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Synthesis and characterization of a novel MPEG–chitosan diblock copolymer and self-assembly of nanoparticles ☆

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ABSTRACT

In this paper, a simple and novel method based on free-radical polymerization initiated by potassium persulfate (KPS) was developed to synthesize the MPEG–chitosan diblock copolymer (MPEG–CS). The obtained MPEG–CS diblock copolymer was characterized by Fourier transform infrared (FTIR), ^{1}H nuclear magnetic resonance (^{1}H NMR), X-ray diffraction (XRD) and differential scanning calorimetry (DSC), respectively. The MPEG–CS copolymer could self-assemble into nanoparticles in aqueous solution. A typical TEM photography indicated that the well-spherical nanoparticles with diameter at about 200 nm were obtained. *In vitro* cell culture assay indicated that MPEG–CS nanoparticles are non-toxic and cell-compatible as the polymer concentration was smaller than 0.6 mg/ml. In conclusion, the obtained MPEG–CS nanoparticles might have great potential application in drug-delivery system.

1. Introduction

Chitosan, the only natural cationic polysaccharide obtained by deacetylation of chitin, has gained considerable attention in pharmaceutical and biomedical field owning to its favorable biological properties, such as low-toxicity, good biocompatibility, biodegradability (Muzzarelli et al., 1988), wound-healing activity (Ueno et al., 1999), antibacterial (Tharanathan & Kittur, 2003), etc. However, chitosan has an apparent pK_a of 5.6, which can only be soluble in few dilute acid solutions such as acetic acid, hydrochloric acid, etc. Poor water solubility had greatly limited its wide applications in pharmaceutical and biomedical field (Ouchi, Nishizawa, & Ohya, 1998). As a result, many attempts have been made in chemical modification of chitosan, aiming at improving its water solubility as well as obtaining water soluble derivatives. Among these derivatives, *N*,*N*,*N*-trimethyl chitosan (Muzzarelli & Tanfani, 1985), *N*-acyl chitosan, *N*-carboxyalkyl chitosan, *O*-carboxyalkyl and *N*-carboxyacyl chitosan are very important examples of successful water solubility modification of chitosan that have hold prominent places in research (Ravi Kumar, 2000). Chemical modification changes the fundamental skeleton of chitosan as well as original physicochemical and biochemical activities (Tanigawa, Tanaka, Sashiwa, Saimoto, & Shigemasa, 1992). However, graft copolymerization of hydrophilic polymer onto chitosan chain has gained considerable attraction due to it is not only could improve the water solubility of chitosan but also maintain the fundamental skeleton of chitosan intact. Although the grafting of chitosan could change its some properties especially in the physical properties such as solubility, crystalline state, etc., it is possible to maintain some interesting inherent characteristics. Recent studies have shown that after primary deviation followed by graft modification, chitosan would obtain much improved water solubility and bioactivities such as antibacterial and antioxidant properties (Xie, Xu, Wang, & Liu, 2002). Many other attempts have also been performed on the graft copolymerization of chitosan in view of preparing polysaccharide-based advanced materials with unique bioactivities and thus widening their applications in biomedicine and environmental fields.

Amphiphilic poly(ethylene glycol) (PEG) can be easily dissolved into aqueous and organic solvents. And PEG could effectively reduce reticuloendothelial system (RES) clearance, and has been approved by Food and Drug Administration (FDA) for human intravenous, oral, and dermal applications (Chan, Kurisawa, Chung, & Yang, 2007). Due to its favorable hydrophilicity and biocompatibility, PEG has been extensively adopted as a soluble polymeric modifier in organic synthesis. Harris et al., (1984) first reported the PEGylation of chitosan with a PEG-aldehyde to yield an imine (Schiff base) that was subsequently reduced with sodium cyanoborohydride ($NaCNBH_3$). Though high degree of PEGylation could be gained, the $NaCNBH_3$ introduced into the

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product was hard to be removed thoroughly. Recently, carbodiimide as a cross-linking agent has been used to conjugate PEG-carboxylic with amino groups of chitosan chain to obtain the PEGlation of chitosan (Aktas et al., 2005; Prego et al., 2006; Rafat et al., 2008). But with the use of carbodiimide, the PEG-grafted chitosan were found to aggregate spontaneously in aqueous solution (Ouchi et al., 1998). Here, we report a novel PEGylation of chitosan via the graft copolymerization based on the MPEG macromonomer and chitosan. Compared with previous reports, the advantage of this method was attributed to the mild condition without the application of harmful organic solvent in the synthesis procedure. The obtained MPEG–CS was a diblock copolymer and could self-assembly into nanoparticles, which could be served as a controlled drug-delivery system.

