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One-step synthesis of amino-reserved chitosan-*graft*-polycaprolactone as a promising substance of biomaterial

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ABSTRACT

One-step approach was developed to synthesize amino-reserved chitosan-*graft*-polycaprolactone (CS-*g*-PCL) by grafting ε-CL oligomers onto the hydroxyl groups of CS *via* ring-opening polymerization by using methanesulfonic acid as solvent and catalyst. The controllable grafting content of PCL within CS-*g*-PCL provided the possibility to manipulate the biodegradation rate, hydrophilicity, and hence the cytotoxicity of CS-*g*-PCL. As a result, the specimen synthesized from the feed molar ratio of glucosamine units in CS *vs.* ε-CL as 1:12 showed equivalent cytotoxicity to the neat CS and PCL against KB cell line, and the cell viability was almost close to 100%. In addition, CS-g-PCL exhibited good solubility in organic solvents, facilitating formation of PCL/CS-g-PCL blend nanofibers *via* electrospinning with the use of DMF/$CHCl_3$ as solvent. Owing to the enhanced cellular attachment results from cationic amino groups, it is promising that these copolymers are ideal substances for developing drug carriers and tissue engineering scaffolds.

1. Introduction

Chitosan (CS) is the fully or partially deacetylated derivative of chitin, the second most abundant natural polysaccharide after cellulose. Due to its biodegradability, biocompatibility, non-toxicity, and bioactivity, much attention has been paid to utilize CS and its derivatives in biomedical applications, such as drug delivery vehicles (Min et al., 2008; Son et al., 2003), gene delivery carriers (Yoo, Lee, Chung, Kwon, & Jeong, 2005), wound healing accelerator (Kweon, Song, & Park, 2003), and tissue engineering scaffolds (Bhattarai, Edmondson, Veiseh, Matsen, & Zhang, 2005; Duan, Dong, Yuan, & Yao, 2004; Ohkawa, Cha, Kim, Nishida, & Yamamoto, 2004; Yagi et al., 1997; Zhang & Zhang, 2001). It is generally believed that the nanofibrous matrixes based on CS are the ideal matrixes as tissue engineering scaffolds (Bhattarai et al., 2005; Duan et al., 2004; Ohkawa et al., 2004). In these cases, the polycationic nature of CS can promote cell attachment (Yagi et al., 2005; Zhang & Zhang, 2001). However, the structural intensity of these CS-based scaffolds can quickly become loose in an aqueous environment due to its over-hydrophility (Bhattarai et al., 2005). In addition, the poor solubility of CS in common organic solvents or in aqueous media with physiological pH is not in favor of the processing of CS-based materials, and hence limits its wide applications. Graft copolymerization of CS is an attractive way to regulate the physical properties of CS. Recently, chitosan-*graft*-poly (ethylene glycol) (Shantha & Harding, 2002), chitosan-*graft*-polylactide (Feng & Dong, 2007), chitosan-*graft*-poly(vinyl acetate) (Don, King, & Chiu, 2002), and chitosan-*graft*-poly(methyl methacrylate) (Jenkins & Hudson, 2002) have been reported, and used for a variety of purpose.

Owing to its excellent mechanical strength, biocompatibility, biodegradability, and good processability, polycaprolactone (PCL) and its copolymers have been widely used as drug delivery systems and tissue-engineering materials (Tan & Teoh, 2007; Wang et al., 2007; Williamson & Coombes, 2004). However, the hydrophobicity of PCL as well as the lack of functional groups is not in favor of cell adhesion, migration, proliferation and differentiation. Therefore, it is promising to combine CS with PCL to yield novel biosynthetic hybrid graft copolymers. This strategy suggests that the hydrophobilization of polysaccharide is possible, and the resultant polysaccharide derivatives show good biocompatibility, full-biodegradability, adequate mechanical strength, and available biofunctions.

