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Synthesis and evaluation of N-succinyl-chitosan nanoparticles toward local hydroxycamptothecin delivery

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ABSTRACT

A new N-succinyl-chitosan derivative (NSC), which could be self-aggregated to form nanoparticles in distilled water, was synthesized by microwave irradiation and characterized by FTIR, element analysis, XRD and TEM. Hydroxycamptothecin (HCPT) used as a modal drug was successfully entrapped into the NSC nanoparticles. The size of NSC nanoparticles and HCPT-loaded NSC (NSCH) nanoparticles were around 30 and 200 nm, respectively. The drug entrapment efficiency of NSCH reached up to 68.5% and the release of HCPT was biphasic with an initial burst effect followed by a subsequent slower release. In vivo studies, the NSCH nanoparticles showed tumor targeting and significant suppression of tumor growth after s.c. injection (close to the tumor) to mice bearing S180 sarcoma tumor. A histopathological analysis of the tumor tissues indicated that NSCH had a lethal effect on the sarcoma cell. The results indicated that NSC nanoparticles had potential as a local sustained delivery system for hydrophobic antitumor drug.

1. Introduction

Chitosan[(1,4)-2-amino-2-deoxy-D-glucan], a linear biopolyaminosaccharide obtained by N-deacetylation of chitin, is an abundant organic materials, which has drawn much attention for its advantageous properties such as biocompatibility, biodegradability (Alves & Manoa, 2008; Kumar et al., 2004) and antibacterial activity, therefore leading to the possibility of synthesizing a variety of chemically modified derivatives for specific uses.

N-succinyl-chitosan has been synthesized by traditional heating method and demonstrated to be safe (Izume, 1998; Song, Onishi, & Nagai, 1993) and to be available as a macromolecular drug carrier showing very long-term retention in the systemic circulation (Kamiyama, Onishi, & Machida, 1999; Kato, Onishi, & Machida, 2000). The conjugates of mitomycin C (MMC) with N-succinyl-chitosan shows good antitumor activities against various tumors such as murine leukaemias (L1210 and P388), B16 melanoma, Sarcoma 180 solid tumor, murine liver metastatic tumor (M5076) and murine hepatic cell carcinoma (MH134) (Kato, Onishi, & Machida, 2004).

Microwave-assisted organic synthesis (Biswal et al., 2007; Takano, Ishikawa, Kamitakahara, & Nakatsubo, 2007), an alternative method compared with conventional heating technique, has been proved to be more rapid and efficient (Cao, Ge, & Lai, 2001; Shao, Yang, & Zhong, 2003). Considerable efforts have been devoted to investigate the microwave-assisted reaction in the synthesis and modification of nature polymers, such as ester-cellulose (Satge et al., 2002), starch acetates (Shogren & Biswas, 2006) and O-alkyl-chitosan.

In the present work, an original N-succinyl-chitosan derivative (NSC) was achieved successfully by microwave irradiation, which can be self-aggregated in distilled water, forming stable nanoparticles after probe sonication; in addition, HCPT was successfully entrapped within the NSC nanoparticles with high drug entrapment efficiency, furthermore, the HCPT-loaded nanoparticles were evaluated based on the in vitro characteristics and in vivo antitumor effects.

2. Experiment

2.1. Materials

Chitosan (CHI), with a deacetylation degree of over 90% and viscosity average molecular weight of 3×10^5D, was obtained from Zhejiang Aoxing Biotech, Ltd., China. Hydroxycamptothecin (HCPT) was supplied by Wuhan Li Shizhen Pharmaceutical Co., Ltd., China. Succinic anhydride was supplied by Sinopharm Chemical Reagent Co., Ltd., China. Dimethyl sulfoxide (DMSO) and other chemicals were analytical reagent purchased from Shanghai Chemical Reagent Co., China.

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2.2. Synthesis of N-succinyl-chitosan derivative (NSC)

Quantitive succinic anhydride and chitosan (0.5 g) with mol ratio of 1:1 were successively dissolved in 10 ml DMSO in a vessel, which was then put into the microwave reaction equipment (ChemPower, produced by Shanghai Zhizhun Sci. Instrument Co., Ltd.) to react according to our optimized procedures (60 °C, 1.5 h, 1200 r/min). The product was washed three times with acetone and vacuum dried at 60 °C for 30 min.

2.3. Preparation of NSC nanoparticles and NSCH nanoparticles

NSC nanoparticles were prepared by sonication. Briefly, water-insoluble NSC was first dispersed in distill water for 24 h by gentle shaking, and then sonicated using a probe type sonifier (Ningbo Scientz Biotechnology Co. Ltd., China) at 50 W for 2 min (with 5-s interval after every 5-s sonication) in an ice bath. The sonication step was repeated five times. The suspension was filtered through a filter (0.8 μm) and collected by ultracentrifugation.

