

Optimization of the preparation process of Xanthoceras sorbifolia Bunge saponin XS-8 chitosan nanoparticles and the study of their properties

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Abstract

In order to improve the bioavailability and ability to cross the blood-brain barrier of the larger molecular weight compound *Xanthoceras sorbifolia* Bunge saponin XS-8, the optimal conditions for the preparation of XS-8 chitosan nanoparticles were established. A series of characterization, property evaluation and cytological studies of XS-8 chitosan nanoparticles were carried out. The results showed that the angeloyl group of XS-8 was involved in the formation of nanoparticles and XS-8 was successfully loaded onto the surface of chitosan nanoparticles with uniform distribution by an encapsulation efficiency of 88.73%, drug loading of 8.97% and mean particle size of 258.4 ± 1.72 nm. The digestion rate of XS-8 chitosan nanoparticles in both intestinal and gastric juices was slower than that of XS-8, which will lead to the reduction of the stimulation damage to the gastrointestinal and digestive systems. PC12 cytology experiments showed that XS-8 chitosan nanoparticles had no significant effect on cell growth and the uptake of XS-8 chitosan nanoparticles by PC12 cells increased significantly in 1h and gradually raised with time. In vivo fluorescence distribution experiments in mice showed that XS-8 chitosan nanoparticles could cross the blood-brain barrier into the brain. All these results provided important data for the application of XS-8 in the treatment of AD and supplied a new solution strategy for the blood-brain barrier restricting the entry of drugs into the central nervous system.

1. Introduction

Alzheimer's disease (AD), commonly known as senile dementia, is a degenerative disease of the central nervous system characterized by cognitive impairment and memory damage[1]. Patients with the disease often suffer from a variety of symptoms including cognitive decline, memory loss, emotional and behavioral abnormalities, difficulty speaking and swallowing, and loss of independent living[2]. AD severely impairs the quality of life of patients and their families. However, existing medications are associated with numerous side effects and their efficacy varies from person to person[3–5]. As a result, more and more research scholars are turning their attention to the development and use of natural plants.

Natural medicines have been used extensively by mankind since ancient times. Today, in the age of nanomedicine, the use of such natural products is increasing. However, their pharmacological activity is hampered by some major barriers such as poor gastrointestinal absorption, rapid humoral degradation, poor water solubility, poor bioavailability and poor intestinal permeability[6]. At this stage of research, nanomedicine has been developed for the treatment of AD and nanocarriers facilitate specific targeting of AD[7, 8]. Nanoparticles can enhance the ability of macromolecular drugs to cross the blood-brain barrier and improve the bioavailability of drugs[9, 10].

Xanthoceras sorbifolia Bunge (Yellow horn) belongs to the Sapindaceae family with only one species in one genus, it is an indigenous and woody shrub mainly distributed in the northeast and north of China. Traditionally, its bark and fruits are used to treat rheumatism and enuresis in children as a folk medicine in China. In addition to its applications in food, ecology and energy, its stem, leaves, fruit shell, stalk and

seed kernel parts can be used as medicine and have good medicinal value[11]. Further studies have shown that the Barrigenol-type triterpenoids in the shell of *Xanthoceras sorbifolia* Bunge have good neuroprotective effects and can improve learning and memory in AD model rats[12–14].

Barrigenol-type triterpenoids are the major constituents of *X. sorbifolia* with a variety of activities[15]. XS-8 ($C_{65}H_{106}O_{29}$) (Fig. 1) is a barrigenol-type triterpenoid with a large molecules isolated from the carpophore and shell of *X. sorbifolia* Bunge by our research team. XS-8 exhibited significant protective effects against learning and memory impairments induced by ICV- $A\beta_{1-42}$ through tested for Y-maze, Morris's water maze, novel object recognition and passive avoidance tests. The mechanism study showed that XS-8 could significantly alter $A\beta$ -induced oxidative defense and pro-inflammatory levels in the hippocampus[16]. However, the large molecular weight of XS-8 and the presence of the blood-brain barrier (BBB) somewhat hindered its effects[17]. Therefore, a new technique is needed to enable the entry and effect of XS-8 across the BBB in the brain. Studies have shown that nanocarriers facilitate specific targeting of AD [8, 18] and enhance the ability of macromolecules to cross the BBB and improve the bioavailability of drugs[9, 10].

In this paper, we design a chitosan nanoparticle loaded with XS-8 to enhance the ability of XS-8 to cross the BBB and improve its bioavailability[19, 20]. The physicochemical properties (size, zeta potential, morphology, encapsulation efficiency, release behavior in simulated gastrointestinal fluid and stability over time) of chitosan nanoparticles loaded with XS-8 were characterized. In addition, the biological properties (cellular uptake and cytotoxicity) of PC12 cells were also assessed.

2. Materials and Methods

2.1 Materials

Chitosan and dialysis bags (MW 8,000–14,000 Da, Biotopped, USA), sodium tripolyphosphate and potassium bromide (Shanghai Maclean Biochemical Technology), concentrated sulphuric acid (analytical grade, Tianjin Fuyu Fine Chemical Corporation). Amylase (A3176), pepsin (P7000) and trypsin (P7545) was bought from Sigma, potassium dihydrogen phosphate was bought from Tianjin Yongda Chemical Reagent Corporation.k65.

Nano-ZS ZEN3700 Malvern Particle Size Analyzer (Malvern UK), Regulus 8100 Scanning Electron Microscope (Hitachi, Ltd.), FTIR-650 Fourier Transform Infrared Spectrometer (Tianjin Gangdong Technology Co.), Tecan M200 PRO Multifunctional Enzyme Labeler (Thermo Forma Corporation, USA), DZKW-D-2 Electric Thermostatic Water Bath (Beijing Yong Guangming Medical Instrument Co.), 0TDZ5-WS Low-speed centrifuge (Xiang Yi Centrifuge Instrument Co.), 100KDa ultrafiltration centrifuge tube (Shanghai McLean Biochemical Technology Co.).

2.2 Animals

All the animal studies were performed in strict accordance with the guidelines of Chinese Society of Laboratory Animal Sciences to minimize the suffering of the animals throughout the study. ICR (22-25g, license No. 210726241100778925) mice were obtained from Changsheng Biotechnology Co., Ltd (China). Mice were kept in a regulated environment ($23 \pm 2^{\circ}\text{C}$, 40–60% humidity and 12 h/12 h light/dark cycle) with free access to food and water. The ethical review number for animal experiments was SYPU-IACUC-S2024-0410-102.

2.3 Blank nanoparticle preparation and screening

An appropriate amount of chitosan (CS) powder was weighed and dissolved with stirring in 0.5% glacial acetic acid. Chitosan solutions were prepared at concentrations of 0.5, 1, 1.5, 2 and 2.5 mg/mL. The appropriate amount of sodium tripolyphosphate (TPP) powder was weighed and dissolved in distilled water to make TPP solutions with concentrations of 0.5, 1, 1.5, 2 and 2.5 mg/mL. According to the different mass ratios of chitosan and sodium tripolyphosphate (2:1, 3:1, 4:1, 5:1, 6:1), the TPP solution of suitable concentration was slowly added into the CS solution of suitable concentration with magnetic stirring (850 r/min) for 30 min to obtain blank chitosan nanoparticles[21].

2.4 Preparation of chitosan nanoparticles with XS-8

The chitosan nanoparticles were prepared by ionic cross-linking method based on the previous blank nanoparticle screening and single-factor investigation of chitosan nanoparticle preparation conditions[22, 23]. An appropriate amount of chitosan powder was weighed and dissolved in 0.5% glacial acetic acid with stirring to make a certain concentration (1, 1.5, 2, 2.5 mg/mL) of chitosan solution. The appropriate amount of sodium tripolyphosphate powder was weighed and dissolved in distilled water to make a solution of sodium tripolyphosphate at a certain concentration (0.5, 1, 1.5, 2 mg/mL). One milliliter of 1 mg/mL XS-8 solution was added to the prepared chitosan solution, and then the appropriate concentration of sodium tripolyphosphate solution was added slowly according to the different mass ratios of chitosan to sodium tripolyphosphate (3:1, 4:1, 5:1, 6:1) with magnetic stirring (850 r/min) for 30 min to obtain XS-8 chitosan nanoparticles.

2.5 Determination of encapsulation rate and drug loading rate

2 mL of chitosan XS-8 nanoparticles was added to a centrifuge tube with a molecular weight of 100 KDa and centrifuged at 4000 r/min for 10 min. Then, 0.5 mL of filtrate was removed with a pipette, 0.5 mL of 8% vanillin ethanol solution was added, 4 mL of 77% H_2SO_4 was added in an ice-water bath, and the mixture was shaken well and heated in a water bath at 60°C . The solution was heated in a water bath at 60°C for 15 min, cooled in an ice-water bath for 5 min and then removed to room temperature. The absorbance values of the filtrates under different conditions of chitosan nanoparticle preparation were measured at the maximum absorption wavelength of 400 nm using an enzyme standardization instrument and substituted into the standard curve to calculate the concentration of free XS-8, and the encapsulation efficiency and the drug loading capacity were calculated according to Eq. 1 and Eq. 2[22, 24].

The encapsulation efficiency is calculated as shown in Eq. 1:

$$\text{encapsulation efficiency}/\% = \frac{M_{\text{total}} - M_{\text{free}}}{M_{\text{total}}} \times 100$$

Note

In Eq. 1: " M_{total} " is the amount of XS-8 added and " M_{free} " is the amount of unloaded XS-8 in the supernatant after centrifugation.

The drug loading rate is calculated as shown in Eq. 2:

$$\begin{aligned} &\text{drug loading capacity}/\% \\ &= \frac{M_{\text{total}} - M_{\text{free}}}{M_{\text{chitosan}} + M_{\text{total}} - M_{\text{free}}} \times 100 \end{aligned}$$

Note

In Eq. 2: " M_{total} " is the amount of XS-8 added, " M_{free} " is the amount of unloaded XS-8 in the supernatant after centrifugation, " M_{chitosan} " is the amount of chitosan added.

