International Journal of Engineering and Science Applications
ISSN: 2406-9833 @2015 PPs-UNHAS
IJEScA

Protein-Benign Microencapsulation with a Water/Oil Microemulsion formed by a Biodegradable Polymer Surfactant

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ABSTRACT

The amphiphilic biodegradable polymer surfactant, poly(ethylene oxide monooleate)-block- poly(*D,L*-lactide) (MOPEO-PLA), was shown to form microemulsions in organic solvent and to dissolve cytochrome c into their microemulsions at high concentration with native form. The microemulsions dissolving cytochrome *c* provided a new preparation route of polylactide microcapsules enclosing cytochrome *c*, which exhibited high entrapment of proteins in the microcapsules and sustained long-term release. A MOPEO-PLA microemulsion system would provide a new encapsulation system with a biodegradable polymer matrix for delivery of proteins and peptides.

Keywords: biodegradable microcapsules, polymer microemulsion, PLA, long-term release.

Article history: Received 15 April 2015, last received in revised 20 April 2015

1. INTRODUCTION

Microencapsulation techniques for water-soluble bioactive agents such as proteins, peptides, nucleic acids, and human growth hormones, which have short half-lives in vivo and are unstable in biological fluids, have been developed for the purpose of long-term sustained-release formulations [1-5]. Biodegradable polymer microcapsules enclosing bioactive agents had mostly been produced by a solvent evaporation method via a double emulsion system, which efficiently traps hydrophilic agents in microcapsules by trapping an inner aqueous phase with the agents in the oil phase compartments [6, 7]. However, double emulsions are thermodynamically unstable due to coalescence and the large size distribution of the inner aqueous phase; therefore protein-loaded microcapsules release protein uncontrollably [8]. Furthermore, it has been demonstrated that sensitive proteins lost their activity at the W/O interface [9, 10], and produced a denatured protein through shear-induced stress with homogenization when an inner aqueous phase was dispersed in the oil phase [11].

W/O microemulsions, which are self-assemblages of amphiphilic molecules in a nonpolar organic solvent, can solubilize hydrophilic macromolecules and bioactive agents in their micro water pool [12-16]. Therefore, W/O microemulsions solubilizing hydrophilic bioactive agents are uniform and thermodynamically stable. A bioactive agent can be uniformly distributed in a microcapsule by adapting bioactive agents solubilized in a uniform W/O microemulsion to the O/W emulsion system. Furthermore, it is considered that this microencapsulation system is benign for bioactive agents because the microemulsion solubilized bioactive agents form spontaneously without intensive agitation. Hayashi *et al.* reported on microencapsulation of bioactive agents using a W/O microemulsion system using di-2-ethylhexyl sodium sulfosuccinate (AOT) and a sucrose ester of fatty acids [17]. However, these surfactants were not suitable because of their immiscibility with the polymer matrix of the microcapsules such as polylactide (PLA) and poly(lactide-co-glycolide). We designed a hydrophobic biodegradable polymer surfactant, poly(ethylene oxide monooleate)-block-poly(D,L-lactide); (MOPEO-PLA), whose chemical structure is shown in Figure 1, for the use of accurate-release polymer microcapsules [18].

MOPEO-PLA can form microemulsions in chloroform solution and to extract water and protein into their microemulsions from the aqueous phase. The largest amount of cytochrome *c* in the MOPEO-PLA microemulsion phase was extracted when the pH of the aqueous phase was close to the pI value of cytochrome *c*, and the degree of extraction increased at higher temperatures [19]. The MOPEO-PLA microemulsion provides a benign extraction process for proteins. The MOPEO-PLA microemulsion system would provide a new encapsulation system with a biodegradable polymer matrix for delivery of proteins and peptides. In this paper, we made an attempt to prepare PLA microcapsules enclosing cytochrome *c*, which is a model protein in the first case of this study, by the solvent evaporation method applying the MOPEO-PLA microemulsion system.

