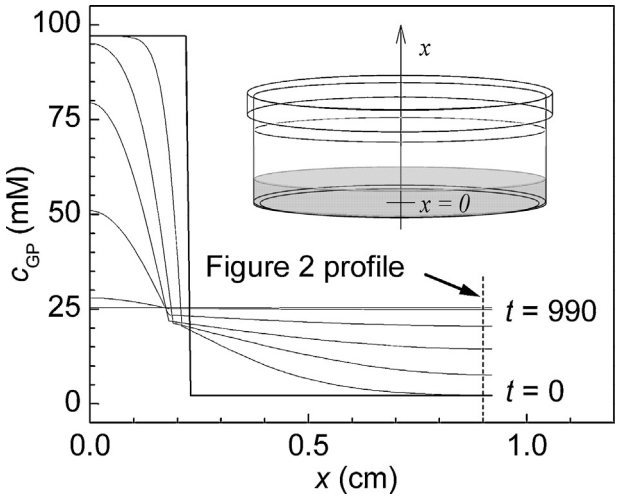


**Fig. 1.** Predicted GP concentration profiles at a position $x$ where the bottom of the Petri dish is $x = 0$ and the gel liquid-medium (DMEM) interface is at $x = 0.92$ cm, for the times $t = 0$, 10, 40, 90, 190, 490 and 990 min.

of the Petri and at $x = 0.22$ cm is the initial top surface of the gel that contacts the liquid medium. The ratio of the height to diameter of the gel (0.9 cm/5.3 cm = 0.17) permits neglecting edge effects and analyzing the diffusion process as a 1-dimensional problem.

We defined $c(x,t)$ as the concentration of GP at position $x$ and time $t$. We also defined the gel–liquid interface position as $x = h(t)$ so that the height of the gel can vary with time $t$ to model gel shrinkage. Gel height was calculated using the Petri radius $r$, the gel mass $m$, which varies with time, and an approximate volumetric density $\rho$ of 1 g/mL via

$$h = \frac{m}{\rho \pi r^2} \tag{1}$$

The total height $\delta$ of the gel and liquid medium was given by the initial gel height $h_i$ plus the washing (bulk) solution height calculated from the volume $V$ added and Petri radius $r$ via

$$\delta = h_i + \frac{V}{\pi r^2} \tag{2}$$

After 30 min in the incubator, 15.5 mL of DMEM (pH 7.4) was added. This time of addition of DMEM determined the time $t = 0$ min and successive samples (50 μL of washing solution) were taken at pre determined times (2.5, 6.5, 14.5, 28.0, 46.5, 79.0, 240.0 and 1080.0 min). A preliminary experiment (data not shown) allowed us to approximate the time to equilibrium at about 18 h (1080 min).

To account for gel shrinkage during the experiment (gel–liquid interface movement), we removed the liquid at time 1080 min and measured the final mass of the gel $m_f$ in order to find the final gel height $h = h_f$ (see Eq. (1)). Knowing the initial gel height $h_i$ and final height $h_f$, we approximate the gel height $h$ as a function of time $t$ by the following function

$$h(t) = h_f + (h_i - h_f)e^{-t/\tau} \tag{3}$$

where the variable $\tau$, a time decay constant, is left to be fit with experimental data. This function was taken from Tanaka (Tanaka, Sato, Hirokawa, Hirotsu, & Peetermans, 1985).

The function $c(x,t)$ is a solution of the diffusion equation

$$\frac{\partial c(x,t)}{\partial t} = D(x,t)\frac{\partial^2 c(x,t)}{\partial x^2} \tag{4}$$

where the diffusion constant $D(x,t)$ is different in the gel versus in the washing solution. We therefore have

$$\begin{aligned} D(x,t) &= D_g \quad \text{for} \quad x < h(t) \\ &= D_s \quad \text{for} \quad x > h(t) \end{aligned} \tag{5}$$

where $D_g$ and $D_s$ are the diffusion constant in the gel and in the washing solution respectively.

Initial conditions are

$$\begin{aligned} c(x,0) &= c_g \quad \text{for} \quad x < h(0) \\ &= c_s \quad \text{for} \quad x > h(0) \end{aligned} \tag{6}$$

where $c_g$ and $c_s$ is the concentration of GP in the gel and in the washing solution respectively.

