In Vitro Release of Rifampicin and Biocompatibility of Oleoylchitosan Nanoparticles

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Received 16 January 2008; accepted 24 July 2008
DOI 10.1002/app.29175
Published online 18 November 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Oleoylchitosan (OCS) self-assembled nanoparticles as a carrier system for hydrophobic drug delivery was proposed. The OCS nanoparticles were prepared by an o/w emulsification method. Mean diameter of the OCS nanoparticles was around 275.3 nm. All the OCS and OCS nanoparticles have good biocompatibility from the cytotoxicity testing and erythrocyte toxicity assay. And the biocompatibility of OCS nanoparticles was better than OCS. Rifampicin, as a model drug, was investigated for its release properties *in vitro*. The release of rifampicin from solution with pH 6.0 and 6.8 was characterized by a faster release than from solution with pH 3.8. The increase of sodium tripolyphosphate could slower the release of drug. The sample with low concentration of rifampicin, released faster and entirely. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 2269–2274, 2009

Key words: oleoylchitosan; nanoparticle; rifampicin; hemolysis; cytotoxicity

INTRODUCTION

Chitosan (CS), α-(1-4)-2-amino-2-deoxy-β-D-glucan, is a deacetylated form of chitin, an abundant natural polysaccharide present in crustacean shells. In its structure, CS is very similar to cellulose, except for the amino group replacing the hydroxyl group on the C-2 position.[1] Recently, CS has drawn increasing attention as a drug[2,3] or gene[4] carrier because of its advantages for biomedical applications such as biocompatibility, biodegradability, and biological activities.[5,6]

Polymeric amphiphiles consisting of hydrophilic and hydrophobic segments have also received increasing attention because they can form self-assembled nanoparticles and because they exhibit unique physicochemical characteristics such as a nanoparticle structure and thermodynamic stability.[7–9] Self-assembled nanoparticles, composed of polymeric amphiphiles, have been considered to provide opportunities for the site-specific delivery of drugs because they can solubilize various hydrophobic drugs,[10] increase bioavailabilty, and stay unrecognized during blood circulation. In the aqueous phase, the hydrophobic cores of polymeric nanoparticles are surrounded by hydrophilic outer shells. Thus, the inner core can serve as a nanocontainer for hydrophobic drugs. Among polymeric micelle systems, biodegradable polymeric micelles are most extensively utilized.

There had been many reports on the CS-derived nanoparticles used as drug carrier[11–14] or gene carrier.[15,16] Few studies on the CS amphiphilic derivatives used as the carrier of hydrophobic drug have been carried out. In our previous paper, we modified the CS moiety with a long chain alkyl group as hydrophobic function, and addition of an oleoyl group to the amino group providing the hydrophilic moiety.[17] The oleoylchitosan (OCS) can form nanoparticles in aqueous media which could be used as hydrophobic drug carrier.

In this article, OCS nanoparticles were prepared by an o/w emulsification method. Hydrophobic drug rifampicin was selected as model drug. The biocompatibility was evaluated using erythrocyte toxicity assay test and cytotoxicity assay. The drug release profile from the OCS nanoparticles were investigated by changing the pH, concentration of rifampicin as well as concentration of sodium

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Contract grant sponsor: NSFC; contract grant number: 30670566.
Contract grant sponsor: ISTCP; contract grant number: 2006DFA33150.
Contract grant sponsor: The Shandong Science Foundation; contract grant number: 2004BS08007.

tripolyphosphate (STPP) to evaluate the potential of the loaded nanoparticles as delivery system.

