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# Chitosan-dibasic orthophosphate hydrogel: A potential drug delivery system

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ABSTRACT

Injectable thermo-activated hydrogels have shown great potential in biomedical applications including use in therapeutic delivery vehicles. In addition to their biocompatibility, the feasibility of these delivery systems is significantly contributed by their ability to gel at physiological conditions and to release entrapped molecules in a sustained manner. In this study, parameters affecting the gelling behavior and the release characteristics of a neutral hydrogel system based on chitosan and an inorganic orthophosphate salt have been investigated. Monobasic and tribasic phosphate salts were not effective in inducing gelation of chitosan solution. However, in the presence of dibasic phosphate salt such as dipotassium hydrogen orthophosphate (DHO), the acidic chitosan solution was neutralized and gelling at temperature and time regulated by varying chitosan and salt concentrations in the formulation. The release rate of the entrapped macromolecules depended on chitosan concentration, DHO concentration, structural conformation and molecular weight of entrapped agents. The relationship between the morphology of the hydrogel and the release profiles are discussed. Chitosan/DHO (Chi/DHO) hydrogels were found to be cytocompatible as evaluated in an in vitro study using a human cell line. These results indicate the potential of Chi/DHO hydrogels as delivery systems for different therapeutic agents with controlled release kinetics.



## 1. Introduction

In a variety of therapeutic contexts, it is necessary to administer therapeutic agents to patients parentally, and in a manner that allows for sustained local delivery of the active agent. For example, local release of therapeutic agents may be desirable in the case where an agent has a narrow therapeutic index, as a means of maintaining efficacy at the organ or tissue where an effective dose is required, while avoiding systemic toxicity and unwanted side-effects. One of the most efficient methods of parental delivery of macromolecules is via thermo-sensitive in-situ gels (Ruel-Gariepy et al., 2000). These gels avoid the need for encapsulation of the active compound in organic solvents or heat treatment which often reduces drug activity (Martini and Lauria, 2003). As well, they have a lower cost and associated risk compared to surgical implantation of drug delivery devices in the body. Recently, much attention has been paid towards the application of chitosan in such drug delivery systems due to its many desirable properties.

Chitosan is derived from chitin, a naturally occurring amino polysaccharide found in various organisms including the cell walls of fungi and exoskeletons of arthropods such as crabs, shrimps and insects (Kumar, 2000; Chenite et al., 2001). Chitosan is a polymer composed of β-(1–4)-linked glucosamine residues and it is cationic, nontoxic, biocompatible and biodegradable (Dhanikula and Panchagnula, 2004; Kashyap et al., 2007; Weska, 2007). In addition, chitosan is mucoadhesive, susceptible to enzymatic degradation and has antibacterial activity and intrinsic wound healing properties (He et al., 1998; Helander et al., 2001; Ueno et al., 2001). Due to these characteristics and the fact that chitosan is abundant and economical to produce (Peter, 1995; Kumar, 2000), it has attracted a wide range of medical and pharmaceutical applications (Oungbho and Muller, 1997; Dodane and Vilivalam, 1998; Bernkop-Schnurch, 2000; Borchard, 2001; Shu et al., 2001; Okamoto, 2002; Shu and Zhu, 2002; Khor and Lim, 2003).

At pH below its p*K*a (pH 6.2), chitosan is water-soluble and positively charged due to protonation of amine groups present on the polymeric chains (Cho et al., 2005). This results in electrostatic repulsion between the polymeric chains and hence chitosan is retained in its solution form. When the pH exceeds 6.2, a gel-like precipitate forms from chitosan aqueous solutions due to the neutralization of chitosan amine groups, leading to the removal of repulsive interchain electrostatic forces, allowing for hydrogen bonding and hydrophobic interactions between chains. Chenite et al. (2001) have reported the neutralization of chitosan solution by a organic polyol counterionic monohead salt, β-glycerophosphate (βGP), leading to the gelation of this polymer system.

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The purpose of this study was to evaluate the feasibility of developing a thermo-activated in-situ gelling chitosan delivery system using an inorganic salt present in the body fluid such as potassium phosphate as the gelling agent. Parameters affecting the gelation properties and the release characteristics were investigated. The evaluation on the cytotoxicity of these hydrogels is also reported.

## 2. Materials and methods

### 2.1. Materials

Low molecular weight (MW) chitosan was obtained from Fluka BioChemika (Switzerland). Analytical grade potassium dihydrogen phosphate ($KH_2PO_4$), dipotassium hydrogen orthophosphate ($K_2HPO_4$) and tripotassium phosphate ($K_3PO_4$) were acquired from GPR (BDH, England). Acetic acid ($CH_3COOH$) was purchased from Ajax Finechem (Australia). FITC-dextran, β-lactoglobulin and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (Australia). Micro BCA Protein assay kit was purchased from Pierce, Australia. The human SaOS-2 osteoblast-like cell line attained from the American Type Culture Collection (ATCC) was employed and cultured in α-MEM (Invitrogen, Australia) supplemented with 10% fetal calf serum (FCS, Invitrogen, giving complete medium, CM) in a humidified 5% $CO_2$ atmosphere. Cells were used within 20 passages. A seeding cell population of greater than 95% viability was used for cytocompatibility assay. Cell Titer Blue (CTB) assay kit was purchased from Promega (Australia).

### 2.2. Preparation of chitosan solution

2.5% (w/w) chitosan solution was prepared by dissolving low MW chitosan flakes in 0.1 M acetic acid overnight at room temperature under constant magnetic stirring. The solution (pH ~5.5) was then filtered through 100 μm pore membranes and heated at 85 °C for 9 h to reduce its viscosity from 600 to 200 cps, measured at shear rate of 10 $s^{-1}$ (Carri-Med $CSL^2$ 100 controlled stress rheometer). The resultant solution was finally stored under refrigeration at 4 °C.

### 2.3. Preparation of hydrogel solution

An appropriate amount of orthophosphate salt in either powder or solution form was added to cold chitosan solutions. The mixture was then magnetically stirred at room temperature until homogeneous.

