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Preparation and characterization of glycol chitin as a new thermogelling polymer for biomedical applications

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ABSTRACT

In this study, a new thermo-sensitive polymer, glycol chitin, was synthesized by controlled *N*-acetylation of glycol chitosan and evaluated as a thermogelling system. The physico-chemical properties of glycol chitins with different degrees of acetylation (DA) were investigated in terms of degradation, cytotoxicity, rheological properties, and *in vitro* and *in vivo* gel formation. Aqueous solutions of glycol chitins were flowable freely at room temperature but quickly became a durable gel at body temperature. Thermo-reversible sol–gel transition properties were observed with fast gelation kinetics. Glycol chitins with higher DA showed faster degradation in the presence of lysozyme. They exhibited no significant biological toxicity against human cell lines. An anti-cancer drug, doxorubicin, could be incorporated into the hydrogel by a simple mixing process and released in a sustained pattern over 13 days. Our findings suggest that glycol chitins could be useful as a new thermogelling biomaterial for drug delivery and injectable tissue engineering.

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1. Introduction

A thermogelling polymer that undergo sol–gel transition in water when the temperature increases, have been extensively studied over the past few years for various biomedical applications, especially for drug delivery and injectable tissue engineering (Jeong, Kim, & Bae, 2002; Kim & Park, 2002; Shim, Yoo, Bae, & Lee, 2005). The thermogelling polymers have various advantages compared to other polymeric hydrogel systems, such as easy preparation, no need for organic solvents, site-specific delivery of drugs, prolonged action periods and improved patient compliance, which offers the opportunity to perform a less invasive surgical procedure (Hatefi & Amsden, 2002; Langer, 1990). In clinical applications, injectable hydrogels are also especially suitable for treating irregularly shaped tissue sites, eliminating the need for custom produced scaffold designs (Yu & Ding, 2008). These thermogelling polymers have been applied to entrap pharmaceutical agents or cells by simple mixing in a solution state, followed by a syringe injection into the target site where they formed hydrogel depots and served as carrier matrices for localized drug delivery or cell growth (Lee, Joo, Oh, Sohn, & Jeong, 2006; Potta, Chun, & Song, 2010). The injectable hydrogel can be formed by UV-irradiation, ionic cross-linking, pH or temperature changes, according to the different materials used. Among them, physically cross-linked thermo-sensitive hydrogels, which are free liquid at low temperature but form hydrogels at physiological temperature, have been the focus of previous investigation (Ruel-Gariepy & Leroux, 2004).

Several synthetic polymers are known to possess a thermo-sensitive property, often a sol–gel transition behavior. Copolymers of poly(ethylene oxide) and poly(propylene oxide) (known as poloxamer), and copolymers of *N*-isopropylacrylamide have been widely studied as commercially available thermo-sensitive synthetic polymers (Jeong, Bae, Lee, & Kim, 1997). They could demonstrate excellent thermo-sensitive properties, but their clinical applications have been limited to their lack of biodegradability, biocompatibility and stability (Lee & Tae, 2007; Missirlis, Hubbell, & Tirelli, 2006). Therefore, there has been a continuing need to develop a new biodegradable thermogelling system with enhanced biocompatibility and physical stability.

Polysaccharides as natural polymers, such as alginate, hyaluronic acid, dextran and chitosan, have been proposed for biomedical purposes due to their biodegradability and biocompatibility (Muzzarelli et al., 2007; Sonia & Sharma, 2011; Tan & Marra, 2010). In particular, chitosan, has been widely investigated for drug delivery, cell encapsulation and tissue engineering,

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because of its antibacterial, biodegradable, biocompatible, and mucoadhesive properties (Bhattarai, Gunn, & Zhang, 2010; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Chitosan-based hydrogels have been prepared by chemical or physical cross-linking of the polymer chains. Several chitosan based thermogelling systems have been prepared, such as combinations of chitosan and glycerol-phosphate, poly(ethylene glycol)-grafted chitosan, poly(vinyl alcohol)/chitosan blended hydrogels and so on (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005; Cao et al., 2007; Chenite et al., 2000; Tang, Du, Hu, Shi, & Kennedy, 2007). Although these hydrogels can be formed at physiological temperature, the excess use of glycerol-phosphate salt and complicated multi-step reactions using organic solvents or chemical agents may practically limit their biomedical applications. Also, the poor water solubility of chitosan itself under physiological conditions is one of the major drawbacks for its extensive use as hydrogel materials (Dash, Chiellini, Ottenbrite, & Chiellini, 2011).

As a water-soluble chitosan derivative, glycol chitosan is easily soluble in water under physiological conditions due to glycol residues and retains the favorable non-toxic and biodegradable properties of chitosan, which makes it more suitable for pharmaceutical and biomedical applications (Knight, Shapka, & Amsden, 2006, 2007). Here, we synthesized a novel glycol chitosan-based thermogelling polymer (*N*-acetylated glycol chitosan, named glycol chitin), and investigated the physico-chemical properties of the glycol chitin as a new thermogelling polymer.

2. Experimental

2.1. Materials

Glycol chitosan (M_W 400 kDa, DA 9.34 ± 2.5% as determined by ^{1}H NMR) was purchased from Sigma (Japan). Acetic anhydride (Ac_2O, 99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lysozyme (63,564 units per mg solid) was purchased from Sigma-Aldrich (Canada). Doxorubicin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Acetone, methanol, sodium hydroxide solution (1 M) and sodium chloride were supplied from Samchun Chemical (Korea). All chemicals were analytical grade and used as received without further purification.

2.2. Synthesis of glycol chitin

Glycol chitin was synthesized by *N*-acetylation of glycol chitosan under mild conditions. Briefly, 0.2 g of glycol chitosan was dissolved in 25 mL distilled water and then diluted by 25 mL methanol. A predetermined amount of Ac_2O was added into the glycol chitosan solution under magnetic stirring. After continuous stirring at room temperature for reaction (0.5–72 h), the polymer was purified by precipitation in acetone, followed by centrifugation to obtain a white solid. The polymer was then treated with sodium hydroxide solution (1 M) for 12 h to remove *O*-acetylation and dialyzed against distilled water for 3 days using a dialysis membrane (molecular weight cut-off, 2000 Da). Glycol chitin was obtained by lyophilization in a powdered form.

2.3. Characterization

Glycol chitin was characterized by ^{1}H NMR spectroscopy using a JNM-AL400 spectrometer (Jeol Ltd., Akishima, Japan) operating at 400 MHz. Sample was prepared by dissolving glycol chitin in D_2O (1.0 wt%). The degree of acetylation (DA) was calculated from ^{1}H NMR spectra by comparing the integrals of signals at δ 3.55 (protons of the glucopyranosyl ring) and δ 1.89 (methyl protons of acetyl group). FT-IR spectra of glycol chitins were recorded on a MAGNA 560 spectrometer (Nicolet, USA) with 32 scans and 4 cm^{-1} resolution. Sample was prepared by the KBr pellet method.

