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# Biological evaluation of *N*-octyl-*O*-sulfate chitosan as a new nano-carrier of intravenous drugs

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ABSTRACT

An amphiphilic chitosan derivate, *N*-octyl-*O*-sulfate chitosan (NOSC) was prepared by octylation of amino group at C-2 position and sulfonylation at C-6 position. Micelle formed by NOSC has great capability in solubilization of water-insoluble drug paclitaxel. Enormous attention was attracted by the potential application of NOSC as a new drug delivery system. Tritium labeled NOSC ($^3$H NOSC) was injected by tail vein at dose of 13.44 mg/kg in mice; kidney retained the maximum amount of NOSC all the time even after 24 h following the injection. Pharmacokinetic parameters (the area under the plasma concentration–time curve, maximum plasma concentration, apparent plasma half-life of distribution phase and elimination phase, mean residence time, apparent volume of distribution, total body clearance) were obtained by fluorometric method in rats. The results showed a linear pharmacokinetics proceeding of FITC-NOSC in vivo. 75.4 ± 11.6% $^3$H NOSC of dose was excreted in urine over a 7-day period, urinary excretion was the predominant way of excretion of NOSC compared with bilary or fecal pathway. A series of safety studies consisted of acute toxicity study, intravenous stimulation study, injection anaphylaxis study, hemolysis study and cell viability assay were performed to warrant the biocompatibility of the NOSC as intravenous materials. The $LD_{50}$ value of NOSC administrated by i.v. and i.p. were calculated as 102.59 and 130.53 mg/kg, respectively. No intravenous stimulation, injection anaphylaxis, hemolysis and cytotoxicity were observed in the safety studies. The tissue distribution, pharmacokinetics, excretion and safety study were persuasive for the potential application of NOSC as a new drug carrier.

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## 1. Introduction

Chitosan is a cationic aminopolysaccharide in neutral or basic pH conditions which is easily obtained by partial deacetylation of chitin. Second only to cellulose, chitin is one of the most abundant polysaccharides in nature, which is available in marine crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of fungi and insects. Because deacetylation of chitin is not complete, chitosan is a copolymer composed of glucosamine and *N*-acetylglucosamine (2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose units linked with β-(1→4) bonds). The solubility of chitosan is

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affected by both pH of the medium and its deacetylation degree. It can be dissolved in dilute inorganic and organic acid solutions, but precipitate when pH is higher than 6.0. The major difference between chitosan and other polysaccharides is the presence of affluent primary amine groups at C-2 position of the glucosamine residues in chitosan; therefore, chitosan is one of the parvus polysaccharides which has a positive charge. The strong functionality of chitosan (two hydroxyl groups and one primary amine group per-repeat unit) renders it considerable opportunity of chemical modification with a wide range of compounds and consequently extensive applications in pharmaceutical field and other industries (Ravi Kumar et al., 2004; Giunchedi et al., 1998; Lee et al., 1998; Shahidi and Abuzaytoun, 2005; Thanou et al., 2000; Aiedeh and Taha, 1999; Säkkinen et al., 2002; Chen et al., 2003; Shanta and Harding, 2002; Huang et al., 2006). Chitosan is generally regarded as non-toxic, biocompatible and biodegradable (Chandy and Sharma, 1990; Hirano et al., 1990; Knapczyk et al., 1989; Bersch et al., 1995) and listed in the standard guides of American Standard Testing Materials (ASTM) for use in food preparations and in the pharmacopoeias of various countries. Now, more and more attention was devoted to the application of chitosan and its derivates in the development of new drug delivery systems, e.g. gels, tablets capsules, microspheres and microcapsules (Ravi Kumar et al., 2004). An amphiphilic chitosan derivate, *N*-octyl-*O*-sulfate chitosan (NOSC) was synthesized by our previous work in two steps, first, *N*-octyl chitosan was prepared by octylation of amino group at C-2 position, and then sulfonylation at C-6 position of *N*-octyl chitosan was achieved. NOSC can be dissolved in aqueous solution to form micelle in self-assembly process. The micelle formed by NOSC has great capability in solubilization of water-insoluble drugs, such as paclitaxel with the entrapment efficiency (%) of 59.11 and loading (%, w/w) of 25.25 (Zhang et al., 2004, 2003). Thus it is possible to exploit NOSC as a versatile water-insoluble drug delivery system in parenteral or oral administration. Overall evaluation consisted of toxicity, quality criteria, tissue distribution, pharmacokinetics, metabolism, excretion and so on had been carried out to ensure the safety and efficacy. In this report, we describe these biological effects of NOSC in rodents as an initial step towards understanding its behaviors in vivo. At the same time, a series of the biocompatibility evaluations, such as acute toxicity, injection irritation, anaphylaxis, hemolysis and cell viability are performed.

## 2. Materials and methods

### 2.1. *Materials*

Chitosan was provided by the Nantong Suanglin Biochemical Ltd., China, with deacetylation degrees of 97% and viscosity average molecular weight of 65 000 Da. Fluorescein isothiocyanate (FITC) and pyrene (>99%) were purchased from Fluka (Milwaukee, WI, USA). Methanol, Hydrogen peroxide, Perchloric acid and other reagents were analytical grade. Double distilled water was used in this study. All commercially available solvents and reagents were used without further purification.

