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Carbohydrate Polymers 66 (2006) 274–279
Carbohydrate Polymers
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Synthesis and characterization of *N*-succinyl-chitosan and its self-assembly of nanospheres

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Received 21 February 2006; received in revised form 6 March 2006; accepted 8 March 2006
Available online 10 July 2006

Abstract

A simple and novel method was developed to synthesize successfully the *N*-succinyl-chitosan (NSCS). The NSCS was characterized by FTIR, ^{1}H NMR, element analysis and XRD. NSCS can be self-assembly of well-dispersed and stable nanospheres in distilled water. The morphology of the nanosphere was investigated by transmission electron microscope (TEM) and shows 50–100 nm in diameter. The steady-state fluorescence spectroscopy indicates that the hydrophobic domain has been formed within these NSCS nanospheres. The assembly mechanisms are believed to be the intermolecular H-bonding of NSCS and hydrophobic interaction among the hydrophobic moieties in NSCS macromolecules. The in vitro cell culture indicates that NSCS has non-toxicity and cell-compatibility. Our results demonstrate that NSCS has great potential in the drug controlled release delivery.

Keywords: Chitosan; *N*-Succinyl-chitosan; Self-assembly; Nanosphere

1. Introduction

Conventional drug therapy has relied on the use of small molecules based on reversibility at the site of action. However, biotechnology has provided, and will do so even more in the future, new types of medical agents like peptides, proteins and DNA for different types of new therapies, e.g., as vaccines and in gene therapy. Such substances call for new types of delivery systems, e.g., for inhalation, for injections, and perhaps also for oral administration.

Increasing attention has been shifted to designing polymeric drug carriers that are degradable, non-toxic, tissue-compatible, and can be chemically modified if so desired. Chitosan is one of the candidates, which possesses these characteristics.

Chitosan is a natural, biodegradable polysaccharide derived from chitin. A co-polymer of glucosamine and *N*-acetyl-D-glucosamine (NAG), the polymer is considered chitosan if the ratio of glucosamine to NAG is greater than 1. In the field of drug delivery, chitosan has been used in the form of tablets, gels, micro- and nanoparticles (Gupta & Ravi Kumar, 2000; Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001; Leong et al., 1998; Lim, Martin, Berry, & Brown, 2000). In the recent years, chitosan microspheres and beads have been investigated as drug delivery systems for anticancer drug or protein (Gupta & Ravi Kumar, 2000; Hari, Chandy, & Sharma, 1996; Lim et al., 2000). Many approaches have been developed to prepare the chitosan beads including water in oil method (Fwu-Long, Chih-Yang, Shin-Shing, Sung-Tao, & Shon-Foun, 2000; Onishi, Shimoda, & Machida, 1996), emulsion-droplet coalescence technique (Tokumitsu, Ichikawa, & Fukumori, 1999), microemulsion (Andersson & Löfroth, 2003), emulsion cross-linking technology (Genta, Costantini, Asti, Conti, & Montanari, 1998) and spray drying process (He, Davis, & Illum, 1999). Usually, these preparation procedures are complex and need to use some organic solvents or surfactants. In addition, by these techniques, the beads are not appropriate for the routes of

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doi:10.1016/j.carbpol.2006.03.014

administration; For example, vein injection due to their large size (larger than 2 mm) (Fwu-Long et al., 2000; He et al., 1999; Onishi et al., 1996).

To overcome these drawbacks, many works have been done. Mao et al. (2001) reported CS–DNA nanoparticles prepared by coacervation of CS and DNA in acidic solution. The size of the CS–DNA nanoparticles was in the range of 100–250 nm, and such nanoparticles can protect the encapsulated plasmid DNA from nuclease degradation. Calvo, Remunan-Lopez, Vila-Jato, and Alonso (1997) developed a kind of hydrophilic chitosan–polyethylene oxide nanoparticle prepared by the ionic interaction between positively charged CS and negatively charged polymer-tripolyphosphate (TPP), and the nanoparticles showed a great protein loading capacity and sustained release ability. Hu et al. designed chitosan (CS)–poly (acrylic acid) (PAA) complex nanoparticles prepared by template polymerization of acrylic acid (AA) in chitosan solution and showed a continuous release of the entrapped silk peptide (SP) (Hu et al., 2002).

