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Synthesis and characterization of PEGylated carboxymethylchitosan nanoparticles

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ABSTRACT

Two types of novel biodegradable nanoparticles, mPEG (monomethoxypoly(ethylene glycol)) grafted with carboxymethylchitosan (mPEG-g-CMCTS) and PEG cross-linked with carboxymethylchitosan (PEG-CMCTS), were prepared. Both carboxymethylchitosan derivatives were synthesized by grafting or cross-linking reaction of carboxymethylchitosan with mPEG-aldehyde or PEG-bisaldehydes to form Schiff base. The structure of the products was determined by FTIR and NMR measurement. These two copolymers could self-assemble into nanoparticles in aqueous solution. Particle size of mPEG-g-CMCTS measured by TEM varied in the range of 300 nm to 1.1 μm depending on the graft volume of $mPEG_{-2000}$. The average size of PEG-CMCTS measured by DLS was in the range of 122–500 nm depending on the molecular weight of PEG. A typical TEM micrograph indicated that nearly spherical nanoparticles can be obtained, the diameter depending on the length and flexibility of the cross-linking and grafting agent. Micelles can remain stable in aqueous solution for more than a month.

1. Introduction

Carboxymethylchitosan is an important derivative of chitin which has found applications in biomedical areas because of its biocompatibility and nontoxicity. Besides, due to the presence of reactive amino groups, hydroxyl groups and carboxyl groups, it can be modified easily to create nano- and microparticles or porous hydrogels (AL-Kahtani Ahmed, Bhojya Naik, & Sherigara, 2009; Kadnaim, Janvikul, Wichai, & Rutnakornpituk, 2008; Mi, Kuan, Shyu, Lee, & Chang, 2000) which can be employed in a wide range of biomedical applications, such as drug- or gene-delivery systems (Li et al., 2009; Zhang et al., 2008).

Many recent attempts have been made to create carboxymethylchitosan particulate systems. Covalently cross-linked nanoparticles can be prepared by several different methods: emulsion cross-linking, reverse micellar, solvent evaporation, or spray-drying (Gorochovceva & Makuvska, 2004; Park, Cho, Chung, Kwon, & Jeong, 2003). Ionotropic gelation methods are based on the interaction between carboxymethylchitosan and various anions (Peng & Zhang, 2007; Zhao, Sun, & Liu, 2009), such as the emulsion cross-linking method and the reverse micellar medium method. However, organic solvent as a dispersion medium in these methods, not only result in wasting a lot of organic solvents, but also result in solvent residues. Therefore, these nanoparticles are not suitable for drug delivery systems. In addition, the safety of most common cross-linkers of carboxymethylchitosan, aldehyde, epoxide, cyanate and other agents (Boudier, Aubert-Pouesel, Gérardin, Devoisselle, & Bégu, 2009; Brack, Tirmizi, & Risen, 1997; Crini, 2005; Kadnaim et al., 2008; Lin-Gibson, Walls, Kennedy, & Welsh, 2003; Mi et al., 2000; Monteiro & Airoldi, 1999; Mukoma, Jooste, & Vosloo, 2004; Qin, Xiao, Du, Shi, & Chen, 2002), has not been confirmed. So the carboxymethylchitosan derivatives formed by these cross-linkers could not be applied to drug delivery systems. Therefore, choosing an effective and safe method for modification of carboxymethylchitosan is important.

Poly (ethylene glycol) (PEG) is one of the most popular macromolecules for the chemical modification of biomaterials because of its unique physicochemical and biological properties, including hydrophilicity, solubility, biocompatibility and ease of chemical modification (Sheng et al., 2009). The introduction of PEG could effectively extend the life time in the body of polysaccharides and hence improve drug delivery properties. A variety of studies have focused on the modification of chitosan by using PEG (Fernandez-Megia, Novoa-Carballal, Quiñoá, & Riguera, 2007; Ganji & Abdekhodaie, 2008; Gorochovceva & Makuška, 2004; Sheng et al., 2009; Zhao et al., 2009) as water-soluble linkages via esterification.

The present investigation reports a mild method for the preparation of PEGylated carboxymethylchitosan nanoparticles via amino groups grafted or cross-linked with mPEG-aldehyde and PEG-bisaldehyde in aqueous media. The grafted copolymer could then self-assemble into micelles and the cross-linked polymer could then form a network structure in water. Different molecular weight

