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Stimuli-responsive materials prepared from carboxymethyl chitosan and poly(γ-glutamic acid) for protein delivery

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ABSTRACT

The development of stimuli-responsive materials in response to the molecules involved in biological processes has gained increased attentions. In this work, carboxymethyl chitosan (CM-chitosan) and poly(γ-glutamic acid) (pGlu) were reacted with a naturally occurring compound, genipin, leading to the formation of genipin-crosslinked CM-chitosan/pGlu conjugates with fluorescence emissions. The genipin-conjugated polymers were sensitive to the oxidation product of glucose, gluconic acid and hydrogen peroxide (H_2O_2). Fluorescence emissions of the polymers were quenched by gluconic acid and H_2O_2. An increase in the hydrodynamic diameter together with the quenching of fluorescence indicated that the genipin-conjugated polymers were self-aggregated into nanoparticles, in response to the stimulus of gluconic acid (but not for H_2O_2). Bovine serum albumin (BSA) could be loaded in the self-aggregated nanoparticles, and the incorporated BSA slowly released from the nanoparticles under hyper-gluconic acid conditions. This material is hence proposed as a stimuli-responsive material for optical sensing and protein delivery purposes.

1. Introduction

Genipin, the aglycone of geniposide extracted from gardenia fruits, has been shown to react with the primary amino groups in chitosan for preparing hydrogels and microspheres (Chen et al., 2004; Mi, Tan, Liang, & Sung, 2002). Genipin might replace glutaraldehyde, with the advantages of stability and safety, to cross-link chitosan for biomedical and pharmaceutical applications (Muzzarelli, 2009). Previous studies found that genipin could react with chitosan to create fluorescence emission (Mi, 2005). The fluorigenic properties of genipin cross-linked type I collagen gels have also been studied (Sundararaghavan et al., 2008).

Carboxymethyl chitosan (CM-chitosan) is an amphoteric chitosan possesses many attractive chemical and biological properties, in addition to its low toxicity, good biocompatibility and high solubility in water (Muzzarelli, 1988). All these features make it very promising for pharmaceutical and biomedical applications (Jayakumar et al., 2010). CM-chitosan-based nanoparticles were used for drug delivery (Anitha et al., 2011; Zheng et al., 2011), specific active targeting (Prabaharan & Gong, 2008), free radicals scavenging (Yu et al., 2011), and antimicrobial application (Anitha et al., 2009). Especially, CM-chitosan-modified quantum dots have been proven to be suitable for live cell imaging (Mathew et al., 2010).

Poly(γ-glutamic acid) (pGlu) is a polypeptide produced by some microorganisms such as *Bacillus subtilis*. Previous studies showed that CS/pGlu self-assembled nanoparticles facilitating gene, insulin and growth factor delivery (Mi et al., 2008; Peng et al., 2011; Tang et al., 2010) because pGlu enhanced the incorporation of biological molecules and intracellular trafficking. Therefore, a combination of pGlu with CM-chitosan is desired to improve the performance of CM-chitosan-based polymer materials for drug delivery.

Stimuli-responsive materials have been receiving much attention due to their potential applications in biomedicine, such as probing biomolecules or delivering drugs (Rapoport, 2007). In this work, genipin was reacted with CM-chitosan and pGlu to prepare genipin-crosslinked CM-chitosan/pGlu conjugates with stimulus-response properties. Self-aggregation and fluorescence quenching

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[1] The contributions by the two collaborating parties are equal.

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properties of the stimulus-responsive polymers, in response to the stimulus of hydrogen peroxide (H_2O_2) and gluconic acid, were respectively investigated by dynamic light scattering (DLS), turbidity and fluorescence spectral analysis. Bovine serum albumin (BSA) release properties of the self-aggregated nanoparticles, under hyper gluconic acid conditions were also studied.

2. Materials and methods

2.1. Materials

Genipin (MW is 226.3) was purchased from Challenge Bioproducts Co. (Taichung, Taiwan). pGlu (MW 60 kDa) was a product of Vedan Bioproducts Co. (Taichung, Taiwan). Chitosan (MW 60 kDa) with a degree of deacetylation of approximately 85% was acquired from Koyo Chemical Co. Ltd. (Japan). Gluconic acid and hydrogen peroxide were all purchased from Sigma–Aldrich Co. Ltd. (USA). All other reagents and solvents used were of reagent grade.

