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Synthesis of Cationic Chitosan Hydrogel with Long Chain Alkyl and Its Controlled Glucose-responsive Drug Delivery Behavior

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Abstract: A novel glucose-responsive controlled drug release system based on cationic chitosan derivative (HDCC) with long chain alkyl was synthesized by etherification with glycidyl trimethylammonium chloride (GTMAC) and (2,3-epoxypropoxy) dodecyldimethylammonium chloride (EDC). The composition of HDCC was characterized by FTIR and ^{1}HNMR analysis. An improved water solubility and a relatively wide pH buffering path can be achieved for HDCC. With increasing substitution degree of long chain alkyl groups (DS_A) of HDCC hydrogels, the modulus and crosslinking density increased, while the swelling ratios decreased, suggesting the formation of more compact structure due to the physical entanglement of the long alkyl chains. The encapsulation efficiency (EE) and drug loading capacity (LC) of HDCC hydrogels increased with increasing DS_A suggesting the enhancement of interactions between the long alkyl chains and bovine serum albumin (BSA). By increasing pH value from 6.8 to 7.4, a considerable decrease in the cumulative release was observed for all hydrogels because the acidic environment could promote the hydrolysis of their cationic groups. Introduction of long alkyl chains slowed down the initial burst release rate of the hydrogels, and better pH and glucose sensitive release behavior of BSA and insulin can be observed. Drug release kinetic data under different pH values and glucose concentration for BSA-loaded hydrogels almost presented a Fickian release behavior and the kinetic constant k decreased with increasing DS_A, indicating HDCC hydrogels may achieve a better slow-release effect.

Keywords: cationic chitosan with long chain alkyl (HDCC); degree of substitution of alkyl groups (DS_A); pH sensitivity; glucose sensitivity; slow-release effect

1. Introduction

Diabetes mellitus, a disorder of glucose regulation, is a global burden affecting 366 million people across the world [1]. For tight control of hyperglycemia and prevention of the resulting complications in diabetic patients, it is highly desirable to develop a simple, effective, and continuous self-regulated drug delivery systems.[2] Glucose-responsive hydrogels, known as stimuli-responsive or "intelligent" systems, can adapt the rate of drug release in response to changes in glucose concentration in order to keep the blood glucose levels within the normal range.

Several examples of glucose-responsive hydrogels have already been reported using natural receptors such as the enzyme glucose oxidase (GOD) [3-5] or lectin concanavalin A (Con A) [6-7] as well as synthetic ones such as phenylboronic acid (PBA). [8-9] GOD modified materials, which was first used in the field of glucose responsive systems, have gained plenty of interest from researchers all over the world because of its convenience. In glucose-sensitive controlled delivery systems, GOD is entrapped or immobilized within a pH-sensitive matrix, which results in the enzyme-catalyzed conversion of glucose to gluconic acid, thereby lowering the pH in the microenvironment of the hydrogel, and causing drug releasing(as shown in Fig.1).

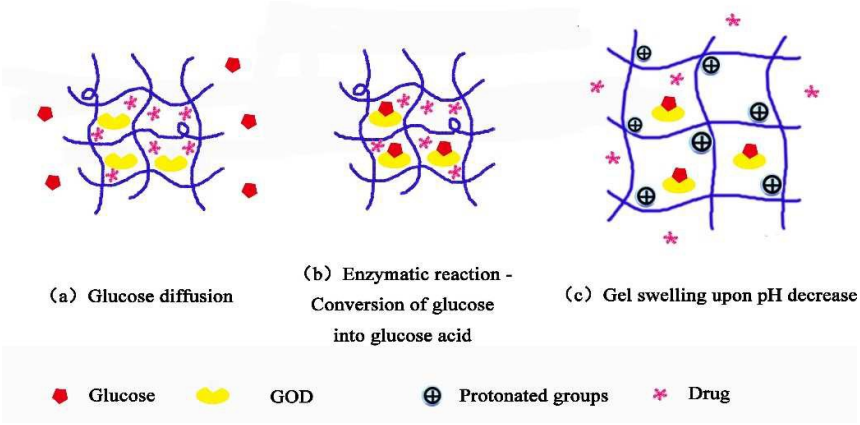


Fig.1 Schematic representation of pH-responsive hydrogel with entrapped GOD.

Chitosan (CS) is a copolymer of d-glucosamine and *N*-acetylglucosamine derived from chitin, which is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity. [10-12] In our previous work, it was demonstrated that the chitosan microspheres based on the amino group were pH sensitive in a wide range of pH 1.0 to 9.0, which was not suitable in the field of glucose responsive drug release systems with such narrow physiological pH-sensitive variation range from 7.4 to 6.8 for the conversion of glucose to gluconic acid through GOD. The quaternized chitosan (HTCC) synthesized by the grafting reaction with glycidyltrimethylammonium chloride (GTMAC) showed more distinct pH sensitivity as compared with chitosan [13], and satisfied the above narrow physiological pH variation for glucose responsive drug release systems. However, the initial burst effect and low drug loading efficiency were still two problems for the application of such HTCC hydrogels system. [14-16]

Burst release is considered to be a negative effect under most of the circumstances in drug delivery. The importance of avoiding burst release can be seen in the number of publications which focused on developing methods to prevent or minimize the burst effect in a wide range of polymer/drug systems. For instance, Wenlong Wang et al. [17] prepared docetaxel-loaded chitosan modified poly (lactic acid) (PLA) nanoparticles by anti-solvent precipitation method. Investigation of in vitro release study illustrated that PLA/chitosan nanoparticles prolonged drug release and decreased the burst release compared to the unmodified PLA nanoparticles. Yongmei Xu et al. [18] obtained modified HTCC nanoparticles by adding polyethylene glycol (PEG) and sodium alginate. The incorporation promoted the stability of nanoparticles surface, which obviously reduced the burst release of BSA

from 42% to 18%. Unfortunately, the methods that have been given to prevent burst effect, almost involve introduction of additional materials and additional costly steps, and also result in reduced drug loading efficiency.

In this work, chitosan was modified by etherification with glycidyl trimethylammonium chloride (GTMAC) and (2,3-epoxypropoxy) dodecyldimethylammonium chloride (EDC) as etherifying agents together, to yield a novel cationic chitosan derivatives (HDCC) with long chain alkyl. By introduction of the cationic groups, the modified chitosan hydrogel may be expected to behave distinct pH- and glucose-sensitivity, and by introduction of long chain alkyl with strong physical entanglement, the more compact network structure and stronger interaction with drug may be expected to form for the hydrogel, resulting in the reduction of the initial burst effect and improvement of the drug encapsulation efficiency so as to realize tight control of hyperglycemia for diabetic patients. The effect of different molar ratio of GTMAC/EDC on the structure and drug release behavior of HDCC hydrogels was studied.

2 Experimental

2.1 Materials

Chitosan (molecular weight 1×10^6 Da, degree of deacetylation 85%) was purchased from Zhejiang Jinke Biochemical (Zhejiang, China). Epichlorohydrin and sodium tripolyphosphate (TPP) were purchased from Tianjin Tianda Chemical Reagent Co. (Tianjin, China). Glycidyl trimethylammonium chloride (GTMAC) and dodecyldimethylamine were obtained from Dongying Guofeng Fine Chemical Co. Ltd. (Shandong, China). Bovine serum albumin (BSA) was provided by Huayi Bioengineering Co. Ltd. (Hubei, China). Glucose, GOD and insulin were purchased

from Baoxin Biotechnology Co. Ltd. (Chengdu, China).All other reagents were of analytic reagent grade. Double distilled water was used throughout.

2.2 Synthesis of (2,3-epoxypropoxy) dodecyldimethylammonium chloride (EDC)

Epichlorohydrin (2.0g) was slowly added to the mixture of the dodecyldimethylamine (1.5g) and isopropanol (30ml). The mixture was stirred at 50^{o}C for 36 h. The solvent and unreacted epichlorohydrin were evaporated off under reduced pressure at room temperature. A transparent and very viscous liquid was obtained.