Generally, CS-*g*-PCL copolymer is synthesized from ring-opening polymerization of ε-CL with stannous (II) octoate (Liu, Tian, & Hu, 2004), $Ti(OBu)_4$ (Luckachan & Pillai, 2006), enzymes (Fujioka et al., 2004), and 4-dimethylaminopyridine (Feng & Dong, 2006) as catalysts, where the hydroxyl or/and amine groups of CS were used as initiating species. However, it is still important to maintain

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various specific functions of the aminosaccharide units, including biological activities and polycationic nature (Muzzarelli & Peter, 1997). To achieve this goal, Fang et al. used phthaloyl-CS as an intermediate to synthesized amphiphilic cationic CS-*g*-PCL *via* a protection-grafting-deprotection route (Liu, Li, Liu, & Fang, 2004). In this case, the amino groups of CS were first protected, and only the hydroxyl groups at C-3 and C-6 were remained as active initiating points for graft modification. After grafting, the phthaloyl groups were deprotected to regenerate free amino groups.

In this work, another one-step approach was developed to synthesize amino-reserved CS-*g*-PCL by grafting ε-CL oligomers onto the hydroxyl groups of CS *via* ring-opening polymerization. Methanesulfonic acid plays a dual solvent and catalyst role in the graft polymerization process. Due to protective protonation of amine groups of CS in acidic medium, grafting of ε-CL takes place mainly on hydroxyl groups of CS. The resultant CS-*g*-PCL showed improved solubility in a variety of organic solvents, and easy to be processed into nanofibers *via* electrospinning. Owing to the negative membrane potentials of cells, it was promising that these amino-reserved CS-*g*-PCL copolymer nanofibers could generate a cationic surface to promote cell adhesion when used as tissue engineering scaffolds. Cytotoxicity tests using KB cell line (human carcinoma cell line) showed that the sample synthesized from the feed molar ratio of glucosamine units in CS *vs.* ε-CL as 1:12 was the most promising materials for tissue engineering purposes due to its relatively low cytotoxicity.

2. Experimental

2.1. Materials

Chitosan (M_w = 60 kDa, degree of deacetylation (DD) = 91%, calculated from ^{1}H NMR spectra) was purchased from Golden-shell Biochemical Co. Ltd. (Zhejiang, China), and dried in vacuum at 50 °C for 24 h before use. ε-Caprolactone (ε-CL) was purchased from Aldrich, and distilled under reduced pressure from calcium hydride. Methanesulfonic acid ($MeSO_3H$, >99.5%, Aldrich) and all other reagents were of analytical grade and used without further purification.

2.2. Cell line and culture

KB (human carcinoma cell line) was supplied from Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (Gibco-BRL, Paris, France), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah), streptomycin at 100 U/mL, and penicillin at 100 U/mL. All cells were incubated at 37 °C in humidified 5% CO_2 atmosphere. Cells were splited by using trypsin/EDTA solution when almost confluent.

2.3. Characterization

IR spectra were recorded on Fourier-transform infrared (FT-IR) spectrometer (Nicolet 6700, United States) in the range of 4000–400 cm^{-1}. CS and its graft copolymers were mixed with KBr and pressed into a slice for measurement. UV–vis spectra were recorded using a Yayan 1900PC spectrophotometer. ^{1}H and ^{13}C NMR were performed on a Bruker Avance 500 NMR spectrometer operated at 500 and 125 MHz, respectively. CS was dissolved in mixed solvents of D_2O and CF_3COOD (D_2O/CF_3COOD = 2:1 v/v). The CS-*g*-PCL and PCL were dissolved in DMSO-d_6 and $CDCl_3$, respectively. Relative molecular weights and molecular weight distributions were measured at 30 °C by gel permeation chromatography system (GPC, Agilent 1200 series) equipped with differential refractive index detector. A mobile phase of THF at a flow rate of 1 mL/min was used, and injection volume was 20 μL. Polystyrene were used as standard for molecular weight calculation. Scanning electron microscopy (SEM) images of electrospun fibers were obtained with a JSM-5610LV field emission scanning electron microscope at an accelerating voltage of 3.0 kV and viewed at magnifications between 5000 and 25,000 times than their original sizes.