As with most of the biopolymers, chitosan is an amphiphilic macromolecule in which the proportion between acetylated and deacetylated residues (DA) is responsible for the balance between hydrophilic and hydrophobic interaction (Schatz, Pichot, Delair, Viton, & Domard, 2003). Chitosan with high DAs can not soluble in water for the strong intermolecular hydrogen bonding (H-bond), however some modification can occurs on the –OH and $–NH_2$ positions of chitosan to improve its water solubility (Chen & Park, 2003; Lillo & Matsuhiro, 1997; Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982; Xie, Xu, Wang, & Liu, 2002). Some of these chitosan derivatives have aggregation behavior in dilute aqueous solution and can be used as the matrix of anticancer drug (Zhu, Chan, Dai, & Li, 2005, 2006). The physicochemical and biomedical properties and final biomedical applications depend strongly on the chemical structure of chitosan derivatives.

In the present work, we report a simple and novel approach to synthesize the *N*-succinyl-chitosan (NSCS) with well-designed structure. NSCS can self-assembly in regular nanospheres in distilled water. The NSCS nanospheres are obtained spontaneously under very mild conditions without the need of high temperature, organic solvent, surfactant and some other special experimental technology; NSCS has non-toxicity and cell-compatibility. Herein, we demonstrate a potential chitosan-based drug matrix for the controlled released delivery.

2. Experimental

2.1. Materials

Chitosan powder was supplied by Lianyungang Biologicals Inc., China, which has a deacetylation degree of 90% and viscosity average molecular weight of 20,000 Da. All commercially available solvents and reagents were used without further purification.

2.2. N-Succinyl-chitosan (NSCS) synthesis

One gram chitosan was dissolved into 200 ml of 1 wt% HAc solution and then transferred into a flask. Succinic anhydride (0.2 g) was dissolved in acetone (20 ml), and added into the flask by drop-wise for 30 min at room temperature, and then the reaction was allowed for 4 h at 40 °C. The reaction mixture was cooled to room temperature. The mixture precipitated in an excess of acetone, filtered to remove the solvent and then washed with 70%, 80%, and 100% acetone, respectively. Finally, the product was dried at 40 °C under vacuum for 24 h. The obtained white powder *N*-succinyl-chitosan (NSCS) was 1.1 g.

2.3. Characterization

The Fourier transform infra red (FTIR) spectrum of NSCS was recorded using a Bio-Rad FTS 6000 spectrometer at room temperature using KBr pallet. The ^1H NMR spectra were determined on a Bruker ACF 300 spectrometer using D_2O as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference. Transmission electron microscopy (TE CHAI-12) (philips) was used to observe the morphology of the NSCS nanospheres. Samples were placed onto copper grill covered with nitrocellulose. They were dried at room temperature, and then were examined using a TEM with being negative stained. Element analysis (Perkin-Elmer 2400 Series II) was used to characterize the element composition. X-ray diffraction spectrometry was obtained by using XD-3A powder diffraction meter with CuKa radiation in the range of 5–40° (2θ) at 40 kV and 30 mA.

2.4. Steady-state fluorescence spectroscopy

Steady-state fluorescence spectra were recorded on a Shimadzu RF 5301 spectrometer equipped with a quartz fluorescence cell. Pyrene, from an acetone stock solution of 1×10^{-3} M, was used as a micropolarity sensitive probe in a final concentration of 6.0×10^{-7} M in water. The excitation wavelength was set to 335 nm. The changes in the intensity ratio of the first and the third vibronic band (I_1/I_3), namely 373 and 384 nm (I_1 andI_3, respectively) in the emission spectra were used to detect the shift of the hydrophobic microdomain.

2.5. In vitro cell toxicity

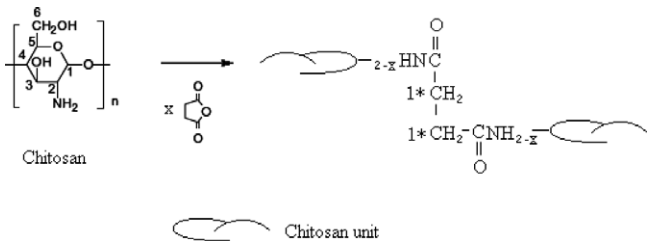
Fibroblast cells (3T3) (NIH/3T3) (ACTT CRL-1658) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum supplemented

with 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C under a 5% CO_2 containing atmosphere. Cells were seeded into 96-well plate at 5000/well and grown to 50% confluence. NSCS colloidal dispersions with 0.5 mg/ml concentration were diluted with OPTI-MEM I (serum-reduced medium, Gibco). The growth medium was removed from the 96-well plate and 100 μl of above diluted solution was added and incubated at 37 °C under a 5% CO_2 atmosphere. Then 100 μl of growth medium containing 20% FBS was added to each well, and incubated at 37 °C under a 5% CO_2 atmosphere. After incubation, 20 μl combined MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/PMS (phenazine methosulfate) solution (Promega) was added to each well. After incubating the plate for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere, the absorbance of each well at 490 nm was recorded by using a 96-well plate reader (Bio-Rad Labs). Six samples were used, and this in- vitro experiment was performed in twice independently.

