Open Access Library Journal

Endostatin-Encapsulated N-Succinyl-Chitosan Nanoparticles Inhabited the Growth of MCF-7 Cell

Yanrong Fan*, Ye Li, Xuefei Xia

Nanjing University of Science and Technology, Nanjing, China
Email: *yr_fan@sina.com

Received 18 November 2015; accepted 5 December 2015; published 10 December 2015

Abstract

A simple method was developed to synthesize the N-succinyl-chitosan (NSC) successfully. NSC was characterized by FTIR, ^{1}H NMR and element analysis. The NSC can form nanoparticles with TPP through electrostatic interaction. The morphology, particle size and zeta potential of the nanoparticle were investigated and showed a 100 - 200 nm in diameter with a positive potential 9.99 mv. The encapsulation efficiency of NSC-ES-NPs was 94.76 ± 0.05. The *in vitro* cell culture indicates that NSC has non-toxicity and cell-compatibility. NSC-ES-NPs show antitumor activity against MCF-7 cell. Our results demonstrate that NSC has great potential in the drug controlled release delivery.

Keywords

N-Succinyl-Chitosan, Nanoparticle, Endostatin

Subject Areas: **Biological Materials, Nanometer Materials**

1. Introduction

Nowadays, chemotherapy is still one of the most important treatments available for cancer diseases. But severe side-effects of chemotherapy and finite efficiency sometimes limited the widely application of chitosan [1]. Therefore, interests have been focus on the idea of developing drug delivery systems more and more frequently to overcome this critical issue [2]. Nanoparticles have become attraction for which can provide a controlled and targeted way to deliver the encapsulated antitumor drugs and results in high efficacy with low side effects [3].

Chitosan is the deacetylated product of chitin, the second most abundant natural polysaccharide next to cellulose [4]. As a non-toxic, biodegradable, biocompatible and novel natural resource material with antibacterial and

*Corresponding author.

How to cite this paper: Fan, Y.R., Li, Y. and Xia, X.F. (2015) Endostatin-Encapsulated N-Succinyl-Chitosan Nanoparticles Inhabited the Growth of MCF-7 Cell. *Open Access Library Journal*, **2**: e2083. http://dx.doi.org/10.4236/oalib.1102083

antitumor activities, chitosan is also easily modified owing to the -OH and NH_2 positions [5]. N-succinyl-chitosan (NSC), synthesized via introduction of succinyl groups at the N-position of the glucosamine unit of chitosan, is a water-soluble derivative with the same bioactivity of chitosan. Many reports focused their attention on the application of NSC as a carrier for protein, peptide and gene in cancer therapy [6] [7].

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors [8]. In malignant tissue growth, angiogenesis is also stimulated by VEGF. As a factor that expression has been found to be high in various malignant tumors, it serum levels correlate with disease stage and prognosis in breast cancer, nasopharyngeal carcinoma, colorectal cancer, prostate cancer and non-Hodgkin's lymphoma [9]-[11]. It has been reported that high serum VEGF levels in cancer patients predict less effectual chemotherapy and poorer prognosis [12].

Angiogenesis inhibitors for the treatment of cancer have now been approved by the FDA in the US and in 28 other countries [13]. Endostatin, the 20 kD internal fragment of the carboxyl terminus of collagen XVIII, was first identified in the conditioned media of hemangioen-dothelioma cells as an antiangiogenic molecule in 1997 by O'Reilly *et al*. [14]. Endostatin causes tumor regression by acting an inhibitor of endothelial cell proliferation and migration and inducing apoptosis in proliferating endothelial cells [15]. Furthermore, endostatin may also down-regulate vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling by a direct action on tumor cells or up-regulate other antiangiogenic genes [16] [17].

In the present study, we investigated the antitumor effects of N-succinyl-chitosan nanoparticles carried endostatin (NCS-ES-NPs) against MCF-7 cell. Our results suggest that NSC combined with endostatin shows a significant Inhibition efficiency on MCF-7 cell.

2. Materials and Methods

2.1. Materials

Chitosan with a deacetylation degree of over 85% and viscosity average molecular weight of 2×10^5 D, was obtained from Shanghai Bio Science & Technology, Co. Ltd., China. After deacetylation and degradation, chitosan was obtained with a DD of over 97% and viscosity average molecular weight of 2×10^4 D. Succinic anhydride was supplied by Aladdin Industrial Inc., China. FITC, BCA kit for Protein Determination and Polymer-tripolyphosphate (TPP) was supplied by Sigma Chemical Co. Endostatin was supplied by Jiangsu Engineering Research Center for Gene Pharmaceutical. Pyridine and other chemicals were analytical reagent purchased from Shanghai Shengbao Chemical Industrial Co., China.