EXPERIMENTAL

Materials

CS, degree of deacetylation 82%, molecular weight 38 KDa, was made from crab shell and obtained from Biotech Co. Methylene chloride, ethanol, acetic acid, STPP, methyl thiazolyl tetrazolium (MTT), and DMSO were purchased from Sigma Chemicals and used without further purification. Rifampicin was kindly donated by the Jiangbei Pharmaceutical Factory (Zhejiang, China). OCS was prepared by reacting CS with oleoyl chloride, as described previously and the degree of substitution was 5%.[18]

Preparation of OCS nanoparticles and drug-loaded nanoparticles

OCS nanoparticles were prepared using an o/w emulsification method. Briefly, 10 mg of OCS was dissolved in 2 mL of 0.1*M* acetic acid solution. Methylene chloride (3%, v/v) were added to the OCS acetic acid solution while stirring and homogenized (5 min, 13,000 × g) with an ULTRA-TURRAX T-25 dispersing machine. The solution was held under vacuum for 30 min at 20°C to remove methylene chloride and then STPP solution was added as a crosslinking reagent.[19] The rifampicin-loaded nanoparticles was prepared by dissolving rifampicin in methylene chloride and subsequently emulsifying as described earlier.

Characterization of OCS nanoparticles

The morphology of the nanoparticles was observed by TEM with a JEM-2010. Solution of OCS nanoparticles was placed onto copper grill covered with nitrocellulose. It was dried at room temperature, and then was examined using a TEM by negative staining with an aqueous solution of STPP.

The size distribution of nanoparticles was measured by the DLS with a Zetasizer 3000. The DLS measurements were done with a wavelength of 632.8 nm at 23°C.

The Fourier transform infrared (FTIR) spectra of CS, OCS nanoparticles, and rifampicin-loaded nanoparticles were recorded on an Avater-360 FTIR spectrometer (Nicolet) at 20°C following the method of Shigemasa et al.[20] For the IR spectroscopic analysis, the solution of samples was freeze-dried. Then 2 mg of the samples was mixed with 100 mg of KBr and made into pellets.

Biocompatibility of OCS nanoparticles

Cytotoxicity testing

The cell viability was determined using fibroblasts by the MTT method.[21] The experiment was carried out according to the method described earlier. Briefly, the OCS nanoparticles sample was diluted for use with DMEM, 10% (v/v) BCS, penicillin (100 U ml^{-1} culture media), and streptomycin (100 U ml^{-1} culture media). L929 cells at logarithmic growth phase (3×10^4 cells/mL) were added to 96-well culture plates, respectively, at 100 μL/well, and incubated overnight to allow attachment. The medium was then replaced with the appropriate test medium containing each kind of sample and the plates were incubated at 37°C in a 5% CO_2, 95% air atmosphere for the specified time. Later 20 μL of MTT solution was transferred to each well and the plates were incubated for 4 h at 37°C and 5% CO_2. After incubation, supernatants were removed and 100 μL DMSO was added. Plates were placed on a shaking water bath at 37°C for 10 min to solubilize the formazan products and the absorbance was recorded at 490 nm. Cells treated with blank medium, without any sample, were used as the negative control. The percent of viability was expressed as the relative growth rate (RGR) as follows.

$$\mathrm{RGR} = \frac{D_{\mathrm{sample}}}{D_{\mathrm{control}}} \times 100\% \quad (1)$$

Blood compatibility

The hemolytic activities of the materials were investigated according to Parnham and Wetzig.[22] Whole blood was obtained from two healthy male adults (22–30 years). Then 8 mL human blood was diluted with 10 mL normal saline. OCS (0.2%) was prepared in the normal saline, and 10 mL such solution was added to the empty tubes. In brief, diluted blood (0.2 mL) was added to each sample that had been equilibrated in normal saline for 30 min at 37°C and then all the tubes were incubated for 60 min at 37°C in a shaking water bath. The release of hemoglobin was determined after centrifugation (700 × g for 10 min) by photometric analysis of the supernatant at 545 nm.

The erythrocyte toxicity assay of OCS nanoparticles was conducted as described by Bock and Muller.[23] To 1 mL of the nanoparticle suspension, 100 μL of erythrocyte stock solution was added into 1 ml of nanoparticles suspension. The mixtures were left for 2 h and centrifuged. Then 100 μL of the resulting supernatant was dissolved in 2 mL of an ethanol/HCl mixture [200 : 5 mL (v/v)]. The absorption of the mixture was determined at 399 nm by an UV spectrophotometer with a S2000.

