Carbohydrate Polymers 82 (2010) 316–322

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Effects of interpenetration of thermo-sensitive gels by crosslinking of chitosan on nasal delivery of insulin: *In vitro* characterization and *in vivo* study

Tze-Wen Chung[a,*], Der-Zen Liu[b], Juin-Sen Yang[a]

[a] *Department of Chemical and Material Engineering, National Yunlin University of Science and Technology, Dou-Liu, Yunlin, Taiwan, ROC*
[b] *Institute of Biomedical Materials, Taipei Medical University, Taipei, Taiwan, ROC*

ARTICLE INFO

Article history:
Received 19 January 2010
Received in revised form 21 April 2010
Accepted 22 April 2010
Available online 26 May 2010

Keywords:
Chitosan
Crosslinking
Sustained release
Poloxamer gel
Insulin delivery
Nasal drug delivery

ABSTRACT

This investigation examines the *in vitro* release of insulin in new thermo-response P-CS/GA/Gly gels, in which a chitosan (CS), crosslinked using glutaraldehyde (GA), interpenetrates Poloxamer (P) gels, and glycine (Gly) is further added to inhibit the crosslinking reactions of GA. The delivery of insulin by nasally administering P-CS/GA/Gly gels in diabetic rats was also examined. The release of insulin from P-CS/GA/Gly gels was significantly sustained ($P < 0.01$) for about six times longer than release from P gels. The mechanisms of the release of insulin from the gels were consistent with a Fickian diffusion model. The nasal administering of insulin using P-CS/GA/Gly gels in diabetic rats is associated with a greatly prolonged hyperglycemic effect and a pharmacological efficiency (involving the maintenance of the lowest possible blood glucose levels (C_{min}, $55.5 \pm 2.8\%$ of original values, $n = 6$) for 3 h, with a pharmacological efficiency of $18.0 \pm 1.8\%$ ($n = 6$)) that significantly exceeded those achieved using other gels.

1. Introduction

Chitosan (CS), an amino polysaccharide (poly-1,4-D-glucoamine) has been extensively applied in drug delivery and tissue engineering (Chung, Wang, & Tsaia, 2008; Kumar, Bakowsky, & Lehr, 2004; Lanza et al., 2000) because of its non-toxicity and biocompatibility. CS exhibits a positive potential in weak acid buffer solutions, which has been exploited to deliver proteins or DNA (Chung et al., 2008; Kumar et al., 2004; Lanza et al., 2000). The cationic property of chitosan has been exploited to deliver a tissue plasminogen activator to substrates of the fibrin network or insulin to mucosa surfaces (Chung et al., 2008; Yu et al., 2004). The nasal delivery of insulin using chitosan solutions has been demonstrated to increase greatly trans-mucosal absorption in rats and sheep (Illum, Farraj, & Davis, 1994; Yu et al., 2004). A formula that contained 1% chitosan and 5% hydroxypropyl-β-cyclodextrin (HP-β-CD) for nasal insulin delivery was recently explored (Yu et al., 2004).Poloxamer block copolymers comprise various poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) blocks that are arranged in a tri-block polymer structure, PEO/PPO/PEO. Since Poloxamers comprise various numbers of hydrophilic PEO and hydrophobic PPO copolymers, various segments of copolymers interact and aggregate into micelles in aqueous solution (Dumortier, Grossiord, Agnely, & Chaumeil, 2006). At sufficiently high concentrations and temperatures, Poloxamer micelles pack in an order that results in a transition of the sol to the gel state (Dumortier et al., 2006; Juhasz, Lenaerts, Raymond, & Ong, 1989; Liu & Chu, 2000), in which they are the so-called thermo-response gel. The sol–gel property of Poloxamers is of particular interest to numerous researchers who have examined their potential use in various pharmaceutical applications (Amiji, Lai, Shenoy, & Rao, 2002; Caffaggi et al., 2008; Liu & Chu, 2000; Roques, Salmon, Fiszman, Fattal, & Fromes, 2007). For example, Poloxamer 407 has been applied as a carrier for the intra-pericardial administering of plasmid DNA for gene therapy (Roques et al., 2007). However, as a drug carrier, Poloxamer gels typically sustain the release of drugs for a short period (such as <5 h) in an aqueous environment (Barichello, Morishita, & Nagai, 1999; Jeong, Kim, & Bae, 2002; Ricci, Lunardi, Nanclares, & Marchettia, 2005), since packed Poloxamer micelles dissociate rapidly in an aqueous environment. Although several chemical modifications of the Poloxamer (Cho et al., 2003; Chung et al., 2005; Sosnik & Cohn, 2004), including amine-termination or the grafting of hyaluronic acid or chitosan to mono-carboxyl Poloxamers (Cho et al., 2003; Chung et al., 2005), can reduce the critical gelation concentration and dissolution rate in aqueous solution, a simple method that, unlike the aforementioned approaches, does not require complex chemical modifications of Poloxamers to reduce the dissolution rate and improve the sustained-release characteristic of drugs, is sought. Accordingly, P-CS/GA gel (Fig. 1) that combines P gels, CS and GA

* Corresponding author. Tel.: +886 5 5342601x4610; fax: +886 5 5342071.
E-mail address: twchung@yuntech.edu.tw (T.-W. Chung).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.04.068

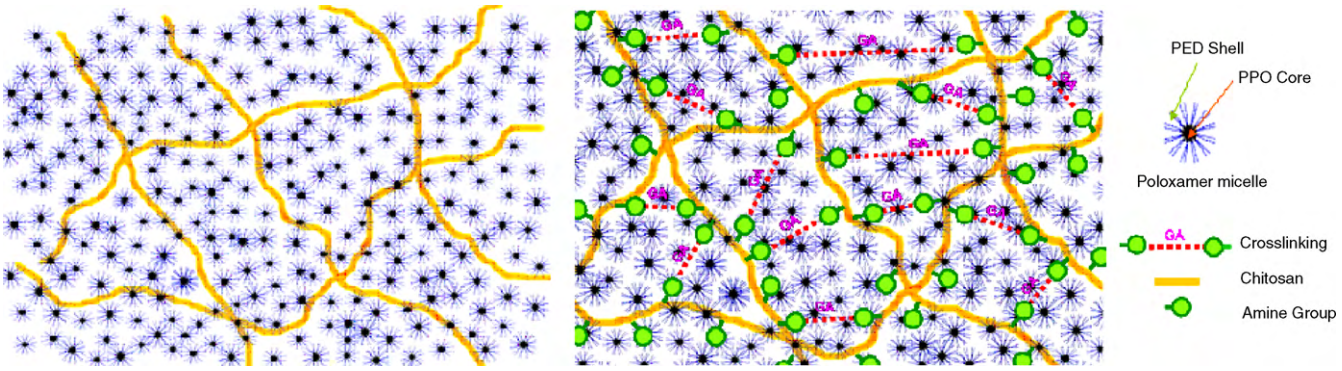

Fig. 1. Proposed schematic diagrams of (a) P-CS and (b) P-CS/GA gels which CS polymers are crosslinked by GA and interpenetrate within the gels.

