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Sustained release of ATP encapsulated in chitosan oligosaccharide nanoparticles

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ABSTRACT

The chemical cross-linked chitosan oligosaccharide (CSO) nanoparticles containing ATP/CSO ionic complex nano-components were prepared using combination techniques of W/O miniemulsion, chemical cross-linking and ionic complexation. The resulted nanoparticles had about 110 nm diameter and 20 mV surface zeta potential. The ATP loading efficiencies in nanoparticles could reach up to 40.6–69.5%. It was found that the ATP loading efficiency increased with increasing the amount and the molecular weight of chitosan oligosaccharide, and decreased with increasing molar ratio of glutaraldehyde to chitosan oligosaccharide. *In vitro* ATP release from chemical cross-linked CSO nanoparticles could continue for 24 h, and could also be adjusted by the amount and molecular weight of CSO, and the molar ratio of glutaraldehyde to CSO. The higher molecular weight and smaller amount of CSO, and the lower molar ratio of glutaraldehyde to CSO led the slower ATP release rate. Furthermore, it was also found that the CSO nanoparticles could be uptaken by HepG-2 tumor cells, and could be applied for intracellular drug delivery.

1. Introduction

In recent years, the developments of ingenious nanoparticles with special structure and component have attracted increasing attention for their potential applications in medical (Sengupta et al., 2007; Kim et al., 2008; Peer et al., 2008; Wei et al., 2008; Griset et al., 2009; Rosenholm et al., 2009), diagnose (Gao et al., 2008; Qian et al., 2008) and biotechnological area (Du et al., 2004, 2005).

Magnetic resonance imaging is an important approach of molecular images for disease diagnosis (Zabow et al., 2008). Magnetic resonance spectroscopy (MRS) is a main progress of magnetic resonance technology (Solga et al., 2005; Cecil, 2006). ^{31}P-MRS diagnoses the disease of liver through examining the energy metabolism change of liver, which displays the change of phosphide metabolism (Niemann et al., 2005). Because there was no obvious difference of the content of phosphomonoesterase (PME), phosphodiesterase (PDE) and adenosine triphosphate (ATP) between normal hepatic tissue and hepatopathy tissue *in vivo*, the ^{31}P-MRS was only used *in vitro* (Corbin et al., 2003, 2004). Targeting delivery is an effective approach for molecular imaging (Jaffer et al., 2006; Geninatti Crich et al., 2006; Kobayashi et al., 2006; Schroder et al., 2006). Targeting delivery of ATP to normal hepatic tissue or hepatopathy tissue may be an effective technology for the diagnosis of liver disease by ^{31}P-MRS. The hepatic targeting could be reached by galactosylation of active reagent or carrier material, due to the less expression of asialoglycoprotein receptor on the cellular membrane of hepatoma carcinoma cell (Kim et al., 2005, 2006). However, the sustained release of hydrophilic drug with low molecular weight such as ATP is still a challenge (Ubrich et al., 2004), which can reduce the loss of active reagent in the systemic circulation, and improve the targeting efficiency.

Many efforts were made to improve the poor drug encapsulation efficiency and rapid release of the water-soluble drug with the help of the nanoparticles or microspheres, however only few works were achieved. Chavanpatil et al. (2007) investigated a novel polymer-surfactant nanoparticle formulation, using the anionic surfactant Aerosol OT^{TM} (AOT) and polysaccharide polymer alginate, for sustained release of water-soluble drugs. Weakly basic molecules like methylene blue, doxorubicin, rhodamine, verapamil and clonidine could be encapsulated efficiently in AOT-alginate nanoparticles. *In vitro* release studies indicated that nanoparticles released 60–70% of the encapsulated drug over 4 weeks, with near zero-order release during the first 15 days. Studies with anionic drug molecules demonstrated poorer drug encapsulation efficiency and more rapid drug release than those observed with basic drugs.