2.2. Synthesis of N-Succinyl-Chitosan

1 g chitosan was dissolved into 10 ml of 2% wt% HAc solution, after dissolved, 20 ml EtoH was added to precipitating chitosan. Succinic anhydride (2 g) was dissolved in acetone (30 ml), and added into the reaction system by drop-wise for 30 min at ambient temperature, and then the reaction was allowed for 4 h at ambient temperature. The mixture was precipitated in an excess of acetone, filtered to remove the solvent and then repeated several times. Finally, the product was dried at 60˚C under vacuum for 24 h. The obtained white powder N-succinyl-chitosan (NSC) was 1.4 g.

2.3. Synthesis of FITC-Labeled N-Succinyl-Chitosan

The synthesis of FITC-labeled chitosan was based on the reaction between the isothiocyanate group of FITC and the primary amino group of chitosan. Dehydrated methanol (10 mL), followed by 2.0 mg/mL of FITC in methanol (10 mL), was added into 1% w/v NSC in 0.1 M acetic acid solution (10 mL). After 3 h of reaction in the darkat ambient temperature, the FITC-labeled chitosan was precipitated in 0.2 M NaOH. To remove unconjugated FITC, the precipitate wassubjected to repeated cycles of washing and ultrafiltration (7500 g for 15 min) until no fluorescence was detected in the supernatant (Perkin-ElmerLS-5B, Beaconsfield, Buckinghamshire, UK, λexc 490 nm, λemi 520 nm) and dialyzed in distilled water for 3 days under darkness before freeze drying.

The labeling efficiency (% w/w FITC to FITC-labeled N-succinyl-chitosan) was determined by fluorescence measurements against FITC standard solutions. FITC-labeled N-succinyl-chitosan dissolved in 0.1 M acetic acid. The standard curve of FITC solutions was calibrated with standard solutions of 0.001 to 0.005 mg/mL of FITC prepared by diluting 2 mg/mL methanolic solutions of FITC with methanol.

2.4. Uptake Studies

MCF-7 cells plated at a density of 4×10^3 cells/well in Multiwell 96-well plates, were used for uptake studies when they formed confluent monolayers. Dosing solutions consisted of freshly prepared FITC-labeled N-succinyl-chitosan solution and diluted with the medium to give equivalent concentrations of 75 to 200 μg/mL. The dosing solutions were adjusted to pH 6.2 with 1 M NaOH. Uptake was initiated by exchanging the transport medium with 0.2 mL of specified dosing solution and incubating the cellsat 37˚C for 24 h to 48 h (each group seted a control group adding the same dosing of NSC). The experiment was terminated by washing the cell monolayer three times with PBS and lysing the cells with 20 μL of RIPA Lysis Buffer. Cell-associated chitosan was quantified by analyzing the cell lysate in a fluorescence platereader calibrated with standard solutions containing 2 to 14 μg/mL of FITC-labeled N-succinyl-chitosan in a cell lysate solution (control group cells dissolved in 20 μL of RIPA Lysis Buffer). Uptake was expressed as the amount (μg) of chitosan associated with unit weight (mg) of cellular protein. The protein content of the cell lysate was measured using the BCA protein assay kit.

2.5. Preparation of NSC Nanoparticles and NSC-ES Nanoparticles

NSC nanoparticles were prepared by ionic interaction. Briefly, 0.25 g NSC was dissolved into 50 ml of 2% wt% HAc solution, filtered by sand core funnel, then obtained clear NSC/HAc (5 mg/ml) solution. 20 ml mixture solution was transferred into a flask. 12 ml TPP (2 mg/ml) was added into the flask by drop-wise for 12 min, and stirred at ambient temperature for 3 min.

Endostatin was loaded by covalent interaction. Briefly, 10 ml endostatin was transferred into a flask. 5 ml NSC was slowly added into the flask by drop-wise while stirring. After 5 min stirring, the product was obtained.

The drug entrapment efficiency was determined indirectly by measuring the amount of free endostatin using BCA protein assay kit in the supernatant recovered after ultracentrifugation using a Millipore (Mw = 15,000). The drug entrapment efficiency was expressed as percentage of the endostatin difference between the initial amount of endostatin and the free amount in the supernatant relative to the total amount used for the nanoparticles preparation.