2.2. Synthesis and characterization of CM-chitosan

CM-chitosan was synthesized as per a procedure described in the literature (Chen et al., 2004). The obtained CM-chitosan used for the FT-IR analysis first was dried and ground into a powder form. The CM-chitosan powder then was mixed with KBr (1:100) and pressed into a disk. Analysis was performed on an FT-IR spectrometer (PerkinElmer Spectrum RX1 FT-IR System, Buckinghamshire, England). The sample was scanned from 400 to 4000 cm^{-1}.

2.3. Preparation of genipin-crosslinked CM-chitosan/pGlu conjugates

Genipin-crosslinked CM-chitosan/pGlu conjugate polymers were prepared as a procedure described in the literature with some modification (Mi, 2005). A pGlu aqueous solution (1 mg/mL in deionized water) was added into the CM-chitosan solution (1 mg/mL in deionized water), which contained 0.5 mg/mL of genipin, and was incubated at room temperature for the preparation of genipin-crosslinked CM-chitosan/pGlu conjugates. The CM-chitosan-to-pGlu weight ratios in the mixtures were 1:2, 1:1 and 2:1. The genipin-crosslinked, fluorescent CM-chitosan/pGlu conjugates (GPF_{CGs}) were respectively noted as GPF_{C1G2} (CM-chitosan/pGlu ratio = 1:2), GPF_{C1G1} (CM-chitosan/pGlu ratio = 1:1) and GPF_{C2G1} (CM-chitosan/pGlu ratio = 2:1). Specifically, CM-chitosan or pGlu (1 mg/mL in deionized water) alone was conjugated with genipin, and was noted as $GPF_{CM\text{-}chitosan}$ or GPF_{pGlu}.

2.4. Stimulus-response of genipin-crosslinked CM-chitosan/pGlu conjugates

Stimulus-responses were studied by examining the self-aggregation and fluorescence quenching properties of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs}, under various concentrations of gluconic acid or H_2O_2.

2.4.1. Self-aggregation of genipin-crosslinked CM-chitosan/pGlu conjugates

$GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions were prepared in dilution form as the aforementioned procedure. The gluconic acid was added into the solutions to examine the stimuli-responses of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs}. The concentrations of gluconic acid and H_2O_2 solutions added to the $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions were 1–10 mM and 1–40 mM, respectively. After the addition of gluconic acid or H_2O_2 solution, the turbidity of each sample was assayed by light transmittance measurement at a wavelength of 500 nm using a UV–vis spectrophotometer (Uvikon923, Kontron Instruments, Italy). The variation of the mean particle sizes, light scattering intensities and zeta potentials of nanoparticles were measured using a Zetasizer 3000HS (Malvern Instruments Ltd., Worcestershire, UK).

The morphology of the self-aggregated nanoparticles in response to different concentration of the gluconic acid solution (10, 25 and 50 mM) was studied by transmission electron microscopy (TEM). The TEM sample was prepared by placing a drop of the nanoparticle suspension onto a 400 mesh a carbon-coated copper grid. After deposition, the grid was tapped with a filter paper to remove surface water, followed by air-drying. The dried samples were observed by TEM (Hitachi H-600, Japan).

2.4.2. Fluorescence quenching of genipin-crosslinked CM-chitosan/pGlu conjugates

Fluorescence spectra of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions were recorded at room temperature on a fluorescence spectrophotometer (Hitachi, F-2500, Japan) equipped with a FL Solutions Program. The excitation and emission wavelengths were 375 nm and 454 nm, respectively. The gluconic acid and H_2O_2 were used to examine the emission quenching. The concentrations of gluconic acid and H_2O_2 solutions added to the $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions were 1–10 mM and 1–40 mM, respectively. After the addition of gluconic acid or H_2O_2, the fluorescence intensities of the solutions at maximum emission were determined. $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions respectively added by the same amount of deionized water were used as control groups. Fluorescence quenching was expressed by the formula $E_{eff} = F/F_0$, where E_{eff} is the efficiency of emission quenching, F denotes the fluorescence intensity after adding gluconic acid or H_2O_2 and F_0 is the intensity of fluorescence emission before adding the compounds.

2.5. Protein-loading capacity of self-aggregated nanoparticles

A sample of 100 mg of bovine serum albumin was dissolved in 1 mL of deionized water. The BSA stock solution (0.1 mL) was mixed with $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions (4.9 mL) under magnetic stirring at room temperature. Gluconic acid was added to the mixture (a final concentration of 10 mM) and the genipin-crosslinked CM-chitosan/pGlu conjugates were self-aggregated into nanoparticles for incorporating BSA. To determine the BSA loading efficiency, the nanoparticles were collected by ultracentrifugation at 12,000 rpm, 4 °C for 30 min and the BSA concentration in the supernatant was assayed by Brandford protein assay kit (Bio-Rad protein dye reagent; Bio-Rad) (Mi et al., 2003). The protein loading efficiency of the nanoparticles was determined as described in the literature (Lin et al., 2007).

