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# Design of genipin-crosslinked microgels from concanavalin A and glucosyloxyethyl acrylated chitosan for glucose-responsive insulin delivery

Ruixue Yin [a], Kemin Wang [b,*], Shuang Du [c], Lu Chen [c], Jun Nie [c,*], Wenjun Zhang [a,d]

[a] Complex and Intelligent Research Center, School of Mechanical and Power Engineering, East China University of Science and Technology, Shanghai, PR China
[b] School of Materials Science and Engineering, Changzhou University, Changzhou 213164, PR China
[c] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, PR China
[d] Division of Biomedical Engineering, University of Saskatchewan, Canada

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ABSTRACT

Glucose-responsive systems are significant for self-regulated insulin delivery. The aim of this study was to assess the potential of genipin-crosslinked concanavalin A/GEA-chitosan microgels as a glucose-responsive insulin delivery system. A chitosan derivative (GEA-chitosan) was designed in this study as the polymer ligand of concanavalin A (Con A), which not only exhibits a strong affinity to Con A, but also could be directly crosslinked with Con A by genipin, thus avoiding the modification of Con A during an immobilization process. Glucose responsive microgels were fabricated by the reversed-phase emulsion crosslinking method. The *in vitro* release of insulin indicated that the insulin release was influenced by glucose concentrations, and a desired pulsatile release behavior was detected in response to stepwise glucose challenges for more than eight cycles. The release data were fitted well to an exponential model, without any significant influence of the surface effect. The released insulin was proved to remain active without destruction of the tertiary structure. The analysis of L929 cells viability suggested that these microgels possessed no *in vitro* cytotoxicity. The obtained genipin crosslinked Con A/GEA-chitosan microgels might be a potential candidate for self-regulated insulin delivery.



## 1. Introduction

Self-regulated insulin delivery systems are very helpful for insulin-dependent diabetes mellitus to control the blood glucose level within a normal range, since traditional subcutaneous injection of insulin often leads to inadequate glycemic control along with poor patient compliance (Gordijo, Shuhendler, & Wu, 2010). Glucose-responsive carriers on the basis of glucose oxidase, concanavalin A (Con A) and phenylboronic acid (Chen et al., 2012; Gu et al., 2013; Kawamura, Hata, Miyata, & Uragami, 2012; Kost & Langer, 2012; Roy, Cambre, & Sumerlin, 2010; Yao, Zhao, Yang, & Yang, 2012) can exhibit swelling changes in response to different glucose concentrations and thus could be used for the development of self-regulated insulin delivery systems. However, the specificity and responsiveness of these systems are still challenges to the clinical application.

The glucose-responsive system based on Con A, a lectin exhibiting reversible strong affinity for pyranose ring with unmodified —OH at C-3, C-4 and C-6, is the most specific system to glucose among the present systems. The reactivity of Con A with non-reducing α-D-mannose and α-D-glucose is stronger than that with other ring forms (Edelman et al., 1972), which makes it capable of causing affinity gelation of polysaccharide or glucose moieties containing polymer. Previous researches (Ballerstadt & Schultz, 1998; You, Lu, Li, Zhang, & Li, 2002) have used dextran and PGEA to react with Con A, thus forming glucose-responsive hydrogels.

However, these hydrogels are vulnerable to leaching Con A, leading to weak glucose sensitivity and undesirable biocompatibility. Therefore, it is necessary to covalently immobilize Con A onto the polymer matrix. Reactable double bond modification and crosslinking of Con A is an efficient way for Con A immobilization (Kawamura et al., 2012; Tanna, Joan, Sahota, & Sawicka, 2006). Our previous investigations (Yin, Wang, Han, & Nie, 2010; Yin, Tong, Yang, & Nie, 2012) also synthesized methacrylated Con A and fabricated glucose-responsive microgels from the crosslinking of methacrylated Con A and reactable dextran, and the reduced leakage amount of Con A after crosslinking was confirmed. Nevertheless, the chemical modification of Con A called for high cost,

* Corresponding authors. Fax: +86 1064421310.
*E-mail addresses:* wangkm61@gmail.com (K. Wang), niejun@mail.buct.edu.cn (J. Nie).

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and there was inevitable Con A loss during the modification process. Hence we intended to develop Con A covalent-binding microgels by direct crosslinking without Con A modification. To achieve this goal, a novel polymer ligand with both Con A affinity and crosslinking functional groups need to be designed.

In this study, we designed GEA-chitosan as Con A ligand containing both reactable amino groups and glucose residues, and microgels based on GEA-chitosan and Con A were fabricated to assess the potential for glucose-responsive insulin delivery. Insulin loading and release behavior were investigated in detail. The leakage of Con A, the activity of released insulin and the *in vitro* cytotoxicity were also measured.