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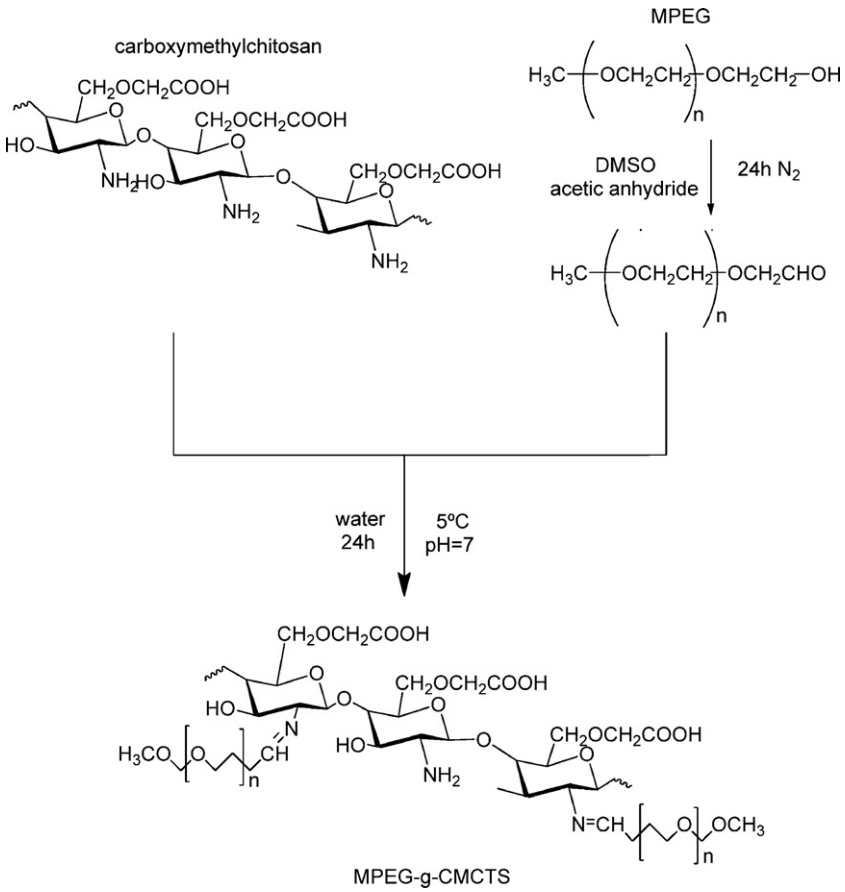


Scheme 1. Synthesis scheme of mPEG-g-carboxymethylchitosan.

PEGs were used to show the correlation of particle size of cross-linked copolymers with PEG molecular weight. These nanoparticles are attractive candidates as delivery vehicles because of their colloidal stability in aqueous media at neutral pH.

2. Experimental

2.1. Materials

Carboxymethylchitosan (Mw = 360,000) was synthesized using a previously reported method (Chen & Park, 2003). Poly (ethylene glycol) methyl ether (mPEG, Mn = 2000) and poly (ethylene glycol) (PEG, Mn = 800, 1500, 2000, 4000, 6000) were purchased from the Sinopharm Chemical Reagent Co. Ltd. All these materials and other chemicals used in this article were of analytical reagent (AR) grade, and used as received without further purification.

2.2. Carboxymethylchitosan modification

Cross-linked and grafted carboxymethylchitosan nanoparticles were prepared by Schiff base formation. PEG-aldehyde and mPEG-aldehyde were used as the cross-linking and grafting agent.

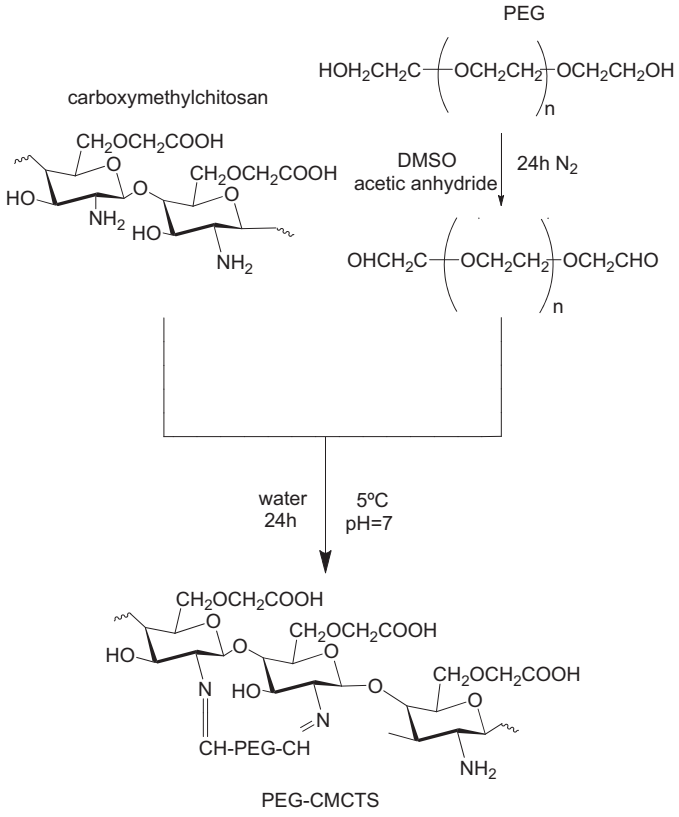
2.2.1. Synthesis of grafted carboxymethylchitosan nanoparticles

mPEG-g-CMCTS was prepared by the same method with little modification as shown in Scheme 1. Briefly, mPEG-aldehyde was first prepared by oxidation of mPEG with DMSO/acetic anhydride. After mPEG was completely dissolved into anhydrous DMSO/chloroform solution (90/10, v/v), acetic anhydride was added gradually into the reaction system under a nitrogen atmosphere. The molar ratio of acetic anhydride to mPEG was 12. The reaction system was kept at ambient temperature for 24 h under the nitrogen atmosphere and finally precipitated with excess prechilled diethyl ether. The mixture was filtered and the obtained crude product of mPEG-aldehyde was re-precipitated twice from chloroform solution with prechilled diethyl ether. After the drying process, the white mPEG-aldehyde powder was obtained and kept in desiccators for further use.

mPEG-g-CMCTS was synthesized by alkylation of carboxymethylchitosan followed by Schiff base formation. Carboxymethylchitosan was dissolved in 100 mL water, mPEG-aldehyde was added to the solution, and the solution was then adjusted to pH 7 with saturated sodium carbonate. The reaction mixture was stirred at about 5 °C for 24 h. The solution containing carboxymethylchitosan nanoparticles was purified by dialysis for 3 days against distilled water and freeze-dried. In the reaction, CMCTS (amino content) and mPEG-aldehyde (aldehyde content) were mixed together at the molar ratio of 3/1, 1.5/1, 0.75/1, 1/1.5, and 1/3.