2.6 Optimization of encapsulation rate of chitosan nanoparticles

The chitosan concentration (A), sodium tripolyphosphate concentration (B) and mass ratio of chitosan and sodium tripolyphosphate (C) were used as the factors under investigation, and the high, medium and low levels of each factor were expressed as 1, 0 and – 1, respectively. The encapsulation efficiency was used as the response value to establish the mathematical model for optimizing the prescription, and the specific design scheme is shown in Table 1.

Table 1
Factors and coded levels of
Y used in experimental
design for response surface
methodology

Level	Factors		
	A	B	C
-1	1.0	0.5	5:1
0	1.5	1.0	6:1
1	2.0	1.5	7:1

2.7 Particle size and potential

The Nano-ZS ZEN3700 Malvern Particle Size Analyzer was used for the analysis. The average particle size and polydispersity index (PDI) of the samples were recorded by taking 1 mL of the sample to be measured into a cuvette at 25°C. The sample to be measured was aspirated with a syringe and injected into a filled potentiometric cup to determine the zeta potential of the sample[25].

2.8 Scanning electron microscopy studies

The morphology of the screened XS-8 chitosan nanoparticles (1.5 mg/mL chitosan, 0.5 mg/mL sodium tripolyphosphate, 6:1 mass ratio of chitosan to sodium tripolyphosphate) was observed by scanning electron microscopy (SEM). XS-8 chitosan nanoparticle powder was fixed to the sample port using carbon double-sided tape and covered with gold (100 s) at 10–25 kV to obtain conductive samples[21].

2.9 FTIR studies

Fourier transform infrared spectroscopy (FTIR) was used to investigate the interaction of XS-8 and XS-8 chitosan nanoparticles. 1 mg of CS, TPP and XS-8 and XS-8 NPs and a physical mixture of CS, TPP and XS-8 were weighed and mixed with approximately 200 mg of spectrally pure KBr in a mortar, pressed into tablets at an ambient temperature of 25°C and humidity of < 50%, and placed in the FTIR for analysis, scanning wavelengths from 400–4000 cm^{-1} .

2.10 Evaluation of storage stability

The liquid and lyophilized powdered chitosan nanoparticles were stored at room temperature (25°C) and low temperature (4°C) respectively, protected from light, and all samples were placed in airtight glass bottles, and each sample was taken in parallel three times. Samples were taken at 0, 3, 5, 10, 15, 20, 25 and 30 days, the changes in the particle size of XS-8 chitosan nanoparticles and in the content of XS-8 were detected to determine its storage stability.

2.11 Evaluation in vitro release characteristics

1 mL 1 mg/mL of XS-8 and chitosan nanoparticles loaded with equal concentrations of XS-8 were placed in a dialysis bag (MW 8,000–14,000 Da) and tied at both ends. The bags were placed in dilution tubes containing 20 mL of pH 7.4 PBS solution, shaken at 37°C and 100 rpm, and 1 mL of release medium was removed at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h, and 1 mL of fresh isothermal release medium was added promptly. The released medium was color developed using vanillin-concentrated sulfuric acid, and the absorbance was measured at a maximum absorption wavelength of 400 nm using an ELIASA. The concentrations of XS-8 in the release medium at each time point were calculated by substituting the standard curve of XS-8 and then the cumulative percentage release of the drug was calculated with the help of Python software according to the following Eq. 3 to plot the in vitro release curve of XS-8.

Equation 3:

$$Q_n (\%) = (C_n V + V_i \sum_{i=1}^{i=n-1} C_i) / M_{XS-8}$$

Note

Eq. 3 in which Q is the cumulative release rate, n is the number of samples taken, C_n is the concentration of XS-8 in the release medium at the n_{th} sample, C_i is the concentration of XS-8 at the i_{th} sample, V is the total volume of release medium (20 mL), V_i is the volume of release medium removed at the i_{th} sample (1 mL), and M_{XS-8} is the total mass of XS-8 in the dialysis bag.

2.12 In vitro digestive stability studies

The in vitro digestibility stability of the XS-8 and XS-8 chitosan nanoparticles was investigated separately. The samples were mixed 1:1 with saliva, 5 mL of sample and 5 mL of simulated oral fluid, the pH of the mixture was adjusted to 6.8, and an equal amount (1 mL) of the component samples was removed after magnetic stirring for 3 min at 37°C. The remaining component samples were added to 8 mL of gastric fluid, and the pH of the mixture was adjusted to 2.0 after the addition of gastric fluid. The pH of the mixture was adjusted to 2.0 and kept at 37°C for 3 h. 20 mL of intestinal fluid was added to the remaining samples and the pH of the mixture was adjusted to 7.0 and kept at 37°C for 2 h. The sampling times for the simulated gastric fluid were 0.5, 1, 1.5, 2, 2.5 and 3 h. The sampling times for the simulated intestinal fluid were 0.5, 1, 1.5 and 2 h. The volume of each sample was 1 mL. After each digestion step, the enzyme was inactivated by adding ethanol to the removed samples three times in parallel for each group. The inactivated samples were centrifuged in ultrafiltration tubes, and the samples were then centrifuged and tested for the determination of XS-8 using the concentrated sulfuric acid-vanillin color development method. And the particle size of XS-8 chitosan nanoparticles was measured by DLS.

2.13 MTT cytotoxicity assay

PC12 cells were used as a cell model and were raised in a sterile cell compartment incubator at 37°C containing 5% CO₂. DMEM medium with 10% bovine serum FBS and 1% double antibodies (100 U penicillin and 100 U streptomycin) was used[26, 27]. PC12 cells were cultured using 96-well plates with

approximately 1×10^4 cells added to each well for 24 h at 37°C. Cells were then treated with a gradient of 0.5, 1, 5, 10, 50 and 100 $\mu\text{mol/L}$ concentrations of XS-8 and XS-8 nanoparticles for 20 h. 10 μL of 5 mg/mL MTT solution was added to the wells to give a final concentration. For the assay, the MTT medium was removed from the 96-well plate and 100 μL of DMSO was added and incubated for 20 min. The absorbance of each well was measured at 490 nm using an enzyme marker and cell viability was calculated using Eq. 4.

Equation 4:

Cell viability (%) = (mean OD of dosed wells/mean OD of control) $\times$ 100%

2.14 In vitro evaluation of the effect of crossing the BBB

2.14.1 Preparation of fluorescein isothiocyanate-labelled chitosan nanoparticles

Prepare 6 mL of 1.5 mg/mL solution of chitosan and add 1 mL of a 1 mg/mL solution of XS-8 to it. FITC (3 mg) was dissolved in 3 mL of methanol and added dropwise to the above mixture of XS-8 and CS under magnetic stirring conditions, and the reaction was carried out for 4 h at 25°C and protected from light to allow the carbon atoms on FITC to react with the amino groups on chitosan for labeling. The pH was adjusted to 9.0 with 10 mol/L NaOH. A mixture of methanolic water with dibasic product was added to the reaction solution, stirred thoroughly and centrifuged at 4000 r for 10 min. The supernatant was discarded, and the resulting precipitate was washed repeatedly with methanolic water until no fluorescence was detected under a fluorescence microscope. The precipitate obtained by centrifugation was then redissolved in 0.5% glacial acetic acid and 0.5 mg/mL TPP solution was slowly added to it according to the mass ratio of CS to TPP of 6:1. The isothiocyanate fluorescein-stained chitosan nanoparticles were obtained by magnetic stirring (850 r/min) for 30 min.

2.14.2 Quantitative evaluation of uptake by PC12 cell

After digestion with trypsin, PC12 cells were inoculated at a density of 5×10^4 cells/well in a prelined 6-well plate and incubated at 37°C in 5% CO_2 incubator for 24 h. After the cells were grown against the wall in the culture dish, the medium was aspirated, the cells were washed three times with PBS, and a suspension of FITC-labeled chitosan nanoparticles at 20 $\mu\text{g/mL}$ was added to the cells for 1 h, 2 h, and 4 h. The cells were then removed and rinsed twice with PBS, fixed in 4% paraformaldehyde for 30 min, and rinsed twice with PBS. The coverslips were removed from the bottom of the 6-well plates with forceps, inverted onto slides dripped with anti-fluorescence quencher containing DAPI, stained for nuclei and sealed, and allowed to dry away from light.

2.14.3 Qualitative evaluation of PC12 cell uptake

PC12 cells were inoculated at a density of 5×10^4 cells/well in a 6-well plate and incubated at 37°C in a 5% CO_2 incubator for 24 h. The cells were then incubated with 20 $\mu\text{g/mL}$ FITC-labelled nanoparticles for

1, 2 and 4 h. The cells were washed with PBS at the end of the incubation period, 200 μ L of trypsin was added to each well to digest the cells, and the supernatant was discarded after centrifugation and washed three times. After centrifugation, the supernatant was discarded and washed three times with PBS slow medium solution. Finally, 500 μ L of PBS was added and mixed to prepare a single cell suspension and quantify the fluorescence of FITC by flow cytometry.

2.15 In vivo evaluation of the effects of crossing the BBB

Male ICR mice were randomly divided into two groups after one week of feeding: free drug group and nano-preparation group. Their tail veins were injected with 500 μ L of free FITC and XS-8-CS-NP/FITC, respectively. Mice were photographed in vivo with a small animal live imaging system under excitation wavelength of 480 nm and emission wavelength of 520 nm at 4, 8, 12 and 24 h after injection. After 24 h of drug administration, the mice were executed, and the heart, liver, spleen, lung, kidney organs and brain tissues were quickly dissected out, cleaned with PBS and placed in the in vivo imager for observation and photography.

3. Results and discussion

3.1 Preparation of blank chitosan nanoparticles

The emulsion phenomenon was a sign of the formation of chitosan nanoparticles. By examining the effects of CS concentration, TPP concentration and the mass ratio of them on the state of the resulting solution (clarification, emulsion or precipitation), the preparation conditions of chitosan nanoparticles were preliminarily screened. From Table 2, the concentrations of CS and TPP as well as the mass ratio of them had an effect on the formation of nanoparticles.

