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ORIGINAL CONTRIBUTION

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# A thermosensitive amphoteric microsphere and its potential application as a biological carrier

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**Abstract** Thermosensitive poly(*N*-isopropylacrylamide) moieties were introduced onto amphoteric styrene/glycidyl methacrylate copolymer seed microspheres prepared by use of amphoteric initiators. The resulting microspheres exhibited thermosensitive and amphoteric behavior, so dual sensitivity to both pH and temperature was observed. The colloidal properties of the microspheres before and after seeded polymerization were characterized by varying the temperature and the pH. The results indicated that the specific surface structure emerged when the environmental conditions were changed. In addition, the reactive epoxy groups on the microsphere surface could be utilized to immobilize the protein molecules. The behavior of protein adsorption and immobilization onto the microspheres was examined in order to understand their potential applications in biological areas.

**Keywords** Soap-free emulsion polymerization · Amphoteric initiator · Amphoteric microsphere · Poly(*N*-isopropylacrylamide) · Protein adsorption/immobilization

## Introduction

Functional monodispersed particles with submicron sizes exhibit valuable properties, such as large surface areas and high mobility as well as quick response [1, 2]; therefore, such microspheres have many potential applications, especially in biological fields [3, 4, 5, 6].

As is well known, a microsphere with glycidyl ester groups at the surface is an excellent carrier owing to its nonspecific adsorption of proteins and moderate reactivity to immobilize bioligands compared to carboxyl- or amino-containing microspheres [7].

As an amphoteric initiator is adopted in an emulsifier-free emulsion polymerization, the resulting colloidal microspheres, which exhibit amphoteric nature, have high stability in low and high pH ranges and moderate stability in an intermediate pH range [8, 9]. Thus, those who use such particles need not worry about the problem of colloidal stabilization in strong acid and base ranges. Moreover, it seems essential to prevent the adsorption of proteins onto the surfaces if the microsphere is expected to be applicable in a biological area [10]. Since the adsorption of protein onto solid surfaces includes many complex events [11], the surfaces of the microspheres need to have suitable compositions and structures.

In this work, we first prepared epoxy-group-containing seed particles by soap-free emulsion polymerization in the presence of amphoteric initiators. Then, thermosensitive poly(*N*-isopropylacrylamide) (PNIPAM) chains were introduced onto such amphoteric seed particles to obtain the final microspheres with dual sensitivity towards temperature and pH. Finally, both protein adsorption and immobilization onto the resulting microspheres are examined and discussed.

## Experimental

### Materials

The monomers, styrene (St) and glycidyl methacylate (GMA), both from Wako Pure Chemicals Co., Tokyo, were distilled under reduced pressures. The three kinds of amphoteric initiators shown in Table 1 were provided by Wako Pure Chemicals Co. and were used in this study without further treatment. Deionized and distilled water was used in all the experiments. NIPAM monomer (Kojin Co., Tokyo) was purified by recrystallization from hexane/toluene 1/1 (v/v) before use.

### Preparation and characterization of seed microspheres

The seed microspheres were prepared by the emulsifier-free emulsion polymerization of St and GMA at 70 °C. The resulting microspheres were purified by means of centrifugation, decantation and redispersion, and further dialysis for about 2 days. Then, the microspheres were observed by transmission electron microscopy (TEM) and scanning electron microscopy, and their sizes were measured by photon correlation spectroscopy. The electrophoretic mobility of the particles and the critical flocculation concentration of the latexes were determined using the procedure indicated in a previous article [12].

### Seeded emulsion polymerization

The seeded emulsion polymerization was carried out without any additional emulsifier at 70 °C according to the basic recipes indicated in Table 1. After the polymerization, the microspheres were purified in the same manner as described earlier in order to remove the water-soluble homopolymers. It was verified by TEM that there were no tiny particles consisting of homo-PNIPAM.