has been developed without the aforementioned disadvantages to extend substantially the release of 5-FU (Chung, Liu, Tyan, & Yang, 2009).

Millions of diabetic patients receive subcutaneous injections of insulin to maintain their blood glucose levels in the normal range. Relieving the pain and inconvenience of injections of insulin is important. The nasal delivery of insulin to diabetic patients using gels with an absorption enhancer, such as CS, generally deposits insulin-loaded gels in regions of the nasal epithelium with high permeability, where the drug absorption rate is high (Ungaro et al., 2009). Additionally, the delivery of insulin using gels can prolong the contact time between the drug and the nasal epithelia of the nasal cavity, further increasing the permeation of the drug (Ungaro et al., 2009). Accordingly, the nasal administering of insulin is a means of improving the control of blood glucose levels in patients and has been extensively investigated (Illum et al., 1994; Ungaro et al., 2009; Wang, Tabata, & Morimoto, 2006; Yu et al., 2004). For example, various formulations of microspheres, including bio-adhesive powders and lipid emulsions (Krauland, Leitner, Grabovac, & Schnch, 2006; Wang et al., 2006) have been examined to evaluate their potential to deliver insulin nasally. Moreover, attempts have been made to deliver insulin using various nasal gels (D'Souza, Mutalik, Venkatesh, Vidyasagar, & Udupa, 2005; Wu, Wei, Wang, Su, & Ma, 2007). A thermo-response hydrogel that is based on quaternized chitosan and PEG has been developed to sustain the release of insulin for approximately a month *in vitro* and, as a nasal gel, effectively to reduce the blood glucose level for several hours *in vivo* (Wu et al., 2007). However, the quaternized chitosan hydrogel needs to be chemically synthesized and is not easily commercially available.This study elucidates the *in vitro* release of insulin from P, P-CS and P-CS/GA/Gly gels, following the addition of a large excess of glycine (Gly) to P-CS/GA to inhibit GA reactions. The *in vivo* nasal delivery of insulin with P, P-CS and P-CS/GA/Gly gels in diabetic rats was adopted as a model to evaluate the hypoglycemic effect and the pharmacological efficiency of the gels. The results of this work suggest that the new P-CS/GA/Gly gels may be effective for the nasal delivery of drugs.

2. Materials and methods

Adequate amounts of F127 and F68 (BASF laboratory, Wyandoote, USA) were dissolved in distilled water to prepare a Poloxamer (P) solution of F127 (18 wt%) and F68 (15 wt%) at 4 °C. To prepare a P solution that contained CS (Sigma Corp., USA), sufficient F127 and F68 were dissolved together in 1% acetic acid solution that contained 1.75% of CS (A solution). To prepare P solution that contained 1% of CS (P-CS solution), 4.5 ml of solution A was diluted in distilled water and stirred. To prepare 0.1% GA in P-CS/GA solutions, 0.5 ml of various concentrations of GA solutions was added to 4.5 ml of solution A, and stirred. To prepare P-CS/GA/Gly solution, GA crosslinking reactions proceeded in P-CS/GA solution for 10 min, and then a large amount of Gly (with a mole ratio of Gly to GA of 20:1) was added to the solution (Chen, Chen, Chen, Hsieh, & Lin, 2006), as described elsewhere, to block the un-reacted aldehyde groups of the residual GA. To prepare insulin-loaded P-CS/GA/Gly gel, insulin solution was loaded into a well-mixed P-CS/GA/Gly solution, mixed and heated to LCST. A temperature of 4 °C was used to prepare the aforementioned solutions. According to an earlier investigation that was conducted at this laboratory (Chung et al., 2009), P-CS/GA/Gly gels can maintain their thermo-response property for longer than 30 min in experimental testing.

2.1. Rheological measurements of P-CS and P-CS/GA solutions

To determine the viscosities of P-CS and P-CS/GA/Gly solutions, 1 ml of each solution was added to a cone/plate viscometer (Rheometer RS-100, HAAKE Mess-Technik, Karlsrhue, Germany) that contained a cone with an angle of 1°, inverted on a stainless steel plate. The viscosities of the solutions at 30 °C were continuously measured at a constant shear rate (75 s^{-1}) and data were automatically logged from 60 s to 900 s. The effects of shear rate and temperature on the viscosities of P-CS or P-CS/GA solutions were determined by making the aforementioned measurements while varying the shear rate from 30 s^{-1} to 300 s^{-1} or the temperature from 25 °C to approximately 35 °C, which is close to the LCST of the gels. To measure the elastic modulus, G', and the viscous modulus, G'', of P, P-CS and P-CS/GA solutions under oscillatory shearing, 4 ml of each solution was loaded into a double-cone sensor (CD60/1), which had a radius of 5.0 cm and a cone angle of 1°, and measurements were made using a viscometer in oscillatory mode (Rheometer RS-1, HAAKE Mess-Technik, Karlsruhe, Germany). The G' and G'' of the solutions in the linear deformation region were determined in dynamic frequency-sweep mode from 0.2 Hz to 5.0 Hz at a constant strain, $\gamma^0 = 0.01$, using a computer-controlled device (Liu, Huang, Jeng, Kao, & Yu, 2004; Vanderhooft, Alcoutlabi, & Magda, 2009).