*N*-Octyl-*O*-sulfate chitosan (NOSC) was prepared following a procedure shown in Fig. 1 which was reported previously by our group (Zhang et al., 2004, 2003). The chemical structure and substitution degree of the derivative were determined by Nicolet 2000 FT-IR spectrophotometer (Thermo Electron Corp., Masison, WI, USA), BRUKER AVANCE 500 AV system (Bruker Biospin GmbH, Rheinstetten, Germany) and the Vario EL III Element analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) with the substitution degree of octyl and sulfonic group of 0.38 and 2.56, respectively.

The molecular weight of NOSC was analyzed with Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) with refractive index detector using a TOSOH TSK-G4000 PWxl column (7.8 mm × 30 cm) (TOSOH Corp., Tokyo, Japan). Number-average molecular weight (Mn) and weight-average molecular weight (Mw) were 9.23 × 105 and 1.03 × 106, respectively. *D* (Mw/Mn) = 1.17. The critical micelle concentration (CMC) value was calculated to be 0.45 mg/ml in fluorescence spectroscopy using pyrene as a hydrophobic probe (Zhang et al., 2004).

Fluorescein isothiocyanate (FITC)-NOSC, abbreviated to FITC-NOSC, was prepared as the marker of NOSC in vivo as follows: 2 g of NOSC was dissolved in 50 ml of carbonate buffer (pH 9.0, 50 mM), 70 mg of FITC was dissolved in 50 ml of the

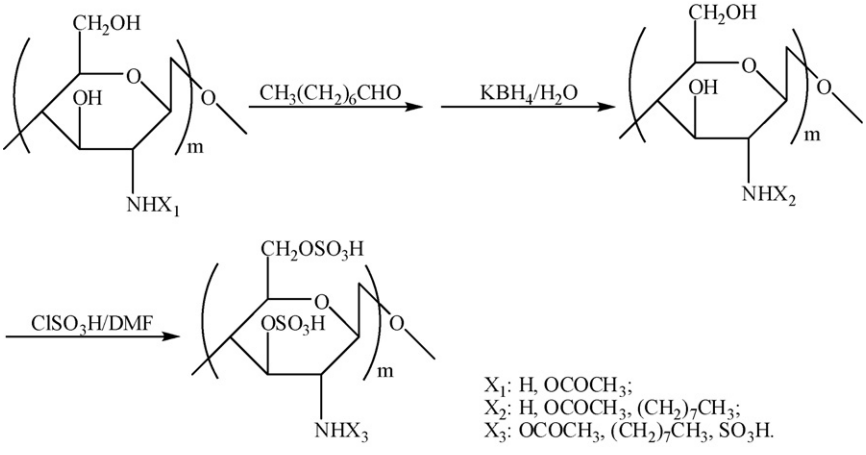


**Fig. 1 – Synthetic scheme of NOSC.**

same buffer, the mixture of the two solutions was stirred at room temperature in dark for 24 h. The product, FITC-NOSC, was dialyzed with Hollow Fiber tube against water for 10 h, and then lyophilized. Fluorescence purity of FITC-NOSC and metabolization of FITC-NOSC in rat plasma was analyzed with Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) at excitation wavelength of 495 nm (ex. 495 nm), emission wavelength was 520 nm (em. 520 nm) for excitation spectra. Separation was achieved using a TOSOH TSK-G4000 PWxl column (7.8 mm × 30 cm) (TOSOH Corp., Tokyo, Japan) at 25°C. The eluting reagent was water at a flow rate of 0.8 ml/min. A 100% fluorescence purity of NOSC-FITC was calculated from the absorbance at 495 nm in HPLC analysis. The FITC content of NOSC-FITC was calculated from the absorbance at 495 nm using a UV9100 spectrophotometer (Beijing Rayleigh Analytical Instrument Corp., Beijing, China) in phosphate buffered solution (PBS) (pH 7.4, 50 mM). The FITC content of NOSC-FITC was 0.58% (w/w). The HPLC analysis of blank plasma, FITC, blank plasma + FITC, FITC-NOSC and rat plasma after injecting were also achieved.

Tritium labeled NOSC, abbreviated to $^3$H NOSC, was prepared by China Institute of Atomic Energy. The $^3$H NOSC was separated and purified by HPLC-UV/Winflow system which consists of a TOSOH TSK-G4000 PWxl column and a winflow isotope on-line detector, $H_2O$ was used as mobile phase at the flow rate of 0.8 ml/min. The radiochemical purity of $^3$H NOSC after separation and purification was 100% approximately.

### 2.2. Experimental animals

Sprague-Dawley (SD) rats were obtained from the Shanghai Silaike Laboratory Animal Limited Liability Company with the certificate number of SCXK (Shanghai) 2003-0003 and Nanjing Medical University Laboratory Animal Center with the certificate number of SCXK (Jiangsu) 2003-00031. ICR mice were obtained from the Zhongmu Enterprise Corporation Nanjing Pharmaceutical Machinery Plant with the certificate number of SCXK (Jiangsu) 2002-00030. New Zealand albino rabbits ranging in weight from 1.8 to 2.0 kg and guinea-pigs weight from 280 to 330 g were obtained from the Laboratory Animal Center of Nanjing General Hospital with the certificate number of SCXK (Jiangsu) 2003–2004. All the animals were pathogen free and allowed to food and water freely. The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals.

