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Synthesis and properties of *O*-carboxymethyl chitosan/methoxy poly(ethylene glycol) graft copolymers

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Abstract *O*-carboxymethyl chitosan/methoxy poly(ethylene glycol) graft copolymers (OCMCS-g-MPEGs) with different degrees of substitution (DS) were synthesized by reductive *N*-alkylation of chitosan with poly(ethylene glycol) aldehyde. The properties of OCMCS-g-MPEGs, including the solubility, structure, hydrodynamic behaviors, isoelectric point (IEP) and interaction with water-soluble chitosan, were investigated. As a PEGylated polyampholyte, OCMCS-g-MPEGs can resolve in water over all pH range and the pH value at IEP (pH_{IEP}) decreases when DS increases. The hydrodynamic behaviors of OCMCS-g-MPEGs in deionized H_2O are markedly affected by DS and pH_{IEP} in the experiment concentration range. The particle size of the complexes of OCMCS-g-MPEGs with water-soluble chitosan is strongly affected by the concentration of water-soluble chitosan and the pH value.

Introduction

Chitosan is a cationic copolymer of glucosamine and *N*-acetylglucosamine, a partially deacetylated derivative of natural chitin, which is one of the most abundant polysaccharides in nature and is mostly derived from the exoskeleton of crustaceans [1–3]. Chitosan and its derivatives are used in various fields: water treatment [4, 5], biomedicine [6–8], cosmetics [9, 10], agriculture [11], food industry [12–15]. However the application of chitosan suffers severe limitations since chitosan is normally insoluble in neutral or alkaline pH because of its very stable crystalline structure due to strong hydrogen bonds. While the solubility of chitosan can be improved by chemical modification of chitosan [16]. Chitosan has reactive amino and hydroxyl groups, both of which can be used to chemically alter chitosan's properties under mild reaction conditions. Therefore many water-soluble derivatives have been prepared by introducing hydrophilic groups like carboxymethyl, dihydroxyethyl, sulfate, phosphate, hydroxyalkylamino [17–21], or by grafting water-soluble polymer [22–25] in the macromolecular chain of chitosan.

Compared with other water-soluble chitosan derivatives, carboxymethyl chitosan (CMCS), an amphiprotic ether derivative [26–29], has many excellent properties: (1) greater solubility in neutral and alkaline conditions; (2) antibacterial activity [30, 31]; (3) nicer chelating agent for heavy metal [32, 33]; (4) better membrane properties [34, 35]; (5) non-toxic. Therefore CMCS is broadly applied in biomedicine [36–39], water treatment [40], and food industry [41, 42]. However, one drawback of CMCS is that the ionization degree of carboxyl groups decreases in acidic conditions. Due to strong hydrogen bonds, a large amount of hydrogel forms in acidic conditions, which can only be dispersed by strongly stirring. This problem may be resolved by grafting methoxy poly(ethylene glycol) (MPEG) into chitosan because MPEG can destroy the strong inter- or intra-molecular hydrogen bonds.

At present, the water-soluble, biocompatible and non-toxic poly(ethylene glycol) (PEG) is frequently used in

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chemical modification of chitosan for biomedical applications [43–45]. PEG, as one of the very few synthetic polymers approved by the FDA for injection, has been widely used in biomedicine [46]. The preparation of chitosan/PEG graft copolymers is a two-step procedure [47–51]: (1) The first step is preparing an activated PEG with a reactive functional group; (2) The second step is anchoring activated PEG to amino, hydroxyl groups in native chitosan, leading to formation of amide, ester linkages.

In this article, the OCMCS/MPEG graft copolymers (OCMCS-g-MPEGs) were synthesized from *O*-carboxylmethyl chitosan (OCMCS) and methoxy poly(ethylene glycol) (MPEG). The properties of OCMCS-g-MPEGs, including the structure, hydrodynamic behaviors, isoelectric point and interaction with water-soluble chitosan, were investigated.

Experimental

Materials

OCMCS (Mn = 56 kDa) was purchased from Yuhuan Ocean Biochemical Co. Ltd. (China) with deacetylation degree of 92% and carboxylation degree of 63.46%. MPEG (Mn = 2 kDa) was purchased from Aldrich Chemical Co. Water-soluble chitosan (M_n = 5 kDa) was purchased from Yuhuan Ocean Biochemical Co. Ltd. (China) with deacetylation degree of 92%. All other chemicals used were of analytical grade and were used without further purification.