2.4. In vitro biodegradation

The biodegradation of glycol chitosan and glycol chitins with various DA was estimated by measuring the viscosity change of the polymer solution in the presence of lysozyme from chicken egg white. Enzymatic degradation experiments were carried out at 37 °C. Glycol chitosan and glycol chitins (40 mg) were dissolved in 20 mL phosphate-buffered saline (PBS, pH 7.4, 0.01 M) solution separately and incubated in a shaking bath (Series BS-21; Lab companion, Korea) at 37 °C for 30 min. Lysozyme was added with a final concentration of 55 μg/mL. The viscosity change of the polymer solution was measured by a Schott-Gerate automatic viscometer (AVS350) as a function of time. The results are representative of triplicate experiments and are presented as the mean value with standard deviation (mean ± SD).

2.5. In vitro cytotoxicity test

HeLa (human cervical cancer) cells were purchased from the American Type Cell Culture Collection (CCL-2, ATCC, Manassas, VA, USA). The cells were cultured in Eagle's minimum essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (Denville, Metuchen, NJ, USA) under 5% CO_2 in a humidified atmosphere. The cells were seeded into a 96-well tissue culture plate (Costar, Corning, NY, USA) at a density of 1×10^4 cells/well and incubated in 100 μL of EMEM/well overnight. Glycol chitins with various DA were dissolved in PBS and serially diluted with PBS. The cells were fed with 100 μL culture media containing a serial dilution of the glycol chitins and cultured for 72 h. Then, 20 μL MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) (Invitrogen, Gaitherburg, MD, USA) was added to each well of the plate with a final concentration of 50 μg/mL in culture media solution. The plates were incubated in a cell culture incubator at 37 °C for 4 h. After removing the culture media, formazan crystals were completely dissolved in 100 μL DMSO and the absorbance was measured using a microplate reader (Spectramax 250, Molecular Device Inc., Sunnyvale, CA) at a wavelength of 540 nm. The relative cell viability (%) was calculated from [ab] test/[ab] control, which refers to cells cultured in media without glycol chitins, respectively. The experimental results were obtained from the average values measured from three independent experiments in triplicate.

2.6. Thermo-sensitive sol–gel transition

Thermo-sensitive sol–gel transition behaviors of the glycol chitin solutions were investigated by the tube inverting method, with temperature increase of 0.2 °C/min. The polymer solutions with a given concentration (3–7 wt%, 1 mL) were prepared by dissolving the glycol chitins in PBS solution (pH 7.4, 0.01 M) at 4 °C. The sol–gel transition temperature was determined by a flow (sol) or no-flow (gel) criterion over 30 s with the tube inverted. Each data point is an average of three measurements with standard deviation (mean ± SD). The sol–gel transition phase diagram obtained using this method is known to have a precision of ±1 °C.

2.7. Rheological studies

Rheological experiments were carried out by measuring changes in viscosity with a rheometer (RVDV-III+; Brookfield Instruments, USA). The temperature range was 15–75 °C with a heating rate of 0.34 °C/min and a fixed shear rate of 0.1/s. The reversible sol–gel transition behavior of glycol chitin in PBS solution

(pH 7.4, 0.01 M, 7 wt%) was confirmed using a rotating, temperature controlled rheometer (Bohlin Advanced Rheometer, Malvern Instruments, UK). The temperature was cycled between 37 °C and 4 °C. The contribution of solid-like behavior (elastic modulus G') was recorded with changing temperature using a parallel plate (20 mm). Frequency was optimized to 1 Hz as determined using a frequency sweep experiment. A constant stress of 25 Pa was used for the measurement.

2.8. In vivo gel formation

To confirm the *in vivo* applicability of glycol chitin as a new thermogelling biomaterial, 9-week-old female nude mice (Jackson Lab, Sacramento, CA, USA) were anesthetized by an intraperitoneal (i.p.) injection of ketamine/xylazine (300 mg ketamine combined with 20 mg of xylazine in a 4 mL volume) into each mouse (1 μL per g body weight). Glycol chitin (91.59 DA) was dissolved in 0.9% NaCl solution and 10 μL of 0.4% trypan blue solution (Invitrogen, Carlsbad, CA, USA) was added to produce glycol chitin solution with blue color. Mice were then treated with 0.2 mL of glycol chitin or 0.9% NaCl solution as a control *via* subcutaneous (s.c.) injection. The injection site was surgically accessed after 15 min and observed for stable gel formation at the injection site.

2.9. Swelling behavior

Glycol chitin hydrogel samples (91.59 DA, 7 wt%, 1 mL) were prepared in 5 mL vials and an excess of 3 mL PBS was gently added to the top of the glycol chitin hydrogel at 37 °C. At predetermined time intervals, PBS was fully removed from the swollen glycol chitin hydrogels, and the resulting hydrogels were weighed. The PBS was freshly replaced after every weight measurement. The weight of the swollen glycol chitin hydrogels (W_s) was measured to calculate the swelling ratio (SR). The SR of the hydrogel was calculated using the following equation, where W_s and W_i represent the weights of the swollen hydrogel samples and the initial hydrogel samples, respectively. The results are representative of triplicate experiments and are presented as mean ± SD.

$$\mathrm{SR} = \frac{W_s - W_i}{W_i} \times 100\%$$

2.10. Hydrogel morphology

The morphology of the glycol chitin hydrogels was observed under field emission scanning electron microscopy (FE-SEM; JSM-7000F; JEOL, Japan) at 15 kV. Freeze-dried hydrogels were prepared by quenching the glycol chitin hydrogel in liquid nitrogen, followed by lyophilization. The freeze-dried glycol chitin hydrogel was coated with platinum by sputtering for 40 s at 20 mA before observing the hydrogel morphology.

2.11. In vitro gel stability and injectability

DOX (0.3 wt%) containing glycol chitin solution (91.59 DA, 7 wt%) was prepared in a 5 mL vial. After increasing the temperature to 37 °C, the aqueous solution turned into a gel state. An excess amount of PBS was added to the top of the gel to investigate gel stability. DOX containing glycol chitin solution was injected into excess PBS at 37 °C through a 21-gauge syringe needle to evaluate the feasibility of glycol chitin for use as an injectable drug carrier.