2.4. Synthesis of CS-g-PCL

The CS-*g*-PCL was synthesized by grafting ε-CL monomers onto chitosan *via* ring-opening polymerization (Sashiwa et al., 2002; Skotak, Leonov, Larsen, Noriega, & Subramanian, 2008). In a typical reaction procedure, thoroughly vacuum-dried CS (1.0 g, 6.05 mmol of glucosamine units, taking into account % DD) and $MeSO_3H$ (15 mL) was charged into a flame-dried 50 mL round bottom flask equipped with a Teflon-coated stir bar and gas adaptor, and rubber septum. The mixture was stirred at 45 °C for about 30 min to allow CS to dissolve, followed by the injection of ε-CL monomer (5.93 g, 52 mmol, 8 equiv). The reaction mixture was stirred under nitrogen atmosphere at 45 °C, and the reaction process was monitored by using UV–vis spectroscopy. After the repeated occurrence of similar UV–vis absorption curves, about 4–5 h, the mixture was filtered. Filtrate was dropped into the solution containing 100 mL of 0.2 M KH_2PO_4, 16 mL of 10 M NaOH, and 50 g of crushed ice. The precipitate was collected by filtration, washed with 100 mL of distilled water several times, and then thoroughly dialyzed using Spectra/Pro membrane (MWCO = 3500) against deionized water at 4 °C for 24 h. The lyophilized product was stored at −20 °C, and labeled as CS-*g*-PCL(1:8) in a 74% yield.

Graft copolymers, CS-*g*-PCL(1:6), CS-*g*-PCL(1:12), CS-*g*-PCL(1:18), and CS-*g*-PCL(1:24) were also synthesized according to the similar procedure by using corresponding feed molar ratio of glucosamine units in CS *vs.* ε-CL (Table 1). Average number of CL units grafted onto one glucosamine unit in CS (CL_n) was calculated from following equation:

Table 1
Characterization of CS-*g*-PCL.

Sample No.	FMR[a]	Yield[b] (%)	M_w[c] ($\times 10^4$)	PDI[c]	CL_n[d]	Amino group[e] ($\times 10^{-3}$) (mol/g)
CS-*g*-PCL(1:6)	1:6	52	1.84	1.89	0.9	4.12
CS-*g*-PCL(1:8)	1:8	74	4.45	3.82	2.7	2.42
CS-*g*-PCL(1:12)	1:12	81	4.92	3.28	2.5	1.92
CS-*g*-PCL(1:18)	1:18	86	2.76	2.99	3.0	1.68
CS-*g*-PCL(1:24)	1:24	88	6.33	7.70	5.6	0.94

[a] Feed molar ratio of glucosamine units in CS *vs.* ε-CL.

[b] Yield (%) = $[(W_{CS\text{-}g\text{-}PCL})/(W_{CL} + W_{CS})] \times 100\%$.

[c] Measured by GPC, polystyrene samples were used as standard.

[d] Average number of ε-CL units per glucosamine unit in CS, $CL_n = A_{[(\alpha)/2]}/A_{[(3,4,5,6,6')/5]}$, calculated from ^{1}H NMR.

[e] Determined by ninhydrin assay.

$$\mathrm{CL}_n = A_{[(\alpha)/2]}/A_{[(3,4,5,6,6')/5]} \tag{1}$$

where $A_{[(\alpha)/2]}$ and $A_{[(3,4,5,6,6')/5]}$ represent the half of the integral area of α protons in PCL units at 2.2 ppm, and the one fifth of the total integral area of proton 3, 4, 5, 6, and 6′ of saccharine units in CS at 3.4–3.9 ppm.