2.2 Synthesis of modified chitosan

Chitosan was modified in neutral aqueous system. Chitosan (2g, 12.4mmol) was dispersed in water/isopropanol media (30ml) at 37^{o}C. The mixture was stirred for 30 min prior to dropwise addition of GTMAC and EDC. After reaction for 6 h at 60^{o}C, the turbid and yellowish reaction solution was poured into cold acetone and stirred in refrigerator overnight. After washed by acetone several times, the white precipitated product was collected by filtration. To obtain purer HTCC, the product was purified by dialysis for 2 to 3 days. By changing the molar ratio of GTMAC/EDC from 5:0, 4:1, 3:2 to 5:2, four modified chitosan samples were obtained, and coded as HTCC, HDCC-1, HDCC-2, and HDCC-3, respectively.

2.3. Preparation of modified chitosan hydrogels

Four modified chitosan solution (2%) containing various concentrations of BSA (or insulin) (5, 10, 15, 20mg) or GOD (0.6mg) at room temperature. Then the TPP aqueous solution (0.16g of TPP in 2.0ml water) was added to the modified chitosan aqueous solution under stirring and incubated at 37^{o}C for 2 h to form hydrogels. The samples were freeze-dried for 24h and stored at 4^{o}C before use.

2.4 Measurements

2.4.1 Water solubility

Aqueous solution of chitosan and the modified chitosan samples (2 mg/ml) was prepared by dissolving them in 2% CH_3COOH aqueous solution, respectively. Either HCl solution (1 M) or NaOH solution (1 M) was slowly added to adjust the pH. The transmittance of the solutions was recorded on an Alpha-1860 UV spectroscopy (China) at the wavelength of 600 nm.

2.4.2 Potentiometric titration

The titration curve of chitosan and the modified chitosan samples were obtained by an acid–base titration method. The specific progress was as follows: chitosan and modified chitosan samples (40mg) were dissolved in 2% CH_3COOH aqueous solution, respectively. Then the pH of this solution was adjusted to 2.5. The increase of the pH value of the samples solution was recorded using an Ohaus Starter 3C pH meter (USA) after adding a NaOH aqueous solution with a concentration of 0.1 mol L^{-1}.

2.4.3 FTIR analysis

The samples of chitosan and the modified chitosan samples were dried and analyzed with a Nicolet-560 FTIR spectrometer (USA). The samples were prepared by mixing with KBr. The scanning rate is 20 min^{-1} and the differentiate rate is 4 min^{-1}.

2.4.4 ^{1}H NMR analysis

^{1}H NMR spectra were recorded with a Mercury VX-300 spectrometer (USA). The samples of chitosan and the modified chitosan samples were dissolved in CH_3COOD/D_2O and D_2O to prepare a 10wt% solution for NMR measurement, respectively.

2.4.5 Rheological evaluation

The rheological properties were examined by Stress Tech rheometer (Sweden) with the standard steel parallel-plate geometry of 20-mm diameter. The test methods

employed were oscillatory frequency sweep.[19] The modified chitosan hydrogel samples were subjected to a frequency sweep in the range from 0.01 to 100 Hz at a fixed shear stress (10 Pa) and temperature (25^{o}C).

2.4.6 Swelling ratio

The swelling properties of the modified chitosan hydrogels were investigated in PBS buffer solutions with pH 6.8. The modified chitosan hydrogels of a known weight (W_o) were immersed in PBS buffer solutions at 37^{o}C. And then the hydrogels were taken out from the PBS buffer solution at predetermined intervals and weighed after removing excess solution from the surface with a wet filter paper. The swelling ratio can be determined with Eq. (3):

$$\% \text{ swelling} = \frac{W_t - W_0}{W_0} \times 100\% \qquad (3)$$

where, W_o is the dried weight of the modified chitosan hydrogels and W_t is the weight of the hydrated hydrogels at time t. Experiments were performed in triplicate.

2.4.7 SEM analysis

The fractured surface morphology of the modified chitosan hydrogels was observed with a JEOL JSM-5900LV scanning electron microscope (SEM) (Japan) under an acceleration voltage of 20 KV. The samples were incubated in PBS buffer solution with different pH at 37^{o}C for 1 h and then instantaneously plunged into liquid nitrogen to be freeze-dried and freeze-fractured.

2.4.8 Drug loading and in vitro drug release

The drug loading capacity (LC) and encapsulation efficiency (EE) of the modified chitosan hydrogels were calculated by using Eq. (4) and Eq. (5), respectively.

$$\text{LC}(\%) = \frac{\text{total drug weight} - \text{free drug weight}}{\text{hydrogel weight}} \times 100\% \qquad (4)$$

$$EE\ (\%) = \frac{\text{total drug weight} - \text{free drug weight}}{\text{total drug weight}} \times 100\% \qquad (5)$$

In vitro pH-sensitive drug release testing was carried out by incubating 10mg BSA-loaded modified chitosan hydrogels samples in PBS buffer solutions with pH values of 6.8.and 7.4. Glucose-sensitive drug release behavior was analyzed by incubating 10mg modified chitosan hydrogels samples in PBS buffer solutions (pH7.4) with glucose concentration values of 0, 1, 4 mg/ml, respectively.

To further investigate the glucose-sensitivity of BSA-loaded modified chitosan hydrogels, the BSA release at alternant glucose concentrations (1 or 4 mg/ml) was also tested. Simply, the BSA release from HDCC hydrogels was conducted in PBS buffer solutions at pH 7.4 containing 1 mg/ml glucose during the first 3 h, then the samples were transferred into the4 mg/ml glucose medium for the second 3 h. At the third 3 h, the release sample was put back into 1 mg/ml glucose medium. After that, the release sample was transferred into the 4 mg/ml glucose medium again. At specified time intervals, 2 ml of this solution was taken out and assayed by an Alpha-1860 UV spectroscopy (China) at the wavelength of 214 nm. The amount of BSA released from the testing hydrogels was then calculated from the standard BSA calibration curve. Samples in triplicate were averaged for each experiment.

The cumulative release (%) was determined using Eq. (6):

$$\text{Cumulative release (\%)} = \frac{V_e \sum_{i=1}^{n-1} C_i + V_o C_n}{W_o} \times 100\% \qquad (6)$$

where V_e was the replaced volume of PBS buffer (2 ml), V_o was the total volume of PBS buffer (10 ml), C_i and C_n were the drug release concentration (mg/ml) at different time, and W_o was the amount of the drug loaded onto the hydrogels (mg).

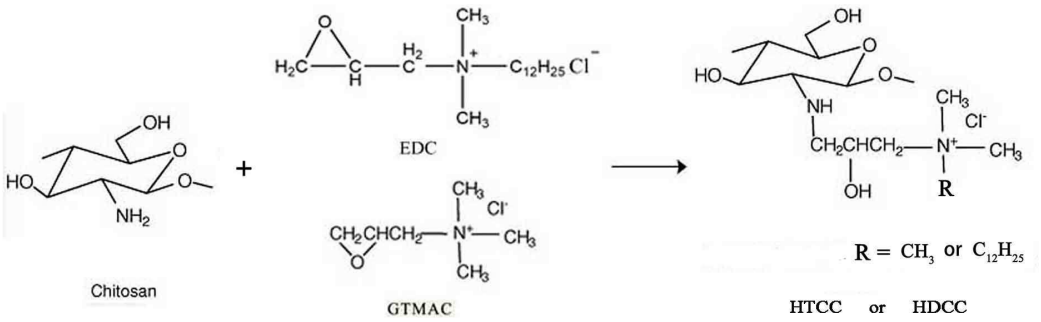
2.5. Statistical analysis

The quantitative results were obtained from triplicate samples and the data was expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way analysis of variance, followed by post hoc Student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

3 Results and discussion

3.1 Characterization of HDCC synthesized with different molar ratio of GTMAC/EDC

As illustrated in Scheme 1, the chitosan were modified by etherification with GTMAC/EDC as etherifying agents in water/isopropanol environment (pH 6.5). In an acidic or neutral environment, the epoxy groups prevalently react with the amine groups of the chitosan backbone, whereas under an alkaline condition, conjugations predominantly occur to the hydroxyl groups of chitosan. [20] In the present study, GTMAC and EDC were mostly grafted onto the amine groups of chitosan in acidic water/isopropanol media.