### 2.4. Gelation determination

Samples containing different molar ratios of chitosan to salt were prepared such that they can be physically tested for gelation under three different temperatures: room temperature, 37 °C and 50 °C. Water bath was used to heat and maintain the samples at 37 °C and 50 °C. At specified time intervals within 1 h, samples were observed for gelation by checking the flowability of the solution. pH of Chi/DHO solutions was measured at 25 °C using Carri-Med $CSL^2$ 100 controlled stress rheometer with cone (4 cm diameter, 1.59° angle) and plate geometry.

### 2.5. Rheology analysis

Dipotassium hydrogen orthophosphate (DHO) was added to low molecular weight chitosan solutions with various chitosan/orthophosphate molar ratios. The mixtures were stirred to allow the salt to totally dissolve. Rheological measurements were performed using a Carri-Med $CSL^2$ 100 controlled stress rheometer with cone (4 cm diameter, 1.59° angle) and plate geometry. The temperature of the plate was controlled by a Peltier unit. For all measurements, approximately 1 mL of each freshly prepared sample was introduced onto the plate at 18 °C. Evaporation of sample was prevented by using a solvent trap in conjunction with silicon oil sealing. All the measurements were performed in oscillation mode at a fixed frequency of 1 Hz and a strain of 1%, which is well within the measured linear viscoelastic region. The temperature evolution of G′ and G″ moduli were measured with change in temperature at a rate of 1 °C/min. The time evolution of G′ and G″ were measured at constant temperature of 37 °C. The gelation temperature and the gelation time were taken as the temperature and the time at which G′ and G″ were equivalent in value (Winter and Chambon, 1986).

### 2.6. Scanning electron microscopy (SEM)

Hydrogel samples were gelled at 37 °C for 1 h before being quenched with liquid nitrogen and freeze dried under vacuum for 24 h in a LABCONCO Lyph-Lock 6 Freeze Dry System. The samples were then adhered onto 25 mm aluminium stubs with carbon tabs, and gold coated with an Edwards S150B sputter coater before SEM was performed using a Philips XL30 FEG scanning electron microscope with an accelerating voltage of 20 kV and a magnification of 500 times.

### 2.7. In vitro release studies

Either FITC-dextran of different molecular weights or β-lactoglobulin or BSA was added to low MW chitosan solutions and mixed thoroughly. The mixtures were kept cold on ice. Appropriate amounts of ice-cold DHO solution were added into ice-cold chitosan solution and mixed until homogeneous. 20 μL of the resultant Chi/DHO solution containing either FITC-dextran or protein was pipetted into a 1.5 mL eppendorf tube which was incubated at 37 °C for 1 h at which temperature the solution gelled. 1 mL PBS pH 7.2 was added. The formed gels occupied the bottom of the tubes and exposed to the release buffer by the same surface areas (~19.6 $mm^2$). The tubes were then placed in a shaking incubator maintained at 37 °C under gentle shaking at 100 rpm. At predetermined time intervals, 900 μL of the release buffer was sampled. Subsequently, 900 μL of fresh buffer was added to the tubes in order to maintain constant volume of release medium. The amount of macromolecules released from the gel matrix was determined as below. The percentage cumulative release was calculated based on the total macromolecule content.

### 2.8. Determination of released FITC-dextran amount

The amount of FITC-Dextran released was determined by measuring the fluorescence intensity of release buffer using Carry fluorimeter with excitation wavelength at 490 nm and emission wavelength at 593 nm. Briefly, a standard curve with fluorescence intensity plotted versus FITC-dextran concentration was prepared and the amount of FITC-dextran released was determined based on this curve.

### 2.9. Determination of released protein amount

β-Lactoglobulin and BSA released was quantified using Micro BCA Protein assay kit (Pierce, Australia). Briefly, Micro BCA working reagent was prepared according to the manufacturer's instructions. 500 μL of reagent solution was added to a tube containing 500 μL of collected release buffer. The tube was covered and incubated at 60 °C for 1 h. Absorbance of the resultant solution was measured at 562 nm using a spectrophotometer. Amount of released protein in buffer was calculated based on a standard curve prepared by plotting absorbance reading at 562 nm of protein standards versus their concentrations.

### 2.10. Cytotoxicity studies

Cytotoxicity of Chi/DHO hydrogels was studied by using SaOS-2 cells. The extent of toxicity was evaluated by measuring the viability of cells cultured in the media containing Chi/DHO hydrogels. Different hydrogels were prepared by varying either chitosan concentration (2%, 2.5%, and 2.9%) or phosphate concentration (1.75%, 2.5%, and 3.5%) in the formulations. The solutions were pipetted into 24-well plates and allowed to gel at 37 °C. 250 μL of medium containing SaOS-2 cells was pipetted into each well (on top of Chi/DHO hydrogels). Cells were seeded at 20,000 cells/well. After 7 days of incubation in a humidified atmosphere and 5% $CO_2$ at 37 °C, cell viability in each well was measured by the CTB assay (Promega, Australia) according to manufacturer's instruction. Briefly, 50 μL of CTB reagent solution was added to cells in each well. The wells were then incubated at 37 °C for 1 h. 100 μL of resulting solution in each well was transferred to 96-well plate. The absorbance was read at 570 nm with 600 nm as a reference wavelength (Promega, 2006) using BioRad Model 680 Microplate Reader. Ratio of OD570/OD600 was plotted versus concentration of chitosan or orthophosphate salt.

### 2.11. Statistical analysis

Data were reported as mean ± SEM. Statistical significance ($P < 0.05$) was determined using Student's *t*-test (1-tailed).

## 3. Results

### 3.1. Effect of basic degree of orthophosphate salt on gelation

It was found that samples containing $KH_2PO_4$ at molar ratios tested could not gel at any of the evaluated temperature settings. The pH of these solutions was between 5.0 and 5.2. In contrast, upon heating, chitosan/$K_2HPO_4$ solutions became more viscous and gelation occurred with gel strength observed to be proportional to temperature (data not shown). The pH range of these solutions was found to be between 7.0 and 7.6. For samples containing $K_3PO_4$, a strong basic salt, local precipitation of chitosan occurred instead of the homogeneous gelation. A pH range of 11.9–12.7 was found for these chitosan/$K_3PO_4$ solutions. pH values and ability to gel of different chitosan/salt solutions are presented in Table 1.