Since there is no concentration gradient at the bottom of the Petri and at the liquid-air interface, the diffusion equation is restricted to the boundary conditions

$$\frac{\partial c(0,t)}{\partial x} = \frac{\partial c(\delta,t)}{\partial x} = 0 \tag{7}$$

To numerically solve the diffusion equation (Eq. (4)) subject to Eqs. (5)–(7), we used the function *pdepe* from Matlab (The Mathworks™).

### 2.4. Calculation of ionic fixed charge concentrations

Gel disks were initially weighed ($m_g^i$) and then incubated in 1.3 mL of the primary buffer solution overnight. Disks were then removed from the solution, weighed again ($m_g^i$) to determine any change in mass:

$$\Delta m = \frac{m_g - m_g^i}{m_g^i} \tag{8}$$

and then put into the corresponding radioactive buffer solutions overnight. Aliquots of 100 μL from these buffer solutions were taken for isotope concentration measurements.

For the single isotope technique, the isotope concentration $i_{Cl}$ was determined using

$$i_{Cl} = \frac{n^\beta}{n^r} i_{Cl}^r \tag{9}$$

where, $n^\beta$ is the number of counts detected (in cpm) from the tested aliquot, $n^r$ is the number of counts detected (in cpm) from the reference solution of known isotope concentration $i_{Cl}^r$.

For the dual isotope technique, the radioactive solution $Na^{22}Cl$ emits one $\gamma$ and one $\beta$ for every emission while the $NaCl^{36}$ only emits $\beta$. The linear relation between isotope concentration $i$ (in μCi/mL) in the aliquoted volume (the aliquot volume was always 100 μL) and the number of counts per minute $n^\beta$ detected by the $\beta$ counter (Packard Tri-Carb,1900 TR, Liquid Scintillation Analyzer) and $n^\gamma$ counts (Beckmann, Gamma 5500) detected by the $\gamma$ counter is given by

$$\begin{bmatrix} n^\beta \\ n^\gamma \end{bmatrix} = \begin{bmatrix} \varepsilon_{Cl}^\beta & \varepsilon_{Na}^\beta \\ \varepsilon_{Cl}^\gamma & \varepsilon_{Na}^\gamma \end{bmatrix} \begin{bmatrix} i_{Cl} \\ i_{Na} \end{bmatrix} \tag{10}$$

where the $\varepsilon_j^k$ (in cpm/(μCi/mL)) values are detection coefficients relative to ion species $j$ and radiation type $k$.

The $\varepsilon_j^k$ values were determined with known isotope concentration solutions, using

$$\begin{bmatrix} i_{Cl} \\ i_{Na} \end{bmatrix} = \begin{bmatrix} \varepsilon_{Cl}^\beta & \varepsilon_{Na}^\beta \\ \varepsilon_{Cl}^\gamma & \varepsilon_{Na}^\gamma \end{bmatrix}^{-1} \begin{bmatrix} n^\beta \\ n^\gamma \end{bmatrix} \tag{11}$$

The ionic concentration of species $j$ is then found using:

$$c_j = \frac{c_j^r i_j}{i_j^r}\left(1 + \frac{V\rho_g}{m_g}\right) \tag{12}$$

where the subscript $j$ is used for ion species (either Na or Cl), $c_j$ is the ion concentration in the gel, $i_j$ is the isotope concentration of the tested aliquot (found using Eq. (11)), $c_j^r$ is the reference solution ionic concentration, $i_j^r$ is the reference solution isotope concentration, $V$ is the total dilution solution volume (the bulk solution of 1.3 mL), $\rho_g$ is the gel density (approximated to 1 g/mL) and mg is the gel mass. The first factor $c_j^r i_j / i_j^r$ is the aliquot concentration and the second factor $1 + V\rho_g/m_g$ takes in account the gel and bulk solution volumes in which ions are now diluted. Note that $c_j^r$ was calculated taking into account the ion content of added Hepes, $NaHCO_3$, NaOH or HCl to the NaCl wash concentration.

To determine the fixed charge concentration $\rho$, we use Donnan equilibrium (Donnan, 1911) (approximating that activity coefficient as 1) for the single isotope technique in which case, $n^\gamma = 0$

$$\rho = c_{Cl} - \frac{(c^b)^2}{c_{Cl}} \tag{13}$$

where $c^b$ is the mean ionic concentration in the bulk solution. For the dual isotope technique, we used the electroneutrality relation (neglecting the presence of the buffer and water ions)

$$\rho = c_{Cl} - c_{Na} \tag{14}$$

The dual isotope technique permits direct calculation of ion concentrations in the gel, thus allowing the calculation of activity coefficient in the gel $\gamma_\pm^g$, using (Donnan, 1911)

$$\gamma_\pm^g = \sqrt{\frac{(\gamma_\pm^b c^b)^2}{c_{Cl} c_{Na}}} \tag{15}$$

where $\gamma_\pm^b$ is the activity coefficient in the bulk solution.