Table 2
Screening of blank nanoparticles

mass ratio (CS:TPP)	CS potency (mg/mL)	TPP potency(mg/mL)				
		0.5	1.0	1.5	2.0	2.5
6:1	0.5	clarification	clarification	clarification	clarification	clarification
	1.0	clarification	emulsion	emulsion	emulsion	emulsion
	1.5	emulsion	emulsion	emulsion	emulsion	emulsion
	2.0	emulsion	emulsion	emulsion	emulsion	emulsion
	2.5	emulsion	emulsion	emulsion	emulsion	precipitation
5:1	0.5	clarification	clarification	clarification	clarification	clarification
	1.0	clarification	emulsion	emulsion	emulsion	emulsion
	1.5	emulsion	emulsion	emulsion	emulsion	emulsion
	2.0	emulsion	emulsion	emulsion	emulsion	emulsion
	2.5	emulsion	emulsion	emulsion	emulsion	precipitation
4:1	0.5	clarification	clarification	clarification	emulsion	emulsion
	1.0	emulsion	emulsion	emulsion	emulsion	emulsion
	1.5	emulsion	emulsion	precipitation	emulsion	emulsion
	2.0	emulsion	emulsion	emulsion	precipitation	emulsion
	2.5	emulsion	emulsion	emulsion	emulsion	precipitation
3:1	0.5	emulsion	emulsion	emulsion	emulsion	emulsion
	1.0	emulsion	emulsion	emulsion	emulsion	emulsion
	1.5	emulsion	emulsion	precipitation	precipitation	emulsion
	2.0	emulsion	precipitation	emulsion	precipitation	emulsion
	2.5	precipitation	emulsion	precipitation	emulsion	precipitation
2:1	0.5	precipitation	precipitation	precipitation	precipitation	precipitation
	1.0	precipitation	precipitation	precipitation	precipitation	precipitation
	1.5	precipitation	precipitation	precipitation	precipitation	precipitation
	2.0	precipitation	precipitation	precipitation	precipitation	precipitation
	2.5	precipitation	precipitation	precipitation	precipitation	precipitation

When the TPP concentration was constant, as the CS concentration increased, emulsification and precipitation became more frequent and clarification became less frequent. This was because the higher the concentration of CS in the TPP solution, the more it will lead to the cross-linking of the CS molecules with the small amount of TPP molecules, so that a single TPP molecules can cross-link multiple CS molecules. As a result, the average particle size in the suspension became larger and larger, which led to the changing of the solution from emulsion to precipitation.

When the CS concentration was constant, the emulsion phenomenon increased significantly as the TPP concentration increased. This was because the relatively high concentration of TPP could lead to the excessive local solution concentration, which can aggravate the degree of opalescence when a certain speed was not reached. Relatively excessive TPP could trigger CS to agglomerate into larger particles and precipitate down. while when the TPP was insufficient, it was difficult to cross-link with CS into nanoparticles, and the solution was in a clarified state.

Among the concentrations examined, the emulsion was more likely to appear when the mass ratio of CS to TPP is 4:1–3:1. When the mass ratio is higher than 4:1, fewer nanoparticles were formed and the solution was clarified; when the mass ratio was lower than 3:1, the emulsion suspension will precipitate, which was considered to be the aggregation of nanoparticles caused by too much cross-linking between TPP and CS.

3.2 Optimization of XS-8 chitosan nanoparticles by response surface methodology

The response surface design scheme and results from the Design-Expert 12 software were illustrated in Table 3 and Table 4. The multiple linear regression analysis was carried out with a linear regression equation:

$$Y = 95.586 + 0.351A - 2.386B + 4.985C + 0.875AB - 0.438AC - 1.233BC - 12.328A^2 - 3.318B^2 - 9.276C^2$$

($R^2 = 0.9870$, $p < 0.0001$).

Table 3
Box-Behnken experimental design and results

group	A	B	C	Pack rate%
1	1	0.5	6	83.10
2	2	0.5	6	82.67
3	1	1.5	6	75.46
4	2	1.5	6	78.53
5	1	1	5	67.98
6	2	1	5	68.94
7	1	1	7	79.90
8	2	1	7	79.11
9	1.5	0.5	5	79.14
10	1.5	1.5	5	77.95
11	1.5	0.5	7	90.50
12	1.5	1.5	7	84.38
13	1.5	1	6	94.71
14	1.5	1	6	93.27
15	1.5	1	6	97.01
16	1.5	1	6	95.21
17	1.5	1	6	97.73

Table 4

ANOVA of the regression model for the response variables (Y) and independent variables (A, B, C)

Factor	Encapsulation rate (%)						
	Sum of Squares	Df	Mean Square	Coefficient	f-value	p-value	
Intercept	1397.48	9	155.28	95.59	58.85	< 0.0001	significant
A	0.9870	1	0.9870	0.35	0.3741	0.5601	
B	45.55	1	45.55	-2.39	17.27	0.0043	
C	198.80	1	198.80	4.99	75.35	< 0.0001	
AB	3.06	1	3.06	0.87	1.16	0.3170	
AC	0.7656	1	0.7656	-0.44	0.2902	0.6068	
BC	6.08	1	6.08	-1.23	2.30	0.1729	
A ²	639.91	1	639.91	-12.33	242.54	< 0.0001	
B ²	46.35	1	46.35	-3.32	17.57	0.0041	
C ²	362.25	1	362.25	-9.28	137.30	< 0.0001	
Residual	18.47	7	2.64				
Lack of Fit	5.57	3	1.86		0.5760	0.6607	not significant
Pure Error	12.90	4	3.22				
Cor Total	1415.95	16					
$R^2 = 0.9870$, $R_{Adj}^2 = 0.9702$, $R_{Pred}^2 = 0.9228$							
Note: $p < 0.05$ indicates significant difference, while $p > 0.05$ indicates insignificant difference.							

As shown in Table 4, the back model developed for this test was reasonably highly significant ($F = 58.85$, $p < 0.0001$). The unfit term of this regression model was 0.6607 with $p > 0.05$, indicating that the model accurately predicts the optimal conditions for the chitosan nanoparticle encapsulation efficiency. The coefficient of determination of the regression model was 0.9870 with R^2 , and the corrected coefficient of determination was 0.9702 with R_{Adj}^2 , indicated that the independent terms in the model correlate well with the response values and that the predictions were in good agreement with the experimental results. The coefficient of variation of this regression model was less than 5% (C.V. = 1.94%), which fully demonstrated the reliability and reproducibility of this test. Therefore, this model can be used to optimize

the preparation conditions of chitosan nanoparticles and predict the encapsulation rate of chitosan nanoparticles.

The contour plots (2D) and 3D response surface plots of the interactions between various factors according to the regression model was illustrated in Fig. 2. The 3D plots more intuitively exhibit interactions between the factors. The surfaces of chitosan to sodium tripolyphosphate mass ratio (C) and sodium tripolyphosphate concentration (B) were steeper, and chitosan concentration (A) were slower, indicating that C and B have a greater influence on the encapsulation efficiency of chitosan nanoparticles compared A, which was consistent with the results shown in Table 4. For better passage of XS-8 chitosan nanoparticles across the blood-brain barrier, the preparation conditions of chitosan nanoparticles were further optimized by combining particle size. The results were shown in Table 5. Therefore, the optimal conditions for the preparation of XS-8 chitosan nanoparticles were chitosan concentration of 1.50 mg/mL, sodium tripolyphosphate concentration of 0.5mg/mL, and the mass ration of chitosan to sodium tripolyphosphate of 6:1.

Table 5

Entrapment efficiency, particle size and dispersion coefficient of nanoparticles under the optimal conditions corresponding to different concentrations of TPP

NO.	TPP concentration (mg/mL)	CS concentration (mg/mL)	Mass ratio (CS:TPP)	Average sealing rate (%)	Average particle size (nm)	Average dispersion coefficient
1	0.5	1.5	6:1	88.73	258.40	0.33
2	1.0	1.5	6:1	97.01	492.67	0.32
3	2.0	1.5	6:1	94.40	665.10	0.47

3.3 Determination of nanoparticle size and potential

The optimum conditions for the final XS-8 chitosan nanoparticles were obtained as follows: CS concentration of 1.5 mg/mL, TPP concentration of 0.5 mg/mL and CS to TPP mass ratio of 6:1. The results were shown in Fig. 3. The potential of the XS-8 chitosan nanoparticles under these conditions was measured at 54.6 ± 0.48 mV and the nanoparticle size was 258.4 ± 1.72 nm.

3.4 Scanning electron microscopy

The SEM result showed that (Fig. 4) the XS-8 nanoparticles were spherical in shape with smooth edges and a largely uniform distribution.

3.5 Fourier transform infrared spectroscopy

As shown in Fig. 5(A), 3424.00 cm^{-1} was attributed to the telescopic vibrations of hydroxyl, amino and amide groups in CS. 2925.54 cm^{-1} was $-\text{CH}_2-$ asymmetric telescopic vibrations. 1651.69 cm^{-1} was attributed to the telescopic vibrational peak of the amide I region of chitosan ($\text{C}=\text{O}$ telescopic

vibrational peak of acetyl group), 1600.00 cm^{-1} was attributed to the vibrational absorption peak of the amide II region (telescopic vibrational peak of C-N stretching vibration peak and N-H bending vibration peak), 1422.77 cm^{-1} was attributed to the amide III region vibration peak (C-N stretching vibration peak and N-H bending vibration peak). 1155.23 cm^{-1} and 1075.69 cm^{-1} were C-O and C-C stretching vibration peaks. 577.23 cm^{-1} is the KBr stretching vibration peak.