2.12. In vitro drug release behavior

The *in vitro* release of DOX using the glycol chitin hydrogel was studied to evaluate its potential application in drug delivery. Glycol chitin (91.59 DA, 7 wt%) and DOX (0.3, 0.6 wt%) were dissolved in PBS solution (pH 7.4, 0.01 M) at 4 °C, respectively. The DOX containing glycol chitin solutions (1 mL) were prepared in 5 mL vials, which were thermostated in a shaking water bath with gentle shaking at 37 °C for 30 min to allow gel formation. After the DOX containing glycol chitin solutions turned into gels, 3 mL fresh release medium (PBS, pH 7.4, 0.01 M, 37 °C) was gently added to each vial, which was incubated at 37 °C and shaken at 20 rpm. At selected time intervals, 3 mL PBS was removed, and an equivalent volume of fresh PBS was added to compensate for the release medium. The DOX concentration in the release media was calculated against the standard curve of DOX using a UV–Vis spectrophotometer (Mini-1024, SHIMADZU, Japan; absorption wavelength: 480 nm). The absorbance comparison was used to calculate the DOX concentration and cumulative release (Al-Abd, Hong, Song, & Kuh, 2010; Huynh et al., 2011). All procedures were performed under light-protected conditions. The results are representative of triplicate experiments and are presented as mean ± SD.

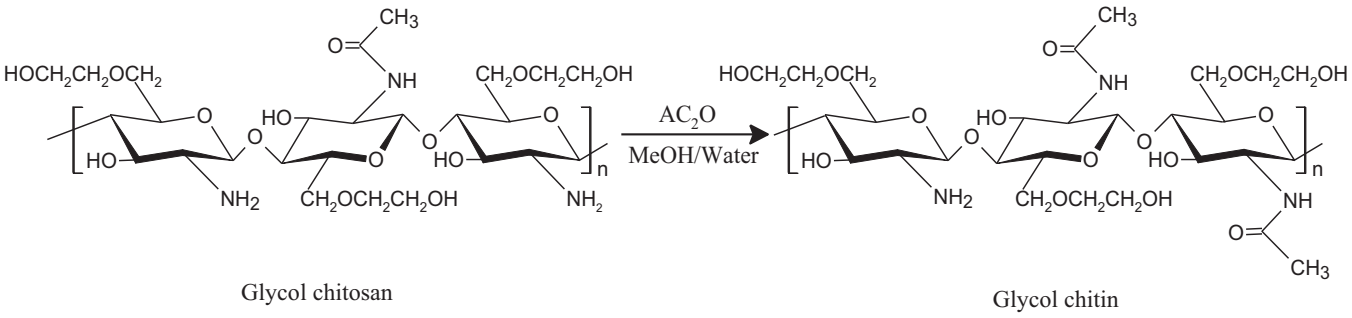
3. Results and discussion

3.1. Synthesis and characterization of glycol chitins

Various glycol chitins with different DA were synthesized by *N*-acetylation of glycol chitosan using a one-step reaction procedure under mild conditions (Scheme 1). The chemical composition of the glycol chitins was confirmed by ^{1}H NMR and FT-IR measurements. Fig. 1a shows the ^{1}H NMR spectra of glycol chitosan and glycol chitins. The polymers were dissolved in D_2O at a concentration of 1.0 wt%. The D_2O peak was used as a reference peak at δ 4.65. The peak at δ 1.89 was assigned to the methyl protons of the acetyl group in glycol chitin. The peak at δ 2.6 arose from the protons of the primary amine residue. The overlapped peaks at δ 3–4 were contributed to the protons of the glucopyranosyl ring at positions 2–8 (H-2 through H-8). Based on the assignments, the average DA of glycol chitosan and glycol chitins was calculated by comparing the integrated signal area of the protons of the glucopyranosyl ring (δ 3.55) with that of the methyl protons of the acetyl group (δ 1.89).

The chemical structure of glycol chitosan and glycol chitin was also confirmed by FT-IR spectroscopy, as presented in Fig. 1b. The broad band at 3400 cm^{-1} was assigned to the stretching vibration of hydroxyl groups, which overlapped the N—H stretching vibration in the same region. The absorption peaks at 2890 cm^{-1} were associated with the C—H stretching of methylene and methyl groups of glycol chitosan and glycol chitins. The appearance of absorption bands at 1655 cm^{-1} and 1555 cm^{-1} were corresponded to the carbonyl stretching and the amide II bending vibration of the aminoacetyl group of the glycol chitins, respectively. Additionally, the absorption peak at 1596 cm^{-1}, which was attributed to the amino bending vibration of glycol chitosan, was not observed after acetylation. The disappearance of the amino vibration band at 1596 cm^{-1} and the appearance of the amide II band at 1555 cm^{-1} indicated that the glycol chitins were successfully synthesized. No distinct ester carbonyl bands were observed, indicating that the acetylation of glycol chitosan mainly occurred at the amino group, instead of the *O*-position on the hydroxyl group (Wang, Liu, & Chi, 2008).

Glycol chitins with controlled DA ranging from 27.55% to 91.59% were synthesized as shown in Table 1 (yields, 73.24–82.59%). The reaction time and feed molar ratio of Ac_2O to the amino groups of glycol chitosan were monitored to investigate the factors controlling DA of glycol chitins. As shown in Fig. 2a, *N*-acetylation of glycol chitosan in aqueous solution seemed to be almost instantaneous. With the same feed molar ratio of Ac_2O to the amino groups of glycol chitosan, the DA increased linearly at the initial time and



Scheme 1. Synthetic route of glycol chitin.

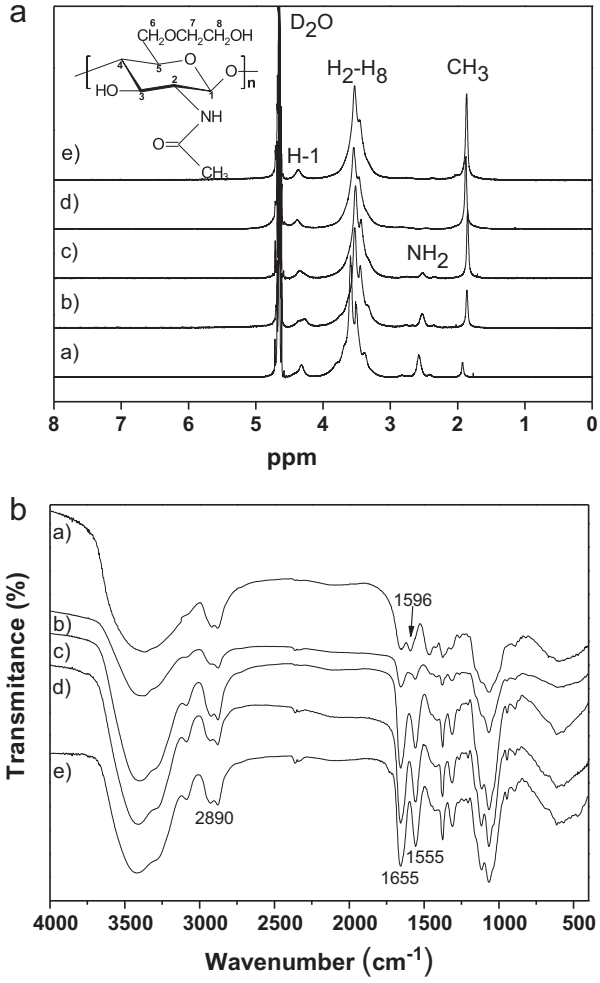


Fig. 1. ^{1}H NMR and FT-IR spectra of glycol chitosan and glycol chitins with different degree of acetylation (DA), (a) glycol chitosan; (b) glycol chitin with 27.55 DA; (c) glycol chitin with 54.09 DA; (d) glycol chitin with 84.45 DA; (e) glycol chitin with 91.59 DA.