### 2.5. Calculation of $pK_{ap}$

In order to calculate the apparent $pK_a$ ($pK_{ap}$) the concentration of amine groups on chitosan, $c_{amine}$, is determined using

$$c_{amine} = \frac{m_c}{MW}\frac{(1 - L_D)f_D}{V} \tag{16}$$

where $m_c$ is the dissolved mass of chitosan of molecular weight MW, dissolved in the volume $V$. $L_D$ refers to the loss on drying measured as the ratio of weighted loss over the weight including volatiles at room temperature and normal pressure.

The fraction of protonated amine groups, $\alpha$, is determined using

$$\alpha = \frac{\rho(1 + \Delta m)}{c_{amine}} \tag{17}$$

Using Eq. (17) and the measured pH, one can calculate the apparent dissociation constant from

$$pK_{ap} = pH - \log\left(\frac{1 - \alpha}{\alpha}\right) \tag{18}$$

## 3. Results

### 3.1. Diffusion of glycerol phosphate from chitosan–GP gels

Measurement of GP in the chitosan–GP gel and in media wash solutions via the Kjeldahl digestion indicates that most of the GP diffuses out with only a small fraction remaining in the gel after 3 washes (~3% or 3 mM of the initial 97 mM) and after 6 washes (~1.5% or 1.5 mM of the initial 97 mM) (see Table 1).

**Table 1**
Phosphorus concentration of different gel components and the washing solution (DMEM).

| Sample | $c_P$ (mM) |
|---|---|
| GP solution (38.8%, w/v) | 1252 |
| Initial gel | 97 ± 2 |
| DMEM initial (pH = 7.4) | 2.16 |
| Gel after 3 washes | 2.97 |
| Gel after 6 washes | 1.56 |

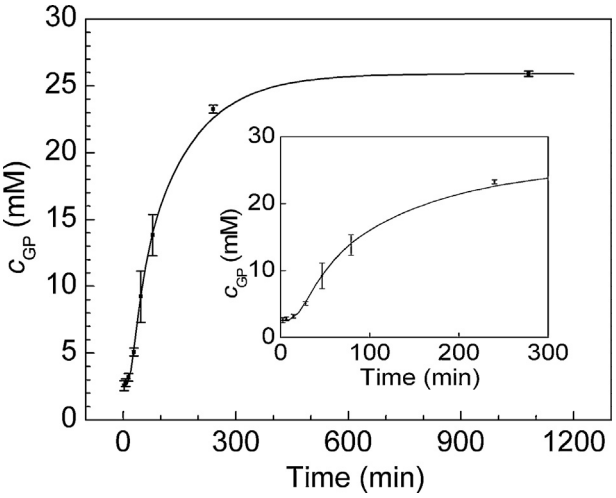


**Fig. 2.** Profile of GP concentration versus time in the bulk of the medium outside the gel at position $x = 0.9$ cm from the center of the Petri dish. GP concentration is obtained via phosphorus measurements on medium aliquots taken at different times. SD represents minimum and maximum values. The model (line) was obtained by fitting Equations 4 to 7 to the data and adjusting diffusion coefficients.

The quantitative diffusion model presented in Eqs. (1)–(7) estimates the concentration profile of GP in the gel and in the wash solutions over time (Fig. 1). The initial gel height was set to 0.22 cm based on its initial mass and the height of the top of the solution to 0.92 cm based on added solution volume (Eqs. (1) and (2)). Final gel height is set to 0.18 cm as estimate using final gel mass following gel contraction at 18 h. An initial GP (phosphorus) concentration of 97 mM in the gel and 2.16 mM in the DMEM solution were used based on phosphorus content analysis (see Table 1), although a direct calculation based on solution composition suggested the GP concentration in the gel to be about 9% lower (88 mM). By comparing the model prediction of GP concentration in the medium (at $x = 0.9$ cm in Fig. 1) to that measured directly from the medium, we found the model to substantially agree with experimental data when diffusion coefficients were chosen appropriately (Fig. 2). Good fits to the data were obtained with a GP diffusion constant of $0.153 \times 10^{-5}$ cm$^2$/s in the gel and $5 \times 10^{-5}$ cm$^2$/s in the washing solution where this latter value approximated a factor due to stirring of the solution. This free diffusion model predicts accurately the GP concentration in medium over time, further confirming that GP is freely diffusible in the chitosan–GP gel. A value of $\tau = 30$ min was used to describe gel shrinkage from 0.22 cm height to 0.18 cm in Eq. (3).