Scheme 1 Synthesis scheme of HTCC or HDCC

The FTIR spectra of chitosan and the modified chitosan were shown in Fig.2. The broad band at around 3432 cm^{-1} was attributed to N–H, O–H stretching vibration, and inter-and intra-molecular hydrogen bond of chitosan molecules. The weak band at 2921 cm^{-1} was ascribed to –CH– stretching of chitosan. The characteristic peak at 1600 cm^{-1} was attributed to the $–NH_2$ bands of chitosan. The peaks matched with

saccharide backbone were easily viewed at 1153 cm^{-1} (anti-symmetric stretching of the C–O–C).

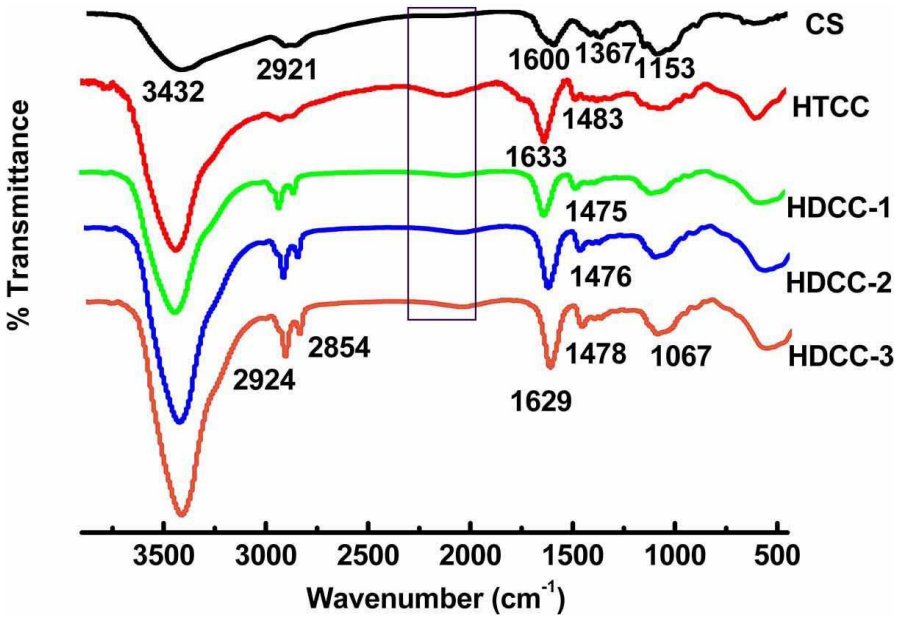


Fig.2. FTIR spectra of chitosan and the modified chitosan

In comparison with chitosan, several noticeable changes of absorption peaks occurred in the spectra of HTCC samples. The new peak at 1483cm^{-1} of HTCC corresponded to the C–H bending of trimethylammonium group, confirming the existence of the quaternary ammonium salt. [21-23] It should be also noted that the peak corresponding to the primary amine (1600cm^{-1}) of chitosan disappeared and a new peak at around 1633cm^{-1} for HTCC was recorded, revealing the change of the primary amine to the secondary amine structure due to the reactions at $–NH_2$ sites on the chitosan backbones. A weak and broad band at 2000-2200 cm^{-1} was observed, potentially assigned to a combination of the asymmetrical $-N^+(CH_3)_3$ bending vibration and the torsional oscillation of the trimethylammonium group.

Compared with HTCC, the FTIR spectra of HDCC showed two new peaks at 2924 cm^{-1} and 2854cm^{-1}, which were attributed to the long alkyl chain, indicating the attachment of long chain alkyl groups onto amino groups of chitosan.

Fig.3 (A) showed the ^{1}H NMR spectra of chitosan and the modified chitosan at room temperature, respectively. It can be seen that NMR chemical shift of hydroxyl and amino hydrogen on chitosan chain disappeared because the active hydrogen was exchanged by D_2O. The protons in methenyl group (H-2) connected to amine group in chitosan were evidenced by the chemical shift at 3.18 ppm. The protons in methylene and methenyl group (H-6,4,3,5) on chitosan backbone could be found at 3.74-3.87ppm.

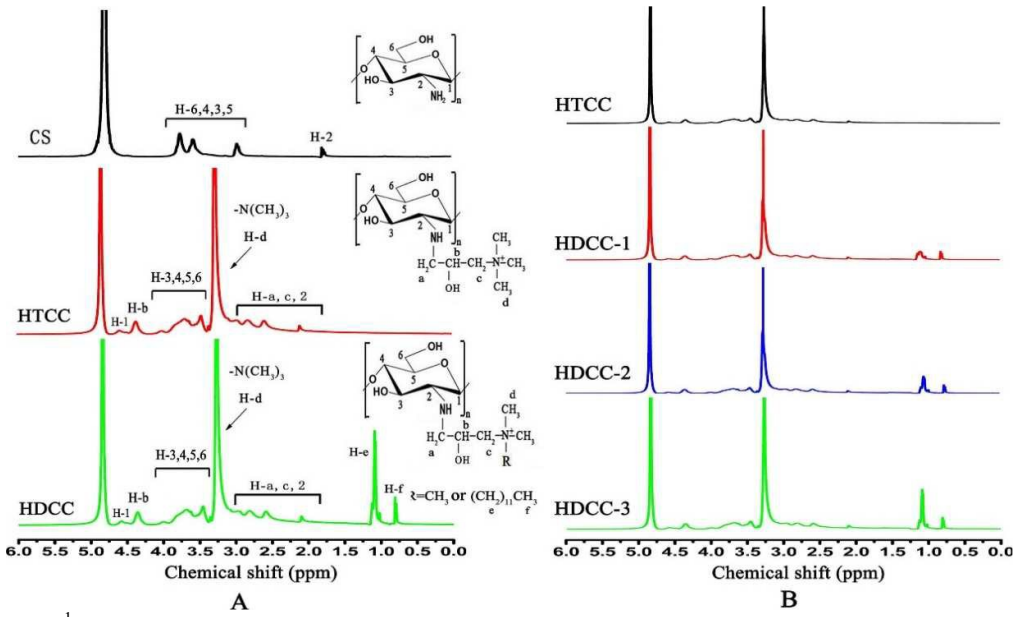


Fig.3. ^{1}H NMR spectra of chitosan and the modified chitosan samples: (A) Chitosan, HTCC and HDCC-3; (B) HTCC, HDCC-1, HDCC-2 and HDCC-3.

Different from chitosan, the spectra of HTCC showed a new characteristic chemical shift at 3.27 ppm attributed to the protons in $-N^+(CH_3)_3$ groups due to introduction of quaternary ammonium group. The proton in methenyl group (H-b) on the side chain of HTCC was exhibited at 4.58 ppm. The protons in methylene group (H-a and H-c) on the side chain of HTCC could be found at 2.84 and 3.35 ppm. The chemical shift at 4.35 ppm was attributed to the proton in hydroxyl groups on the side chain of HTCC.