As shown in Fig. 5(B), 3405.54 cm^{-1} was the stretching vibration attributed to O-H in the XS-8. 2932.92 cm^{-1} was the $-\text{CH}_2-$ asymmetric stretching vibration. 1703.38 cm^{-1} and 1614.77 cm^{-1} were angelica acyl stretching vibrations. 1456.00 cm^{-1} , 1382.15 cm^{-1} and 1241.85 cm^{-1} were the C-H and O-H absorption peaks. 1079.38 cm^{-1} and 1038.77 cm^{-1} were due to C-O stretching vibrations. 595.69 cm^{-1} are KBr stretching vibration peaks.

The results in Fig. 5(C) showed that the vibrational absorption peaks of 1651.69 cm^{-1} , 1600.00 cm^{-1} and 1422.77 cm^{-1} amides in CS became weaker in the nanoparticles, which confirmed that both $-\text{CONH}$ and $-\text{NH}_2$ groups of CS were involved in electrostatic interactions with the phosphate group of TPP. The characteristic vibrational frequencies of angeloyl group of XS-8 shifted from 1703.38 cm^{-1} and 1614.77 cm^{-1} to 1644.31 cm^{-1} and 1570.64 cm^{-1} in nanoparticles, which suggested that both XS-8 and CS contained multiple hydrogen bond donors and acceptors and the angeloyl group of XS-8 was involved in hydrogen bonding., while the IR spectra of the blank nanoparticles showed no peak near this wave number. Besides that, the intensity of the C-O stretching vibration peak near 1083.08 cm^{-1} was increased. All the above data indicated that the XS-8 was successfully loaded into the chitosan nanoparticles.

In Fig. 5(C), Fig. 5(D) and Fig. 5(E), it was clear that the IR spectra of the XS-8 nanoparticles were distinctly different from the mixture of CS, TPP, and XS-8. The disappearance of the characteristic absorption peak $500\text{--}1000\text{ cm}^{-1}$ of TPP further suggested that TPP cross-linked with CS to form nanoparticles.

3.6 Evaluation of storage stability

The storage stability results for the liquid and lyophilized powder of XS-8 chitosan nanoparticles at 4°C and 25°C were shown in Fig. 6(A) and (B). For liquid chitosan XS-8 nanoparticles at 25°C , the encapsulation efficiency decreased significantly from 89.93% to 86.48% after 3 days of storage, and then the decreasing trend of encapsulation efficiency was less, but still decreasing. After 30 days storage, the encapsulation efficiency decreased to 82.35%. The encapsulation efficiency of chitosan nanoparticles in powder form at this temperature was almost stable at 92% for 30 days without any significant change. It was worth mentioning that the liquid and powder form of chitosan nanoparticles at 4°C was relatively stable, with encapsulation efficiency of 90% and 92% respectively for 30 days storage. In addition, the results of Fig. 6(C) indicate that the particle size of the liquid XS-8 chitosan nanoparticles was stable at around 250 nm without relatively obvious changes during 30 days of storage at 4°C and 25°C .

The experimental results suggested that the storage stability of powdered XS-8 chitosan nanoparticles was good, and it can be stored at room temperature while it was more suitable for liquid XS-8 chitosan nanoparticles to be stored at low temperature. Lyophilization of liquid XS-8 chitosan nanoparticles into powder further improved their preservation and enhanced the storage stability of liquid XS-8 chitosan nanoparticles at room temperature, avoiding the leakage of the loaded drug to the maximum extent.

3.7 In vitro release studies

The in vitro release curves of XS-8 and XS-8 nanoparticles were plotted based on the arithmetic results. As shown in Fig. 6(D), the release of XS-8 was basically complete after 6 h, and the cumulative release rate tended to be stable afterwards when the PH was 7.4. In contrast, the cumulative release rate of XS-8 nanoparticles was close to 100% near 12 h, indicating that chitosan nanoparticles played a buffering role on the release of XS-8. The uniform and almost linear release of the drug from the nanoparticles during the abrupt release phase may be due to the XS-8 adsorbed on the surface of the nanoparticles; the long duration of the slow release phase may be due to the slower dissociation of the drug dispersed between the polymer chains as they have intermolecular van der Waals forces and hydrogen bonds[28].

Further, release profiles of XS-8 (Fig. 7) and XS-8 chitosan nanoparticles (Fig. 8) were fitted by the zero-level model, the first-order model, and the Higuchi model. It could be seen that XS-8 chitosan nanoparticles might improve the stability of XS-8 to some extent and both XS-8 and XS-8 chitosan nanoparticles fitted the first-order release model (Table 6).

Table 6
Fitting results of in vitro release model of XS-8 and XS-8 nanoparticles

model	parameter	XS-8	XS-8 nanoparticles
Zero Order	R ²	0.5141	0.7654
	a	4.00	4.30
	b	41.49	15.78
First Order	R ²	0.9871	0.9148
	a	101.37	109.12
	b	0.37	0.11
Higuchi	R ²	0.8262	0.8701
	a	24.16	22.35
	b	15.74	-4.55

3.8 Evaluation of the stability of in vitro digestion

As shown in Fig. 6(E), the retention of XS-8 nanoparticles in simulative gastric juice decreased from 100% to 67.20% in the first 2 hours and remained at that level for the next one hour. In contrast, the retention rate of XS-8 in gastric juice decreased gradually from 100% to 41.72% over in the first 3 hours and did not plateau. The results in Fig. 6(F) showed that the particle size of XS-8 nanoparticles always remained around 220 nm with little variation as the digestion time proceeded. This was due to the high degree of ionization of chitosan in chitosan nanoparticles and the strong electrostatic interaction with sodium tripolyphosphate under acidic conditions. Therefore, the nanoparticles were more stable, to some extent, slowed down the digestion of XS-8 and improved its stability in the digestive fluid.[29, 30].

In the simulated intestinal fluid, the retention of XS-8 gradually decreased from 41.72% to 25.76%. The solubility of chitosan decreased at this pH and with the increase in incubation time, a sudden decrease in the retention of XS-8 nanoparticles occurred from 70.03% to 50.54% and the particle size of XS-8 nanoparticles occurred from 200 nm to 100 nm in the simulated intestinal fluid at 4-4.5 h. It might be that the low ionization of chitosan at this pH and the low cross-linking with sodium tripolyphosphate, which decreased the stability of the nanoparticles.

In summary, XS-8 chitosan nanoparticles were stable in acidic conditions (gastric simulated digestive solution) in vitro digestion stability evaluation. Moreover, the overall digestion rate of XS-8 nanoparticles was slower than that of XS-8, and the retention rate was better at the same time, which slowed down the damage of the drug to the gastrointestinal digestive system to a certain extent and improved the bioavailability of XS-8.

3.9 MTT cytotoxicity assay

The result of in vitro toxicity assay of PC12 cells in Fig. 9(A) showed that there was no significant decrease or increase in cell survival at 0-100 $\mu\text{mol/L}$ of XS-8 and XS-8 nanoparticles on PC12 cells except the cell survival rate increased from 100% to 113.24% at 50 $\mu\text{mol/L}$ of XS-8. Therefore, the concentration of 0-100 $\mu\text{mol/L}$ of XS-8 and XS-8 nanoparticles could be used in vitro study for further.

3.10 Quantitative evaluation of PC12 cell uptake

To examine the uptake of XS-8 chitosan nanoparticles by PC12 cells in response to XS-8, the mean fluorescence intensity of PC12 cells was quantified after coincubation with nanoparticles for 1, 2 and 4 h by flow cytometry. Additionally, the entry of chitosan nanoparticles into the cells was determined based on the magnitude of the fluorescence intensity[26]. The results were shown in Fig. 9(B). It was noteworthy that chitosan nanoparticles stained by fluorescence could enter PC12 cells after 1, 2 and 4 h. The average fluorescence intensity value at 1 h was much higher than that of 0 h, indicating that more nanoparticles had entered the cells at this time. And the uptake of chitosan nanoparticles by PC12 cells was gradually increased with the time. It was also evident from Fig. 9(C) that the average fluorescence intensity of the cells gradually increased from 1419 to 1948 as the coculture time was extended from 1 h to 4 h, which also indicated that the amount of nanoparticles taken up by the cells gradually increased.

3.11 Qualitative evaluation of PC12 cell uptake

The intracellular fluorescence of the FITC-labelled XS-8 chitosan nanoparticles was observed by inverted fluorescence microscopy after 1 h, 2 h and 4 h of coculture with PC12 cells. As shown in Fig. 10, the fluorescence signal of each group of FITC-labelled XS-8 chitosan nanoparticles was consistent with the results of flow cytometry quantification, and it could be seen more visually that there were fluorescently stained chitosan nanoparticles entering PC12 cells after 1, 2 and 4 h, and the number of nanoparticles entering the cells gradually increased.

3.12 In vivo distribution studies in mice

To evaluate the trans-BBB penetration and brain targeting ability of the nano-agents in mice, the biodistribution of the fluorescent substances in mice was examined using a small animal in vivo imaging system. The results of in vivo imaging were shown in Fig. 11(A) and (B). Compared with free FITC, the XS-8-CS-NP/FITC group showed a stronger fluorescence intensity in mice at the same time. In addition, it can be clearly seen that a more obvious fluorescence appeared in the brains of mice in the XS-8-CS-NP/FITC group at 24h of drug administration, which indicated that the nano-preparation gradually entered the mouse brain with gradual increase in time. Moreover, to further verify the biodistribution and brain-targeting ability of the nanomedicine, after 24 h of drug administration, the major organs (heart, liver, spleen, lung and kidney) and brain tissues were further stripped for ex vivo fluorescence imaging. As shown in Fig. 11(D), the nano-preparation were distributed throughout the body of the mice, with the liver being the strongest, followed by the brain and kidney. The results further confirmed the large accumulation of XS-8-CS-NP in the brain. In conclusion, the nano-preparation can assist the drug to effectively cross the BBB and thus accumulate in the brain, which helps to increase its therapeutic effect.