Distilled water (100% hemolysis) and normal saline (0% hemolysis) were used as positive and

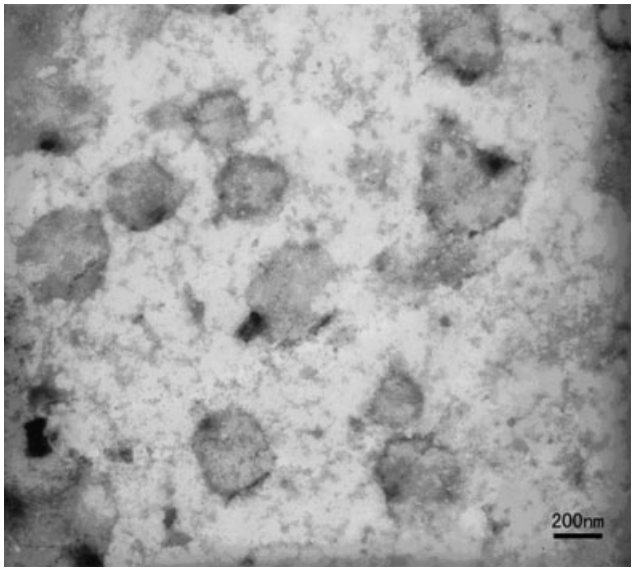

Figure 1 TEM photograph of OCS (DS 5%) nanoparticles (30,000×).

negative controls for this work, respectively, and they were treated in the same way as earlier. The hemolysis rate (HR) was calculated as follows:

$$\mathrm{HR} = \frac{D\mathrm{t} - D\mathrm{nc}}{D\mathrm{pc} - D\mathrm{nc}} \times 100\% \quad (2)$$

where Dt, Dnc, and Dpc are the absorbance of the sample, the negative control and the positive control, respectively. The experiments were run in triplicate and were repeated twice.

Evaluation of *in vitro* drug release

The drug entrapment efficiency (EE) was calculated from the ratio of the drug amount in the nanoparticles to the total drug amount added in the system. And the loading efficiency (LE) was calculated from the ratio of the drug amount in the nanoparticles to the weight of drug-loaded nanoparticles. A cellulose membrane (8000–10,000) tube containing 2 mL of the rifampicin-loaded nanoparticles solution was placed in 100 mL of acetate-buffer saline (pH 3.8) and phosphate-buffer saline (pH 6.0 and 6.8) at 37°C for 1 day under protection from light. Incubations were carried out in a water bath at 37°C while gentle stirring. Periodically, the whole media was removed and replaced with fresh media to maintain sink conditions. The amount of released rifampicin was assayed by spectrophotometry at 475 nm in comparison with the standard curve.

Statistical analyses

The assays were performed at least in triplicate on separate occasions. The data collected in this study were expressed as the mean value ± standard deviation.

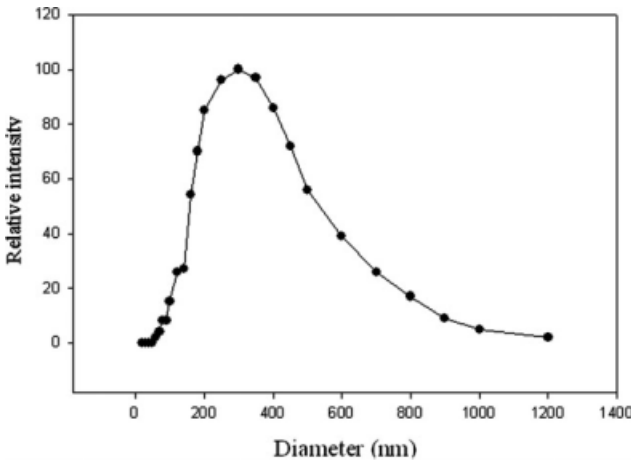

Figure 2 Size distribution of OCS (DS 5%) nanoparticles.