## 2. Materials and methods

### 2.1. Materials

Chitosan ($\bar{M}w$ 50 kDa, DA 88%) was purchased from Zhejiang Golden-Shell Biochemical Co. (Yuhuan, Zhejiang, China). Methyl α-D-glucopyranoside (99%) and fluorescamine was obtained from J&K chemical, Ltd. (Beijing, China). Glucosyloxyethyl acrylate (GEA) was synthesized from α-D-glucopyranoside with HEA by using phosphomolybdic acid as a catalyzer according to Sadaya, Masakazu, Keisuke, and Toshiyuki (1990), with a yield of 67.6%. Concanavalin A (Con A; type IV, extracted from Jack Bean, $\bar{M}w$ 102 kDa, BR) and D-glucose anhydrous (AR) were purchased from Yuanju Bio-Tech Co., Ltd. (Shanghai, China). Genipin was obtained from Zhi Xin Biochemical Co. (Fuzhou, Jiangxi, China). Insulin (bovine pancreas, >27 USP U/mg, Sigma I 5500) and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was supplied by Sigma (USA). Other reagents were all obtained from Beijing Chemical Agent Co. (Beijing, China) and used as received. Mouse fibroblast cells (L929) were obtained from the Department of Microbiology, Peking University Health Science Center.

### 2.2. Synthesis of glucosyloxyethyl acrylated chitosan

Glucosyloxyethyl acrylated chitosan (GEA-chitosan) was designed here as the polymer ligand of Con A to form Con A-immobilized microgels by a direct crosslinking method. The synthesis of GEA-chitosan was achieved by Michael-addition reaction of chitosan and acrylic glycomonomer GEA as has been reported in our previous work (Wang, Yin, Tong, Yu, & Nie, 2011). Details for synthesis and characterization of GEA-chitosan used in this study can be seen in Supplementary data.

### 2.3. Preparation of genipin-crosslinked microhydrogels

Microgels with different compositions were fabricated by using reversed-phase emulsion crosslinking method according to the present literature (Karewicz et al., 2010). The formulations of microgels were listed in Table 1. Using microgel-2 as an example, the detailed preparation procedures were as follows. GEA-chitosan (50 mg) was dissolved in 1 mL deionized water; then the aqueous solution was mixed with 1 mL of 10 mg/mL Con A phosphate buffer solution (PBS, pH 7.0, 0.1 M KCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$, 6 h before utilization to allow the reactivation of the denatured protein by $Ca^{2+}$ and $Mn^{2+}$) for 3 h to make Con A interact with GEA-chitosan.

The round-bottom flask (100 mL) was charged with 45 mL of cyclohexane to which 0.7 g of Tween 85, an emulsion stabilizing agent, was added and stirred for 30 min to assure complete dissolution of the stabilizer. Then the GEA-chitosan/Con A mixture was added dropwise and stirred at 600 rpm to get a milk-like yellow emulsion. 1 h later, 0.565 mL of genipin (10 mg/mL) solution was slowly dropped to react with the amino-groups of GEA-chitosan and Con A. The resulting mixture was stirred in dark conditions for 48 h to allow for microgels hardening. Finally, after standing for 1 h, the mixture was allowed to remove the upper oil, followed by precipitated by isopropanol, centrifuged, and washed with water for at least three times. The obtained microgels were freeze dried and stored at 4 °C before use.

### 2.4. Characterization

The morphology of blank microgels was visualized by a Hitachi S-4700 field-emission scanning electron microscope (SEM) at an accelerating voltage of 20 kV. Prior to the observation, specimen was fixed on stubs with sputter coated with gold. The average diameter of microgels and diameter distribution were obtained by randomly analyzing 100 microgels from several SEM images. The images of microgels after insulin-loading were taken by an inverted fluorescence microscope (Olympus IX81) excited in long wavelength light (>500 nm).

### 2.5. Con A leakage assay

Concanavalin A was apt to leach out from the microgels during swelling process. Leached Con A from immobilized and unimmobilized microgels (microgel-2) was measured by UV–vis spectrophotometer at an absorption wavelength of 276 nm. The Con A leakage was calculated as the ratio of absorbance at 276 nm in the medium of PBS (pH 7.4) with glucose concentrations of 4 and 0 mg/mL.

### 2.6. Insulin loading

Insulin-loading into microgels was taken by diffusion loading method in mediums with different pH values (5.5, 6.5 and 7.4). Since insulin and chitosan had different isoelectric point (PI) (5.3 and 6.8), pH value of loading condition could affect the electrostatic interactions between negative insulin and positive GEA-chitosan, thus having influence on insulin-loading efficiency. A 10 mg-quantity of microgels produced as in Table 1 was placed in 25 mL 1 mg/mL insulin solution (dissolved in 0.01 N HCl, and then adjusted to fixed pH values: 5.5, 6.5 and 7.4) and allowed to stand for 5 h at room temperature. The microgels were then rinsed with distilled water for three times, freeze-dried for 12 h and finally stored at 4 °C until used.

The entrapment efficiency of insulin (EE) and loading capacity (LC) were calculated according to

$$\mathrm{EE}(\%) = \left[\frac{(\text{total insulin} - \text{free insulin}) \times 100}{\text{total insulin}}\right] \quad (1)$$

$$\mathrm{LC}(\%) = \left[\frac{(\text{total insulin} - \text{free insulin}) \times 100}{\text{microgels weight}}\right] \quad (2)$$

where total insulin is the initial amount of insulin added in the loading process and free insulin represents insulin in the solution after insulin-loading.