2.2.2. Synthesis of cross-linked carboxymethylchitosan nanoparticles

PEG-CMCTS was prepared by a similar method to mPEG-g-CMCTS as shown in Scheme 2. For the difference, PEG was used and it was cross-linked with carboxymethylchitosan. In the reaction, CMCTS (amino content) and PEG-bisaldehyde (aldehyde content) were mixed together at a molar ratio of 1/1.The molecular weights of PEG used in the reaction were 800, 1500, 2000, 4000 and 6000.



Scheme 2. Synthesis scheme of PEG-carboxymethylchitosan.

2.3. Characterization of carboxymethylchitosan nanoparticles

2.3.1. ^1H nuclear magnetic resonance analysis (NMR)

^1H NMR spectra were obtained on a Bruker DRX 400 MHz instrument. The samples were dissolved in D_2O. The chemical shifts were represented in ppm, based on the signal from TMS (tetramethylsilane) as a reference.

2.3.2. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were performed at room temperature using Nicolet-5700 Infrared Spectrophotometer (USA). The characteristic absorption bands were detected at wavenumbers ranging from 500 to 4500 cm^{-1} using a KBr-pellet method.

2.3.3. Transmission electron microscopy (TEM)

An H-800 transmission electron microscope was used to characterize the size and morphology of the dried carboxymethylchitosan nanoparticles. For TEM observation, all the carboxymethylchitosan nanoparticles mentioned in the article were prepared from the reaction mixture after dialysis at an appropriate concentration. The sample for TEM analysis was obtained by placing a drop of the colloid dispersion containing the chitosan nanoparticles onto a carbon-coated copper grid. It was dried at room temperature and then examined using TEM without any further modification or coating.

2.3.4. Dynamic laser light scattering (DLS)

The hydrodynamic diameters of the cross-linked carboxymethylchitosan nanoparticles were measured by using a Malvern Instruments Zetasizer Nano series instrument (ZS90) equipped with a 22 mV He–Ne laser operating at a wavelength of 633 nm. Measurements of the average size of nanoparticles were performed at 25 °C with an angle detection of 90° in optically homogeneous quartz cylinder cuvettes. The samples were prepared from the reaction mixture after dialysis. The concentration of the carboxymethylchitosan derivative solutions was 1 mg/mL. Each sample was measured three times, and the average was calculated.

2.4. Determination of molecular weight

The viscosity average molecular weight was determined by measuring the relative viscosity with an Ostwald viscometer. The solvent system used was 0.10 M CH_3COOH/0.20 M NaCl. Molecular weight was calculated from the intrinsic viscosity based on the Mark–Houwink equation. The values for the constants K and a were 1.81×10^{-3} and 0.93 (Roberts & Domszy, 1982).

3. Results and discussion

3.1. Synthesis of nanoparticles

Schiff base is a chemical bond which is acid-sensitive at pH = 5.4 consistent with the pH of tumor tissue. These formed nanoparticles might give the anti-tumor drug passive targeting after combining. Therefore, they have great potential for the further use of anti-tumor drug carriers in the drug delivery and release fields. Here we report our successful quest for synthesis of two nanoparticles in water without the addition of any surfactant or organic solvent; Previously similar nanogels have almost always been synthesized in an inverse emulsion polymerization system with a large amount of surfactant. Also, many researches have been carried on the PEGylated chitosan, but most reaction conditions are not appropriate, such as high temperature during esterification (Gorochovceva & Makuška, 2004; Hu, Jiang, Xu, Wang, & Zhu, 2005) and are hard to process for the clearance of DCU (dicyclohexyl urea) after the use of DCC (dicyclohexylcarbodiimide) in esterification or amidation.

Grafted nanoparticles (mPEG-g-CMCTS were prepared by the chemical modification of carboxymethylchitosan linear chains using mPEG-aldehyde at different ratios (Table 1). With the increasing amount of mPEG, namely the molar ratio of aldehyde content/amino group content increases from 1:3 to 3:1, graft volume gave out an upward trend which was from 76.5% from 89.75%. Also, the grafting degree of substitution increases from 16.23% to 96.12%, the grafting reaction rate decreased from 55.19% to 27.54% with the increasing of mPEG-CHO. The main reason is that carboxymethylchitosan and mPEG were both macromolecules which were hard to diffuse in the water solution. This leads to the Schiff-base reaction not being fully completed. An increase in aldehyde content increased the reaction probability of aldehyde with the amino, making the graft volume increase.

Cross-linked nanoparticles (PEG-CMCTS) were prepared by the chemical modification of carboxymethylchitosan linear chains using PEG-bisaldehyde as cross-linking agent. In this case, the aldehyde groups and residual free amino groups of the carboxymethylchitosan chain were bound covalently. The stoichiometric ratio of cross-linking was less than 100%. PEGs with different molecular weights were used in the reaction giving cross-linked polymers with different properties.