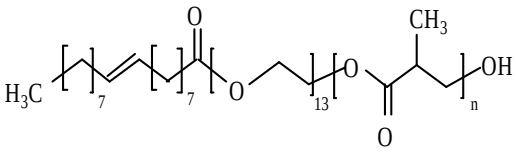

Figure 1. Molecular structure of MOPEO-PLA block copolymers.

2. THE STUDY METHOD

A. Reagents

D, L-lactide was purchased from Purac and was used by recrystallization from toluene and dried for over 24 hr in vacuo at room temperature. Polyethylene oxide monooleate (Mw: 860) was purchased from Aldrich. Cytochrome *c* from horse heart was purchased from Sigma. Anhydride chloroform, ethyl acetate, isopropanol and methanol were purchased from Wako Pure Chemical Industries, Ltd. MOPEO-PLA was synthesized by bulk ring-opening polymerization of D, L-lactide initiated with MOPEO in the presence of a stannous 2-ethylhexanoate catalyst according to the procedure given in our previous paper [18]. The polymerization was carried out in a sealed glass ampoule at 403 K for 24 hours. After 24 hours of polymerization, the product dissolved in chloroform was precipitated by an excess amount of hexane. Finally, MOPEO-PLA was isolated and dried at 323 K under vacuum. The obtained polymer showed a single peak with the GPC trace and a polydispersity of less than 1.4 [18]. The molecular weight of MOPEO-PLA used in this study was about $2.0x10^3$-$7.0x10^3$.

B. Preparation of Boidegradable PLA Microcapsules

PLA microcapsules enclosing cytochrome c were prepared by solvent evaporation method via O/W emulsification. The dispersed organic phase was a MOPEO-PLA (Mw 4,755) microemulsion solution solubilizing cytochrome *c*. The concentration of MOPEO-PLA in the organic phase was fixed at 10 wt%. Ethyl acetate was used as a solvent of organic phase, and 20 wt% of isopropanol was added as a co-surfactant. After the organic phase was contacted with an equal weight of phosphate buffer solution, the clear organic phase containing water dissolved by the formation of W/O microemulsion was obtained by centrifugation at 6,000 rpm for 10 min and a solid state of cytochrome c was added in the organic phase at desired concentration. Then, the ethyl acetate solution of D, L-PLA (Mw 29,342 or 7,189) was mixed with the microemulsion solution solubilizing cytochrome c. The concentration of D, L-PLA in the dispersed organic phase was fixed at 11.3 wt%. The mixed solution was poured to the aqueous phase, which was composed of 4 wt% of poly(vinyl alcohol) (n=2,000) and 1.0 wt% of Q12S, and agitated at to form the O/W emulsion. The solvent evaporation was carried out for 4 hr at 298 K under

reduced pressure. After the solvent evaporation process, PLA microcapsules collected by filtration were washed with distilled water and lyophilized for 24 hr.

The enclosing efficiency of cytochrome c into the microcapsules was determined from the concentration of cytochrome c released in the aqueous phase after the preparation and the following equation:

Enclosing efficiency [%] = 100·(Corg,0·Vorg,0 – Caq·Vaq) / (Corg,0·Vorg,0) (1); where Corg, 0, Vorg, 0 , Caq and Vaq are the initial concentration of cytochrome c in the organic phase of the O/W emulsions, the initial volume of the organic phase, the concentration of cytochrome c released in the aqueous phase after the preparation, and the volume of the aqueous phase.

C. Release of cytochrome c from Biodegradable PLA Microcapsules

The PLA microcapsules of 0.1 g were put into 30 ml of pH 7.2 phosphate buffer solution, and then placed in a thermostatted bath at 310 K with shaking at 150 spm. After appropriate intervals, the amount of cytochrome c released in the buffer solution was measured.

D. Observation and measurement

The morphology of the PLA microcapsules was observed by scanning electron microscopy (SEM: S-3500N, Hitachi, Ltd.) at an intensity of 5 kV under various magnification. A sputter-coater (E-1030 Ion Sputter, Hitachi, Ltd.) was used to coat the samples with Pt-Pd.