Direct encapsulation of water-soluble drug into silica microcapsules was facilely achieved by a sol-gel process of tetraethoxysilane (TEOS) in W/O emulsion with hydrochloric acid (HCl) aqueous solution containing Tween 80 and drug as well as cyclohexane solution containing Span 80 (Wang et al., 2008). Two water-soluble drugs of gentamicin sulphate (GS) and salbutamol sulphate (SS) were successfully encapsulated into the voids of silica microcapsules. *In vitro*

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release behavior of drug in simulated body fluid (SBF) revealed that such system exhibited excellent sustained release properties.

Composite double-walled microspheres with biodegradable poly(L-lactic acid) (PLLA) shells and poly(D, L-lactic–co-glycolic acid) (PLGA) cores were fabricated with highly water-soluble etanidazole entrapped within the core as solid crystals (Lee et al., 2002). The delivery system was able to achieve higher entrapment efficiency for a highly water-soluble drug along with release profiles that contained a time lag.

Chitosan, a natural polysaccharide, consists of 2-amino-2-deoxy-(1-4b)-D-glucopyranose residues (D-glucosamine units) and N-acetyl-D-glucosamine units, which were obtained from chitin by deacetylation (Kean et al., 2005). Chitosan was generally regarded as non-toxic, biocompatible and biodegradable, and was widely accepted as a material of drug delivery carriers. In recent years, many researchers focused on the water-soluble chitosan with lower molecular weight, chitosan oligosaccharide (CSO), to reduce the viscosity, toxicity, and improve the solubility under physiological condition (Feng and Dong, 2006; Zhang et al., 2004).

In this study, the chemical cross-linked chitosan oligosaccharide (CSO) nanoparticles encapsulating ATP were prepared by combination techniques of water-in-oil (W/O), miniemulsion, chemical cross-linking and ionic complexation to gain the controlled and sustained release of ATP. The amount and molecular weight of CSO, and the molar ratio of crosslinker (glutaradehyde) to CSO were taken into account to the controlled release of ATP from nanoparticles. Using HepG-2 cells as the model cell, the cellular uptake ability of the nanoparticles was also evaluated.

2. Materials and methods

2.1. Materials

95% Deacetylated chitosan (Mw = 450 kDa) was supplied by Yuhuan Marine Biochemistry Co., Ltd., Zhejiang, China. Adenosine triphosphate (ATP) and hydrochloric acid were purchased from Hangzhou Meiya Biotechnical Co. Ltd. and Hangzhou Chemical Reagent Co. Ltd., China, respectively. Sodium hydroxide, dichloromethane, petroleum and benzene were obtained from Hangzhou Xiaoshan Chemical Reagent Co. Ltd., Hangzhou Shuanglin Chemical Reagent Co. Ltd. and Hangzhou Petroleum Refinery, China, respectively. Potassium dihydrogen phosphate, diethyl ether anhydrous, n-hexane and glutaraldehyde solution 25% were supplied by Sinopharm Chemical Reagent Co.,Ltd. Span 80 and Tween 80 were both purchased from Wenzhou Qingming Chemical Co., Ltd., China. All other solvents were of analytical or chromatographic grade.

2.2. Preparation of chitosan oligosaccharide (CSO)

Chitosan oligosaccharide (CSO) was obtained by enzymatic hydrolysis of chitosan (Du et al., 2009). 50 g of chitosan was dispersed in 2 L deionized water (DI water), and 18 mL of 36.5% (w/v) hydrochloric acid was added, then the temperature of the mixture was raised up to 55 °C and kept for 2 h, and 1 g chitosanase was then added. The reaction time of hydrolysis was controlled by molecular weight measurement of chitosan, performed by gel permeation chromatography (GPC). After the reaction, the temperature of the mixture was raised up to 80 °C, stirred for 30 min to completely inactivate the chitosanase. 0.3% (w/v) active carbon was then added. After stirring another 30 min, the mixture was centrifuged for 10 min under 4000 rpm. The supernatant was filtered through the Millipore filter (0.45 μm), and the low molecular weight chitosan, chitosan oligosaccharide (CSO) was obtained by lyophilization. The molecular weight of final chitosan oligosaccharide (CSO) was determined by gel permeation chromatography (GPC) with TSK-gel column (G3000SW, 7.5 mm I.D. × 30 cm) at 25 °C. Master samples of polysaccharide with different molecular weight (Mw = 1.0, 5.9, 11.8, 22.8, 47.3, 112, 212 kDa) were dissolved in acetate buffer solution (pH 6.0, the mobile phase), and their final concentrations were set to 1.0 mg mL^{-1}. Calibration curve was performed by means of polysaccharide samples using the integral molecular weight distribution method. The weighted lyophilized powder of CSO was dissolved in acetate buffer solution (pH 6.0) with a final concentration of 1.0 mg mL^{-1}. 10 μL of the sample was chromatographed with a flow rate of 0.8 mL min^{-1}. The molecular weight of CSO was then calculated from the calibration curve. The CSO with 5, 11 and 18 kDa weight average molecular weight were used in the preparation of chitosan oligosaccharide nanoparticles.