NSCH nanoparticles were prepared by dialysis. Briefly, NSC nanoparticles (0.5 g) and HCPT (0.05 g) was co-mixed in 10 ml DMSO. Then the suspension was placed into a 10 ml dialysis bag (MWCO: 14,000) for dialysis against 100 ml distilled water for 12 h at room temperature. The obtained suspension in dialysis bag was centrifuged at 10,000 rpm for 30 min and vacuum dried at 40 °C for 1 h.

The drug entrapment efficiency was determined indirectly by measuring the amount of free HCPT using UV/vis spectrometer at 390 nm in the supernatant recovered after ultracentrifugation and the outer solution of dialysis bag. The drug entrapment efficiency was expressed as percentage of the HCPT difference between the initial amount of HCPT and the free amount in the supernatant and outer solution of dialysis bag relative to the total amount used for the nanoparticles preparation.

2.4. Characterization of NSC and NSCH

The Fourier transform infra-red (FTIR) spectrum of NSC was recorded at room temperature on a Nicolet AVATR 360 spectrometer. The sample was in the form of a KBr pellet. Elemental analysis (Vario EL III) was used to detect the composition of NSC and chitosan. X-ray diffraction spectrometry of NSC was obtained by using Philips PANalytical X'Pert powder diffraction meter with CuK_α radiation. Transmission electron microscopy (TEM, Tecnai F30) was used to observe the morphology and size of the NSC and NSCH nanoparticles.

2.5. Drug release study

The in vitro release of HCPT from the NSCH nanoparticles was measured in PBS at pH 7.4 by dialysis method. Briefly, NSCH nanoparticles (10 mg) were suspended in 10 ml of PBS at pH 7.4 and the suspension was injected into a dialysis bag of 3500 molecular weight cut-off. This mixture was diluted with 50 ml of the medium. And then incubated at 37 °C and shaken horizontally at 72 rpm. Aliquots of the reservoir were removed periodically for assaying the drug released. In a typical test, 0.5 ml of the buffer solution was removed and 0.5 ml of fresh buffer solution was added to maintain a constant volume of the release medium. All release tests were run in triplicate.

2.6. In vivo imaging and fluorescence microscopy experiment

The tumor targeting of NSCH nanoparticles was investigated by in vivo imaging system (CF Pro/F, Carestream Health Co. Toronto, Canada) and fluorescence microscopy (Axiovert 200, Zeiss, Germany). S180 sarcoma tumor model was constructed by the animal service of antitumor center of Xiamen University, China. After subcutaneous injection of 0.2 ml 10 mg/ml NSCH nanoparticles close to the tumor, sarcoma bearing mice was narcotized and positioned on an animal plate at normal temperature before being placed into the imaging chamber for scanning. The setting of count time was optimized at 2 min per point. λ_{ex} (excitation wave) 400 nm was set to excite HCPT molecules, so λ_{em} (fluorescence emission wave) at 535 nm was collected and detected.

For visualizing NSCH nanoparticles within the tumor, the tumor tissue was excised from mice and rinsed with PBS, fixated with 4% formaldehyde in PBS, dehydrated with ethanol, embedded in paraffin blocks, sectioned (3 μm) and observed by fluorescence microscopy.

2.7. In vivo evaluation of antitumor efficiency of NSCH nanoparticles

The antitumor efficiency of NSCH nanoparticles was investigated by injected subcutaneously (close to the tumor) into mice bearing S180 sarcoma tumor. Four-week old male mice (18–22 g body weight) were provided by the animal service of antitumor center of Xiamen University, China. Murine S180 sarcoma was injected intraperitoneally into mice. After about 7 days, ascites with ivory white could be extracted. Ascites containing about 5×10^6 sarcoma cells were injected subcutaneously between the shoulder blades of the experimental mice. The tumors were allowed to grow for 6 days. The highly metastatic mice models of S180 sarcoma tumor were obtained. All the mice were divided into three groups with six mice each. Groups 1, 2 and 3 (control group) were injected subcutaneously with 0.3 ml of 10 mg/ml NSCN nanoparticles, NSC nanoparticles and physiological saline respectively. Day 10 was selected to allow the tumor growing into an easily measurable size within the ethical limit. All tumor tissues were harvested and weighed. The efficacy of the NSCH for tumor growth regression in mice was evaluated with inhibiting tumor growth rate expressed as percentage of the average tumor weight difference between the control group and the experimental group. The cytotoxicity of NSCH in S180 sarcoma was assessed by measuring the necrosis number of S180 sarcoma cell in tumor tissue 10 days post-injection with pathological examination. Tissue specimens were rinsed with PBS, fixated with 4% formaldehyde in PBS, dehydrated with ethanol, embedded in paraffin blocks, sectioned (3 μm) and stained with hematoxylin–eosin (H&E).