**Table 1.** Recipes of seed preparation and seeded emulsion polymerization. Ingredients: styrene (*St*); glycidyl methacylate (*GMA*); 2,2′-azobis(*N*-(1-caboxyethyl)-2-methylpropionamidine) (*1CE*); 2,2′-azobis(*N*-(2-caboxyethyl)-2-methylpropionamidine) (*2CE*); 2,2′-azobis(methyl-*N*-(2-hydroxyethyl)-propionamidine) (*VA-086*); *N*,*N*′-methylenebisacrylamide (*MBAAm*); *N*-isopropylacrylamide (*NIPAM*)

| Preparation of seed microspheres | | | |
|---|---|---|---|
| Ingredients | S-01 | S-02 | |
| St (g) | 1.2 | 1.2 | |
| GMA (g) | 3.0 + 0.8 | 3.0 + 0.8 | |
| 2CE (g) | 0.1 | | |
| 1CE (g) | | 0.1 | |
| $H_2O$ (g) | 100 | 100 | |
| $T$ (°C) | 70 | 70 | |
| $D_w$ (nm) | 351 | 436 | |
| $D_w/D_n$ | 1.01 | 1.03 | |
| Seeded polymerization | | | |
| Ingredients | SGN01 | SGN02 | SGN03 |
| Seed no. | S-01 | S-02 | S-01 |
| Seed (g) | 1.9 | 1.9 | 1.9 |
| NIPAM (g) | 1.08 | 1.08 | 1.08 |
| MBAAm (g) | 0.02 | 0.02 | 0.02 |
| 2CE (g) | 0.03 | 0.03 | |
| VA-086 (g) | | | 0.03 |
| $H_2O$ (g) | 60 | 60 | 60 |
| $T$ (°C) | 70 | 70 | 80 |

As for the microspheres obtained by the seeded polymerization, the temperature dependence of the hydrodynamic diameter was examined besides similar measurements for the seed particles.

### Adsorption/immobilization of protein

The latex particles (about 10 mg) were first washed with the buffer at least three times. Then, protein solution was added to the dispersion of the microspheres and the mixture was kept at the desired temperatures for the required time. The protein adsorption on the microspheres was carried out by incubating the mixture of microspheres and proteins at 0 °C for 2 h, and protein immobilization was at 37 °C for 20 h [13]. After the adsorption/immobilization of protein, the microspheres were separated from the medium via centrifugation and the concentration of protein remaining in the medium was determined using a bicinchoninic acid protein assay kit in order to calculate the adsorption/immobilization amount of protein onto the microsphere surfaces.

## Results and discussion

### Properties of thermosensitive amphoteric microspheres

TEM views of amphoteric microspheres with thermosensitive moieties, which were prepared by two-step soap-free emulsion polymerization, are shown in Fig. 1. It can be seen that the microspheres had a narrow size distribution.

It was confirmed by titration that an appreciable number of epoxy groups resided on the microsphere surfaces even after the seeded emulsion polymerization in the acidic range (data not shown) [7].

The hydrodynamic diameter of the microspheres is shown in Fig. 2. The diameter changed with temperature and the phase transition occurred at about 32 °C [14]. These results indicated that the thermosensitive PNIPAM moieties have been introduced successfully onto the microspheres. The microspheres prepared by using different initiators had different sizes because of the different water solubility and decomposition rate constants of the three initiators [8]. However, the temperature dependence of the hydrodynamic size was not different among the microspheres. Therefore, SGN01 was exclusively used in the subsequent experiments.

The pH dependence of the hydrodynamic diameter of SGN02 microspheres seems insignificant. This might be because the highly thermosensitive PNIPAM moieties masked the difference in the degree of swelling between dissociated and undissociated ionic groups just attached at the surfaces of the seed (or core) microspheres. The hydrodynamic diameter of shrunken microspheres at high temperature is supposed to reflect the difference in dissociation of COOH caused by different pH, but this was not the case. The unexpected fact that even shrunken microspheres had almost the same hydrodynamic diameter irrespective of pH suggested that the