2.6. Characterization

^{1}H NMR experiments were recorded using a Unity Inova 300 spectrometer (Varian, Palo Alto, California) in D_2O with 10% of CF_3COOD. The Fourier transform infra-red (FTIR) spectrum of NSC was recorded using a Nicolet lS-10 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. Particle size and zeta potential of nanoparticles were measured by laser Doppler anemometry using a Zetasizer Nano ZS instrument (Malvern, Zetasizer 3000 HS, UK).

2.7. Cell Culture

NIH-3T3 cell, a standard fibroblast cell line and MCF-7 cell, a human breast cancer cell line were obtained from Shanghai cell bank of Chinese Academy of Sciences. All cells were cultivated at 37˚C under humidified 5% CO_2 in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The medium was replenished every 1days, and cells were subcultured after reaching confluence.

2.8. Cytotoxicity Assay

NIH-3T3 cell were seeded in 96-well tissue culture plates at a density of 4×10^3 cells per well in DMEM medium containing 10% FBS. The cytotoxicity of NSC was evaluated by determining the viability after 48 - 72 hours' incubation with various concentrations of polymers (25 - 500 μg of polymer per milliliter). The number of viable cells was determined by estimation of their mitochondrial reductase activity using the tetrazolium-based colorimetric method (MTT conversion test).

2.9. Antitumor Effect

MCF cell were seeded in 96-well tissue culture plates with the same condition of NIH-3T3 cell. After 24 h incubation, the initial medium was abandoned, 100% of DMEM medium mixed with NSC-ES was added to incubating the cell. Set various concentrations of NSC-ES, each group set negative control (only added 100% of

DMEM) and positive control (added same concentrations of ES and 100% of DMEM). The cytotoxicity of NSC was evaluated by determining the viability after 48.72 hours' incubation using MTT conversion test.

2.10. Statistical Analysis

All data were expressed as mean standard deviation (S.D.). Data were analyzed by ANOVA; paired two-sample t-tests were applied for paired comparisons (SPSS 19; SPSS, Chicago, IL). Statistical significance were represented as $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results and Discussion

3.1. The Synthesis and Characterization of NSC

The synthetic scheme of NSC was presented in **Figure 1(a)**. Succinic anhydride dissolved in acetone could react with the hydroxyl and amino groups of chitosan in the presence of EtOH under stirring at ambient temperature, resulting in formation of the derivative. **Figure 2** showed the FTIR spectra of CHI and NSC. From the CHI spectrum, it was found that distinctive absorption bands of CHI appeared at 3355 cm^{-1} (the combination of stretching of -OH- and -NH-), 1585 cm^{-1} (ν = (C=O), Amide I) and 1378 cm^{-1} (ν = (C-N), Amide III). Compared with that of chitosan, the peaks at 1652 cm^{-1} (ν = (C=O), Amide I) 1557 cm^{-1} (ν = (N-H), Amide II) and 1403 cm^{-1} (ν = (C-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.

The ^{1}H NMR spectrum of the NSC was given in **Figure 3**. The ^{1}H NMR assignments of NSC was as follows: δ = 1.93(-NH-CO-); δ = 2.36(-NH(CO)-CH_2); δ = 2.55(-CH_2-COOH); δ = 2.867(H2) δ = 2.26 - 3.66 (H_1,H_3, H_4, H_5, H_6). According to the ratio of the integral peak of -CH_2-CH_2- of NSC and H_2 in chitosan structure, it can be

