Journal of Colloid and Interface Science 371 (2012) 46–51

Contents lists available at SciVerse ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Size-controlled and monodisperse enzyme-encapsulated chitosan microspheres developed by the SPG membrane emulsification technique

Kazuki Akamatsu [a,*], Yuto Ikeuchi [a], Aiko Nakao [b,1], Shin-ichi Nakao [a]

[a] Department of Environmental and Energy Chemistry, Faculty of Engineering, Kogakuin University, 2665-1 Nakano-machi, Hachioji-shi, Tokyo 192-0015, Japan
[b] Cooperative Support Team, RIKEN, ASI, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

ARTICLE INFO

Article history:
Received 23 November 2011
Accepted 30 December 2011
Available online 12 January 2012

Keywords:
Membrane emulsification
Microspheres
Shirasu porous glass (SPG) membranes
Chitosan
Lysozyme
Activity

ABSTRACT

Lysozyme-encapsulated chitosan microspheres with micron-size diameters were successfully prepared for the first time by employing the Shirasu porous glass (SPG) membrane emulsification technique followed by cross-linking with glutaraldehyde, and the relationships between the preparation conditions and characteristics of the microspheres were studied in detail. This preparation method provided size-controllability and monodispersity of the microspheres, owing to the sharpness of the pore sizes of the SPG membranes. It was also possible to predict the average diameters of the enzyme-encapsulated microspheres using no fitting parameters, on the basis that each microsphere is prepared in an emulsion containing chitosan and lysozyme, without any collisions or aggregation occurring. X-ray photoelectron spectroscopy measurements indicated that the amount of encapsulated lysozyme was controlled by the concentrations of chitosan and lysozymes in the dispersion phase used for preparing the emulsions from which the enzyme-encapsulated microspheres are formed. Finally, the apparent activity of the encapsulated lysozymes was measured by the viscosimetric method, using ethyleneglycolchitin. Results showed that about half of the activity of the encapsulated lysozymes was maintained during the preparation procedure when employing the SPG membrane emulsification technique.

1. Introduction

Chitosan, one of the most well-known biopolymers, is a linear polysaccharide composed of *N*-acetyl-D-glucosamine and β-(1–4)-linked D-glucosamine. Due to its very good biocompatibility and biodegradability, and its abundance, chitosan is useful for the development of many kinds of biomaterials [1–4].

Among such biomaterials, studies on drug delivery systems using chitosan microspheres or microcapsules have been extensively conducted. In particular, the development of chitosan microspheres encapsulating some proteins or drugs has gained much attention [5–13]. Suitable methods to prepare such microspheres require controllability of the diameters of the microspheres and the monodispersity. This is because the body distribution of the microspheres and the interaction with cells are greatly affected by the diameter of the microspheres [14,15]. Furthermore, the activity of the encapsulated proteins or drugs should be retained. For this, a facile loading procedure is preferable.

There are mainly two types of protein or drug loading methods: one is the loading with microsphere formation and the other is the loading after microsphere formation by usage of drug diffusion into microspheres. In terms of loading efficiency, the former method is preferable. This is because when using the former method, the amount of protein or drug loss can, in principle, be reduced [16]. However, it has often been suggested that a significant loss of activity of the encapsulated proteins or drugs could occur. This is usually attributed to the preparation conditions. Therefore, to prevent such deactivation, efforts have been made to determine the best preparation method.

In recent years, the membrane emulsification technique for preparing functional microspheres has attracted much attention. The technique is actually used for preparing emulsions, and then, by using the emulsions as reaction space, microspheres are formed in the emulsions. In particular, the membrane emulsification technique using Shirasu porous glass (SPG) membranes is one of the well-known methods [17–20]. Owing to the sharp pore-size distribution of the SPG membrane, the diameters of the emulsions obtained when using this method can be strictly controlled by manipulating the pore size, and highly monodisperse emulsions are easily obtained. There are reports on the preparation of

Abbreviations: FE-SEM, field-emission scanning electron microscopy; SPG, Shirasu porous glass; TGCR, tetraglycerol condensed ricinoleate; XPS, X-ray photoelectron spectroscopy.

* Corresponding author. Fax: +81 42 628 4542.
E-mail address: akamatsu@cc.kogakuin.ac.jp (K. Akamatsu).
[1] Present address: Industrial Cooperation Team, RNC, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

0021-9797/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jcis.2011.12.078

microspheres or microcapsules by employing the SPG membrane emulsification technique [21,22].

We have successfully prepared chitosan microspheres and chitosan microcapsules with hollow structures by this method [23,24]. The diameter of the microspheres/microcapsules was controlled in the submicron to 10 μm range by the pore sizes of the SPG membranes, and the obtained microspheres/microcapsules were monodisperse. Furthermore, we demonstrated that the diameter of the microspheres/microcapsules could be successfully predicted by considering the mass balance of the chitosan in one emulsion droplet, based on the assumption that one chitosan microsphere is formed in one W/O emulsion droplet. Using the same technique, silver nanoparticles with diameters as small as 10 nm were successfully prepared, and the average diameter was well predicted using the same assumption [25]. We therefore consider that it will be a major breakthrough when it can be successfully demonstrated that size-controlled and monodisperse enzyme-encapsulated chitosan microspheres can be prepared, without losing their activities, by using the SPG membrane emulsification technique. In addition, from an engineering viewpoint, the membrane emulsification technique is more suitable for scale-up than the microsphere/microcapsule preparation technique using microfluidics [26–34].