2.2. In vitro release of insulin from P, P-CS and P-CS/GA/Gly gels

The procedure for investigating the release of insulin from gels was similar to that employed in the authors' earlier work, but with a few modifications (Chung et al., 2009). Briefly, bovine insulin (Sigma, MO, USA) was dissolved in 0.1 M HCl and adjusted to a pH of 4.5 by adding 0.1 M NaOH solution. This solution was then transferred to P-CS solution (pH 6.0) to yield 100 IU/ml or 3.45 mg/ml insulin solutions. To examine the release of insulin (100 IU/ml) from P-CS/GA/Gly gels, these gels were placed in a dialysis membrane with a cut-off molecular weight of 300 kDa (Spectrum Medical

Industries Inc., USA) and suspended in vials that contained 30 ml of distilled water as the dissolution medium. The vials were shaken at 60 rpm at 37 °C (Chung et al., 2009). 0.5 ml of the dissolution medium was periodically withdrawn to determine the insulin concentration using the Brandford method using a UV spectrometer (Jasco-530, Kobe, Japan) (Wu et al., 2007). The dissolution medium was replaced with an equal volume of fresh medium following each withdrawn.

Although many simple mathematical models describe the release of drugs from swellable polymeric systems, including gels (Peppas, Gurny, Doelker, & Buri, 1980; Ritger & Peppas, 1987), none completely predicts all associated experimental observations. Generalized empirical equations, including a power law model, have been extensively adopted to evaluate the release of drugs from swellable systems (Ritger & Peppas, 1987). In this study, the empirical power law equation, Eq. (1), is employed to analyze the release of insulin from gels with various formulations:

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

where M_t/M_∞ is the fractional release of the drug at time t; 'k' is the characteristic constant of the drug-gel systems, and 'n' is the diffusion exponent that characterizes the release mechanism. The equation applies until 60% of the drug has been released and effectively describes the release of the drug from discs of gels and other swellable materials, as well as that from non-swellable matrices. To evaluate the effectiveness of the mechanism of the release of insulin from each gel, k values were calculated using Eq. (1).

2.3. In vivo evaluation of effectiveness of nasal administering of gels in diabetic rats

Male Wistar rats were obtained from the Animal Center of Taipei Medical University, Taipei, Taiwan. They were housed individually in wire-bottomed stainless steel cages in an air-conditioned room (21 ± 2 °C, 50–70% relative humidity) with a 12 h light–dark cycle and free access to the basal diet and water for one week before diabetes was induced.

All experimental procedures that involved the animals followed the guidelines of the National Science Council of Taiwan and were approved by the Institutional Animal Care and Use Committee of Taipei Medical University, Taipei, Taiwan. The details of the procedure for inducing diabetes in rats can be found elsewhere (Chen & Cheng, 2006). Briefly, rats were made diabetic by intra-peritoneally administering streptozotocin (STZ, 45 mg/kg BW, in 0.9% sodium chloride solution) and injecting them 15 min later with nicotinamide (200 mg/kg BW) solution. These steps were then repeated two days later. A rat was identified as diabetic when its fasting plasma glucose level exceeded 10 mmol/l (or 1.8 mg/ml) after the administering of streptozotocin (Chen & Cheng, 2006). Only diabetic rats with plasma glucose levels from 2.0 mg/ml to 5.0 mg/ml were used.

Diabetic rats (250–300 g) fasted for 24 h before the experiments were conducted, but were allowed free access to water during that time. They were divided into five groups of six rats each, and anesthetized using gaseous ether. One group was the control group while the other four groups were the nasal delivery groups. At the beginning of each experiment, the initial blood glucose level of the rats was determined and designated as the reference level at zero time, 100%. The rats in the control group were subcutaneously injected (sc) with insulin (1.0 IU/kg) solution. 30 μl of insulin-free P-CS solution, and solutions of 10 IU/kg insulin in P, P-CS and P-CS/GA/Gly were loaded into separate micropipettes for nasal administering to the rats of the four nasal delivery groups. The solutions were administered into one nostril of each rat by carefully inserting a micropipette 3–5 mm into the nasal cavity. 200 μl blood samples were taken from the tail vein at 0.5 h and every half hour between 1 and 8 h following dosing. The glucose levels in the blood were determined by the glucose oxidase method using a glucose kit; glucose in blood reacts with an enzyme to produce a number of electrons that is correlated with the glucose level in the blood (Chen & Cheng, 2006; Krauland et al., 2006). For this work, the 200 μl blood samples from the rats were placed on a strip that was coated with the enzyme glucose oxidase for subsequent measurement of the glucose concentration using a blood glucose meter (One Touch blood glucose meter, LifeScan Inc., a Johnson & Johnson Company, Milpitas, CA, USA). The areas over the glucose level/time curves but below the baseline, defined as AOC, were calculated using the trapezoid rule. AOC is an index of the deviation of blood glucose level from the baseline. The relative pharmacological efficiency (Fr) was calculated using the following equation (Krauland et al., 2006; Wu et al., 2007; Yu et al., 2004):

$$\text{Fr}\ (\%) = \frac{\text{AOC}_{\text{nasal}}/\text{Dose}_{\text{nasal}}}{\text{AOC}_{\text{sc}}/\text{Dose}_{\text{sc}}} \times 100$$

All of the calculations were made using Sigmastat statistical software (Jandel Science Corp., San Rafael, CA, USA). Student's t-test and ANOVA with a confidence level of at least 95% were used to determine statistical significance ($P < 0.05$). Data were measured at least in triplicate and presented as mean ± standard deviation.

3. Results and discussion

3.1. Thermo-responses of P-CS and P-CS/GA/Gly gels

The lower critical solution temperature (LCST) of Poloxamer thermo-response gel depends on the ratio of P407 (or F127) to P188 (or F68) in the gel (Dumortier et al., 2006). In this work, the P solution comprised F127/F68 (18%/15% in wt %), yielding an LCST value of 34.5 °C. The small amount of CS only slightly changed the LCST value of the P-CS gels. For example, the LCST value of the P-CS gels with 1% CS was around 33.5 °C, which was 1 °C lower than that of P gels, perhaps because the CS may be present outside the packed micelles of the Poloxamer gel, since no specific interaction occurs between CS and Poloxamer polymers (Fig. 1(a)). The effect of GA concentration in P-CS/GA gels on its thermo-response is examined (Chung et al., 2009). The adopted concentrations of GA and Gly in P-CS/GA/Gly gels herein sustained their thermo-responses for over 0.5 h at 25 °C.