2.5. Determination of amino groups in CS-g-PCL by ninhydrin assay

Amino groups in CS-*g*-PCL were examined by the ninhydrin assay according to the method described in literatures (Leane, Nankervis, Smith, & Illum, 2004; Liu, Xu, Guo, & Han, 2009). Briefly, the ninhydrin reagent was freshly prepared by adding 10 mL of lithium acetate buffer (pH = 5.2) to the solution of 0.8 g of ninhydrin and 0.12 g hydrindantin in 30 mL of DMSO. For the assay, 0.5 mL of the ninhydrin reagent was added to 0.5 mL of the suspension of CS-*g*-PCL copolymers with 1.0 mg/mL in deionized water in a glass vial. The vial were immediately capped, briefly shaken by hand and heated in boiling water for 30 min to accelerate the reaction between ninhydrin and amino groups. After cooling, 15 mL of a 50:50 mixture of ethanol and water was added to each vial. The absorbance of each solution was measured on a UV–vis spectrophotometer at 570 nm, using similarly treated water as the blank. The molar content of amino groups in the sample was obtained from a standard calibration curve, using glycine as standard.

2.6. Cell viability assay

A total of 200 mg of each sample was contacted with 5 mL of RPMI1640 cell culture medium in a 15 mL centrifuge tube and placed in a shaker incubator (37 °C, 60 rpm) for 3 days. After then, the medium was filtered with 0.22 μm sterile filter into a sterile container. Filtrate was stored in a refrigerator at 4 °C before use.

KB cells were seeded in a 96-well plate with a cell density of 1.0×10^4 per well. Cells were left in the culture incubator overnight to settle and attach to the bottom of the plate. Cell culture medium was replaced by 100 μL of the extracted leached medium from the corresponding copolymer materials. After 48 h incubation, the well was washed twice with PBS solution. A total of 10 μL of MTT solution (5 mg/mL in PBS) was added to each well. Plates were incubated for 4 h in a CO_2 cell culture incubator, MTT solution was removed, and 100 μL of DMSO was added to each well. Plates were incubated for 10 min in the incubator, and then for 15 min at room temperature. The optical density (OD) at 570 nm was measured using an automatic BIO-TEK microplate reader (Powerwave XS, USA) to evaluate the metabolic activity of the cells. Cell viability was calculated from following equation:

$$\text{Cell viability } (\%) = (\mathrm{OD}_{\text{sample}}/\mathrm{OD}_{\text{control}}) \times 100 \tag{2}$$

where $\mathrm{OD}_{\text{sample}}$ represented an OD value from a well treated with samples and $\mathrm{OD}_{\text{control}}$ from a well treated with PBS buffer only. Each experiment was carried out in triplicate. Means and corresponding standard deviations (mean ± SD) were shown as results.

2.7. Electrospinning of nanofibers

Electrospinning of nanofibers was performed by the method described by Skotak et al. (2008). The weight concentration of sample dissolved in DMF and chloroform (1:4, v/v) was set as 45%. The feed rate of spinning solution was 1.5 mL/h, and the voltage was 13 kV. A flat aluminum foil was kept 12 cm from the syringe needle tip for collecting the fibers. After electrospinning process, the fibers were dried in a vacuum at room temperature for 48 h before imaging on a JSM-5610LV field emission SEM.

3. Results and discussion

3.1. Synthesis of CS-g-PCL

To synthesize amino-reserved CS-g-PCL, PCL was grafted onto the hydroxyl groups of CS *via* the ring-opening reaction of ε-CL as shown in Fig. 1. Methanesulfonic acid was chosen as solvent and catalyst. It was promising that the grafting reaction of ε-CL predominantly occurred on the hydroxyl groups due to the protective protonation of amino groups onto CS in strong acidic media. To reduce the hydrolizablity of CS in methanesulfonic acid, thoroughly vacuum-dried CS was used as raw material. In this case, grafting reaction was initiated at rather mild temperature of 45 °C due to the enough activity of methanesulfonic acid as catalyst. After 15 min, the light yellow to dark brown color change and viscosity increase in the reaction mixture was observed. Moreover, the growth of side chains was assumed to be completed after 4–5 h until the repeated occurrence of similar UV–vis absorption (Fig. 2). In order to avoid excessive heat release and the degradation of polymer chains, an excess of cold buffer solution containing 100 mL of 0.2 M KH_2PO_4, 16 mL of 10 M NaOH, and 50 g of crushed ice was used to neutralize methanesulfonic acid. The buffering properties of the monobasic phosphate ion avoid rapid and uncon-

