Preparation of Amphiphilic Phenyl–Polysucrose Microspheres for Protein Adsorption

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ABSTRACT: In this study, we prepared novel amphiphilic phenyl–polysucrose microspheres by a two-step pathway. Crosslinked polysucrose microspheres were synthesized with soluble polysucrose and epichlorohydrin by inversed suspension polymerization first. Then, phenyl–polysucrose microspheres were obtained through the reaction between the polysucrose microspheres and glycidyl phenyl ether. Fourier transform infrared spectrometry and X-ray photoelectron spectroscopy proved that the microspheres had both hydroxyl groups and phenyl ligands. The quantitative determination of the phenyl groups indicated the optimal conditions for synthesis of the phenyl–polysucrose microspheres. The properties of the phenyl–polysucrose beads showed that the dry density increased from 1.38 to 2.29 g/mL, the equilibrium water content decreased from 77.30 to 33.68%, and the hydroxyl content remained at about 41.70 mmol/g when the phenyl content was increased from 0.00 to 2.64 mmol/g. The results of protein adsorption showed that the saturated adsorption capacities of the phenyl–polysucrose microspheres increased with increasing ion strength compared with the polysucrose microspheres.
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Key words: adsorption; hydrogels; microgels

INTRODUCTION

Recently, for good performances, such as high functionality and purity, low toxicity, and good biocompatibility, much attention has been paid to materials relevant to natural polysaccharides, which include tissue engineering scaffolds,[1–3] functional membranes,[4,5] and microspheres.[6–8] Polysaccharide microspheres with low toxicity and high functionality, including agarose and chitosan, have been modified to obtain hydrophobic interaction media by the hydrophobic groups, and amphiphilic microspheres with both hydrophilic and hydrophobic groups have broadened the applications of polysaccharides at the same time.[9–11]

Sucrose, a disaccharide, is liable to react because it has eight chemically active hydroxyl groups. Sucrose polymers with good properties of low toxicity and good biocompatibility have been widely applied in biomedical, pharmaceutical, and related fields.[12,13] Crosslinked polysucrose microspheres have been synthesized by a two-stage polymerization with sucrose and epichlorohydrin.[14] Further studies have shown that polysucrose microspheres have a high adsorption capacity for protein. However, there are only hydrophilic hydroxyl groups in polysucrose microspheres, so the saturated adsorption capacities of the polysucrose microspheres decrease with increasing ion strength, which limits their application.[15] Although polysaccharide microspheres have been used as hydrophobic interaction media, there have been no reports on amphiphilic polysucrose beads. In this study, we attempted to synthesize novel microspheres based on polysucrose microspheres coupled with glycidyl phenyl ether, as shown in Figure 1. Because of the hydrophilic and hydrophobic groups, the phenyl–polysucrose microspheres had amphiphilicity and could retain a high saturated adsorption capacity with increasing ion strength, which indicated that the microspheres could be used as hydrophobic interaction media in the separation and purification of proteins.

EXPERIMENTAL

Materials and apparatus

The polysucrose microspheres were made through inversion suspension polymerization with soluble polysucrose, epichlorohydrin, and dimethyl ether of poly(ethylene glycol).[16] Glycidyl phenyl ether (chemically pure) was purchased from Jiangsu Resin Factory (Wuxi, Jiangsu Province, China). Sodium hydroxide (NaOH; analytically pure), acetone (analytically pure), methanol anhydrous (analytically pure), and concentrated hydrochloric acid (HCl; analytically pure) were purchased from Tianjin Kewei Co. (Tianjin, China).

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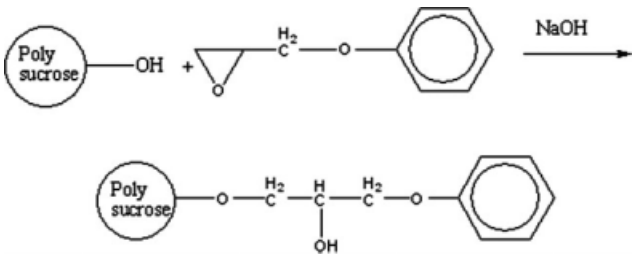

Figure 1 Scheme of the synthesis of the phenyl–polysucrose microspheres.

Preparation of the phenyl–polysucrose microspheres

Dried polysucrose microspheres (5 g, dry density = 1.38 ± 0.15 g/mL, equilibrium water content = 77.30 ± 7.50%, hydroxyl content = 41.78 ± 4.23 mmol/g) were first dipped in 40 mL of a mixed solvent for complete swelling. The solvent was a mixture of 10 mL of acetone and 30 mL of distilled water. Then, 5 mL of glycidyl phenyl ether was added to the solution under the continuous mechanical stirring. The catalyst (5 mL of 3 mol/L NaOH solution) was dropped into the solution with an increasing water bath temperature (60°C) under continuous mechanical stirring. After 5 h, the microspheres were obtained by several filtrations with acetone and distilled water under normal pressure. At last, the microspheres were stored in a mixed solvent of acetone and distilled water (volume ratio = 1 : 3).