2.6. Protein release from self-aggregated nanoparticles

The release profiles of BSA from the self-aggregated nanoparticles were investigated alternately in various gluconic acid-containing dissolution media (10, 25 and 50 mM) at 37 °C under agitation (100 rpm, DISTEK-2230A, North Brunswick, NJ). At particular time intervals, the samples were taken out and centrifuged and the supernatants were used for the protein analysis. The amount of BSA released was expressed as a percentage of the total BSA associated with the nanoparticles as calculated from the loading efficiency determined by the aforementioned method (Lin et al., 2007; Mi et al., 2003).

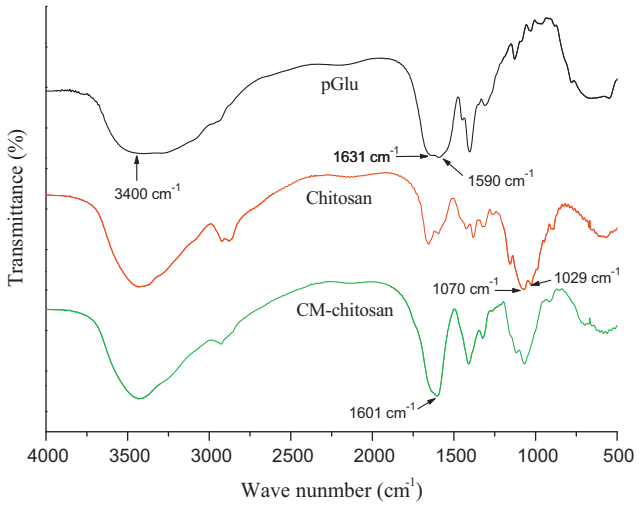

Fig. 1. FT-IR spectra of chitosan, CM-chitosan and pGlu.

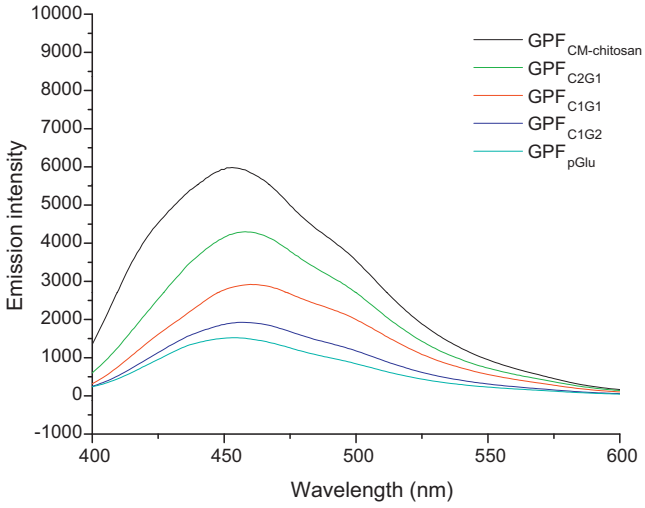

Fig. 2. Emission spectra of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions.

3. Results and discussion

3.1. Characterization of CM-chitosan and pGlu

Fig. 1 shows the FT-IR spectra of chitosan, CM-chitosan and pGlu. The spectra of chitosan displayed peaks around 905 cm^{-1} and 1153 cm^{-1} of assigned saccharine structure, and a strong amide characteristic peak at 1650 cm^{-1} as well as a characteristic peak assigned to protonated amine, at around 1570 cm^{-1}. The spectrum of CM-chitosan showed a characteristic peak (1601 cm^{-1}) for associated carboxylic acid salt, suggesting that there were carboxymethyl groups existing on CM-chitosan. pGlu showed a characteristic band at 1631 cm^{-1} for the associated carboxylic acid salt (–COO^- antisymmetric stretch). The characteristic absorption due to the –C=O in secondary amides (amide I band) was overlapped by the characteristic band of –COO^-. Additionally, the characteristic peak observed at 1590 cm^{-1} and 3400 cm^{-1} was the C–N and N–H stretch of amide II bands.

3.2. Fluorescent properties of genipin-crosslinked CM-chitosan/pGlu conjugates

The genipin-crosslinked CM-chitosan/pGlu conjugates exhibit special fluorescent properties. Fig. 2 shows the fluorescence emission spectra of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} where the excitation wavelength was 375 nm. $GPF_{CM\text{-}chitosan}$ exhibited a strong fluorescence emission with maximum intensity at around 454 nm. The emission intensity of GPF_{pGlu} was weak as compared with that of $GPF_{CM\text{-}chitosan}$ (maximum intensity at 454 nm). The emission intensity of GPF_{CGs} increased with increasing the CM-chitosan-to-pGlu weight ratio ($GPF_{C2G1} > GPF_{C1G1} > GPF_{C1G2}$).