In the ^{1}H NMR spectra of HDCC, a new strong signal at 1.09 ppm was assigned to the methylene groups sequence (-N-CH_2 (CH_2)$_{10}CH_3$) that was attached to the methyl group of dodecyl groups. The protons in methylene group (-N-CH_2 (CH_2)$_{10}CH_3$) adjacent to the secondary amino groups could be found at 2.94 to 3.07 ppm. The methyl group of long alkyl chain (-N-(CH_2)$_{11}$-CH_3) showed a shift at 0.78 ppm. As seen in Fig.3 (B), the integral areas of the methyl groups in long alkyl chain increased with increasing molar ratio of EDC/GTMAC and total amount of cationic etherifying agents.

The degree of substitution of quaternary ammonium groups (DS_Q) and the degree of substitution of long chain alkyl cationic groups (DS_A) of modified chitosan were further determined. The number of the protons in methenyl (H-2) on the backbone of chitosan chain was a constant before or after grafting reaction, and it could be used as the internal standard. After grafting, the DS_Q and DS_A could be calculated from the integral curve area of the protons in $-N^+(CH_3)_3$ groups and protons in grafted alkyl chain. The DS_Q and DS_A of the modified chitosan samples were calculated with Eq. (7) and Eq. (8):

$$\%DS_Q = \frac{\frac{I_{N^+(CH_3)_3}}{9} + \frac{I_{N^+(CH_3)_2(CH_2)_{11}CH_3}}{6}}{I_{H-2}} \times 100\% \qquad (7)$$

$$\%DS_A = \frac{I_{(CH_2)_{11}/22}}{I_{H-2}} \times 100\% \qquad (8)$$

Where $\frac{I_{N^+(CH_3)_3}}{9} + \frac{I_{N^+(CH_3)_2(CH_2)_{11}CH_3}}{6}$, $I_{(CH_2)_{11}/22}$ and I_{H-2} represented integral area of the signal of protons in -N $(CH_3)_3^+$ groups and $-N^+(CH_3)_2(CH_2)_{11}CH_3$ groups, the protons of grafted long chain alkyl groups and the protons in methenyl

(H-2) on the backbone of chitosan chain.

The results were presented in Table 1. It was concluded that for the modified chitosan hydrogels samples HTCC, HDCC-1 and HDCC-2 with molar ratio of chitosan/etherifying agents was 1:5, the DS_Q values of HDCC decreased while the DS_A values increased with decreasing GTMAC / EDC ratio. It could be interpreted that introduction of EDC with relatively larger steric hindrance effect among the long alkyl chains resulted in the lower activity of the reaction system. As for HDCC-3 with 1:7 molar ratio of chitosan/etherifying agents, higher values of DS_Q and DS_A can be obtained due to the increase of total amount of etherifying agents.

Table 1 The DS_Q and DS_A of the modified chitosan samples synthesized with different molar ratio of GTMAC/EDC

Samples	GTMAC / EDC ratio	DS_Q%	DS_A%
HTCC	5:0	197	--
HDCC-1	4:1	173	12
HDCC-2	3:2	167	27
HDCC-3	5:2	179	38

Fig.4 exhibited the solubility of the chitosan and the modified chitosan samples at different pH values. The transmittance of the chitosan significantly decreased when pH was above 6, however, HTCC and HDCC samples all exhibited higher transmittance value (about 90%) over a wide pH range. Introduction of large amount of cationic groups made chitosan become more hydrophilic, and the steric hindrance effect of quaternary amino groups weakened the inter- and intra-molecular hydrogen bonding, resulting in the great improvement of the water solubility of chitosan. On the other hand, the water solubility of HDCC was slightly lower than that of HTCC due to the hydrophobicity of long chain alkyl groups.

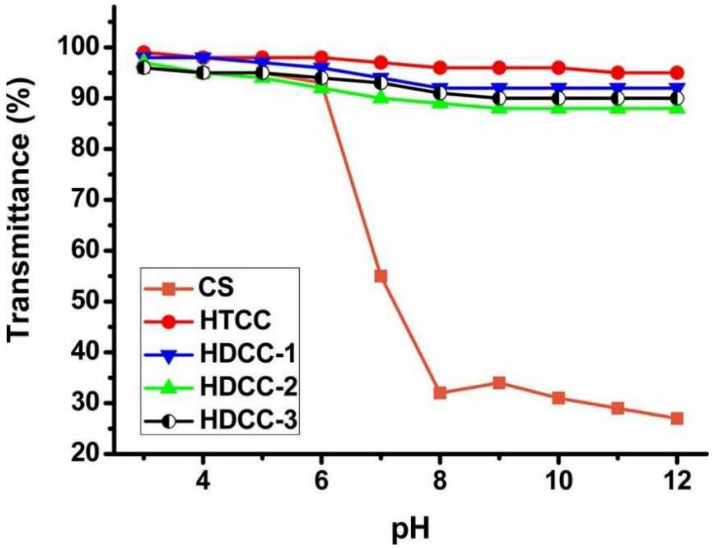


Fig.4. The UV transmittance of different chitosan samples at the wavelength of 600 nm

To better understand the pH-sensitive behavior of chitosan and the modified chitosan in aqueous solution, the acid–base titration curve of the hydrogels was presented macroscopically in Fig.5. In contrast, pure water, chitosan and modified chitosan were tested simultaneously with a concentration of 1 mg/ml. As shown in Fig.5, compared with pure water and chitosan, the modified chitosan both showed an obvious pH buffering path which ranged from pH 5.3 to pH 7.6, and the buffering path of HDCC samples was wider than that of HTCC. This was due to the protonation of the trimethyl ammonium chloride groups and physical entanglement of long chain alkyl groups for HDCC, revealing that HDCC were more pH-sensitive than HTCC.

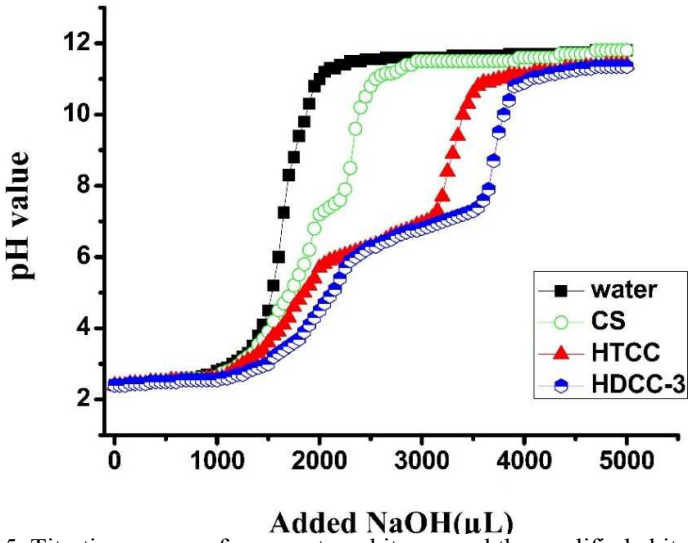


Fig.5. Titration curves of pure water, chitosan and the modified chitosan

3.2 Network structure of HDCC hydrogels synthesized with different molar ratio of GTMAC/EDC

The HTCC and HDCC were then cross-linked by TPP to form hydrogels. Frequency sweep tests are widely used to obtain information about the stability of three dimensional cross-linked networks. The frequency dependence of both storage modulus (G') and loss modulus (G") was plotted in Fig.6 for HTCC and HDCC hydrogels. Both figures showed typical gel spectra, with only very slight frequency dependence of the modulus, which could be asserted that the modulus would have a stable value even at low frequencies. The modulus of HDCC hydrogels increased as the DS_A increased, which indicated that more alkyl group substitution resulted in more compact and stable hydrogels.