3.2. Characterization of carboxymethylchitosan nanoparticles

3.2.1. NMR results

The ^1H NMR spectrum of grafted polymer is presented in Fig. 1, which made further confirmation -of its molecular structure. Typical peaks at 3.5–3.8 ppm (H3, 4, 5) and 3.4 ppm (H8) are assigned to the ring methane and methylene protons of carboxymethylchitosan saccharide units and methylene groups of mPEG (Kong et al., 2010; Li et al., 2010). Peaks at 2.9 ppm (H2) is attributed to

Table 1
Effect of molar ratio (amino content/aldehyde content) on the property of products.

Molar ratio	Graft volume[a] (%)	Grafting reaction rate[b] (%)	Grafting degree of substitution[c] (%)
3:1	76.5	55.19	16.23
1.5:1	60.32	46.40	33.18
0.75:1	81.7	37.80	47.65
1:1.5	89.68	28.09	93.51
1:3	89.75	27.54	96.12

[a] The quantity of mPEG in the nanoparticle/the quantity of the nanoparticle × 100%.
[b] The molar volume of mPEG in the nanoparticle/the total molar volume of aldehyde × 100%.
[c] The molar volume of mPEG in the nanoparticle/the molar volume of carboxymethylchitosan unit.

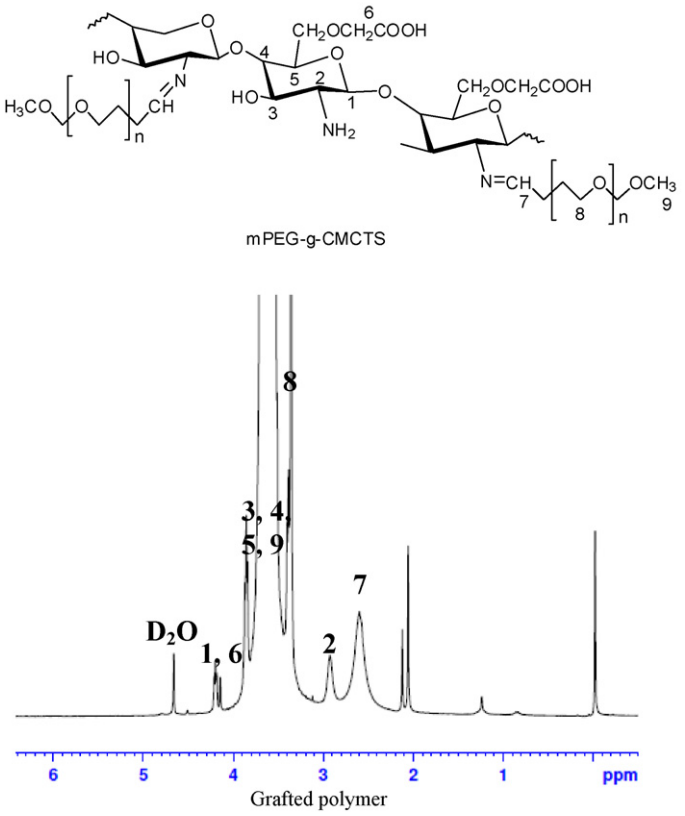


Fig. 1. ^{1}H NMR of grafted polymer.

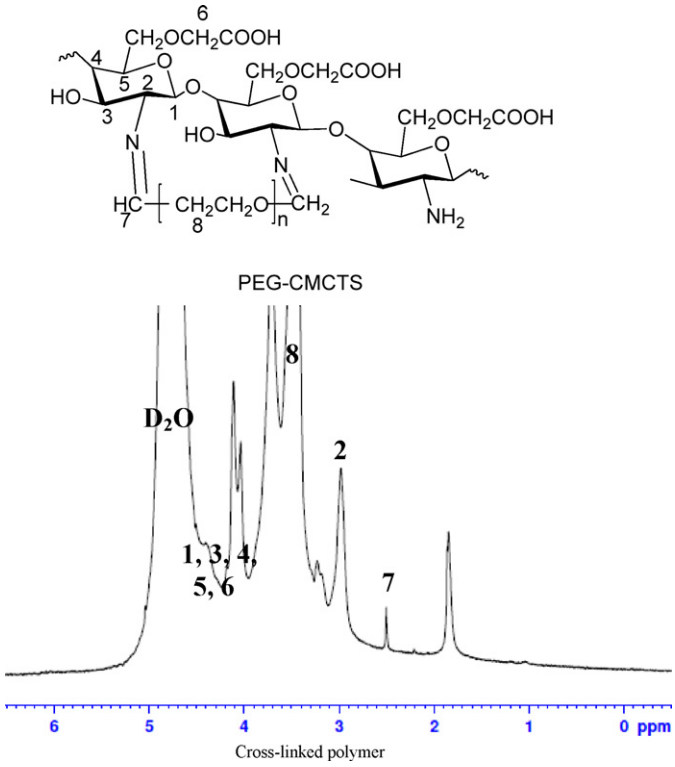


Fig. 2. ^{1}H NMR of cross-linked polymer.

–$CHNH_2$ from carboxymethylchitosan. The linkage between carboxymethylchitosan and mPEG was confirmed by the appearance of a peak of –N=$CHCH_2O$– at 2.59 ppm (H7). The characteristic spectrum of cross-linked polymer is similar to the grafted polymer as shown in Fig. 2.