Concentrations of insulin were determined by fluorescamine method (Basan, Gümüşderelioğlu, & Tevfik, 2007; Tadashi, 2007). Briefly, boric acid buffer (pH 9.0), fluorescamine acetone solution (0.125 mg/mL) and the sample insulin solution were rapidly agitated. The intensity of fluorescence was then measured by using a Hitachi F-4500 fluorescence spectrophotometer at an excitation wavelength of 390 nm and emission wavelength of 475 nm for the fluorescamine labeled protein.

**Table 1**
Formulation, average size and insulin-loading properties of microgels.

| Sample code | Formulation | | | Average size (μm) | Drug loading content (wt%) | | | Encapsulation efficiency (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CS-GEA (mg) | Con A (mg) | Genipin/—$NH_2$ (mol ratio) | | pH 5.5 | pH 6.5 | pH 7.4 | pH 5.5 | pH 6.5 | pH 7.4 |
| Microgel-1 | 30 | 10 | 1/10 | 4.0 ± 1.3 | 5.7% | 5.4% | 5.7% | 56.2% | 52.5% | 54.0% |
| Microgel-2 | 50 | 10 | 1/10 | 2.9 ± 1.0 | 7.8% | 6.6% | 6.7% | 68.2% | 70.8% | 61.7% |
| Microgel-3 | 80 | 10 | 1/10 | 4.6 ± 1.1 | 6.7% | 7.2% | 6.9% | 53.1% | 56.3% | 48.6% |

### 2.7. In vitro insulin release experiments

The weighted amount (2 mg) of insulin-loaded microgel was placed in a 7 mL centrifuge tube and 4 mL of release medium solution (PBS, pH 7.4, with different glucose concentrations) was added. Insulin release was analyzed by incubating these insulin-loaded microgels at 37 °C (±0.5 °C) while shaking (100 r/min) as a function of time. Pulsatile release of insulin from microgels was achieved in response to stepwise changes of glucose concentration (the concentration was changed between 0 and 4 mg/mL in cycle).

At appropriate time intervals, after centrifuging at 10,000 r/min for 30 s, the released insulin in the supernatant was fluorescent labeled by fluorescamine and the concentration was measured by using a Hitachi F-4500 fluorescence spectrophotometer at an excitation wavelength of 390 nm and emission wavelength of 475 nm. The release study was continued after replacement with an equal volume of fresh solution to maintain a constant volume. The release data were expressed as mean ± standard deviation (S.D.) based on three independent measurements.

### 2.8. Insulin activity measurement

The activity of the released insulin was determined by analysis of the structure stability by using fluorescence emission spectrum at an excitation wavelength of 276 nm and an emission wavelength of 305 nm. The resulting spectrum was compared to standard insulin. The standard insulin solution was prepared in PBS (pH 7.4) to a final concentration of 0.01 mg/mL.

### 2.9. MTT assay

MTT assay was carried out following a similar procedure reported previously (Dev et al., 2010; Zhou et al., 2008). Mouse fibroblast cell line (L929) was cultured on a 96-well tissue culture plates at $1 \times 10^5$ cells/mL in 100 μL of growth medium DMEM containing 10% fetal bovine serum. Microgels were dispersed in the DMEM medium in the range of 2–8 mg/mL. Cells were seeded to medium containing 0.64% phenol (positive control) and a fresh culture medium (negative control) for references. The cell viability was evaluated after incubation for 24, 48 and 72 h by measuring the absorbance of the solution using a microplate reader (Model 680, Bio-Rad) at a wavelength of 490 nm.

Cell viability (%) was calculated from $[A]_{test}/[A]_{n\text{-}control} \times 100\%$, where $[A]_{test}$ and $[A]_{n\text{-}control}$ are the absorbance values of samples and negative control. For each sample, the final absorbance was the average value measured from six wells in parallel.