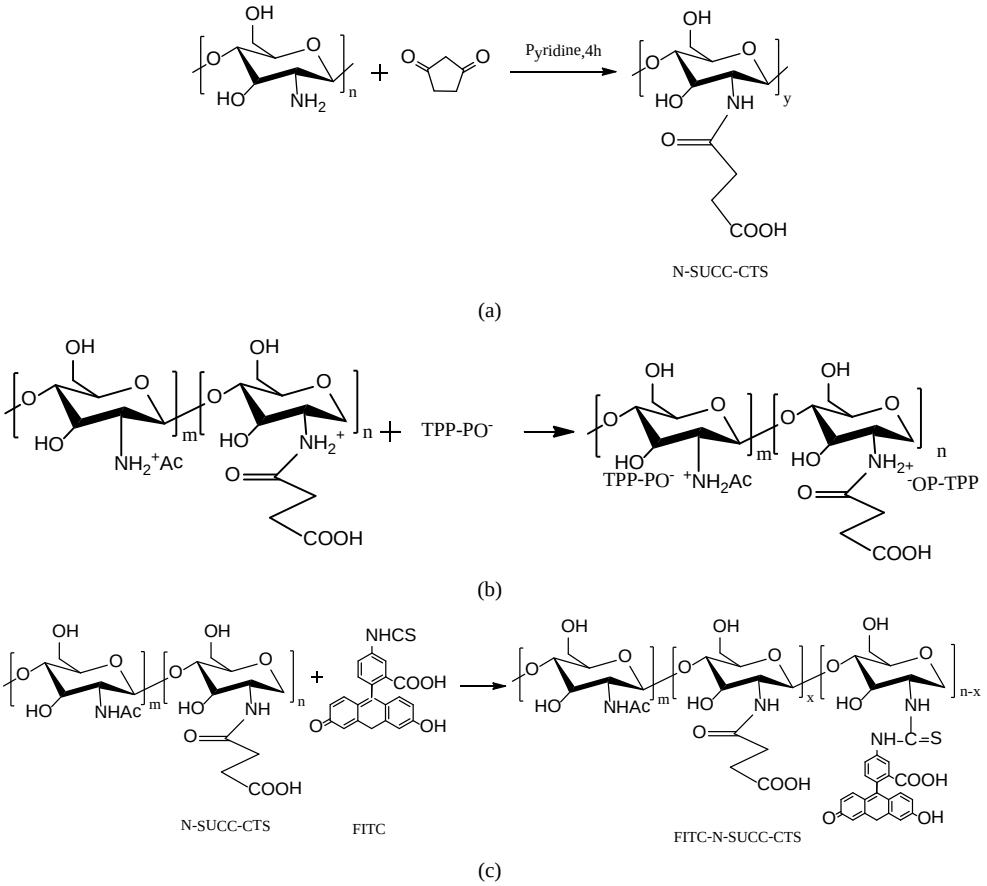

Figure 1. (a) Synthesis scheme of N-succinyl-chitosan, (b) synthesis scheme of Nanoparticles, (c) synthesis scheme of FITC-labeled N-succinyl-chitosan.

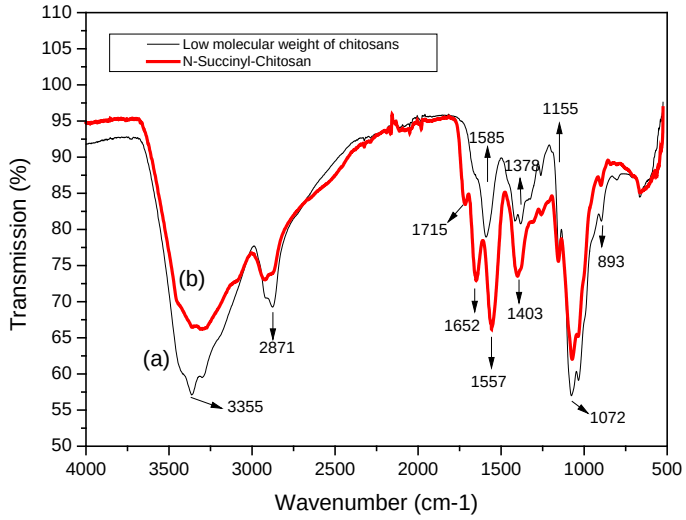

Figure 2. FITR spectra of (a) low molecular weight of chitosans and (b) N-succinyl-chitosan.

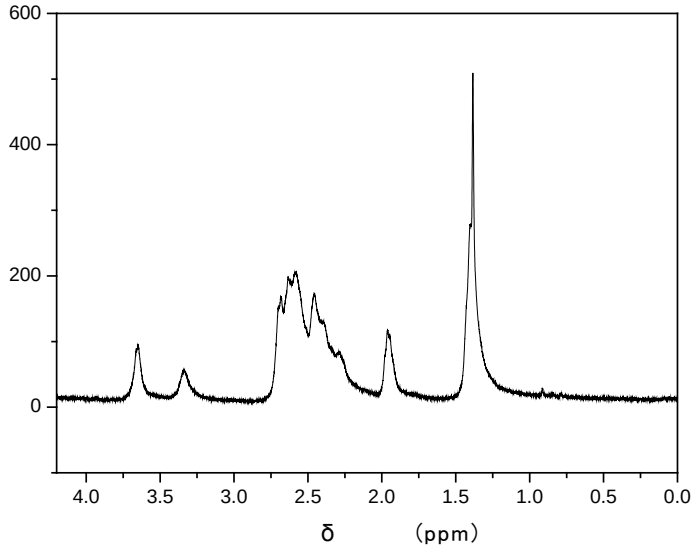

Figure 3. ^{1}H-NMR spectra of N-succinyl-chitosan.

known that the substitution degree (x) of NSC is 0.58. This result indicates that 0.58 "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 NSC. Found: C, 42.73%; H, 5.95%; and N 6.7%, respectively. From elemental composition, it can be calculated the substitution degree (x) of succinyl, which is 61.2% and is good consistent with that of ^{1}H NMR result.

