International Journal of Pharmaceutics 414 (2011) 6–15

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Smart gelation of chitosan solution in the presence of $NaHCO_3$ for injectable drug delivery system

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ARTICLE INFO

Article history:
Received 10 January 2011
Received in revised form 31 March 2011
Accepted 19 April 2011
Available online 28 April 2011

Keywords:
Gelation
Chitosan
In situ gel
Drug delivery

ABSTRACT

In situ gelling systems are attractive as injectable vehicles for drug delivery. The present work described a novel gelation process of acidic chitosan solution in the presence of sodium bicarbonate ($NaHCO_3$). The $NaHCO_3$ concentration played an important role in this gelling system. When it came within the appropriate range, the chitosan/$NaHCO_3$ system would stay at sol state in certain condition and showed sol–gel transition from the top to the bottom after heating. The rheological properties of the gelling system, as well as the morphology and erosion behavior of the formed chitosan hydrogels were evaluated as a function of the $NaHCO_3$ concentration in sols. The hydrogels showed porous morphologies with some diversification depending on the $NaHCO_3$ concentration, which also affected their erosion behaviors and drug release rates. Moreover, the gelation mechanism of such chitosan/$NaHCO_3$ system was studied and proposed as the formation of three-dimensional chitosan network with physical junctions thanks to the deprotonation of $-NH_3^+$ in chitosan accompanying with the gradual neutralization between HCO_3^- and acid. In vivo gelation test was also performed by the dorsal subcutaneous injection of chitosan/$NaHCO_3$ solution in rat. The formation of in situ gels suggested such system promising applications in injectable drug delivery system.

1. Introduction

In situ gelling polymers have become increasingly attractive as carrier matrices for injectable drug delivery system and tissue engineering. Such system would stay in sol state before administration and become hydrogel in situ after injection into the body. The formed hydrogels are promising in trapping pharmaceuticals or bioactive agents, such as proteins and cells, and presenting sustained release at the target site. Especially, increasing attention has been paid to those gelling systems exhibiting smart sol–gel transitions without any toxic cross-linkers or organic solvents for in vivo applications, such as the thermogelling system of amphiphilic block copolymers (Fang et al., 2009; Gong et al., 2009a,b,c; Jeong et al., 1997; Nagahama et al., 2008), and the stimuli–responsive hydrogels based on grafting/blending of polysaccharides (Prabaharan and Mano, 2006; Ta et al., 2008).

Chitosan, an amino polysaccharide obtained from the N-deacetylation of chitin, is known to have good biocompatibility, biodegradability, low immunogenicity, and biological activities (Rinaudo, 2006). A lot of physical (Bhattarai et al., 2005; Chen and Cheng, 2006; Chenite et al., 2006; Chiu et al., 2009; Dang et al., 2006) and chemical (Chen et al., 2004; Poon et al., 2007) hydrogels based on chitosan have been reported with promising utilizations in the biomedical field. Among them, a thermosensitive gelling system of chitosan/glycerophosphate salt (GP) combination with the sol–gel transition at a temperature close to 37 °C (Chenite et al., 2000; Ruel-Gariepy et al., 2000) has gained much attention recently. A series of similar systems based on chitosan and polyol salt have been investigated regarding the solution properties (Chenite et al., 2001; Cho et al., 2006; Filion et al., 2007; Zhou et al., 2008), the thermosensitive mechanisms (Berger et al., 2005; Cho et al., 2005; Kempe et al., 2008), the effects of sterilization and storage on thermogelling characteristics (Jarry et al., 2001; Schuetz et al., 2008), the application in drug delivery (Hoemann et al., 2007; Kashyap et al., 2007; Ruel-Gariepy et al., 2002; Ruel-Gariepy et al., 2004; Wu et al., 2006, 2007) and tissue engineering (Crompton et al., 2007; Ngoenkam et al., 2010; Richardson et al., 2006).

Hydrogel is known to be an enormous three-dimensional network of polymer chains holding a mass of water. For the processing of physical hydrogels of chitosan, polymer network is expected from the physical junctions between chitosan macromolecules. Chitosan is typically soluble in acidic aqueous media due to the protonation of amino group. Recently, Domard's group (Montembault et al., 2005) reported an ingenious gelation of aqueous chitosan solution by contacting with gaseous ammonia. The sol–gel transition occurred from the surface of a sample to the bottom of reactor. The physical junctions of polymer network were

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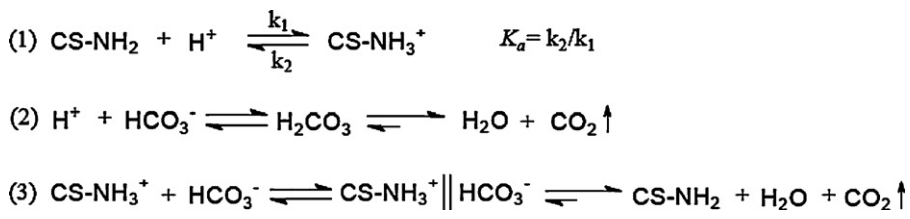
doi:10.1016/j.ijpharm.2011.04.052



Scheme 1. The main ionization equilibriums existed in the chitosan/$NaHCO_3$ system.

Table 1
Characteristics of aqueous chitosan/$NaHCO_3$ mixtures with different concentrations of $NaHCO_3$.

Trial	Con. of $NaHCO_3$ (mol/L)	Gelation time at 37 °C (h)	Transmittance (%)		pH values	
			Before gelation	After gelation	Before gelation	After gelation
Control	0	/	79.1	/	5.10	/
A	0.07	/	78.9	/	6.18	/
B	0.08	20	74.7	56.9	6.28	6.66
C	0.10	7	79.5	3.9	6.42	6.78
D	0.12	0.5	53.4	1.5	6.78	7.34
E	0.13	↓[a]	/	/	6.79	/

[a] Cotton-like precipitation appeared.

demonstrated from a homogeneous neutralization of amine groups and the resultant inter-chain entanglements through hydrogen bonding and hydrophobic interactions. It is a successful case to get physical hydrogels of chitosan without any cross-linking additive or organic solvent, though the hydrogels need to be washed with water to eliminate ammonium acetate and excess ammonia in the end. Nevertheless, this case provides a facile and effective way to build three-dimensional polymer network via the neutralization of chitosan. Compared with the ordinary case, a hydrated precipitation often appeared rather than a gel when the acidic chitosan solution was neutralized by an alkaline aqueous solution, like sodium hydroxide. This phenomenon may arise from a sharp increase of pH values in the bulk, where the neutralization reaction between proton and hydroxyl group occurred too rapidly and irreversibly.