To investigate the relation between the degrees of phenyl ligand and the reaction conditions, a series of the phenyl–polysucrose microspheres were prepared through changes in the experimental parameters, such as the hydroxyl content of the polysucrose microspheres, temperature, reaction time, and NaOH solution volume. The synthesized microspheres were named PSH, PST, PSI, and PSC. The PSH, PST, PSI, and PSC were respectively represented the microspheres prepared under different hydroxyl contents, temperatures, reaction times and NaOH solution volumes.

Characterization of the phenyl–polysucrose microspheres

Fourier transform infrared (FTIR) spectra

FTIR spectra were obtained with a Bio-Rad FTS 135 FTIR (Bio-Rad, Hercules, CA); the dry samples were powdered and mixed with KBr and then pressed into pellets under reduced pressure.

X-ray photoelectron spectroscopy (XPS)

XPS was performed with a PerkinElmer (Waltham, MA) 5600 spectrometer equipped with a spherical capacitor with Mg Kα radiation (1253.6 eV). The samples were dried *in vacuo* at 25°C for 48 h to ensure completely drying. The positions of the peaks were determined with reference to the carbon peak (referred to as the C1s peak) at 284.5 eV. The binding energies were measured within an accuracy of ±0.2 eV.

Morphology of the microspheres

Scanning electron microscopy (SEM; XL-30, Philips Co., Eindhoven, Netherlands) was used to determine the morphology of the phenyl–polysucrose microspheres. The samples were sputter-coated with a thin layer of gold to enhance the surface contrast and reduce surface charging before SEM examination.

Quantitative determination of the phenyl groups

The degree of phenyl ligand was determined by UV spectrophotometry according to the method of Johansson and Nystrom.[17] The phenyl-substituted and unsubstituted microspheres were dried and then hydrolyzed with concentrated HCl (25°C, 10 min). After the microspheres were diluted with anhydrous methanol, their UV spectrum was recorded at 270.5 nm with a 756MC ultraviolet–visible spectrophotometer (Shanghai The Third Analytic Instrument Factory, Shanghai, China). The methanol anhydrous solution of the hydrolysis product from the unsubstituted microspheres was used as a blank. The degree of phenyl substitution was calculated with the molar absorptivity for 2-phenoxyrthanol at 1760 L $mol^{-1} \cdot cm^{-1}$ at 270.5 nm.

Densities of the phenyl–polysucrose microspheres

To estimate the density of the dried phenyl–polysucrose microspheres, the beads (0.5 g) were first dried to a constant weight. Then, they were placed into a 10-mL volumetric flask. *N*-Heptane was added to the flask, and the mixture was kept at 25°C for 24 h. The density of the porous beads (d) was calculated according to the following equation:[18]

$$d = \frac{W_0}{10 - (W_1 - W_0)/d_s} \quad (1)$$

where W_1 is the total weight of the resin and the solvent, W_0 is the weight of the dry resin, and d_s is the density of the solvent.

Equilibrium water content of the phenyl–polysucrose microspheres

We determined the swelling ability of the phenyl–polysucrose microspheres by monitoring the equilibrium water content. The sample microspheres were immersed in distilled water at 25°C for 24 h. The

excessive surface-adhered water was removed by blotting. Then, the microspheres were dried in a vacuum oven at 60°C for 10 h until a constant mass was reached. The equilibrium water content [X (%)] was calculated according to the following equation:[14]

$$X(\%) = \frac{m_2 - m_3}{m_2 - m_1} \times 100\% \quad (2)$$

where m_1 is the mass of the empty container, m_2 is the total mass of the microspheres and the container, and m_3 is the constant mass of the microspheres and the container after drying.

Hydroxyl content[19]

The acid number was determined in the following way: 0.1-g dried samples were introduced into 20 mL of ethanol. A solution of NaOH (1.0 mol/L) was used to titrate the excess of acetic acid with a phenolphthalein solution as an indicator. A blank titration was done in the same way to prevent systematic errors. The acid number was calculated according to the following equation:

$$\text{Acid number (mg/g)} = \frac{40 \times (V - V_0) \times c}{m} \quad (3)$$

where V and V_0 are the volumes of the NaOH solution for the experimental and blank titrations, respectively; c is the concentration of the NaOH solution (mol/L); and m is the mass of the sample (g).

Nonaqueous titration was used to determine the hydroxyl content in the microspheres. To a pyridine solution of acetic anhydride (20 mL, 25% v/v) were added 0.30-g samples at room temperature; the solution was then heated to 100°C to acetylate for 1 h. Afterward, 5 mL of distilled water was added to the reaction system, and the reaction time was extended 30 min. An aqueous solution of NaOH (1.0 mol/L) was used to titrate the excess of acetic acid with phenolphthalein solution as an indicator. A blank titration was performed in the same way to prevent systematic errors. The hydroxyl content was calculated according to the following equation:

$$\text{Hydroxyl number (mg/g)} = \frac{40 \times (V_1 - V_2) \times c}{m} + \text{Acid number} \quad (4)$$

$$\text{Hydroxyl content (mmol/g)} = \frac{\text{Hydroxyl number}}{N} \quad (5)$$

where V_2 and V_1 are the volumes of the NaOH solution for the experimental and blank titrations, respectively, and N is the molar mass of NaOH (g/mol).