### 3.2. Adjustable gelation temperature and gelation time

The gelation temperature and gelation time of Chi/DHO hydrogels are adjustable and depended on the concentrations of chitosan and DHO in their formulations. Decreasing either chitosan concentration or DHO concentration led to the increase of both gelation temperature and time (Fig. 1A and B). Therefore, by varying the combinations of chitosan and DHO in the formulations, the gelation properties of these hydrogels can be controlled and adjusted to be acceptable for a particular utility.

**Table 1**
pH and ability to gel of chitosan/orthophosphate solutions with different molar ratios of chitosan to salt.

| Molar ratio of chitosan to salt | pH of chitosan/salt solution at 25 °C | | |
|---|---|---|---|
| | $KH_2PO_4$ | $K_2HPO_4$ | $K_3PO_4$ |
| 1:1 | 5.2 | 7.1* | 11.9 |
| 1:1.5 | 5.2 | 7.2* | 12.3 |
| 1:2 | 5.2 | 7.3* | 12.5 |
| 1:2.5 | 5.2 | 7.6* | 12.7 |

* Solutions were able to gel.

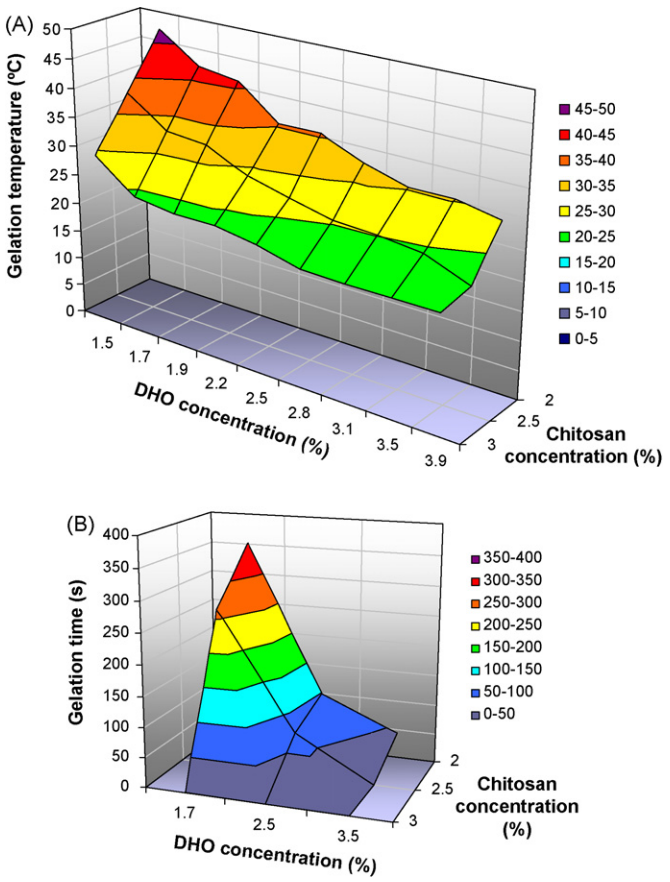


**Fig. 1.** Dependence of (A) gelation temperature and (B) gelation time (at 37 °C) of Chi/DHO solutions on chitosan concentration and DHO concentration.

### 3.3. In vitro release of macromolecules in PBS (pH 7.2) at 37 °C

The release of protein such as β-lactoglobulin was significantly affected by chitosan concentration: the higher the concentration of chitosan, the slower the release of protein and the lower the initial burst (Fig. 2A). Formulations Chi/DHO-2 and Chi/DHO-2.5 released 35% protein within 1 day while formulation Chi/DHO-2.9 released only 25% protein. The cumulative percentage of protein released from Chi/DHO-2, Chi/DHO-2.5, and Chi/DHO-2.9 after 10 days were about 60%, 53%, and 35%, respectively. These results were in agreement with the morphology study (Fig. 2B). As the chitosan concentration increased, the pore size of gel matrix became smaller, which led to the slower release of entrapped protein.

In contrast, salt concentration did not significantly influence the release profile of β-lactoglobulin (Fig. 3A) but it did affect the morphology of the hydrogel matrix. Much larger outer pores were observed in hydrogels with a smaller salt content (Fig. 3B1 and B2). While the size of outer pores was significantly different between hydrogels containing different salt amounts, that of inner pores were not. As the amount of orthophosphate increased, the outer pores of Chi/DHO matrix became smaller. For example, size distribution of outer pores in Chi/DHO-70 was from 50 to 200 μm while that in Chi/DHO-100 was from 10 to 70 μm. Smaller outer pores were thought to be associated with the lower initial burst effect. However, in this study, the formulation containing a higher concentration of salt exhibited a slightly larger initial burst effect although much smaller outer pores were observed in this hydrogel. At day 4 of the study, approximately 50% of β-lactoglobulin was released from both hydrogel formulations and afterwards, a plateau was reached.