Methods

Preparation of MPEG-aldehyde

MPEG-aldehyde (MPEG-CHO) was prepared by the oxidation of MPEG with anhydrous dimethylsulfoxide/acetic anhydride [52, 53]. Acetic anhydride (AcO_2, 5.1 mL) was added into 30 mL MPEG (Mn = 2 kDa, 10 g)/anhydrous dimethylsulfoxide (DMSO) solution containing 2 mL chloroform under a N_2 atmosphere. The mixture was stirred for 9 h at 20 °C. The mixture was then poured into 400 mL anhydrous diethyl ether. The precipitate was filtered with a paper filter (No.2) and reprecipitated twice from chloroform solution with diethyl ether. The precipitate was dried in a vacuum at room temperature for 24 h. The degree of conversion (DC) from hydroxy group to aldehyde group, estimated by hydroxylamine method, was 60%. The unreacted mPEG does not affect the synthesis and properties of OCMCS-g-MPEGs.

Preparation of OCMCS-g-MPEGs

OCMCS-g-MPEGs was prepared according to Harris's method [53]. OCMCS (0.25 g, 1.3 mmol amino group) was dissolved in deionized H_2O and MPEG-CHO (1.25 g, –CHO: 0.375 mmol) was then added to the OCMCS solution. The pH value of OCMCS/MPEG-CHO solution was adjusted to 6.5 by NaOH solution (1 mol/L). After the solution was stirred for 60 min at room temperature, $NaCNBH_3$ ($NaCNBH_3$/MPEG-CHO = 10/1 (mol/mol)) was added to the solution and the solution was stirred for 24 h at room temperature. The solution was dialyzed with dialysis membrane (12 kDa molecular weight cut-off) against water for 48 h to remove the unreacted MPEG. At last, the dialyzed solution was freeze-dried and washed twice with 100 mL acetone to remove the residually unreacted MPEG. After drying in vacuo, the white powder obtained was OCMCS-g-MPEG. In this article, three OCMCS-g-MPEG samples were prepared by changing the ratios of OCMCS to MPEG, i.e. OCMCS-g-MPEG1, OCMCS-g-MPEG2 and OCMCS-g-MPEG3 with different degrees of substitution (DS), 22.6 mol, 38.6 mol and 54.8 mol%, respectively. DS of OCMCS-g-MPEGs was evaluated from Formula 1 given below.

$$\mathrm{DS} = \frac{(m - m_1)/\mathrm{M}_2}{m_1/\mathrm{M}_1} \times 100\% \qquad (1)$$

where m is the weight of OCMCS-g-MPEGs (g); m_1 is the weight of OCMCS (g); M_1 is the molecular weight of OCMCS sugar unit (g/mol); M_2 is the molecular weight of MPEG-CHO (g/mol).

Hydrodynamic studies

A series of aqueous OCMCS-g-MPEG solutions, using deionized H_2O as the solvent, were prepared. The reduced viscosities (η_{sp}/C) of OCMCS-g-MPEGs in aqueous solutions were measured by a Ubbelohde viscometer at 25 °C as shown in Formula 2.

$$\eta_{sp}/C = \left(\frac{t}{t_0} - 1\right)\Big/C \qquad (2)$$

where t_0 is the outflow time of the solvent, t is the outflow time of polymer solution, and C is the concentration of polymer solution.

Complexes of OCMCS-g-MPEGs with water-soluble chitosan

OCMCS and OCMCS-g-MPEG aqueous solutions with the same mass concentration of OCMCS (0.28 g/L) were prepared. Specified amount of the water-soluble chitosan solution was added to OCMCS and OCMCS-g-MPEG aqueous solutions. The final total volume of solution should be the same for all samples.

Characterization

Fourier transform infrared (FTIR) spectroscopy (FT3000, Bio-Rad, Hercules, CA) was used to confirm the structure of OCMCS-g-MPEGs at a resolution of 2 cm^{-1} at room temperature. Polymer samples were pressed into KBr pellets (1:100 copolymer/KBr ratio) and analyzed with IR data manager software.