3. Results and discussion

3.1. The synthesis and characterization of NSC

The synthetic scheme of NSC was presented in Fig. 1. Succinic anhydride could reacted with the hydroxyl and amino groups of chitosan in the presence of DMSO under microwave irradiation, resulting in formation of the derivative. Fig. 2 showed the FTIR spectra of CHI and NSC. From the CHI spectrum, it was found that distinctive absorption bands of CHI appeared at 3440 cm^{-1} (the combination of stretching of –OH– and –NH–), 1630 cm^{-1} (υ(C=O), Amide I) and 1380 cm^{-1} (υ(C–N), Amide III). Compared with that of chitosan, the peaks at 1642 cm^{-1} (υ(C=O), Amide I) 1570 cm^{-1} (δN–H, Amide II) and 1380 cm^{-1} (υC–N, Amide III) increased in the NSC spectrum, these results indicated that the succinyl derivation reaction took place at the N-position and –NH–CO– groups have been formed. Meanwhile, the appearance of the peaks around 1700–1740 cm^{-1} suggested that the reaction occurred at the O-position resulting in the formation of –C–O–CO– groups.

Elemental analysis showed that the C/N ratio of NSC and chitosan were 8.34 and 6.22, respectively. The elemental composition, also confirmed the hypothesis that the reaction occurred between hydroxyl group and acid anhydride group in accord with synthetic scheme of NSC (Fig. 1). According to the results of elemental analysis and FTIR, the suggested chemical structure of N-succinyl-chitosan was confirmed.

X-ray diffraction spectra of chitosan and its derivatives (Fig. 3) showed that chitosan exhibited two reflection peaks at $2\theta = 11.6^\circ$ (crystal form I) and $2\theta = 20^\circ$ (crystal form II) (Samuels, 1981). However, the peak at $2\theta = 6^\circ$ emerged for the NSC, indicating a new crystal form was produced, which might be due to the connection of succinic anhydride bridge.

3.2. The morphology and size of NSC and NSCH nanoparticles

From Fig. 4(a), it could be known clearly that NSC nanoparticles showed spherical morphology, of which the size was about 30 nm. In the present system, the amino groups were transformed into –NH–CO– groups, and thus there were few groups that dissociated in water. Therefore, the electrostatic interactions were not the main factor in the formation of self-aggregated NSC. The decrease in the intermolecular H-bonding promoted NSC to dispersion in distilled water. However, the remaining intermolecular H-bond and the new hydrophobic moieties ($-CH_2-O-CO-CH_2-CH_2-$) and glucosidic rings in chitosan inhibited complete dissolution of NSC in water to form a real solution. Therefore, NSC self-aggregated to form nanoparticles in distilled water after probe sonication should be induced by the weak intermolecular H-bonding between the –NH–CO– and –OH groups and hydrophobic interaction among the hydrophobic moieties in NSC. From Fig. 4(b), it was seen that after loading of HCPT, the size of new nanoparticles grew to around 200 nm demonstrated that HCPT was successfully entrapped into the NSC nanoparticles. The entrapment efficiency of drug reached up to 68.5%. It was well known that chitosan is a liner biopolyaminosaccharide, however, after cross-linking by succinic anhydride, the chitosan derivative contained both hydrophobic and hydrophilic domain and could be self-aggregated in distilled water after probe sonication to form stable nanoparticles, in which the hydrophobic drug HPTC was entrapped. The present result indicated that the NSC could be loaded with a hydrophobic antitumor drug.

3.3. Drug release

The in vitro release profiles of HPCT from the NSCH nanoparticles were monitored as a function of time. The amount of MMC released at pH 7.2 medium was 20.76% over 24 h, the burst effect was mainly due to the unencapsulated drug on the surface of nanoparticles. Following the initial rapid release phase, NSCH nanoparticles exhibited a controlled HPCT release, a cumulated drug release over 11 days was 75.26%, afterward, a much slow drug release phase was observed. We suspected that the controlled drug released from these nanoparticles via diffusion through the interconnecting channels and pores formed within the nanoparticles, and the release of drug was associated with formation of channels and pores and they decreased in number and size as the size of nanoparticles decreased, resulting in subsequent slower release rates of the drug.

3.4. Targeting to tumor tissue in vivo

Fig. 5(a–d) showed the time (corresponding to 12, 14, 20, 30 min)-correlated HCPT fluorescence photos. It was clearly to see a strong tumor targeting character of NSCH nanoparticles, seen as the decrease of the fluorescence intensity at the injected region and the increase at the tumor tissue. Moreover, HCPT fluorescence within the tumor tissue become apparent (seen from Fig. 6), these observations suggested that the NSCH nanoparticles tumor targeting after local injection and displayed its great potential as a local drug delivery system for the treatment of tumor. The strong tumor targeting character of NSCH nanoparticles are mainly due to passive targeting to tumor sites that is attributed to high vascularization and enhanced permeability of tumor blood vessels combined with limited lymphatic clearance of nanoparticles from the tumor environment.