3.3. Stimuli-response of genipin-crosslinked CM-chitosan/pGlu conjugates

Glucose-sensitive materials have gained increasing attention in biomedical research for sensing blood glucose or releasing insulin in response to the raised levels of blood glucose (Bajpai, Shukla, Bhanu, & Kankane, 2008). Glucose can be catalyzed by enzyme and oxidized into D-glucono-1,5-lactone, which then hydrolyzes to gluconic acid and H_2O_2. Therefore, the stimuli-responsive properties, such as fluorescence quenching and self-aggregation of the genipin-crosslinked CM-chitosan/pGlu conjugates, were preliminary studied in the presence of gluconic acid or H_2O_2, to examine the sensitivity of these materials to the glucose-oxidation products. Immobilization of glucose oxidase (GOD) in the genipin-conjugated polymers for glucose oxidation and their stimuli-responsive properties will be examined in future works, if the polymers are able to respond to gluconic acid or H_2O_2 in this work.

3.3.1. Effect of gluconic acid and H_2O_2 on self-aggregation

Self-aggregation of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} into nanoparticles in response to external stimuli under varied concentration of the glucose oxidized products, gluconic acid and H_2O_2, was examined by turbidity measurement (light transmittance). $GPF_{CM\text{-}chitosan}$ was self-aggregated into nanoparticles via the formation of hydrophilic–hydrophobic balanced regions because the protonated carboxyl groups of $GPF_{CM\text{-}chitosan}$ could form hydrogen bonds with its heterocyclic amines. As shown in Fig. 3, light transmittance of $GPF_{CM\text{-}chitosan}$ decreased after adding the gluconic acid solutions. Optically clear $GPF_{CM\text{-}chitosan}$ solutions abruptly become turbid after adding 10 mM of gluconic acid. The increased turbidity indicates the formation of micelles or nanoparticles in the $GPF_{CM\text{-}chitosan}$ solutions. Additionally, the hydrodynamic diameter increased with the increase of gluconic acid concentrations in

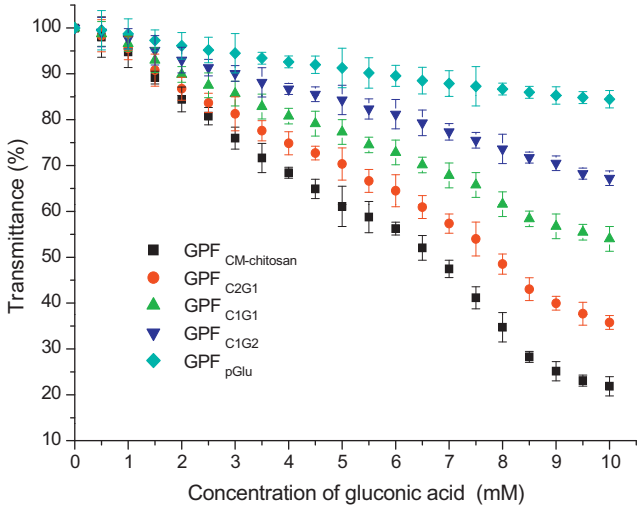

Fig. 3. Turbidity studies (light transmittance at λ = 500 nm) of the self-aggregated $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions after the addition of 1–10 mM gluconic acid.