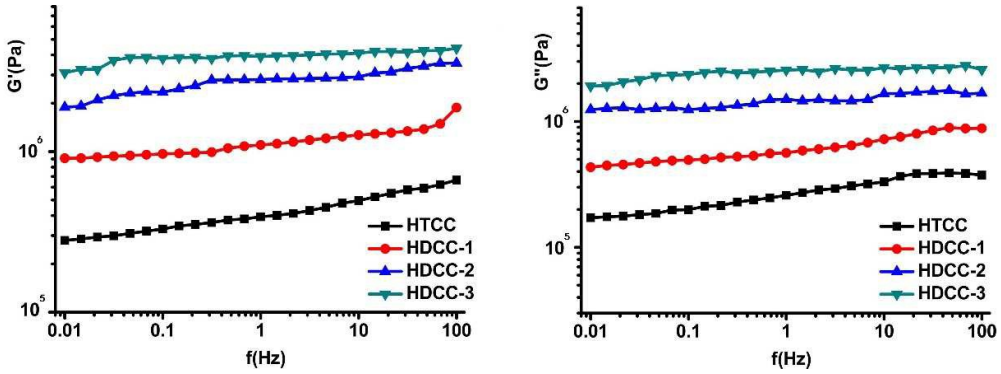


Fig.6. Variations with frequency of the shear modulus (G' and G") of the modified chitosan hydrogels

In the hypothesis that the hydrogels may be regarded as a classic network, in spite of the fact that they presented a complex hierarchical structure, the storage modulus (G') determined at low frequency may be related to the average number of equivalent units in a "network strand", N, connecting two "ideal" junctions and to the approximate, the value of the network mesh size L_c was calculated with Eq. (8) and Eq. (9). [19] In the meantime, crosslinking density (X) of hydrogels can be calculated with Eq. (10).

$$G' = RT\Phi^{1/3}/N_{Av}\, a^3\, N \qquad (8)$$

$$L_c = (\Phi)^{-1/3}(C_\infty N)^{1/2} a \qquad (9)$$

$$X = G'Q^{1/3}/RT \qquad (10)$$

In Eq. (8) N_{Av} is Avogadro number, G' is the shear modulus of modified chitosan hydrogels samples, R is the gas constant, T is the absolute temperature, Φ is the polymer volume fractions of gel in the swollen state, $N_{Av}a^3$ is the molar volume of the solvent, N is the average number of equivalent units with volume equal to the solvent volume (a^3), comprised between two junctions (in a "network strand"), Q is the

equilibrium swelling ratios of modified chitosan hydrogels. In Eq. (9) C_∞ is the characteristic ratio of modified chitosan in relation to the molecular weight.

Table 2 The network parameters of the modified chitosan hydrogels synthesized with different molar ratio of GTMAC/EDC

Samples	G'(Pa), Storage modulus	N, Average number of equivalent units	X(mol /cm^3), Crosslinking density	Lc (nm), Average length of network strands
HTCC	393000	28.578	0.1203	10.721
HDCC-1	1100000	9.382	0.3095	4.676
HDCC-2	2810000	3.234	0.6962	2.163
HDCC-3	3902400	2.218	0.9210	1.598

As shown in Table 2, the values of L_c thus obtained were in the range of 1.598-10.678 nm. With increasing DS_A, G' increased, L_c decreased and the crosslinking density of HDCC hydrogels increased, due to the enhancement of the physical entanglement interactions among the long chain alkyl groups of HDCC hydrogels, resulting in the formation of more compact structure which made the hydrogel network shrink.

3.3 Swelling behavior and pH-sensitive drug release of HDCC hydrogels synthesized with different molar ratio of GTMAC/EDC

The swelling ratios of modified chitosan hydrogels by being immersed in buffer solutions (pH6.8) versus time were plotted in Fig.7 (A). It can be seen that in the initial swelling stage, all samples absorbed water rapidly. The swelling ratio increased gradually with time, and reached equilibrium in about 12h. Besides, the swelling ratios of HDCC hydrogels decreased with increasing DS_A. This decrease can be attributed to the higher crosslinking density and more compact structure of HDCC hydrogels.

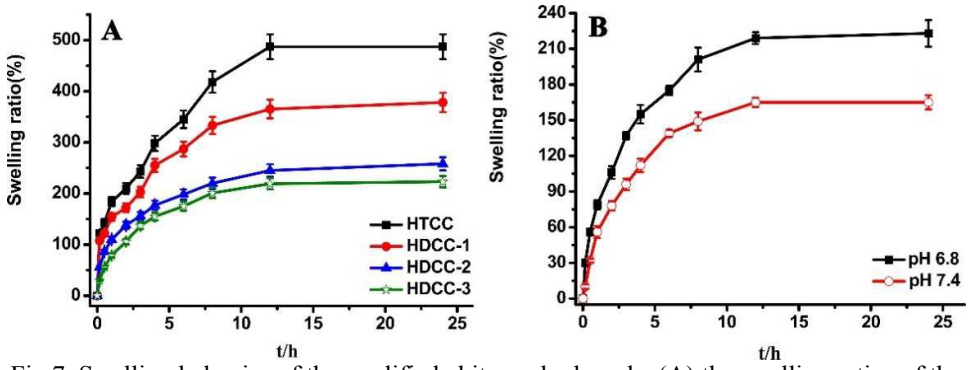


Fig.7. Swelling behavior of the modified chitosan hydrogels: (A) the swelling ratios of the modified chitosan hydrogels by being immersed in buffer solutions (pH6.8) (B) the swelling ratios of HDCC-3 hydrogels in different pH conditions at 37◦C. Data points represent mean ± SD (n= 3)

As can be seen from Fig.7 (B), the swelling ratio of HDCC-3 hydrogels exhibited higher value at acidic pH (pH6.8) than that at basic pH (pH7.4), which was attributed to the HDCC chains bearing positive charges. The expansive network allowed more acid solution to enter the interior. The results showed that HDCC hydrogels had good pH sensitivity.

In order to investigate the effect of pH value of the external medium on the drug release behavior of BSA-loaded modified chitosan hydrogels samples, cumulative release amount of BSA in PBS buffer solution with pH 6.8 and 7.4 was measured.

Cumulative release data (as shown in Fig.8) indicated that by increasing pH value from 6.8 to 7.4, a considerable decrease in the cumulative release was observed for all hydrogels. It suggested that the drug release profiles of all modified chitosan hydrogels were pH-sensitive. Besides, pH-sensitivity of modified chitosan hydrogels increased with the increase of DS_A. Noticeably, when the DS_A of HDCC was 38% (HDCC-3), there was a significant difference between BSA release profiles in the medium of pH 6.8 and 7.4, especially at the first 24h.

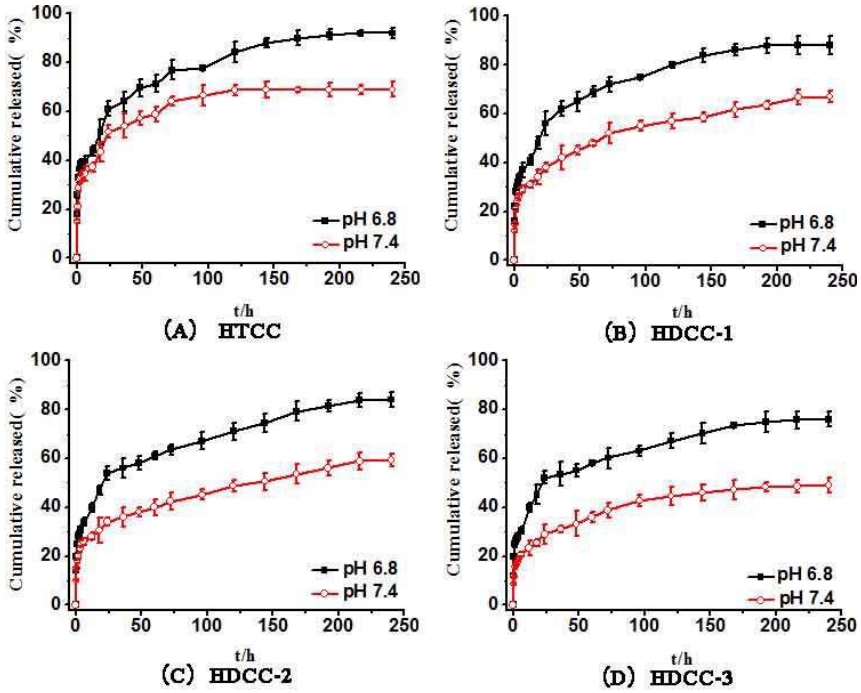


Fig.8. PH responsive release behavior of the modified chitosan hydrogels in different pH conditions (pH6.8 and 7.4) at 37°C. Data points represent mean ± SD (n= 3)