In this study, sodium bicarbonate ($NaHCO_3$) was chosen to neutralize the acidic chitosan solution. $NaHCO_3$ is a weak base. As shown in Scheme 1, the reaction between HCO_3^- and H^+ will produce carbon dioxide (CO_2), resulting in an increase of pH value. It is important that this conversion is mediated through the equilibrium via carbonic acid. And there is also an equilibrium between protonated aminosaccharide unit ($CS\text{-}NH_3^+$) and deprotonated unit ($CS\text{-}NH_2$) via the weak acid and mild base salt of [$CS\text{-}NH_3^+$][HCO_3^-], where the generation of CO_2 would lead to the conversion from $CS\text{-}NH_3^+$ to $CS\text{-}NH_2$. If the CO_2 emitting could be modulated in a controlled mild way, gelation of chitosan solution would occur, similar to the gelation process through gaseous ammonia diffusing described by Domard (Montembault et al., 2005). It is worth noting that the sol–gel transition in this case was driven by an endogenic motility of self-generated CO_2, which is the metabolism product in body and nontoxic.

Herein, we explored the gelation of chitosan aqueous solutions containing varying $NaHCO_3$ concentration. The corresponding chitosan hydrogels were investigated on the morphology, the erosion and drug delivery behaviors, as well as their rheological properties. Then in vivo gelation test was also carried out on this chitosan/$NaHCO_3$ system.

2. Materials and methods

2.1. Materials

Chitosan was purchased from JinKe Biochemical Co. Ltd. (Zhejiang, China), with average molecular weight of 500,000 Da and degree of deacetylation of 95%. All other chemicals were of analytical grade.

2.2. Preparation of chitosan/$NaHCO_3$ mixture

A clear solution of chitosan was obtained by dissolving chitosan in 1% acetate acid aqueous solution, following filtration on a G2 sand core funnel. All solutions were chilled in an ice bath for 15 min before use. Then the $NaHCO_3$ solution was slowly added to the chitosan solution in an ice bath under magnetic stirring to get homogeneous mixture, containing 2% (w/v) chitosan and $NaHCO_3$ in range from 0.07 to 0.13 mol/L. Here, the concentration of chitosan and $NaHCO_3$ referred to their concentrations

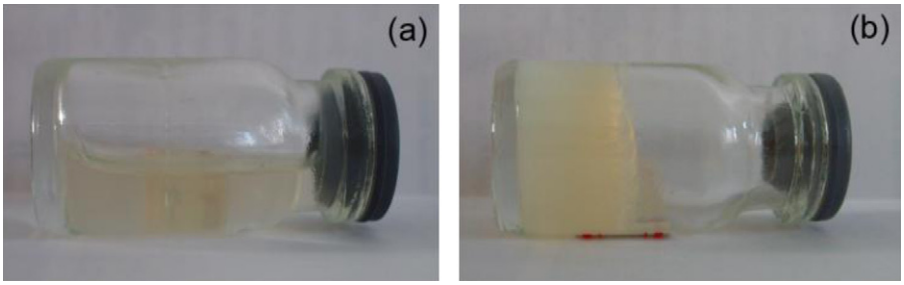


Fig. 1. (a) The chitosan/$NaHCO_3$ solution at low temperature of 4 °C and (b) the formed hydrogel at 37 °C [chitosan concentration = 2% (w/v), concentration of $NaHCO_3$ (C_{NaHCO_3}) = 0.10 mol/L].