3.2. Rheology of P, P-CS and P-CS/GA/Gly solutions

The viscosities of P-CS/GA solutions at a fixed temperature are reportedly a function of time, whereas that of the P-CS solution is constant (Chung et al., 2009). The viscosity of P-CS/GA/Gly solution is also a function of time, as is that of P-CS/GA solution, but that of the latter is higher and rises more quickly than that of the former (515.6 ± 0.7 and 575.4 ± 0.3 mPa, respectively, at 10 min and 30 °C). Interestingly, at a give temperature (30 °C), the viscosity of the P-CS solution (300.0 mPa) was approximately three times that of the P solution, possibly because the tangled CS polymers hindered the flow of micelles in the P-CS solution (Chung et al., 2009). Table 1 presents the percentage increases in the viscosities of P, P-CS and P-CS/GA/Gly solutions with temperature from 25 to 35 °C, which is approximately the LCST of the P, P-CS and P-CS/GA/Gly gels. The viscosities of P, P-CS and P-CS/GA/Gly (0.1% GA) solutions rise with temperature in a similar manner up to approximately the LCST of each solution (Table 1). Hence, large changes in viscosities of P, P-CS and P-CS/GA/Gly solutions were observed as the measuring temperature was increased to approximately the corresponding LCST (34.5, 33.5 and 32.5 °C, respectively). For example, the viscosities of the P-CS solution increased from 145.0 to 448.1 mPa as the

Table 1
The viscosities of P, P-CS and P-CS/GA/Gly (0.1% GA) solutions were function of increasing temperature of the solutions. The viscosity of each solution at 25 °C was assigned as a basis value (100%) and the percentages of changes in viscosities for each solution at different temperature were presented. (*Note*: "*" P, P-CS and P-CS/GA/Gly (0.1% GA) solution would transform to gel at 34.5, 33.5 and 32.5 °C (or LCST value of each gel), respectively).

Temperature	25.0 °C	27.0 °C	29.0 °C	31.0 °C	33.0 °C	35.0 °C
P solution	100%	120%	180%	210%	340%	*770%
P-CS solution	100%	110%	130%	160%	240%	*480%
P-CS/GA/Gly solution	100%	130%	160%	210%	*370%	590%

temperature of the solution increased from 25 to 34.5 °C, which is the LCST of the P-CS gel. Oscillatory shear measurements of the elastic modulus, G', and the viscous modulus, G'', of P, P-CS and P-CS/GA solutions at 30 °C, at a constant strain of $\gamma^0 = 0.01$ but with dynamic frequencies from 0.2 to 5.0 Hz, revealed that the values of G' of those solutions ranged from 0.02 Pa or less, 0.2 to 3.4 Pa, and 0.9 to 20.5 Pa, respectively. The G' values of the P-CS/GA solution increased by much more than those of the P and P-CS solutions with increasing dynamic frequency, because CS was crosslinked by GA (Fig. 1(b)).

In contrast, the values of G'' of P, P-CS and P-CS/GA solutions ranged from 0.7 to 10.7 Pa, 3.8 to 48.6 Pa and 5.3 to 53.0 Pa, respectively, indicating only a small difference between P-CS and P-CS/GA solutions. Although the value of G' of the P-CS/GA/Gly solution (i.e., adopted for nasal administration herein) was a little lower than that of the P-CS/GA solution, it still exceeded that of the P-CS solution (data not shown). The viscous moduli, G'', of the tested solutions exceeded the elastic moduli, G', since oscillatory shear measurements of the solutions were made below the LCST of the gels. Notably, at a constant strain, the larger increases in the G' values of GA-containing P-CS solutions than of those without added GA suggests that CS polymers are crosslinked by GA and interpenetrate the solutions.

Fig. 1a schematically depicts the P/CS solutions. CS polymers freely disperse within the CS solutions and P/CS gels at temperatures above LCST (33.5 °C) without the formation of chemical bonds. In P-CS/GA solutions, numerous crosslinking reactions between CS and GA are assumed to yield a CS crosslinked network, which interpenetrates the solutions and P-CS/GA gels at temperatures higher than LCST (32.5 °C) (Fig. 1b). Fluorescent images of entangled CS polymers or CS network-like configurations that formed within P-CS/GA gels were obtained (Chung et al., 2009). P-CS/GA/Gly solutions are assumed to have a similar structure to that in P/CS/GA solutions, except that further extension of CS network were inhibited after large amounts of Gly were added into the P-CS/GA solutions, because GA molecules react with the amine groups of Gly molecules. The formation of an interpenetrating CS network in P-CS/GA/Gly gels (Fig. 1) may alter the rheological properties (Table 1) and the insulin release properties of the solutions (as will be described below).

3.3. Swelling ratios and dissolutions of P-CS/GA/Gly gels

The dissolution of Poloxamer gel (or packed micelles) is well known to proceed rapidly (Jeong et al., 2002; Lin & Sung, 2000). For example, 25 wt% of Poloxamer 407 gel was completely dissolved in a release medium in 4 h *in vitro* (Lin & Sung, 2000). Without further processing, the swelling ratios of P and P-CS gels in excess in the aqueous state were very low, since the inflow of water resulted in the rapid dissolution of gels. Detailed data on the swelling ratios of P-CS/GA gels can be found elsewhere (Chung et al., 2009). Interestingly, the maximum swelling ratio of the P-CS/GA/Gly gel with 0.1% GA was 10.9 ± 0.4, which was obtained after 18 h (Fig. 2). The maximum swelling ratios and the time of dissolution of P-CS/GA/Gly gels (42 h) were substantially lower than those of P-CS/GA (0.1 wt%) (Fig. 2), suggesting that the crosslinking reactions between CS and GA in P-CS/GA/Gly gels were effectively inhibited. The high swelling ratios of the P-CS/GA/Gly gels may be associated with the interpenetration of crosslinked CS within the gels, binding the aggregates of P micelles inside the domains of crosslinked CS, preventing the rapid dissolution of the gels.