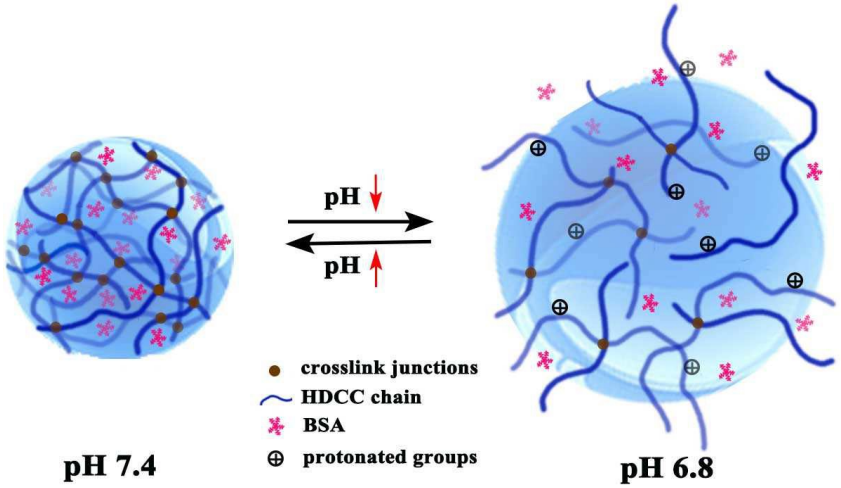


Fig.9. Schematic mechanism of pH-responsive release behavior of the modified chitosan hydrogels

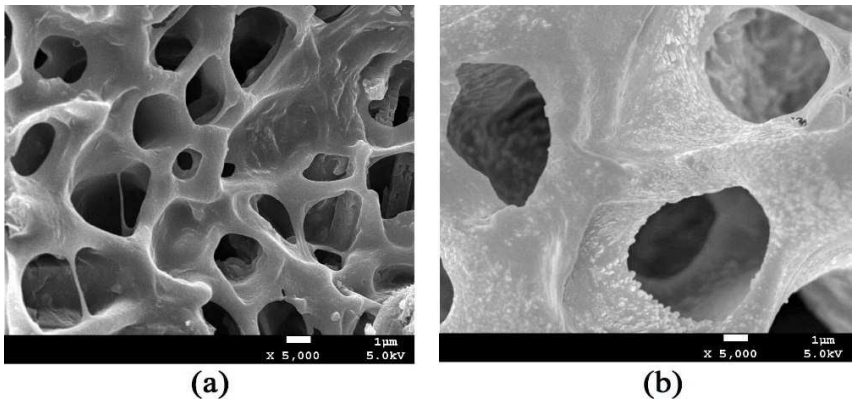


Fig.10. SEM photographs (×5000) of HDCC-3 hydrogels after immersion in PBS buffer solution (pH 7.4 or pH 6.8 37^{o}C) for 1 h. (a) HDCC hydrogel at pH 7.4; (b) HDCC hydrogel at pH 6.8

The schematic mechanism of pH-responsive release behavior of modified chitosan hydrogels was shown in Fig.9. It should be attributed to a different hydrolysis degree in PBS buffer solution with pH 6.8 and pH 7.4 because the acidic environment could promote the hydrolysis of the cationic groups of hydrogels. At physiological condition (pH7.4), the weak degree of ionization along with the less positive-charged amine groups of modified chitosan and more hydrogen bonds resulted in a relatively dense network structure with pore size 0.5-2μm (Fig.10(a)) and restricted the BSA release from its carrier. In contrast, the amine groups on the chitosan and trimethyl ammonium chloride groups of modified chitosan became protonated, forming the hydrophilic $-NH_3^+$ groups and $-N^+(CH_3)_3$ groups under subacid environment (pH 6.8). The resulting electrostatic repulsion between the protonated cationic groups weakened the intermolecular and intramolecular hydrogen bonding interaction of chitosan molecules, and the obvious loose network structure with pore size 4-6μm (Fig.10(b)) can be observed, as a result, the PBS buffer solution can diffuse into modified chitosan network easily which would facilitate the swelling and drug release. Moreover, the introduction of long chain alkyl groups slowed down the initial burst release rate (especially in pH 7.4) of the chitosan hydrogels, and better pH sensitive release behavior can be observed.

Encapsulation efficiency (EE) and drug loading capacity (LC) are two critical characteristics for evaluating the capacity of a selected polymer to entrap and carry a selected drug. EE and LC of drug may be altered by several factors such as the chemical structure of the polymers and drug, and the interactions between the polymers and drug. In the present study, DS_A of HDCC hydrogels was investigated for its possible influence on the EE and LC by entrapping BSA.

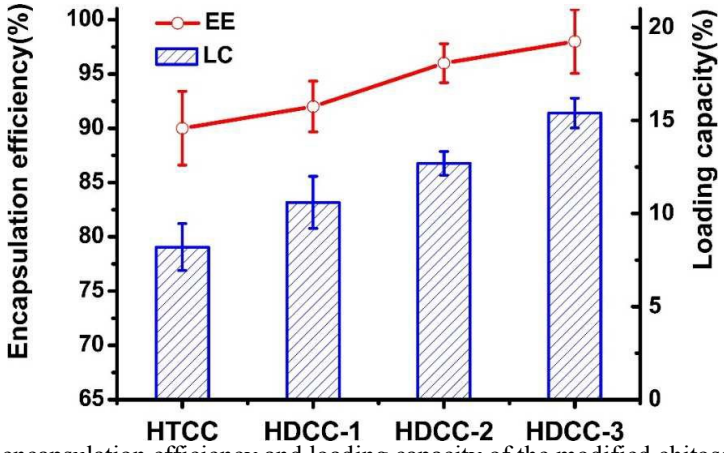


Fig.11. BSA encapsulation efficiency and loading capacity of the modified chitosan hydrogels. Data points represent mean ± SD (n= 3)

As revealed in Fig.11, the BSA loading capacity for HTCC and HDCC hydrogels samples was 8.2%, 10.6%, 12.7% and 15.4%, respectively. The BSA encapsulation efficiency for HTCC and HDCC hydrogels samples was 90.1%, 92.3%, 96.5% and 98.4%, respectively. The EE and LC of modified chitosan hydrogels increased with increasing DS_A, which suggesting that the interactions between the long alkyl chains of HDCC hydrogels and BSA played an important role in determining the encapsulation efficiency and drug loading capacity.

3.4 Glucose-responsive drug release behavior of HDCC hydrogels

With respect to the in vitro release behavior of polysaccharides delivery system, the release rate is highly affected by the nature of the interactive forces between the

drug and hydrogel, as well as by the characteristics of the release medium. PH-sensitive modified chitosan hydrogels entrapped with GOD may be used as glucose responsive drug release system. As shown in Fig.12 (A), the conversion of glucose to gluconic acid through GOD resulted in a decrease of pH value of PBS buffer solution from 7.4 to 6.8.