2.3. Preparation of chitosan oligosaccharide nanoparticles loading ATP

The chemical cross-linked chitosan oligosaccharide (CSO) nanoparticles encapsulating ATP were prepared by combination techniques of water-in-oil (W/O), miniemulsion, chemical cross-linking and ionic complexation. 0.1–0.2 g chitosan, 0.01 g ATP and 0.1 g Tween 80 were dissolved in 10 mL phosphate buffered saline (PBS) solution (pH 7.4). The PBS solution was added into 90 mL hexane solution containing 1% (w/v) Span 80, and the mixture was agitated for 10 min under 400 rpm by magnetic stirrer to prepare W/O pre-emulsion. The pre-emulsion was then treated by ultrasonic for 20 circulations (600 W, working 2 s following stopping 3 s) to prepare W/O miniemulsion. After the addition of glutaraldehyde (the amounts were 10, 20, 30 times of the molar number of chitosan), the crosslink reaction of chitosan was conducted under magnetic stirrer at 60 °C for 4 h. In order to form ATP/chitosan complex in cross-linked chitosan oligosaccharide nanoparticles, the pH of intra water phase was adjusted by the addition 500 μL of 1N chlorhydric acid solution. The nanoparticles dispersion was then washed thrice with petroleum benzene by the help of centrifugation (20,000 rpm, 15 min) to remove the hexane and water, and used for the further research.

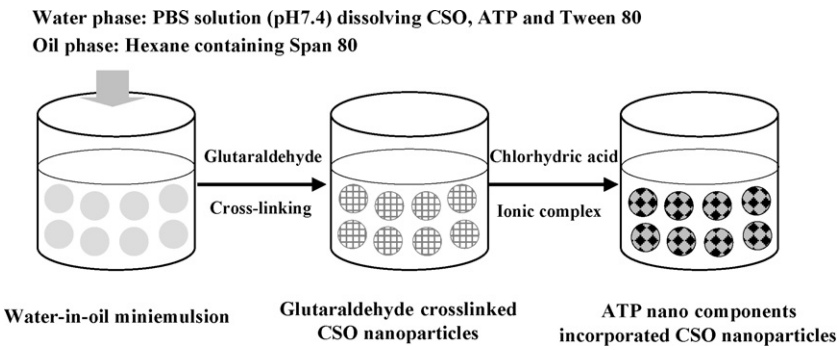
As a control, the chitosan oligosaccharide microparticles were prepared in the same preparation condition without the ultrasonic treatment.

2.4. Determination of particle size and zeta potential

The size and zeta potential of W/O miniemulsion and final chitosan oligosaccharide nanoparticles were determined by Zetasizer (3000HS, Malvern Instruments Ltd., UK). The size of chitosan oligosaccharide microparticles was determined by laser particle diameter (Winner 2000Z, Jinan Micro-nano instrument LTD. Co., China). The samples of miniemulsion were diluted by hexane. The samples of CSO nanoparticles and microparticles were prepared after the nanoparticles' dispersion washed thrice with petroleum benzene by the help of centrifugation, and re-dispersed in DI water.