According to the dependence of conductivity on the pH, the isoelectric points (IEPs) were determined by the conductometric titration method at 25 °C, using a conductometer (DDS-312, Shanghai DaZhong Technology Co, China) and a pH meter (PHS-3C, Shanghai LeiCi Technology Co, China).

The transmission electron microscopy (TEM) specimens for the complexes of OCMCS-g-MPEGs with water-soluble chitosan were observed under a JEM-100CX II instrument. The samples were prepared by adding a drop of the complexes dispersion on the Formvar-coated copper TEM grid, and then dyed by phosphatotungstic acid.

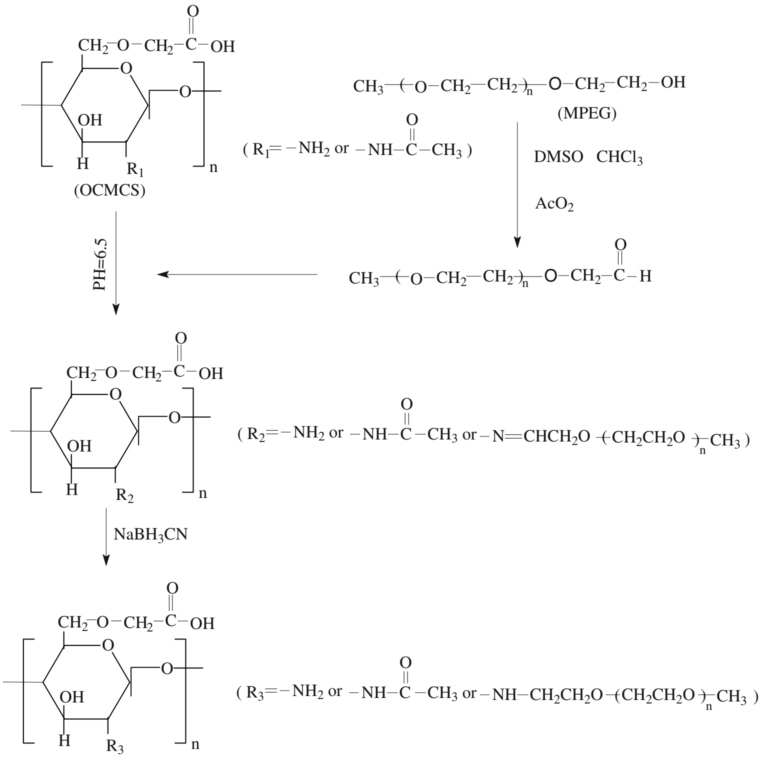
The size and distribution of the complexes of OCMCS-g-MPEGs with water-soluble chitosan were determined by BI-90Plus laser particle size analyzer (LPSA, Brookhaven Instruments, USA). In all cases, λ of measurement was 678 nm, the angle of measurement was 90° and the temperature of measurement was 25 °C. Effects of pH value on particle size of complexes were evaluated by adjusting pH of OCMCS/chitosan and OCMCS-g-MPEG/chitosan solutions.

Results and discussion

Mechanism of graft reaction

The graft reaction mechanism of OCMCS with MPEG is shown in Scheme 1. The preparation of OCMCS-g-MPEGs was performed according to Harris's method. The reaction procedure is divided into two-step. Firstly, the hydroxy groups of MPEG are oxidated into aldehyde groups by the anhydrous DMSO/AcO_2; secondly, the reductive *N*-alkylation of chitosan forms, i.e. the aldehyde groups of MPEG-CHO react with amino groups of native chitosan to form the schiff-base and then the reductive reaction of schiff-base by $NaCNBH_3$ leads to formation of amido linkages.

The pH value can significantly affect DS of OCMCS-g-MPEGs. As shown in Fig. 1, the maximum DS of OCMCS-g-MPEGs is 22.6 mol% at pH 6.5. When the nucleophilic reaction of –CHO takes place under acid condition, the combination of the carbonyl oxygen atoms with protons can enhance the activity of the carbonyl