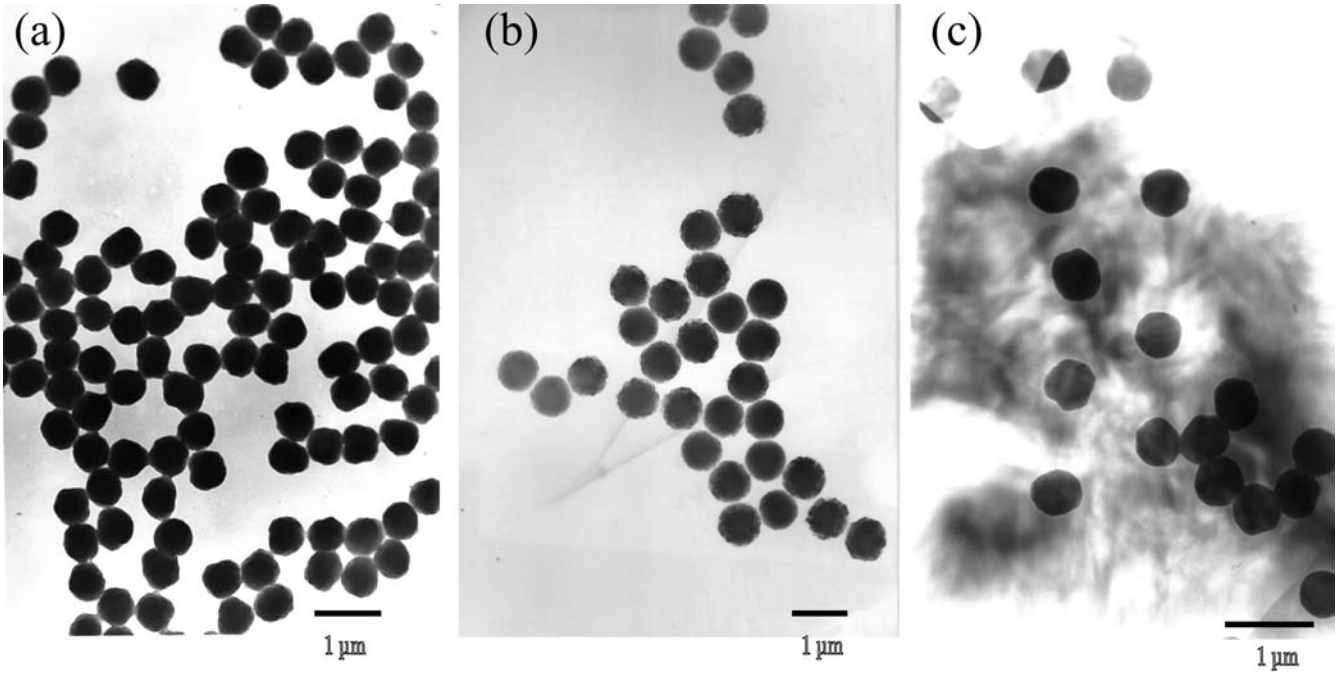


**Fig. 1.** Transmission electron microscopy views of thermosensitive amphoteric microspheres: **a** SGN0l; **b** SGN02; **c** SGN03

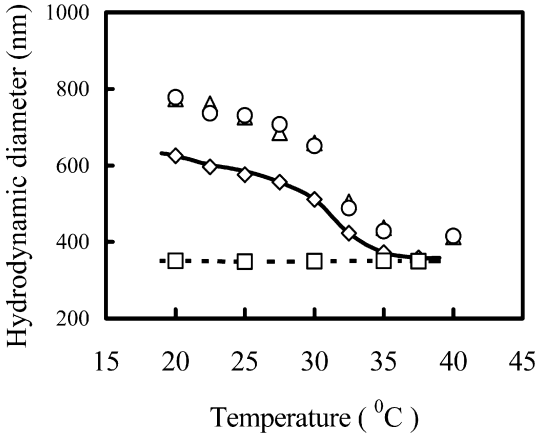


**Fig. 2.** Hydrodynamic diameters of the microspheres before and after seeded emulsion polymerization: S-01 (*squares*); SGN01, pH 6.8 (*diamonds*); SGN02, pH 3.0 (*circles*); SGN02, pH 10.0 (*triangles*)

hydrodynamic diameter measured by photon correlation spectroscopy might not be absolute but was affected by the surface structure of the microspheres.

The zeta potentials (or electrophoretic mobility) of the core microspheres and PNIPAM-coated microspheres were determined for an ionic strength of 0.001 M NaCl, and are shown in Fig. 3. Because the thermosensitive PNIPAM surface layer covered the seed amphoteric microspheres and buried ionic groups inside, the absolute value of the zeta potential of the PNIPAM-coated microspheres was smaller than that of seed particles at various pHs below the phase-transition temperature (about 32 °C) [15]. However, above this temperature, the zeta potential of the microspheres containing shrunken PNIPAM moieties was almost the same as that of seed particles. This was attributed to the exposure and concentration of ionic groups after the shrinkage of the PNIPAM layer. It is worth mentioning that all the microspheres in Fig. 3 had the same isoelectric points. The isoelectric points of the microspheres in the present cases only depended upon the residual amphoteric fragments from the decomposed initiator.