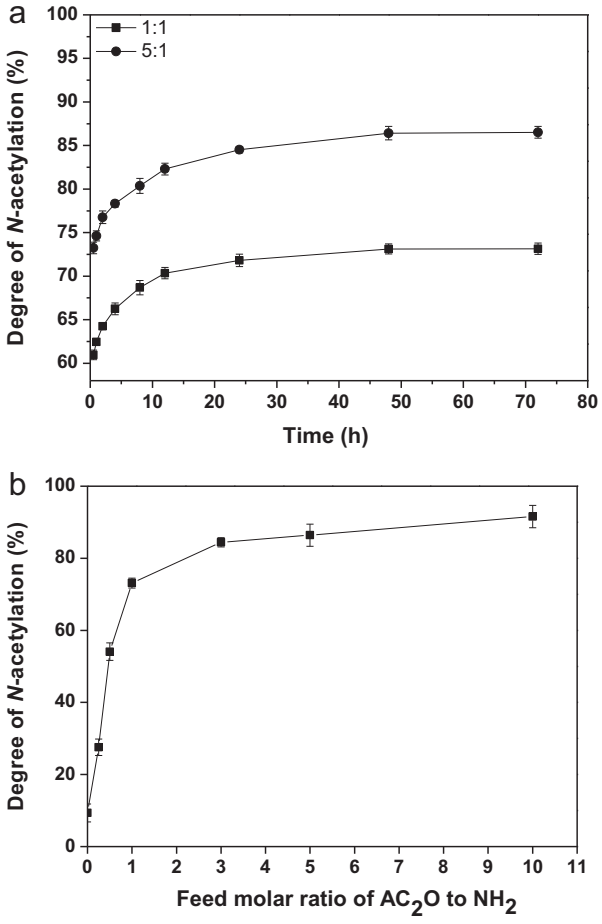


Fig. 2. (a) Dependence of the degree of acetylation (DA) on the reaction time (■ feed molar ratio fixed at 1:1; ● feed molar ratio fixed at 5:1). (b) Dependence of the DA on the feed molar ratio of AC_2O to the amino group of glycol chitosan (reaction time fixed at 48 h).

Table 1
Chemical data for glycol chitins.

Sample	Ac_2O/NH_2[a]	DA[b] (%)	Yield (%)
Glycol chitosan	–	9.34 ± 2.50	–
Glycol chitin 1	0.25	27.55 ± 2.30	82.59
Glycol chitin 2	0.5	54.09 ± 2.43	80.37
Glycol chitin 3	1.00	73.12 ± 1.37	73.24
Glycol chitin 4	3.00	84.45 ± 1.28	78.61
Glycol chitin 5	5.00	86.42 ± 3.07	77.39
Glycol chitin 6	10.00	91.59 ± 3.10	79.46

[a] Feed molar ratio of Ac_2O to the amino group of glycol chitosan.
[b] Degree of *N*-acetylation determined by the peak integration of ^{1}H NMR spectra.

reached a maximum conversion at 48 h. Besides, it could be found that glycol chitins with higher DA were produced from a higher feed molar ratio of Ac_2O to the amino groups of glycol chitosan at the same reaction time, which indicated that the DA could be effectively controlled by varying the feed molar ratio. When the reaction time was fixed to 48 h, the DA of glycol chitin increased. The DA showed a saturated result with a maximum of 91.59% at a feed molar ratio of 10, where the available amino groups were almost substituted with acetyl groups and so the reaction could not process any further (Fig. 2b).

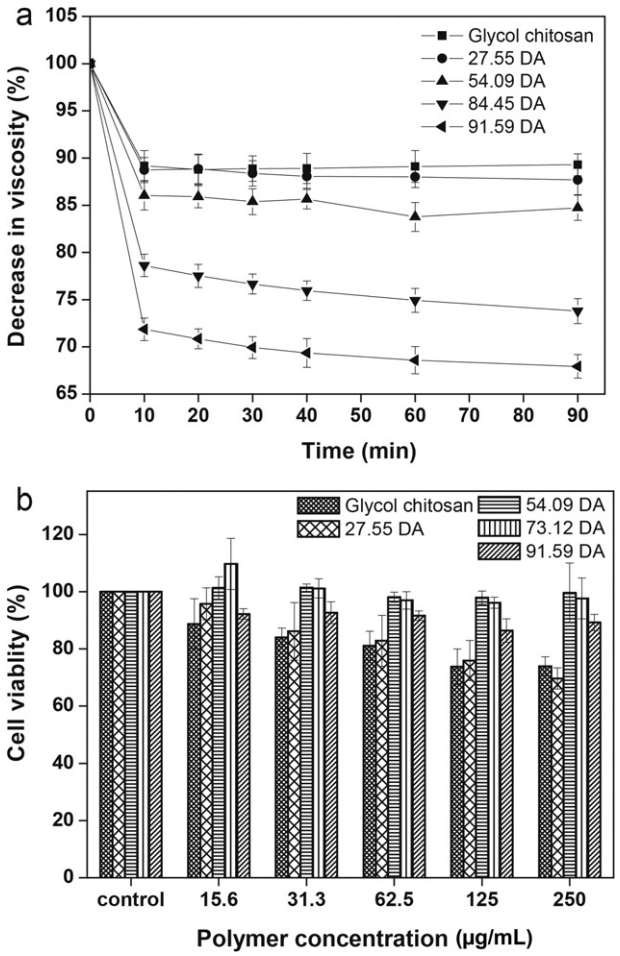


Fig. 3. (a) *In vitro* biodegradation of glycol chitosan and glycol chitins in PBS (pH 7.4, 0.01 M) at 37 °C. (b) MTT-cell cytotoxicity assay of glycol chitosan and glycol chitins in Hela cells.