Determination of protein adsorption

Bovine serum albumin (BSA) was used as a model protein to test the adsorption of the phenyl–polysucrose microspheres compared with the polysucrose microspheres. All of the adsorption experiments were conducted at 25°C in a 0.01-mol/L trishydroxymethylaminomethane–HCl buffer solution. Typically, 0.05-g sample microspheres were added to 5.0-mL aliquots of BSA solution with different concentrations (ranging from 0.25 to 2.5 mg/mL) for 24 h in a shaking incubator (pH 4.0, 0.0 mol/L NaCl). The BSA standard curve was determined first. After centrifugation, the optical density at 280 nm of the supernatant solutions was recorded, and the amount of adsorbed protein was calculated by a mass balance with the following equation:[20]

$$q\ (\text{mg/g}) = (C_0 - C_1) \times V/W \quad (6)$$

where q is the amount of adsorbed protein per mass beads, C_0 is the concentration of BSA in the liquid phase before adsorption (mg/mL), C_1 is the concentration of BSA in the liquid phase after adsorption (mg/mL), V is the volume of the solvent (mL), and W is the mass of the beads (g). Afterward, the protein adsorptions of the phenyl–polysucrose microspheres and polysucrose microspheres were investigated under different ion strengths.

RESULTS AND DISCUSSION

FTIR analysis

The FTIR spectra of the polysucrose microspheres and phenyl–polysucrose microspheres are shown in Figure 2. In comparison with the polysucrose microspheres, the peaks at 3448 and 2874 cm^{-1} for the phenyl–polysucrose microspheres, which were attributed to the hydroxyl groups and methylene groups, respectively, showed little decrease in intensity. Compared with the FTIR spectra of the polysucrose microspheres, the peaks at 3039, 1597, 1496, and 690 cm^{-1}, which were attributed to ν(C—H), ν(C—C), β(C—H), and γ(C—H) of the aromatic ring in the phenyl–polysucrose, respectively, only appeared in phenyl–polysucrose microspheres.[21] These results indicate that the phenyl groups of glycidyl phenyl ether were coupled to the polysucrose microspheres successfully.

XPS analysis

The XPS spectra of the soluble polysucrose, polysucrose microspheres, and phenyl–polysucrose microspheres are shown in Figure 3. The spectra showed that the ratio of carbon atoms to oxygen atoms increased with the step reactions. The reason for this may have been the following:

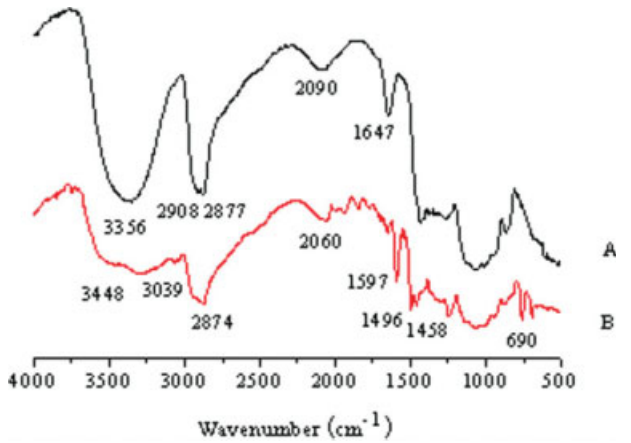

Figure 2 FTIR spectra of the (A) polysucrose microspheres (PS) and (B) phenyl–polysucrose microspheres (PSI3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1. Epichlorohydrin reacted with the soluble polysucrose, which made the ratio of carbon atoms to oxygen atoms increase from 66.8 : 30.6 to 69.8 : 28.9.
2. The glycidyl phenyl ether molecule, in which the ratio of carbon atoms to oxygen atoms was 9 : 2, was coupled onto the polysucrose microspheres, so the ratio of carbon atoms to oxygen atoms increased from 69.8 : 28.9 to 79.9 : 20.1.

The XPS C1s spectra of the soluble polysucrose, polysucrose microspheres, and phenyl–polysucrose microspheres are shown in Figure 4. The C1s spectra were divided into four different peaks, which responded to the carbon atoms in four different chemical states, as shown in Table I and Figure 5. The four different chemical states of the carbon atoms did not change during the reaction of soluble polysucrose and epichlorohydrin. The intensity of the band at 284.82 eV of the phenyl–polysucrose microspheres increased compared with that of the polysucrose microspheres. The reason was that the C1s spectrum of the phenyl groups was at 285 eV,[22] which indicated that the phenyl groups were coupled to the polysucrose microspheres.