Scheme 1 Mechanism of the graft reaction of OCMCS with MPEG

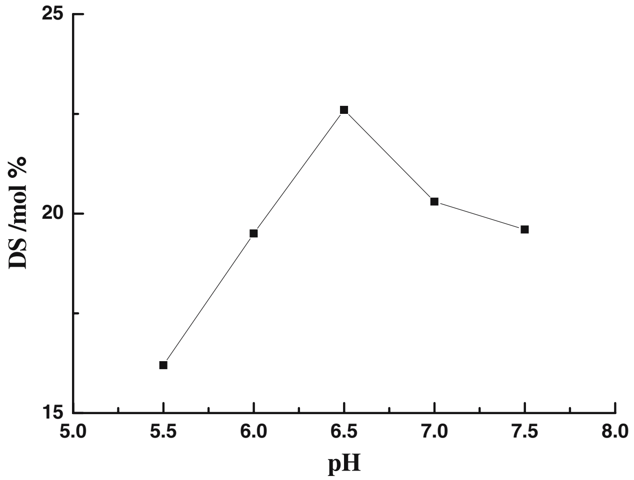


Fig. 1 Effect of the pH value on DS of OCMCS-g-MPEGs. The reaction took place at room temperature under –CHO/–NH_2 of 0.375/1.3 (mol/mol), $NaCNBH_3$/ –CHO of 10/1 (mol/mol)

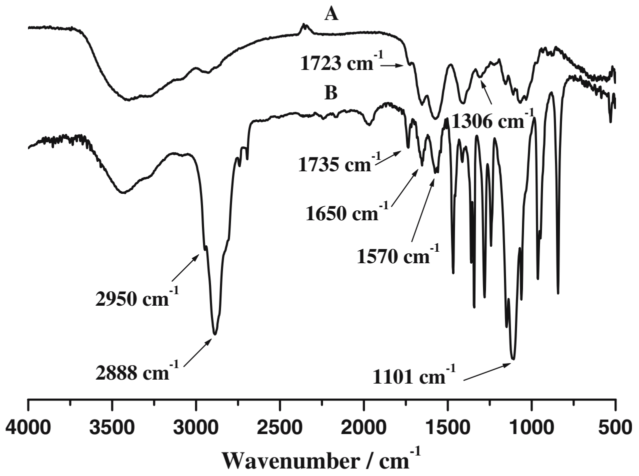
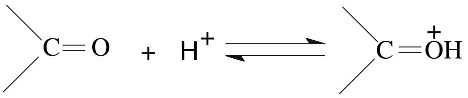


Fig. 2 IR spectra of OCMCS (**A**) and OCMCS-g-MPEGs (**B**). For all cases, the resolution was 2 cm^{-1}, the temperature of measurement was the room temperature, and the copolymer/KBr ratio was 1/100 (wt/wt)

groups as shown in Reaction 1. But the acidity of the reaction system is not very strong. Because the salts formed by the combination of H_2NY (alkali compound) with protons make their nucleophilicity lose as shown in Reaction 2. Therefore the reaction is suggested to take place under weaker acid condition.



(1)

$$H_2NY + H^+ \rightleftharpoons H_3\overset{+}{N}Y \tag{2}$$

As a reductive agent produced by chemically modified $NaBH_4$, the advantages of $NaCNBH_3$ are listed as follows: (1) it is stable under acidic condition and can quickly reduce aldehyde to alcohol when the pH value is below 3.2; (2) the reductive ability to aldehyde is much lower than that to –N = CH– under close neuter condition. Therefore DS is maximal at pH 6.5.

Structure of OCMCS-g-MPEGs

The IR spectra of OCMCS and OCMCS-g-MPEGs are shown in Fig. 2. In the IR spectrum of OCMCS (Fig. 2(A)), the strong characteristic bands at 1650, 1570 and 1306 cm^{-1} are assigned to the amide I, II and III vibration bends and the carboxylic hydroxyl band appears at 3700–2700 cm^{-1}. Because of strong amide bands, the carboxylic carbonyl band at 1723 cm^{-1} is very weak. Compared to OCMCS, in the IR spectrum of OCMCS-g-MPEGs (Fig. 2(B)), the intensity of the amide-characteristic bands obviously decreases and an obvious carboxylic carbonyl band appears at 1735 cm^{-1}, which is due to the introduction of MPEG. The hydrogen bonds between the carboxyl groups are destructed and the carboxylic carbonyl band moves to the higher wavenumber. In addition, the methylene-characteristic bands at 2950 and 2888 cm^{-1} and the C–O–C stretching band at 1101 cm^{-1} become very strong. Therefore the results of the IR spectroscopy confirm the success of grafting PEG on chitosan.