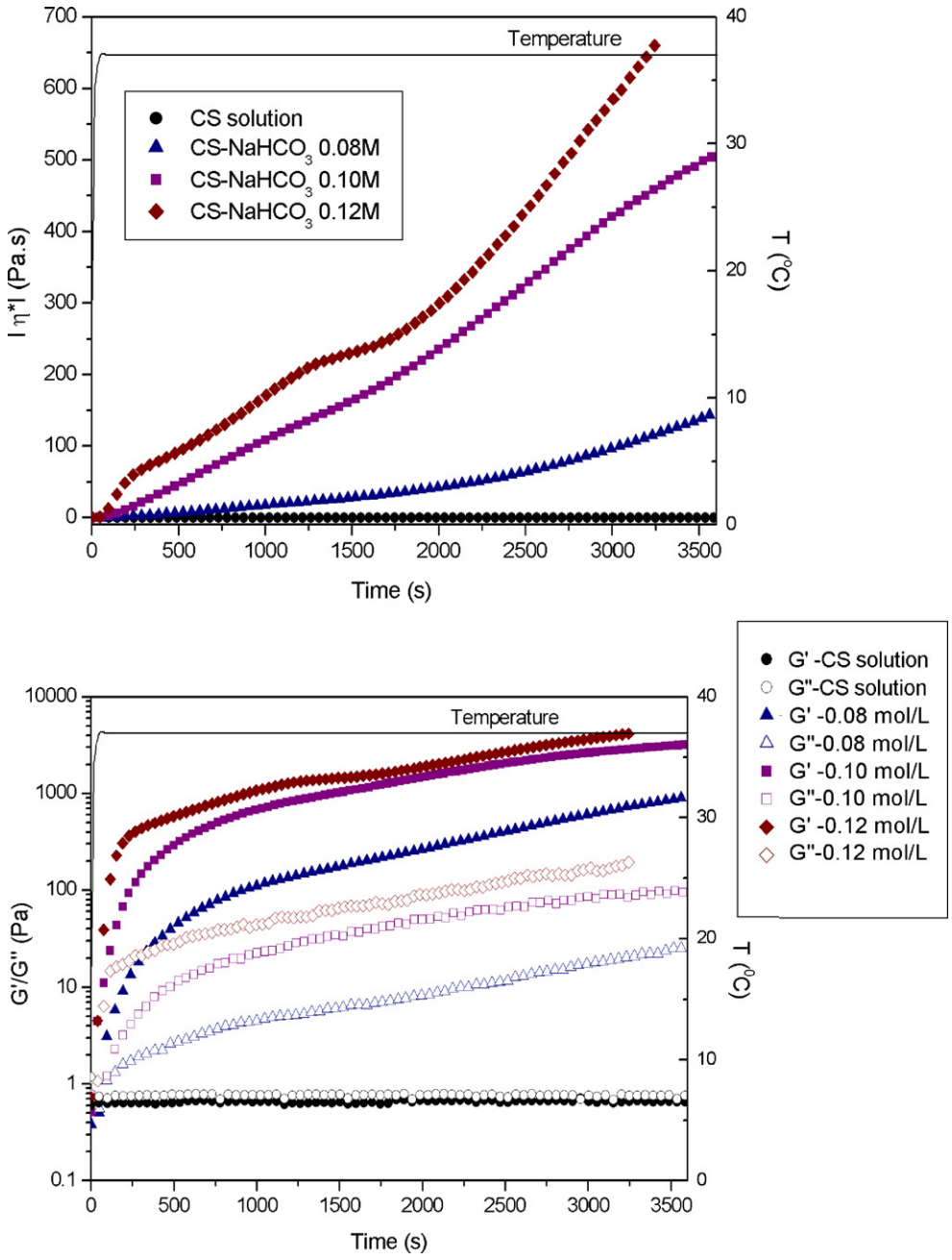


Fig. 2. (a) Plots of the dynamic viscosity (η^*) versus time at a frequency of 6.28 rad/s, during the gelation of chitosan solutions. (b) Plots of the elastic modulus (G') and viscous modulus (G'') versus time at a frequency of 6.28 rad/s, during the gelation of chitosan solutions.

in the mixed system, respectively, hypothesizing no release of CO_2.

2.3. Characterization of gelling system

2.3.1. Sol-to-gel transition behavior

The sol-to-gel transition was determined by the test tube inverted method. Vials containing 3 ml of fresh chitosan/$NaHCO_3$ mixture were immersed in a water bath at 37 °C, allowing gelation. Gelation time was determined by tilting the vials with 90° for 1 min till no flow.

2.3.2. pH measurements

The pH of sample was measured directly with a contact electrode, model pHS-25 from Shanghai Weiye Instruments.

2.3.3. Transparence measurements

The transparence of samples including sol and gel was performed on a Spectrumlab 54 UV/Vis spectrometer (Lengguang Tech) at the wavelength $\lambda = 600$ nm. The transmittance of light passed through distilled water was defined as 100%.

2.3.4. Rheological measurements

The rheological properties were performed on a rotational rheometer (AR GZ, TA Instruments, USA) fitted with a plate–plate configuration. The diameter of the plate was 50 mm. Samples were piped between the plates, and mineral oil covered the marginal surface of chitosan solutions to prevent water evaporation during the tests. A dynamic mode was used and all oscillatory shear measurements were performed within the linear viscoelastic regime. A constant-strain frequency sweep was per-

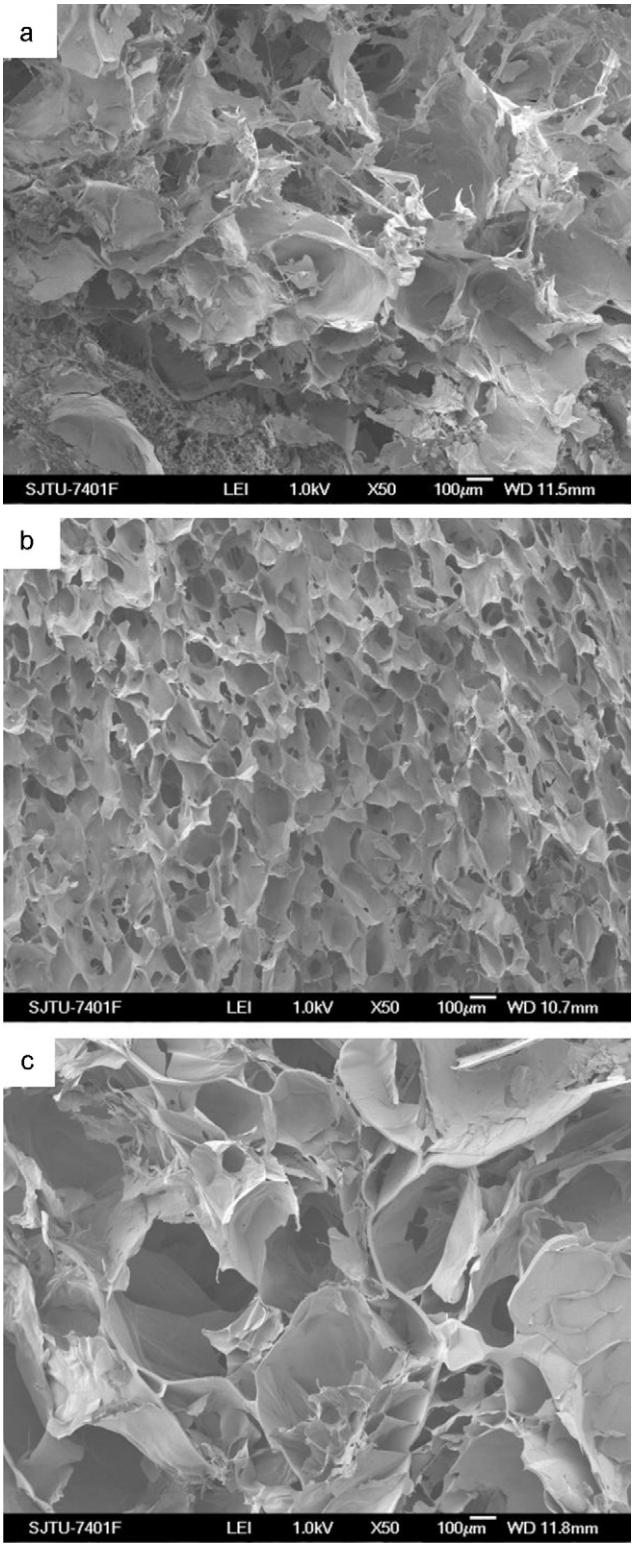


Fig. 3. SEM photographs focused on the cross-section of chitosan hydrogels originated from gelling systems with varying $NaHCO_3$ concentration. $C_{chitosan}$ = 2% (w/v), C_{NaHCO_3} = 0.08 mol/L (a); 0.10 mol/L (b); 0.12 mol/L (c).

formed for chitosan/$NaHCO_3$ solutions within frequency range of 100–0.1 rad/s at low temperature of 5 °C. Then the evolution of rheological properties was monitored by a time sweep at 6.28 rad/s, while the temperature was fast increased and maintained at 37 °C.