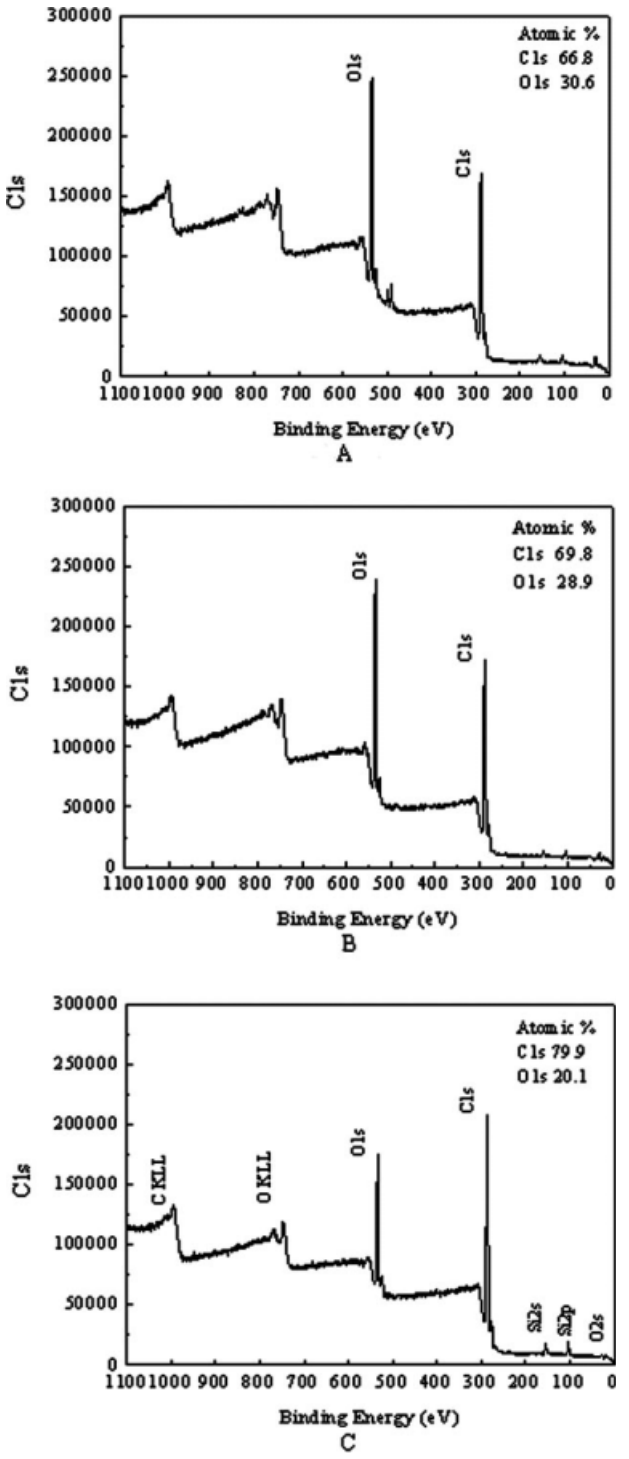

Figure 3 XPS spectra of the (A) soluble polysucrose, (B) polysucrose microspheres (PS), and (C) phenyl–polysucrose microspheres (PSI3).

Morphology of the phenyl–polysucrose microspheres

Figure 6 shows the morphology of the polysucrose microspheres and phenyl–polysucrose microspheres in SEM images. As shown, the phenyl–polysucrose microspheres were round, and the morphology did not change much after the reaction, compared with that of the polysucrose beads. The amount of grafted phenyl ligands was relatively lower on the surface of the microspheres, so the coupled phenyl groups did not have much influence on the morphology of the microspheres. However, the particle size of the microspheres decreased after the reaction. The main reason may have been that the glycidyl phenyl ether and acetone made the hydrophilic polysucrose microspheres shrink in the experiment.