3.2. In vitro biodegradation and cytotoxicity of glycol chitin

Chitosan is biodegradable by lysozyme, which is widely present in the human body fluids (*e.g.*, serum, saliva, and tears) *in vivo* (Cho, Cho, Chung, Yoo, & Ko, 1999; Tomihata & Ikada, 1997). To ascertain whether glycol chitosan and glycol chitins were biodegradable by lysozyme, the viscosity change of the aqueous glycol chitosan and glycol chitin solutions was observed in the presence of lysozyme. As shown in Fig. 3a, the viscosity of glycol chitin solutions decreased to 67–87% of initial viscosity after 90 min, whereas the viscosity of the glycol chitosan solution decreased slightly to 89% by lysozyme-catalyzed biodegradation. The glycol chitins exhibited a larger decrease in viscosity than that of glycol chitosan at all time points, indicating that the glycol chitin structure, an *N*-acetylated form of glycol chitosan, had better biodegradability than glycol chitosan, probably due to the higher content of *N*-acetyl glucosamine units that are more susceptible to lysozyme. The extent of lysozyme-catalyzed degradation of glycol chitins was proportionally dependent on the DA, which was similar with previous studies on chitosan (Freier, Koh, Kazazian, & Shoichet, 2005).

To investigate whether the glycol chitins exhibit cytotoxicity, we performed a MTT-based cytotoxicity assay with HeLa cells. Based on the DA, we divided the glycol chitins into three different groups, which are group I (glycol chitosan and glycol chitin with 27.55 DA), group II (glycol chitins with 54.09 DA and 73.12 DA, respectively), and group III (glycol chitin with 91.59 DA). The *in vitro* cytotoxicity test revealed that the glycol chitins exhibited significantly low cellular toxicity to the cultured cancer cells, showing at least 70% of cell viability following exposure to the highest polymer concentrations tested (Fig. 3b). In detail, group I showed about 74 ± 3.3% and 70 ± 3.7% cell viability at 250 μg/mL, and the cell viability increased gradually up to 88 ± 8.1% and 96 ± 5.6% at 15.6 μg/mL. Interestingly, group II demonstrated a much lower toxicity profile compared to that of group I with cell viabilities of 99 ± 10.4% and 98 ± 7.1% at 250 μg/mL and the viability of group II was over 100% at 15.6 μg/mL. In contrast, group III showed cell viabilities of 89 ± 2.9% and 92 ± 1.9% at 250 μg/mL and 15.6 μg/mL respectively, which decreased slightly compared to that of group II. The increase in DA of the glycol chitins gradually reduced their cytotoxic effect toward cultured cancer cells. Although the actual mechanism explaining the effect of DA against cytotoxicity is unknown, the present findings indicated the glycol chitins could be useful as generally biologically safe and non-toxic materials.

3.3. Thermo-sensitive sol–gel transition of glycol chitin in vitro

Thermo-sensitive properties of the glycol chitins were investigated by the tube inverting method and rheological studies. Fig. 4a shows a sol–gel transition phase diagram obtained by the tube inverting method. Glycol chitins with various DA were dissolved in PBS solution (pH 7.4, 0.01 M) at a concentration range of 3–7 wt%. With the DA ranging from 84.45% to 91.59%, glycol chitin solutions demonstrated a phase transition from a transparent, flowing sol state to a non-flowing transparent gel state as temperature increased (Fig. 4a, inset), whereas no thermo-sensitive gelation behavior was observed for glycol chitosan and glycol chitins with a lower DA. The sol–gel transition temperature could be controlled from 23 °C to 72 °C by varying the DA and concentrations of the glycol chitins. The phase diagram shifted to a lower temperature by increasing the DA of the glycol chitin. An increase in the concentration of glycol chitin from 3 to 7 wt% led to a decrease in sol–gel transition temperature, which was contributed to higher physical cross-linking density formed by the hydrophobic interactions among acetyl groups and enhanced chain entanglement. The sol–gel transition temperature was slightly affected by adding salts. The presence of sodium chloride, as a water-structure breaking salt, is known to decrease the sol–gel transition temperature in typical thermogelling polymer systems of polyesters and polyphosphazenes (Lee, Lee, Sohn, & Song, 2002; Xu & Li, 2005). As the sodium chloride concentration increased to 2.5 M, the sol–gel transition temperature decreased by 3–15 °C depending on the glycol chitin concentration (Fig. 4b). These results suggest that the fine-tuning of the sol–gel transition temperature could be realized by adjusting the DA, polymer concentration, and salt concentration.

Rheological studies were carried out to investigate the sol–gel transition behavior of the glycol chitins in response to temperature change (15–75 °C), as dynamic rheometry was identified as a more reproducible and quantitative method compared to the tube inverting method (Jeong, Wang, & Gutowska, 2001). As shown in Fig. 4c, the higher DA of the glycol chitin (86.42 DA and 91.59 DA) led to sharp increases in viscosity as the temperature increased. However, glycol chitosan and glycol chitins with lower DA at the same concentration (7 wt%) did not show an obvious increase in viscosity as the temperature increased, which coincided with the result from the tube inverting method. Moreover, a concentration dependent sol–gel transition behavior of glycol chitin was confirmed. As shown in Fig. 4d, a sharp increase in the viscosity of glycol chitin solutions (91.59 DA) with concentrations of 3, 5, and 7 wt% was observed at 51.80 °C, 35.80 °C, and 20.90 °C respectively. These data demonstrated that both the DA and concentration of glycol chitins collectively affected the sol–gel transition properties of the glycol chitins. The sol–gel transition temperatures of glycol chitin (91.59 DA, 5 and 7 wt%) were below body temperature, allowing for