### 2.3. Tissue distribution study of radioactively labeled NOSC ($^3$H NOSC) in mice

The tissue distribution of NOSC was investigated after intravenous administration of $^3$H NOSC dissolved in normal saline via tail vein of mice. This animal experiment was carried out with ICR mice around 30 g. Thirty-two mice were randomly divided into four groups. Each group consists of half-female and half-male. A tracer dose of $^3$H NOSC of 13.44 mg/kg (Equivalent to $2.53 \times 10^8$ dpm/kg) was injected. At different time, i.e. 10 min, 30 min, 4 h and 24 h post-injection, mice were sacrificed by cervical dislocation and blood was withdrawn from the heart (about 50 μl of whole blood was obtained), and following closely, the blood was immediately centrifuged to obtain plasma at 4000 rpm for 15 min using Anke TGL-16B desk centrifuge (Shanghai Anting Scientific Instrument Co., Ltd., Shanghai, China). The organs of heart, liver, spleen, lung, stomach, intestine, small intestine, brain, genital, muscle and kidney were excised, thoroughly washed with double distilled water, blotted and weighed. The excised tissue samples were then homogenized in a tissue homogenizer (Tearork™, BioSpec Products Inc., Bartlesville, OK). Fifty milligram of solid tissue or 50 μl of blood plasma sample was mixed with 200 μl Perchloric acid and 300 μl hydrogen peroxide, and then incubated at 70°C for 30 min. Six millilitre liquid scintillation cocktail was added into each sample. The radioactivity levels were determined using scintillation counter (Model LB5801, Beckman Coulter Inc., Fullerton, California, USA). The blank sample was obtained in the way described above but by injecting normal saline alone into mice instead of $^3$H NOSC solution, the concentration of $^3$H NOSC in the sample was determined from the net intensity of radioactivity obtained by subtracting the intensity of radioactivity of the blank based on the standard calibration curve.

### 2.4. Pharmacokinetic study with fluorometric method in rats

Eighteen rats were divided into three groups randomly. Each group consists of half-female and half-male. FITC-NOSC was injected via the tail vein at three dose levels of 16.8 mg/kg, 8.4 mg/kg and 4.2 mg/kg, respectively. At each sampling time (0 min, 2 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, 72 h), 0.5 ml blood sample was drawn from retro-orbital plexus under ether anesthesia. Subsequently, blood samples were centrifuged at 4000 rpm for 15 min using Anke TGL-16B centrifuge and the plasma was separated and stored at −20°C. The plasma were thawed at ambient temperature and diluted appropriately with PBS (pH 7.4, 50 mM), and their fluorescence intensities were determined (ex. 495 nm, em. 520 nm). The blank blood sample from rats was obtained and treated in the same way by injecting normal saline alone instead of FITC-NOSC solution. Pharmacokinetic analysis was performed on the plasma concentration-time profile. The area under the plasma concentration-time curve (AUC) was calculated using linear trapezoidal method with extrapolation to infinity and individual profiles were analyzed by compartment model method and non-compartment model according to the statistical moment principle using Pharmacokinetics statistical software of Drug and Statistics for Windows (DAS ver1.0) to obtain pharmacokinetic parameters. Compartment model was determined by AIC (Akaike's Information Criterion) rule.

### 2.5. Bilary, urinary and fecal excretion studies of $^3$H NOSC in rats

Bilary excretion study was performed on eight healthy SD rats. Under etherization the common bile duct were dissected and cannulated. Then, the animals were administered with $^3$H NOSC at a dose of 20 mg/kg (equivalent to $2.6 \times 10^7$ dpm/kg) by tail vein injection. The bile samples were collected at 0 h, 3 h, 9 h, 18 h, 24 h, 36 h, and the total volume was measured. Fifty microlitres of bile sample was placed into 6 ml of liquid

scintillation cocktail, and the total radioactivity in the samples was determined based on standard curve. Same to the body distribution studies mentioned above, background level of radioactivity in bile was obtained from control animals. Background levels of radioactivity were subtracted before calculations.

Six SD rats were performed in urinary/fecal excretion study. $^{3}$H NOSC was administered at a dose of 120 mg/kg ($5.4 \times 10^{7}$ dpm/kg) via tail vein. Animals were kept separately in metabolite cages with free access to food and water after administration. Urine and feces were collected cumulatively before administration and at 6 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d after i.v. administration, at every sampling time, urine volume and feces weight were measured. The urinary samples were prepared by an addition of 6 ml liquid scintillation cocktail and 50 μl urine samples into a scintillation vial, and the total radioactivity in the samples were determined.