2. Experimental

2.1. Materials

Chitosan (medium molecular weight) with deacetylation of 92%, acryloyl chloride, potassium persulfate (KPS) and monomethoxy poly(ethylene glycol) (MPEG, Mn = 2000) were purchased from Sigma–Aldrich (USA). Triethylamine and pre-dried 1,2-dichloromethane were obtained from Chengdu KeLong Chemicals (Chengdu, China). All other chemicals used in this work were analytical grade. Ultrapure water from Milli-Q water system was used to prepare the aqueous solutions.

2.2. Synthesis of MPEG–Chitosan diblock copolymer

The MPEG–chitosan diblock copolymer was prepared at two steps, which were shown as follows.

2.2.1. Preparation of MPEG macromonomer

Briefly, 20 g of MPEG was first dissolved in 50 ml of pre-dried dichloromethane (CH_2Cl_2) with continuous stirring. And 2–3 drops of triethylamine (TEA) were added to the above MPEG solution. Subsequently, acryloyl chloride (0.5 ml) was added dropwisely to the reaction system with continuous stirring, and the mixture was refluxed for 4 h at 40 °C. Finally, the system was evaporated to remove the solvent. After dried under vacuum, white powder product was obtained. The purified MPEG macromonomer was kept in air-tight bags prior to use.

2.2.2. Synthesis of MPEG–CS diblock copolymer

First, 1 g of chitosan was dissolved in 1% of aqueous acetic acid (100 ml) under nitrogen atmosphere. Then, 0.25 g of potassium persulfate (KPS) was added to the chitosan acetic aqueous solution and the mixture was stirred at 50 °C for 4 h under nitrogen atmosphere. Finally, the whole mixture was poured into 300 ml of acetone, and the precipitate was separated by centrifugation. The obtained precipitate was re-dissolved in distilled water and dialyzed with a dialysis membrane (MWCO = 8000–14,000) against distilled water for 3 days. The final products were obtained by freeze–drying and stored in desiccator for further use. The obtained product of MPEG–CS diblock copolymer in this paper was named MPEG–CS.

2.3. Characterization of the diblock copolymer

2.3.1. Fourier transform infrared (FTIR) measurements

The Fourier transform infrared (FTIR) spectra were detected on a NICOLET 200SXV Infrared Spectrophotometer (USA) at room temperature. All samples were cast on KBr pallet before the measurement.

2.3.2. ^{1}H Nuclear magnetic resonance analysis (^{1}H NMR)

The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were determined on Varian 400 spectrometer (Varian, USA) at 400 MHz using D_2O as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane (TMS) as an internal reference.

2.3.3. Crystallographic assay

X-ray diffraction spectrometry was obtained by using X-ray Diffractometer (DX-2000, DanDong Fangyuan Instrument Company, China) using Cu Kα radiation.

2.3.4. Thermal properties

The thermal properties of MPEG–CS and CS were characterized on a differential scanning calorimeter (DSC, NETSCZ 200, Germany). The purified and dried samples were used for DSC test. Sample was first heated from 20 to 200 °C under nitrogen atmosphere at a heating rate of 10 °C /min, and reheated to 200 °C at the same rate after quenched to 20 °C, at last sample was cooled to 20 °C again at the cooling rate of 10 °C/min.

2.4. Preparation of MPEG–CS nanoparticles by self-assembly

A calculated amount of MPEG–CS copolymer was dispersed into water under gentle agitation at 50 °C until a clear solution was obtained. The solution of self-aggregates was passed through membrane filter (pore size: 0.45 μm, Millipore) and stored at room temperature before further characterization.