According to the results of elemental analysis, FTIR, 1H NMR, the suggested chemical structure of N-succinyl chitosan was confirmed and the substitution degree (x) was near 0.6.

3.2. Evaluation of NSC-E Nanoparticles

The preparation scheme of NSC was presented in **Figure 1(b)**. The mean particle size of NSC-ES-NPs was 178.4 ± 7.3 nm as **Figure 4**, PDI = 0.3. EE% was 94.76 ± 0.05 reveal a good drug capacity. The zeta potential of NSC-ES-NPs was +9.99 mv showed in **Figure 5** reveals that the morphology of NSC-ES-NPs formed stable

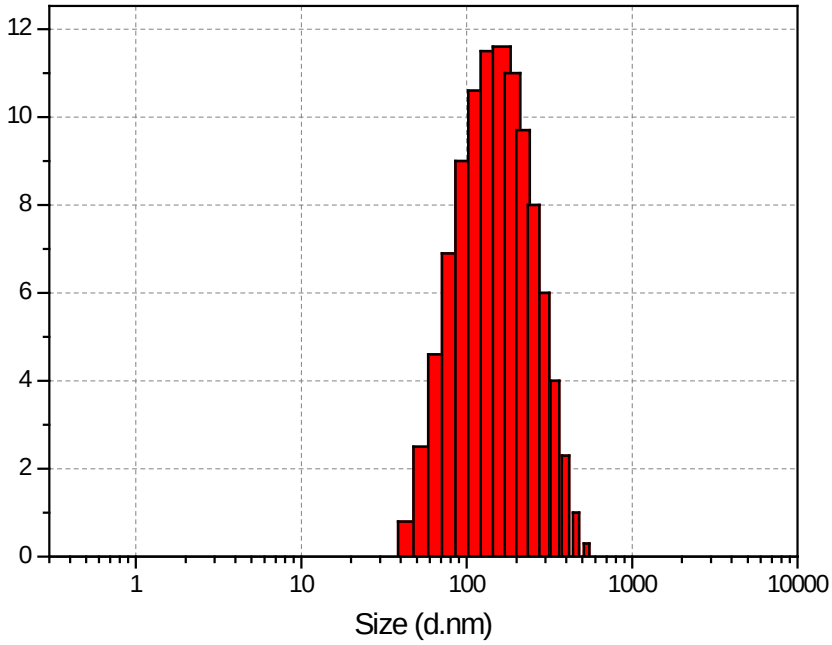

Figure 4. NSC-ES size distribution by intensity.

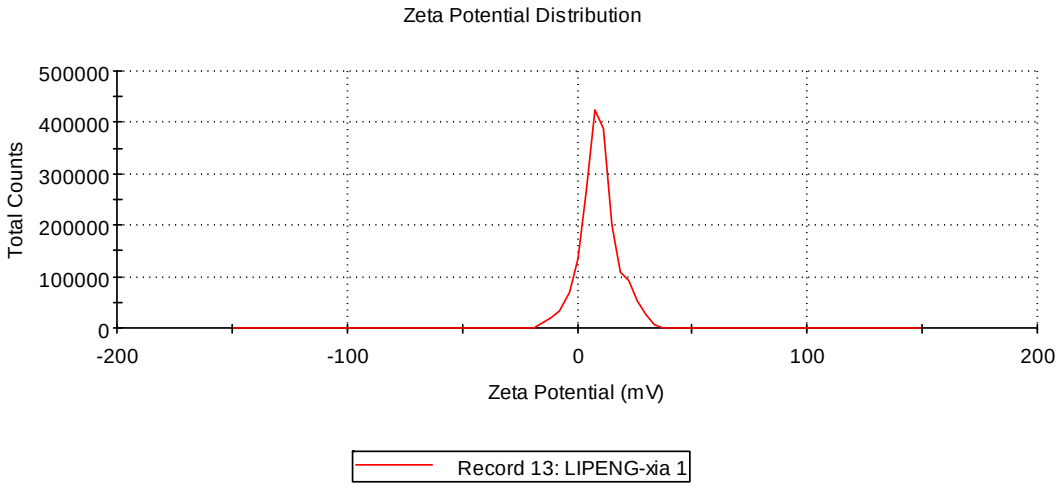

Figure 5. Zeta potential distribution.