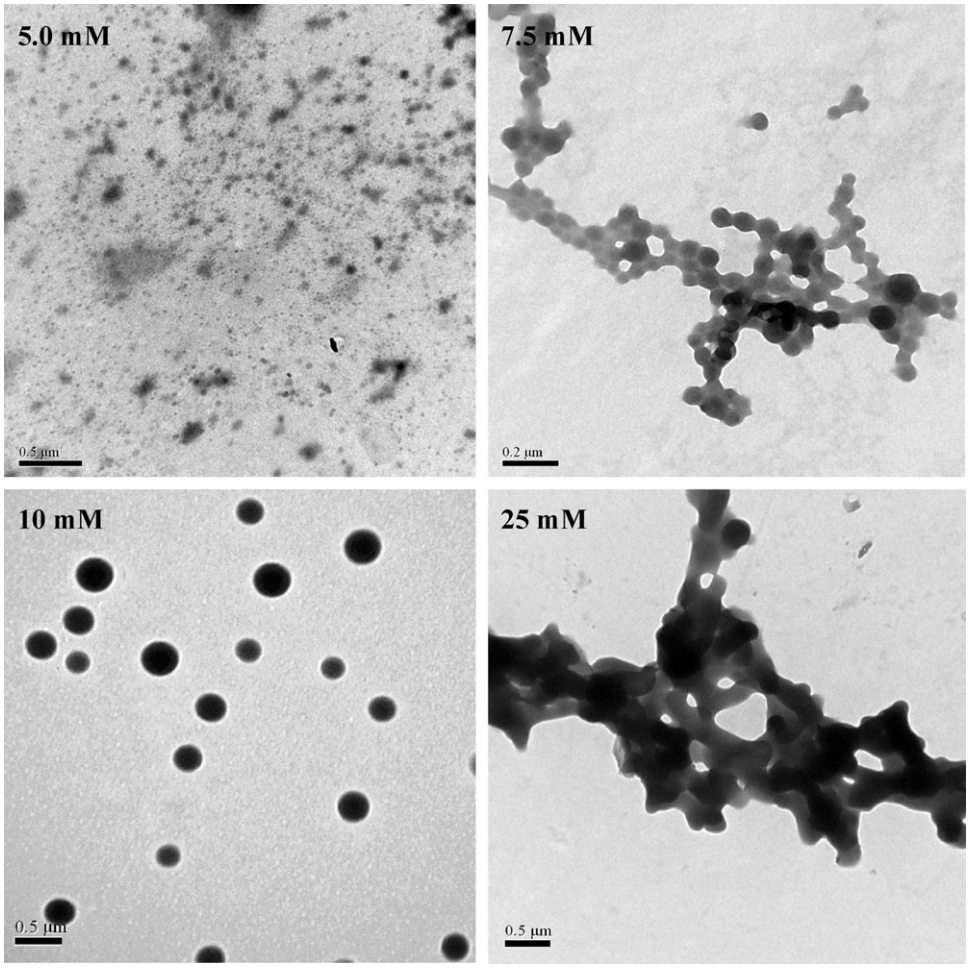

Fig. 4. TEM micrographs of the self-aggregated $GPF_{CM\text{-}chitosan}$ solutions after adding 5 mM, 7.5 mM, 10 mM and 25 mM gluconic acid.

the $GPF_{CM\text{-}chitosan}$ solution. (Table 1), suggesting the occurrence of self-aggregation. In contrast, H_2O_2 did not show obvious effect on the decrease of light transmittance of $GPF_{CM\text{-}chitosan}$ solutions. This result suggested that $GPF_{CM\text{-}chitosan}$ was not responsive to H_2O_2 for self-aggregation.

The mean particle sizes, zeta potentials, and light scattering intensity (indices of particle concentration) of the self-aggregated nanoparticles prepared by adding 10 mM of gluconic acid in the $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions are also shown in Table 1. The mean particle size, zeta potential and light scattering intensity (k_{counts}/s) of $GPF_{CM\text{-}chitosan}$ nanoparticles were 413.7 nm, −18.1 mV and 325.6, suggesting that $GPF_{CM\text{-}chitosan}$ was sensitive to gluconic acid and could be easily assembled into nanoparticles with negative charges on the surface. However, the mean particle size and light scattering intensity of GPF_{CGs} nanoparticles decreased with the increase of conjugated pGlu. GPF_{pGlu} seems to be insensitive to gluconic acid because the light scattering intensity was very low (Table 1). These data could be correlated to the result of turbidity studies (Fig. 3), indicating that $GPF_{CM\text{-}chitosan}$ was sensitive to gluconic acid but GPF_{pGlu} was inert.

Table 1
Mean particle sizes, polydispersity indices and zeta potential values of the self-aggregated nanoparticles after the addition of different concentrations of gluconic acid into $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions.

	Particle size (nm)	Scattering intensity (k_{counts}/s)	Zeta potential (mV)	Transmittance (%)
$GPF_{CM\text{-}chitosan}$[a]				
1.0 mM	16.4	26.0[b]	−31.6	94.7
5.0 mM	45.2	42.2[b]	−28.4	61.1
7.5 mM	118.5	116.7	−23.3	41.2
10 mM	413.7	325.6	−18.1	21.9
15 mM	526.6	372.3	−15.6	10.5
GPF_{C2G1}[a]	371.6	196.8	−18.6	35.8
GPF_{C1G1}[a]	198.4	101.4	−21.2	54.1
GPF_{C1G2}[a]	96.7	49.6[b]	−23.2	67.3
GPF_{pGlu}[a]	56.4	36.0[b]	−31.6	84.5

[a] The concentrations of gluconic acid added in $GPF_{CM\text{-}chitosan}$ solutions were 1.0, 5.0, 7.5, 10 and 15 mM while those added in GPF_{C2G1}, GPF_{C1G1}, GPF_{C1G2} and GPF_{pGlu} were all kept at 10 mM.