The fecal sample were prepared as follows:100 mg feces was put into a scintillation vial and then 200 μl perchloric acid and 300 μl of hydrogen peroxide were added into the sample for 30 min incubation at 70 °C. The radioactivity levels were determined after an addition of 6 ml liquid scintillation cocktail. The concentration of $^{3}$H NOSC was measured from the net radioactive intensity using a calibration curve in the same manner as in the body distribution studies and bilary excretion study. The total amounts excreted in urine and feces were calculated from the NOSC concentration in samples and urinary volume/fecal weight. HPLC analysis condition of $^{3}$H NOSC has been described in Section 2.3.

### 2.6. *Safety study*

#### 2.6.1. *Acute toxicity study*

Different concentrations of NOSC solution were obtained with geometric proportion dilution at a dose-ratio of 1:0.7 and used after totally dissolved in 5% glucose injection. Five doses, 240.0, 168.0, 117.6, 82.3 and 57.6 mg/kg body weight, were administered i.v. to mice in five groups and i.p. to mice in another five groups, respectively. Every group had five males and five females. The volume of NOSC solution was 0.5 ml/20 g body weight. The animals were carefully observed for any toxic effect and distribution of death rate, immediately after dosing, during the subsequent 14 days. Median lethal dose ($LD_{50}$) and 95% confidence limit were determined using Bliss method. At the end of the observational period, the animals were sacrificed by cervical dislocation. A thorough autopsy was carried out on all the animals for pathological examination.

#### 2.6.2. *Intravenous stimulation study*

One male and two female rabbits weighing 1.8–2.0 kg were used for this study. NOSC was dissolved in 5% glucose injection at the concentration of 1.5 mg/ml. Each rabbit was injected with a daily dose of 9 mg/kg body weight of NOSC solution from the vein at the edge of the left ear at the injection rate of 1 ml/min for 3 days. And equivalent volume of 5% glucose injection was injected from the right ear-border vein at the same time with the injection of NOSC solution as control. After injection, paradoxical reaction at the injection site was recorded. The rabbits were sacrificed by blood letting at 24 h after the last administration and the ears were cut and fixed in 10% liquor formaldehyde for histological examination. At the localizations of 1.3 cm, 2.6 cm and 4.0 cm from the injection site to proximal part, histological sections were prepared for histopathological examination.

#### 2.6.3. *Injection anaphylaxis study*

Eighteen guinea-pigs weighted 220–270 g were randomly assigned 3 groups, treated group, positive control group and negative control group, every group consisted of three males and three females. NOSC dissolved in 5% glucose injection with concentration of 3.7 mg/ml, 4% egg albumen solution and 5% glucose injection were intraperitoneally injected to guinea-pigs of treated group, positive control group and negative control group, respectively at the same volume of 0.5 ml once every other day for three times. Then every group was divided into two groups randomly, each group contained three guinea-pigs. The two treated groups were intravenously injected 1 ml of NOSC solution with concentration of 3.7 mg/ml at 14 and 21 days after the first intraperitoneal injection, respectively. The control groups were treated same to the medication administration team, 1 ml of the solution used in the first intraperitoneal injection were intravenously injected at 14 and 21 days after first injection. The anaphylactic response was recorded in 30 min after the second injection.

#### 2.6.4. *Hemolysis study*

Hemolysis experiments were done using rabbit blood (Garrec et al., 2004). Briefly, 10 ml of rabbit blood was obtained from arteria cruralis and the fibrinogen was removed by stirring with glass rod. 10 ml of 5% glucose injection was added into the defibrinogen blood sample, and supernatant was removed after centrifugation at 3000 rpm for 5 min. The obtained erythrocyte pellets at the bottom of centrifuge tube were washed four times (centrifugation followed by re-dispersion) with 5% glucose injection. Finally, after repeated washing and centrifugation, an adequate amount of 5% glucose injection was added to the erythrocyte pellets to give a 2% erythrocyte standard dispersion stored at 4 °C. NOSC was dissolved in 5% glucose injection at the concentration of 1.5 mg/ml, and different amounts of NOSC solution with volume of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml were respectively added into six tubes with 2.5 ml of 2% erythrocyte standard dispersion in each. Then adequate amounts of 5% glucose injection were added in every tube to obtain a total volume at 5 ml. Positive control was prepared by addition of 2.5 ml of water into 2.5 ml of 2% erythrocyte standard dispersion instead of 5% glucose injection and NOSC solution. After misce bene, the tubes were incubated at 37 °C and observed microscopically from 15 min to 4 h.

#### 2.6.5. *Cell viability assay*

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium inner salt (MTS) assay which works on the principle that the mitochondrial dehydrogenase enzyme of surviving cells reduces the salt to a colored formazan product that can be read directly at 490 nm (Brunot et al., 2007; Goodwin et al., 1995; Buttke et al., 1993; Chao et al., 1999; Mao et al., 2005) was utilized in this study. Mouse primary hepatocytes were plated in flat-bottomed 96-well plates (Costar, USA) with a density of $10^{4}$/well in triplicate. The medium was replaced after 24 h. NOSC was diluted