The UV and visible spectrum and the concentration of cytochrome c in the organic and the buffer solutions were measured with using a UV spectrophotometer (U-2000A, Hitachi, Ltd.)

3. RESULT AND DISCUSSION

A. Solubilization of cytochrome c in biodegradable W/O microemulsion

The solid cytochrome *c* was directly added and dissolved in the MOPEO-PLA microemulsion solution at various concentrations. After dissolution, the clear reddish solution was obtained. The absorption spectra of cytochrome *c* dissolved in the microemulsion and the buffer solutions are illustrated in Figure 2. An absorption peak at 408 nm was observed in both spectra with almost same shape and indicated that cytochrome *c* was dissolved in the MOPEO-PLA microemulsion. The absorbance of cytochrome c at 408 nm in the microemulsion and the buffer solutions was plotted against the concentration of cytochrome *c* in Figure 3. The both plots were plotted on a same straight line until high concentration range. This suggests that the structure around heme in cytochrome *c* dissolved in the MOPEO-PLA microemulsion was kept the native structure of that in the buffer solution. From these results, the MOPEO-PLA microemulsions have ability to dissolve cytochrome *c* at high concentration with keeping its native structure.

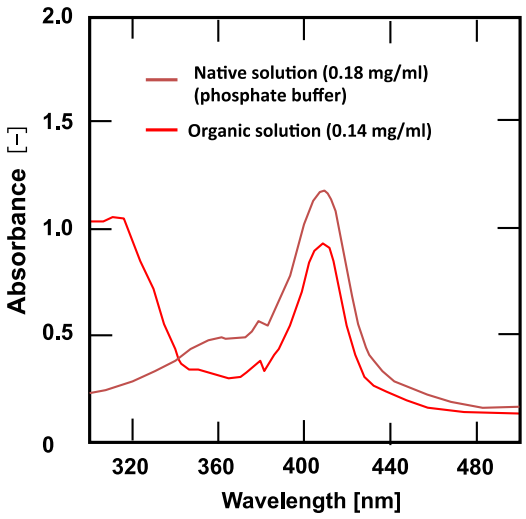

Figure 2. Absorption spectra of cytochrome c dissolved into the MOPEO-PLA microemulsion organic solution and native cytochrome *c* in the phosphate buffer.

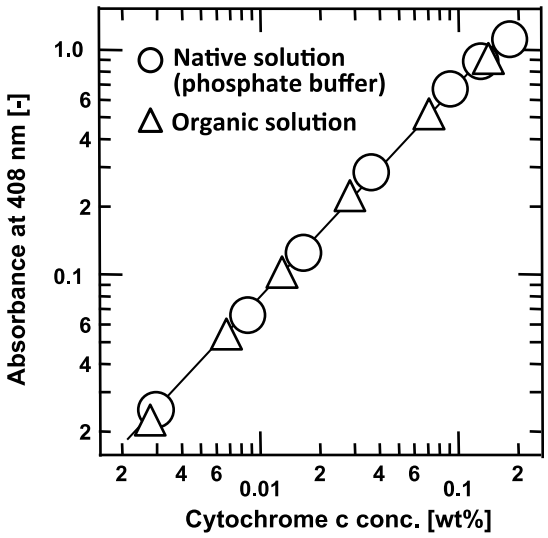

Figure 3. Reatioship between absorbance at 408 nm and the concenreation of cytochrome *c* dissolved into the MOPEO-PLA micro-emulsion organic solution and native cytochrome c in the phosphate buffer.