particles and not easily aggregated, showed a good dispersibility. Owing to the positive zeta potential of NSC-ES-NPs, it could interact with the membrane which has a negative zeta potential more easily. **Figure 6** shows the transmission electron microscopy (TEM) morphology of the nanoparticles of NSC-ES. The NSC-ES-NPs were nearly spherical with a size of 200 nm and independently distributed.

3.3. Uptake Studies

Figure 1(c) shows the synthetic scheme of FITC-labeled NSC. The weight fraction of FITC per unit weight of chitosan was 2.67% ± 0.02%. Fluorescence intensity (F) in the cell lysate solution varied linearly with concentration (C) of the FITC-labeled NSC in the range of 2 to 14 μg/mL, obeying the relation of $F = 5.46\ C - 57.82$ ($R^2 = 0.995$). Uptake of the FITC-NSC by the MCF-7cells depended on concentration and time of incubation, the uptake amount increasing by 3.3-fold when dosing concentration was increased from 75 to 200 μg/mL, and the highest uptake amount is 6.805 μg/mg (**Figure 7**).

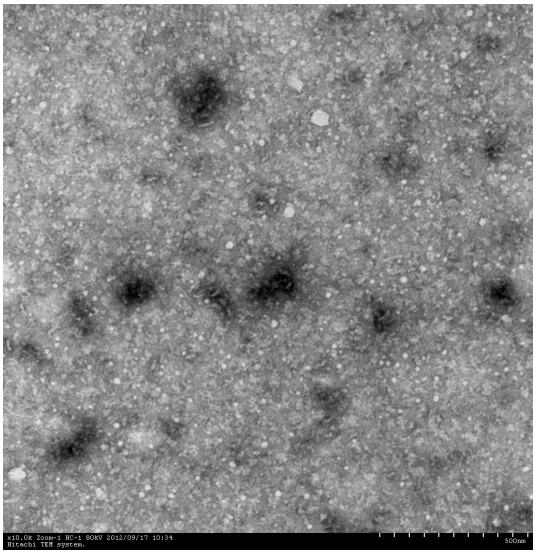

Figure 6. TEM micrograph of NSC-ES-NPs.

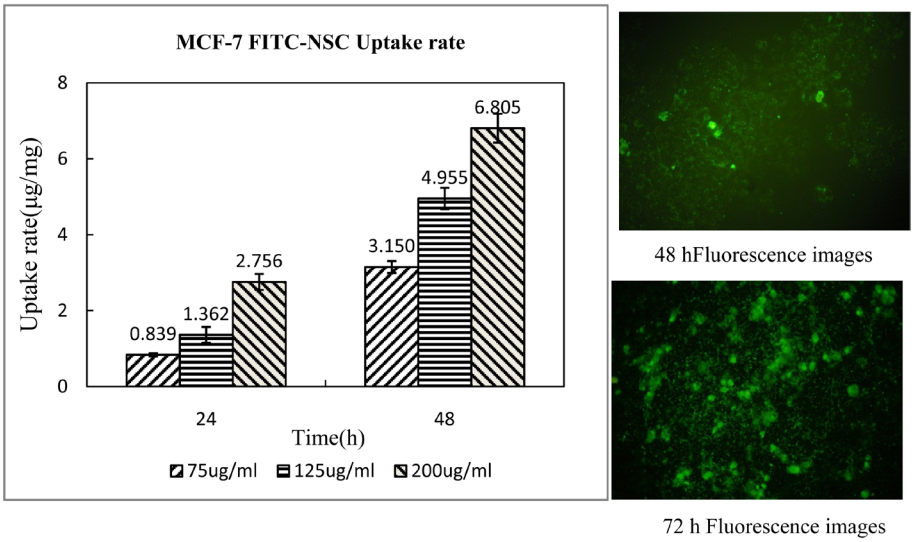

Figure 7. The uptake rate of FITC-NSC against MCF-7 (n = 6).