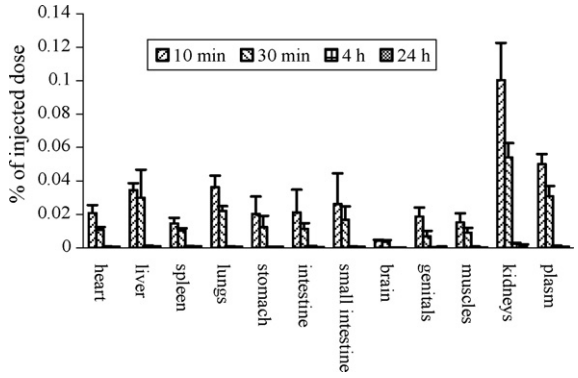


**Fig. 2 – Plasma concentration and tissue distribution of NOSC among various organs (hearts, livers, spleens, lungs, stomachs, intestines, small intestines, brains, genitals, muscles and kidneys) at different sampling time after i.v. administration at a dose of 13.44 mg/kg in rats. Each column represents the mean ± S.D. ($n = 8$).**

in complete medium and added to cell cultures with five different concentrations of 0, 10, 50, 100 and 200 μg/ml. After incubation at 37 °C, 5% carbon dioxide for 24 h, 10 μl/well of CellTiter 96®$AQ_{ueous}$ One Solution Reagent (Promega, Madison, WI, USA) was added. The mixtures were further incubated at 37 °C in a humidified 5% $CO_2$ atmosphere for 1 h and the optical density at 490 nm ($OD_{490nm}$) was recorded using a Victor 3 V multiplate reader (PerkinElmer Life and Analytical Sciences, USA). Control refers to incubation in the presence of medium only and was considered as 100% of viable cells. Results were expressed as the mean of percentage of viable cells.

#### 2.6.6. *Statistical analysis*

All the experiments data was presented as the mean ± S.D. A statistically significant difference was considered at $p < 0.05$ using one-way analysis of variance (one-way ANOVA).

## 3. Results and discussion

### 3.1. *Tissue distribution of $^3$H NOSC*

Tissue distribution study was performed to assist in providing a comprehensive understanding of affinity between $^3$H NOSC and various tissues. The recoveries of $^3$H NOSC from each tissue homogenate and plasma were determined. All the recoveries of six different concentrations suffused the whole linear range fitted with the limitation of technology. Previous studies have revealed the bio-distribution and excretion of some copolymers in rodents (Wang and Stern, 1975; Moghimi, 1999; Leu et al., 1984; Clarke et al., 1999), which indicated that these agents were distributed in extracellular fraction and excreted mainly from the kidneys. In our studies the similar results were obtained as shown in Fig. 2. The maximum concentration was in kidney at 10 min followed by plasma, lung, liver, small intestine, heart, intestine, stomach, genital, spleen, muscle, and brain in decreasing order. The exposure of the organs to NOSC at 24 h decreased in the following order: kidney > liver > spleen > genital > small intestine > plasma > lung > intestine > stomach > heart > muscle > brain. Accumulation amounts of $^3$H NOSC in the kidney was approximately 4.15 ± 1.12 μg/g tissue at 24 h post-injection which showed a long-term retention in the kidney compared with other tissues or organs and decrease of tissue concentrations over time in kidney was shown in Fig. 3. Lower radioactivity was detected in plasma, liver, lung, and intestines. And, the concentrations of NOSC were very low in muscle, genital and brain.

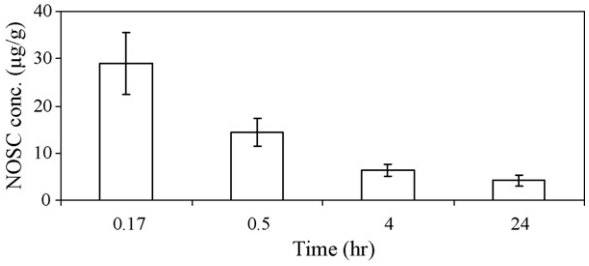


**Fig. 3 – The time course of NOSC accumulation in kidney at four different times after administration at a dose of 13.44 mg/kg in rats. Each point represents the mean ± S.D. ($n = 8$).**

Generally, concentrations of NOSC in various tissues decreased over time, and the tissue with the highest concentration of $^3$H NOSC in mice and dogs (3.12 ± 0.58 μg/g at 24 h after injection) was the kidney which was well perfused organ. The observation of the highest concentration of $^3$H NOSC in kidney in the tissue distribution study with mice prognosticated that this polymer maybe excrete from urine mainly.

### 3.2. *Pharmacokinetic behavior of NOSC in mice*

DAS software was used to calculate pharmacokinetic parameters of compartment model and non-compartment model pharmacokinetic parameters. The plasma concentration–time

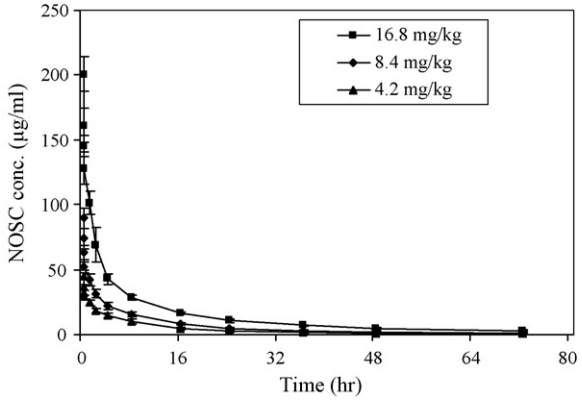


**Fig. 4 – NOSC plasma concentrations–time profiles following i.v. administration at various doses of 16.8 mg/kg (■), 8.4 mg/kg (♦) and 4.2 mg/kg (▲) in rats. Each point represents the mean ± S.D. ($n = 6$).**