B. Preparation of PLA microcapsules enclosing cytochrome c

The PLA microcapsules enclosing cytochrome *c* were prepared by the solvent evaporation method via O/W emulsification, where the organic phase of the O/W emulsions was a mixed solution of the ethyl acetate solution of D, L-PLA and the MOPEO-PLA microemulsion solution solubilizing cytochrome *c*. The SEM photograph of the prepared PLA microcapsules enclosing cytochrome *c* using the MOPEO-PLA microemulsion system is shown in Figure 4. It was clear that the prepared microcapsules were spherical and had a smooth surface. The diameter of the microcapsules was 5-50 μm. The enclosing efficiency of cytochrome *c* into the microcapsules was found to be 92.1 %. This high entrapment was due to the fact that cytochrome *c* was retained in the dispersed phase during the solvent evaporation process by strongly forming a complex with the MOPEO-PLA microemulsions.

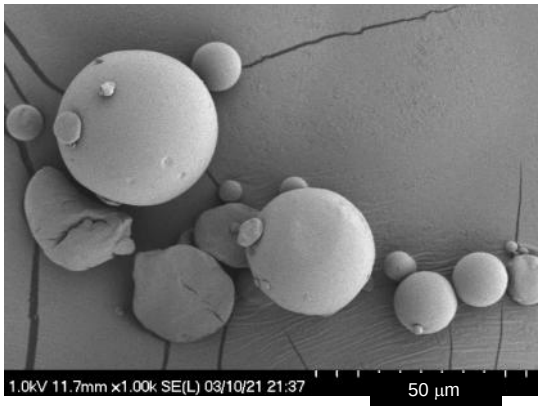

Figure 4. SEM image of the PLA microcapsules enclosing cytochrome *c*. Cytochrome *c* concentration was 20 mg/g-microcapsules. Mw of PLA was 29,343.

C. Release profile of cytochrome c from PLA microcapsules

We have performed the evaluation of the *in vitro* release profile of cytochrome *c* from the PLA microcapsules. The release profile of cytochrome *c* from the PLA microcapsules is shown in Figure 5. Cytochrome *c* was successfully released at constant for 2 month with a slight initial burst. This constant release is due to that the matrix with high *Mw* of PLA had a dense structure. Furthermore, suppression of initial burst was successfully achieved. This is attributable to uniform distribution of cytochrome *c* in the microcapsules. This is an advantage of this process to utilize the microemulsion system in order to solubilize proteins homogeneously in oil droplets. In addition, the circular dichroism spectra of cytochrome *c* released in the aqueous media from the PLA microcapsules had the same spectrum as that of native one. Indicating that the cytochrome *c* was not denatured during the storage in the microcapsules until release and the release process from the microcapsules. These results indicate indefectible encapsulation of cytochrome *c* by solvent evaporation process with MOPEO-PLA microemulsion system.

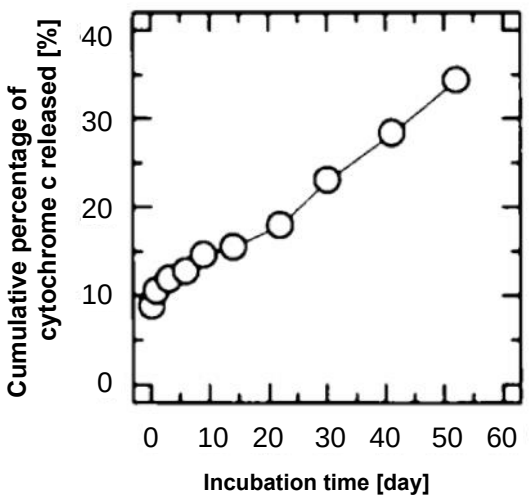

Figure 5. Release profile of cytochrome c from the PLA microcapsules. Cytochrome c concentration was 5.0 mg/g-microcapsules. Mw of PLA was 7,189.

4. CONSLUSION

We have found that the MOPEO-PLA, which is a biodegradable polymeric surfactant, provided a microemulsion system, which solubilized cytochrome *c* in organic phase with the native form. Cytochrome *c* was successfully microencapsulated in the PLA microcapsules using a MOPEO-PLA microemulsion system and was constantly released from the microcapsules for a long time. This encapsulation system would have a good potential for delivery of various bioactive agents.

5. REFERENCES

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