2.5. TEM observation of nanoparticles

The morphology of CSO nanoparticles was examined by transmission electronic microscopy (TEM, TECNAI 10, PHILIPS, Dutch). A drop of nanoparticles' dispersions was dropped onto a copper grid without any staining. The air-dried samples were then directly observed under the transmission electronic microscopy.



Scheme 1. Preparation process of ATP nano-component incorporated chitosan oligosaccharide nanoparticles.

2.6. Determination of ATP content and ATP loading efficiency in CSO nanoparticles

The ATP content was determined by ultraviolet spectrophotometry. The UV wave length was set at 259 nm. The calibration curve of UV absorbance against ATP concentration was obtained using ATP PBS solution (pH 7.4). The UV absorbance of PBS was used as a blank. The good linear correlation was obtained in the range of 0.05–0.035 mg mL^{-1}. The regression equation was: $y = 23.58x + 0.007$ ($R^2 = 0.999$). The ATP loading efficiency was then calculated from the ATP content in the water phase (PBS) during the separation process of nanoparticles and the charged amount of ATP.

2.7. In vitro ATP release experiments from CSO nanoparticles

In vitro ATP release behaviors from chitosan oligosaccharide nanoparticles were performed using PBS (pH 7.4) as a dissolution medium. After the CSO nanoparticles' dispersion was washed thrice with petroleum benzene by the help of centrifugation (20,000 rpm, 15 min) to remove hexane and water, the CSO nanoparticles were re-dispersed in 25 mL PBS (pH 7.4) solution. The CSO nanoparticles PBS dispersion was then shaken horizontally (SHELLAB1227-2E, SHELLAB, USA) at 37 °C and 60 strokes/min. One millilitre of the dispersion was withdrawn from the system at definite time interval, and the dispersion was centrifuged (20,000 rpm) for 15 min, following filtrated with 100 nm filter. The ATP content in filtrate was determined by ultraviolet spectrophotometry as described above. All the ATP release tests were performed thrice.

2.8. Cellular uptake tests

HepG-2 cells were seeded in a 24-well plate at a seeding density of 10,000 cells per well in 1 mL of growth medium and allowed to attach for 24 h. Cells were then incubated with FITC labeled CSO nanoparticles dispersion (the concentration was 100 μg/mL) in growth medium for different time. The cells were then washed twice with PBS and directly observed under a fluorescence microscope (OLYMPUS America, Melville, NY).

3. Results and discussions

In this study, the chemical cross-linked chitosan oligosaccharide (CSO) nanoparticles encapsulating ATP were prepared by combination techniques of water-in-oil (W/O), miniemulsion, chemical cross-linking and ionic complex, to gain the controlled and sustained release of ATP. The preparation process was shown in Scheme 1. The recipes and properties of prepared chemical cross-linked CSO nanoparticles loading ATP were shown in Table 1. The chitosan oligosaccharide, low molecular weight chitosan was firstly prepared by enzyme degradation method (Du et al., 2009). CSO with 5, 11 and 18 kDa weight average molecular weight were used in the preparation of chitosan oligosaccharide nanoparticles. For the preparation of chitosan oligosaccharide nanoparticles encapsulating ATP, 0.1–0.2 g chitosan, 0.01 g ATP and 0.1 g Tween 80 were dissolved in 10 mL phosphate buffered saline (PBS) solution (pH 7.4). The PBS solution was added into 90 mL hexane solution containing 1% (w/v) Span 80, and agitated for 10 min under 400 rpm by magnetic stirrer to prepare W/O pre-emulsion. The pre-emulsion was then treated by ultrasonic for 20 circulations (600 W, working 2 s following stopping 3 s) to prepare W/O miniemulsion. After the addition of glutaraldehyde (the amounts were 10, 20, 30 times of the molar number of chitosan), the crosslink reaction of chitosan was conducted under magnetic stirrer at 60 °C for 4 h. In order to form ATP/chitosan complex in cross-linked chitosan oligosaccharide nanoparticles, the pH of water phase was adjusted by the addition 500 μL of 1 N hydrochloric acid solution. The nanoparticles' dispersion was then washed thrice with petroleum ether by the help of centrifugation (20,000 rpm, 15 min) to remove hexane and water, and used for the further research.