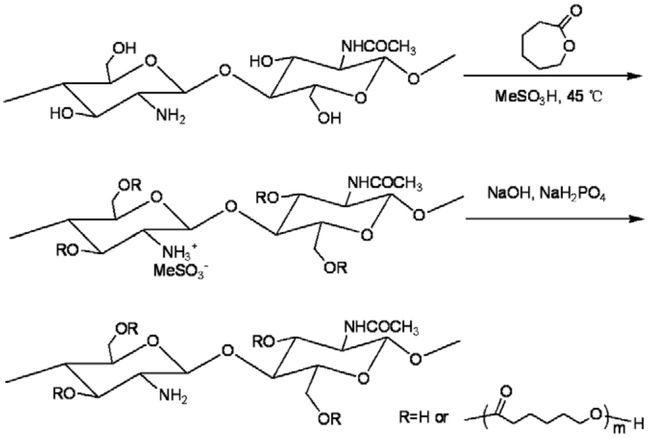

Fig. 1. Synthesis scheme of CS-*g*-PCL.

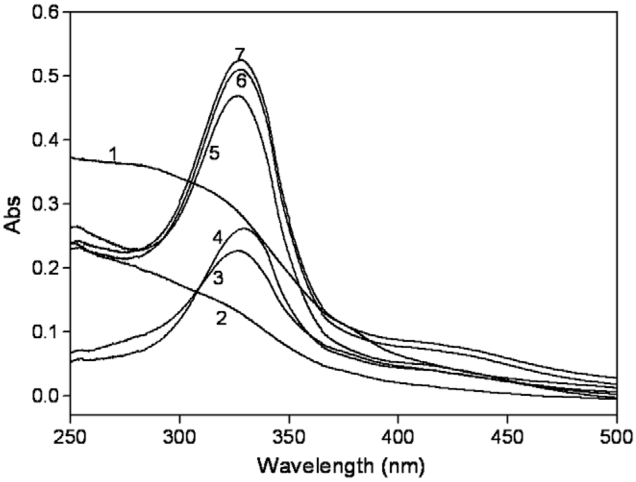

Fig. 2. Ex situ reaction screening of CS-*g*-PCL(1:8) by UV–vis spectroscopy.

trolled pH changes during the quenching of the reaction. After dialysis in ice-cold water, the CS-*g*-PCL coploymers were lyophilized as light-yellow solid powders. The CS-*g*-PCL copolymers with various molecular weights, shown in Table 1, were synthesized by varying the feed molar ratio. With a change in feed molar ratio (glucosamine units in CS *vs.* ε-CL) from 1:6 to 1:24, the yield and CL_n of CS-*g*-PCL copolymers showed a tendency to increase from 52% to 88% and 0.9 to 5.6, respectively. The molecular weights of the CS-*g*-PCL copolymers showed changeful character with the increase of feed molar ratio from 1:6 to 1:24, and ranked at CS-*g*-PCL(1:6) < CS-*g*-PCL(1:18) < CS-*g*-PCL(1:8) < CS-*g*-PCL(1:12) < CS-*g*-PCL(1:24). Due to grafted PCL chains, all the CS-*g*-PCL copolymers showed good solubility in common organic solvent, such as DMF, acetone and chloroform, etc., facilitating the formation of nanofibers *via* electrospinning.