## 3. Results and discussion

### 3.1. Synthesis and structural characterization of chitosan derivative

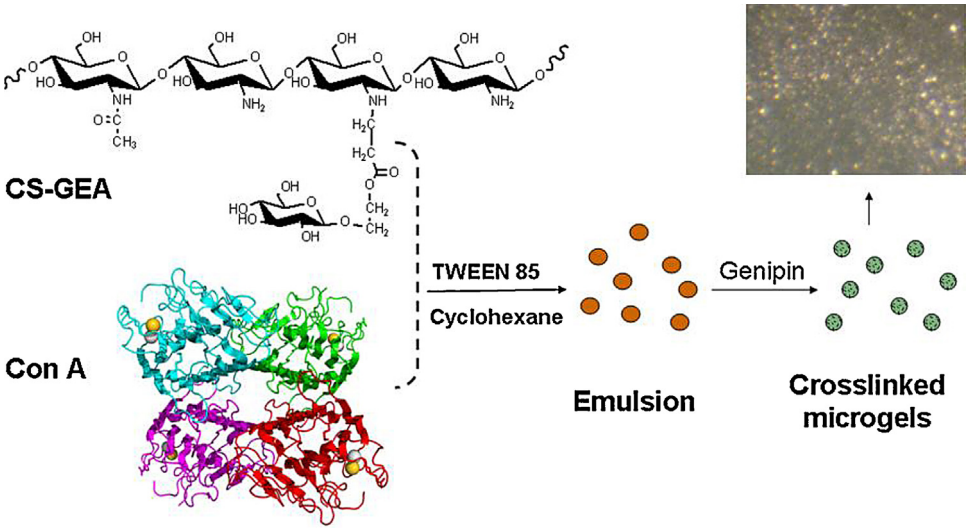
Glucosyloxyethyl acrylated chitosan (GEA-chitosan) was synthesized here as the polymer ligand of Con A by Michael addition reaction of chitosan and GEA. The reactable amino groups of chitosan, a widely investigated polyelectrolyte (Muzzarelli, 2009), supported the crosslinking by genipin and the glucose residues from GEA, a kind of acrylic glycomonomer, supplied the strong affinity to Con A (Takashi, Atsushi, Katsuhiko, & Allan, 1996; You et al., 2002). Moreover, the positive charged chitosan would improve insulin entrapment efficiency under a certain pH condition. The successful synthesis of GEA-chitosan was confirmed by FT-IR, $^1$H NMR, XRD and elemental analysis as shown in Supplementary data. The breakage of crystalline structure made GEA-chitosan soluble in neutral aqueous medium, thus expanding the applications of chitosan which could only dissolve in acidic solution. The DS of GEA-chitosan here was 0.19 calculated by elemental analysis according to previous research (Inukai, Chinen, Matsuda, Kaida, & Yasuda, 1998; Justi, Fávere, Laranjeira, Neves, & Casellato, 2005), which was slightly higher than that synthesized in our previous work using chitosan with higher molecular weight 200 kDa (Wang et al., 2011). More details of characterization part were shown in Supplementary data.

### 3.2. Preparation and characterization of microgels

The insulin delivery microgels based on GEA-chitosan and Con A were prepared through reversed-phase emulsion crosslinking using genipin as crosslinking agent in cyclohexane stabilized by Tween 85 for 48 h. Genipin is an aglucone of geniposide, which is a component of the Chinese medicine: gradeniae fructus, and a variety of pharmacological activities of genipin has been reported (Mi, Sung, & Shyu, 2000). Genipin can spontaneously react with primary amine to form dark blue pigments (Chen et al., 2004) and the cytotoxicity of genipin might be about 5000–10,000 times less than that of glutaraldehyde (Sung et al., 1999). Hence, genipin was used to form intermolecular cross-links among the primary amine groups of Con A and polymer ligand, thus immobilizing Con A to the microgel systems in order to prevent the leakage of lectin.

The fabrication process of microgels was schematically shown in Scheme 1. The complex of Con A and GEA-chitosan was first formed before emulsification in virtue of the affinity links between lectin sites and sugar units. Crosslinking of GEA-chitosan and Con A was observed as the color changed from light yellow to deep blue within several hours on treatment with genipin. The color change was due to the establishment of intermolecular cross-links produced by the reaction of genipin with the primary amine groups of both Con A and GEA-chitosan with a heterocyclic structure. The amount of genipin had significant influences on the formation and morphology of microgels. The size shrinked from more than 50 μm to less than 5 μm and the shape became regular as the ratio of genipin to amino groups (genipin/—$NH_2$) increased from 1/25 to 1/10 (Fig. 1).

As shown in Table 1, the average size of microgel-1, microgel-2 and microgel-3 was 4.0 μm, 2.9 μm and 4.6 μm, respectively, displaying no dramatic difference, for the same speed of the dispersion of polymers in the oil phase and the similar viscosity of polymers mixture. Using microgel-2 as a typical example, the SEM image and size distribution before insulin loading were presented in Fig. 2a and b. The microgels existed individually and the surface was generally rounded and smooth (see Fig. 2a). The diameter of microgels ranged from 1.3 μm to 6.7 μm, however, microgels of diameter within 2–4 μm accounted for more than 80% (see Fig. 2b). Representative fluorescence micrograph of genipin-crosslinked GEA-chitosan/Con A microgels (microgel-2) after insulin loading




**Scheme 1.** A schematic presentation of the fabrication process of the blank microgels.

was presented in Fig. 2c. The crosslinking reaction of genipin and primary amine generated the fluorescent conjugates (Muzzarelli, 2009), which could be observed from the red color of microgels as the fluorescence intensity excited by long wavelength light (>500 nm). The fluorescence of insulin also contributed a little to the fluorescence intensity. Besides, the main fluorescence emission of the image came from the whole microgels, indicating cross-links occurred homogeneously inside the microgels and insulin could enter the inside.

### 3.3. Comparison of Con A leakage

The leakage of Con A component was a primary problem which restricted the glucose sensitivity of hydrogels obtained from Con A and polymer ligand. Tanna and Taylor showed Con A leakage from dextran-Con A hydrogels in the receptor fluid, as monitored at 276 nm in the *in vitro* diffusion experiment (Tanna, Taylor, & Adams, 1999). Our previous research proved that photo-crosslinking of methacrylated Con A and dextran derivatives could effectively reduce the leakage of Con A (Yin et al., 2010). In this study, Con A was directly immobilized into microgels by the crosslinking reaction of genipin with amino groups. The effect of glucose stimulation on Con A leakage from microgels was determined here. Microgels without Con A immobilization were used as comparison. We assumed that there was no leakage of Con A or the leakage amount was insignificant without glucose stimulation, so the ratio of Con A leakage amount in the medium of PBS (pH 7.4) with glucose concentrations of 4 and 0 mg/mL could represent the relative leachability with glucose stimulation.