Using Tween 80 and Span 80 as surfactant for water and oil phase, and hexane as oil phase, respectively, the stable W/O miniemulsion with about 120 nm droplet size could be prepared by ultrasonic treatment, and the droplet size could be kept for further 12 h. After the cross-linking by glutaraldehyde, the sizes of chitosan oligosaccharide nanoparticles became about 110 nm, which were slightly smaller than those of miniemulsion droplets. It was also found that the sizes of W/O miniemulsion and chitosan oligosaccharide nanoparticles were not affected by the changes of amount

Table 1
Recipes and properties of chemical cross-linked CSO nanoparticles loading ATP.

Recipes no.	Amount of CSO (g)	M_W of CSO (kDa)	Molar ratio of Glu to CSO	Particle size (nm)	Zeta potential (mV)	ATP encapsulating efficiency (%)	ATP loading (%)
1	0.1	5	20:1	108 ± 1.00	21.0 ± 0.45	46.3	4.4
2	0.1	11	20:1	104 ± 2.64	20.6 ± 0.25	49.7	4.7
3	0.1	18	20:1	107 ± 3.06	20.3 ± 1.33	58.1	5.5
4	0.15	18	20:1	105 ± 3.21	21.6 ± 0.79	44.9	2.9
5	0.2	18	20:1	111 ± 4.51	22.6 ± 1.25	42.3	2.1
6	0.1	18	10:1	109 ± 2.64	23.8 ± 1.14	69.5	6.5
7	0.1	18	30:1	111 ± 4.51	18.2 ± 1.28	40.6	3.9

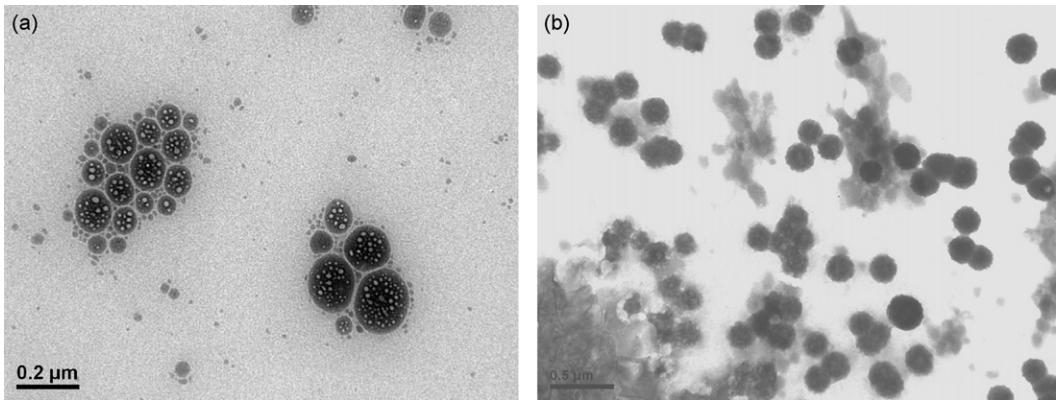


Fig. 1. TEM images of chitosan oligosaccharide nanoparticles loading ATP nano-components before (a) and after (b) ATP release test.

and molecular weight of chitosan oligosaccharide, and the molar ratio of glutaraldehyde to chitosan oligosaccharide.

The zeta potentials of prepared chitosan oligosaccharide nanoparticles were near 20 mV, and were slightly influenced by changing the amount and molar ratio of glutaraldehyde to chitosan oligosaccharide. The zeta potential decreased from 23.8 ± 1.1 to 18.2 ± 1.3 mV as the molar ratio of glutaraldehyde to chitosan oligosaccharide increased from 10:1 to 30:1. Because the increased amount of glutaraldehyde would reduce the primary amino group number of chitosan oligosaccharide in nanoparticles. However, the zeta potential could not be changed by altering the molecular weight of chitosan oligosaccharide when the molar ratio of glutaraldehyde to CSO was fixed.