RESULTS AND DISCUSSION

Characterization of OCS nanoparticles

The morphology of the OCS nanoparticles was investigated by the TEM technique. Figure 1 shows the TEM image of polymeric micelles. It could be confirmed that polymeric micelles are spherical in shape.

Figure 2 shows the size distribution of the micelle particles formed by OCS (5 g/L) in the 0.1*M* acetic acid solution after o/w emulsification with methylene chloride (1%, v/v) and crosslinking with STPP. The majority number of the particles was around 275.3 nm in size.

FTIR studies

The FTIR spectra of CS and OCS nanoparticles were shown in Figure 3. Compared with CS, the absorption of OCS nanoparticles at 3000–4000 cm^{-1} (OH, NH_2) decreased, and the band at 1570 cm^{-1} (δ *N*—H of

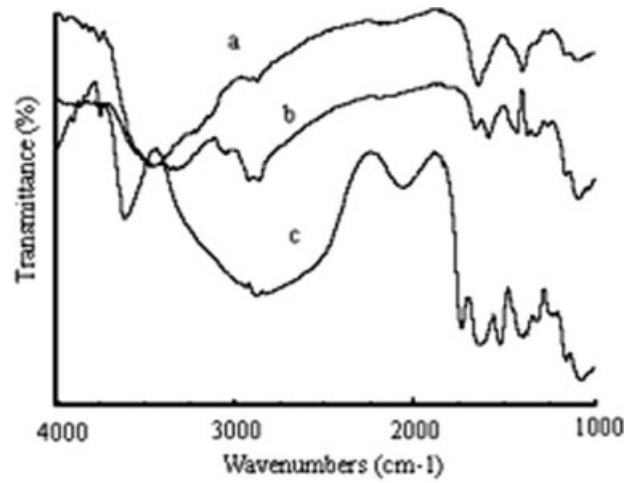

Figure 3 FTIR of CS, OCS nanoparticles, and rifampicin-loaded OCS nanoparticles. a, CS; b, OCS nanoparticles; and c, rifampicin-loaded OCS nanoparticles.

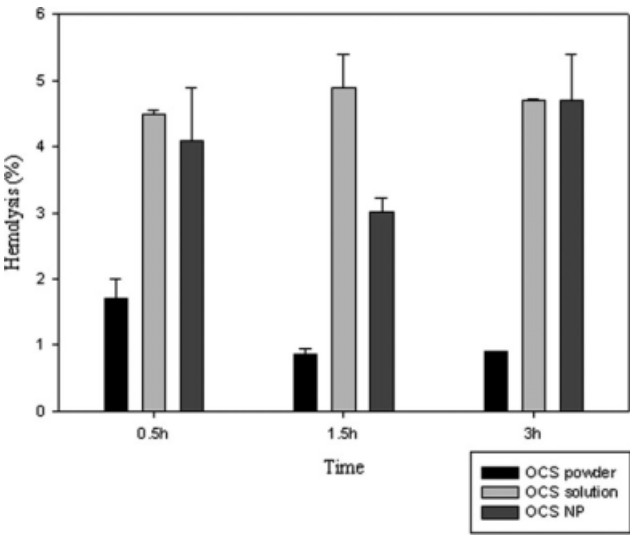

Figure 4 Red blood cell lysis by OCS and OCS nanoparticles.

amide) decreased, whereas prominent bands at 1655 cm^{-1} (ν C═O) and 1555 cm^{-1} (δ *N*—H of amide II) were observed. The peaks at 2924 cm^{-1} (ν_{as} CH_2), 2854 cm^{-1} (ν_s CH_2), 1464 cm^{-1} (δ CH_2), and 1182 cm^{-1} (twisting vibration of CH_2) were stronger and shaper.

FTIR spectra of rifampicin-loaded nanoparticles were shown in Figure 3. The rifampicin-loaded nanoparticles had characteristic peaks at definite wavenumber respectively. Rifampicin-loaded nanoparticles had characteristic peaks of rifampicin at 3580 cm^{-1} (—OH) and 1738 cm^{-1} (C—O). These results indicated the success of model drug rifampicin to OCS nanoparticles.