As shown in Fig. 3, Con A-immobilized microgels displayed decrease tendency of Con A leakage during swelling of 24 h, while the leakage amount of Con A increased a lot from Con A-unimmobilized microgels, and the leachability of Con A from Con A-immobilized microgels was much lower than that from Con A-unimmobilized microgels. The results indicated genipin crosslinking of Con A and GEA-chitosan ligand could efficiently restrict Con A leakage.

### 3.4. Insulin loading efficiency

Insulin was entrapped in the microgels under three different pH conditions: pH 5.5, 6.5 and 7.4. The corresponding loading content (LC) and encapsulation efficiency (EE) were shown in Table 1. The EE of microgel-2 and microgel-3 in pH 6.5 and 5.5 were much higher than those in pH 7.4, furthermore, they achieved the highest in pH 6.5, while the EE of microgel-1 achieved the highest in pH 5.5. However, the EE of microgel-2 were the highest among all these microgels no matter what the pH value of loading medium was. The LC of microgel-2 and microgel-3 were higher than those of microgel-1 under all these pH conditions. In general, insulin was oppositely charged to the GEA-chitosan/Con A microgels under the condition of optimized pH value, so it was loaded in the microgels mainly through electrostatic attraction. With the increase of GEA-chitosan content, the insulin loading efficiency was improved and the optimized pH value changed from 5.5 to 6.5. However, more GEA-chitosan could result in a denser gel network inside the microgels, which prevented insulin from entering into the microgels.