Structural conformation of entrapped compounds played an important role on the release rate. Initially, the release rates of

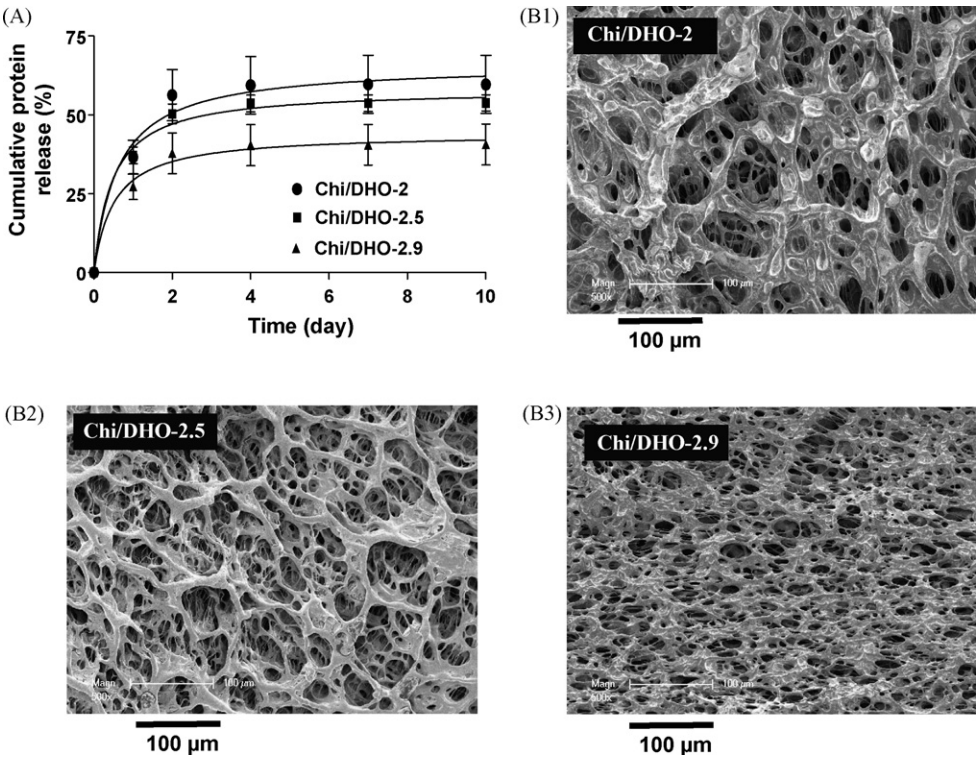


**Fig. 2.** Influence of chitosan concentration on (A) in vitro release profiles of β-lactoglobulin at 37 °C as function of time; and (B) morphology of Chi/DHO hydrogels. Chi/DHO formulations were prepared with different low MW chitosan concentrations (w/w): 2% (Chi/DHO-2), 2.5% (Chi/DHO-2.5), and 2.9% (Chi/DHO-2.9). DHO concentrations in these hydrogels were kept constant at 2.5%. Each formulation contained 1 mL of chitosan solution and 100 μL of 25% DHO solution. Protein was incorporated in chitosan component at concentration of 5 mg protein per 1 mL Chi/DHO solution.

FITC-dextran (FD) molecules with different MWs (4 kDa FD4, 10 kDa FD10, and 20 kDa FD20) were the same but later on they varied significantly (Fig. 4A). FD20, a flexible polymer, was released fastest with the cumulative release percentage at nearly 90% after 6 days of the study. FD10, existing as random flexible chain/coil, diffused out of the gel matrix faster than FD4 but less than FD20. And FD4, a coil polymer in solution, was released at the slowest rate.

The molecular weight of loaded macromolecules also affected the release profile. The release profiles of globular proteins including β-lactoglobulin (MW: 36.6 kDa, Stokes' radius: 1.83 nm) and BSA (MW: 66.4 kDa, Stokes' radius: 3.18 nm) were presented in Fig. 4B. It was found that β-lactoglobulin, a lower-MW protein, was released faster than BSA, a higher-MW protein. After 10 days of the experiment, approximately 55% and 45% of entrapped β-lactoglobulin and BSA, respectively, was detected in the release buffer.

### 3.4. Cytotoxicity of CHOR hydrogels

Fig. 5 shows the metabolic activity of cells cultured for 7 days in media containing CHOR hydrogels formed by various concentrations of chitosan (Fig. 5A) and various concentration of DHO (Fig. 5B). As shown in the figure, cells grew well in the presence of CHOR hydrogels. Increasing the concentration of chitosan and/or phosphate salt did not affect the viability of these cells. Fig. 5C shows the photomicrograph of SaOS-2 cells in direct contact with a CHOR hydrogel. As can be seen in this figure, cells looked healthy and normal and grew around the hydrogel.

## 4. Discussion

Chitosan is a pH-dependent cationic, non-toxic, antibacterial, easily bioabsorbable, biodegradable, biocompatible (Chandy and Sharma, 1990; Hirano et al., 1990) and mucoadhesive biopolymer (Henriksen et al., 1996; He et al., 1998). It was ranked as GRAS (generally recognised as safe) by FDA (Food and Drug Administration). Therefore, chitosan can be widely used in pharmaceutical preparations and food products without concern for patient or consumer safety. Chitosan remains dissolved in aqueous solutions up to a pH of 6.2. When the pH exceeds 6.2, a gel-like precipitate forms from chitosan aqueous solutions. This gel formation is due to neutralization of chitosan amine groups, leading to the removal of repulsive interchain electrostatic forces, allowing for hydrogen bonding and hydrophobic interactions between chains. Therefore, in addition to the use of poly- or multivalent anions (such as polyphosphate) to develop ionically cross-linked chitosan hydrogels, increasing the pH of chitosan solution above 6.2 is another way to induce the physical gelation of chitosan. Organic phosphate such as β-glycerophosphate was investigated for this purpose. In this study, we investigated the use of inorganic phosphate salts such as potassium phosphate salts on the development of thermal-activated neutral injectable chitosan solutions. Both potassium and phosphate ions are naturally present in the body fluid. Therefore, the combination of chitosan and this salt are expected to be biocompatible.

Monobasic, dibasic and tribasic potassium orthophosphate salts were investigated for their abilities to form chitosan hydrogels. Only dibasic orthophosphate salt could induce the gelation of chitosan solution. Samples containing monobasic phosphate salt could not gel at any temperature settings. For samples containing tribasic phosphate, local precipitation of chitosan occurred spontaneously upon salt addition instead of a homogeneous gelation. These observations indicate a strong relationship between pH and gelation ability of chitosan/orthophosphate solutions.