### 3.2. Assessment of fixed charge from amine groups in chitosan–GP gels

#### 3.2.1. Single isotope method

The mass variation $\Delta m$ from overnight incubation (Eq. (8)) and fixed charge concentration $\rho$ (Eqs. (9), (12) and (13)) found using

**Table 2**
Concentration of fixed charge $\rho$ at different pH, temperature, $T$, and ionic strength, $c_{NaCl}$, conditions calculated using the single isotope method and Donnan equilibrium relation. $\Delta m$ is defined in eq 8. $n$ is the number of samples per condition.

| | pH | $T$ (°C) | $c_{NaCl}$ (mM) | $\Delta m$ | $\rho$ (mM) | $n$ |
|---|---|---|---|---|---|---|
| Chitosan gels | 6.0 | 4 | 50 | NA | 78 ± 6 | 2 |
| | 6.6 | 37 | 50 | −0.07 | 28 ± 16 | 3 |
| | 6.9 | 4 | 50 | 0.22 | 31 ± 2 | 3 |
| | 7.0 | 4 | 50 | NA | 31 ± 8 | 2 |
| | 7.0 | 4 | 50 | 0.05 | 29 ± 9 | 3 |
| | 7.5 | 4 | 150 | −0.06 | −1 ± 11 | 3 |
| Cartilage discs | 6.0–7.0 | 4 | 50 | 0.14 | −60 ± 6 | 4 |
| Agarose gels | 6.0–7.0 | 4 | 50 | 0.17 | 1 ± 2 | 3 |

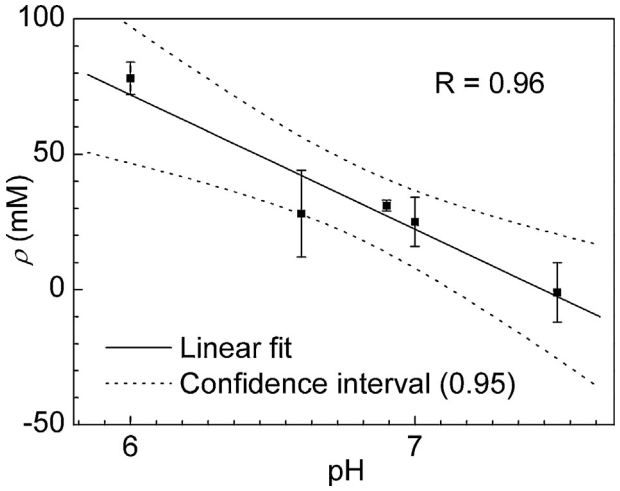


**Fig. 3.** Linear dependence of the fixed charge concentration $\rho$ with pH. $\rho$ was obtained from the single and dual isotope methods (see Tables 2 and 3). SD represents minimum and maximum values. The best linear fit of the data (solid line) and the confidence interval of 0.95 (dash lines) are shown.

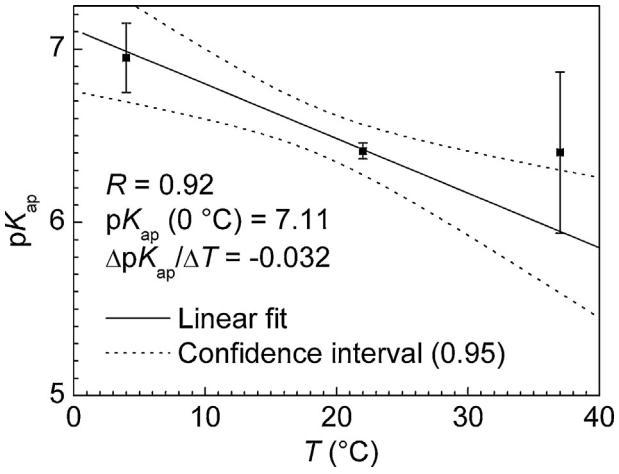


**Fig. 4.** Experimental chitosan gel p$K_{ap}$ measurements as a function of temperature. $\rho$ and pH measurements ($n$ = 3 at each temperature) from the single and dual isotope methods were used to derive p$K_{ap}$ using equations 16 to 18. The best linear fit of the p$K_{ap}$ (solid line) and the confidence interval of 0.95 (dashed lines) are shown.

the single isotope method are presented in Table 2. Chitosan gels present positive fixed charge from amine groups, except for the high salt (NaCl = 150 mM) and high pH (7.5) condition where they were essentially neutral, consistent with previously measured p$K_a$ of chitosan in the 6.5–7 range (Filion et al., 2007). Cartilage disks present negative fixed charge disk while agarose gels were mainly neutral as expected.