The pH and temperature dependences of the critical flocculation concentration of the microspheres are illustrated in Fig. 4. It was clear that the microspheres

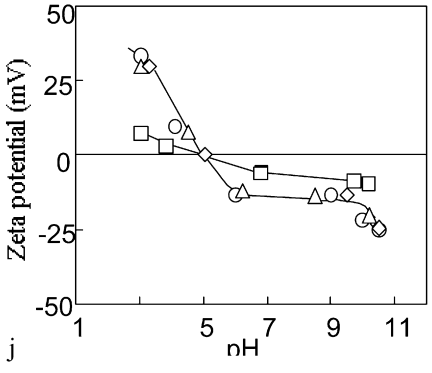


**Fig. 3.** pH,dependence of the electrophoteric mobility of microspheres before and after seeded emulsion polymerization: S-01 (*circles*); SGN01, 20 °C (*squares*), 35 °C (*triangles*), 40 °C (*diamonds*)

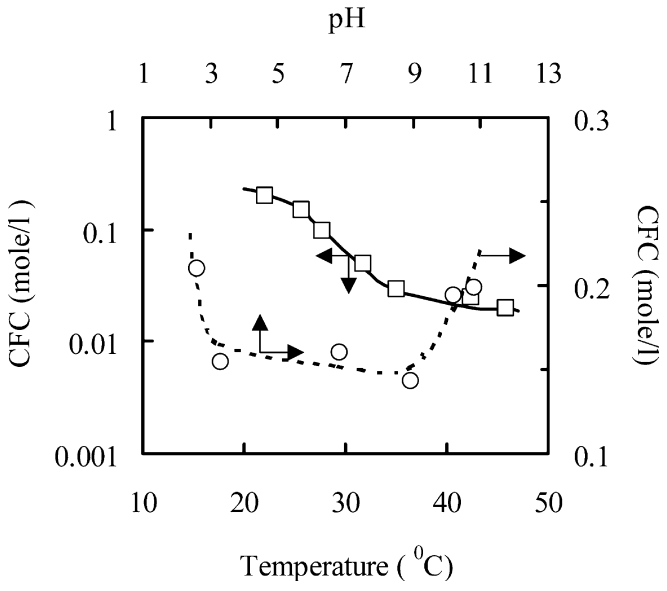


**Fig. 4.** Critical flocculation concentration (*CFC*) of the latexes after seeded polymerization: SGN01, 20 °C (*circles*); pH 6 8 (*squares*)

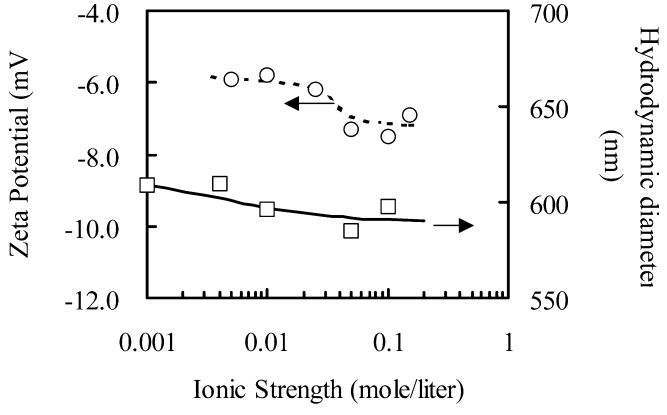


**Fig. 5.** Effect of ionic strength on hydrodynamic diameters of microspheres, after seeded polymerization. SGN01, 20 °C

had much higher stabilization in low and high pH ranges than in a medium pH range. This is due to the ionization of the amphoteric groups attached at the surfaces in low and high pH ranges. Moreover, the colloidal microspheres tended to become unstable when the temperature was raised. This is because PNIPAM chains were shrunken and the particles lost the steric stabilization force. Thus, it is concluded that the electrostatic effect from amphoteric ionic groups at the seed surface layer and the steric/solvation effect of the thermosensitive moieties introduced after seeded polymerization seemed to operate independently on the colloidal stability, so the microspheres exhibited the pH and temperature dependence of the critical flocculation concentration.