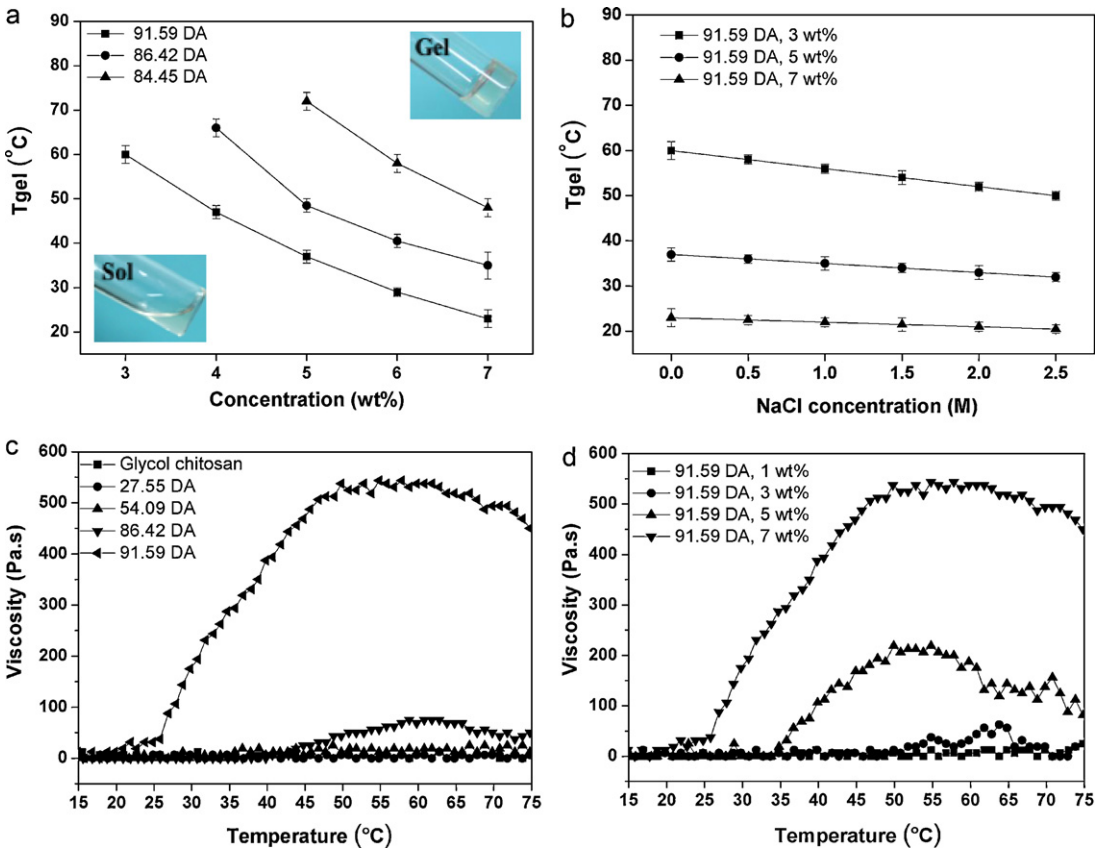


Fig. 4. (a) Sol–gel transition phase diagram of glycol chitin measured by the tube inverting method. (b) Dependence of sol–gel transition temperature of glycol chitin on the NaCl concentration measured by the tube inverting method. (c) Dependence of viscosity changes of the glycol chitin solution on the degree of acetylation (DA) measured by rheometer (concentration fixed at 7 wt%). (d) Dependence of viscosity changes of glycol chitin solution (91.59 DA) on the concentration measured by rheometer.

the potential use of glycol chitin as a new thermogelling biomaterial. It is also significant to note that distinctively from chitosan and other polysaccharide based thermogelling systems, the gelation of glycol chitin could be observed even at a relatively low concentration and triggered only by temperature change without the use of any chemical crosslinkers or additives such as glycerol-phosphate salt (Chenite et al., 2000).

3.4. Thermo-reversible and fast gelation kinetics

To evaluate the thermogelling kinetics of glycol chitin, we used a glycol chitin solution (91.59 DA, 7 wt%) to determine the time for change in the elastic modulus (G') as the temperature changed using a rotating, temperature controlled rheometer. As shown in Fig. 5, the sol–gel transition of glycol chitin was so fast and reversible in response to temperature. The G' showed cyclic changes as the temperature was cycled between 4 °C and 37 °C, matching with the reversible sol–gel transition behavior. Fast gelation is preferred for practical applications, particularly for thermogelling systems, as slow gelation *in vivo* may result in undesired diffusion of hydrogel precursors or bioactive molecules into surrounding tissues or failure of gel formation (Lee et al., 2010). Our glycol chitin based thermogelling system exhibited a fast thermoreversible sol–gel transition kinetic, which is very promising for use in localized drug delivery as well as tissue engineering.

3.5. Thermo-sensitive sol–gel transition of glycol chitin in vivo

We tested the thermo-sensitive sol–gel transition of the glycol chitins *in vitro* and demonstrated that the sol–gel transition occurred below 37 °C. To further confirm the thermo-sensitive

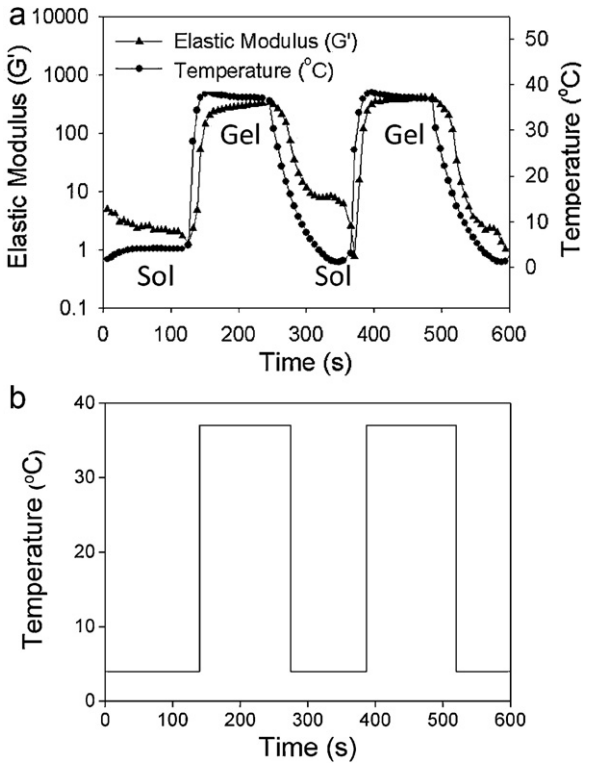


Fig. 5. Elastic modulus (G') changes of glycol chitin hydrogel (91.59 DA, 7 wt%) with thermal cycles of heating (37 °C) and cooling (4 °C).