2.5. Morphology study of the self-assembled nanoparticles

The morphology of self-assembled nanoparticles was observed on a transmission electron microscopy (TEM) (H-6009IV, Hitachi, Japan) as following: sample was diluted with distilled water and placed on a copper grid covered with nitrocellulose. The sample was negatively stained with phosphotungstic acid and dried at room temperature before observation.

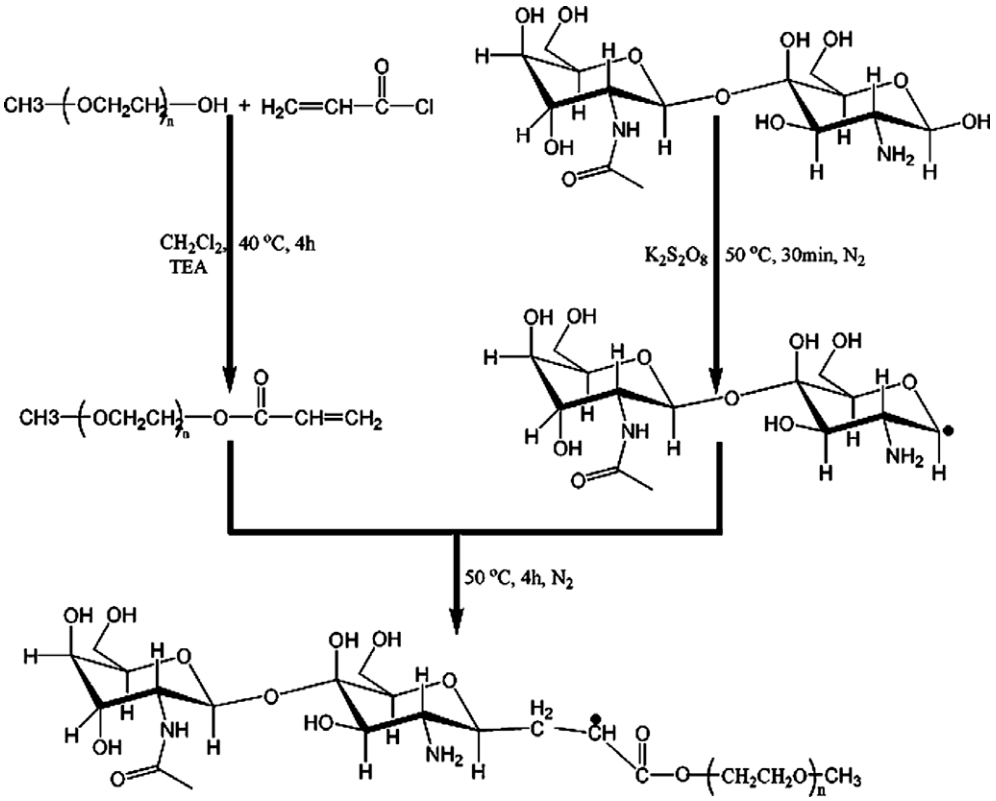
2.6. In vitro cell toxicity

The cytotoxicity of MPEG–CS nanoparticles and F127 solution was determined by MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 (HEK 293) cells/well in 0.1 ml of growth medium and incubated overnight, and then added 0.1 ml of fresh DMEM growth medium containing a series of concentrations of MPEG–CS nanoparticles or F127 solution to each well. Untreated cells in growth media were used as the blank control. Cells were incubated for 48 h and then followed by addition of 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml). After further incubation for 2–4 h, the MTT solution (0.5 mg/ml) was carefully removed from each well, and 150 μl of DMSO was added to dissolve the MTT formazan crystals. The absorbance was recorded at 570 nm by an ELISA microplate reader (Bio-Rad). The cell activity (%) was related to the control wells containing untreated cells with fresh cell culture medium and was calculated according to the following: Cell activity (%) = absorption test/absorption control × 100%. All data are presented as the mean of six measurements (±SD).