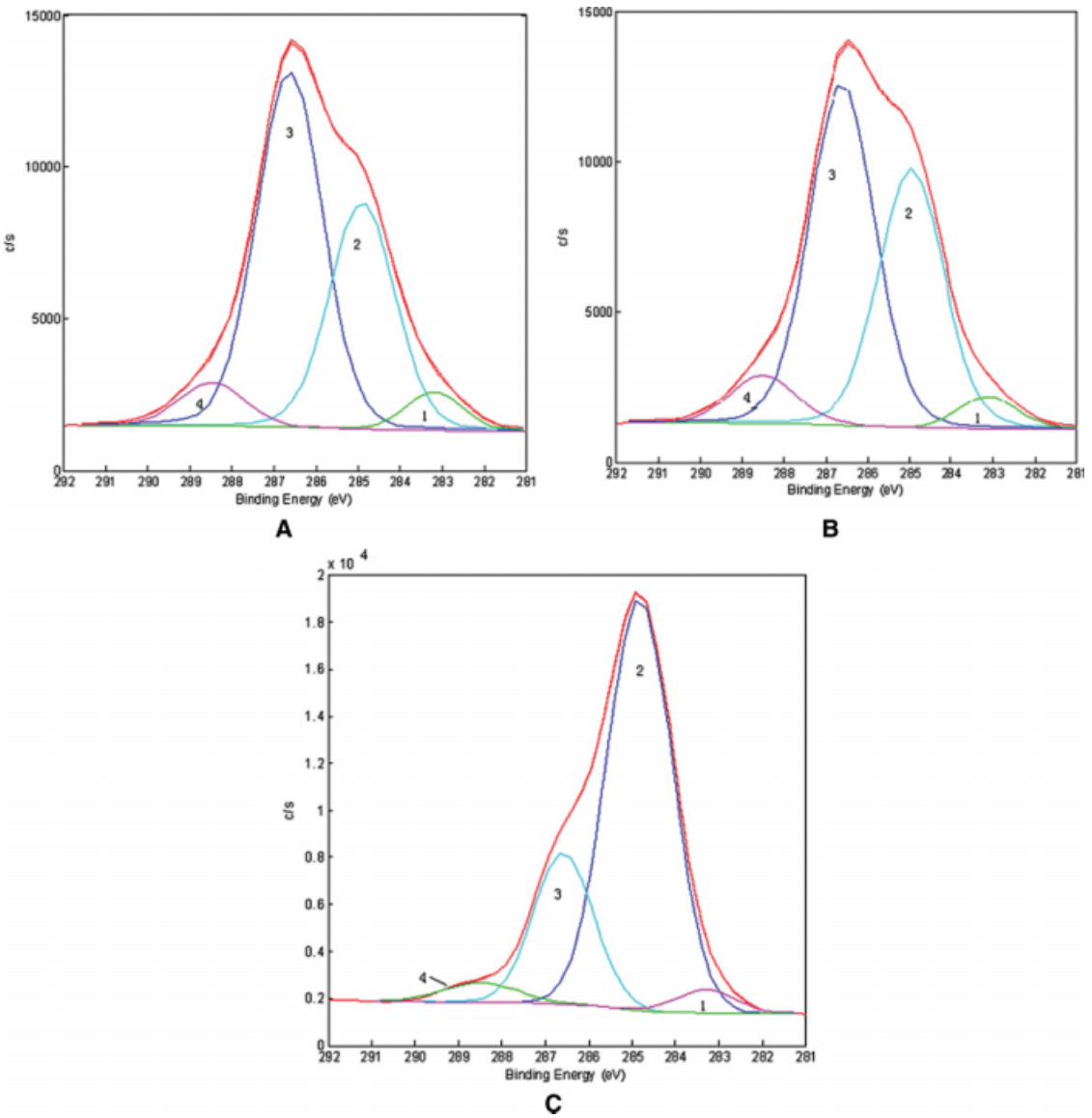

Figure 4 XPS C1s spectra of the (A) soluble polysucrose, (B) porous polysucrose microspheres (PS), and (C) phenyl–polysucrose microspheres (PSI3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I
Chemical States of the Soluble Polysucrose, Polysucrose Microspheres (PS), and Phenyl–Polysucrose Microspheres (PSI3) by XPS

		C1s			
		1 (C—O—C)	2 (CH_2—OH)[a]	3 (CH—OH)	4 (O—C—O)
Soluble polysucrose	Binding energy (eV)	283.2	284.9	286.63	288.48
	Area (%)	5.09	34.45	53.95	6.51
PS	Binding energy (eV)	283.12	284.96	286.61	288.5
	Area (%)	4.08	38.11	50.63	7.18
PSI3	Binding energy (eV)	283.29	284.82	286.57	288.5
	Area (%)	3.63	69.25	23.34	3.78

[a] The number 2 carbon atom represents CH_2—OH in the soluble polysucrose and polysucrose microspheres and CH_2—OH and phenyl in the phenyl–polysucrose microspheres.

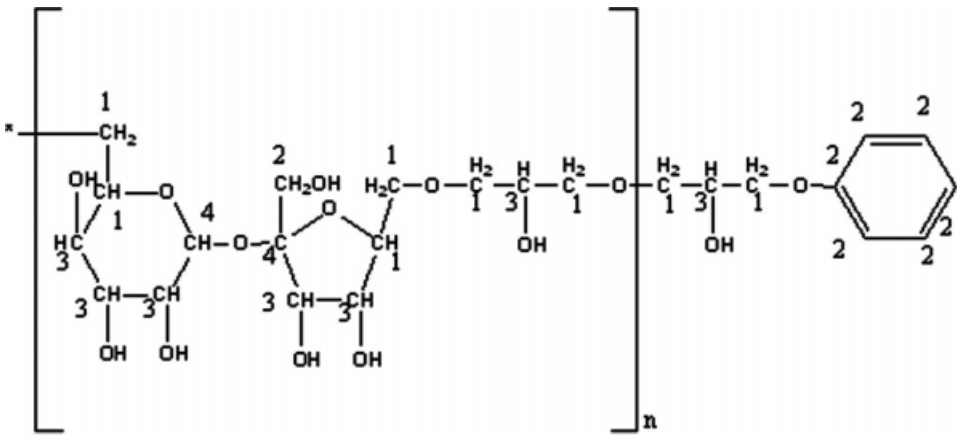

Figure 5 Representation of the C1s peaks.