4. Conclusion

In summary, the chitosan nanoparticles could enhance the stability and bioavailability of XS-8. Besides that, the digestion rate of XS-8 chitosan nanoparticles in both intestinal and gastric juices was slower than that of XS-8 which suggested that XS-8 chitosan nanoparticles could decrease the stimulation damage to the gastrointestinal and digestive systems. Further study showed that XS-8 chitosan nanoparticles almost completely passed through PC12 cells within 4 h. This was a further possibility for the successful crossing of the BBB by the XS-8. In vivo fluorescence distribution experiments in mice showed that XS-8 chitosan nanoparticles could cross the blood-brain barrier into the brain. All these results provide important data for the application of XS-8 in the treatment of AD.

Declarations

Ethical Approval

All experimental procedures met the recommendations of the Chinese Society of Experimental Animals with the approval of the Animal Ethics Committee of Shenyang Pharmaceutical University. The ethical review number for animal experiments was SYPUIACUC-S2024-0410-102.

Conflicts of interest

The authors report no conflicts of interest.

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Author Contribution

Yaming Guo and Yang Wang wrote the main manuscript text ,Tianjiao Yan prepared figures and Bingjing Ma prepared . All authors reviewed the manuscript.

Data Availability

The data and material will be available on reasonable request.

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Figures

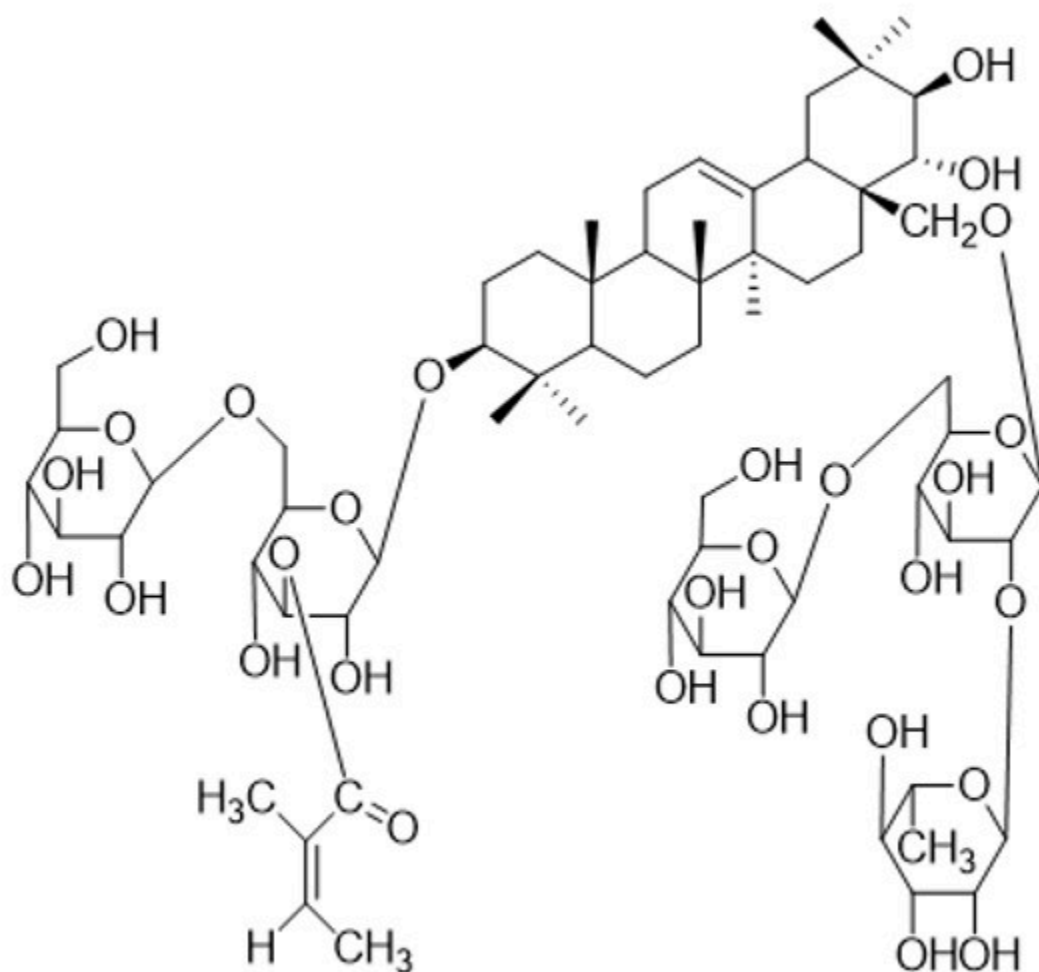


Figure 1

The structure of XS-8

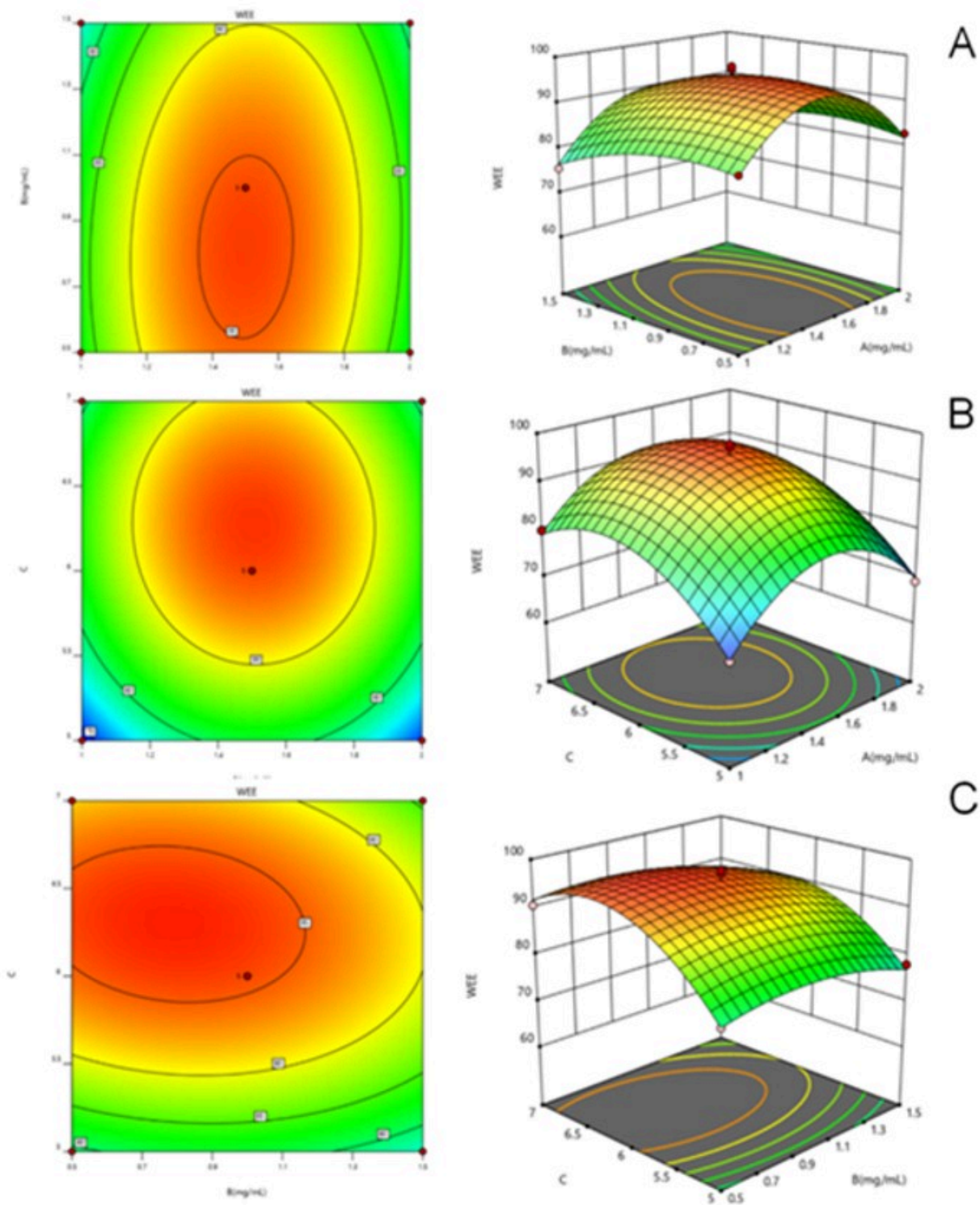


Figure 2

3D response surface plots and 2D contour plots for the influence of A and B on Y.(A); A and C on Y(B); B and C on Y(C)