Influences of DS on IEP of OCMCS-g-MPEGs

The aqueous OCMCS-g-MPEG solution with deionized H_2O as the solvent was prepared. By adjusting the pH value of the aqueous OCMCS-g-MPEG solution, we have found that OCMCS-g-MPEGs can be resolved in aqueous solution and no hydrogel forms over all pH range, especially in acidic conditions. This phenomenon suggests that the strong inter- or intra-molecular hydrogen bonds can be destroyed by MPEG.

According to the dependence of the conductivity on pH, IEP was determined by the conductometric titration method and the results are shown in Fig. 3 and Table 1. The pH value at IEP (pH_{IEP}) of OCMCS-g-MPEGs decreases as DS increases. This is because a part of amine groups of the OCMCS macromolecules are consumed during the graft reaction, which leads to the increment of the ratio of carboxyl groups to amine groups.

Hydrodynamic behaviors of OCMCS-g-MPEGs

During the investigation on the hydrodynamic behaviors of OCMCS-g-MPEGs, it is found that the polyampholyte concentration can affect the pH value of aqueous solution

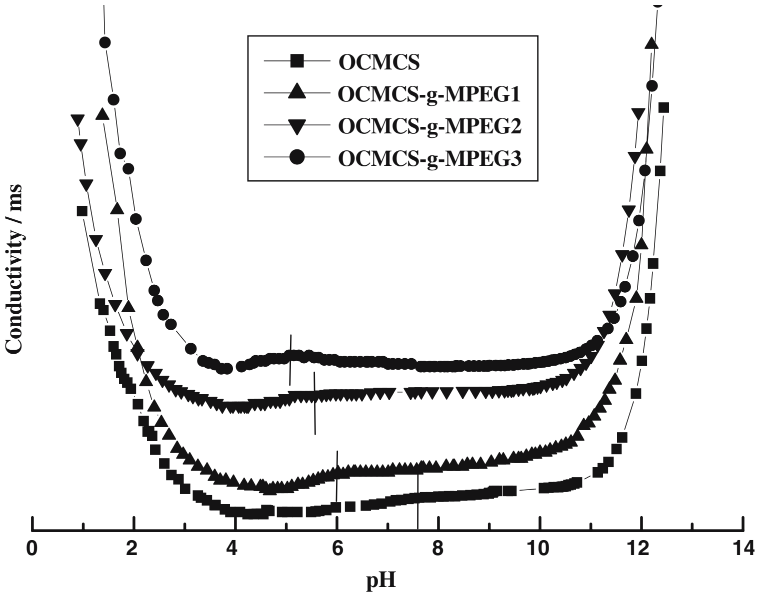


Fig. 3 Influences of the pH value on the conductivity of OCMCS and OCMCS-g-MPEG aqueous solution. DS of OCMCS-g-MPEG (1, 2, 3) is 22.6, 38.6 and 54.8 mol%, respectively. The temperature of measurement was 25 °C

Table 1 pH_{IEP} of OCMCS and OCMCS-g-MPEGs

Polymer	DS/mol%	pH_{IEP}
OCMCS	0	7.6
OCMCS-g-MPEG1	22.6	6.0
OCMCS-g-MPEG2	38.6	5.5
OCMCS-g-MPEG3	54.8	5.0