### 3.2.2. Dual isotope method

The mass variation $\Delta m$ from overnight incubation (Eq. (8)), fixed charge concentration $\rho$ (Eqs. (11), (12) and (14)), and activity coefficients $\gamma_{\pm}^{g}$ (Eq. (15)) found using the dual isotope method are presented in Table 3. Detection coefficient values $\varepsilon_{Cl}^{\beta}$, $\varepsilon_{Na}^{\beta}$, $\varepsilon_{Cl}^{\gamma}$ and $\varepsilon_{Na}^{\gamma}$ were found to be 203.0, 202.2, 2.4 and 144.8 kcpm/(μCi/mL), respectively. Chitosan gels present positively charged discs, cartilage, negatively charged and agarose gels, mainly neutral. The fixed charge concentration at pH 7 with 50 mM NaCl found with the dual isotope method was comparable but slightly lower than that found with the single isotope method (18 mM vs. 30 mM).

### 3.3. pH and p$K_{ap}$ dependence of fixed charge

Titration of chitosan gels revealed a linear relationship between $\rho$ and pH (Fig. 3). Charge concentration varied from nearly +80 mM at pH = 6.0 to 0 mM (neutral) at pH = 7.5. Thus chitosan amine groups in chitosan–GP gels remain largely titratable as they are not bound to GP. Using Eqs. (16), (17) and (18) we also found the apparent dissociation constant p$K_{ap}$ from the single and dual isotope methods. Interestingly pKap of amine groups in the gel depends on temperature in a manner similar to that found for chitosan in solution (Filion et al., 2007) where increasing temperature reduces p$K_{ap}$ (Fig. 4).

## 4. Discussion

Our main objective was to assess the gelation mechanism of the thermosensitive chitosan–GP system proposed previously (Filion et al., 2007; Lavertu et al., 2008) by testing some underlying hypotheses. Our first hypothesis was that GP is free to diffuse out of the gel since the model proposes that chitosan releases protons upon heating and these protons are taken up by temperature insensitive GP permitting heat induced neutralization of chitosan, reduction of electrostatic repulsion between chitosan chains, bulk precipitation of chitosan in a hydrogel form and subsequent free diffusion of GP out of the gel. GP is therefore not bound to chitosan after gel formation either by electrostatic or hydrophobic interactions. This hypothesis was confirmed through experimental assessment of GP diffusion to adjacent medium, compared to a free diffusion model. Moreover, phosphorus measurements (Table 1) clearly reveal that GP is entirely equilibrated and released from the chitosan gel after 3 to 6 washes. The free diffusion model also adequately predicts the GP concentration profile (Fig. 2). A diffusion coefficient of $0.153 \times 10^{-5}$ cm$^2$/s was obtained from the best fit of this model to data and is close to expected values in the range of $0.38–0.51 \times 10^{-5}$ cm$^2$/s for sucrose in gellan gels at 37 °C (Bayarri, Rivas, Costell & Duran, 2001) and $0.11 \times 10^{-5}$ cm$^2$/s for $KNO_3$ in polyacrylamide gels (1%, w/v) at 20 °C (Shavit, Shaviv & Zaslavsky, 1995). The diffusion coefficient of $KNO_3$ in water is $1.85 \times 10^{-5}$ cm$^2$/s.

The model of heat-induced proton transfer from chitosan to GP would also imply that chitosan amino sites are free to obey

**Table 3**
Fixed charge concentration $\rho$ at pH = 7.0, $T$ = 22 °C, in a 50 mM NaCl solution using the direct dual isotope method. The values of $c_{Cl}$, $c_{Na}$ and $n$ represent respectively the concentration of Cl and Na ions in the discs and the number of samples measured. $\Delta m$ is defined in Eq. (8). $\gamma_{\pm}^{b}$ and $\gamma_{\pm}^{g}$ are the activity coefficients in the bulk solution and in the gel, respectively.