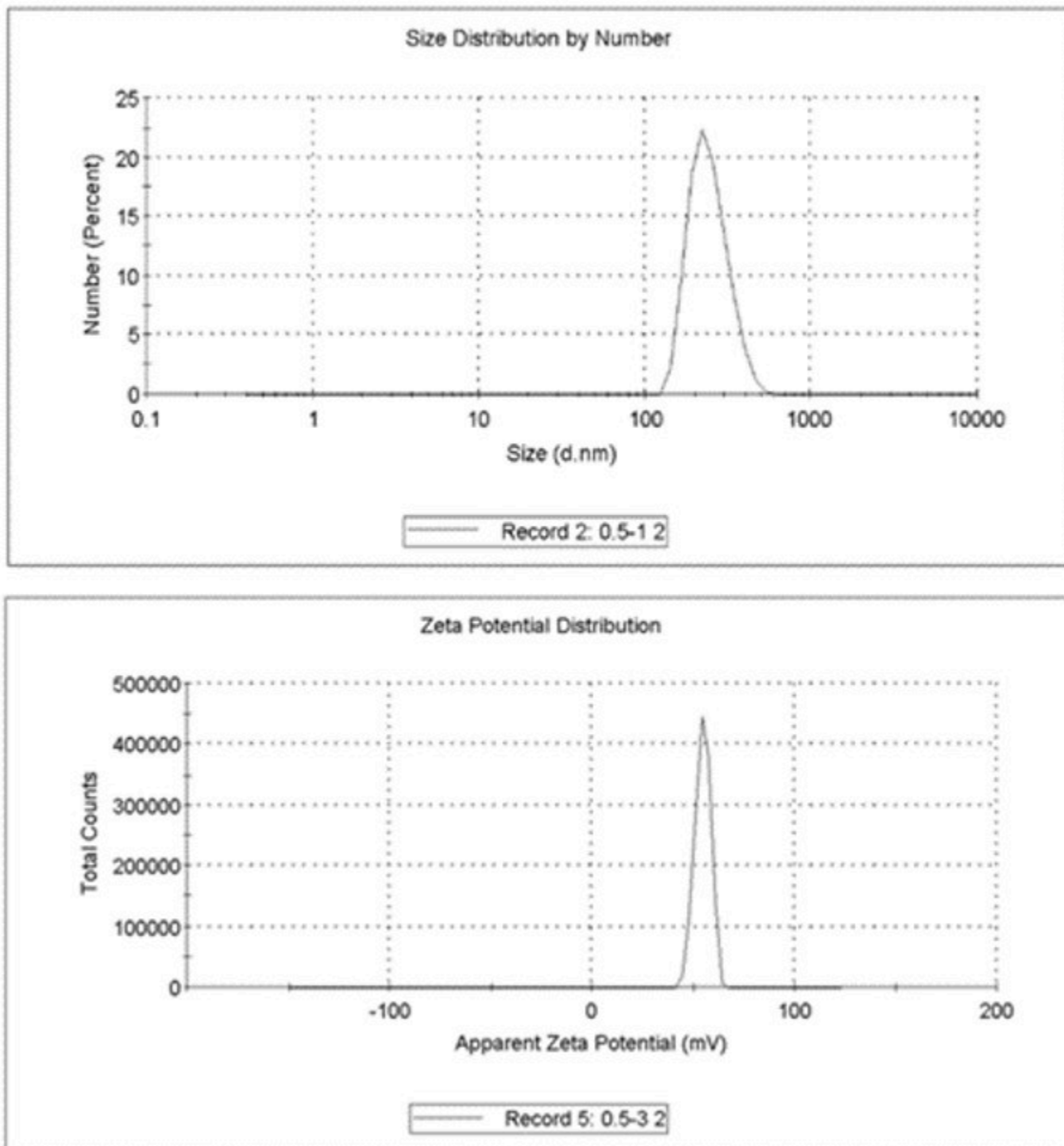


Figure 3

Particle size, dispersion coefficient and zeta potential of XS-8 nanoparticles

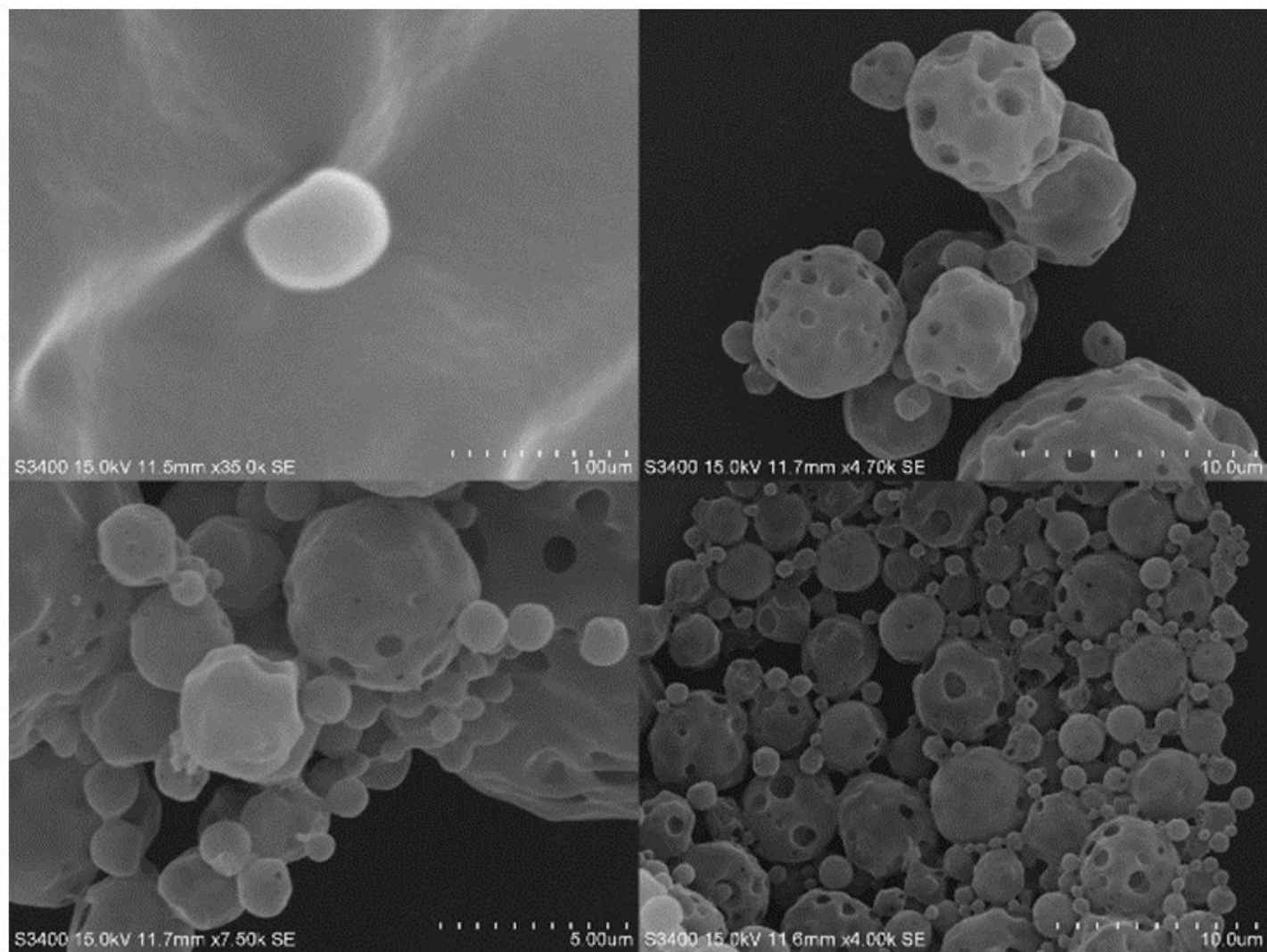


Figure 4

Scanning electron microscopy of XS-8 nanoparticles

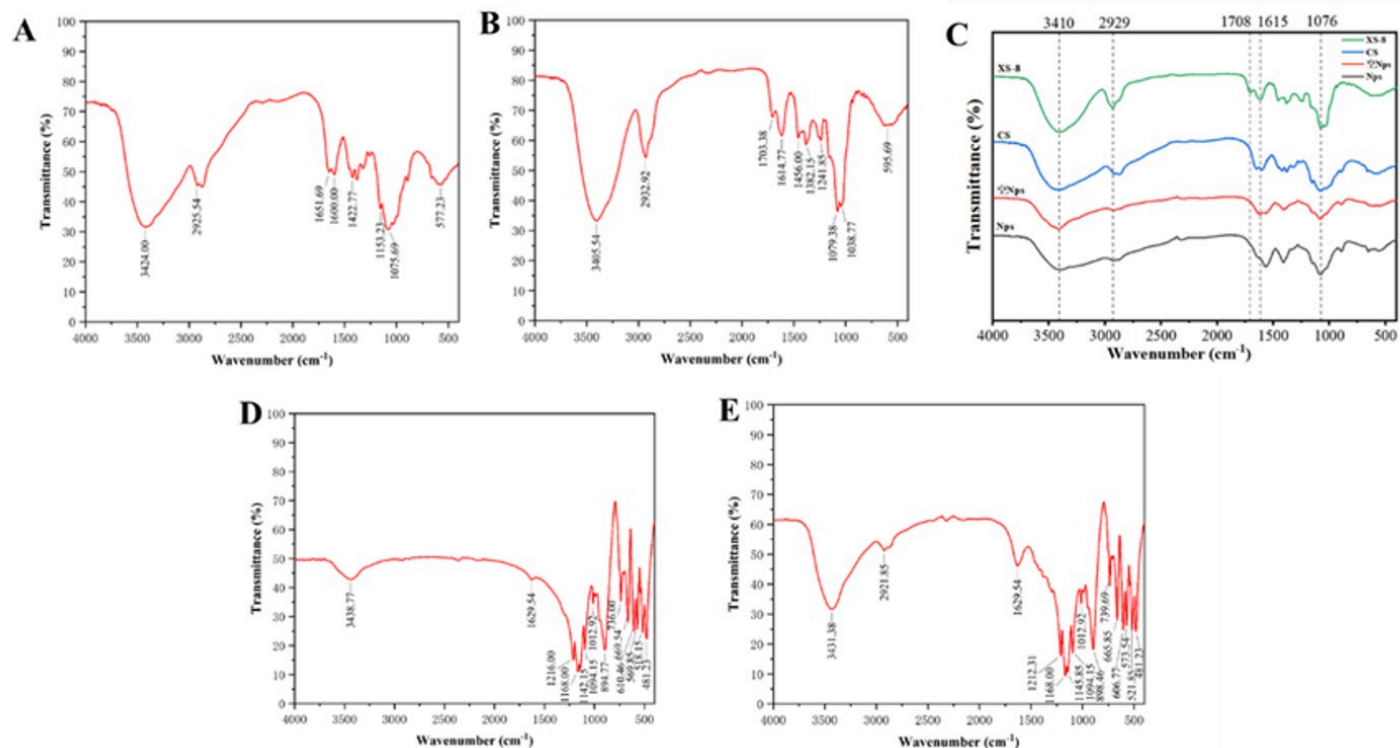


Figure 5

Fourier transform infrared spectroscopy of chitosan **(A)**; Fourier transform infrared spectroscopy of XS-8 **(B)**; Fourier transform infrared spectroscopy of XS-8, chitosan, blank nanoparticle and XS-8 nanoparticle **(C)**; Fourier transform infrared spectroscopy of TPP **(D)**; Fourier transform infrared spectroscopy of physical mixture of chitosan, TPP and XS-8 **(E)**