The morphologies of chitosan oligosaccharide nanoparticles were observed by transmission electron microscope. The TEM image of chitosan oligosaccharide nanoparticles prepared by using CSO with 18 kDa molecular weight and 20:1 molar ratio of glutaraldehyde to chitosan oligosaccharide was shown in Fig. 1a. It could be found many of smaller components existed in the chitosan oligosaccharide nanoparticles. The smaller components inside the chitosan oligosaccharide nanoparticles could be caused by the formation of ATP/CSO ionic complex under the acid condition, because the pK_a of chitosan was about 6.5. In the miniemulsion and chemical cross-linking stages, the pH of water phase was 7.4, so the primary amino groups of chitosan oligosaccharide did not protonate. The CSO presented in PBS solution as molecular form, and did not complex with ATP. After the cross-linking by glutaraldehyde, pH was adjusted by HCl. Consequeantly the ATP could complex with some uncross-linked chitosan oligosaccharide to form ATP/CSO nano-components.

The ATP content and ATP loading efficiency in chitosan nanoparticles were determined by ultraviolet spectrophotometry. The ATP loading efficiency was calculated from the ATP content in the water phase during the separation process of nanoparticles and the charged amount of ATP. It was found (Table 1) the ATP loading efficiencies could reach up to 40.6–69.5%, and it was affected by the amount and molecular weight of chitosan, and the molar ratio of glutaraldehyde to chitosan oligosaccharide. The ATP loading efficiency increased with increasing the amount and the molecular weight of chitosan oligosaccharide, and decreased with increasing molar ratio of glutaraldehyde to chitosan oligosaccharide.

In vitro ATP release behaviors from chitosan oligosaccharide nanoparticles were then performed using PBS (pH 7.4) as a dissolution medium. Fig. 2 showed the *in vitro* ATP release behaviors from chitosan oligosaccharide nanoparticles comparing with that from chitosan oligosaccharide microparticles. The chitosan oligosaccharide microparticles were prepared by the same preparation condition without the ultrasonic treatment. The chitosan oligosaccharide microparticles had a D_{50} of 2.22 μm. Usually, the instantaneous dissociation of the ionic complex formed by ionic polymer with active reagent having opposite charge and low molecular weight occurred under the dissociation pH condition, and led to the rapid release rate of active reagent. From Fig. 2, it was found the ATP was completely released from chitosan oligosaccharide microparticles in 4 h, however, the ATP release from chitosan oligosaccharide nanoparticles could continue for 24 h. Although the chitosan oligosaccharide microparticles were also cross-linked by glutaraldehyde, comparing with chitosan oligosaccharide nanoparticles the polymeric network was relatively loose. The PBS solution and ATP could diffuse through the cross-linked polymeric network easily and consequently led to the faster dissociation of ATP/CSO complexes and ATP release rate. Comparing with ionic complex nanoparticles, the solid ATP/CSO nano-component existing in chemical cross-linked chitosan oligosaccharide nanoparticles could delay the dissociation of ATP/CSO ionic complex, and result in the slow ATP release rate from chemical cross-linked chitosan oligosaccharide nanoparticles.

Fig. 1b presented the TEM image of chitosan oligosaccharide nanoparticles after *in vitro* ATP release test. It was clear that on the surface and inside of chitosan oligosaccharide nanoparticles remained many smaller cavities after the ATP was completely released from chitosan oligosaccharide nanoparticles. The smaller cavities were remained after the dissociation of ATP/CSO complex and the release of ATP from chitosan oligosaccharide nanoparticles. This result demonstrated the ATP was encapsulated in chemical cross-linked CSO nanoparticles as ATP/CSO ionic complex form.