Quantitative determination of the phenyl groups

In this experiment, one hydroxyl group of polysucrose reacted with one epoxy group of glycidyl phenyl ether, so the contents of the two functional groups were important factors. The phenyl–polysucrose microspheres were prepared with polysucrose microspheres with different hydroxyl contents, whereas the amount of glycidyl phenyl ether was invariable. The phenyl contents of the phenyl–polysucrose microspheres are shown in Table II and Figure 7. As shown, the phenyl content increased from 2.01 to 2.70 mmol/g with increasing hydroxyl content. The reaction shown in Figure 1 shifted positively with increasing hydroxyl content in the experiment, so there were more coupled phenyl groups in the microspheres.

The phenyl contents of the phenyl–polysucrose microspheres under different conditions are shown in Table II and Figures 8–10. The samples were prepared at different temperatures, times, and catalyst contents with a constant hydroxyl content. The phenyl content showed different trends under different preparation conditions: (1) the phenyl content increased from 1.28 to 2.70 mmol/g with increasing temperature, (2) the phenyl content increased from 1.52 to 2.70 mmol/g with increasing time, and (3) the phenyl content first increased from 2.09 to 2.38 mmol/g and then decreased from 2.38 to 0.54 mmol/g with increasing catalyst content.

The reasons for the aforementioned phenomena may have been that (1) the reaction shifted positively with increasing temperature and time, which made the phenyl content increase and (2) when the catalyst content was lower, the NaOH solution mainly catalyzed the reaction. However, when the amount of catalyst was greater than 5 mL, the NaOH solution mainly hydrolyzed the glycidyl phenyl ether. These results indicate that the phenyl content was influenced by the hydroxyl content, reactive time, reactive temperature, and catalyst content. According to our experiments, the optimum preparation conditions occurred when the polysucrose microspheres with a 41.78-mmol/g hydroxyl content reacted with glycidyl phenyl ether (5 mL) under catalyst (5 mL) at 70°C for 6 h.

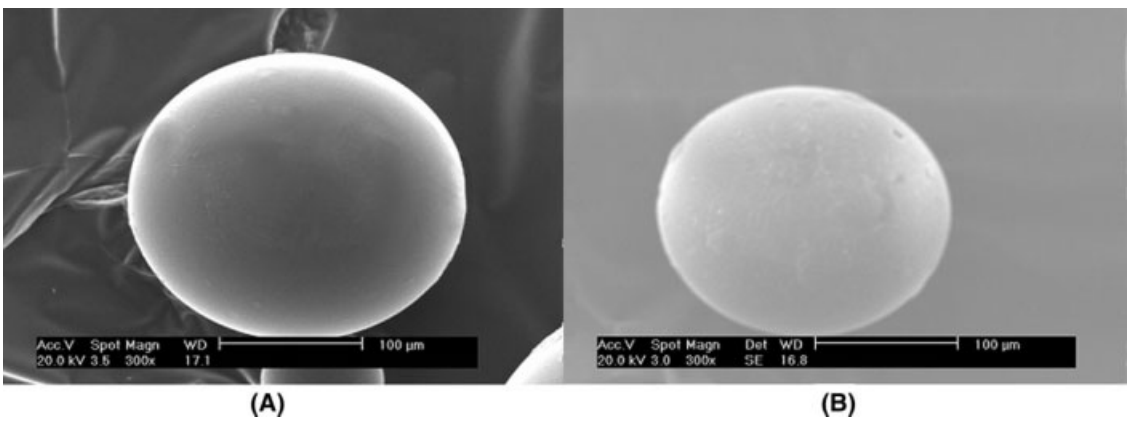

Figure 6 SEM images (300×) of the (A) polysucrose microspheres (PS) and (B) phenyl–polysucrose microspheres (PSI3).

TABLE II
Phenyl Contents of the Microspheres Under Different Preparation Conditions

Microsphere	Hydroxyl content (mmol/g)	Temperature (°C)	Time (h)	Catalyst content (mL)	Phenyl content (mmol/g)
PSH1	28.93	70	6	5	2.01 ± 0.10
PSH2	32.42	70	6	5	2.39 ± 0.12
PSH3	38.92	70	6	5	2.66 ± 0.13
PSH4	39.92	70	6	5	2.69 ± 0.13
PSH5	41.78	70	6	5	2.70 ± 0.14
PST1	41.78	60	6	5	1.28 ± 0.06
PST2	41.78	65	6	5	1.24 ± 0.06
PST3	41.78	70	6	5	2.66 ± 0.13
PHT4	41.78	75	6	5	2.61 ± 0.13
PHT5	41.78	80	6	5	2.70 ± 0.14
PSI1	41.78	70	3	5	1.52 ± 0.08
PSI2	41.78	70	4	5	1.95 ± 0.10
PSI3	41.78	70	5	5	2.38 ± 0.12
PSI4	41.78	70	6	5	2.66 ± 0.13
PSI5	41.78	70	7	5	2.64 ± 0.13
PSI6	41.78	70	8	5	2.70 ± 0.14
PSC1	41.78	70	6	3	2.09 ± 0.21
PSC2	41.78	70	6	4	2.37 ± 0.24
PSC3	41.78	70	6	5	2.38 ± 0.24
PSC4	41.78	70	6	6	1.14 ± 0.12
PSC5	41.78	70	6	7	1.11 ± 0.11
PSC6	41.78	70	6	8	0.92 ± 0.091
PSC7	41.78	70	6	10	0.54 ± 0.05

The amount of glycidyl phenyl ether was 5 mL in all of the experiments.