3. Results and discussion

3.1. The synthesis and characterization of NSCS

The synthesis of NSCS was shown in Scheme 1. Fig. 1 shows the FTIR spectra of (a) chitosan, (b) NSCS. From



Scheme 1. Synthesis of NSCS.

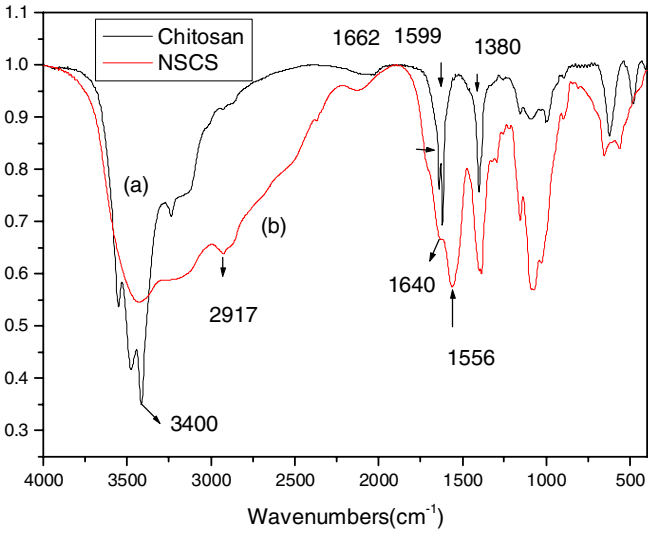


Fig. 1. FTIR spectra of (a) chitosan, (b) NSCS.

the chitosan spectrum, it was found that distinctive absorption bands appear at 1662 cm^{-1} (Amide I), 1599 cm^{-1} (–NH_2 bending) and 1380 cm^{-1} (Amide III). The absorption bands at 1156 cm^{-1} (asymmetric stretching of the C–O–C bridge), 1075 and 1033 cm^{-1} (skeletal vibration involving the C–O stretching) are the characteristics of its saccharine structure. Compared with that of chitosan, 2917 cm^{-1} appears (stretching of –CH_2–), the peaks at 3400 and 1599 cm^{-1} (amino group characteristics) decrease greatly, and the peak at 1656 cm^{-1} (Amide I) and 1380 cm^{-1} (Amide III) increase, these results indicated the succinyl derivation reaction took place at the N-position (Scheme 1) and –NH–CO– groups have been formed.

The ^{1}H NMR spectrum of the NSCS was given in Fig. 2. The ^{1}H NMR assignments of NSCS was as follows: ^{1}H NMR (D_2O) $\delta = 2.95$ (H_2); $\delta = 3.\ 51$–3.82 (H_1,H_3, H_4, H_5, H_6); $\delta = 2.3$ (H 1*) (labeled in Scheme 1). According to the ratio of the integral peak of H 1* of NSCS and H_2 in chitosan structure, it can be known that the substitution degree (x) in the Scheme 1 is 0.95. This result indicates that 0.95 "H" in amino group has been substituted by succinyl, and the new bonds formed in the NSCS macromolecules are almost –NH–CO– structure. The ^{1}H NMR result confirms the FTIR result.

Elemental analysis was also done to further characterize the composition of NSCS. Found: C, 41.23%; H, 7.13%; and N 5.8%, respectively. From elemental composition, it can be calculated the substitution degree (x) of succinyl, which is 96.5% and is good consistent with that of ^{1}H NMR result.

According to the results of elemental analysis, FTIR, ^{1}H NMR, the suggested chemical structure of *N*-succinyl-chitosan was confirmed and the substitution degree (x) in Scheme 1 is near 1.