3.2.2. FTIR results

According to the characteristic spectra of the mPEG-CHO, CMCTS and grafted polymer in Fig. 3, an attempt was made to determine the eventual presence of interactions (possible interactions) between the polymers. The characteristic absorption bands (1737 cm^{-1}) of mPEG-CHO disappeared, indicating that aldehyde has been successfully involved in the reaction. The characteristic absorption bands of mPEG-g-CMCTS were around 1638 cm^{-1} which confirmed the presence of C=N in polymer. And it (1638 cm^{-1}) merged with carboxyl stretching bands (1620 cm^{-1}) of carboxymethylchitosan. This illustrates the success of the Schiff-base reaction. The characteristic spectrum of the cross-linked polymer is almost the same as the grafted polymer.

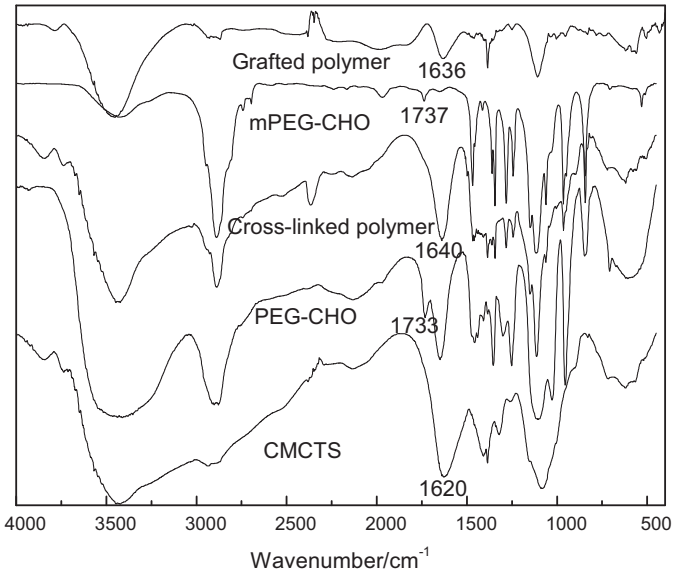


Fig. 3. FTIR spectra of Grafted polymer, mPEG-CHO, cross-linked polymer, PEG-CHO and CMCTS.

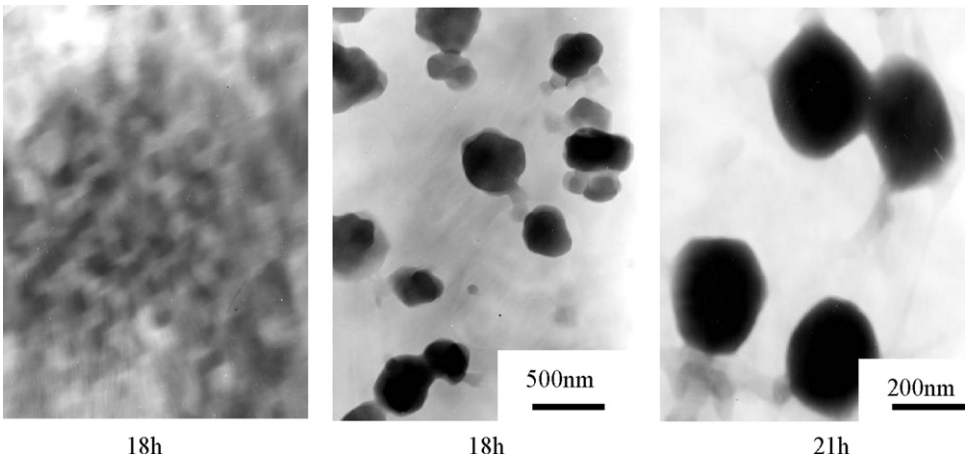


Fig. 4. TEM images of the formation of the grafted polymer.

3.3. Morphological characterization

Water is a good solvent for hydrophilic mPEG, whereas carboxymethylchitosan has poor solubility in water, so the grafted polymer mPEG-g-CMCTS could assemble as nanoscale micelles in water. The hydrophobic segments were self-assembled to form the core of the micelles and the hydrophilic segments attached to the surface of micelles. The morphologies and particle size of the macromolecular micelles depend on the reaction time, reaction temperature and molar ratio (amino content/aldehyde content) in the reaction.

The cross-linked carboxymethylchitosan nanoparticles separated into square particles in an aqueous environment. TEM micrographs confirmed the nanosize of the cross-linked carboxymethylchitosan particles. The morphologies and particle size of the nanoparticles depend on the reaction time and molecular weight of PEG in the reaction.

Fig. 4 presents the TEM images of the formation of the grafted polymer. The molar ratio of amino content to aldehyde content in the reaction is 1:1. Within the first 18 h, nothing could be seen in the TEM micrographs. Then a large number of reticular structures appear in the view, at longer times, the reticular structures began to specific morphologies with sizes of 300–500 nm. Spherical shapes, ellipsoid and square shapes were observed; in the following 3 h, all the ellipsoids and square shapes change into spherical shapes with a diameter of 300 nm. Due to the introduction of mPEG, these spherical micelles have good dispersion in water. mPEG attached to the surface of micelles which is constant with the model of the micellar structure.

Fig. 5 presents the TEM images of the grafted polymer reacted under different temperatures. The molar ratio of amino content to aldehyde content in the reaction is 1:1. The micrographs show that only the grafted polymer reacted under 5 °C could assemble to spherical micelle. Views of the other two reaction solutions (reacted under 30 °C and 60 °C, respectively) were empty in TEM test. Interestingly, after 2 days dynamic dialysis under 5 °C, the TEM test results showed that a large number of spherical structures formed. This phenomenon indicates that low temperature is conducive to self-assembled to form a certain shape. To sum up, the micelles formed at low temperature are spherical, well dispersed in water and have a uniform particle size distribution, mPEG attached to the surface of the sphere displaying a light-colored hydrophilic layer. In addition, only the mPEG layer of micelles reacted under 60 °C is burr-like. Others are all very smooth tightly wrapped outside the surface of the sphere. The main reason maybe the excessive differences between reaction temperature and dialysis temperature in the formation of micelles.