Properties of the phenyl–polysucrose microspheres

The properties of the phenyl–polysucrose microspheres with different phenyl contents are listed in Table III. The table shows that the dry density increased from 1.38 to 2.29 g/mL with increasing phenyl content, whereas the equilibrium water content decreased from 77.30 to 33.68% at the same time. The hydroxyl content almost remained the same when the phenyl content increased from 0.00 to 2.64 mmol/g. The main reasons for the previous phenomena could have been that

1. The phenyl groups were grafted onto the polysucrose beads after the reaction, so the mass of the microspheres increased. Meanwhile, the volumes of microspheres decreased because of the solvent. These led an increase in the dry density of the beads.
2. As Figure 1 shows, the reaction consumed one hydroxyl group of polysucrose beads and

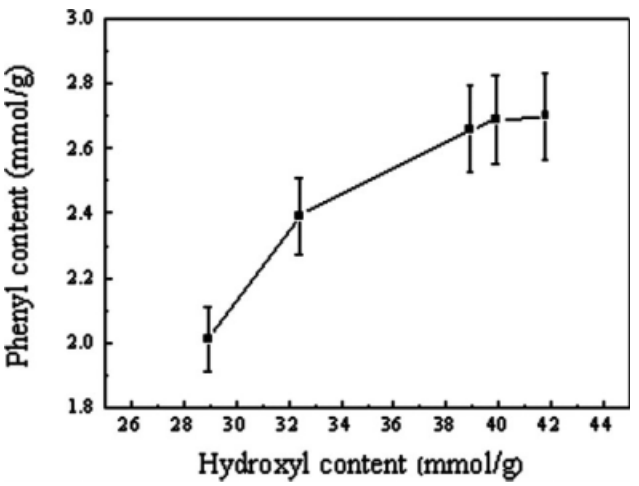

Figure 7 Effects of the hydroxyl content on the phenyl content of the polysucrose microspheres.

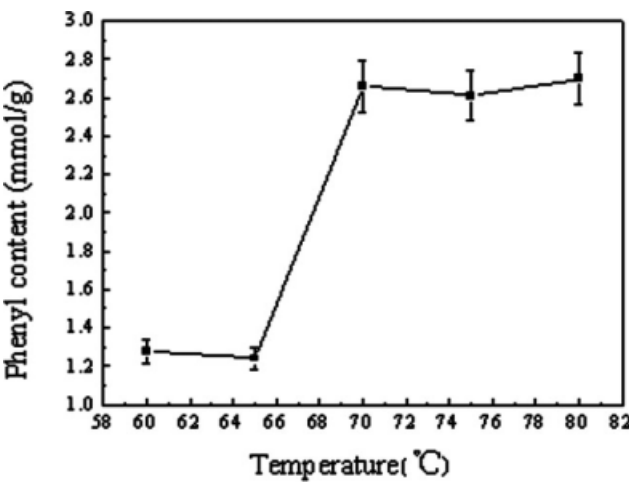

Figure 8 Effects of the reactive temperature on the phenyl content of the polysucrose microspheres.

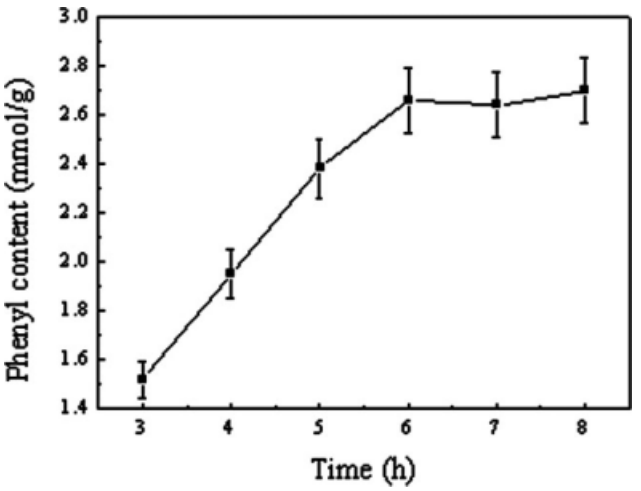

Figure 9 Effects of the reaction time on the phenyl content of the polysucrose microspheres.

generated a new one at the same time; therefore, the hydroxyl content of the beads did not change after the reaction.

3. The hydroxyl content did not change after the reaction, but the grafted phenyl groups were hydrophobic and sterically hindered for the adsorption of water. So the equilibrium water content decreased.

Compared with the polysucrose microspheres, the equilibrium water content of the phenyl–polysucrose microspheres obviously decreased, which indicated that the phenyl–polysucrose microspheres took advantage of the separation and purification of proteins in the salt solution.