It is well known that chitosan with high DAs can not be soluble in water because of the strong intermolecular hydrogen bonding (H-bond). X-ray diffraction spectra of chitosan and its derivative (Fig. 3) show that chitosan exhibits two reflection fall at $2\theta = 11°$, $2\theta = 20°$. Samuels et al. reported that the reflection fall at $2\theta = 11°$ was assigned to crystal form I and the strongest reflection appears at $2\theta = 20°$ which corresponds to crystal forms II. Therefore, chitosan has half-crystal property (Samuels, 1981). However, with the *N*-succinyl substitution, there is only one broad peak at around $2\theta = 20°$ in the XRD spectrum of NSCS. This result indicates that crystal forms have been destroyed in NSCS macromolecules. This result suggests that intermolecular hydrogen bondings (H-bonds) in NSCS have been greatly decreased in comparison with that of chitosan. As a result, the solubility of NSCS is better than that of chitosan, NSCS can be disperse into distilled water and obtain a transparent and stable system. From the broad peak at around $2\theta = 20°$ in the XRD spectrum of NSCS (Fig. 3(b)), which is indicated that there are still weak intermolecular H-bonds existed in NSCS.

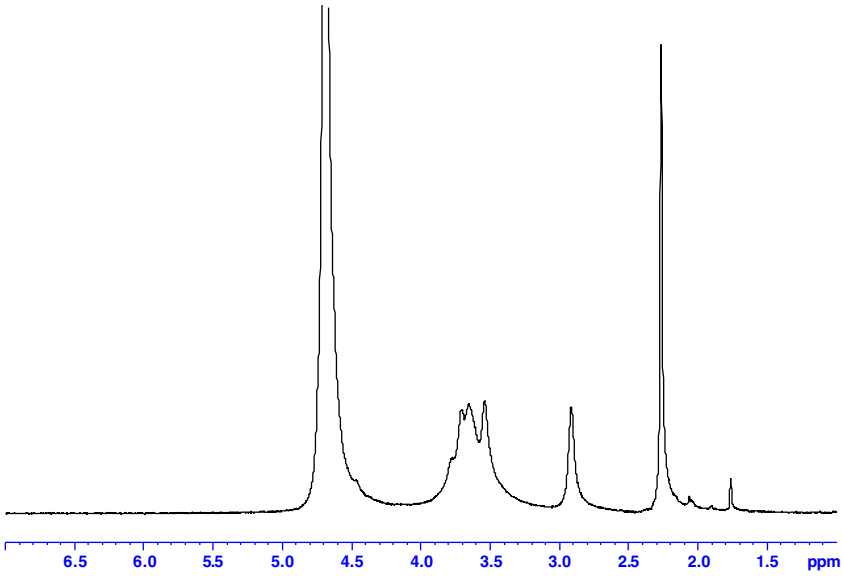


Fig. 2. ^{1}H NMR spectra of NSCS.

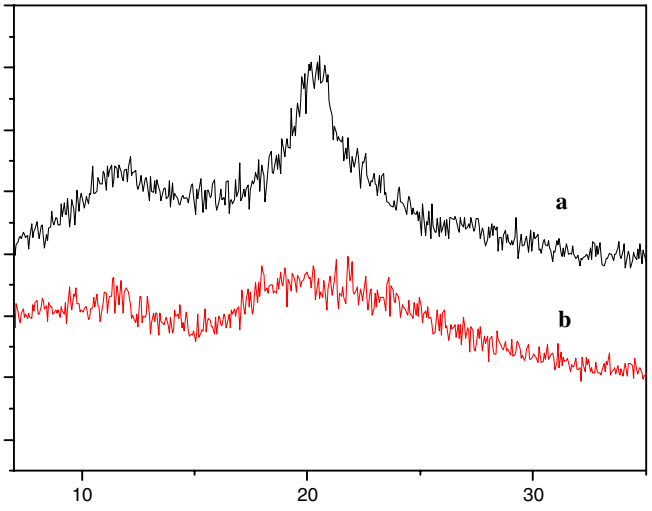


Fig. 3. WAXD patterns of (a) chitosan, (b) NSCS.

3.2. Physicochemical properties

3.2.1. Self-assembly behavior

From XRD analysis, it can be known that the crystal behavior of NSCS becomes weaker than that of chitosan, which is due to the decrease of the intermolecular H-bond. As a result NSCS is easy to disperse in the distilled water and obtain the stable and transparent colloidal dispersion. Fig. 4 shows the transmission electron microscopy (TEM) morphology of the colloid of NSCS, Fig. 4(b) is the magnification of Fig. 4(a). From the Fig. 4, it can be known clearly that NSCS colloids show the regular sphere morphology. The size of the NSCS nanospheres was in the range of 50–100 nm. This result indicates that NSCS can self-assembly in narrow-distributed nanospheres in distilled water. Different from the other technologies (Andersson & Löfroth, 2003; Calvo et al., 1997; Genta et al., 1998; Hu et al., 2002; Onishi et al., 1996), herein, we demonstrate a very simple and novel method to obtain chitosan-based nanospheres without the need of high temperature, organic solvent, surfactant and some other special experimental technology.