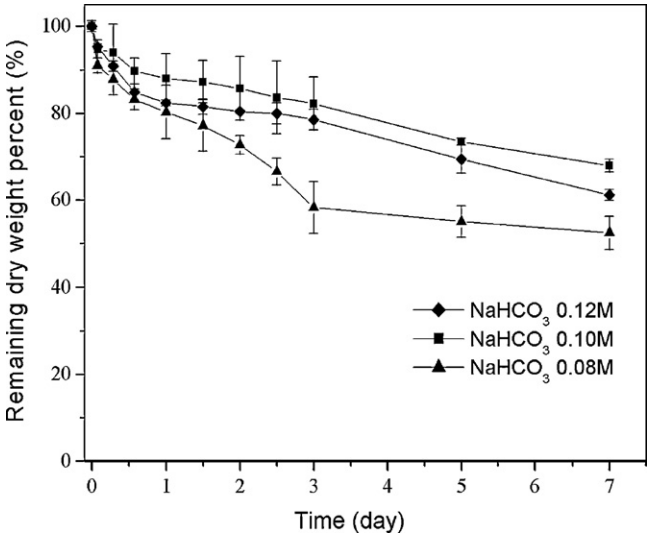


Fig. 4. Mass remain (dry weight percent) of chitosan hydrogels as a function of immersion time in PBS buffer with pH = 7.4 at 37 °C.

2.3.5. Morphological studies

The formed chitosan hydrogel was frozen at −75 °C for 24 h and then lyophilized for 48 h. The lyophilized sample was fractured in liquid nitrogen to obtain the cross-section, which was coated (Emitech K575 Sputter Coater) with gold and then examined by a scanning electron microscopy (SEM) (JEOL, Tokyo, Japan), operated at 1 kV accelerating voltage and 20 mA current.

2.3.6. In vitro erosion testing

Each chitosan hydrogel was prepared from 2 ml of chitosan/$NaHCO_3$ mixture in a 15 ml tube at 37 °C. Then 10 ml of PBS (pH = 7.4) solution was added on the top of hydrogel, which was incubated at 37 °C. At various intervals, PBS was removed and the remaining hydrogel was lyophilized until the constant weight was achieved. The remaining weight percentage of hydrogel was calculated based on the weight ratio of dry matrix:

$$\text{Remaining weight percent } (\%) = (W_t/W_o) \times 100$$

where W_o is the weight of initial dry hydrogel and W_t is the weight of dry hydrogel after different incubation times with PBS.

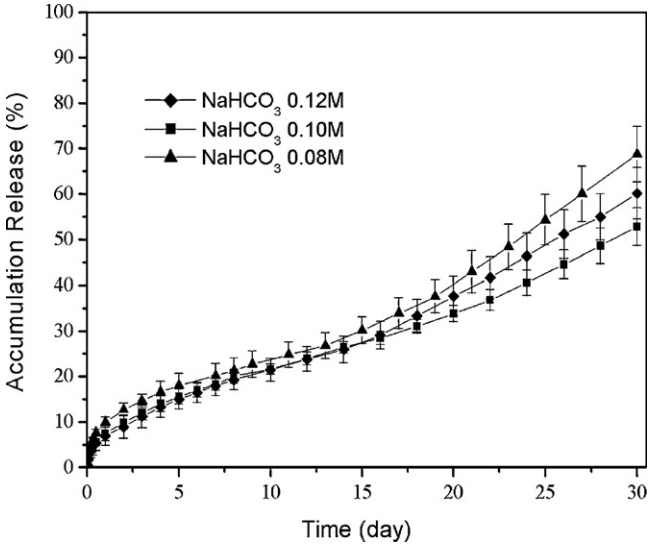


Fig. 5. In vitro accumulative release of dipyridamole (DP) from chitosan hydrogels.