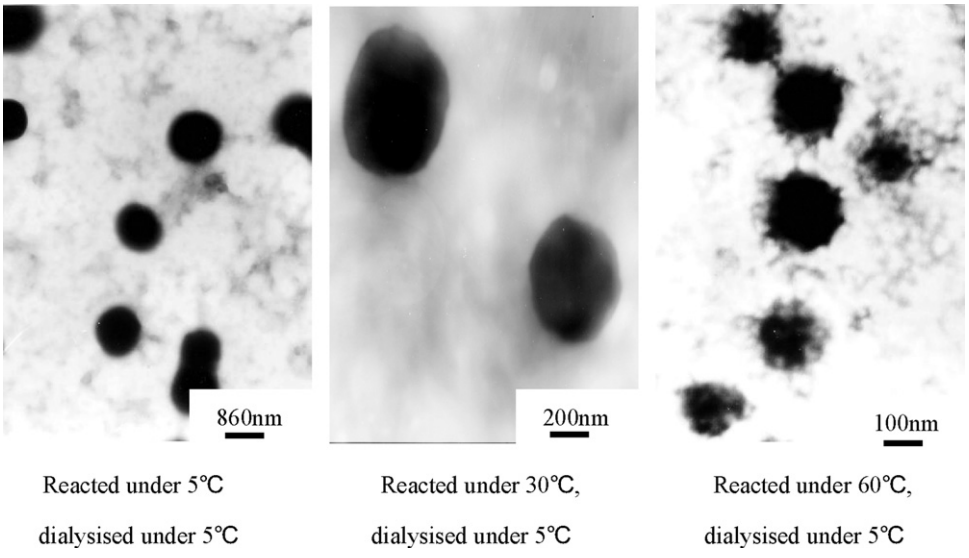


Fig. 5. TEM images of the grafted polymer reacted under different temperatures.

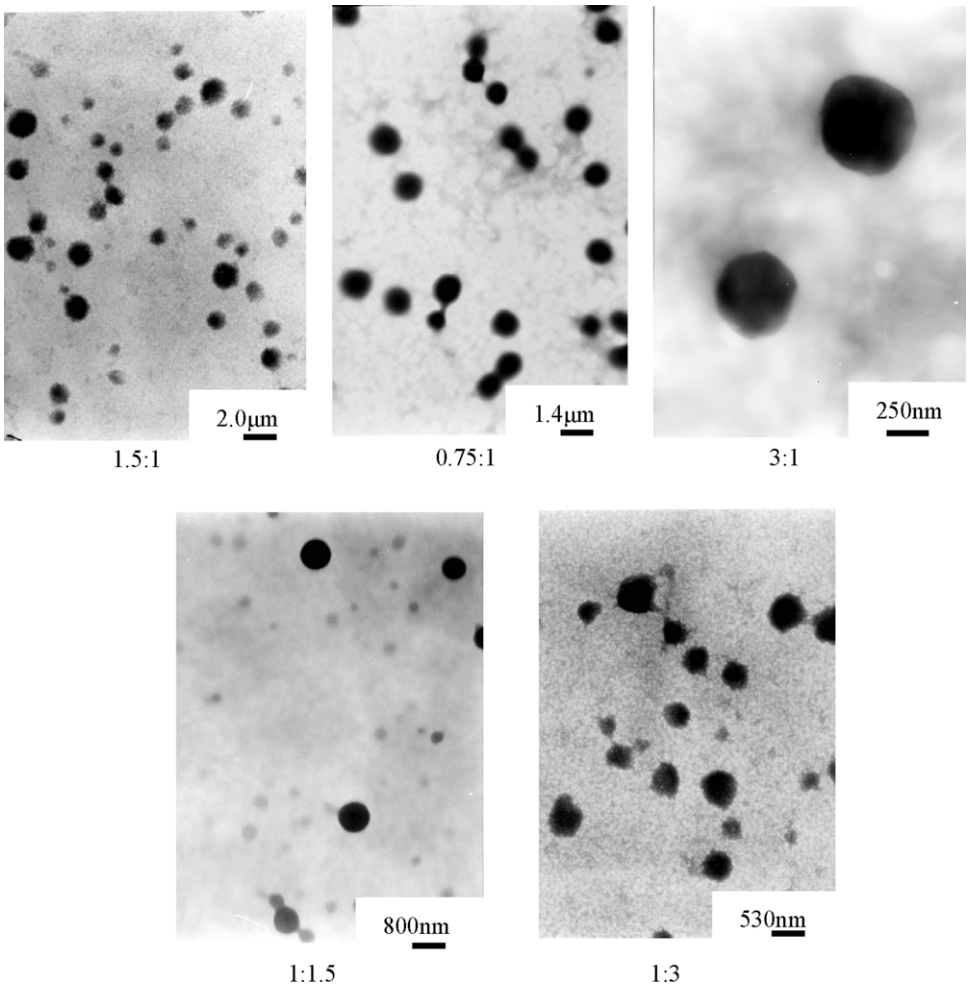


Fig. 6. TEM images of the grafted polymer with different molar ratios (amino content/aldehyde content) in the reaction.