**Table 1 – Pharmacokinetic parameters for FITC-NOSC after i.v. administration at three doses in rats ($n = 6$)**

| Model type | Dose (mg/kg) | 16.8 | 8.4 | 4.2 |
|---|---|---|---|---|
| Two compartment model | Vd[a] (L/kg) | 0.477 ± 0.014 | 0.549 ± 0.117 | 0.457 ± 0.031 |
| | $T_{1/2}\alpha$[b] (h) | 1.329 ± 0.30 | 2.08 ± 0.79 | 3.59 ± 0.84 |
| | $T_{1/2}\beta$[c] (h) | 20.9 ± 2.0 | 22.0 ± 3.9 | 21.4 ± 2.7 |
| | CL[d] (L/h/kg) | 0.016 ± 0.001 | 0.017 ± 0.002 | 0.015 ± 0.002 |
| Non-compartment model | $AUC_{0\text{-}tn}$[e] (mg h/L) | 1030.2 ± 75.4 | 485.4 ± 55.3 | 278.1 ± 32.1 |
| | $AUC_{0\text{-}\infty}$[f] (mg h/L) | 1048.1 ± 78.3 | 505.2 ± 55.8 | 290.5 ± 32.7 |
| | $MRT_{0\text{-}tn}$[g] (h) | 16.2 ± 0.7 | 15.2 ± 0.8 | 13.6 ± 0.7 |
| | $MRT_{0\text{-}\infty}$[h] (h) | 17.3 ± 0.7 | 18.1 ± 0.6 | 17.2 ± 0.8 |

[a] Apparent volume of distribution.
[b] Apparent plasma half-life ($Tt_{1/2}$) of distribution phase (α-phase).
[c] Apparent plasma half-life ($T_{1/2}$) of elimination phase (β-phase).
[d] Total body clearance.
[e] The area under the plasma concentration-time curve from time 0 to the last time point examined.
[f] The area under the plasma concentration–time curve from time 0 to time infinity.
[g] Mean residence time from time 0 to the last time point examined.
[h] Mean residence time from time 0 to time infinity.

curves of FITC-NOSC were shown in Fig. 4 and fitted to two-compartment model determined by AIC rule. The pharmacokinetic parameters were shown in Table 1. The apparent distribution volume (Vd), the half-life and CLs were calculated according to two-compartment model. The mean residence time (MRT) and AUC were calculated according to non-compartment model. The apparent distribution volume (Vd) calculated from three dose groups were 0.447 ± 0.014, 0.549 ± 0.117 and 0.457 ± 0.031 L/kg, respectively which were not markedly different indicating that FITC-NOSC mainly distributed inside extracellular fluid and plasma.

The $t_{1/2}\alpha$ of NOSC were 1.329 ± 0.30, 2.08 ± 0.79 and 3.59 ± 0.84 at the doses of 16.8, 8.4 and 4.2 mg/kg, whereas the $t_{1/2}\alpha$ of paclitaxel-associated NOSC micelle (PTX-M) were 0.0891 ± 0.0602, 0.237 ± 0.183 and 0.133 ± 0.110 at the paclitaxel doses of 14, 7 and 3.5 mg/kg (Zhang et al., 2007). It is unlikely that PTX-M based on NOSC distribute to a large extent in tumours, but the comparable anti-tumor efficacy of PTX-M and Taxol® (clinical formulation of paclitaxel) were observed at the same dose. The half-life of elimination phase ($T_{1/2}\beta$) calculated from three dose groups were 20.9 ± 2.0, 22.0 ± 3.9 and 21.4 ± 2.7 h ($p > 0.05$), respectively. In plasma, fluorescence intensity was detectable up to 48 h post-injection. We considered that the slow metabolism and excretion were due to its large molecular weight of NOSC. The mean residence time (MRT) calculated from three dose groups were 16.2 ± 0.7, 15.2 ± 0.8 and 13.6 ± 0.7 h ($p < 0.05$), respectively. The slow metabolism and long MRT may contribute to the good anti-tumor efficacy. AUC0-tn of high, middle and low dose group were 1030.2 ± 75.4, 485.4 ± 55.3 and 278.1 ± 32.1 mg h/L, respectively, it is noteworthy that the increase in dose of FITC-NOSC leads to a proportional increase of the AUC values. This linearity is shown in Fig. 5. Linear pharmacokinetics proceeding of FITC-NOSC in vivo was indicated by the linearity between AUC and dose shown in Fig. 5. The same results were obtained in the research of Pluronic P85 (Batrakova et al., 2004). CLs of different groups were quite low and close to each other which demonstrated a slow and linear elimination also. Whereas, non-linear pharmacokinetic behavior of PTX-M based on NOSC was observed in rats in our study (Zhang et al., 2007).