3.4. Release of insulin from P-CS or P-CS/GA/Gly gels

Since residues of GA in biomaterials may cause un-wanted effects *in vivo*, large amounts of glycine are added to block GA residues when GA is used as a crosslinking agent in biomaterials (Chen et al., 2006). Similar approaches were employed in P-CS/GA solutions prior to the addition of insulin. Accordingly, large amounts of glycine (more than 20 times the amount of GA) were added to the P-CS/GA solutions at 10 min after 0.1 wt% GA had been added to the P-CS solutions. Fig. 3a plots the *in vitro* cumulative releases of insulin in P, P-CS and P-CS/GA/Gly gels. When the insulin solution was present only in membranes, the burst release of insulin was observed at 0.5 h, and the release of insulin ended within 1.5 h (Fig. 3a). When insulin was trapped in the P and P-CS gels, less was burst-released than from the insulin solution, but the releases were sustained to 3.0 and 3.5 h, respectively. Notably, the burst release of insulin from P-CS/GA/Gly gel was significantly reduced ($P < 0.01$, $n = 3$) (from 70% of the total amount of insulin to 20% at 0.5 h) and the duration of the release of insulin was substantially extended to 20 h, which is approximately six or more times the duration of insulin release from P or P-CS gels (Fig. 3a). The sustained release of insulin from the P-CS/GA/Gly gel was probably caused by an lengthening of diffusion paths of insulin, because the gel was still swelling (Fig. 2) during the period of release, causing the slow diffusion of insulin. Furthermore, the high viscosity of P-CS/GA/Gly solution and its gel may have reduced the diffusion rate of insulin into the dissolution medium during release.

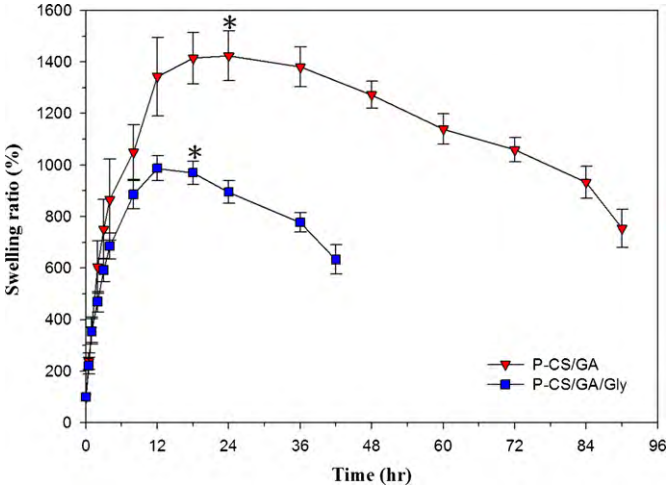

Fig. 2. Swelling ratios of P-CS/GA and P-CS/GA/Gly gels as functions of time. The maximum swelling ratios of the various gels were marked "*", and the measurements were terminated when the gels were dissociated to 50% of their maximum values.

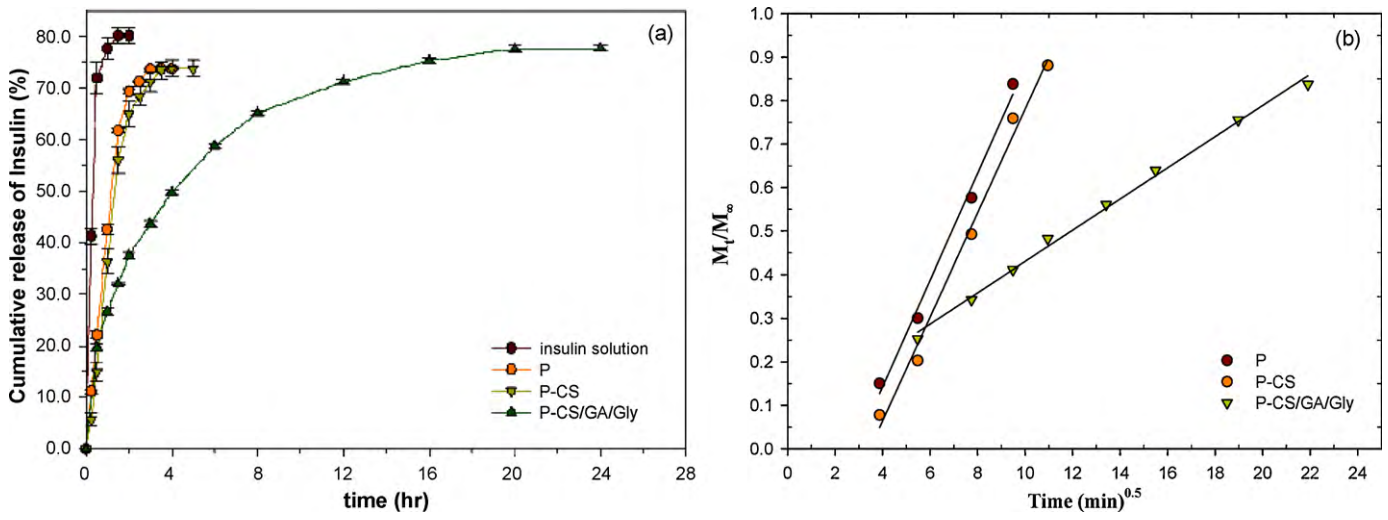

Fig. 3. (a) *In vitro* cumulative release of insulin in P, P-CS and P-CS/GA/ Gly gels. Initial burst releases of insulin were observed in P and P-CS gels was significantly reduced ($P < 0.01$, $n = 3$) in P-CS/GA/Gly gels. Insulin release in P-CS/GA/Gly gels was markedly sustained up to 20 h which was approximately six or more times the period of insulin release from P or P-CS gels. (b) Plots of M_t/M_∞ versus t^n; $n = 0.5$, the best-fitted value of n with a coefficient of correlation, R^2, over 0.99, for evaluating the fractional release of insulin from P, P-CS and P-CS/GA/Gly gels.