The effects of ionic strength on the hydrodynamic diameter and zeta potentials of the microspheres are shown in Fig. 5. It can be seen that the hydrodynamic diameter of the microspheres gradually decreased with the increase of ionic strength, whereas the zeta potential of the microspheres at first remained at the same level and then decreased. This result implies that the PNIPAM chains at the surfaces shrank slightly with increasing ionic strength [15]. This can be caused by the dehydration in PNIPAM layers with salt and/or the adsorption of free ions onto the microsphere surfaces in the dispersion at higher ionic concentration.

On the basis of these results, it can be recognized that some colloidal properties of the amphoteric microsphere containing thermosensitive PNIPAM moieties seemed controllable by adjusting either the pH or the temperature, so both the desired colloidal stabilization and the surface structure of the microspheres were easily acquired for their applications.

## Adsorption and immobilization of proteins

It has been reported that the adsorption of proteins is a result of many complex events that include conformational change, protonation, electrostatic interaction and so on [11]. Thus, one would expect that the immobilization of protein should be more complicated because this process still includes some chemical reactions at the surfaces. To clearly elucidate the mechanism of protein adsorption/immobilization seems very difficult now.

In this work, we investigated the effect of pH on the adsorption of protein onto the microsphere surfaces. The adsorption experiments were carried out at 0 °C in order to eliminate the possibility of the covalent coupling of protein. On the other hand, the immobilization of proteins was attained by the reaction of epoxy groups on the microsphere surfaces with amino groups of protein molecules at 37 °C and 20 h.

The pH dependence of the adsorption of various proteins is shown in Fig. 6. The black bars indicate the amount of bovine serum albumin (BSA) adsorption and the open bars represent that of human gamma globulin ($\gamma$-HG).

According to the results in Fig. 6, the amounts of proteins adsorbed depended slightly upon the pH, and the maxima for BSA and $\gamma$-HG adsorption appeared around pH 5 and 7, respectively, These pHs were close to the isoelectric points of the respective proteins. pH 5 was also the isoelectric point of the amphoteric microspheres (SGN01). Therefore, the pH dependence of BSA adsorption may be explained solely by the electrostatic force between BSA and the microspheres, i.e., a BSA molecule and a microsphere are positively charged below pH 5 and negatively charged above pH 5, so they

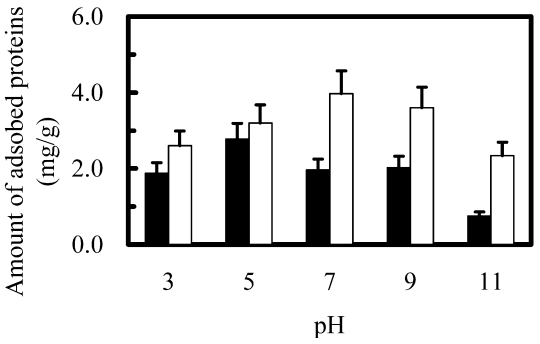


**Fig. 6.** pH dependence of the protein adsorption onto amphoteric microspheres containing thermosensitive moieties (SGN01). Bovine serum albumin (*BSA*) (filled squares); human gamma globulin (open squares); 0 °C, initial amount of protein charged 1000 ppm

repel electrostatically at any pH except for pH 5, and the adsorption was a maximum at pH 5.

However, the electrostatic interaction between a protein molecule and a microsphere cannot fully explain the pH dependence of adsorption as shown in the case of $\gamma$-HG, in which some electroattractive force can work between a protein molecule and a microsphere in the pH range from 5 to 7 and the maximum adsorption might be expected to be observed around pH 6, but this is not the case. Therefore, some other effective force to give the maximum adsorption at pH 7 for $\gamma$-HG must exist. It is intermolecular interaction of proteins having no charge, i.e., no electrorepulsive force between neighboring protein molecules, that causes the largest amount of adsorption at their isoelectric points [16]. Comparing $\gamma$-HG and BSA adsorption, $\gamma$-HG was adsorbed more than BSA because the former is more hydrophobic.