$KH_2PO_4$ was incapable of causing gelation due to their low pH ranges of 5.0–5.2. These pH ranges are below the p*K*a value of chitosan (6.2). Hence chitosan is still positively charged due to the ammonium group ($-NH_3^+$) which leads to an electrostatic repul-

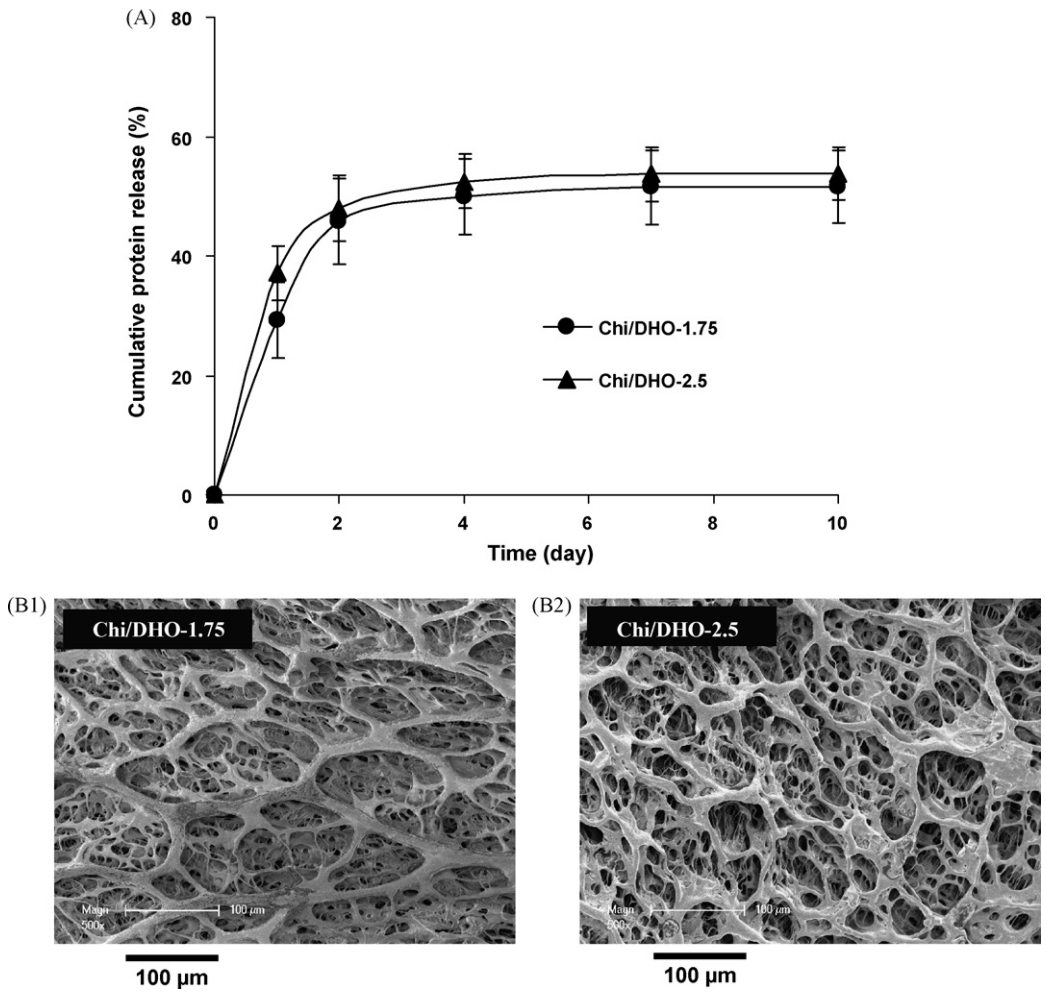


**Fig. 3.** Influence of DHO concentration on (A) in vitro release profiles of β-lactoglobulin at 37 °C as function of time; and (B) morphology of Chi/DHO hydrogels. Chi/DHO formulations were prepared with different DHO concentrations: 1.75% (Chi/DHO-1.75) and 2.5% (Chi/DHO-2.5). Chitosan concentrations in these hydrogels were kept constant at 2.5%. Each formulation contained 1 mL of 2.5% low MW chitosan solution and 70 or 100 μL of 25% (w/w) DHO solution. Protein was incorporated in chitosan component at concentration of 5 mg protein per 1 mL Chi/DHO solution.

sion between the protonated amines and consequently between the molecules (Vachoud et al., 1997). Chitosan/$K_2HPO_4$ solutions have pH range between 7.0 and 7.6. This pH range is above the p*K*a value for chitosan (6.2), therefore leading to a reduction in electrostatic repulsion between the chitosan molecules. Upon increase in temperature, transfer of protons occurs from the chitosan amine groups to the $HPO_4^{2-}$ anions resulting in a diminution in chitosan charge density and allowing for a preponderance of attractive interchain hydrophobic interactions and hydrogen-bonding forces between chains (Chenite et al., 2001). Thus, temperature-dependent gelation was observed in the rheological analysis of chitosan/$K_2HPO_4$ solutions. The pH of chitosan/$K_3PO_4$ solutions were measured to be in the range of 11.9–12.7. At this high pH, instant gelation resulted around the site where the salt solution was added to the chitosan solution. Thus, local heterogeneous gelation was observed instead of the desired homogeneous gelation.

It was found that the addition of DHO into chitosan solution at low temperature increased the pH of chitosan solution above 6.2 without spontaneous gelation. It transformed to gel as the temperature of the solution was increased. The temperature at which chitosan solution gels and the heating time required for gelation to occur depended on the concentration of chitosan. Increasing chitosan concentration led to a decrease in both gelation temperature and gelation time. It was hypothesized that increased density of chitosan chains and increased pH of chitosan solution at higher concentration were responsible for the lower energy (lower heating temperature and shorter heating time) required to induce gelation. Stronger produced gel was also a consequence. Gelation temperature and gelation time were also inversely proportional to DHO concentration. The higher the concentration of DHO, the lower the gelation temperature and the shorter the gelation time. This may be due to the increase of pH near precipitation point of chitosan as the orthophosphate moles increased, leading to lower energy or temperature requirement for gelation. Although there are a number of factors which can influence the gelation behaviour of chitosan solutions such as molecular weight of the polymer, degree of deacetylation, crystallinity of the polymer, concentration of acetic acid, and pH, chitosan concentration and orthophosphate concentration are two critical parameters determining the gelation properties of chitosan solutions.