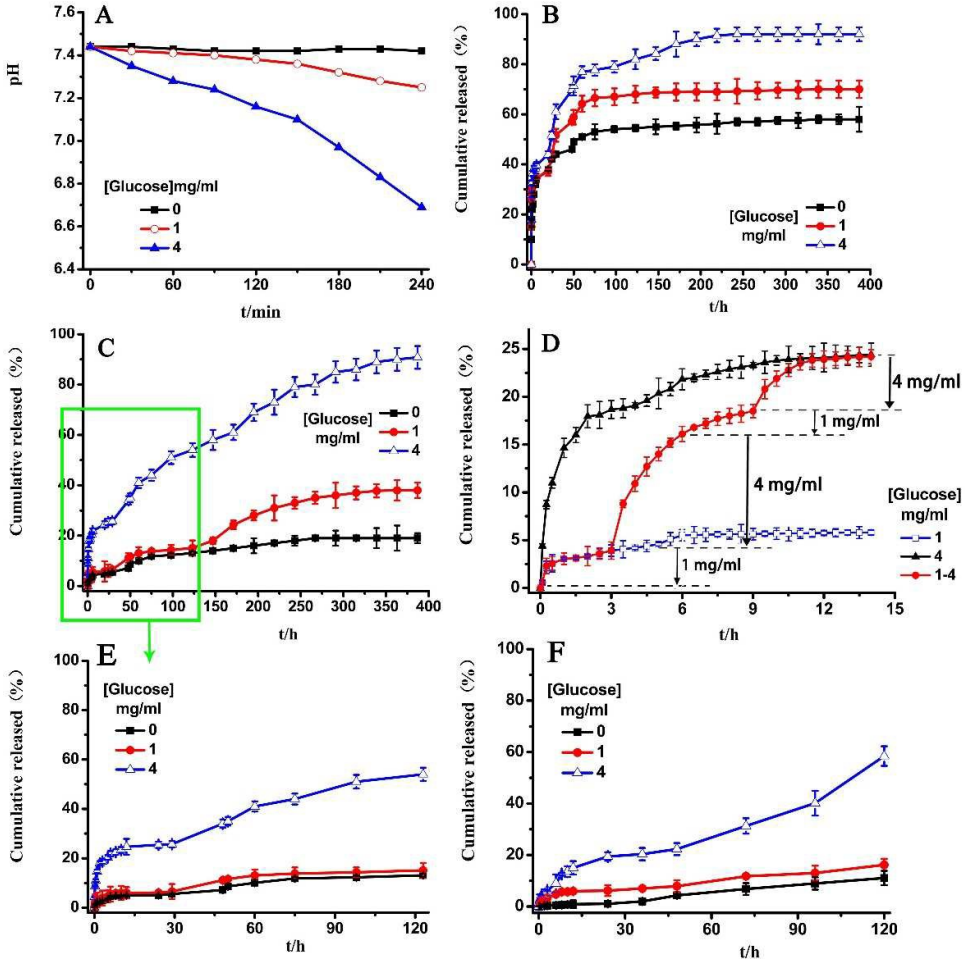


Fig.12. (A) Relevant pH changes in different incubation solutions. (B) Glucose responsive release behavior of HTCC hydrogels under different glucose concentrations: 0, 1, and 4 mg/ml at 37°C. (C)Glucose responsive release behavior of HDCC-3 hydrogels under different glucose concentrations: 0, 1, and 4 mg/ml at 37°C. (D) Glucose responsive release behavior of BSA-loaded HDCC-3 hydrogels under alternant glucose concentrations at pH 7.4, 37°C. The marker of 1-4 represented the cumulative BSA release in PBS with alternant glucose concentrations 1 or 4 mg/ ml. (E) Glucose responsive release behavior of BSA-loaded HDCC-3 hydrogels for 120h under different glucose concentrations: 0, 1, and 4 mg/ml at 37°C. (F) Glucose responsive release behavior of insulin-loaded HDCC-3 hydrogels for 120h under different glucose concentrations: 0, 1, and 4 mg/ml at 37°C. Data points represent mean ± SD (n= 3)

Fig.12 (B) and (C) showed the cumulative release profiles of BSA from HTCC and HDCC-3 hydrogels in response to different concentrations of glucose at pH 7.4, 37^{o}C. The results indicated a burst release behavior for HTCC hydrogels with different glucose concentrations during the initial 10h. After initial release, the release rate slowed down. However, it was observed that a much slower release rate was obtained when HTCC hydrogels were exposed to the basal glucose levels (1mg/ml) and control solutions (0mg/ml) than that at hyperglycemic glucose levels (4mg/mL). These results were consistent with the observed pH response. (Fig.7)

As seen in Fig.12(C), the glucose-sensitivity of HDCC hydrogels was more distinct than that of HTCC hydrogels. Moreover, Compared with HTCC hydrogels, HDCC-3 hydrogels exhibited a low initial burst and a slow release rate. This could be attributed to the strong physical entanglement interactions between the long alkyl chains of HDCC-3 hydrogels and BSA, and the structure of BSA-loaded HDCC hydrogels was more compact and dissociated more slowly. A hydrophobic barrier limited the access of water and dissolution of the drug.

The alternant release ability is considered to be one of the most important requisites for potential clinical used glucose-responsive delivery system. In the present work, the alternant drug release from BSA-loaded HDCC-3 hydrogel was conducted in PBS buffer solution containing 1 or 4 mg/ml glucose at intervals of 3 h (Fig.12 (D)). The BSA-loaded HDCC-3 hydrogel was first placed in PBS buffer solution with 1mg/ml glucose. The release of BSA was slow and only 3.9% of BSA was released in 3 h. After that, the culture medium was changed to PBS with 4 mg/ml

glucose, then, obvious BSA release (about 12.2%) was observed for the subsequent 3 h based on the glucose-sensitive release mechanism. When the release media was switched back to PBS with 1 mg/ml glucose, BSA release was interrupted and only 2.4% release amount was released in the following 3 h. However, the release behavior could be recovered as the release medium was changed from PBS with 1 mg/ml glucose to PBS with 4 mg/ml glucose again, demonstrated by release of 5.4% BSA in the 3 h interval. Therefore, the glucose-sensitive HDCC hydrogel that underwent glucose-switchable release was expected to be a promising therapy approach for diabetes mellitus.

In order to demonstrate the glucose responsive release behavior of insulin-loaded HDCC-3 hydrogels, the cumulative release data of insulin from HDCC-3 hydrogels in response to different concentrations of glucose (0, 1, and 4 mg/ml, at pH 7.4, 37^{o}C) was tested. The results showed that the release behaviors tendency of insulin was consistent with BSA during the first 120h. As shown in Fig.12 (E) and (F), insulin was released slower than BSA under the same glucose concentrations, especially at the first 10h. For example, under glucose concentration of 4 mg/ml, only 13.53% of insulin was released from the hydrogels, while 23.35% of BSA was released. It can be attributed to the larger molecule and slower diffusion rate of insulin, resulting in the enhancement of the physical entanglement interactions between the long chain alkyl groups of HDCC hydrogels and insulin.

3.5 In vitro release kinetic of HDCC hydrogels

To determine the drug release mechanism and compare the release profiles, the amount of drug release versus time was studied with the following Korsmeyer-Peppas models:

$$\ln M_t/M_\infty = n \ln t + \ln k$$

where Mt/M∞ is the fractional active agents release at time t; k is a kinetic constant incorporating the structural characteristics of the matrix; n is the release exponent, indicative of the drug release mechanism. For Korsmeyer-Peppas model, *n* gives an indication of the release mechanism. In case of Fickian release, *n* has the limiting values of 0.43. For case II, transport or relaxation controlled delivery, the exponent *n* is 0.85. [27-28] The non-Fickian release or anomalous transport of drug occurred when the *n* values are between 0.43 and 0.85, which corresponds with coupled diffusion/polymer relaxation.