The grafted carboxymethylchitosan nanoparticles separated into spherical particles in an aqueous environment. TEM micrographs confirmed the nanosize of the micelles. Particle size distribution is as follows: around 1200 nm reacted under 5 °C, around 470 nm reacted under 30 °C and around 100 nm reacted under 60 °C. The results show that with the reaction temperature increasing, the particle size of micelles becomes smaller after dialysis. Generally speaking, polymer chains at high temperature are stretched state; when they enter a new environment where the temperature is much lower. The sudden large temperature difference leads to the phenomenon of self-assembly. And the greater the temperature difference is, the stronger the ability to self-assembly. Large combination forces make the micelles form with high density, small particle size.

Fig. 6 shows the TEM images of the grafted polymer with different molar ratios (amino content/aldehyde content) in the reaction. TEM results show that with the increase of mPEG content in the reaction, the average particle size increased first and then began to decline. Average particle size distribution is as follows: around 500 nm when amino content/aldehyde content is 3:1; around 700 nm when amino content/aldehyde content is 1.5:1; around 1160 nm when amino content/aldehyde content is 0.75:1; around 800 nm when amino content/aldehyde content is 1:1.5; around 333 nm when amino content/aldehyde content is 1:3. Reason for such a phenomenon may be that when there is a relatively high amino group content in the reaction system, the steric hindrance between carboxymethylchitosan and mPEG is relatively small, mPEG could easily react with the amino group, making it possible to assemble into micelles of small particle size. When there is excess aldehyde group, allowing a significant increase in the graft volume of mPEG, the ability to self-assemble into micelles becomes stronger, so the particle size is also small. However, when the aldehyde group content is similar to the amino group content, steric hindrance blocks the reaction between aldehyde group and amino group so that the binding forces between the micelles are weak. Finally, the formed particles are very loose with larger particle size.

Fig. 7 shows the TEM images of the grafted polymer immediately after the reaction, 15 days later and 30 days later. After 30 days, micelles could remain well spherical degree, show stability in water and have little change in particle size (around 450 nm). And with the extension of the immersion time in water, particles could readjust so that the surface is smoother. In addition, the introduction of mPEG can increase the particle dispersion in water, particles will not aggregate together, so sticky phenomenon does not occur.

Fig. 8 presents the TEM images of the cross-linked polymer (the molar ratio of amino content and aldehyde content is 1:1, the molecular weight of PEGs are 800, 1500, 2000 and 4000 in the reaction). Unlike grafted micelles, the cross-linked carboxymethylchitosan nanoparticles separated into square particles in aqueous environment which are well dispersed in aqueous solution. TEM micrographs confirmed the nanosize of the cross-linked carboxymethylchitosan particles. Although the spherical

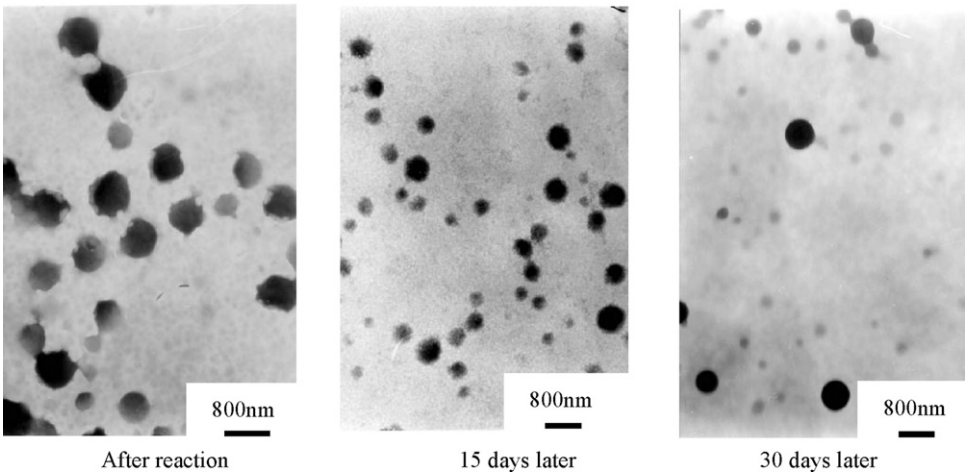


Fig. 7. TEM images of the grafted polymer after the reaction, 15 days later and 30 days later.

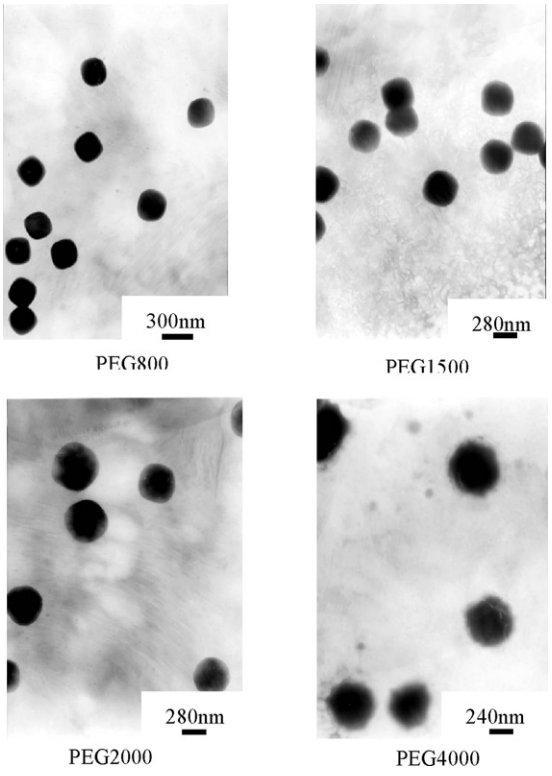


Fig. 8. TEM images of the cross-linked polymer.