and further affect η_{sp}/C of OCMCS-g-MPEGs in aqueous solution. The investigation results are shown in Figs. 4 and 5. As shown in Fig. 4, with increasing the OCMCS-g-MPEG concentration, the pH value of the solution decreases. The pH_{IEP} of OCMCS-g-MPEG1 appears at the concentration of 12 mg/mL. However, the pH_{IEP} of OCMCS-g-MPEG2 and OCMCS-g-MPEG3 does not appear until the concentration reaches 30 mg/mL. These phenomena can significantly affect the hydrodynamic behaviors. As shown in Fig. 5, with decreasing the polyampholyte concentration in the experiment concentration range, η_{sp}/C of OCMCS-g-MPEG2 and OCMCS-g-MPEG3 in deionized H_2O increase, but η_{sp}/C of OCMCS and OCMCS-g-MPEG1 in deionized H_2O decreases and then sharply increases. As shown in Fig. 4, 5 and Table 1, for OCMCS-g-MPEG2 and OCMCS-g-MPEG3, the pH values of polyampholyte solutions are greater than their pH_{IEP} in the experiment concentration range. Therefore the net charges of OCMCS moieties are negative. Like the common polyelectrolyte, OCMCS moieties with negative charges strongly stretch with decreasing the polyampholyte concentration, which makes η_{sp}/C increase. As shown in Fig. 4, 5 and Table 1, for OCMCS and OCMCS-g-MPEG1, the η_{sp}/C–C curves pass through the concentration corresponding pH_{IEP}, which suggests that η_{sp}/C of OCMCS and OCMCS-g-MPEG1 in deionized H_2O will be affected by the pH_{IEP}. At the concentration corresponding pH_{IEP}, the amounts of the positive and negative charge on the polyampholyte molecular chains are equal, i.e. the net charges are zero, which makes the molecular chains shrink. However, at the concentration apart from the concentration corresponding pH_{IEP}, the net charges are positive or negative and the polyampholyte molecular chains stretch like the common polyelectrolyte. When the concentration is close to the concentration corresponding pH_{IEP}, the stretching degree of the polyampholyte molecular chains is

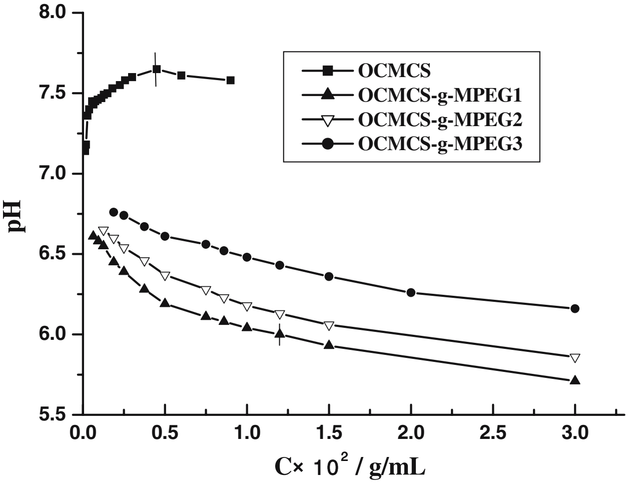


Fig. 4 Influences of the OCMCS-g-MPEG concentration on the pH value of the aqueous OCMCS-g-MPEG solutions. The temperature of measurement was 25 °C. DS of OCMCS-g-MPEG (1, 2, 3) is 22.6, 38.6 and 54.8 mol%, respectively

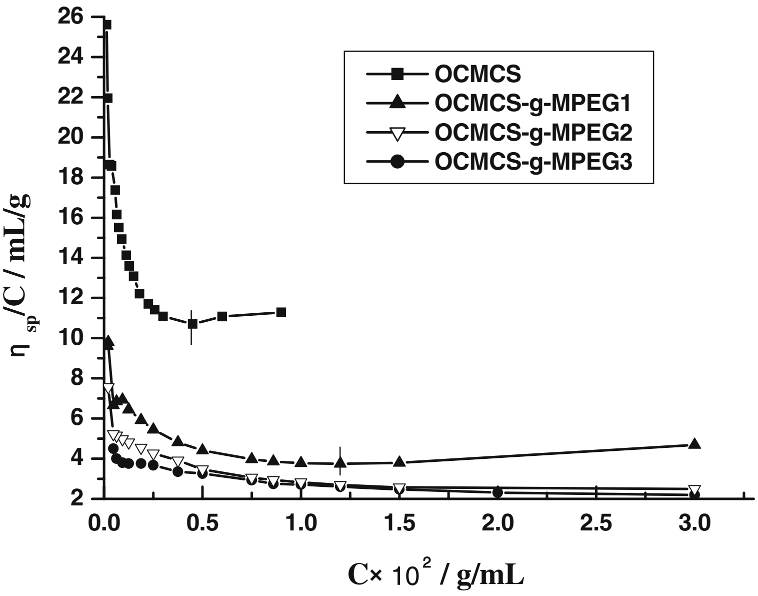


Fig. 5 Influences of the OCMCS-g-MPEG concentration on η_{sp}/C of OCMCS-g-MPEG in deionized H_2O. The temperature of measurement was 25 °C. DS of OCMCS-g-MPEG (1, 2, 3) is 22.6, 38.6 and 54.8 mol%, respectively

gradually decreasing. As a result, η_{sp}/C is the smallest at the concentration corresponding pH_{IEP}.