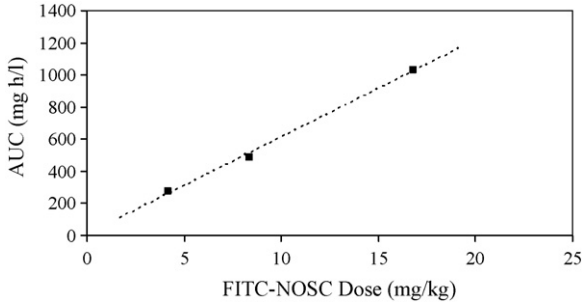


**Fig. 5 – The relationship between FITC-NOSC dose and AUC in rats. Each point represents the mean ± S.D. ($n = 3$).**

### 3.3. *Bilary, urinary and fecal excretion of NOFC in rats*

The cumulative percentage of $^3$H NOSC eliminated in bile over the 36 h period following i.v. administration was shown in Fig. 6. The accumulative excretion amount was 15.6 ± 4.83%

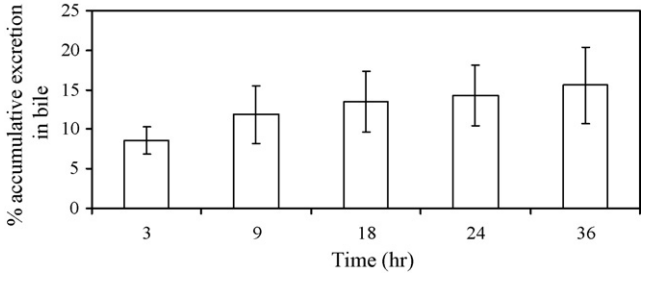


**Fig. 6 – The time course of cumulative percentage of $^3$H NOSC eliminated in bile over the 36 h period following i.v. administration of $^3$H NOSC in rats. Each point represents the mean ± S.D. ($n = 8$).**

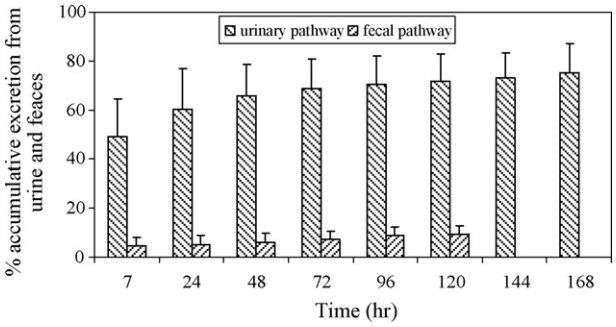


**Fig. 7 – The time course of cumulative percentage of $^{3}$H NOSC eliminated from urine over the 168 h period and from feces over the 120 h period following i.v. administration of $^{3}$H NOSC in rats. Each point represents the mean ± S.D. ($n$ = 6).**

in bile within 36 h. It indicated that the bilary excretion was a subsidiary pathway of $^{3}$H NOSC. The cumulative percentage of $^{3}$H NOSC eliminated from feces and urine were shown in Fig. 7. Identical with the anticipation, the primary pathway of excretion of $^{3}$H NOSC in rats was urine with means of 75.4 ± 11.6% of the dose over 168 h and the fecal accumulative excretion amount was 9.23 ± 3.44% of the dose in 120 h period post-dose, and residual fecas in carcass was not taken, so the total fecal excretion was considered to be higher than the value offered before.

Metabolic product of $^{3}$H NOSC in urine of rat was analyzed by size-exclusion gel-chromatography mentioned in Section 2.1, the components with higher molecular weight were excluded preferentially and first eluted. As shown in Fig. 8, retention time of intact form of $^{3}$H NOSC was 8.45 min, whereas retention time of metabolic product of $^{3}$H NOSC was 17.23 min, which demonstrated that the polymer is eliminated as a degraded form with lower molecular weight in urine of rat.

In a previous study by other workers, the excretion of Purified poloxamer 188 (PP188) was investigated in rats with similar excretion routes to $^{3}$H NOSC. And an available study

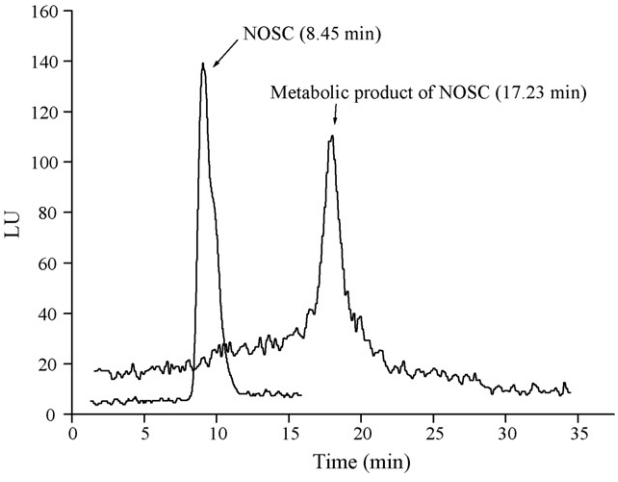


**Fig. 8 – HPLC analysis of $^{3}$H NOSC and its metabolic product in urine of rats.**

demonstrated that Pluronics are excreted mainly via the kidneys with few metabolisms (Grindel et al., 2002).