3.5. Mechanisms of diffusion of insulin from gels

According to the best-fit for the release of insulin in Fig. 3a, the '*n*' value in Eq. (1) in Section 2 is 0.5. Fig. 3b plots M_t/M_∞ versus $t^{0.5}$ for the fractional release of insulin from drug-loaded P, P-CS and P-CS/GA/Gly gels. Moreover, the '*k*' values of P, P-CS and P-CS/GA/Gly gels were 12.3, 11.9 and 3.6×10^{-2}, with a correlation coefficient of over 0.99. The '*k*' values for the release of insulin from P and P-CS gels herein were approximately equal to those of 5-FU in an earlier investigation (Chung et al., 2009), suggesting that the hydrophobic or the hydrophilic property of the drugs only weakly influenced their *in vitro* release in this system. Interestingly, for a particular GA content in the gels, the '*k*' value for P-CS/GA/Gly was lower than that for P-CS/GA (Chung et al., 2009), revealing the effect of adding Gly on the GA and CS crosslinking reactions. Additionally, according to an investigation of 5-FU delivery in a similar gel, the '*k*' value for the release from the gel declines as the viscosity of the corresponding solution increases (Chung et al., 2009). Since the viscosity of the P-CS/GA/Gly solution exceeds those of the other two solutions herein, the '*k*' value of the gel that transforms from P-CS/GA/Gly solution for insulin release is lower than the gel formed from P or P-CS solution.

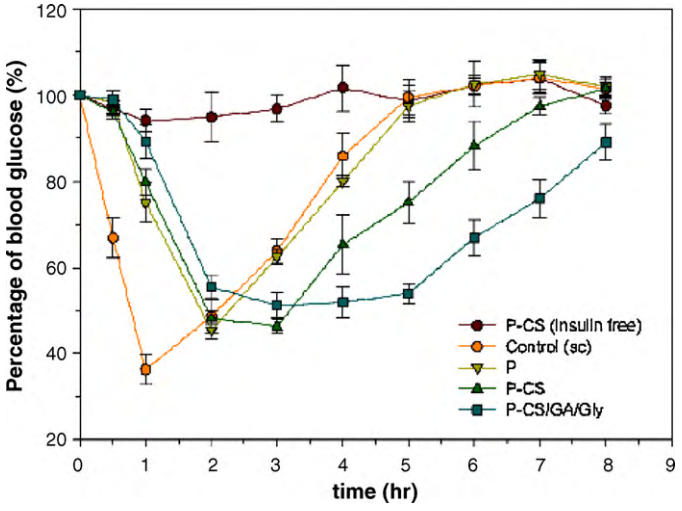

Fig. 4. Decrease in blood glucose levels as biological responses to insulin administered to fasted diabetic rats, after insulin was delivered subcutaneously (sc) at a dose of 1.0 IU/Kg and nasally using P, P-CS, P-CS/GA/Gly gels at a dose of 10.0 IU/Kg. No change in blood glucose level occurred after the administration of plain P-CS gels (insulin-free), as shown for reference.

3.6. Hypoglycemic activity of insulin-loaded P-CS/GA/Gly nasal gels in vivo

The hypoglycemic activity that is associated with the nasal delivery of insulin using P, P-CS and P-CS/GA/Gly gels in diabetic rats was examined. Rats that were injected subcutaneously (sc, 1.0 IU/kg) with insulin comprised the control group, with an Fr value of 100%. In this study, the dosages of insulin that were administered to the rats by subcutaneous injection or nasal delivery were similar to those employed elsewhere, although different carriers were used (Barichello et al., 1999; Wu et al., 2007; Yu et al., 2004). The blood glucose levels of rats following the nasal administering of insulin (10.0 IU/kg) using variously formulated gels, and those of the control group, were monitored (Fig. 4). Animals to which insulin had been administered nasally using only P or P-CS gel exhibited no reduction in blood glucose level (Fig. 4). The blood glucose concentration in the rats in the sc (or control) group was the lowest (C_{min}, $36.3 \pm 3.4\%$ of original values, $n = 6$) 1 h after the insulin was administered; this concentration had returned to its original level at 5 h. The profile of the blood glucose level of the rats to which insulin had been administered nasally using P gel, was similar to that of the sc-injected rats, but with a lag of 0.5 h; their C_{min} ($45.4 \pm 2.2\%$, $n = 6$) was reached 2 h after the insulin was administered. The rats to which insulin had been nasally administered using P-CS gels reached C_{min} ($48.5 \pm 2.0\%$, $n = 6$) 3 h thereafter, and the C_{min} state lasted for about 1 h; the glucose concentration returned to the original level a little more slowly than in the sc group. Notably, the rats to which insulin had been nasally administered using P-CS/GA/Gly gels reached C_{min} at 2 h, and this C_{min} state) ($55.5 \pm 2.8\%$, $n = 6$) lasted for 3 h, although the rate at which the glucose concentration returned to its original level was similar to that in P-CS gels. Moreover, since the C_{min} state lasted for 3 h, the blood glucose level of the group to which insulin had been administered using P-CS/GA/Gly gel was significantly lower ($P < 0.01$, $n = 6$) than that of other groups between 4 h and 8 h. The pharmacological efficiency (Fr) of the insulin that was nasally delivered using P, P-CS and P-CS/GA/Gly gels was $9.3 \pm 0.9\%$, $13.6 \pm 1.8\%$ and $18.0 \pm 1.8\%$ ($n = 6$), respectively, calculated with reference to the results for the

sc group. Hence, statistical analysis revealed no variation in the age, weight or basal blood glucose level of the tested diabetic rats in this investigation, suggesting that the gel dominated the hypoglycemic activity of insulin in diabetic rats.

For comparison, the Fr values for rats that to which insulin had been administered nasally using P and P-CS gels were similar to those of the other rats that were administered insulin using chitosan solution (Yu et al., 2004). Notably, the Fr value of the P-CS/GA/Gly gel significantly ($P < 0.01$, $n = 6$) exceeded those of other formulations herein. The subcutaneous administering of insulin using F127 gels or gels that contain insulin-PLGA nanoparticles in normal rats is associated with a prolonged hypoglycemic effect (Barichello et al., 1999). In this work, P-CS/GA/Gly gels that were used for the nasal delivery of insulin yielded similar results. CS is a well-known drug vehicle, which may open up the tight junctions between epithelial cells and thereby promote trans-mucosal absorption and consequently, the nasal and oral delivery of peptides and proteins (Illum et al., 1994; Kumar et al., 2004; Yu et al., 2004). Furthermore, the intranasal delivery of *Bordetella bronchiseptica* antigens that contain dermonecrotoxin (BBD)-loaded chitosan/F127 microparticles (MP) in mice has revealed that F127 promotes the delivery of BBD-loaded chitosan MP for vaccination (Kang et al., 2007). Accordingly, a drug delivery system that contains chitosan and F127 may promote mucosal delivery.