Due to their thermogelling and injectable properties, Chi/DHO systems have the potential to be used as therapeutic delivery vehicles in animals and humans. It may also have applicability for other warm blooded animals such as avians and marsupials. It was recognized that the physiological temperature may vary somewhat between species, and indeed may vary between animals within a species depending upon individual characteristics and health of the animal. For example, physiological temperature in human is 37–37.5 °C while that in chicken is 38.9–39.4 °C. By varying the concentrations of both chitosan and DHO, gelation temperature of the

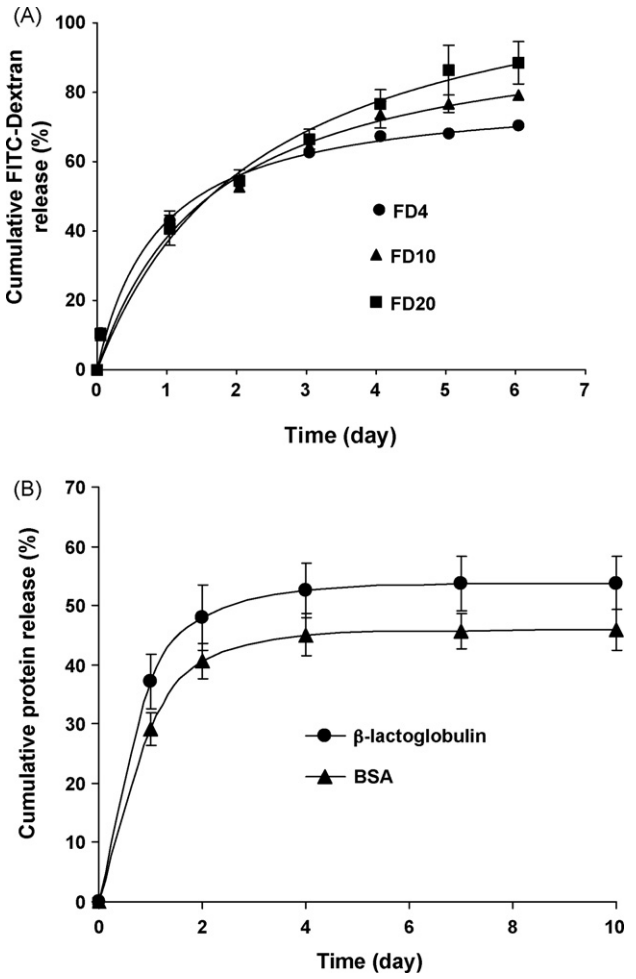


**Fig. 4.** Influence of entrapped macromolecule properties on in vitro release profiles at 37 °C as function of time. (A) Influence of structural conformation of FITC-dextran with different molecular weights: 4 kDa (FD4), 10 kDa (FD10) and 20 KDa (FD20) Formulation contained 1 mL of 2.5% low MW chitosan solution and 100 μL of 25% (w/w) DHO solution. FITC-dextran was incorporated in chitosan component at concentration of 1.5 mg FITC-dextran per 1 mL Chi/DHO solution (30 μg FITC-dextran/20 μL formulation). (B) Influence of molecular weight of proteins including β-lactoglobulin (MW: 36.6 kDa) and BSA (MW: 66.4 kDa). Formulation contained 1 mL of 2.5% low MW chitosan solution and 100 μL of 25% (w/w) DHO solution. Protein was incorporated in chitosan component at concentration of 5 mg protein per 1 mL Chi/DHO formulation (100 μg protein/20 μL formulation).

CHOR compositions can be adjusted to be optimal for the physiological temperature of a particular species. The desired gelation time after having been administered to the patient could be also controlled.

Properties of the hydrogel delivery systems and properties of the drug agents were expected to affect the release of macromolecules from gel matrix under physiological conditions. In this study, the impact of parameters constructing the gel including chitosan concentration and DHO concentration was investigated. Effects of molecular weight and structural conformation of entrapped agents on the release profile were also studied. In each experiment, only the investigated factor was varied, the others were kept constant in the formulations.

Chitosan concentration significantly affected the release of β-lactoglobulin but DHO concentration did not. However, the formulation containing a higher concentration of salt exhibited a slightly larger initial burst effect. These results can be explained by the negative charge of β-lactoglobulin at neutral pH and the procedure used to prepare the hydrogels. At acidic pH, amine groups ($-NH_2$) on the chitosan chain are protonated, leading to an overall positive charge on chitosan. As β-lactoglobulin was mixed with the chitosan solution before the addition of DHO, negatively charged β-lactoglobulin was attracted to positive-charged chitosan due to electrostatic interaction. When salt was added, it neutralized the positive charges of chitosan and free protein. The more salt was added, the more protein was released.

The molecular weight of loaded molecules affected the release profile. However, structural conformation of entrapped compounds also played an important role in the diffusion and release rate. Based on product information (Sigma–Aldrich), dextrans of MW greater than 10 kDa behave as typically branched flexible polymers, while those of 2–10 kDa exhibit the properties of an expandable coil. At MW below 2 kDa, dextran is more stiff chain, rod-like molecules. Dextran with MW at 10 kDa can be best described as random flexible chain/expandable coil. The approximate Stokes' radii (hydrodynamic radii) of FD4, FD10, and FD20 are 14, 23, and 33 Å, respectively. In our study, although MWs and Stoke's radii of FD4, FD10 and FD20 were different from each other, they seemed not to influence the diffusion and release of these dextrans. Few studies on dextran diffusion through a range of membranes also suggested that dextran was less hindered than would be predicted on the basis of Stokes' radius (Bohrer et al., 1984; De Belder, 2003). On the other hand, geometrical structure of dextrans seemed to play an important role in their diffusion and release characteristics. The gel is considered as a heterogeneous network of overlapping or cross-linked chains in which "pore size" or "mesh size" is a defining parameter of the system. Since FD4 exists as coil in solution, it might quickly become and remain trapped in pores. Conversely, as FD20 appeared as flexible polymers, it diffused through the gel matrix more easily. Since FD10 existed as random flexible chain/coil, its diffusion through the gel matrix was better than FD4 but less than FD20. These explanations are supported by a study reported by Pluen et al. (1999) that the diffusion coefficients of flexible macromolecules in agarose gels were greater than those of rigid and/or spherical macromolecules. It has also been demonstrated that flexibility allows rods to escape enmeshment more easily (Cush, 2003).