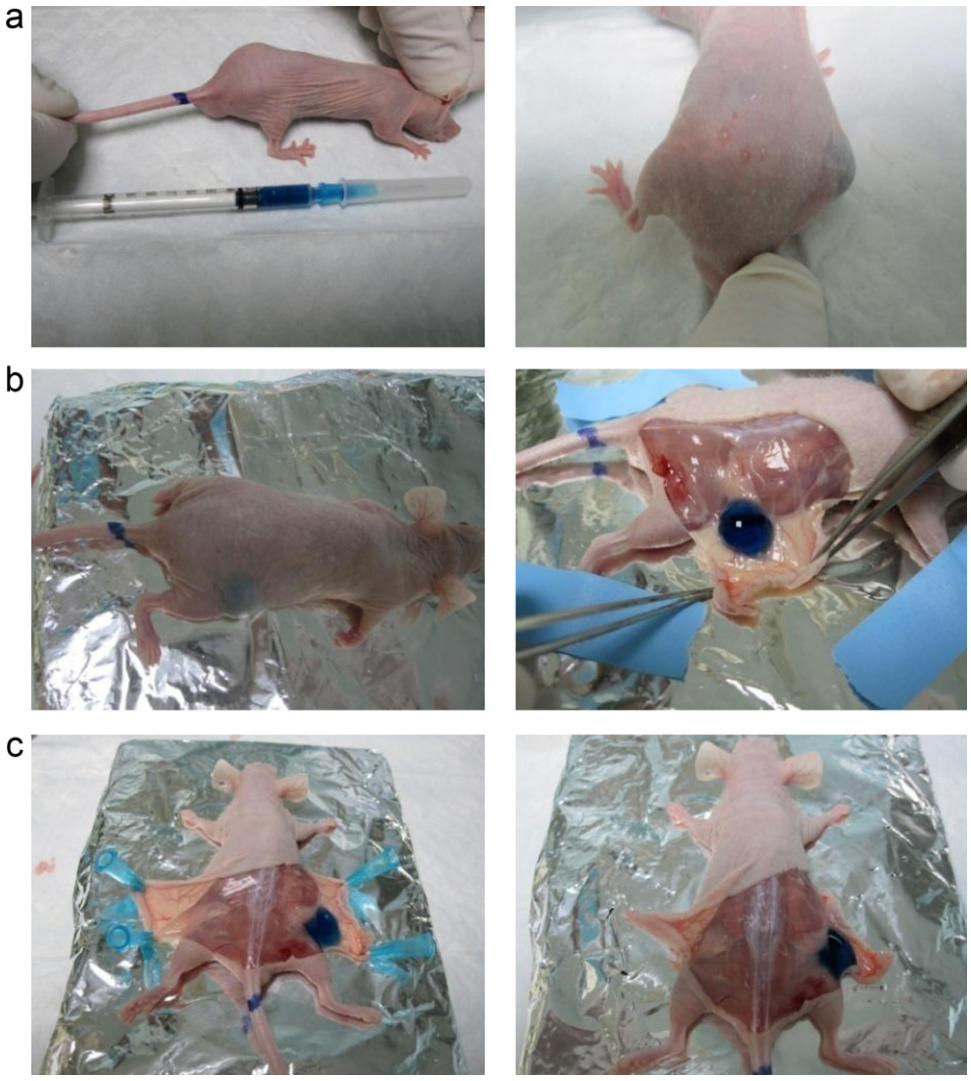


Fig. 6. Thermo-responsive sol–gel transition of glycol chitin *in vivo*. (a) Images before and after subcutaneous injection of glycol chitin (91.59 DA, 7 wt%) and 0.9% NaCl solution. (b) Surgical opening of the injection site and confirmation of gelation of glycol chitin at body temperature (37 °C) of mice 15 min after injection. (c) Loss of gel-like property of glycol chitin 10 min after opening the injection site.

sol–gel transition *in vivo*, we injected an aqueous solution of glycol chitin (91.59 DA, 7 wt%) and 0.9% NaCl as a control solution subcutaneously into the right/left flanks of 9-week-old female nude mice (Fig. 6a). A 10 μL aliquot of 0.4% trypan blue solution was added to produce glycol chitin solution with blue color. We surgically accessed the injection site 15 min after the injection. No control solution was observed at the injection site (Fig. 6b, left). We confirmed that the sol–gel transition had occurred at the injection site by observing local gelation of the glycol chitin solution as a result of the change in temperature under physiological conditions (Fig. 6b, right). Of interest, glycol chitin lost its gel-like properties and converted to a solution-like state within 10 min after opening the injection site (Fig. 6c), suggesting that the sol–gel transition of glycol chitin was reversible depending on the temperature changes. These *in vivo* experiments demonstrated that glycol chitin underwent the sol–gel transition under physiological conditions and this sol–gel transition was reversible depending on the temperature changes.

3.6. Swelling behavior and morphology of glycol chitin hydrogel

The macroscopic properties of glycol chitin hydrogels were studied in terms of their swelling behavior and morphology. We observed that the glycol chitin hydrogel reached its maximum swelling around 8 h with a swelling ratio of 170% while maintaining its swelling state up to 24 h without showing any apparent changes (Supplementary Fig. 1).

To investigate the morphology of glycol chitin hydrogels, we lyophilized the glycol chitin hydrogels formed with different concentrations and observed the surface and cross section morphology using FE-SEM. It was observed that the glycol chitin hydrogels were highly macroporous and the pores were well-interconnected to each other (Fig. 7). The pore sizes of these hydrogels were generally in the range of 5–40 μm. This result suggests that the highly macroporous and well-interconnected structure resulting from the three-dimensional network within the glycol chitin hydrogel can be potentially utilized for producing an injectable hydrogel matrix scaffold supporting cell growth and migration.

3.7. In vitro drug release

DOX is one of the most active and widely used anti-cancer drugs that exert its cytotoxic activity by inhibiting the synthesis of nucleic acids within cancer cells (Deverdiere et al., 1994; Omelyanenko, Kopeckova, Gentry, & Kopecek, 1998). However, the clinical use of

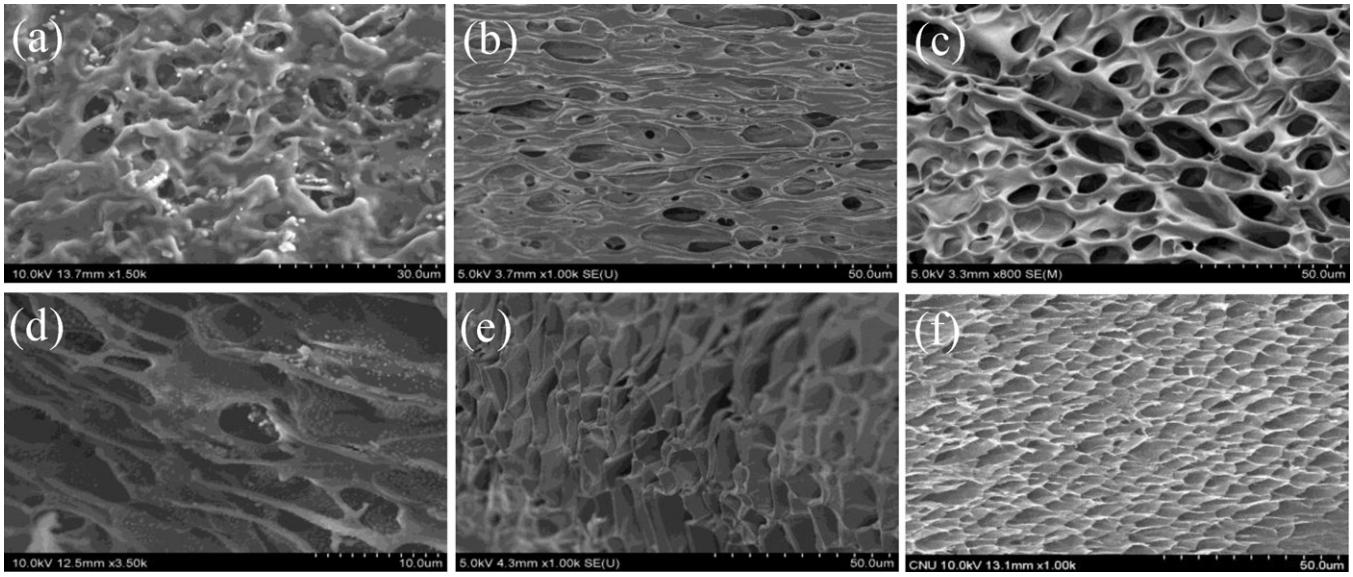


Fig. 7. SEM images of lyophilized glycol chitin hydrogel (91.59 DA) at different concentrations. Surface morphology of glycol chitin hydrogel at concentrations of (a) 3 wt%, (b) 5 wt%, and (c) 7 wt%; cross-section morphology of glycol chitin hydrogel at concentrations of (d) 3 wt%, (e) 5 wt%, and (f) 7 wt%.