To further confirm the formation of solid ATP/CSO nano-component in CSO particles, the observation of optical microscopy

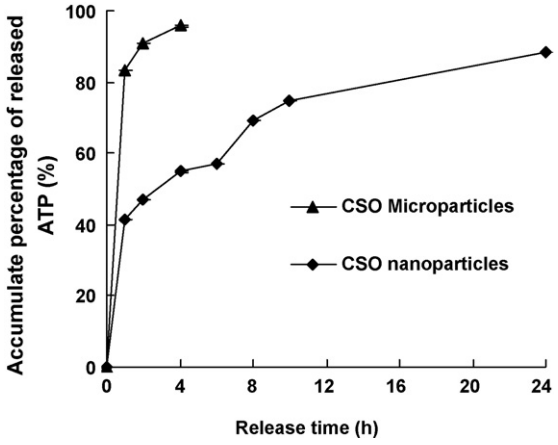


Fig. 2. *In vitro* ATP release behaviors from chitosan oligosaccharide microparticles and nanoparticles.

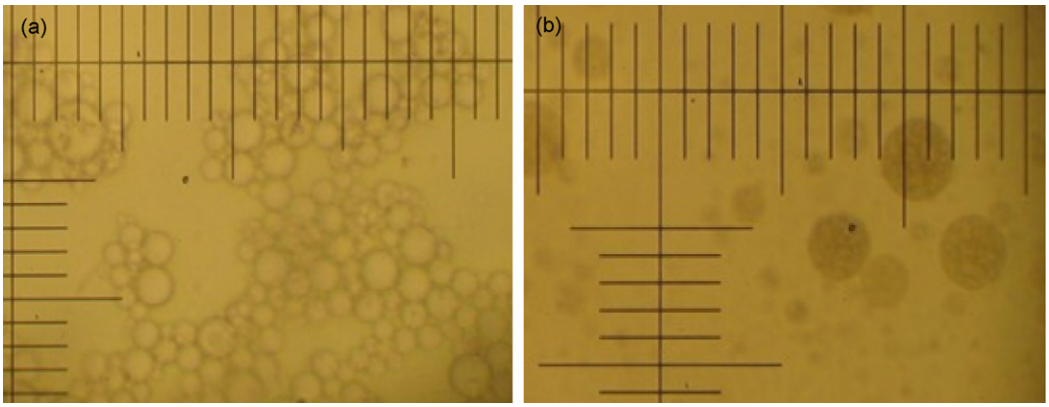


Fig. 3. Optical microcopies of water-in-oil emulsion for the preparation of chemical cross-linked CSO microparticles loading ATP. (a) Before the addition of HCl, and (b) after the addition of HCl.

was used in the preparation of chitosan oligosaccharide microparticles. Fig. 3a showed the chemical cross-linked CSO microparticles loading ATP before the pH adjustment, and Fig. 3b showed the chemical cross-linked CSO microparticles loading ATP after the pH adjustment. Many smaller particles were found in microparticles.

Fig. 4a showed the *in vitro* ATP release behaviors from CSO nanoparticles with different molecular weights of CSO. The molar ratio of glutaradehyde to CSO was fixed at 20:1. It was evident that the ATP release rate decreased with increasing the molecular weight of used CSO, which might be caused from the tight polymeric network structure of nanoparticles formed using CSO with higher molecular weight.

Fig. 4b showed the *in vitro* ATP release behaviors from CSO nanoparticles with different molar ratios of glutaradehyde to CSO. The CSO with 18 kDa molecular weight was used. From Fig. 4b, it could be seen that the ATP release rate was enhanced by increasing the molar ratio of glutaradehyde to chitosan oligosaccharide. Usually, the increase of cross-linking degree would reduce the ATP release rate. The increased ATP rate might be originated from the reduced free CSO molecule which could complex with ATP, by increasing the molar ratio of glutaradehyde to CSO.

Fig. 4c showed the *in vitro* ATP release behaviors from CSO nanoparticles with different amounts of CSO. The molar ratio of glutaradehyde to CSO was fixed at 20:1. It was clear that the ATP release rate increased with the additional amount of CSO. The increasing amount of CSO reduced the cross-linking degree and enhanced the diffusion rate of PBS and ATP via polymeric network.