The CS solution promotes the absorption of insulin in rats or sheep when it is used in nasal delivery (Illum et al., 1994; Krauland et al., 2006; Wu et al., 2007; Yu et al., 2004). For example, a formulation of 1% chitosan and 5% hydroxypropyl-β-cyclodextran effectively reduces blood glucose levels in rats (Yu et al., 2004). The use of chemically modified CS, including insulin-loaded thiolated chitosan-based microparticles (Krauland et al., 2006), as well as quaternized chitosan and PEG hydrogel, to deliver insulin to rats nasally, was recently demonstrated to prolong its hypoglycemic activity and increase its pharmacological efficiency. Hence, the nasal delivery of insulin using CS-containing gels or crosslinked gels may promote (the absorption of insulin while reducing the nasal mucociliary clearance rate. However, the times to reach C_{min}, which is associated with hypoglycemia in rats, when P, P-CS and P-CS/GA/Gly were used herein, were approximately equal (Fig. 4), indicating that the promotion of the absorption of insulin by nasal delivery using CS may be insignificant. Interestingly, the period for which the blood glucose of rats exhibits the C_{min} state is 1 and 3 h, for the delivery of insulin by P-CS and P-CS/GA/Gly gels, respectively, whereas C_{min} was not maintained at all when P gel was used. These results are probably associated with the sustained release of insulin of the P-CS and P-CS/GA/Gly gels *in vitro* (Fig. 3a). Comparing the results in this work with those in other studies in which CS or modified CS was used to facilitate nasal insulin delivery (Illum et al., 1994; Krauland et al., 2006; Wu et al., 2007; Yu et al., 2004), demonstrates that the P-CS/GA/Gly gels that were developed herein had unique characteristics, including the ability to maintain glucose levels at C_{min} for 3 h. Furthermore, the Fr value of P-CS/GA/Gly gels for nasal insulin delivery exceeded those of other nasal delivery systems (Krauland et al., 2006; Yu et al., 2004). The use of a solution or gel that contains CS can enhance the permeation of drugs by opening up the tight junctions in the nasal epithelia (Wu et al., 2007), enhancing the rate of drug absorption. Notably, mucosal clearance importantly influences the efficiency of the nasal administering of insulin. In this study, the high viscosities of gels such as P-CS/GA/Gly gel may have promoted the adhesion of gel in the epithelium and the absorption of insulin, while reducing the nasal mucosal clearance rate.

Notably, no macroscopic change in the morphology of the nasal cavity or any other side effect was observed in diabetic rats beyond two weeks after the rats were subjected to the nasal delivery of insulin using P, P-CS and P-CS/GA/Gly gels (data not shown). Therefore, the gels were generally biocompatible with the animals. Since nasal insulin delivery was conducted in a rat model, whether the insulin dosage in the rat model can be related to that required for humans is of interest. Although this investigation did not address this issue, the relationship between the nasal administering of insulin in an animal model and that in human subjects will be considered in the near future.

4. Conclusion

In this work, CS in P-CS solution was simply crosslinked using 0.1% GA and then by the addition of glycine to yield P-CS/GA/Gly solution for insulin delivery. Insulin delivery using P-CS/GA/Gly gels *in vitro* significantly reduced the burst release of insulin ($P < 0.01$) below those achieved using P or P-CS gels, and greatly sustained the release of insulin ($P < 0.01$) for up to 20 h. The release of insulin from the gels was well modeled by the Fickian diffusion model, and the crosslinking of CS by GA within the P-CS/GA/Gly gels reduces the characteristic constant '*k*' of the drug–gel systems. P-CS/GA/Gly gels were used to deliver insulin nasally in diabetic rats, which method is associated with a highly prolonged hyperglycemic effect and improved pharmacological efficiency (Fr) ($18.0 \pm 1.8\%$), suggesting that such gels are potentially effective carriers of insulin for nasal delivery.

Acknowledgements

The authors would like to thank the National Science Council of the Republic of China, Taiwan, for financially supporting this research under Contract No. 96-2221-E-224-077-MY3 and 98-2221-E-224-009-MY2. Ted Knoy is appreciated for his editorial assistance.

References

Amiji, M. M., Lai, P. K., Shenoy, D. B., & Rao, M. (2002). Intratumoral administration of Paclitaxel in an in situ gelling Poloxamer 407 formulation. *Pharmaceutical Development and Technology*, *7*, 195–202.

Barichello, J. M., Morishita, M., & Nagai, K. T. T. (1999). Absorption of insulin from Pluronic F-127 gels following subcutaneous administration in rats. *International Journal of Pharmaceutics*, *184*, 189–198.

Caffaggi, S., Russo, E., Caviglioli, G., Parodi, B., Stefani, R., Sillo, G., et al. (2008). Poloxamer as a solubilising agent for tolfenamic acid and as a base for a gel formation. *European Journal of Pharmaceutical Sciences*, *35*, 19–29.

Chen, M. H., Chen, P. R., Chen, M. H., Hsieh, S. T., & Lin, F. H. (2006). Gelatin tricalcium phosphate membrane immobilized with NGF, BDNF, or IGF-I for peripheral nerve repair: An in vitro and in vivo study. *Journal of Biomedical Materials Research*, *79A*, 846–857.

Chen, C. W., & Cheng, H. H. (2006). A rice bran oil diet increases LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity in rats with streptozotocin/nicotinamide-induced type 2 diabetes. *Journal of Nutrition*, *136*, 1472–1476.

Cho, K. Y., Chung, T. W., Kim, B. C., Kim, M. K., Lee, J. H., Wee, W. R., et al. (2003). Release of ciprofloxacin from poloxamer-graft-hyaluronic acid hydrogels in vitro. *International Journal of Pharmaceutics*, *260*, 83–91.