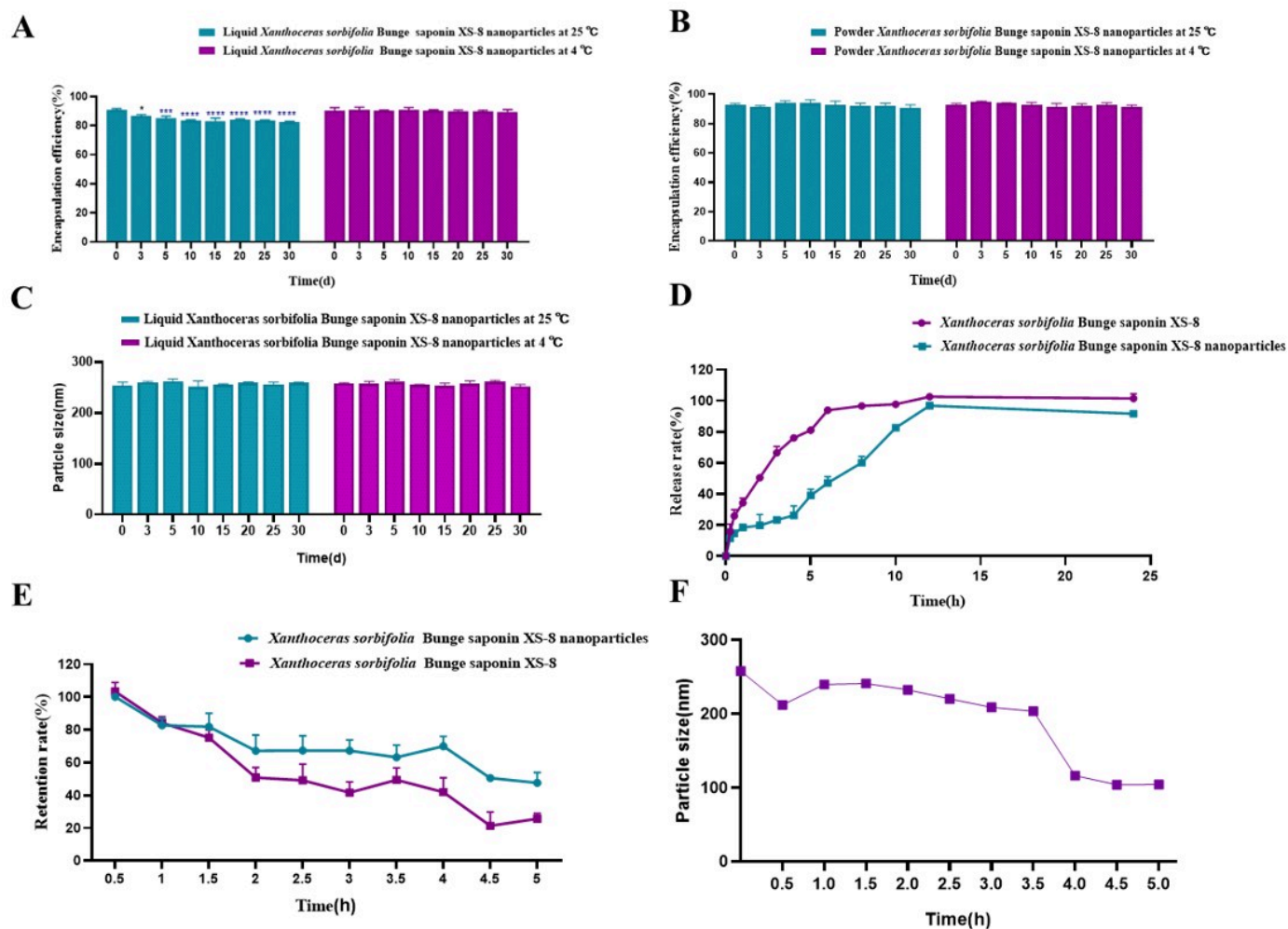


Figure 6

Storage stability evaluation of liquid XS-8 nanoparticles at 25°C and 4°C (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs. 0 group.) (A); Storage stability evaluation of powder XS-8 nanoparticles at 25°C and 4°C (B); Particle size of liquid XS-8 nanoparticles at 25°C and 4°C (C); In Vitro Release Profiles of XS-8 and XS-8 nanoparticles (D); In vitro digestion stability evaluation of XS-8 and XS-8 nanoparticles (E); Particle size of XS-8 nanoparticles in vitro digestion (F)

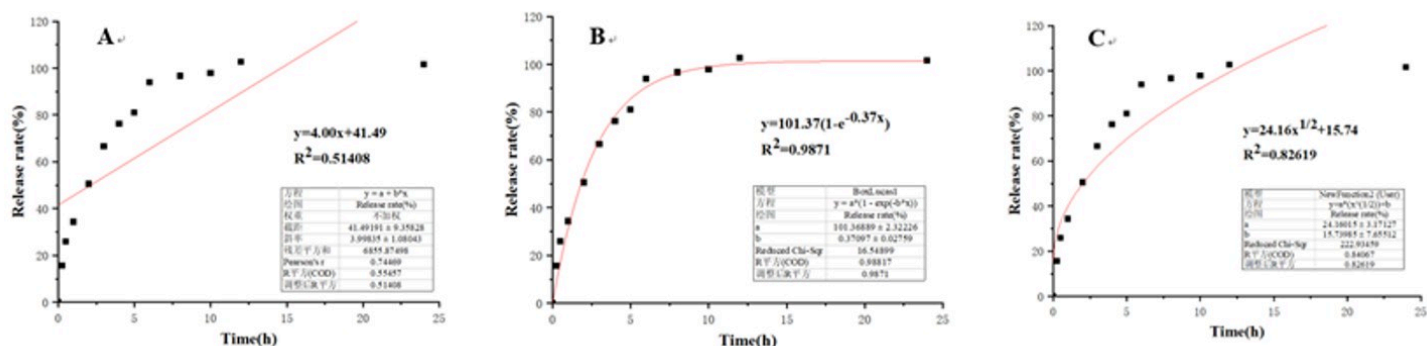


Figure 7

Zero-order release model (A), first-order release model (B) and Higuchi release model (C) of XS-8

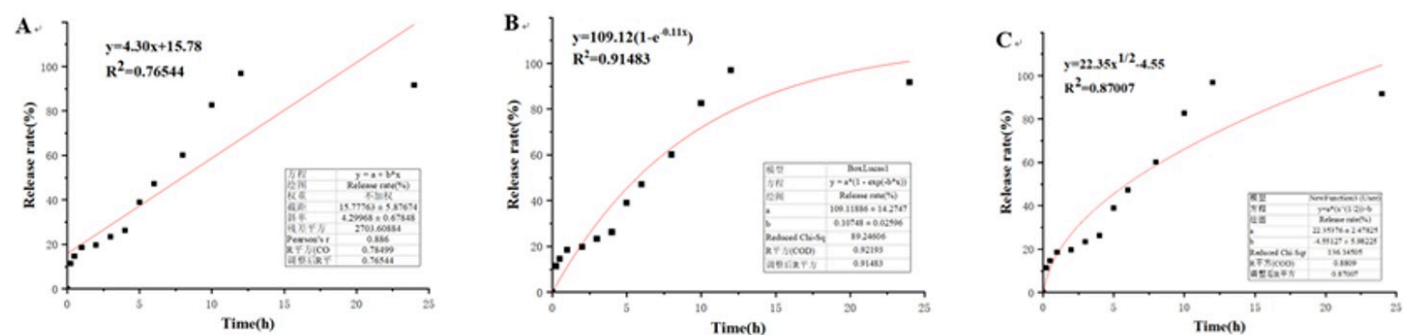


Figure 8

Zero-order release model (A), first-order release model (B) and Higuchi release model (C) of XS-8 nanoparticles