3.2. Chemical structure of CS-g-PCL

The chemical structures of the CS-*g*-PCL copolymers were verified by FT-IR and NMR spectra. As shown in Fig. 3, CS showed the characteristic bands of saccharine structure at 890 and 1147 cm^{-1}. The absorbance of amide I band and amino groups in CS was shown at 1664 and 1616 cm^{-1} (Pearson, Marchessault, & Liang, 1960; Velde & Kiekens, 2004), respectively. The characteristic absorbance of ester in PCL was shown at 1734 cm^{-1}. All the CS-*g*-PCL copolymers presented absorbance bands at 1734, 1665, and 1617 cm^{-1}, which were assigned to the characteristic bands of ester in PCL, amide I band, and amino groups in CS, respectively. This implied the success of grafting PCL onto CS, along with the reservation of amino groups in CS. The ^1H NMR spectra of CS, PCL, and CS-*g*-PCL were shown in Fig. 4. The peaks at 3.4–3.9, 3.2, and 2.05 ppm were assigned to $H_{3,4,5,6,6'}$, $H_{2'}$, and H_7 of pyranose repeat units in CS, respectively. The peaks of H_α, $H_{\beta,\delta}$, H_γ, and H_ε in PCL were shown at 2.2, 1.6–1.7, 1.4, and 4.15 ppm, respectively. The chemical structure of CS-*g*-PCL was clearly confirmed by its ^1H NMR spectrum, due to the presence of the characteristic peaks of CS and PCL (Yu, Wang, Chen, Deng, & Jing, 2006). The chemical structure of CS-*g*-PCL was further characterized by ^{13}C NMR (Fig. 5). The peaks appeared at around 24–35 and 61 ppm was assigned to the methylene of PCL side chains. Several peaks belonging to C_6, C_3, C_5, and C_4 of CS were observed at 60–78 ppm while the characteristic peak of CS acetal carbon (C_1) and carbonyl carbon of PCL located at 101 and 173 ppm, respectively (Guan, Quan, Shuai, Liao, & Mai, 2007; Liu, Wang, Shen, & Fang, 2005).

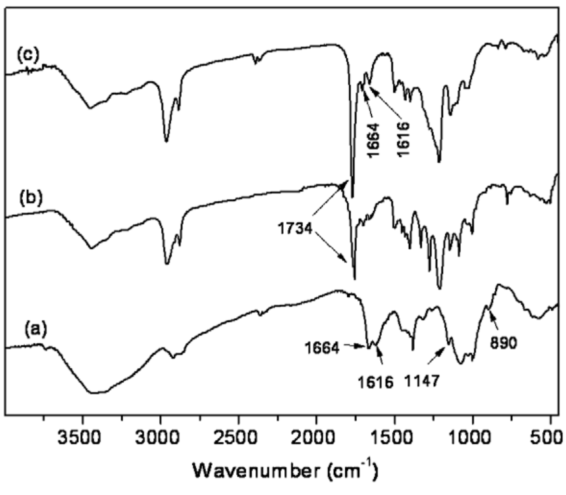

Fig. 3. FT-IR spectra of (a) CS; (b) PCL and (c) CS-*g*-PCL(1:12).

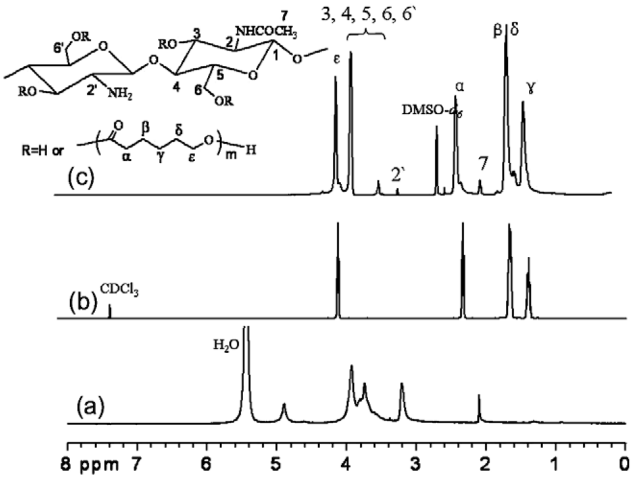

Fig. 4. ^1H NMR of (a) CS, (b) PCL and (c) CS-*g*-PCL(1:12).