### 3.4. *Safety study*

#### 3.4.1. *Acute toxicity study*

The acute toxicity evaluation in vivo was performed on mice with NOSC. In the first day after injecting, no paradoxical reaction was observed in all the groups. The toxic effects, body weight loss, inactivity and ingestion decrease, occurred from the second day and returned to normal 8 days after administrating. Death of mice was observed from the second day, and after 8 days, the survivor recovery from toxic effects gradually and death of mice was not observed until the end of the experiment. The autopsy of the treated animals did not reveal any macroscopic changes in major organ, as heart, liver, spleen, lung and kidney. The $LD_{50}$ value and 95% confidence limit of NOSC administrated by i.v. and i.p. were calculated as 102.59 (78.04–134.85) mg/kg and 130.53 (114.43–148.89) mg/kg, respectively. The dosage of NOSC was very low because of its excellent capacity of solubilization to lipophilic drug, although the $LD_{50}$ values of NOSC was very low, it is a suitable carrier for specified drug. For example, drug loading (%) of the formulation of paclitaxol-loaded NOSC micelle (PTX-M) approached 50%, which bring a 1/1 (w/w) ratio of drug and NOSC in the formulation. In tissue distribution and pharmacokinetics studies of paclitaxol-loaded NOSC micelle (PTX-M) in rats, the dosage of paclitaxol was no more than 15 mg/kg, so the dosage of NOSC was no more than 15 mg/kg, which is much smaller than the $LD_{50}$.

#### 3.4.2. *Intravenous stimulation*

After a 3-day administration of NOSC solution and 5% glucose injection, erythema and edema were not observed at the injection site. In the histopathologic examination of histological section of rabbit ear-border vein, we observed that the vessel wall and endothelial cell structure were distinct and integrity, furthermore, angiectasia and thrombus were not observed in the lumen of vein. The pathological changes, obvious inflammatory cell infiltrate, hemorrhage apomorphosis and necrosis, were not discovered in the vessel wall and surrounding tissue. The histopathologic examination results of ears administrated with NOSC solution were similar to the control group. All the results mentioned above demonstrated that NOSC solution at a dose of 9 mg/kg has no simulative reaction with vein of rabbit.

#### 3.4.3. *Injection anaphylaxis*

Positive reaction was assessmented with the occurrence of the behaviors as convulsion, collapse, circulatory collapse and death or occurrence of at least two kinds of anaphylactic response as piloerection, anhelation, sneeze, retch, begma. Treated group and negative control group did not respond to the provocation. In positive control, typical anaphylaxis, such as anhelation and convulsion, occurred immediately after provocation, and all the three guinea-pigs died within 1 min.

The results of anaphylaxis test demonstrated that the NOSC solution used intravenously at concentration of 3.7 mg/ml did not show the effect of sensitization.

#### 3.4.4. *Hemolysis study*

Complete hemolysis was observed in tube of positive control at 15 min, the solution in it was red clear-diaphanous, and no erythrocyte survived at the bottom of the tube. The erythrocyte precipitated at the bottom of other six tubes and dispersed after shaking, the supernatant was achromatic and transparent in the observation period for 4 h. These results demonstrated that the NOSC solution at concentration of 0.15 mg/ml did not cause haematolysis and erythrocyte agglutination at 37 °C incubation.

#### 3.4.5. *Cell viability assay*

The aim of cell viability assay was to evaluate in vitro cytotoxicity of NOSC towards rat primary hepatocytes. The cell viabilities of primary hepatocytes via MTS assay were $100.0 \pm 4.5\%$, $101.7 \pm 7.8\%$, $96.3 \pm 12.1\%$, $96.1 \pm 10.0\%$ and $87.9 \pm 5.8\%$ at the presence of 0 mg/ml, 10 mg/ml, 50 mg/ml, 100 mg/ml and 200 mg/ml of NOSC solution, respectively. This clearly indicates that NOSC has very low toxicity in rat primary hepatocytes.

## 4. Conclusion

This study presents a comprehensive evaluation of pharmacokinetics, tissue distribution, metabolism, excretion, acute toxicity, safety and cell viability assay of *N*-octyl-*O*-sulfate chitosan. It is technically difficult to characterize the in vivo behavior of macromolecule polysaccharide directly. So, in this study, we evaluated the tissue distribution and excretion of tritium labeled NOSC and characterized principal pharmacokinetic parameter of fluorescein labeled NOSC. After i.v. administration, $^{3}$H NOSC was widely distributed in most tissues of mice and the highest concentration was observed in kidney, the main excretion organ. The primary pathway of excretion of $^{3}$H NOSC in rats post-injection was urine. Pharmacokinetic analysis of FITC-NOSC in rats after i.v. injection showed that the AUC values increased with the increasing dose levels in a linear manner but CL values and Vd values fluctuated a little. These observations illustrated the possibility of a linear pharmacokinetics behavior of FITC-NOSC in rats. Safety study was valuable for the clinic test. There was no acute toxicity, haematolysis, allergy effect, stimulation effect on vessel and cytotoxicity at given respective concentration of NOSC. All these results can present a comprehensive evaluation of NOSC and provide considerable information for the future development of polymeric micelle system.

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