In addition, it can be observed from Fig. 5 that η_{sp}/C decreases with increasing DS. Under the same concentrations, OCMCS-g-MPEGs with higher DS contain less content of OCMCS moieties and ionized groups, while the intensified shielding effect of MPEG weakens the electrostatic interaction of macromolecular networks. These all lead to the lower stretching degree and lower η_{sp}/C.

Complex behaviors between OCMCS-g-MPEGs and water-soluble chitosan

CMCS can be hydrophobically modified by two methods: (1) the hydrophobic groups are introduced into the CMCS macromolecules; (2) the cationic surfactants or polyelectrolytes, as counterions, are added into the CMCS solutions to form surfactant/polyelectrolyte complexes or polyelectrolyte/polyelectrolyte complexes by electrostatic interaction [54]. In this article, we investigated the complexation between OCMCS-g-MPEGs and water-soluble chitosan as well as the effect of pH on the particle size of the complexes.

Water-soluble chitosan solutions were added to the OCMCS-g-MPEG solutions to obtain polyelectrolyte/polyelectrolyte complexes (PECs). Due to the charge neutralization, water-soluble chitosan binds to OCMCS-g-MPEG, forming a neutralized core and a stabilizing shell of the excess component. As shown in Fig. 6, the PECs represent sphere structure and the particle size is approximate 280 nm.

The influences of the water-soluble chitosan concentration on the particle size of PECs are shown in Fig. 7. With increasing the concentration of water-soluble chitosan, the particle size of PECs sharply decreases and then increases. At low concentrations of water-soluble chitosan, PECs are loose aggregates. With increasing the concentrations of water-soluble chitosan, the solvent is gradually excluded from the core of PECs. Therefore the particle size of PECs decreases. As more chitosan is added, the charge neutralization disturbs the hydrophilic–hydrophobic balance, which results in the aggregation of PECs. As a result, the particle size of PECs increases and PECs finally precipitate.

As shown in Fig. 7, with increasing DS, the size sequence of PECs is reversed and the concentration of water-soluble chitosan at the precipitation point increases. As a hydrophilic chain, MPEG can stabilize the aggregates.

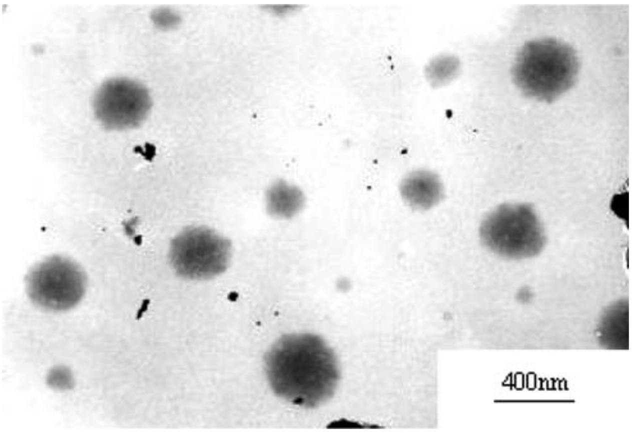


Fig. 6 TEM image of the OCMCS-g-MPEG3 complexes with water-soluble chitosan. The concentration of water-soluble chitosan is 0.042 g/L and the concentration of OCMCS-g-MPEG3 is 0.28 g/L

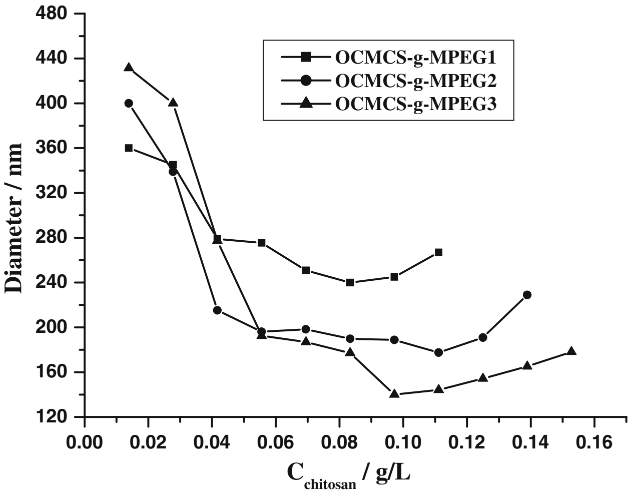


Fig. 7 Influences of the concentration of water-soluble chitosan on the particle size of PECs. The concentration of OCMCS moieties is 0.28 g/L. The size and distribution of the complex were determined by LPSA. For all cases, λ of measurement was 678 nm, the angle of measurement was 90° and the temperature of measurement was 25 °C. DS of OCMCS-g-MPEG (1, 2, 3) is 22.6, 38.6 and 54.8 mol%, respectively