3. Results and discussion

3.1. Characterization of MPEG–chitosan diblock copolymer

MPEG–chitosan was synthesized by the free-radical reaction from MPEG macromonomer and chitosan as shown in Scheme 1. PEG was introduced into chitosan main chain to improve its hydro-



Scheme 1. Schematic representation of polymerization of chitosan and MPEG macromonomer initiated by KPS.

philicity. As the PEG was introduced into polymer, the hydrophilic PEG could form a hydrated outer shell, which protects the nanoparticles from being quickly uptaken by reticuloendothelial system (RES) (Hu et al., 2003). Here, methoxy poly(ethylene glycol) (MPEG) was selected for PEGylation of chitosan instead of PEG in order to avoid cross-linking of the polymers (Gorochovceva, Naderi, Dedinaite, & Makuška, 2005). The chemical structure of MPEG–CS diblock copolymer was characterized by the FTIR, ^{1}H-NMR, XRD and DSC, respectively.

The characteristic IR spectra of unmodified chitosan and MPEG–CS diblock copolymer are presented in Fig. 1. Chitosan spectra have been described by Mi, Sung, and Shyu (2001) and they correspond to: –CO (1660 cm^{-1}), C–O–C (1154 cm^{-1}) and –NH$_2$ (1598 and 1651 cm^{-1}), while the band at 3430 cm^{-1} belongs to the stretching vibrations of the hydroxyl groups via hydrogen bonds. The characteristic amide bands of chitosan at 1651 cm^{-1} (amide I), 1598 cm^{-1} (amide II) were clearly evident in Fig. 1. For MPEG–CS diblock copolymer, the chitosan amide peaks slightly shifted to 1644 and 1537 cm^{-1}, respectively. Meanwhile, the amide I band at 1651 cm^{-1} became less intense and was shifted to 1644 cm^{-1}, possibly due to hydrogen bonding between amide I carbonyl with PEG hydroxyl (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005).

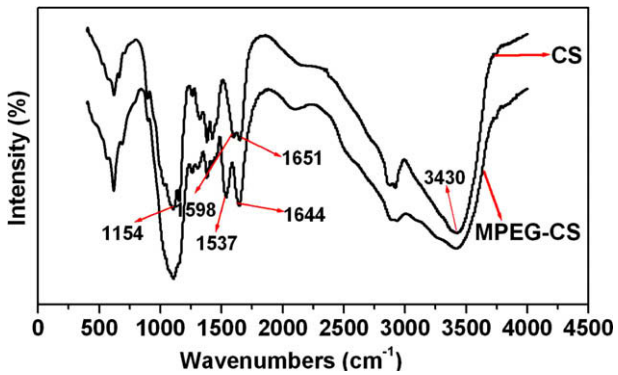


Fig. 1. FTIR spectra of chitosan and MPEG–CS diblock copolymer.

The ^{1}H-NMR spectrum of MPEG–CS diblock copolymer was presented in Fig. 2, which made further confirmation of its molecular structure. Typical peaks at 3.5–3.9 ppm ($H_{3,4,5,}$ H_6) and 3.6 ppm ($H_{11,12}$) are assigned to the ring methane and methylene protons of chitosan saccharide units and methylene groups of MPEG (Ganji & Abdekhodaie, 2008). Peaks at 3.1 ppm (H_2) and 2.1 ppm (H_7) are attributed to –CHNH$_2$ and –COCH$_3$ from chitosan. Methylene protons ($H_{9,10}$) and –OCH$_3$ (H_{13}) peaks from MPEG macromonomer appear at 2.0 ppm and 3.4 ppm, respectively. The bond between chitosan and MPEG macromonomer, which resulted from the breakage of the C–O–C bond in the main chain of chitosan, made the peak of H_1 at 4.6 ppm shift to 3.8 ppm (H_8).