3.4. Cytotoxicity Assay

Figure 8 shows the dependence of the concentration of NSC-NPs against the cell viability for a different period of cell culture. Although chitosan has been proved to be a non-toxic, tissue-compatible polysaccharide, its derivative NSC should be carefully checked before it is used as biomaterials. From the figure, generally speaking, in the range of 0 - 500 μg/mL of NSC-NPs, it does not show the bad effect against the NIH-3T3 cell. These findings demonstrate that NSCS is nontoxic, and cell-compatible.

3.5. Antitumor Effect

The efficacy of NSC-ES-NPs nanoparticles for inhibiting MCF-7 cell was evaluated in **Figure 9**. The results show that only using ES it has a weakly inhibition against MCF-7 and that the inhibition rate increases with the dose concentration. The group incubated with NSC-ES-NPs had an inhabitation rate of up to 45.5% ($P < 0.01$) and all group incubated with NSC-ES-NPs had a higher inhabitation rate than only using ES. It suggests that NSC-NPs can be taken by MCF-7 cell and show a good potential as a drug carrier.

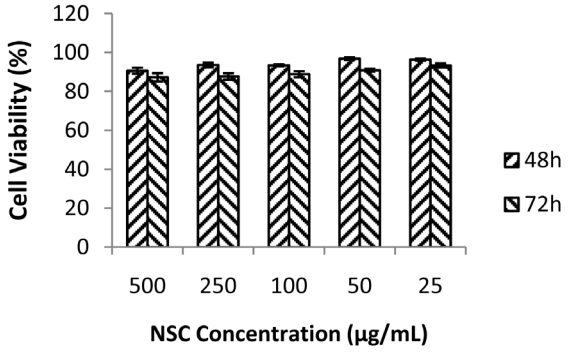

Figure 8. The inhibition effect of NSC-NPs carrier on NIH cells proliferation (n = 6).

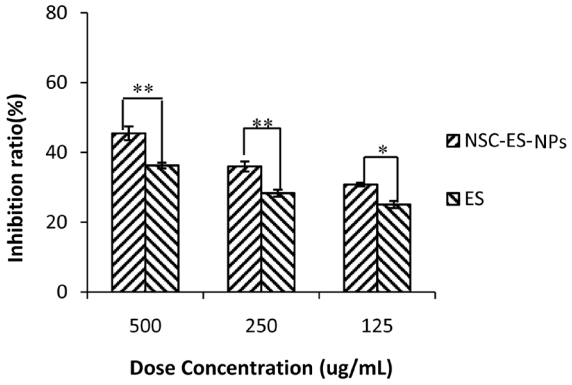

Figure 9. The inhibition effect of NSC-NPs on MCF-7 cells proliferation (n = 6).

4. Conclusion

In this study, a novel biocompatible chitosan derivative, N-succinyl-chitosan (NSC), with DS = 0.58, has been successfully synthesized. NSC was prepared to nanoparticles with TPP. The mechanism is the PO-Na^+ of TPP interacting with the -NH^{2+}- of chitosan. NSC-NPs have a positive zeta potential. It can cross-link endostatin with a negative charge by electrostatic interaction. It is also found that NSC-NPs have nearly non-toxicity. The anti-tumor effect data suggested that NSC-NPs had a great potential to be used as a novel drug matrix in the cancer treatment.

Acknowledgements

This work was supported by the grants of 2014CB744501 from the National Basic Research Program of China(973 Program), grants of BY2012185, BK2011095, BK2011271 and BK2011044 from the Natural Science Foundation of Jiangsu Province, China, and the grant KF-GN-201204 from State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University to GXX.

References

[1] Luo, H., Li, J. and Chen, X. (2010) Antitumor Effect of N-Succinyl-Chitosan Nanoparticles on K562 Cells. *Biomedicine & Pharmacotherapy*, **64**, 521-526. http://dx.doi.org/10.1016/j.biopha.2009.09.002

[2] Park, J.H., Saravanakumar, G., Kim, K., *et al.* (2010) Targeted Delivery of Low Molecular Drugs Using Chitosan and Its Derivatives. *Advanced Drug Delivery Reviews*, **62**, 28-41. http://dx.doi.org/10.1016/j.addr.2009.10.003

[3] Ying, G.Q., Yang, H., Yi, Y., *et al.* (2007) Relationships between the Molecular Structure and Moisture-Absorption and Moisture-Retention Abilities of Succinyl Chitosan. *Polymer Bulletin*, **59**, 509-516. http://dx.doi.org/10.1007/s00289-007-0790-9