3.2.2. Self-assembly mechanism

In the chitosan-based aqueous system, there are electrostatic interaction, hydrophobic interaction and H-bond, which will influence the solubility or assembly behaviors of chitosan derivatives (Zhu et al., 2005). In present system, the amino groups are transformed into –NH–CO– groups, and thus there are little groups that can protonated or dissociated in distilled water. Therefore, the electrostatic interactions are not the main factor of the self-assembly of NSCS. It is the decrease of the intermolecular H-bond that promotes NSCS to disperse into distilled water. 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 NSCS to dissolve into water and form real solution. Therefore, the driven force to induce NSCS self-assembly of nanospheres in distilled water are the weak intermolecular H-bonding because of the –NH–CO– and –OH groups along the NSCS macromolecular chains and hydrophobic interaction among the hydrophobic moieties in NSCS, such as –CH_2CH_2, acetyl groups and glucosidic rings.

3.2.3. Hydrophobic microdomain formation

As it is well known, the proportion between acetylated and deacetylated residues (DA) in chitosan is responsible for the balance between hydrophilic and hydrophobic interactions. When the degree of deacetylation of chitosan is as high as 90%, the hydrophilic interaction is

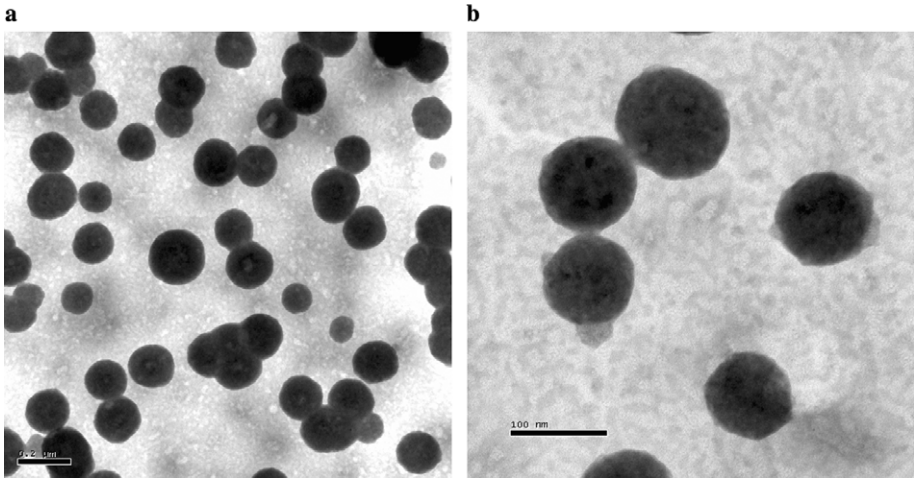


Fig. 4. TEM micrograph of NSCS nanosphere (0.5 mg/ml NSCS colloidal dispersion).

strong, the new hydrophobic moieties (–CH_2CH_2–) in NSCS increase the hydrophobic property in comparison with that of chitosan. These interesting chemical structure and self-assembly properties of NSCS motivate our interest to investigate in the loading of hydrophobic molecules into NSCS nanospheres, which is important for further development the potential application of NSCS as a matrix for loading not only the hydrophilic drugs but also the hydrophobic drugs. To attain this aim, the NSCS nanospheres dispersions were further examined by steady-state fluorescence spectroscopy using pyrene as a fluorescence probe. Fig. 5 shows the fluorescence emission spectra of pyrene incorporated into NSCS in distilled water at 25 °C. The excitation wavelength was 335 nm. If the hydrophobic microdomains are formed in aqueous system, the hydrophobic pyrene preferably lies in these hydrophobic microdomains to increase its solubility in dispersion. In the fluorescence spectra, the intensity ratio of the first and the third vibrational band is sensitive to the circumstance of the pyrene molecules. When the pyrene is dissolved into the NSCS nanospheres, the total emission intensity will increases, which indicates the increase in the pyrene concentration in NSCS colloids. In addition, the value of I_1/I_3 drastically decreases. The I_1/I_3 value is 1.72 for pyrene in water, and the value decreases to 1.54 at 0.050 mg/ml of NSCS. However, the I_1/I_3 value does not decrease with the increasing of the NSCS concentration (The data is not shown). This result indicates that there is not a critical assembly concentration (CAC) for the NSCS colloidal dispersions but the hydrophobic domains in NSCS nanospheres do form. The hydrophobic domains within NSCS nanospheres should be composed of hydrophobic moieties, such as –CH_2CH_2–, acetyl groups and glucosidic rings in NSCS. The present result demonstrates that NSCS can load hydrophobic molecules.