The amount of BSA immobilized with various amounts of charged BSA under the incubation condition of 37 °C and 20 h is shown in Fig. 7. It can be seen from Fig. 7 that the amount of BSA immobilized increases with the increase in the amount of charged BSA and then reached a constant value. This can be because

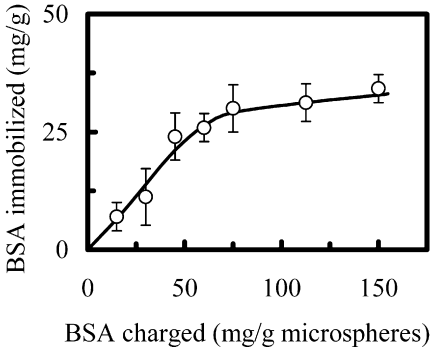


**Fig. 7.** The immobilization of protein onto microspheres with functional epoxy groups (SGN01). Incubation conditions: 37 °C, 20 h

immobilization sites for BSA on the microspheres were limited and the occupied surface area of BSA was calculated from the previously mentioned constant value, assuming that the immobilization took place in a monolayer. The calculated value (about 15 $nm^2$) was reasonable for the occupied surface area of BSA and supported the monolayer immobilization of BSA on the microspheres.

The time course for BSA immobilization is represented in Fig. 8. In the initial stage, the amount of BSA immobilized onto the surfaces quickly increased with the reaction time and then leveled off. The final amount of immobilized BSA was determined after about 15 h under the present experimental condition.

The effect of ionic strength on the adsorption and immobilization of BSA onto the microspheres is shown in Fig. 9. It can be noted that the amount of BSA adsorbed increased slightly and the amount of BSA immobilized decreased slowly with the increase in ionic strength. The results on adsorption can be explained by that, with the increase in ionic concentration, the electrostatic interaction between a microsphere and a

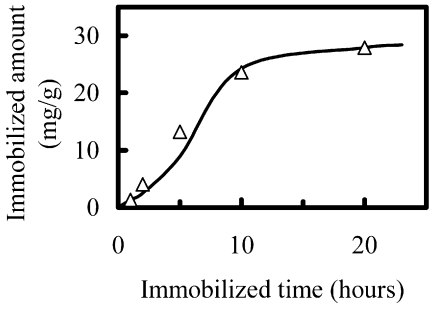


**Fig. 8.** The relationship between the amount of immobilization and immobilization time. SGN01, pH 6.8, 37 °C

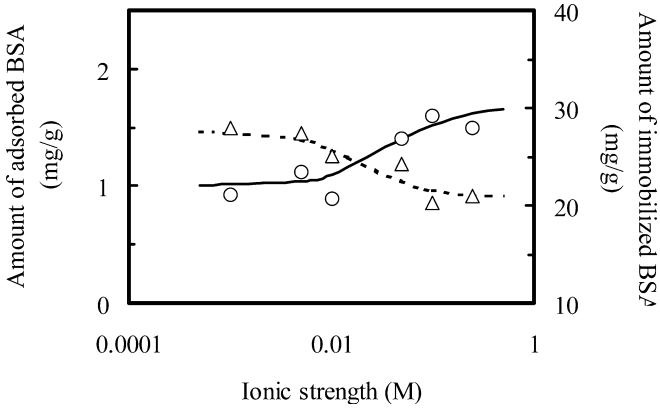


**Fig. 9.** Effect of ionic strength on the protein adsorption and immobilization onto the microspheres. SNG01, adsorption conditions: 0 °C, 2 h (*circles*); immobilization conditions: 37 °C, 20 h (*triangles*)

protein molecule, both are of negative charge, decreased to induce an increase in physical adsorption. As to the results on the immobilization, it is speculated that the higher ionic strength leads to masking the particle surfaces by free ions, interfering with the immobilization reaction, and resulted in the lower amount of protein immobilized onto the microsphere surfaces.

## Conclusions

Thermosensitive amphoteric microspheres were prepared by two-step soap-free emulsion polymerization in the presence of amphoteric initiators. The resulting microspheres exhibited dual response to either pH or temperature. Adsorption of proteins onto the microspheres could be controlled by the pH or the temperature, and immobilization of proteins onto the microspheres was controlled by adjusting the conditions for the reaction of epoxy groups on the microspheres with amino groups of the proteins. Therefore, it is expected that the microspheres have the potential to be versatile biological carriers.

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