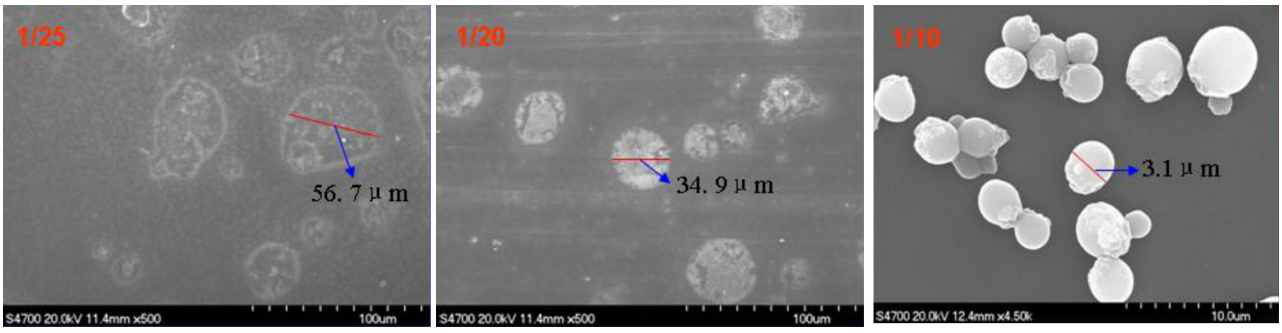



**Fig. 1.** Influence of genipin amount on the formation and morphology of microgels.

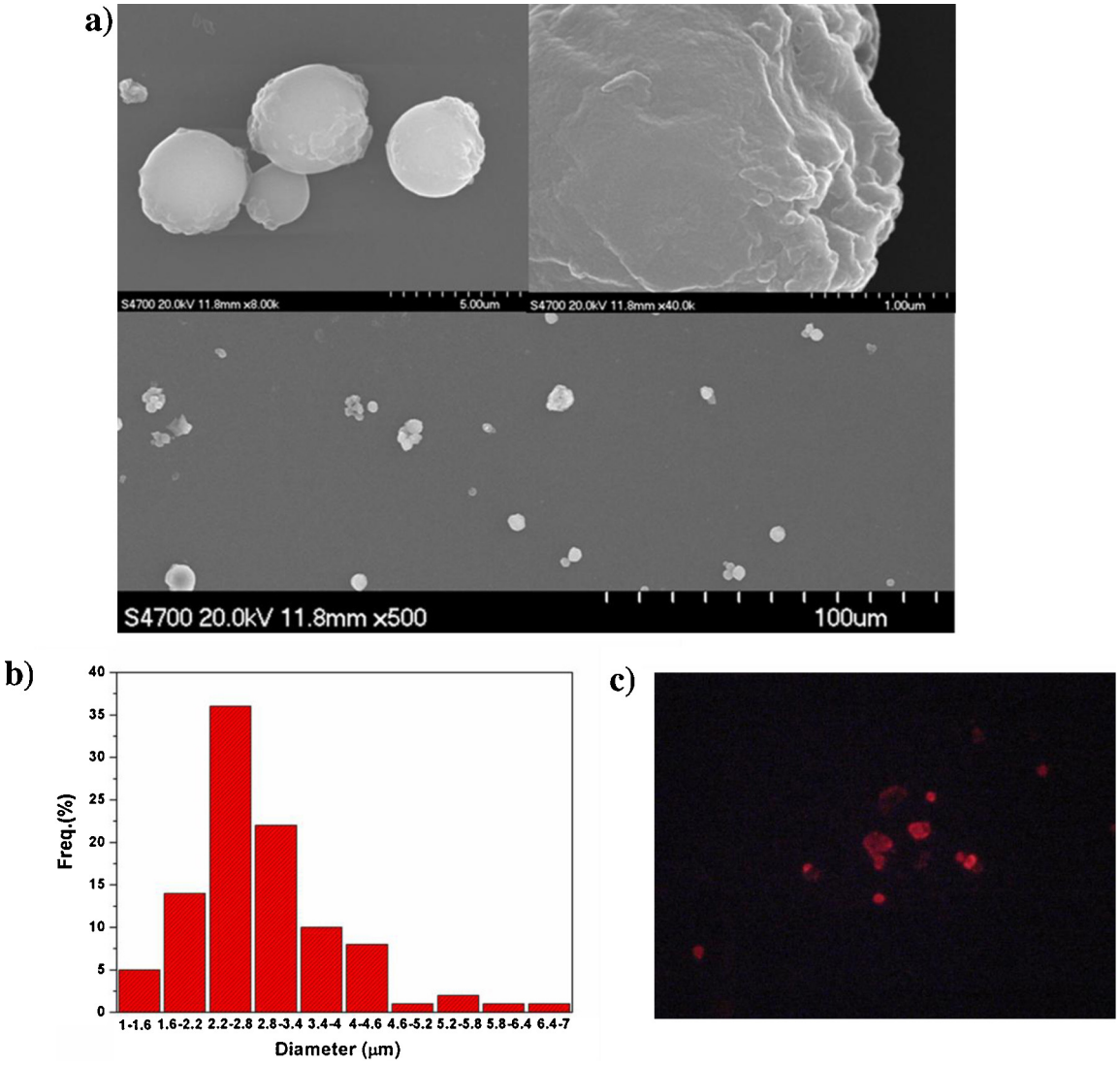



**Fig. 2.** Morphology of microgels observed by SEM with lower and higher magnification after freeze-drying (a), diameter distribution of microgels (b), and fluorescence microscopy images of microgels after insulin loading (c).

### 3.5. Insulin release in vitro

A type I diabetic patient or a serious type II diabetic patient without the function of insulin secretion must have a specific dosage regimen: about 0.2 IU/kg/day of basal insulin, and between 0.05 and 0.1 IU/kg before consuming a meal. For a patient with a body mass of 75 kg, these values correspond to a total daily insulin intake of about 30 mg. The basal dose was about half of the daily value, about 15 mg, while the amount before food intake was between 3.7 and 7.5 mg (Ahmed & Levy, 2004; Marek & Peppas, 2013). Insulin delivery must have a rapid-responsive release after glucose stimulation for several minutes to mimic the first-phase insulin secretion of pancreatic island β-cell and meet the insulin needs after food consumption, and a continued release for more than 1 h until the glucose concentration decreased to base line level, to mimic the second-phase insulin secretion and satisfy the basal insulin requirement (Steil, Panteleon, & Rebrin, 2004).

Insulin release profiles from insulin-loaded microgels (prepared in the loading condition of pH 6.5) with or without glucose stimulation were displayed in Fig. 4. From Fig. 4a, a rapid release could be found in the first ten minutes for all samples with glucose stimulation (4 mg/mL), and then the insulin release in the same medium