Drug release kinetic data for BSA-loaded modified chitosan hydrogels in PBS buffer solution with pH 6.8 and 7.4 obtained from fitting drug release experimental data to the Korsmeyer–Peppas equation were summarized in Table 3. The exponent *n* values for the release of BSA from modified chitosan hydrogels were all less than 0.43, suggesting a Fickian release behavior and the diffusion through the swelling of modified chitosan hydrogels was the main factor in controlling BSA release. According to the correlation coefficient (r^2), the release data fitted well to the exponential model when the surface effect was excluded. From Table 3, it could be found that the constant *k* of hydrogels decreased with increasing DS_A in the same pH, corresponding to the enhanced physical entanglements interaction by long chain alkyl groups and compact network structure with high crosslinking density. Furthermore, Δ*k* (the difference between constant *k* in pH 6.8 and pH 7.4) of the modified chitosan

hydrogels increased with increasing DS_A, which turned out that HDCC-3 were more distinct pH-sensitive than HTCC.

Table 3 Drug release kinetic data for BSA-loaded modified chitosan hydrogels obtained from fitting drug release experimental data to the Korsmeyer–Peppas equation

Samples	pH	Korsmeyer-Peppas model			
		Correlation Coefficient, r^2	Diffusion Exponent, n	Kinetic Constant, k	Δk
HTCC	6.8	0.9763	0.2179	28.9960	3.8484
	7.4	0.9738	0.2085	25.1476	
HDCC-1	6.8	0.9880	0.2277	25.2636	5.2875
	7.4	0.9843	0.1924	19.9761	
HDCC-2	6.8	0.9896	0.2474	22.8535	5.3013
	7.4	0.9901	0.2019	17.5522	
HDCC-3	6.8	0.9808	0.2518	21.5604	7.6949
	7.4	0.9939	0.2236	13.8655	

Note: Δk is the difference between constant k in pH 6.8 and pH 7.4.

Drug release kinetic data for BSA-loaded modified chitosan hydrogels under different glucose concentrations were shown in Table 4. A good correlation coefficient (r^2) approaching 0.98 was obtained in all cases. It was shown that the exponent n values for the release of BSA from HTCC hydrogels under different glucose concentrations were all less than 0.43, suggesting a Fickian release behavior. The exponent n values for the release of BSA from HDCC-3 hydrogels also gave an indication of Fickian diffusion under 0 mg/ml and 4 mg/ml glucose concentrations, and Fickian release behavior and polymer chain relaxation under 1 mg/ml glucose concentrations. Moreover, the constant k of HDCC-3 hydrogels was much lower than that of HTCC hydrogels under different glucose concentrations, indicating that HDCC-3 hydrogels may achieve better slow-release effect.

Table 4 Drug release kinetic data for BSA-loaded modified chitosan hydrogels in different glucose concentration obtained from fitting drug release experimental data to the Korsmeyer–Peppas equation

Samples	Glucose Concentration (mg/ml)	Korsmeyer-Peppas model			Transport Mechanism
		Correlation Coefficient ,r^2	Diffusion Exponent ,n	Kinetic Constant ,k	

HTCC	0	0.9814	0.2445	9.5780	Fickian diffusion
	1	0.9517	0.1581	29.3573	Fickian diffusion
	4	0.9732	0.1881	32.0494	Fickian diffusion
HDCC-3	0	0.9846	0.4178	1.7218	Fickian diffusion
	1	0.9769	0.6422	0.8939	Fickian diffusion /polymer chain relaxation
	4	0.9821	0.4149	7.7292	Fickian diffusion

4 Conclusions

In this work, glucose-responsive controlled drug release system based on HDCC hydrogel with long chain alkyl groups was synthesized, and its composition was analyzed by FTIR and ^{1}H NMR analysis. Introduction of cationic groups resulted in the improvement of water solubility of HDCC, and introduction of long chain alkyl groups resulted in a wider pH buffering path. With increasing $DS_{A,}$ the modulus and crosslinking density of HDCC hydrogel increased, while the swelling ratios decreased, which indicated that more alkyl group substitution resulted in more compact and stable hydrogels. A considerable increase in the cumulative release of BSA was observed by decreasing pH value from 7.4 to 6.8 because the acidic environment could promote the hydrolysis of the cationic groups of hydrogels and pH-sensitivity of the hydrogels increased with the increase of DS_A. The encapsulation efficiency and drug loading capacity of HDCC hydrogels increased with increasing DS_A, which suggesting the enhancement of interactions between the long alkyl chains and BSA. The release profiles revealed that BSA and insulin release from HDCC hydrogels was in response to the glucose concentration and the alternant glucose-switchable release ability was observed. Drug release kinetic data under different pH values and glucose concentration for BSA-loaded hydrogels almost presented a Fickian release behavior and HDCC hydrogels were more distinct pH-and glucose-sensitive than HTCC hydrogels and it can achieve better slow-release effect.

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Figure captions

Fig.1 Schematic representation of pH-responsive hydrogel with entrapped GOD

Scheme 1 Synthesis scheme of HTCC or HDCC

Fig.2 FTIR spectra of chitosan and the modified chitosan

Fig.3 ^{1}H NMR spectra of chitosan and the modified chitosan samples: (A) Chitosan, HTCC and HDCC-3; (B) HTCC, HDCC-1, HDCC-2 and HDCC-3.

Fig.4 The UV transmittance of different chitosan samples at the wavelength of 600 nm

Fig.5 Titration curves of pure water, chitosan and the modified chitosan

Fig.6 Variations with frequency of the modulus (G' and G") of the modified chitosan hydrogels

Fig.7 Swelling behavior of the modified chitosan hydrogels: (A) the swelling ratios of the modified chitosan hydrogels by being immersed in buffer solutions (pH6.8) (B) the swelling ratios of HDCC-3 hydrogels in different pH conditions at 37^{o}C. Data points represent mean ± SD (n= 3)

Fig.8 PH responsive release behavior of the modified chitosan hydrogels in different pH conditions (pH6.8 and 7.4) at 37^{o}C. Data points represent mean ± SD (n= 3)

Fig.9 Schematic mechanism of pH-responsive release behavior of the modified chitosan hydrogels

Fig.10 SEM photographs (×5000) of HDCC-3 hydrogels after immersion in PBS buffer solution (pH 7.4 or pH 6.8 37^{o}C) for 1 h. (a) HDCC hydrogel at pH 7.4; (b) HDCC hydrogel at pH 6.8

Fig.11 BSA encapsulation efficiency and loading capacity of the modified chitosan hydrogels. Data points represent mean ± SD (n= 3)

Fig.12 (A) Relevant pH changes in different incubation solutions. (B) Glucose responsive release behavior of HTCC hydrogels under different glucose concentrations: 0, 1, and 4 mg/ml at 37^{o}C. (C) Glucose responsive release behavior of HDCC-3 hydrogels under different glucose concentrations: 0, 1, and 4 mg/ml at 37^{o}C. (D) Glucose responsive release behavior of BSA-loaded HDCC-3 hydrogels under alternant glucose concentrations at pH 7.4, 37^{o}C. The marker of 1-4 represented the cumulative BSA release in PBS with alternant glucose concentrations 1 or 4 mg/ ml. (E) Glucose responsive release behavior of BSA-loaded HDCC-3 hydrogels for 120h under different glucose concentrations: 0, 1, and 4 mg/ml at 37^{o}C. (F) Glucose responsive release behavior of insulin-loaded HDCC-3 hydrogels for 120h under different glucose concentrations: 0, 1, and 4 mg/ml at 37^{o}C. Data points represent mean ± SD (n= 3)

Table captions

Table 1

The DS_Q and DS_A of the modified chitosan samples synthesized with different molar ratio of GTMAC/EDC

Table 2

The network parameters of the modified chitosan hydrogels synthesized with different molar ratio of GTMAC/EDC

Table 3

Drug release kinetic data for BSA-loaded modified chitosan hydrogels obtained from fitting drug release experimental data to the Korsmeyer–Peppas equation

Table 4

Drug release kinetic data for BSA-loaded modified chitosan hydrogels in different glucose concentration obtained from fitting drug release experimental data to the Korsmeyer–Peppas equation