Protein adsorption

The adsorption capacities of the phenyl–polysucrose microspheres and polysucrose microspheres under different ion strengths are shown in Table IV. These results reveal that the saturated adsorption capacities of the phenyl–polysucrose microspheres increased from 66.14 to 83.33 mg/g when the ion strength increased from 0.00 to 0.20 mol/L, whereas those of the polysucrose microspheres decreased from 69.49 to 42.64 mg/g.

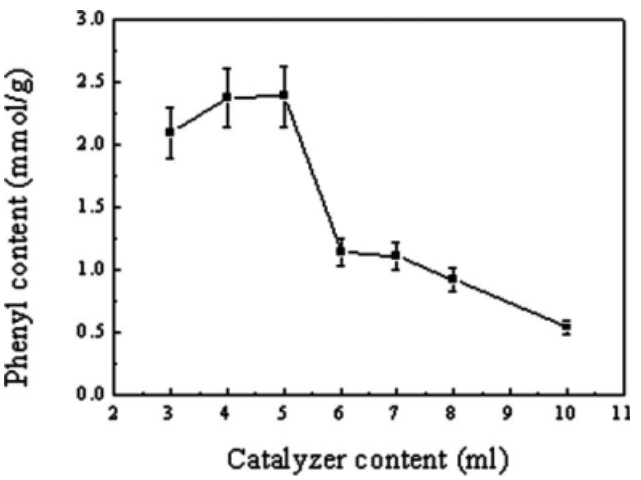

Figure 10 Effects of the catalyst content on the phenyl content of the polysucrose microspheres.

TABLE III
Properties of the Phenyl–Polysucrose Microspheres with Different Phenyl Contents

Microspheres	Phenyl content (mmol/g)	Dry density (g/mL)	Equilibrium water content (%)	Hydroxyl content (mmol/g)
PS[a]	0.00	1.38 ± 0.15	77.30 ± 7.50	41.78 ± 4.23
PSI1	1.52	1.42 ± 0.14	47.44 ± 5.60	41.72 ± 4.18
PSI3	2.38	1.82 ± 0.18	40.35 ± 5.40	41.70 ± 4.15
PHI5	2.64	2.29 ± 0.18	33.68 ± 4.30	41.76 ± 4.20

[a] Polysucrose microspheres without phenyl.

It was reported that a hydrated protein has a hydration shell, which prevents its hydrophobic areas from binding to hydrophobic ligands. The hydrophobic areas of dehydrated-state protein are exposed because of the addition of salts in the solution, so the protein can be absorbed to the hydrophobic ligands as phenyl.[23] In this experiment, the polysucrose microspheres had only hydrophilic hydroxyl groups and hydrophobic interactions, so the saturated adsorption capacities of the polysucrose microspheres decreased because the interactions between the hydrophilic hydroxyl groups and proteins decreased with increasing ion strength. However, the adsorption interactions of the phenyl–polysucrose microspheres contained hydrophobic and hydrophilic interactions due to the hydroxyl groups and phenyl ligands of the amphiphilic polysucrose molecule. The interactions between the proteins and phenyl beads increased with increasing ion strength, which led to an increase in the saturated adsorption capacity. These results indicated

TABLE IV
Adsorption Capacity of the Phenyl–Polysucrose Microspheres Under Different Ion Strengths

pH	NaCl (mol/L)	Microspheres	q_m (mg/g)	K_d (mg/mL)
4.5	0.00	PHI5	66.14	0.83
		PS[a]	69.49	0.71
4.5	0.10	PHI5	72.99	0.64
		PS[a]	45.52	0.45
4.5	0.20	PHI5	83.33	0.53
		PS[a]	42.64	0.75

[a] Polysucrose microspheres without phenyl.

q_m, the saturated adsorption capacities of microspheres; K_d, the dissociation constant of adsorption equilibrium.

that the phenyl–polysucrose microspheres had amphiphilicity and could be used as hydrophobic interaction media in the separation and purification of proteins. These results explain why the phenyl–polysucrose microspheres were synthesized.

CONCLUSIONS

In this experiment, novel amphiphilic microspheres were prepared successfully with polysucrose microspheres and glycidyl phenyl ether; this reaction was proven by FTIR spectrometry and XPS. The quantitative determination of the phenyl groups indicated the optimal conditions for the synthesis of the phenyl–polysucrose microspheres occurred when polysucrose microspheres with a 41.78-mmol/g hydroxyl content reacted with glycidyl phenyl ether (5 mL) under catalyst (5 mL) at 70°C for 6 h. The properties of the phenyl–polysucrose microspheres showed that the dry density increased from 1.38 to 2.29 g/mL when the equilibrium water content decreased from 77.30 to 33.68% with increasing phenyl content from 0.00 to 2.64 mmol/g. The hydroxyl content mostly remained the same when the phenyl content increased from 0.00 to 2.64 mmol/g. The results of the adsorption property measurements show that the saturated adsorption capacities increased from 66.14 to 83.33 mg/g with increasing ion strength, whereas those of the polysucrose microspheres decreased from 69.49 to 42.64 mg/g. These results indicate that the phenyl–polysucrose microspheres had a higher saturated adsorption capacity because of the amphiphilicity and could be used as hydrophobic interaction media in the separation and purification of proteins.

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