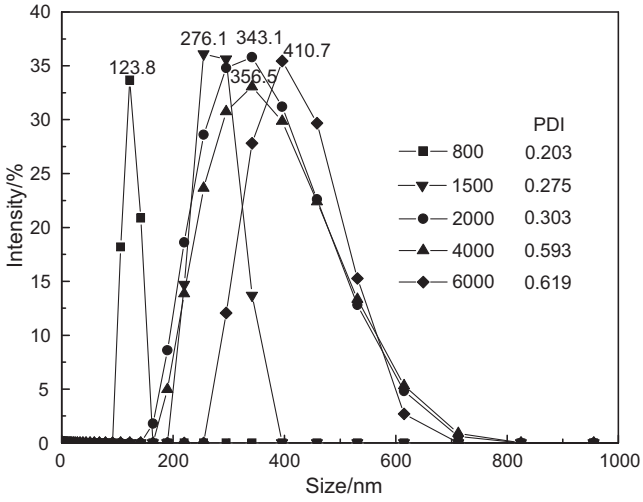


Fig. 9. The size distribution of individual carboxymethylchitosan nanoparticles cross-linked with PEGs with different molecular weights.

degree is not high, the particle size distribution is very narrow. The cross-linked particles are internal cross-linked network structure, creating a hollow structure conducive to drug load.

3.4. Change in product molecular weight

Table 2 shows the molecular weights of carboxymethylchitosan, grafted polymer and cross-linked polymers. In theory, the molecular weight of the products should be increased after the Schiff base reaction. But the result indicates that after the Schiff base reaction with PEG (or mPEG), the apparent viscosity average molecular weight of the products have decreased by an order of magnitude. This phenomenon occurs because of the formation of nanoparticles. Especially the introduction of PEG can increase the particle dispersion in water so that aggregation phenomenon of particles does not occur, leading to reduce viscosity of the product solution significantly. Therefore, the apparent viscosity average molecular weight determined by measuring the relative viscosity with an Ostwald viscometer decreased.

3.5. Particle size of cross-linked particles by DLS

Solution samples were prepared from the reaction mixture after dialysis. The concentration of the polymer solution was 1 mg/mL. The cross-linking agent is a hydrophilic oligomer with a long flexible chain, resulting in a loosely cross-linked carboxymethylchitosan. PEG with different molecular weights was used for the modification. The nanosystems are soluble in aqueous media and the hydrodynamic diameters increase because of the chain length of PEGs. The DLS measurements (Fig. 9) show that the average hydrodynamic diameter of individual nanoparticles is between 122 and 500 nm depending on different PEGs. The numbers plotted in the figure refer to the peak and the polydispersity index of each cross-linked product with different PEGs. But the size distributions appear to be independent of the chain length of the PEGs.

Table 2
Molecular weights of carboxymethylchitosan, grafted polymer and cross-linked polymers.

Molecular weight of carboxymethylchitosan	Molecular weight of grafted polymer[a]		Molecular weights of cross-linked polymers[b]	
	Measured value	Theoretical value	Measured value	Theoretical value
3.6×10^5	5.76×10^4	1.55×10^6	2.03×10^4 [c]	4.67×10^5
			4.20×10^4 [d]	7.54×10^5
			6.97×10^4 [e]	1.36×10^6
			2.49×10^4 [f]	1.63×10^6
			1.11×10^4 [g]	1.67×10^6

[a] Amino content/aldehyde content in the reaction is 0.75:1.
[b] Amino content/aldehyde content in the reaction is 1:1.
[c] Carboxymethylchitosan reacted with PEG800.
[d] Carboxymethylchitosan reacted with PEG1500.
[e] Carboxymethylchitosan reacted with PEG2000.
[f] Carboxymethylchitosan reacted with PEG4000.
[g] Carboxymethylchitosan reacted with PEG6000.

4. Conclusions

We have shown that a nanosized micelle assembled from carboxymethylchitosan using a Schiff base reaction with mPEG oligomer, and a nanosized square particle formed from carboxymethylchitosan using a Schiff base reaction with PEG oligomer as the cross-linking agent. The Schiff base reaction of aldehyde groups and amino groups of carboxymethylchitosan was performed by using Imines. PEGs with short chains were used for the intramolecular grafting and cross-linking of carboxymethylchitosan linear chains. Clear or opalescent stable colloid systems based on carboxymethylchitosan were fabricated in aqueous medium at room temperature. Particle size of mPEG-g-CMCTS measured by TEM varied in the range of 300 nm to 1.1 μm depending on the graft volume of mPEG2000. The average size of PEG-CMCTS measured by DLS was in the range of 122–500 nm depending on the molecular weight of PEG. Both mPEG-g-CMCTS and PEG-CMCTS have good dispersion in water. Strong correlations existed between the size of the nanoparticles and the stoichiometric ratio of crosslinking with PEG or the graft volume of mPEG. Considerable decrease in molecular weight was observed because of the formation of nanoparticles. There was a direct but weak correlation between the particle size and the molecular weight of the PEG used to make the particle.

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