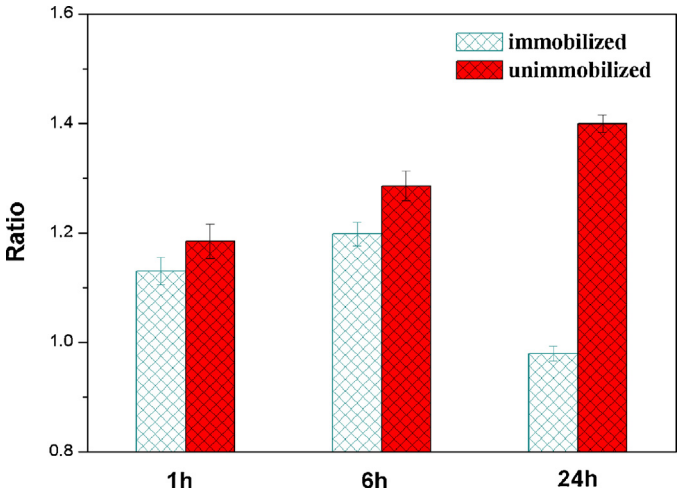



**Fig. 3.** Con A leakage from Con A-immobilized and unimmobilized microgels during swelling process.

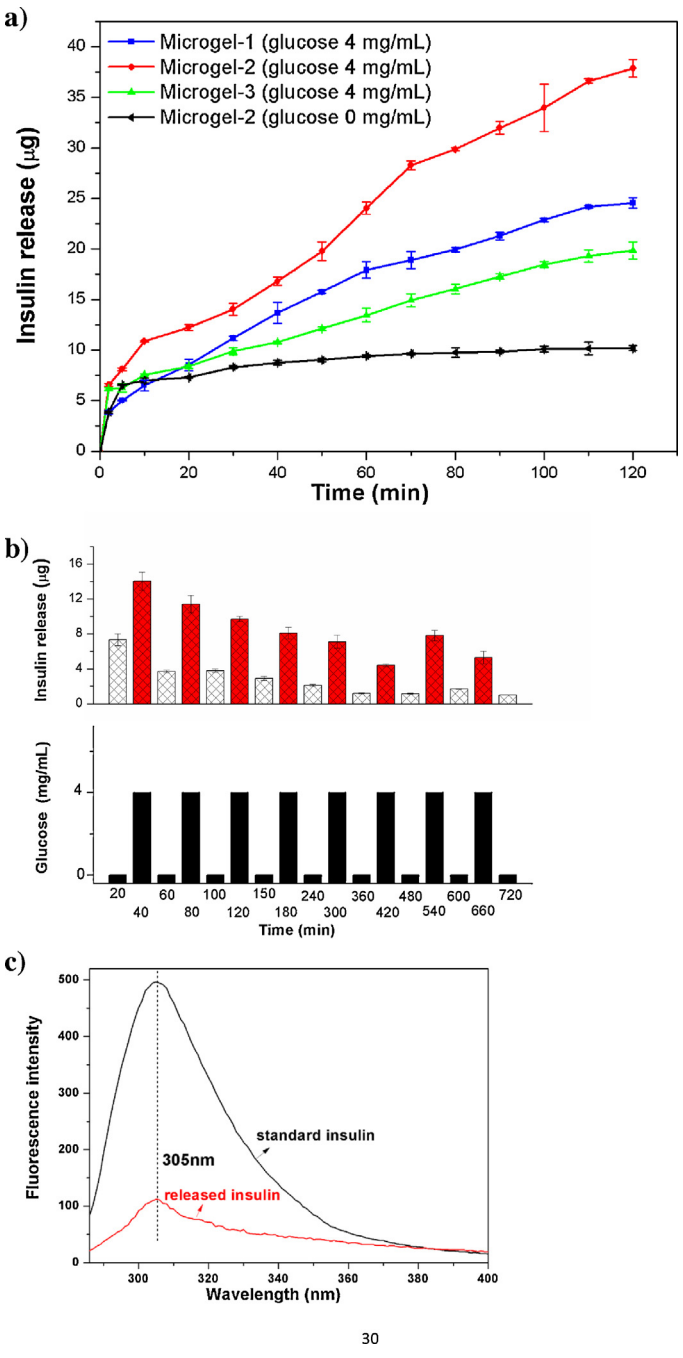



**Fig. 4.** *In vitro* release profiles of insulin from microgels in PBS (pH 7.4) with or without glucose stimulation at 37 °C (a), pulsatile release of insulin from microgel-2 in response to stepwise glucose changes between 0 and 4 mg/mL in PBS (pH 7.4) (b), and fluorescence spectra of standard and released insulin (c).

could be prolonged for more than 2 h, with a highest release rate from microgel-2. Besides, compared with the release profile without glucose (for microgel-2), the cumulate amount in response to glucose stimulation was much higher. Fig. 4b showed insulin release at specific time in response to stepwise changes in glucose concentration. Pulsatile insulin release pattern for more than eight cycles was displayed as the glucose concentration changed between 0 and 4 mg/mL, and in general the released insulin in the medium with glucose was higher than that in the medium without glucose. Moreover, combined Fig. 4a with Fig. 4b, we could conclude that microgel-2 was able to repeatedly fulfill the rapid-responsive release and continued release needs of insulin, which might be very helpful for diabetic patients. The reason for the glucose responsiveness of these microgels was that when free glucose was present in the environment and diffused into the microgel, Con A preferred to complex with free glucose (Kim & Park, 2001) rather than the pendant glucose in the side chain of GEA-chitosan, thus the specific sugar–protein crosslinking points were reduced by dissociation of the complex between the pendant glucose and Con A. Moreover, the complexation and dissociation of GEA-chitosan and Con A were reversible and repeatable, resulting in the reversible glucose-responsive property of the microgels.

**Table 2**
Values of parameters of the kinetic model for the release rate.

| Sample | With surface effect[a] | | Without surface effect[b] | |
|---|---|---|---|---|
| | $B$ | $r^2$ | $B$ | $r^2$ |
| Microgel-1 | 0.0252 | 0.9378 | 0.0288 | 0.9415 |
| Microgel-2 | 0.0264 | 0.9713 | 0.0293 | 0.9734 |
| Microgel-3 | 0.0248 | 0.9537 | 0.0289 | 0.9889 |

*Note*: $r$ is the correlation coefficient. The closer the $r^2$ value approaches to 1, the better the kinetic model fits in with the experimental data.
[a] Experimental data from 0 min are used for fitting the kinetic model.
[b] Experimental data from 2 min are used for fitting the kinetic model.