3.5. The antitumor efficiency of NSCH nanoparticles

The efficacy of NSCH nanoparticles for reducing tumor growth in mice was evaluated in Table 1. The results showed that the group treated with NSCH nanoparticles had an inhibition rate of up to 52.72% ($P < 0.01$). In addition, the mice experienced a slight weight loss as the day of the administration, however, the weight loss was recovered in the following 2 days and the relative average body weight of all groups was above 20 g at 10 days after inoculation. Since the tumor weight in groups treated with NSCH nanoparticles was less than 7% of the initial body weight, the increase in body weight should not be attributed solely to the growth of tumor and implied that NSCH nanoparticles were well-tolerated at the tested dose levels.

Representative microscopic images of paraffin sections of tumor tissue harvested after 10 days of administration of NSCH nanoparticles were shown in Fig. 7. Pathological examination indicated that NSCH nanoparticles resulted in significant S180 sarcoma cell necrosis in tumor tissue at the tested dose levels, while the NSC nanoparticles showed no or little lethal effect to S180 sarcoma cell.

4. Conclusion

In this study, a novel N-succinyl-chitosan derivative has been successfully synthesized. Compared with the untreated chitosan, the NSC derivative could be self-aggregated in distilled water after probe sonication, imbibe hydrophobic drug HPTC and form stable nanoparticles. The NSCH nanoparticles showed a tumor targeting and apparent antitumor effect after s.c. injection (nearby tumor) to mice bearing S180 sarcoma tumor. The results of this work suggested that the NSC nanoparticles could be a promising candidate for local hydrophobic antitumor drug delivery.

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References

Alves, N. M., & Manoa, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. *International Journal of Biological Macromolecules*, *43*, 401–414.

Biswal, J., Kumar, V., Bhardwaj, Y. K., Goel, N. K., Dubey, K. A., Chaudhari, C. V., et al. (2007). Radiation-induced grafting of acrylamide onto guar gum in aqueous medium: synthesis and characterization of grafted polymer guar-g-acrylamide. *Radiation Physics and Chemistry*, *76*, 1624–1630.

Cao, Z. Y., Ge, H. C., & Lai, S. L. (2001). Studies on synthesis and adsorption properties of chitosan cross-linked by glutaraldehyde and Cu (II) as template under microwave irradiation. *European Polymer Journal*, *37*, 2141–2143.

Izume, M. (1998). The application of chitin and chitosan to cosmetics. *Chitin and Chitosan Research*, *4*, 12–17.

Kamiyama, K., Onishi, H., & Machida, Y. (1999). Biodisposition characteristics of N-succinyl-chitosan and glycol-chitosan in normal and tumor-bearing mice. *Biological & Pharmaceutical Bulletin*, *22*, 179–186.

Kato, Y., Onishi, H., & Machida, Y. (2000). Evaluation of N-succinyl-chitosan as a systemic long-circulating polymer. *Biomaterials*, *21*, 1579–1585.
Kato, Y., Onishi, H., & Machida, Y. (2004). N-succinyl-chitosan as a drug carrier: Water-insoluble and water-soluble conjugates. *Biomaterials*, *25*, 907–915.
Shao, J., Yang, Y. M., & Zhong, Q. Q. (2003). Studies on preparation of oligoglucosamine by oxidative degradation under microwave irradiation. *Polymer Degradation and Stability*, *82*, 395–398.
Satge, C., Verneuil, B., Branland, P., Granet, R., Krausz, P., Rozier, J., et al. (2002). Rapid homogeneous esterification of cellulose induced by microwave irradiation. *Carbohydrate Polymers*, *49*, 373–376.
Shogren, R. L., & Biswas, A. (2006). Preparation of water-soluble and water-swellable starch acetates using microwave heating. *Carbohydrate Polymers*, *64*, 16–21.
Samuels, R. J. (1981). Solid state characterization of the structure of chitosan films. *Journal of Polymer Science Polymer Physics*, *19*, 1081–1105.
Song, Y., Onishi, H., & Nagai, T. (1993). Conjugate of mitomycin C with N-succinyl-chitosan: In vitro drug release properties, toxicity and antitumor activity. *International Journal of Pharmaceutics*, *98*, 121–130.
Takano, T., Ishikawa, J., Kamitakahara, H., & Nakatsubo, F. (2007). The application of microwave heating to the synthesis of 6-amino-6-deoxycellulose. *Carbohydrate Research*, *342*, 2456–2460.