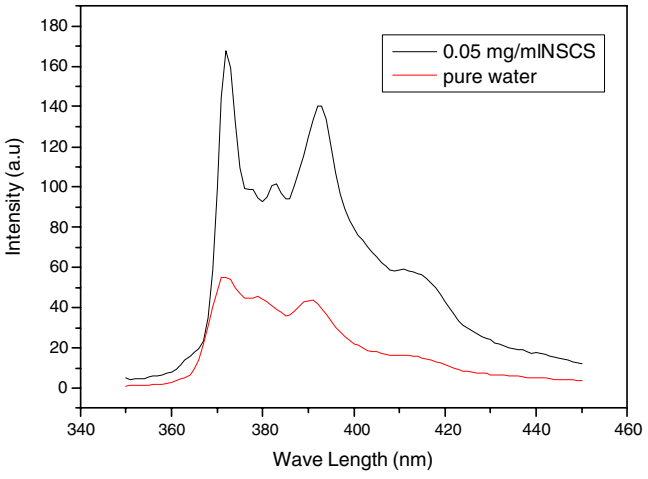


Fig. 5. Effect of NSCS colloidal dispersion on the fluorescence emission spectra of pyrene 6.0×10^{-7} M in water and 0.05 mg/ml of NSCS. The excitation wavelength was 335 nm.

3.3. Cell toxicity

Although chitosan has been proved to have non-toxic, tissue-compatible, its new derivative should be carefully checked before it is used as biomaterials. Fig. 6 shows the dependence of the concentration of NSCS colloidal dispersions on the relative cell activity for a different period of cell culture. From this figure, generally speaking, in the range of 0–0.25 mg/ml of NSCS nanospheres, it does not show the

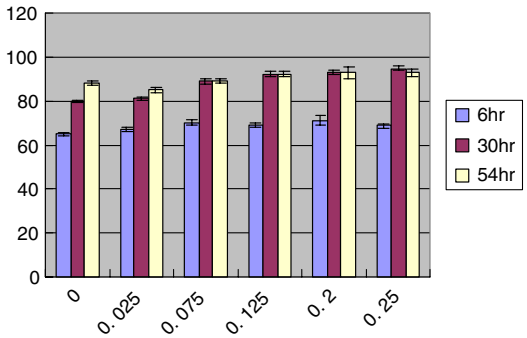


Fig. 6. The dependence of the concentration of NSCS colloidal dispersion on the relative cell activity for a different period of cell culture.

bad effect on the activity of 3T3 fibroblasts. There is little effect on the relative cell activity after short period of cell culture (6 h), however, prolonging the period of cell culture to 30 or 54 h, the higher concentration of NSCS shows higher relative cell activity than that control sample (without adding NSCS). These findings demonstrate that NSCS is non-toxic, and cell-compatible. It can be safely used as the drug matrix. In our future work, we will develop the NSCS nanospheres as the drug matrix not only for the hydrophilic drugs (peptides and protein) but also for the hydrophobic drugs (hydrophobic anticancer drug).

4. Conclusions

In this study, a novel biocompatible chitosan derivative, *N*-succinyl-chitosan (NSCS), with well-designed structure, has been successfully synthesized. NSCS can self-assembly of regular nanosphere morphology in distilled water. The mechanism of self-assembly of NSCS in distilled water are believed to be the intermolecular H-bonding and hydrophobic interaction among the hydrophobic moieties such as $-CH_2CH_2-$, acetyl groups and glucosidic rings in NSCS. Within the nanospheres, there are the hydrophobic domains formed. NSCS has non-toxic and cell-compatible properties. All these results suggest that NSCS nanosphere has great potential to be used as a novel drug matrix in the controlled released delivery.

Acknowledgement

This research was supported by a Natural & Scientific grant of Jiangsu Province (Project No. 05KJB430149. China).

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