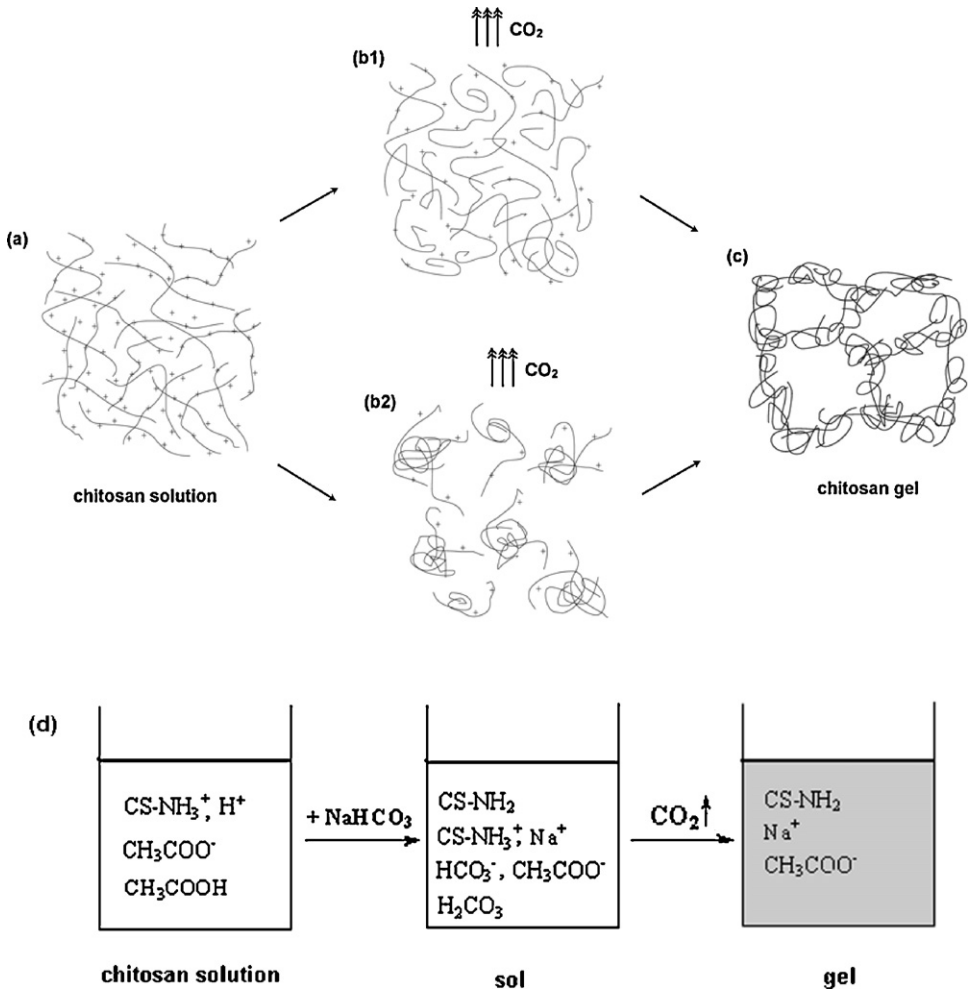


Fig. 6. Schematic representation of the formation of chitosan hydrogel from the chitosan/$NaHCO_3$ system. (a) chitosan solution without $NaHCO_3$; (b1) sol state of chitosan/$NaHCO_3$ system with low $NaHCO_3$ concentration (like trials B and C); (b2) sol state of chitosan/$NaHCO_3$ system with high $NaHCO_3$ concentration (like trial D), where exited polymer aggregates; (c) the formed polymer network of chitosan hydrogel; (d) overview of the gelation process from chitosan solution.

2.4. In vitro drug release

Powdered dipyridamole (DP) was added and dispersed into the stirring chitosan/$NaHCO_3$ solution wherein the drug content was 2.5 mg/ml. The resultant mixture (1 ml) containing DP was incubated at 37 °C to form gel and then 10 ml of PBS was added to each tube. At predetermined collection times, 8 ml of medium was replaced and analyzed in a Spectrumlab 54 UV/Vis spectrometer (Lengguang Tech) at 283 nm. A calibration curve was generated at each time interval using a non-loaded gel in order to correct the intrinsic absorbance of polymer. Samples in triplicate were analyzed for each experiment.

2.5. In vivo injection

Liquid chitosan/$NaHCO_3$ mixtures were administered by dorsal subcutaneous injections in adult Sprague–Dawley rats (~250 g), and 2% (w/v) of chitosan solution without $NaHCO_3$ was also administered as control. Sterile solutions were obtained by ultraviolet sterilization of solid chitosan powder, 0.22 μm filtration of 1% acetate acid solution and $NaHCO_3$ solutions, and sterile preparation of the chitosan solution and chitosan/$NaHCO_3$ mixtures. Rats were anesthetized by intra-peritoneal injection of an aqueous urethane solution, and sterilized locally for dorsal injections. Each injection was 0.4 ml in volume and performed subcutaneously through syringe equipped with a gauge G2 needle.

3. Results and discussion

3.1. Gel formation

To form a physical hydrogel, a basic condition is that the initial polymer concentration must be over the critical concentration of chain entanglement C*. In this work, the chitosan concentration was always 2% (w/v), largely over C* (Boucard et al., 2005). The chitosan solution in control trial without $NaHCO_3$ was quite transparent and maintained fluidity during the investigation, whose pH value was found close to 5.

The mixture of chitosan/$NaHCO_3$ was obtained by adding aqueous $NaHCO_3$ solution of different concentrations to the chitosan solution under stirring at about 4 °C. The low temperature was set to inhibit the generation and release of CO_2 during mixing. Then the mixed solution was placed at 37 °C to allow the gelation. Gelation time was determined by the test tube inverted method. Since the thickness of samples has an effect on this percolating gelation, all measurement of gelation time were investigated in a glass vials (diameter = 1.7 cm) containing 3 ml of the solution. It was observed, as shown in Table 1, that gels could form from the chitosan solutions containing $NaHCO_3$ in a moderate concentration range of 0.08–0.12 mol/L (such as B, C and D trials). Otherwise, the chitosan solution containing a low $NaHCO_3$ concentration at 0.07 mol/L did not form gel, while a precipitate rather than a gel appeared if the $NaHCO_3$ concentration was up to 0.13 mol/L.