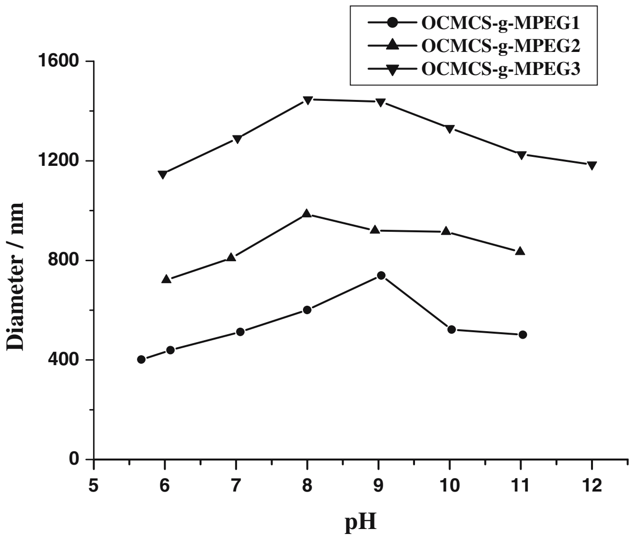


Fig. 8 Influences of the pH value on the particle size of PECs. The mass ratio of chitosan with OCMCS moieties is 0.07. The size and distribution of PECs were determined by LPSA. For all cases, λ of measurement was 678 nm, the angle of measurement was 90° and the temperature of measurement was 25 °C. DS of OCMCS-g-MPEG (1, 2, 3) is 22.6, 38.6 and 54.8 mol%, respectively

On the other hand, MPEG can make the aggregates loose and the aggregation number decrease. At lower concentration of water-soluble chitosan, PECs loosely aggregate. Therefore OCMCS-g-MPEGs with lower DS contain less MPEG chains and the stronger hydrophobicity makes the particle size smaller. At higher concentration of water-soluble chitosan, all of PECs are compact. Therefore the decrease of molecular aggregation number in PECs makes the particle size smaller with increasing DS. In addition, the greater content of MPEG makes PECs more stable and the precipitation point higher.

The influences of pH value on the size of PECs are shown in Fig. 8. With increasing the pH value, there is a maximal diameter point, i.e. OCMCS-g-MPEG1 at pH 9.03, OCMCS-g-MPEG2 at pH 7.99, and OCMCS-g-MPEG3 at pH 8.01. At lower pH values, the ionization of –COOH is restrained and the macromolecular chains shrink due to the stronger hydrogen-bonding interaction. As a result, the size of PECs decreases with the decrease of pH value. At higher pH values, the size of PECs will increase because of more ionization carboxyl groups, while decrease because of the increasing of amine hydrophobicity. Under the co-effects of the two factors mentioned above, the particle size decreases.

Conclusion

OCMCS-g-MPEGs were prepared by the reaction between aldehyded MPEG and OCMCS. As a PEGylated polyampholyte, OCMCS-g-MPEGs can be resolved in water over all pH range. The pH_{IEP} of OCMCS-g-MPEGs lies on the ratio of carboxyl groups to amine groups. The pH_{IEP} decreases with increasing DS. In the experiment concentration range, OCMCS-g-MPEGs with greater DS represent the hydrodynamic behaviors of the common polyelectrolytes. However, η_{sp}/C of the copolymers with lower DS is minimal at the concentration corresponding pH_{IEP}, which is similar to the hydrodynamic behaviors of OCMCS. PECs can be formed from OCMCS-g-MPEGs with water-soluble chitosan. The size of PECs decreases sharply and then increases with the increment of the concentration of water-soluble chitosan, while there is a maximal particle size with increasing the pH value.

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