In this study, glutaraldehyde cross-linked enzyme-encapsulated chitosan microspheres were developed by employing the SPG membrane emulsification technique, and the relationships between the preparation conditions and characteristics of the microspheres were studied in detail. For this purpose, lysozymes were used as enzymes. An aqueous solution containing a mixture of chitosan polymers and lysozymes was emulsified to form W/O emulsions by the SPG membrane emulsification method, followed by a cross-linking reaction with glutaraldehyde. The effects of the pore sizes of the SPG membranes and the concentrations of the lysozymes on the diameters of the microspheres obtained were investigated. Finally, the apparent activity of the encapsulated lysozymes was examined to demonstrate that this proposed method is useful for preparing the enzyme-encapsulated microspheres with the maintenance of their activity.

2. Experimental

2.1. Materials

Chitosan (deacetylation rate > 80.0 mol%; viscosity of an aqueous solution, 5 g/L, 20 °C, 10–100 mPa s), kerosene, acetic acid, glutaraldehyde, sodium hydroxide and ethyleneglycolchitin were purchased from Wako Pure Chemical Industries Ltd., Japan, and used without further purification. Tetraglycerol condensed ricinoleate (TGCR) was kindly supplied by Sakamoto Yakuhin Kogyo Co., Japan.

2.2. Preparation of lysozyme-encapsulated chitosan microspheres

An external-pressure type micro kit (SPG Techno Co. Ltd., Miyazaki, Japan) and three different hydrophobic SPG membranes (SPG Techno Co. Ltd.), with pore sizes 1.1 μm, 5.5 μm and 12 μm, were employed to prepare monodispersed W/O emulsions containing the mixture of chitosan and lysozyme. As the dispersion phase, chitosan and lysozyme were dissolved in a 2.0 wt.% acetic acid solution, in desirable concentrations, followed by addition of NaOH aqueous solution to adjust pH 5. As the continuous phase, kerosene with 1.0 wt.% TGCR was prepared. After pouring the dispersion phase into the dispersion tank and pouring the continuous phase into a beaker, the dispersion phase was slowly pressurized, using nitrogen, into the continuous phase to prepare a W/O emulsion containing chitosan and lysozyme. When the membranes with pore sizes 1.1 μm, 5.5 μm, 12 μm were used, the pressures applied were 70–120, 8–12, 2–5 kPa, respectively. These conditions were determined for stable emulsification by keeping the emulsification rate less than 2 mL h^{-1}. The stirring speed of continuous phase was 250 rpm. Next, a 25% aqueous glutaraldehyde solution was added to the emulsion, with stirring, and the cross-linking reaction carried out over a period of 2 h. After centrifugation, the resulting microspheres were washed with excess amount of distilled water. The microspheres were then observed using field-emission scanning electron microscopy (FE-SEM) (JSM-6701F, JEOL, Japan) and analyzed by X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, Thermo Fisher Scientific K.K., Japan). For XPS studies, the microspheres were brayed by agate mortar, to analyze not only the surface but also the whole of the microspheres.

2.3. Apparent activity assay of the encapsulated lysozymes

The viscosimetric method, using ethyleneglycolchitin, was employed to determine the apparent activity of the lysozymes immobilized in the chitosan microspheres [35,36]. The microsphere tested was prepared from the dispersion phase containing 1.0% chitosan and 1.5% lysozyme. A 0.2% ethyleneglycolchitin solution was prepared with 0.1 M phosphate buffer solution (pH 5.6). Prior to carrying out the activity tests, the solution was filtered through a glass filter. Using an Ostwald viscometer, the plunge times of 10 mL solvent and of the ethyleneglycolchitin solution were measured and expressed as $t_{solvent}$ and t_0. Then the plunge times of the ethyleneglycolchitin solution with 0.2 mL lysozyme solution, of known concentrations, were measured and expressed as t. Using $t_{solvent}$, t_0 and t, the ratio of the viscosity decrease was defined as follows:

$$\text{the ratio of viscosity decrease } (\%) = \frac{t - t_{solvent}}{t_0 - t_{solvent}} \times 100 \quad (1)$$

The relationship between the free lysozyme concentrations and the ratio of the viscosity decrease at 3 min was then obtained and used to create a calibration curve. Subsequently, using the lysozyme-encapsulated chitosan microspheres, the ratio of the viscosity decrease at 3 min was measured.

3. Results and discussion

3.1. Effect of the pore sizes of the SPG membranes

To investigate the effect of the pore size of the SPG membranes on the average diameters of the microspheres obtained, three different SPG membranes, with pore sizes 1.1 μm, 5.5 μm and 12 μm, were employed to prepare the lysozyme-encapsulated chitosan microspheres. Fig. 1a–c shows the FE-SEM micrographs of the microspheres. The pore sizes of the two SPG membranes used are (a) 1.1 μm, (b) 5.5 μm and (c) 12 μm, and the other experimental conditions used to prepare the microspheres were the same. A solution of 1.0% chitosan and 1.0% lysozyme was used as the dispersion phase. The micrographs clearly show that the average diameter of the microspheres became smaller as the SPG membrane with smaller pore size was used. The average diameters of the microspheres and coefficient of variance (CV) were (a) 1.1 μm and 12%, (b) 5.6 μm and 22% and (c) 13.9 μm and 13%, respectively. Hence, it can be said that monodispersed and size-controlled lysozyme-encapsulated chitosan microspheres were successfully prepared. Furthermore, the sharp pore-size distribution of the SPG membranes was reflected to the monodispersity of the lysozyme-encapsulated chitosan microspheres.

In our previous study [23], in the case of the preparation of chitosan microspheres without enzymes, it was demonstrated that