| | $\Delta m$ | $\gamma_{\pm}^{b}$ [a] | $\gamma_{\pm}^{g}$ | $c_{Cl}$ (mM) | $c_{Na}$ (mM) | $\rho$ (mM) | $n$ |
|---|---|---|---|---|---|---|---|
| Chitosan gels | −0.19 | 0.81 | 0.79 | 43.8 | 60.8 | 17 ± 0.8 | 3 |
| Cartilage discs | 0.65 | 0.81 | 0.68 | 109 | 32.7 | −77 | 1 |
| Agarose gel | 0.24 | 0.81 | 0.94 | 41.8 | 44.1 | 2.3 | 1 |

[a] Value obtained from Maroudas and Evans (1972).

equilibrium ionization in the hydrogel. Therefore, our second hypothesis is that the chitosan gel would bear fixed titratable charge groups after gel formation. The radioactive tracer data revealed fixed charge to vary from 0 to nearly 80 mM of positive charge depending on pH, salt and temperature. Cartilage disks gave the expected negative fixed charge between −60 and −77 mM in the range of values obtained previously (Maroudas, 1968; Maroudas et al., 1969). Agarose gels also appeared electrically neutral as expected between 1 and 2.3 mM of fixed charge. Activity coefficients for the case of cartilage and chitosan show that there is a higher content of ions inside the gel than in the bulk solution due to Donnan equilibrium with the charged matrix. In the case of agarose, the presence of fewer ions in the gel matrix than in the bulk solution results in activity coefficients near 1 for the gel (see Table 3). As expected for ionizable amine groups in the chitosan gel, fixed charge concentration was lowered with increasing pH, decreased with high salt concentration and decreased with increased temperature. The dependence of fixed charge in the chitosan gel on pH, salt and temperature are similar to that observed with chitosan in solution (Filion et al., 2007). In particular, the temperature sensitivity of apparent p$K_a$ (p$K_{ap}$) of the amine group of chitosan in the gel displays a linear dependence −0.032 p$K$unit/°C in the gel similar to −0.027 p$K$unit/°C for chitosan in solution (Filion et al., 2007).

The experimental approach used here to assess the diffusion of GP was designed to simulate that which occurs upon implantation of this physiological heat sensitive gel in biomedical applications. The use of DMEM, a cell culture media presenting physiological conditions, allowed us to have a low phosphorus concentration (about 2 mM) and a mostly neutral gel since the pH is about 7.4. Also, for every chitosan gel mixture, we used a non-thermoreversible gel (Hoemann et al., 2007) in order to limit shrinkage and obtain an equilibrium state with a bulk solution. The introduction of a shrinkage function with a form of an exponential decay also allowed us to explain the change of concavity in GP concentration vs. time (Fig. 2) when the diffusion process is starting. This effect of variation in the GP release rate was attributed to a change of the chitosan volume and this change of chitosan volume is most probably due to a pH variation and associated electrostatic swelling forces. Similar results showing this concavity change in the diffusion profile were found (Ruel-Gariépy, Leclair, Hildgen, Gupta & Leroux, 2002 – Fig. 3).

The validation of the free diffusion of GP indicates that GP is not physically part of the gel and is in fact released to the surrounding environment. These results are consistent with the proposed mechanism for thermal gelation where GP needs to be initially present to absorb proton from chitosan and permit its neutralization. Since GP diffuses freely from the gel, one should keep in mind for biomedical applications that gel implantation in situ can present a hyperosmotic shock through release GP when it is in contact with a biological system. Also, glycerol phosphate is well known to be biologically active in promoting osteoblast differentiation in culture (Krawetz et al., 2012) and may have biological effects post-implantation. Thermoreversibility can also be affected when the gel is in contact with another solution since GP can diffuse out and be replaced by other ions which do not present a p$K_a$ appropriate for proton exchange with chitosan. The presence of titratable fixed charge on the resulting chitosan gel can also have significant effects on in vivo applications since binding of encapsulated drugs or therapeutic agents may be affected by pH, salt or temperature induced changes in the charge state of the gel. The gel also swells or shrinks as a result of protonation or deprotonation of amino groups on chitosan. Thus polyanionic proteins or polynucleotides that are initially bound to the gel may be preferentially released as pH rises in situ toward physiological.

## 5. Conclusion

Free diffusion of GP from chitosan–GP gels affirms the thermosensitive gelation mechanism as due to proton transfer from chitosan to GP and resulting neutralization of chitosan and precipitation into a physical hydrogel. Chitosan in the gel state still possesses titratable fixed charge that can be exploited to facilitate controlled release of therapeutic agents delivered using this thermosensitive and biodedegradable system.

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