The crystallographic structure of chitosan and MPEG–CS were determined by XRD. As presented in Fig. 3, the semi-crystalline chitosan exhibited a reflection peak at about 25° and a relatively weak reflection at 10°, which were assigned to two different crystal forms (Prashanth & Tharanathan, 2003). As well known, chitosan with high deacetylation degree could not be soluble in water due to its strong intermolecular hydrogen bond. For the MPEG–CS diblock copolymer, the peak associated with chitosan at 10° was absence and the reflection at about 25° also decreased significantly. This result indicated that crystalline structure has been disrupted to a great extent by the chemical bond between chitosan and MPEG in the prepared MPEG–CS diblock copolymer (Bhattarai, Matsen, & Zhang, 2005). The intermolecular hydrogen bond in MPEG–CS diblock copolymer have decreased obviously in comparison with that of chitosan, and as a result, the water solubility of the material was significantly improved (Zhu, Chen, Yuan, Wu, & Lu, 2006).

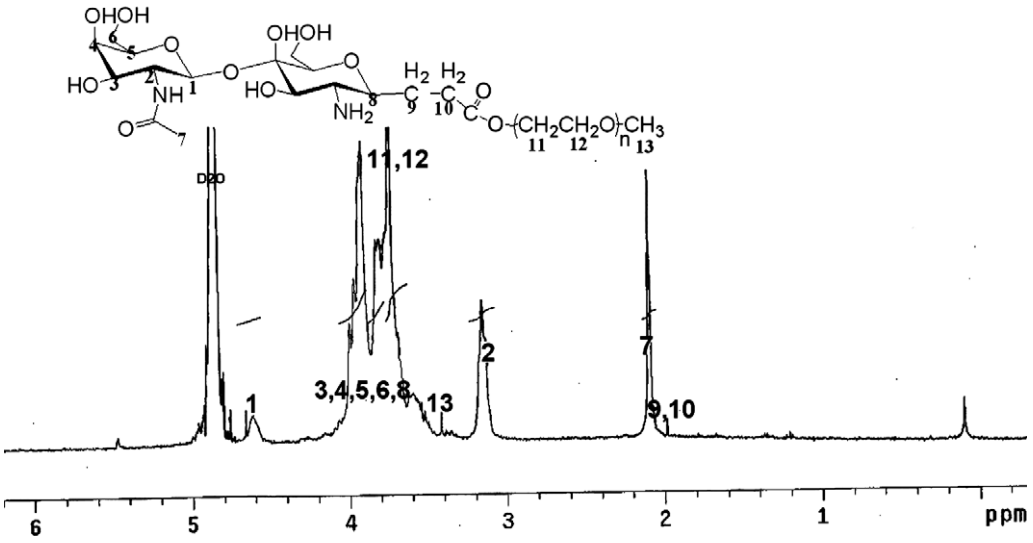


Fig. 2. ^{1}H NMR spectrum of MPEG–CS diblock copolymer in D_2O.

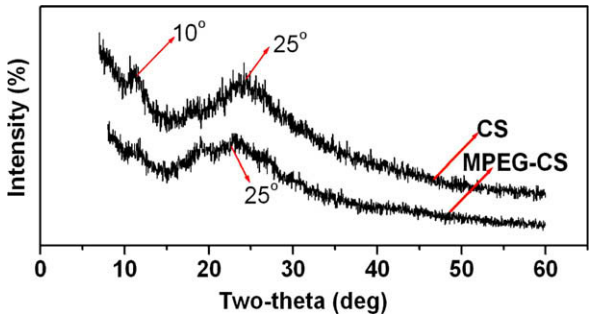


Fig. 3. XRD patterns of chitosan and MPEG–CS diblock copolymer.

The thermal properties of chitosan and MPEG–CS diblock copolymer was also studied by DSC method. Generally, chitosan does not melt or degrade between 20 and 200 °C as it indicated in the DSC curve shown in Fig. 4. Conversely, MPEG–CS diblock copolymer showed an endothermal depression at the 61 °C, which might attribute to the presence of MPEG in polymer (Ganji & Abdekhodaie, 2008). The variance in thermal properties between