Using HepG-2 tumor cells as a model cell of liver disease, the cellular uptake tests of CSO nanoparticles loading ATP were performed. The CSO nanoparticles were firstly labeled with FITC with 2:1 molar ratio of FITC molecules to CSO molecules. Fig. 5 showed the fluorescence image of HepG-2 cells after the cells were incubated with 100 μg/mL FITC labeled CSO nanoparticles containing ATP for 30 h. It was clear that the CSO nanoparticles could be uptaken by HepG-2 cells, which would be used for the further *in vivo* diagnosis of the liver disease.

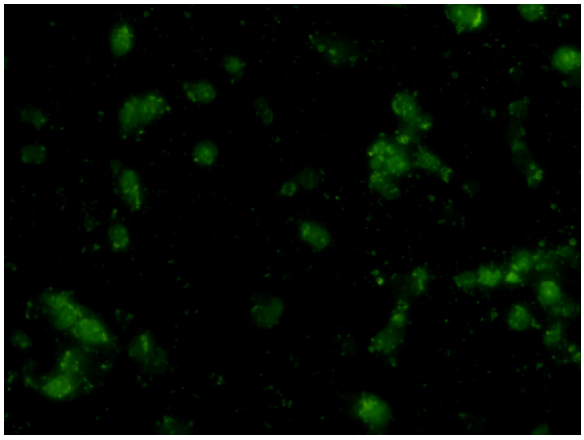


Fig. 5. Fluorescence image of HepG-2 cells after the cells were incubated with 100 μg/mL FITC labeled CSO nanoparticles containing ATP for 30 h.

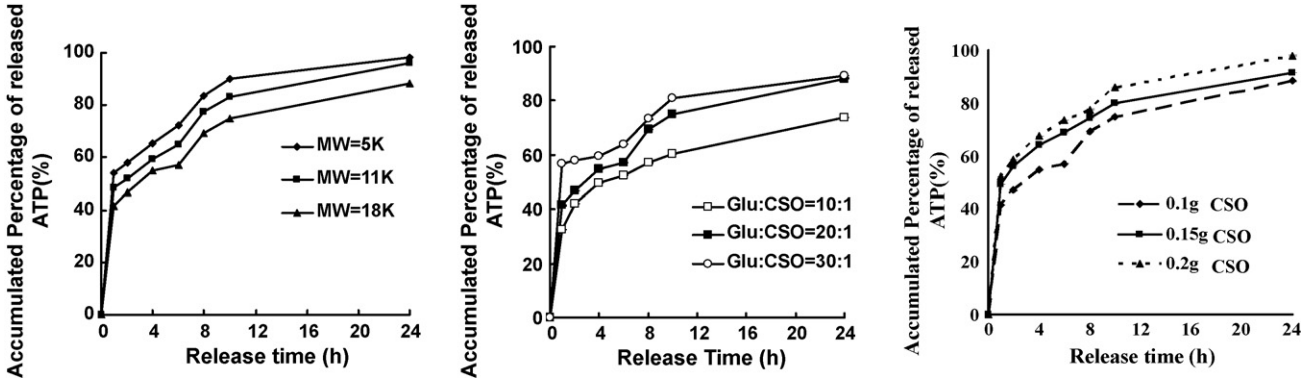


Fig. 4. *In vitro* ATP release behaviors from chitosan oligosaccharide nanoparticles with different molecular weight of CSO (a, in which the molar ratio of glutaraldehyde to CSO was fixed at 20:1), chitosan oligosaccharide nanoparticles with different molar ratio of glutaraldehyde (Glu) to CSO (b, in which the CSO with 18 kDa molecular weight was used) and chitosan oligosaccharide nanoparticles with different amounts of CSO (c, in which the molar ratio of glutaraldehyde to CSO was fixed at 20:1, and the CSO with 18 kDa molecular weight was used).

4. Conclusion

The combination techniques of W/O miniemulsion, chemical cross-linking and ionic complexation could prepare chemical cross-linked CSO nanoparticles encapsulating ATP/CSO nanocomponents. The ATP release from nanoparticles could continue for 24 h, and could be adjusted by the amount and molecular weight of chitosan, and the molar ratio of glutaradehyde to CSO. This kind of CSO nanoparticles also indicated cellular uptake ability by HepG-2 tumor cells.

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