Biocompatibility of OCS nanoparticles

Erythrocyte toxicity assay

Red blood cells (RBCs) hemolyse when they come in contact with water. *In vitro* erythorocyte-induced hemolysis is considered to be a simple and reliable measure for estimating blood compatibility of materials.

Previously, the blood compatibility of unmodified CS in microspheres and emulsions were evaluated in terms of hemolysis.[24] Lee et al. investigated the blood compatibility of acylated CS in the formation of films with the measurement of dynamic viscoelasticity by a rheological method.[25] In this work, hemolysis was used to evaluate the biocompatibility of amphiphilic OCS and the nanoparticles of OCS.

Hemolysis results of human fresh blood with OCS and OCS nanoparticles are shown in Figure 4. The results demonstrated that a slight hemolysis was produced in all OCS power, OCS solution, and OCS nanoparticles systems after 0.5, 1.5, and 3 h incubation. The HR of OCS power after 0.5, 1.5, and 3 h were 1.70, 0.87, and 0.90%, respectively. As for the OCS solution sample, it revealed a relatively higher extent of breakdown of the RBCs. The long hydrophobic acyl groups on the surface of OCS may damage RBCs, leading to enhanced release of hemoglobin. And the surface tension of OCS may also increase the damage. However, the largest observed hemolytic activity was lower than 5% which indicates a wide safety margin in blood-contacting applications and suitability for intravenous administration. The HR of OCS nanoparticles solutions were lower than the OCS solutions. That may be from the o/w emulsification which made some of the hydrophobic groups encapsule the inner hydrophobic core after the formation of nanoparticles. The o/w emulsification made some of the hydrophobic groups encapsule the inner hydrophobic core. And the surface tension decrease with the formation of nanoparticles.[26]

Cytotoxicity testing

The MTT assay was performed, which is a quick effective method for testing mitochondrial impairment and correlates quite well with cell proliferation.[27] In our previous research, we had concluded there was no need to set too high the test concentrations for CS sample, for the real effective concentration would remain relatively constant above certain concentration level. The test concentrations for all the samples in this work were 200 μg ml^{-1}. Cell proliferation results of OCS and OCS nanoparticle are shown in Figure 5. There were no significant differences between the absorbance of the negative controls and the wells treated with CS sample during 5 days. For the OCS sample, it showed an apparent inhibition ($P < 0.05$, Student's *t*-test, data not shown) of cell proliferation with the time elapsing and the decrease degree was as high as 21.60% compared with the negative controls. However, the OCS nanoparticle sample showed no inhibition

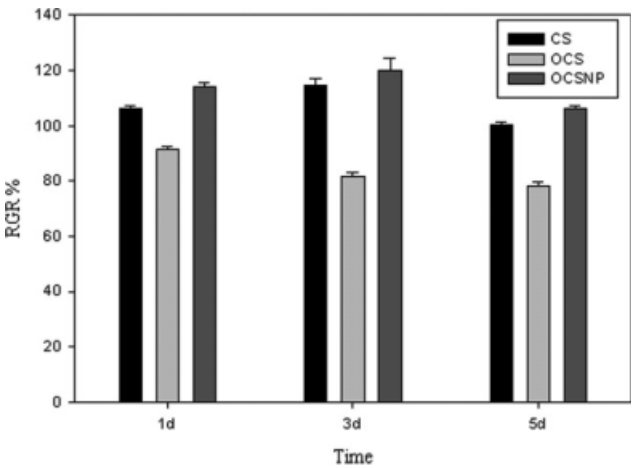

Figure 5 Cell viability of CS, OCS, and OCS nanoparticles (data shown are the mean ± S.D, $N = 3$).