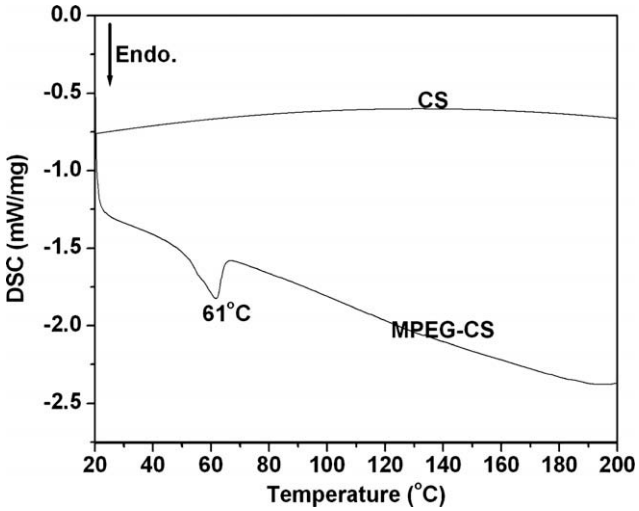


Fig. 4. DSC behavior of chitosan and MPEG–CS diblock copolymer.

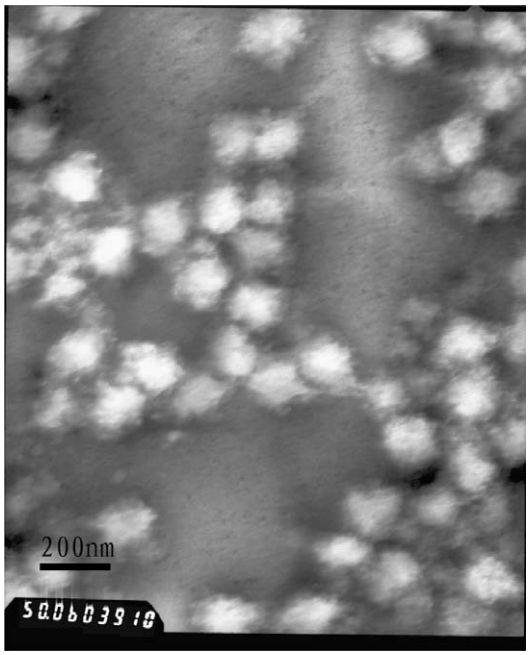


Fig. 5. TEM photograph of the self-assembled nanoparticles of MPEG–CS in aqueous solution.

CS and MPEG–CS diblock copolymer also confirmed the successful synthesis of expected product.

3.2. Preparation and characterization of self-assembled MPEG–CS nanoparticles

According to the result of FTIR and XRD analysis, it can be known that the crystal behavior of MPEG–CS diblock copolymer becomes weaker than that of chitosan, which is attributed to the decrease of the intermolecular hydrogen bond. As the result, MPEG–CS diblock copolymer is easy to be dispersed in the distilled water and form stable and transparent colloidal dispersion. A typical TEM photography of self-assembled MPEG–CS nanoparticles was shown in Fig. 5. From Fig. 5, the self-assembled nanoparticles are roughly spherical. The size of nanoparticle is about 200 nm. In