[b] Low scattering intensity (k_{counts} < 50) means very few nanoparticles could be measured in the solutions. The mean particle size and zeta potential were not reliable.

The morphologies of the self-aggregated nanoparticles prepared by adding gluconic acid into the $GPF_{CM\text{-}chitosan}$ solution were shown in Fig. 4. The TEM micrographs of $GPF_{CM\text{-}chitosan}$ nanoparticles showed the appearance of newly formed nanoparticles. After adding 5 mM of gluconic acid, the particle sizes of the newly formed micelles were almost less than 50 nm because $GPF_{CM\text{-}chitosan}$ was still during nuclear formation. Increasing the concentration of gluconic acid (7.5 mM) involves rapid growth of nanoparticles in the $GPF_{CM\text{-}chitosan}$ solution but the nanoparticles were still not very solid. After continuously adding 10 mM of gluconic acid, the nanoparticles became solid and spherical in shape and the particle sizes of the well-formed nanoparticles were about 400 nm. Under hyper gluconic acid condition (25 mM), the nanoparticles became unstable and subsequently aggregated and precipitated, consequently resulted in the formation of insoluble gels. GPF_{pGlu} in

contrast to $GPF_{CM\text{-}chitosan}$ was not able to form nanoparticles even in the presence of 10 mM of gluconic acid.

3.3.2. *Effect of gluconic acid and H_2O_2 on fluorescence quenching*

Fluorescence quenching of genipin-crosslinked CM-chitosan/pGlu conjugates responding to the stimulus of gluconic acid or H_2O_2 were studied. As shown in Fig. 5A, the fluorescence emission of $GPF_{CM\text{-}chitosan}$ was significantly quenched upon the addition of gluconic acid (65.3% of the fluorescence intensity quenched by 10 mM gluconic acid). Self-aggregation of fluorescence species through hydrogen bonding interaction, electrostatic forces or van der Waals forces has been proved to be accompanied by fluorescence self-quenching (Liu et al., 2011). Such quenching approaches are useful for fluorescent sensing. In contrast to $GPF_{CM\text{-}chitosan}$, the fluorescence quenched by gluconic acid in the GPF_{pGlu} solution was less remarkable than that in the $GPF_{CM\text{-}chitosan}$ solution. Fig. 5A shows typical Stern–Volmer plots obtained for the quenching of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} fluorescence upon the addition of gluconic acid.

The quenching effect of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} shown in Fig. 5A is consistent with the Stern–Volmer equation, which can be expressed by the equation $(F_0/F) = 1 + K_{SV}[\text{gluconic acid}]$, where F_0 and F are the respective fluorescence intensities in the absence and presence of the fluorescence quencher (gluconic acid) and K_{SV} is the apparent Stern–Volmer quenching constant.