Proteins with different MW values but existing in a same conformation were further employed in the release test to investigate the influence of MW of entrapped compounds on the release characteristics. They were globular proteins including β-lactoglobulin and BSA. It was found that β-lactoglobulin was released faster than BSA. However, despite the significant gaps in MWs and Stokes' radii of β-lactoglobulin and BSA, the difference in the release patterns of these proteins was not significant, which was exhibited in the overlap of error bars. It may be due to the much larger pore sizes of hydrogel than the molecular size or Stokes' radius of these proteins.

It was noted that the release of the neutral dextrans from Chi/DHO gel matrix was quicker and better in terms of sustained release rather than that of the negative-charged proteins. β-Lactoglobulin and BSA both carry a net negative charge at pH 7.4 and hence could interact with the cationic chitosan backbone. In addition, it is probably due to the difference in structural conformation of these macromolecules in which dextran existed as flexible chain while β-lactoglobulin and BSA were globular in shape.

It was observed that the release or the diffusion of proteins from the hydrogel matrix to media quickly reached plateau after the initial burst effect within a few days of the study (4 days). Since the three-dimensional network of the gel did not significantly change over time, it indicated that there was no substantial erosion of the hydrogel matrix in PBS (pH 7.4) within 10 days of the study. Thus proteins were released mainly by diffusion mechanism in PBS. Since only 50% of entrapped proteins were released after 10 days, it suggested that part of the protein was linked to the polymeric network. However, in vivo, it was expected that the release rate of proteins would be higher due to the degradation of the chitosan network caused by various enzymes and macrophages present in the body.

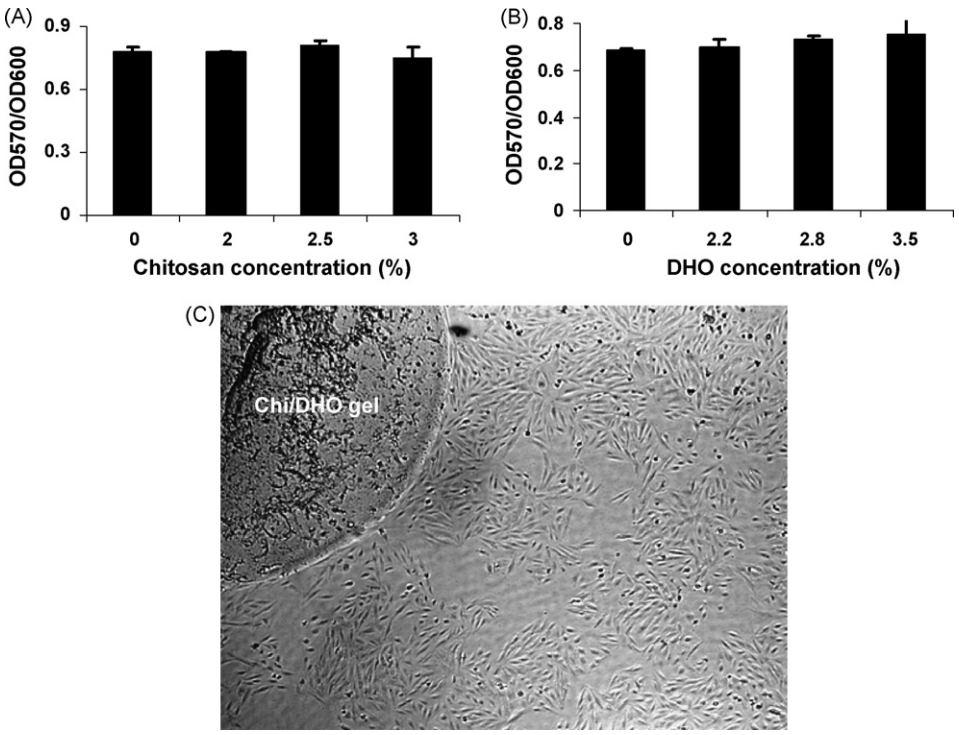


**Fig. 5.** Viability of SaOS-2 cells cultured in media containing various Chi/DHO gels. (A) Chi/DHO gels prepared by different concentrations of chitosan (2%, 2.5% and 3%). (B) Chi/DHO gels prepared by different concentrations of DHO (2.2%, 2.8% and 3.5%). (C) Photomicrograph showing the viability of SaOS-2 cells cultured in direct contact with a Chi/DHO gel containing 2.5% chitosan and 2.5% DHO.

In fact, the addition of lysozyme at concentration of 8 μg/mL – similar to the enzyme concentration found in human serum (Montagne et al., 1998) – at day 10 resulted in a further release of these proteins (data not shown). Chitosan is known to be degraded or metabolized in vivo by certain enzymes, especially lysozyme through the hydrolysis of the acetylated residues (Tomihata and Ikada, 1997). The rate of this degradation inversely depends on the degree of acetylation and crystallinity of the polymer.

The cytotoxicity of gel study using SaOS-2 cells preliminarily demonstrated that Chi/DHO hydrogels are cytocompatible. However, another study using other various cell lines should be conducted to confirm the cytocompatibility of these systems. The gelling Chi/DHO solution is therefore a potential candidate for a wide range of biomedical applications due to its biocompatibility.