[4] Sui, W., Wang, Y., Dong, S, *et al.* (2008) Preparation and Properties of an Amphiphilic Derivative of Succinyl- Chitosan. *Colloids and Surfaces A*: *Physicochemical and Engineering Aspects*, **316**, 171-175.

http://dx.doi.org/10.1016/j.colsurfa.2007.09.016

[5] Zhu, A.P., Chen, T., Yuan, L.H., Wu, H. and Lu, P. (2006) Synthesis and Characterization of N-Succinyl-Chitosan and Its Self-Assembly of Nanospheres. *Carbohydrate Polymers*, **66**, 274-279. http://dx.doi.org/10.1016/j.carbpol.2006.03.014

[6] Kato, Y., Onishi, H. and Machida, Y. (2001) Biological Characteristics of Lactosaminated N-Succinyl-Chitosan as a Liver-Specific Drug Carrier in Mice. *Journal of Controlled Release*, **70**, 295-307. http://dx.doi.org/10.1016/S0168-3659(00)00356-4

[7] Kato, Y., Onishi, H. and Machida, Y. (2001) Lactosaminated and Intact N-Succinyl-Chitosans as Drug Carriers in Liver Metastasis. *International Journal of Pharmaceutics*, **226**, 93-106. http://dx.doi.org/10.1016/S0378-5173(01)00777-3

[8] Asai, K., Kanazawa, H., Otani, K., *et al.* (2002) Imbalance between Vascular Endothelial Growth Factor and Endostatin Levels in Induced Sputum from Asthmatic Subjects. *Journal of Allergy and Clinical Immunology*, **110**, 571-575. http://dx.doi.org/10.1067/mai.2002.127797

[9] Qian, C.N., Zhang, C.Q., Guo, X., *et al.* (2000) Elevation of Serum Vascular Endothelial Growth Factor in Male Patients with Metastatic Nasopharyngeal Carcinoma. *Cancer*, **88**, 255-261. http://dx.doi.org/10.1002/(SICI)1097-0142(20000115)88:2<255::AID-CNCR2>3.0.CO;2-N

[10] Takeda, A., Shimada, H., Imaseki, H., *et al.* (2000) Clinical Significance of Serum Vascular Endothelial Growth Factor in Colorectal Cancer Patients: Correlation with Clinicopathological Factors and Tumor Markers. *Oncology Reports*, **7**, 333-338. http://dx.doi.org/10.3892/or.7.2.333

[11] Jones A, Fujiyama C, Turner K, *et al.* (2000) Elevated Serum Vascular Endothelial Growth Factor in Patients with Hormone-Escaped Prostate Cancer. *BJU International*, **85**, 276-280. http://dx.doi.org/10.1046/j.1464-410x.2000.00432.x

[12] Suzuki, M., Iizasa, T., Ko, E., *et al.* (2002) Serum Endostatin Correlates with Progression and Prognosis of Non-Small Cell Lung Cancer. *Lung Cancer*, **35**, 29-34. http://dx.doi.org/10.1016/S0169-5002(01)00285-9

[13] Folkman, J. (2006) Antiangiogenesis in Cancer Therapy—Endostatin and Its Mechanisms of Action. *Experimental Cell Research*, **312**, 594-607. http://dx.doi.org/10.1016/j.yexcr.2005.11.015

[14] O'Reilly, M.S., Boehm, T., Shing, Y., *et al.* (1997) Endostatin: An Endogenous Inhibitor of Angiogenesis and Tumor Growth. *Cell*, **88**, 277-285. http://dx.doi.org/10.1016/S0092-8674(00)81848-6

[15] Nilsson, U.W. and Dabrosin, C. (2006) Estradiol and Tamoxifen Regulate Endostatin Generation via Matrix Metalloproteinase Activity in Breast Cancer *in Vivo*. *Cancer Research*, **66**, 4789-4794. http://dx.doi.org/10.1158/0008-5472.CAN-05-4012

[16] Abdollahi, A., Hahnfeldt, P., Maercker, C., *et al.* (2004) Endostatin's Antiangiogenic Signaling Network. Molecular *Cell*, **13**, 649-663. http://dx.doi.org/10.1016/S1097-2765(04)00102-9

[17] Hajitou, A., Grignet, C., Devy, L., *et al.* (2002) The Antitumoral Effect of Endostatin and Angiostatin Is Associated with a Down-Regulation of Vascular Endothelial Growth Factor Expression in Tumor Cells. *The FASEB Journal*, **16**, 1802-1804. http://dx.doi.org/10.1096/fj.02-0109fje