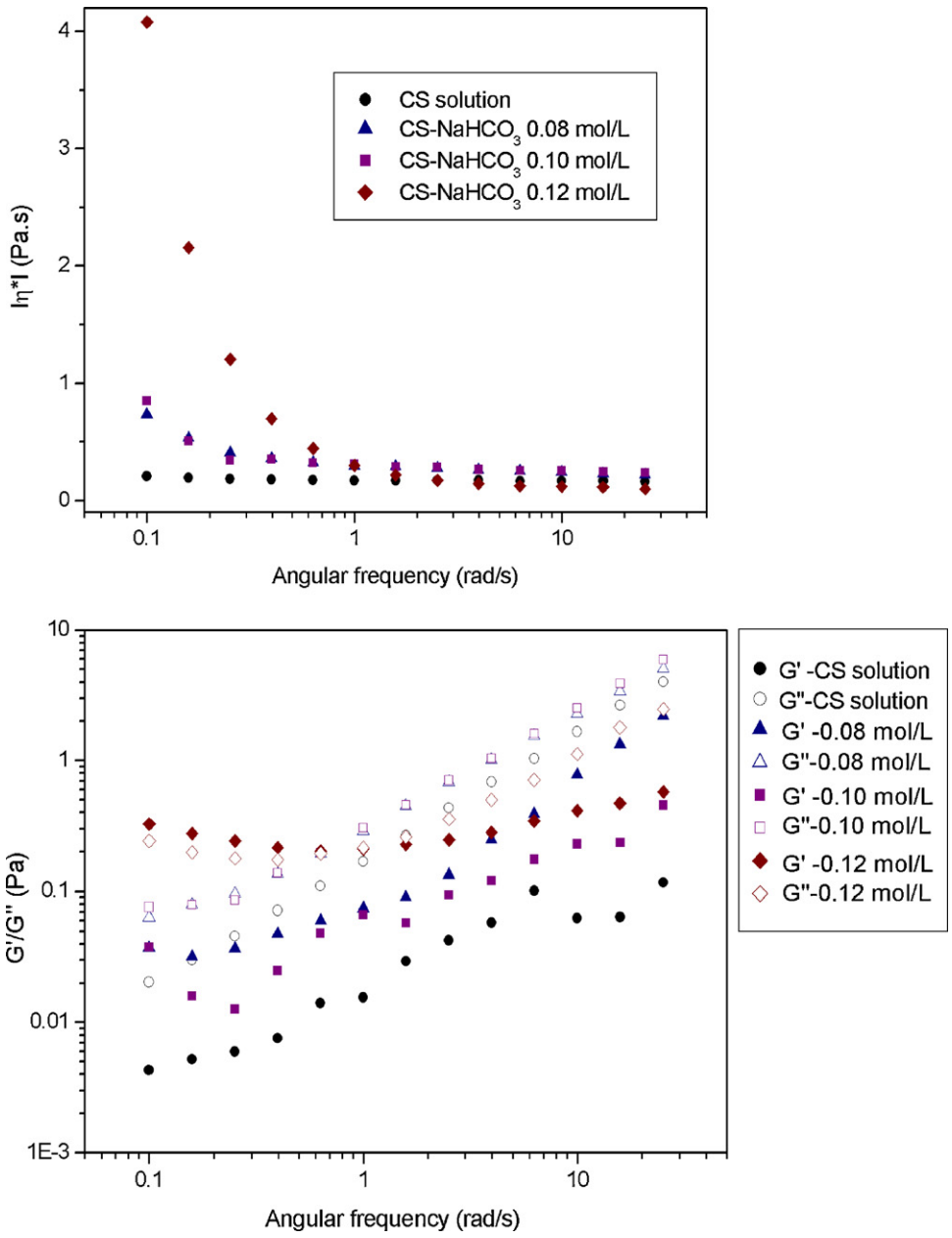


Fig. 7. (a) Variation of the dynamic viscosity (η^*) versus the angular frequency (ω); (b) variation of the elastic modulus (G') and viscous modulus (G'') versus the angular frequency (ω), for the chitosan solutions [$C_{chitosan}$ = 2% (w/v)] with different $NaHCO_3$ concentrations at a low temperature of 5 °C.

Moreover, the gelation time was shortened with the increase of $NaHCO_3$ concentration, which would influence the ionization equilibriums shown in Scheme 1. Fig. 1 gave the representative photographs of the initial sol of chitosan/$NaHCO_3$ solution and the formed gel. It is noted that the chitosan hydrogel became turbid compared with the initial sol.

Rheological measurement of the chitosan/$NaHCO_3$ solution when heating (37 °C) was also performed to study the gelation process. As shown in Fig. 2, the dynamic viscosity (η^*), as well as the elastic modulus (G') and the viscous modulus (G'') were increasing upon time. And G' rapidly rose over G'' and was largely higher than G'' eventually. The data indicated that the elastic response of materials became stronger than the viscous response, which demonstrated the formation of chitosan gel network in relation with the increasing number of physical junctions. Additionally, the final elastic modulus of formed hydrogel increased with the $NaHCO_3$ concentration. It was known that the higher G' value of gel meant the stronger gel intensity, since this modulus had relationship with the number of junctions per unit volume. There would be more physical junctions (H-bonding interaction) between inter or intra chitosan macromolecules along with the neutralization of higher concentration of $NaHCO_3$, thus resulting in the increase of gel intensity.

3.2. Hydrogel morphology

Fig. 3 showed the SEM photographs of freeze-dried hydrogels from different chitosan/$NaHCO_3$ mixtures, which clearly illustrated the effect of $NaHCO_3$ concentration on the hydrogel morphology. The gel network was not evident when $NaHCO_3$ concentration was low at 0.08 mol/L. Furthermore, in Fig. 3(b), it showed a uniformly porous morphology for hydrogel from chitosan/$NaHCO_3$ system with $NaHCO_3$ concentration moderate at 0.10 mol/L. And there were also smaller micropores in the macropore wall, which may

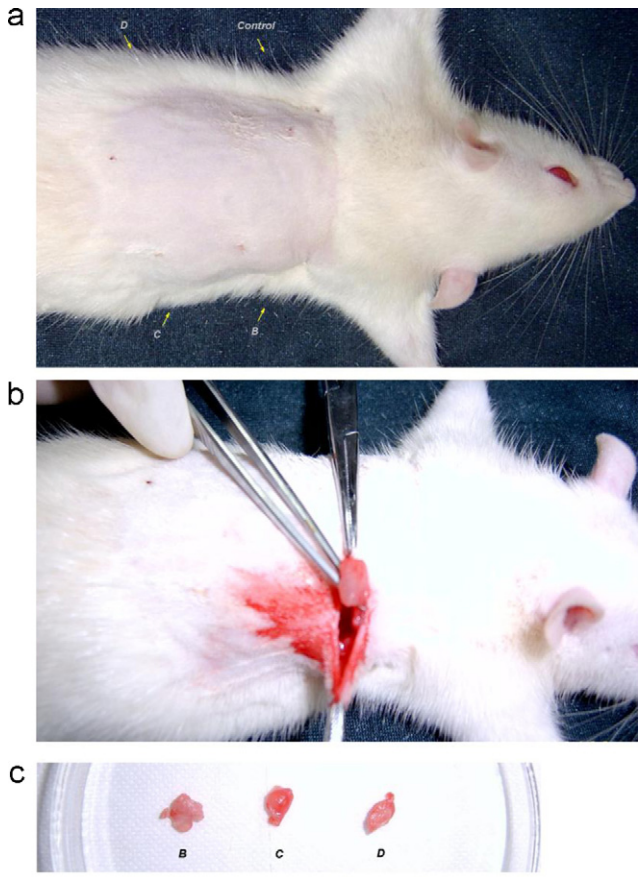


Fig. 8. In situ gel formation after subcutaneous injection of 0.4 ml aqueous chitosan solution and chitosa/$NaHCO_3$ mixtures. (a) Subcutaneous gel-implants remained bean-like shapes after 3 h. (b) The formed gel was visible after anatomy. (c) In situ gels taken out from the rats.

be attributed to the generation of CO_2 gas. However, when $NaHCO_3$ concentration increased up to 0.12 mol/L, the formed hydrogel represented a porous structure more loosely.

3.3. In vitro erosion profile of the hydrogels

The chitosan hydrogels generated from different chitosan/$NaHCO_3$ systems were incubated in PBS at 37 °C, to investigate their erosions. Fig. 4 showed the profiles of time-dependent weight change (dry state) of these hydrogels. All three hydrolgels displayed weight loss gradually during 1 week. Hydrogels from the chitosan solution with higher $NaHCO_3$ concentration at 0.10 mol/L and 0.12 mol/L showed slower erosion rates than the hydrogel with 0.08 mol/L $NaHCO_3$. The reason may lie in the denser network structures of hydrogels due to the increase of physical junctions among chitosan chains. However, the erosion of hydrogel became a little faster on the contrary, when the initial $NaHCO_3$ concentration increased up to 0.12 mol/L. These results could be illustrated by the difference of hydrogel morphologies as described above, that hydrogel formed from chitosan/$NaHCO_3$ (0.12 mol/L) system exhibited a looser porous structure.