Chung, H. J., Go, D. H., Bae, J. W., Jung, I. K., Lee, J. W., & Park, K. D. (2005). Synthesis and characterization of Pluronic® grafted chitosan copolymer as a novel injectable biomaterial. *Current Applied Physics*, *5*, 485–488.

Chung, T. W., Liu, D. Z., Tyan, Y. C., & Yang, J. S. (2009). Sustained release of 5-FU from Poloxamer gels interpenetrated by crosslinking chitosan network. *International Journal of Pharmaceutics*, *382*, 39–44.

Chung, T. W., Wang, S. S., & Tsaia, W. J. (2008). Accelerating thrombolysis with chitosan-coated plasminogen activators encapsulated in poly-(lactide-co-glycolide) (PLGA) nanoparticles. *Biomaterials*, *29*, 228–237.

D'Souza, R. D., Mutalik, S., Venkatesh, M., Vidyasagar, S., & Udupa, N. (2005). Insulin gel as an alternate to parenteral insulin: Formulation, preclinical, and clinical studies. *AAPS PharmSciTech*, *6*, E184–E189.

Dumortier, G., Grossiord, J. L., Agnely, F., & Chaumeil, J. C. (2006). A review of Poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharmaceutical Research*, *23*, 12–25.

Illum, L., Farraj, N. F., & Davis, S. S. (1994). Chitosan as a novel nasal delivery system for peptide drugs. *Pharmaceutical Research*, *11*, 1186–1189.

Jeong, B., Kim, S. W., & Bae, Y. H. (2002). Thermosensitive sol-gel reversible hydrogels. *Advanced Drug Delivery Reviews*, *54*, 37–51.

Juhasz, J., Lenaerts, V., Raymond, P., & Ong, H. (1989). Diffusion of rat atrial natriuretic factor in thermo-reversible poloxamer gels. *Biomaterials*, *10*(4), 265–268.
Kang, M. L., Jiang, H. L., Kang, S. G., Guo, D. D., Lee, D. Y., Cho, C. S., et al. (2007). Pluronic F127 enhances the effect as an adjuvant of chitosan microspheres in the intranasal delivery of *Bordetella bronchiseptica* antigens containing dermonecrotoxin. *Vaccine*, *25*, 4602–4610.
Krauland, A. H., Leitner, V. M., Grabovac, V., & Schnch, A. B. (2006). In vivo evaluation of a nasal insulin delivery system based on thiolated chitosan. *Journal of Pharmaceutical Sciences*, *95*, 2463–2472.
Kumar, M. N. V. R., Bakowsky, U., & Lehr, C. M. (2004). Preparation and characterization of cationic PLGA nano-spheres as DNA carriers. *Biomaterials*, *25*, 1771–1777.
Lanza, R. P., Langer, R., & Vancanti, J. (2000). *Principles of tissue engineering* (2nd ed.). San Diego, CA, USA: Academic Press.
Lin, H. R., & Sung, K. C. (2000). Carbopol/pluronic phase change solutions for ophthalmic drug delivery. *Journal of Controlled Release*, *29*, 379–388.
Liu, T., & Chu, B. (2000). Formation of homogeneous gel-like phases by mixed triblock copolymer micelles in aqueous solution: FCC to BCC phase transition. *Journal of Applied Crystallography*, *33*, 727–730.
Liu, D. Z., Huang, S. Y., Jeng, C., Kao, S. C., & Yu, J. H. J. (2004). Improved haemorrheological properties by Ginkgo biloba extract (Egb 761) in type 2 diabetes mellitus complicated with retinopathy. *Clinical Nutrition*, *23*, 615–621.
Peppas, N. A., Gurny, R., Doelker, E., & Buri, P. (1980). Modelling of drug diffusion through swellable systems. *Journal of Membrane Science*, *7*, 241–253.
Ritger, P. L., & Peppas, N. A. (1987). A simple equation for description of solute release. I. Fickian and non-Fickian release from swellable devices. *Journal of Controlled Release*, *5*, 37–42.
Ricci, E. J., Lunardi, L. O., Nanclares, D. M. A., & Marchettia, J. M. (2005). Sustained release of lidocaine from Poloxamer 407 gels. *International Journal of Pharmaceutics*, *288*, 235–244.
Roques, C., Salmon, A., Fiszman, M. Y., Fattal, E., & Fromes, Y. (2007). Intrapericardial administration of novel DNA formulations based on thermosensitive Poloxamer 407 gel. *International Journal of Pharmaceutics*, *331*, 220–223.
Sosnik, A., & Cohn, D. (2004). Ethoxysilane-capped PEO–PPO–PEO triblocks: A new family of reverse thermo-responsive polymers. *Biomaterials*, *25*, 2851–2858.
Ungaro, F., d'Emmanuele di Villa Bianca, R., Giovino, C., Miro, A., Sorrentino, R., Quaglia, F., et al. (2009). Insulin-loaded PLGA/cyclodextrin large porous particles with improved aerosolization properties: In vivo deposition and hypoglycaemic activity after delivery to rat lungs. *Journal of Controlled Release*, *135*, 25–34.
Vanderhooft, J. L., Alcoutlabi, M., & Magda, J. J. (2009). Prestwich GD Rheological properties of cross-linked hyaluronan–gelatin hydrogels for tissue engineering. *Macromolecular Bioscience*, *9*, 20–28.
Wang, J., Tabata, Y., & Morimoto, K. (2006). Aminated gelatin microspheres as a nasal delivery system for peptide drugs: Evaluation of in vitro release and in vivo insulin absorption in rats. *Journal of Controlled Release*, *113*, 31–37.
Wu, J., Wei, W., Wang, L. Y., Su, Z. G., & Ma, G. H. (2007). A thermo-sensitive hydrogel based on quaternized chitosan and poly(ethylene glycol) for nasal drug delivery system. *Biomaterials*, *28*, 2220–2232.
Yu, S. Y., Zhao, Y., Wu, F., Zhang, X., Lü, W., Zhang, H., et al. (2004). Nasal insulin delivery in the chitosan solution: In vitro and in vivo studies. *International Journal of Pharmaceutics*, *281*, 11–23.