DOX has been severally limited by its serious side effect of toxicity to the patients because of non-specific action (Bally, Nayar, Masin, Cullis, & Mayer, 1990; Rahman, Joher, & Neefe, 1986). Therefore, intratumoral administration of DOX appears to be a safe and potentially effective treatment without severer toxicity (De Groot, Cadee, Koten, Hennink, & Den Otter, 2002).

To check the possibility of glycol chitin hydrogel as a promising thermogelling system for local DOX administration, we first evaluated the gel formation of DOX containing glycol chitin solution and its stability *in vitro*. DOX (0.3 wt%) was dissolved into the glycol chitin solution (91.59 DA, 7 wt%) at 4 °C. By increasing the temperature to body temperature (37 °C), we were able to obtain a hydrogel (Supplementary Fig. 2a and b). The hydrogel maintained its three-dimensional structure without dissolution or disintegration after adding an excess amount of PBS at 37 °C to the top of gel (Supplementary Fig. 2c and d). Furthermore, we injected 0.5 mL DOX containing glycol chitin solution through a 21-gauge syringe needle into PBS at 37 °C to determine the feasibility of the glycol chitin hydrogel as an injectable DOX carrier. The results demonstrated immediate and stable gel formation in PBS at 37 °C (Supplementary Fig. 2e and f). These *in vitro* study results suggest that DOX containing glycol chitin solution could be successfully converted into stable gel state at body temperature, which would be useful as a new thermogelling drug delivery system for local DOX administration.

In vitro release of DOX from glycol chitin hydrogel with different DOX-loaded concentrations was evaluated. We measured the release of DOX (0.3 and 0.6 wt%) from the glycol chitin hydrogel (91.59 DA, 7 wt%) by UV–Vis spectroscopy. The release profiles showed that DOX had undergone a rather sustained release from the glycol chitin hydrogel over 13 days after an initial burst release of 20% of DOX under physiological conditions (Fig. 8a). Although a sustained release may provide long-lasting control, an initial burst effect may also be advantageous for initial tumor growth control (Al-Abd et al., 2010). Both hydrogels with 0.3 and 0.6 wt% drug loading showed similar release kinetics with the maximum accumulated release amount of DOX of 86.4% for 0.6 wt%. In order to evaluate the kinetics and the mechanism of drug release from the glycol chitin hydrogel formulations, the data obtained from the *in vitro* drug release studies were analyzed by the Higuchi

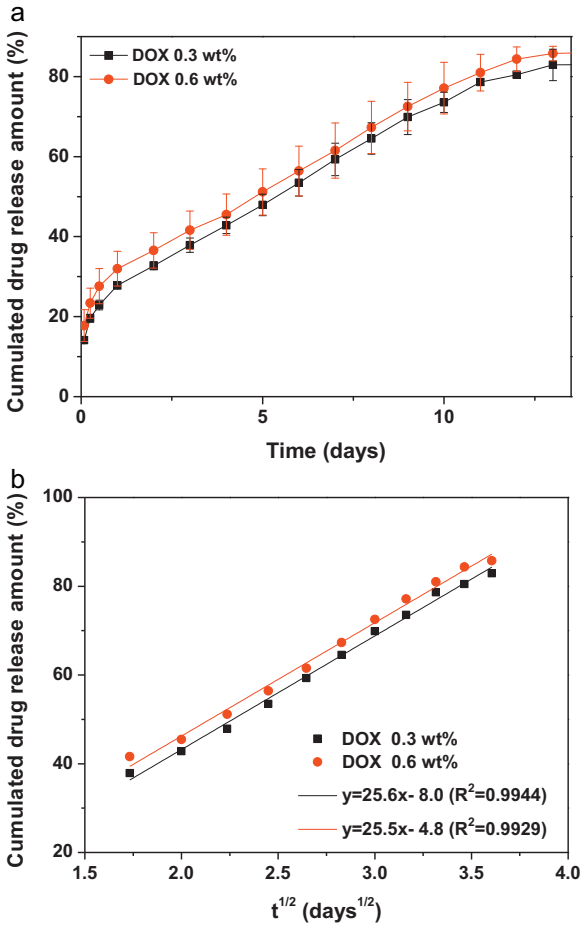


Fig. 8. (a) *In vitro* cumulative DOX release profile from *in situ* formed hydrogel prepared from glycol chitin solution (91.59 DA, 7 wt%) in PBS (pH 7.4, 0.01 M) at 37 °C. (b) *In vitro* cumulative DOX release profile against the square-root of time from the 3nd day to 13th day. R^2 is a measure of goodness of a fit.

model (Higuchi, 1963). The release profile from the 3nd day to 13th day against the square-root of time showed an excellent linear fit with *r*-square values of 0.9944 and 0.9929, indicating that the DOX release from the glycol chitin hydrogel followed a diffusion-dominant mechanism (Fig. 8b). We expect that the glycol chitin hydrogel has great promise as a new thermogelling system for the local drug administration of DOX with sustained release kinetics.

4. Conclusions

A new thermogelling polymer, glycol chitin, was successfully synthesized by selective *N*-acetylation of glycol chitosan and its thermo-sensitive sol–gel transition property was observed by various analytical techniques. The sol–gel transition temperature of glycol chitin could be controlled from 23 °C to 72 °C by varying the DA, polymer concentration, and salt concentration. The glycol chitin hydrogels were biodegradable, biocompatible, and thermo-reversible. The gelation was fast and durable under diluted conditions. As a model drug, the anti-cancer agent, DOX, could be easily loaded by simple mixing at low temperature and was released in a sustained manner over 13 days. These useful features thus render the glycol chitin hydrogel appropriate to serve as a promising thermogelling platform for drug delivery to control various diseases (*i.e.*, skin and cervical cancer) and injectable tissue engineering.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.11.068.

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