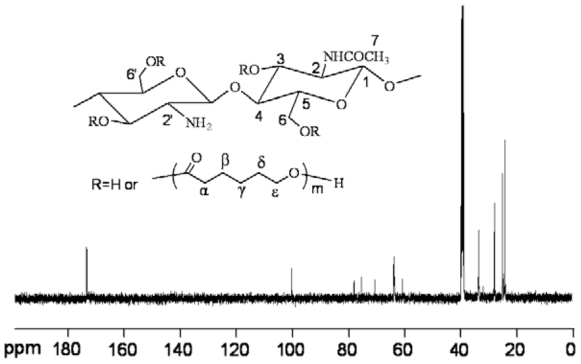

Fig. 5. ^{13}C NMR of CS-*g*-PCL(1:12).

3.3. Determination of amino groups in CS-g-PCL

As mentioned above, amino groups are a beneficial issue when CS was used as tissue engineering scaffold. To confirm the presence of amino groups in CS-*g*-PCL copolymers, ninhydrin assay was performed. As shown in Table 1, all the graft copolymers possess ami-

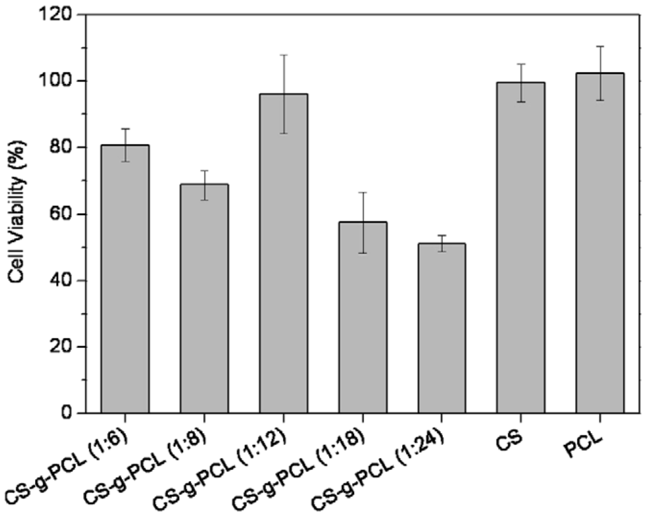

Fig. 6. Cell viability of KB cell line incubated with the extracted leached media of CS-*g*-PCL (mean ± SD, *n* = 3).