3.4. In vitro drug delivery

With dipyridamole (DP) as a model hydrophobic drug, cumulative release of DP from chitosan hydrogel was shown in Fig. 5. The release profiles exhibited a fast release rate in the first day, then a gently stable release in 16 days, followed by an accelerating linear release over 30 days. For the gelling systems of three formulations in this study, the chitosan hydrogel from one with moderate $NaHCO_3$ concentration at 0.10 mol/L had the lowest DP release rate, which was mainly attributed to its stronger gel structure. The drug release behaviors of hydrogels were coincident with their erosion profiles above.

3.5. Gelation mechanism

To further investigate the gelation process of such chitosan/$NaHCO_3$ system, the transparence and pH value of these chitosan/$NaHCO_3$ systems were determined before and after gelation. As summarized in Table 1, the transparence of hydrogel showed an obvious decline compared with the corresponding sol. It indicated the formation and distribution of large domains with chitosan enrichment during gelation, and thus their light-scattering led to the turbid hydrogels. On the other hand, the adding $NaHCO_3$ led to an increase on pH value of chitosan solution as expected, and the initial pH of such chitosan/$NaHCO_3$ system rose with the increase of $NaHCO_3$ concentration. It is known that the apparent pK_a of chitosan is at about 6.5, below which chitosan molecules exist mainly in the protonated form as CS-NH_3^+ and may behave freely like semi-rigid chains. Therefore, the chitosan/$NaHCO_3$ mixture remained fluid state and was relatively transparent at pH < 6.5, while the corresponding gels showed increased pH values over 6.5. This point of view illustrated the sol–gel transition of chitosan/$NaHCO_3$ system represented in trails B and C, where their initial pH values were less than 6.5. Nevertheless, when the $NaHCO_3$ concentration increased up to 0.12 mol/L (trial D), the initial pH of chitosan/$NaHCO_3$ increased over 6.5, accompanying with a drop of its transmittance. Fortunately, this system could maintain homogeneous and fluent in a few days. It was proposed the existence of some micro-aggregates constituted of polymer chain self-associations in this chitosan/$NaHCO_3$ (0.12 mol/L) system. These aggregates may be deemed as the precursors of local gelation originated from the entanglement of fractional chitosan chains. This assumption could be supported by the phenomenon that the turbid chitosan/$NaHCO_3$ (0.12 mol/L) system was not stable, where phase separation appeared after 4 days at 4 °C. The more junctions were initially formed between inter/intra macromolecules with the higher concentrations of $NaHCO_3$. Therefore, for the chitosan/$NaHCO_3$ (0.13 mol/L) system, the excessive neutralization of chitosan amine groups resulted in precipitates immediately, in which the pH value increased sharply over 6.5. The similar trial was mentioned in Montembault's work (Montembault et al., 2005), where to put a chitosan solution in contact with an aqueous ammonia solution instead of gaseous ammonia would generate a precipitate in the bulk rather than a gel.

As discussed above, the sol–gel transition of chitosan/$NaHCO_3$ systems with varying $NaHCO_3$ concentrations could be schematized in Fig. 6. Generally, after mixing acidic chitosan solution with $NaHCO_3$, carbon dioxide would generate and blow off in the open atmosphere (see evidence in supplement information). When $NaHCO_3$, concentration is low, the generation and release of carbon dioxide is mild and would contribute to a gradual increase in the pH value of the system. Consequently, more and more amine fractions of chitosan (CS-NH_3^+) translated to amino (CS-NH_2) fractions. The decrease of the apparent charge density of polysaccharide contributed to the formation of physical junctions of hydrogen bonding between macromolecular chains. Thus the chitosan/$NaHCO_3$ solution first generated a gelling surface in contact with atmosphere. Thereafter, a percolating gelation occurred with the emitting carbon dioxide, where an interphase of a sol–gel transition displaced progressively from the surface of the sample to the bottom of the vital. The appropriate concentration of $NaHCO_3$ to induce gelation of chitosan was in a narrow range. When the concentration of $NaHCO_3$ was at 0.07 mol/L, the neutralization was weak and not

enough to cause the pH value up to the pK_a of chitosan, and no gelation appeared consequently. For the chitosan/$NaHCO_3$ (0.12 mol/L) system, there was a contention between gelation and precipitation. The mixture represented as a homogenous but unstable dispersoid with polymer aggregates at the low temperature of 4 °C after the initial burst of CO_2. The gel could be obtained from such turbid sol when it was heated to 37 °C, where the higher temperature would allow more mobility of chitosan molecules and speed the mild generation of CO_2 at the same time. The reconstruction of three-dimensional polymer network resulted in the formation of hydrogel.

Rheological measurement of the fresh chitosan/$NaHCO_3$ mixtures at low temperature (5 °C) also confirmed this deduction, which was evaluated from a constant-strain frequency sweep. In Fig. 7(a), it was observed that the dynamic viscosity η^* of chitosan/$NaHCO_3$ solutions dropped with the frequency increased, while a relatively steady dynamic viscosity presented in the pure chitosan solution. The decrease of dynamic viscosity indicated the disentanglement of chitosan chains, in relation with the breakdown of the low-energy inter-molecular interactions, which were corresponding to hydrogen bonding here (Montembault et al., 2005). This illustration coincided with the description mentioned above that the addition of $NaHCO_3$ with higher concentration led to higher pH values of the mixture and gave rise to more physical junctions of hydrogen bonding. Therefore, the rheology curves showed more sharp decrease in the dynamic viscosity upon frequency increasing for the chitosan/$NaHCO_3$ solutions with higher $NaHCO_3$ concentration. In Fig. 7(b), the plots of elastic modulus (G') and viscous modulus (G'') versus frequency showed an increase upon frequency and the G' was lower than G'' for the chitosan solution and the chitosan/$NaHCO_3$ (0.08 and 0.10 mol/L) solutions, which were similar as the typical liquids behaved. But for the chitosan solution with 0.12 mol/L $NaHCO_3$, the G' was higher than G'' in the range of low frequency, and G' became lower than G'' till frequency increased over an inflexion point. The different behaviors of G' and G'' versus frequency evidenced the existence of gelling precursors of polymer chain entanglements in this chitosan/$NaHCO_3$ (0.12 mol/L) system.