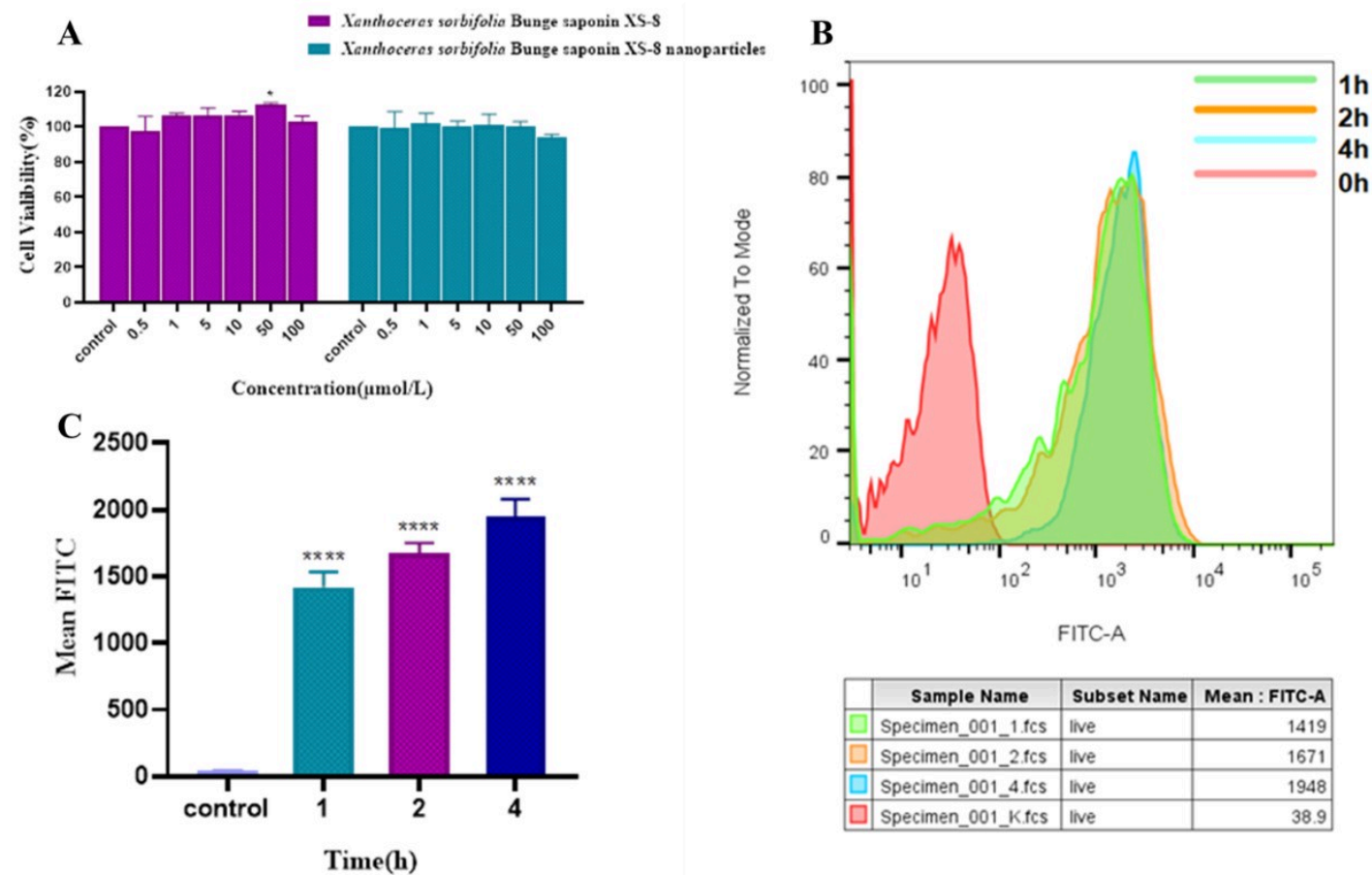


Figure 9

In vitro toxicity of different concentration of XS-8 and XS-8 nanoparticles on PC12 cells. (* $p < 0.05$ vs. control group.) (A); Cellular uptake of XS-8 nanoparticles by PC12 cells under different time (B); Analysis of average fluorescence intensity of PC12 cells at different time by flow cytometry. (**** $p < 0.0001$ vs. control group.) (C)

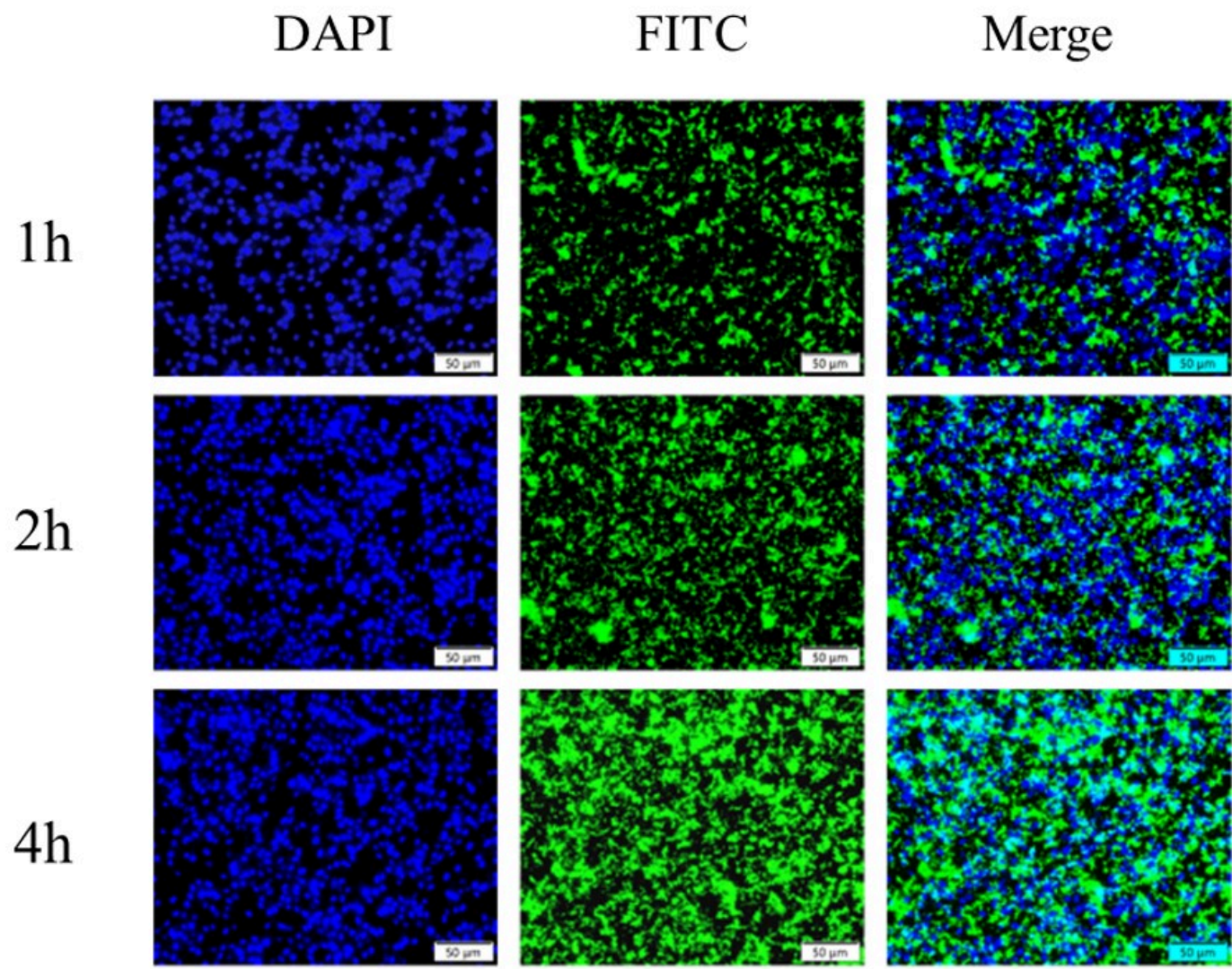


Figure 10

Qualitative evaluation of uptake of chitosan nanoparticles by PC12 cells at different time. (20 times mirror, scale length 50μm)

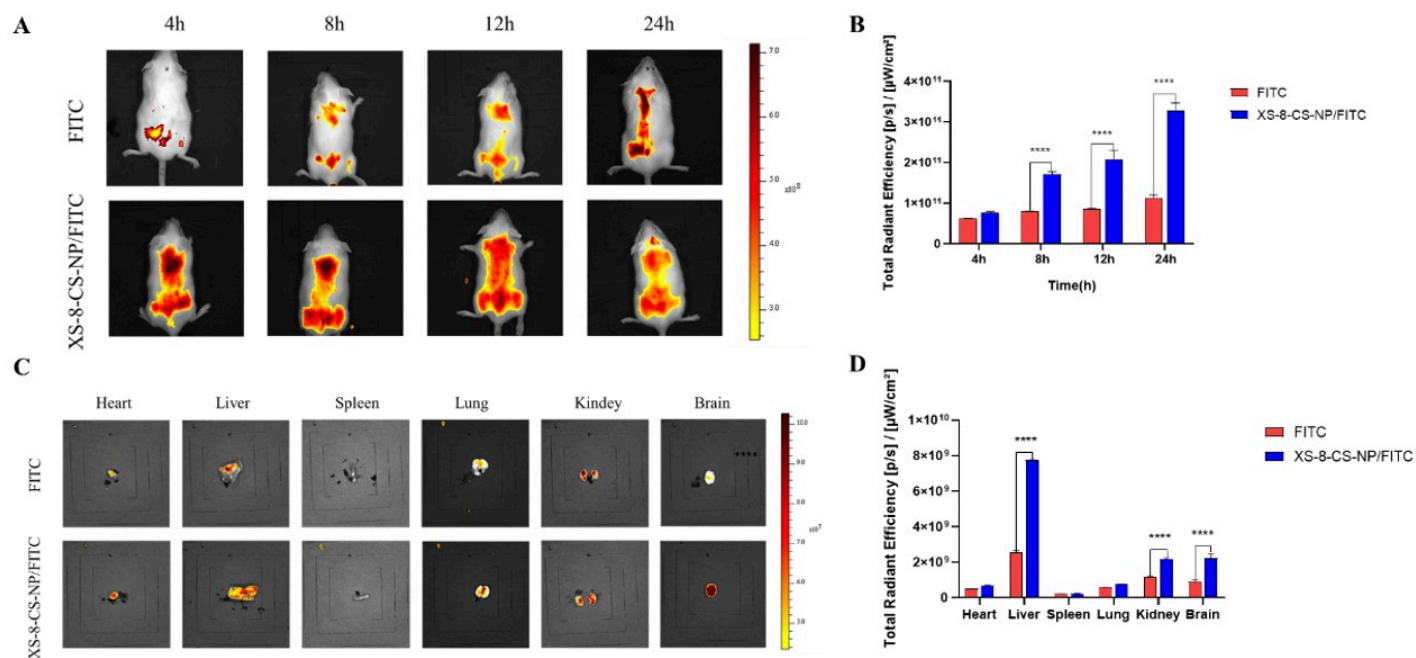


Figure 11

In vivo fluorescence imaging of mice at different time points after tail vein injection of free FITC and XS-8-CS-NP/FITC. **(A)**; Fluorescence intensity analysis of mouse body parts at different time points ($n = 3$. **** $p < 0.0001$). **(B)**; Ex vivo fluorescence imaging of various organs and brain in mice 24 h after tail vein injection of free FITC and XS-8-CS-NP/FITC **(C)**; Fluorescence intensity analysis of each organ and brain after 24 h. ($n = 3$. **** $p < 0.0001$) **(D)**

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