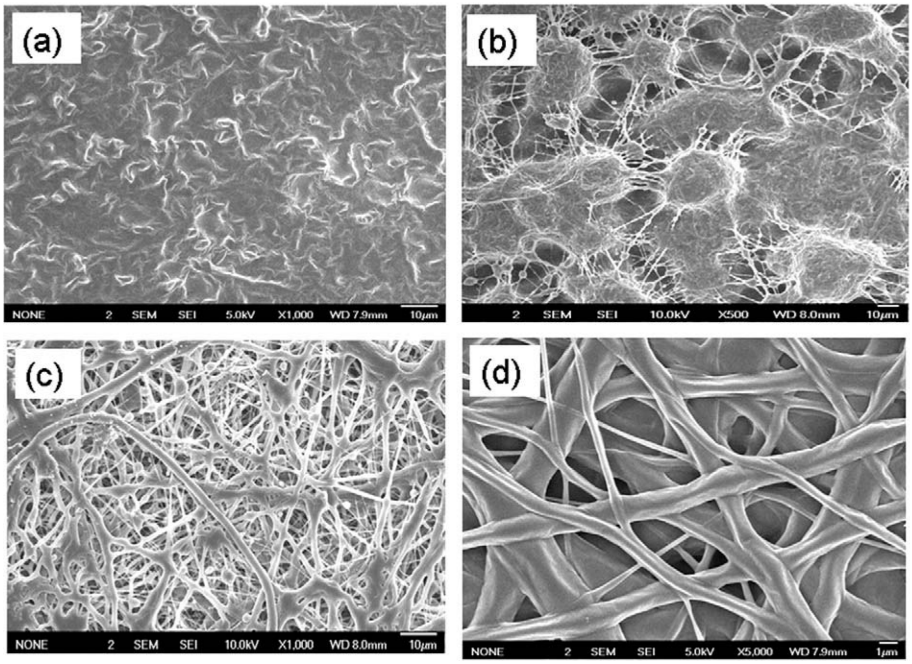

Fig. 7. SEM imagines of electrospun fibers, (a) CS-*g*-PCL(1:12), (b) CS-*g*-PCL(1:12)/PCL = 9:1, (c) CS-*g*-PCL(1:12)/PCL = 7:3, and (d) CS-*g*-PCL(1:12)/PCL = 5:5.

no groups. It suggested the satisfactory one-pot synthesis of amino-reserved CS-*g*-PCL by means of methanesulfonic acid as solvent and catalyst. Furthermore, with a change of feed molar ratio from 1:6 to 1:24, the contents of amino groups in the CS-*g*-PCL copolymers decrease from 4.12×10^{-3} to 9.4×10^{-4} mol/g.

3.4. Cell viability assay

To know the cytotoxicity of CS-g-PCL copolymers, the *in vitro* cell viability of their extracted leached media from corresponding copolymers was evaluated with KB cell line by MTT method, and native CS and PCL were used as controls. As shown in Fig. 6, CS and PCL showed almost non-toxicity to KB cell line, and their cell viability were above 100%. Compared with CS and PCL, all the copolymers showed some toxicity to KB cell line. The order of CS-g-PCL(1:6) < CS-g-PCL(1:8) < CS-g-PCL(1:24) < CS-g-PCL(1:12) was ranked according to their cell viability. It is worth of noting that the cell viability of CS-g-PCL(1:12) was above 98%, equivalent to that of native CS and PCL, which indicated its great potential as tissue engineering scaffolds.

3.5. Electrospinning of nanofibers

Owing to its rather lower cytotoxicity and good solubility in organic solvent, CS-*g*-PCL(1:12) copolymer was selected to electro spin nanofibers as a potential tissue-engineering materials. Our preliminary attempts were to fabricate CS-g-PCL(1:12) nanofibers *via* electrospinning by using DMF and chloroform as mixing solvents over a broad concentration range. However, only "tailed" micro- and nanoparticles were observed. It suggested that CS-g-PCL(1:12) itself was very difficult to electrospin. To obtain a nanofibrous mat, PCL was used as a suitable partner to prepare nanofibers containing CS-*g*-PCL(1:12). Same as previous report (Zhou et al., 2008), the morphology of electrospun nanofibers (presence or absence of defects, shape of the fibers) was strongly influenced by the composition of the spinning solution. Fig. 7b showed that the jet solution was spinnable when the weight ratio of CS-*g*-PCL(1:12) *vs.* PCL was 9:1. With an increase of the PCL content in the blends, the number of spindles among the fibers decreased while the ability of fiber shaping improved (Fig. 7c). When the weight ratio of CS-*g*-PCL(1:12)/PCL reached up to 5:5, the bead-on-string morphology disappeared, and homogeneous smooth fibers were produced (Fig. 7 d).

4. Conclusion

Amino-reserved CS-*g*-PCL copolymers were synthesized with facile one-step manner *via* ring-opening polymerization of ε-CL onto CS by using methanesulfonic acid as solvent and catalyst. Owing to its low cytotoxicity, polycationic nature and excellent electrospinnability when blended with PCL, CS-*g*-PCL (1:12) showed great potential as tissue engineering scaffold materials.

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