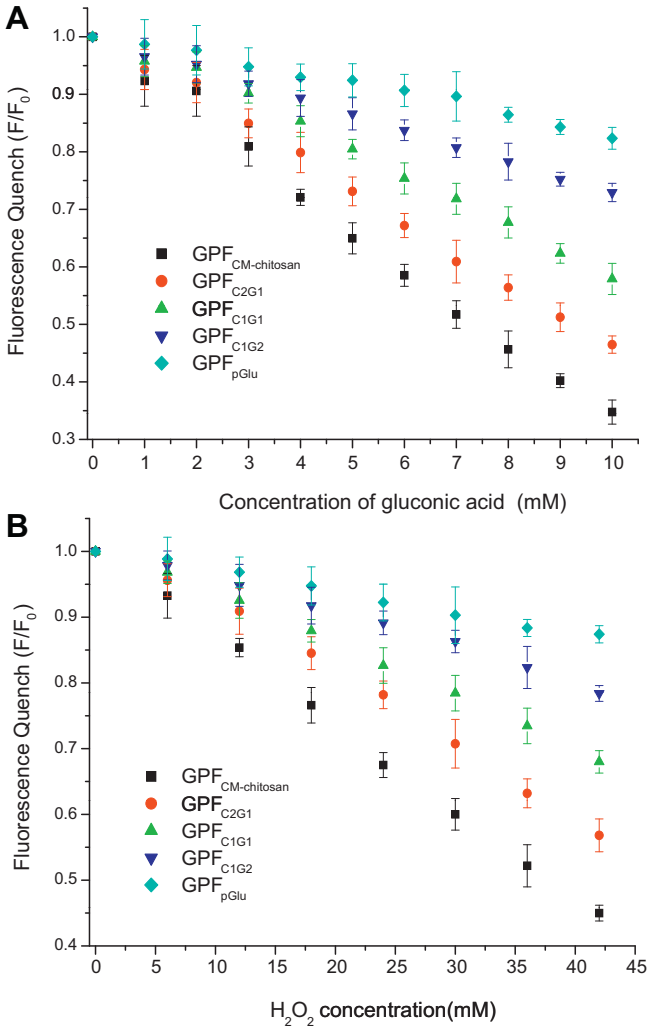

Fig. 5. Fluorescence quenching of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions upon the addition of 1–10 mM gluconic acid (A) or 1–40 mM H_2O_2 (B).

Table 2
UV absorbances at 240 and 290 nm, and the apparent Stern–Volmer quenching constants (K_{SV}) of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions.

Type	UV absorbance[a]		K_{SV}[b]	
	240 nm	290 nm	Gluconic acid	H_2O_2
$GPF_{CM\text{-}chitosan}$	2.619/1.415	0.035/0.821	−0.0919	−0.0140
GPF_{C2G1}	2.627/1.498	0.035/0.805	−0.0761	−0.0119
GPF_{C1G1}	2.628/1.586	0.011/0.703	−0.0637	−0.0106
GPF_{C1G2}	2.613/1.721	0.023/0.573	−0.0295	−0.0075
GPF_{pGlu}	2.627/1.875	0.017/0.355	−0.0188	−0.0042

[a] UV absorbance of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions decreased at 240 nm and increased at 290 nm (original/120 h).
[b] Apparent Stern–Volmer quenching constant (K_{SV}) of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} solutions, respectively quenched by gluconic acid and H_2O_2.

The apparent Stern–Volmer constants calculated from the data in Fig. 5A are summarized in Table 2. The result indicated that the efficiency of fluorescence quench by gluconic acid ($GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs}) decreased with the increase of conjugated pGlu concentrations.

The effect of H_2O_2 on the fluorescence quenching of the genipin-crosslinked CM-chitosan/pGlu conjugates are shown in Fig. 5B. The results showed that H_2O_2 causes significant changes in fluorescence emission spectra of $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs}. Almost 55.2% of the fluorescence intensity of $GPF_{CM\text{-}chitosan}$ was quenched by the addition of 42 mM H_2O_2. However, the fluorescence quenched in the GPF_{pGlu} solution was less remarkable than that in the $GPF_{CM\text{-}chitosan}$ solution. The apparent Stern–Volmer constants calculated from the data in Fig. 5B are also summarized in Table 2. The result suggested that the efficiency of fluorescence quench by H_2O_2 ($GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs}) decreased with the increase of conjugated pGlu concentrations.

3.4. *Protein release from self-aggregated nanoparticles*

Protein drugs, like insulin, could be loaded in nanoparticles for the development of injectable, long-acting formulations. In this work, BSA was loaded in test nanoparticles and the loading efficiency was investigated. In presence of 10 mM gluconic acid, the genipin-crosslinked CM-chitosan/pGlu conjugates were self-aggregated into nanoparticles. The loading efficiency of BSA in self-aggregated $GPF_{CM\text{-}chitosan}$, GPF_{C2G1} and GPF_{C1G1} nanoparticles, were 35.5%, 28.2% and 15.6%, respectively. BSA cannot be incorporated with GPF_{pGlu} and GPF_{C1G2} because of their poor self-aggregation ability. Fig. 6A shows the release profiles of BSA from the self-aggregated $GPF_{CM\text{-}chitosan}$, GPF_{C2G1} and GPF_{C1G1} nanoparticles in the presence of 10 mM GA. As shown in this figure, BSA release rate of the $GPF_{CM\text{-}chitosan}$ nanoparticles was slower than its GPF_{CGs} counterparts (GPF_{C2G1} and GPF_{C1G1}). Additionally, the BSA release rates of GPF_{CGs} nanoparticles decreased with increasing the contents of conjugated CM-chitosan ($GPF_{C1G1} > GPF_{C2G1}$). Therefore, the $GPF_{CM\text{-}chitosan}$ nanoparticle was selected for the following stimuli-responsive release study because of its higher BSA loading efficiency and slower BSA release rate.