## 5. Conclusion

The ability of DHO to induce the thermo-activated gelation of chitosan solutions was demonstrated. Gelation temperature and gelation time of Chi/DHO systems are adjustable and can be controlled for use in a particular utility. The in vitro cytotoxicity study indicates that neutral Chi/DHO hydrogels are non-toxic toward SaOS-2 cells. The potential of Chi/DHO as a prolonged drug delivery vehicle was demonstrated using FITC-dextran, β-lactoglobulin and BSA. The release of these macromolecules depends on key parameters constructing the gel including chitosan concentration and DHO concentration; and the properties of entrapped macromolecules such as structural conformation and molecular weight. The neutral hydrophilic macromolecule FITC-dextran showed a fast release from the gel while proteins showed a slow partial release which quickly reached plateau. Because of the ionic interactions of proteins with the hydrogel polymer, the complete release of proteins might be possible only after the enzymatic degradation of the CHOR matrix. The results obtained from this study show a strong potential of these biocompatible hydrogel systems as sustained drug delivery system.

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## References

Bernkop-Schnurch, A., 2000. Chitosan and its derivatives: potential excipients for peroral peptide delivery systems. Int. J. Pharm. 194, 1–13.
Bohrer, M.P., Patterson, G.D., Carrol, P.J., 1984. Hindered diffusion of dextran and ficoll in microporous membranes. Macromolecules 17, 1170–1173.
Borchard, G., 2001. Chitosans for gene delivery. Adv. Drug Deliv. Rev. 52, 145–150.
Chandy, T., Sharma, C.P., 1990. Chitosan as a biomaterial. Biomater. Artif. Cells Artif. Organs 18, 1–24.
Chenite, A., Buschmann, M., Wang, D., Chaput, C., Kandani, N., 2001. Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. Carbohydr. Polym. 46, 39–47.
Cho, J., Heuzey, M.-C., Begin, A., Carreau, P.J., 2005. Physical gelation of chitosan in the presence of β-glycerophosphate: the effect of temperature. Biomacromolecules 6, 3267–3275.
Cush, R.C., 2003. PhD thesis: Diffusion of a rodlike virus in complex solutions. Department of Chemistry, Louisiana State University, p. 113.
De Belder, A.N., 2003. Dextran. Amersham Biosciences Limited, Uppsala, Sweden.
Dhanikula, A.B., Panchagnula, R., 2004. Development and characterization of biodegradable chitosan films for local delivery of paclitaxel. AAPS J. 6, e27.
Dodane, V., Vilivalam, V.D., 1998. Pharmaceutical applications of chitosan. Pharm. Sci. Technol. Today 1, 246–253.
He, P., Davis, S.S., Illum, L., 1998. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. Int. J. Pharm. 166, 75–88.
Helander, I.M., Nurmiaho-Lassila, E.L., Ahvenainen, R., Rhoades, J., Roller, S., 2001. Chitosan disrupts the barrier properties of gram-negative bacteria. Int. J. Food Microbiol. 71, 235–244.
Henriksen, I., Green, K.L., Smart, J.D., 1996. Bioadhesion of hydrated chitosans: an in vitro study. Int. J. Pharm. 145, 231–240.
Hirano, S., Yamaguchi, R., Fukui, N., 1990. A chitosan oxalate gel: its conversion to an *N*-acetylchitosan gel via a chitosan gel. Carbohydr. Res. 201, 145–149.

Kashyap, N., Viswanad, B., Sharma, G., Bhardwaj, V., Ramarao, P., Ravi Kumar, M.N., 2007. Design and evaluation of biodegradable, biosensitive in situ gelling system for pulsatile delivery of insulin. Biomaterials 28, 2051–2060.
Khor, E., Lim, L.Y., 2003. Implantable applications of chitin and chitosan. Biomaterials 272, 65–78.
Kumar, M.N.V.R., 2000. A review of chitin and chitosan applications. React. Funct. Polym. 46, 1–27.
Martini, A., Lauria, S., 2003. Sustained Release Injectable Products. Am. Pharm. Rev., http://www.americanpharmaceuticalreview.com/current_issue/3_APR_Fall_2003/Martini_article.htm.
Montagne, P., Cuiliere, M.L., Mole, C., Bene, M.C., Faure, G., 1998. Microparticle-enhanced nephelometric immunoassay of lysozyme in milk and other human body fluids. Clin. Chem. 44, 1610–1615.
Okamoto, Y., 2002. Analgesic effects of chitin and chitosan. Carbohydr. Polym. 49, 249–252.
Oungbho, K., Muller, B.W., 1997. Chitosan sponges as sustained release drug carriers. Int. J. Pharm. 156, 229–237.
Peter, M.G., 1995. Applications and environmental aspects of chitin and chitosan. J. Macromol. Sci. Pure Appl. Chem. A32, 629–640.
Pluen, A., Netti, P.A., Jain, R.K., Berk, D.A., 1999. Diffusion of macromolecules in agarose gels: comparison of linear and globular configurations. Biophys. J. 77, 542–552.
Promega, 2006. Cell viability: Protocols and Applications guide, Promega Corporation.
Ruel-Gariepy, E., Chenite, A., Chaput, C., Guirguis, S., Leroux, J.C., 2000. Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. Int. J. Pharm. 203, 89–98.
Shu, X.Z., Zhu, K.J., Song, W., 2001. Novel pH-sensitive citrate cross-linked chitosan films for drug controlled release. Int. J. Pharm. 212, 19–28.
Shu, X.Z., Zhu, K.J., 2002. Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. Int. J. Pharm. 233, 217–225.
Tomihata, K., Ikada, Y., 1997. *In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives. Biomaterials 18, 567–575.
Ueno, H., Mori, T., Fujinaga, T., 2001. Topical formulations and wound healing applications of chitosan. Adv. Drug Deliv. Rev. 52, 105–115.
Vachoud, L., Zydowicz, N., Domard, A., 1997. Formation and characterization of a physical chitin. Carbohydr. Res. 302, 169–177.
Weska, R.F., 2007. Optimization of deacetylation in the production of chitosan from shrimp wastes: use of response surface methodology. J. Food Eng. 80, 749–753.
Winter, H.H., Chambon, F., 1986. Analysis of linear viscoelasticity of crosslinking polymer at gel point. J. Rheol. 30, 367–382.