In order to compare the release rate and the influence of surface effect, insulin release behaviors of microgels-1, 2 and 3 in the first two hours with time intervals of 2 or 5 min (data not shown) were studied by using an exponential model. The release data were fitted to the exponential model as the following equation (Zhang et al., 2009):

$$y = Ae^{-Bx} \quad (3)$$

where $y$ is the insulin retention fraction in the microgels, $A$ and $B$ are two constants, and $x$ is the time. The values of parameters were listed in Table 2. According to the parameter $r^2$, the release data fitted well to the exponential model and the surface burst did not affect the fitting except for microgel-3, indicating that microgel-3 was most significantly influence by the surface burst release among all the samples. The reason might be that with the increase of GEA-chitosan amount, the more cross-links and stronger electrostatic attraction made insulin molecular harder to be entrapped into the inside microgels. According to the characters of exponential function, the larger the constant $B$ was, the faster the release rate was. From Table 2, it could be found that there was no significant difference between the constant $B$ of the three samples, however, the constant $B$ of microgel-2 was a little higher than that of others, indicating the fastest release rate from microgel-2, which was consistent with the results from Fig. 4a.

### 3.6. Activity of released insulin

Fluorescence spectrophotometer was used to evaluate the conformational change of tertiary structure of insulin (Amidi et al., 2008; Murali & Jayakumar, 2005), for the functions and structures of insulin were inherently correlated. Fig. 4c showed the fluorescence spectra of standard and released insulin. The two formulations displayed similar peaks with an emission maximum at 305 nm, indicating that the tertiary structure of insulin was not severely or irreversibly distorted by adsorption in the microhydrogels. It could be inferred that insulin released from the microhydrogels stayed active.

### 3.7. In vitro cytotoxicity

The potential applications of the GEA-chitosan/Con A microgels in drug delivery fields were evaluated by investigating the *in vitro* cytotoxicity using MTT assay (Dev et al., 2010; Zhou et al., 2008).

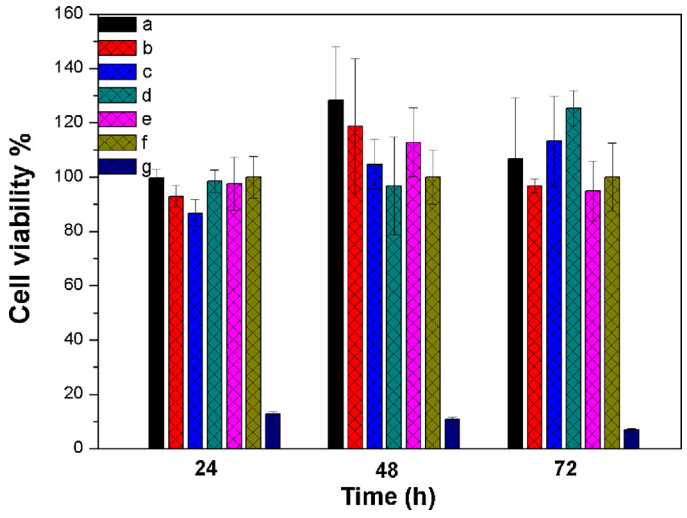



**Fig. 5.** *In vitro* cytotoxicity evaluation of microgels with different composition and concentration (a–c) 2 mg/mL, 5 mg/mL and 8 mg/mL of microgel-3; (d) 5 mg/mL of microgel-1; (e) 5 mg/mL of microgel-2) with negative (f) and positive controls (g) ($p < 0.05$) $^{*}p < 0.05$ when compared to the negative control of indirect cytotoxicity.

The viability of L929 culture for 24, 48 and 72 h was illustrated in Fig. 5. No statistically significant differences were observed in most samples for 24, 48 and 72 h, except for samples a (48 h), b (48 h) and d (72 h). However, the cell viability of a (48 h), b (48 h) and d (72 h) were higher than that of the negative control. Furthermore, the cell viability of all these samples was at a significant different level to that of positive control. Moreover, the L929 cell viability was maintained higher than 90% after 72 h of cultivation at concentrations up to 8.0 mg/mL, while that of phenylboronic acid based polymeric micelles was decreased to less than 40% at the concentration of 0.4 mg/mL as reported by Yao et al. (2012) indicating a better cytocompatibility of Con A based microgels. The nontoxicity of microgels was mainly attributed to the inherent good biocompatibility of natural products chitosan and genipin, combined with the efficiently immobilization of Con A. Clearly, these microgels were good candidates to be used as drug carriers.

## 4. Conclusion

Genipin-crosslinked glucose-responsive microgels based on GEA-chitosan and Con A were successfully fabricated, characterized and used for *in vitro* insulin delivery. The microgels were prepared through a reversed-phase emulsion crosslinking method with sphere shape and diameters less than 5 μm. The genipin crosslinking method to immobilize Con A with polymer ligand could efficiently restrict Con A leakage. Insulin loading of microgels was affected by pH value of the loading condition. Insulin release was reversibly in response to different glucose concentrations, and could repeatedly fulfill the rapid and continued release needs, which might be very helpful for diabetic patients. The surface release effect had no significant influence on the microgels and microgel-2 had the fastest release rate. The released insulin was proved to remain active without destroyed the tertiary structure. The microgels showed no cytotoxicity toward growth of L929 cells and had good *in vitro* biocompatibility. This microgel might be a promising system for self-regulated insulin delivery as well as other applications such as actuators and separation systems with sensitivity to glucose. In the further study, we will pay more attention to the resilience and robustness of the glucose-responsive system, for example, the responsiveness to very low or very high glucose level, and the release stability toward undesired factors. *In vivo* experiments will also perform in our next work.

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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.2013.12.067.

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