3.6. In vivo gelation

As mentioned above, the gelation of chitosan/$NaHCO_3$ system was demonstrated from a gradual neutralization of chitosan triggered by CO_2 generation. So it was assumed that to restrain the CO_2 release would prevent the sol–gel transition. A test on the chitosan/$NaHCO_3$ (0.10 mol/L) solution showed that it could remain in the fluent sol state for more than 180 days in a sealed and CO_2 concentrated vessel, while gelation would occur as soon as the CO_2 gas was permitted to release. Such charming property endowed the system with great potential as an injectable drug carrier.

Here the dorsal subcutaneous injections of these chitosan/$NaHCO_3$ systems were carried through to validate their in situ gelling properties. Several formulations of sol with different $NaHCO_3$ concentrations at 0.08, 0.10 and 0.12 mol/L, as well as chitosan solutions without $NaHCO_3$ were tested in this manner. As shown in Fig. 8, gels formed and retained in situ for all three chitosan/$NaHCO_3$ systems, while no gel was observed at the injection site of pure chitosan solution. The in situ gels were intimately integrated within the subdermal mucous layer, and remained quite morphologically intact when excised at 3 h and even after 1 month. No inflammation was detected at the injection sites of chitosan/$NaHCO_3$ sols. However, there presented a little tissue irritation at the injection site of the pure chitosan solution (the control), which may be attributed to a local acidic media (pH ≈ 5) (Williams, 2008).

The in situ forming beanlike gels were excised after 1 month of subcutaneous implanting, and were lyophilized and focused on the cross-sectional interior structure by SEM (Fig. 9). It exhibited a porous but inhomogeneous structure with multilayer, where the

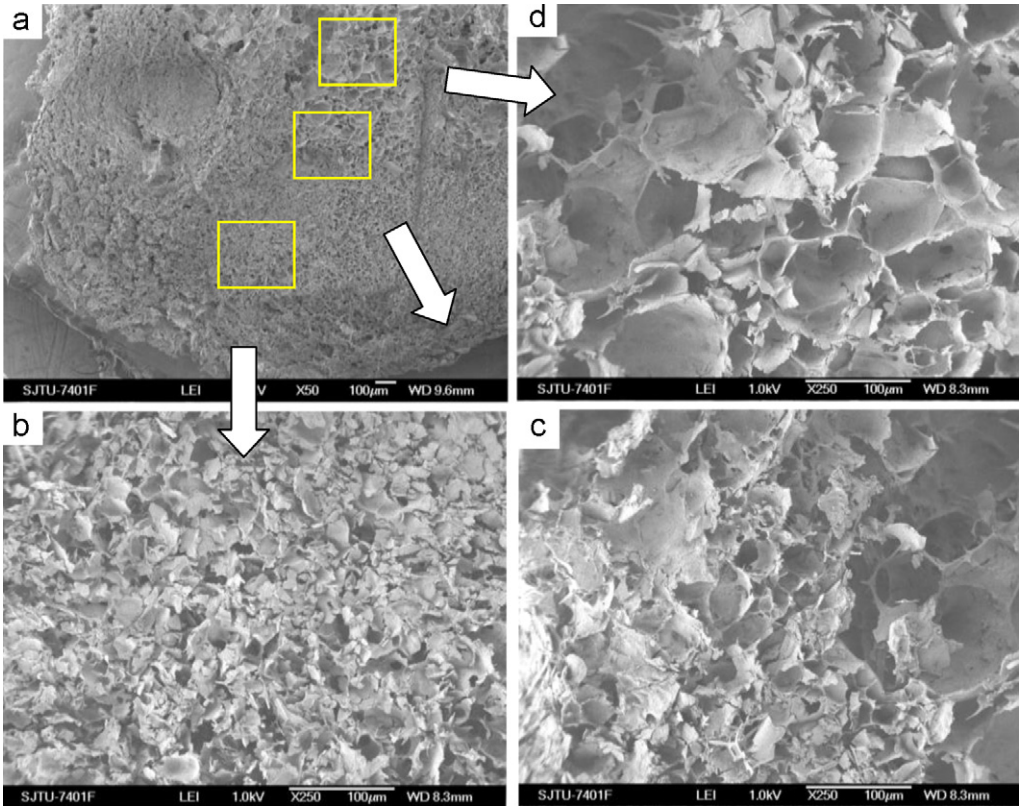


Fig. 9. SEM photographs of chitosan hydrogel formed in vivo for Trial-C, focused on the cross-section (a: magnification 50×; b, c, d: magnification 250×).

outer layer was compact and had smaller pores, while the inner core showed honeycomb-like structure with big pores. This multilayer structure formed in situ would endow the gel with great potential to be a site-specific implant for drug release.

4. Conclusion

Herein the chitosan-based physical hydrogel was achieved from a simple aqueous solution containing chitosan and $NaHCO_3$ with no additives of cross-linking agent or organic solvent. $NaHCO_3$ is known as a weak base and can be used to increase pH via the reaction between HCO_3^- and H^+ and accordingly the CO_2 emitting. The increase in pH value would bring chain entanglements due to the physical junctions of hydrogen bonding between chitosan chains. In certain conditions, the hydrogel of three-dimension polymer network would be generated from the evolution of a sol–gel transition interphase from the surface of the sample to the bottom of the chitosan solution with gradual release of CO_2. Only those chitosan solutions with the $NaHCO_3$ concentration in the range of 0.08–0.12 mol/L can remain in a homogeneous sol state initially and become a hydrogel with CO_2 emitting. The concentration of $NaHCO_3$ plays an important role in the formation of physical junctions between chitosan chains, responsible for gelation and also the formed hydrogel properties, like their morphology, erosion and in vitro drug release behavior as carriers. The in situ sol–gel transition of such chitosan/$NaHCO_3$ system in vivo gives rise to great potential of its application in injectable drug delivery system.

Acknowledgements

Thanks for the financial supporting from Shanghai Science and Technology Committee (08JC1411500). We are grateful to Prof. Sixun Zheng (Shanghai Jiao Tong University) for help in rheology analysis and Prof. Shun-ai Che (Shanghai Jiao Tong University) for help in SEM examination.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.04.052.

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