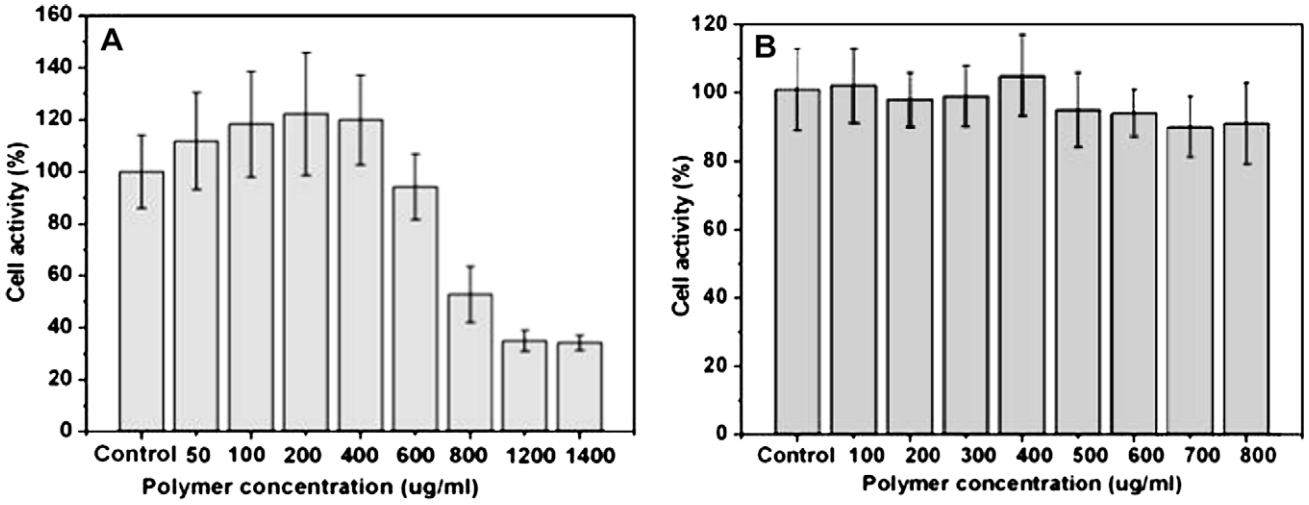


Fig. 6. *In vitro* cytotoxicity of MPEG–CS nanoparticles (A) and F-127 (B) against HEK 293 cells.

the chitosan-based aqueous system, there are electrostatic interaction, hydrophobic interaction and H-bond, which will greatly influence the solubility or assembly behaviors of chitosan derivatives (Zhu, Chan-Park, Dai, & Li, 2005, 2006). In our present system, the degree of deacetylation of chitosan is about 92%. When the obtained MPEG–CS copolymer was dispersed in aqueous solution, amino groups of MPEG–CS can not be protonated or dissociated. Therefore the electrostatic interaction is not the major factor of the self-assembly of MPEG–CS nanoparticles (Yang et al., 2008). According to the result of XRD, MPEG modification could efficiently decrease the intermolecular H-bond of chitosan, which promoted the MPEG–CS copolymer to be easily dispersed into aqueous solution. However, the remained intermolecular H-bond (XRD result) and the new hydrophobic moieties ($-CH_2CH_2-$) besides acetyl groups and glucosidic rings in chitosan inhibit MPEG–CS to be dissolved into water and form real solution. Therefore, hydrophobic interaction among the hydrophobic moieties in MPEG–CS, such as $-CH_2CH_2-$, acetyl groups and glucosidic rings perhaps also play a role in the self-aggregation of MPEG–CS nanoparticles.

3.3. In vitro cell toxicity of MPEG–CS nanoparticles

Although chitosan has been proved to be non-toxic, tissue-compatible, its new derivative should be carefully checked before it is used as biomaterials. Fig. 6A shows the dependence of the concentration of MPEG–CS colloidal dispersions on the relative cell activity of cell culture (HEK293). From Fig. 6A, we could find that it does not show the cytotoxicity against HEK293 in 48 h culture as MPEG–CS nanoparticles concentration is ranging from 0 to 0.6 mg/ml. Meanwhile, F127 which is approved by FDA for adjuvant was employed to investigate the cytotoxicity of MPEG–CS nanoparticles. According to Fig. 6B, we could find that the F127 was non-toxic as polymer concentration was smaller than 0.8 mg/ml. These findings demonstrate that an MPEG–CS nanoparticle is non-toxic, and cell-compatible. It can be safely used as the drug carrier. We will develop the MPEG–CS nanoparticles as the drug carrier not only for the hydrophilic drugs (peptides and protein) but also for the hydrophobic drugs (hydrophobic anticancer drug) in further research.

4. Conclusion

A novel amphiphilic polymer, MPEG–CS was synthesized by using potassium persulfate as a free radical initiator. The chemical structure and properties of obtained product were characterized by FTIR, H^1 NMR, XRD and DSC. The TEM photograph indicated that the synthesized MPEG–CS could self-assemble into nanoparticles in aqueous media. Due to the good cell-compatibility as shown in the *in vitro* cell toxicity test, MPEG–CS nanoparticles might be useful and safety as the drug carrier, and the further investigations as drug carriers are in progress.

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