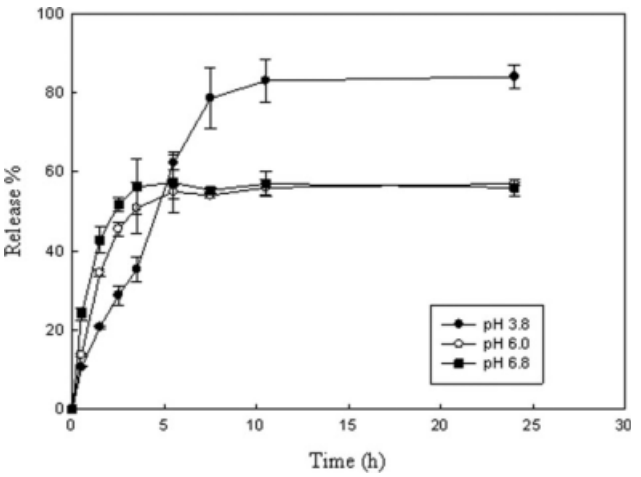

Figure 6 Mean percent drug release from rifampicin-loaded OCS nanoparticles in different pH values release medium (rifampicin concentrations 20 mg/mL and STPP 0.33 mg/mL) (data shown are the mean ± S.D. $N = 3$).

($P < 0.05$, Student's t-test, data not shown) of cell proliferation. Since OCS is a polycationic polymer that can bind substances like protein, it can therefore be assumed to inactivate some proteins and growth factors in fetal calf serum to inhibit fibroblast proliferation. An alternative explanation for the cytotoxicity effect of OCS is that, like most cationic macromolecules such as protamine and polylysine, it probably interacts with anionic components of the glycoproteins on the surface of cells, causing cytotoxic effects. In our previous work, we had got the surface tension of OCS that was larger than CS. The OCS exhibits higher inhibition of cell proliferation than CS since the OCS has higher surface tension surface than CS and can interact and destroy surface of the cell.[28] Therefore, high surface tension and highly flexible polymers may cause higher cytotoxic effects than those with low surface tension and relatively rigid structures. And the presence of primary amines of OCS may enlarge the effect.[29] The o/w emulsification made some of the hydrophobic groups encapsule the inner hydrophobic core. And the surface tension decrease with the formation of nanoparticles. That made the RGR of OCS nanoparticle solution higher than the OCS solution. These phenomena were confirmed by the erythrocyte toxicity assay.

In vitro release of rifampicin from the nanoparticles

The EE of the sample (rifampicin concentration 20 mg/mL and STPP 0.33 mg/mL) was 39.9% and the LE was 19.95%.

The rifampicin release profiles from OCS nanoparticles in acetate-buffer solutions (pH 3.8) and phosphate-buffer saline (pH 6.0 and 6.8) values were shown in Figure 6. The drug release was affected by the pH of buffer solutions. From the release profile, we can see that the release of rifampicin from solution with pH 6.0 and 6.8 was rapid. The release from solution with pH 3.8 had the slower rate. That was related to the stability of the nanoparticles in the solution. The OCS was soluble in an aqueous acidic solution below pH 6.5. However, it was not soluble at neutral or alkali pH. That made the nanoparticles less stable in the system above pH 6.5 than in the system below pH 6.5. And that also made the release of drug from solution below pH 6.5 slower than from solution above pH 6.5.

Figure 7 shows the release profile of rifampicin from OCS nanoparticles prepared from different drug concentrations (10, 20, and 80 mg/mL). The release was affected by the different concentrations of drug. The OCS nanoparticles made from 80 mg/mL rifampicin had a slowest release rate. The nanoparticles prepared with 80 mg/mL drug concentration released only 20% during 24 h and the nanoparticles prepared with 10 mg/mL drug concentration released more than 80% in the same period. The results showed there was an optimum concentration of drug. We think this result could be interpreted by the formation process of nanoparticles. The rifampicin was dissolved in the CH_2Cl_2, when the nanoparticles were forming. There was much of the CH_2Cl_2 that could not form the hydrophobic core after the formation of nanoparticles. That means much of the drug cannot be incapsuled into the hydrophobic core. So the sample with low drug concentration released faster and rapidly.