The release profiles of BSA from the self-assembled $GPF_{CM\text{-}chitosan}$ nanoparticles were investigated in distinct dissolution media containing 10, 25 and 50 mM gluconic acid, which simulated the hyper gluconic acid conditions (Fig. 6B). In the dissolution medium containing 10 mM gluconic acid (simulating gluconic acid generated at the condition of slightly high glucose level) the release rate was quick. There was about 57.3% BSA released from the nanoparticles after 24 h of dissolution test. In contrast, the nanoparticles showed a slow and continuous release of BSA, in the dissolution medium containing 25 or 50 mM gluconic acid (simulating the gluconic acid generated at hyperglucose conditions). Only 38.7% and 24.3% of BSA released from the

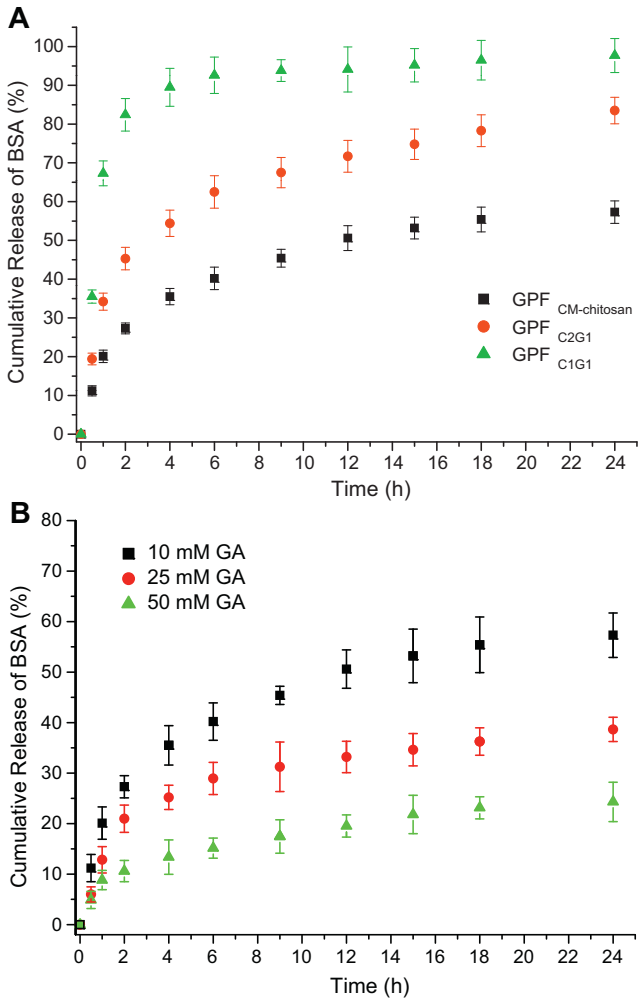

Fig. 6. (A) Release profiles of BSA from the self-aggregated $GPF_{CM\text{-}chitosan}$, GPF_{pGlu} and GPF_{CGs} nanoparticles in presence of 10 mM GA, and (B) release profiles of BSA from the $GPF_{CM\text{-}chitosan}$ nanoparticles in distinct dissolution media containing 10, 25 or 50 mM gluconic acid.

nanoparticles within 24 h, in the dissolution medium containing 25 and 50 mM gluconic acid, respectively. It has been reported that minimum swelling ratios of CM-chitosan-based hydrogels occurred near the isoelectric point of CM-chitosan, under acid conditions (Li, Du, Tang, & Wang, 2009). As shown in Fig. 4, in the presence of 25 mM gluconic acid, the nanoparticles became unstable and subsequently aggregated into insoluble gels, consequently resulted in the slow release of BSA. The result indicated that the $GPF_{CM\text{-}chitosan}$ nanoparticles prepared in the study may be beneficial for long-acting release of protein drugs under hyper gluconic acid condition.

4. Conclusion

We have described the synthesis and properties of genipin-crosslinked CM-chitosan/pGlu conjugates. The conjugation process endows fluorescence properties of the genipin-conjugated polymers. The stimulus-response studies suggested that the genipin-conjugated polymers were sensitive to H_2O_2 and gluconic acid, the major products in GOD-catalyzed oxidation of glucose. The genipin-conjugated polymers could be self-aggregated into nanoparticles and the fluorescent emissions were quenched under the stimulus of gluconic acid and H_2O_2. BSA could be loaded in the self-aggregated nanoparticles and the incorporated BSA slowly released from the nanoparticles, in the presence of 25 or 50 mM gluconic acid. The result indicated that the genipin-conjugated polymers may be potential materials for optical sensing the oxidized products of glucose (gluconic acid and H_2O_2) and long-acting releasing protein drugs.

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