The influence of the mass of STPP on drug release *in vitro* was shown in Figure 8. We could see that the release rate of samples with more STPP was slower than that of with less STPP. The OCS

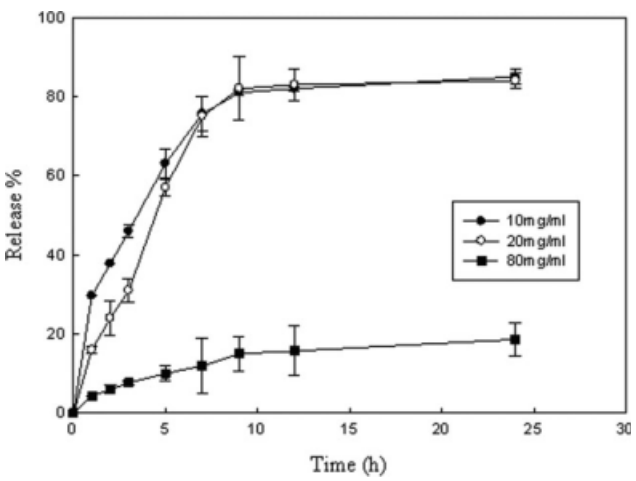

Figure 7 Mean percent drug release from rifampicin-loaded OCS nanoparticles in different drug concentrations (pH 3.8 and STPP 0.33 mg/mL) (data shown are the mean ± S.D. $N = 3$).

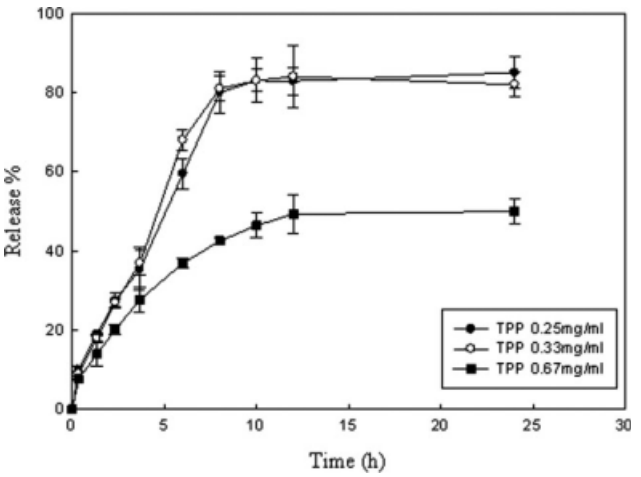

Figure 8 Mean percent drug release from rifampicin-loaded OCS nanoparticles in different STPP concentrations (pH 3.8 and rifampicin concentration 20 mg/mL) (data shown are the mean ± S.D. $N = 3$).

nanoparticles made from STPP (0.67 mg/mL) had a slowest release rate. The nanoparticles prepared with STPP (0.67 mg/mL) concentration released only 40% during 24 h and the nanoparticles prepared with STPP (0.33 mg/mL) concentration released more than 80% in the same period. The reason for the difference in the release rates between nanoparticles prepared with 0.33 and 0.67 mg/mL STPP concentrations might be that the addition of STPP made the nanoparticles more stable and the connecting network was then formed from the percolation of bridges leading to a slow release.

CONCLUSIONS

In summary, new OCS self-assembled nanoparticles as a carrier system for hydrophobic drug delivery was prepared. OCS nanoparticles were prepared by an o/w emulsification method. The TEM image of polymeric micelles confirmed that polymeric micelles are spherical in shape. Mean diameter of the polymeric amphiphilic nanoparticles of OCS was around 275.3 nm. All the OCS and OCS nanoparticles have good biocompatibility from the cytotoxicity testing and erythrocyte toxicity assay. And the biocompatibility of OCS nanoparticles was better than OCS. Rifampicin, as a model drug, was investigated for its release properties *in vitro*. The pH of buffer solution, concentration of rifampicin, and concentration of STPP were changed to evaluate the *in vitro* release behavior of rifampicin. The release of rifampicin from solution with pH 6.0 and 6.8 was characterized by a faster release than from solution with pH 3.8. The addition of STPP could slower the release. The sample with low concentration of rifampicin released faster and entirely and there was an optimum concentration of drug.

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