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In this study, suspension polymerization is described to prepare hydrophilic polystyrene microspheres with casein macromolecules on the surface. In the suspension polymerization, casein w as used as an emulsifier and stabilizer instead of synthetic surfactants. The microspheres had spherical shape and the size distribution was narrow, the average size was about 3.5 μm. The microspheres were characterized with a Fourier transform infrared spectroscopy, revealing the existence of hydroxyl, carboxyl and amino groups. XPS analysis was carried out to study the surface composition, it revealed that the nitrogen concentration was 5.04% on the surface of the particles and it remained almost unchanged after the particles were washed by 2% SDS. The microspheres were confirmed to be hydrophilic due to the casein molecules on the surface.

Keywords: microspheres; suspension polymerization; casein; surface; hydrophilic

1 Introduction

Polymer microspheres have been attracting great attention because of various applications in many areas, especially in life science and medicine. There is a huge amount of materials that can be used to fabricate polymer microspheres through different methods and technology, granting polymer microspheres special physcochemical characteristics and various functions, so polymer microspheres can be widely used in enzyme immobilization, targeted drug delivery, immunoassay, bioseparation, advanced cosmetics etc. (1, 2). Recently, there has been increasing interest in introducing functional groups (such as hydrophilic hydroxyl groups, carboxyl and aldehyde groups) onto the surface of polymer microspheres. It can effectively improve the suspension stability and bio-compatibility of microspheres to introduce functional groups (3), more importantly, it can make functional microspheres. Microspheres containing inorganic semiconductor materials, functional organic molecules or molecules with biological activity (4–7) have been made through functionalization.

Researchers have tried various methods to introduce functional groups onto polymer microspheres by heterogeneous copolymerization of monomers containing the required functional groups. The methods include emulsion polymerization, suspension polymerization, dispersion polymerization and seeded emulsion polymerization. Functional polymer microspheres have also been obtained by surface modification of preformed microspheres (8). The above methods have been reported numerous times and several papers provided comprehensive reviews on them (1, 2, 9, 10).

Another way to introduce functional groups onto the microspheres surface is physical adsorbing. Coombes AGA et al. (11) have prepared poly(lactice-co-glycolide) (PLG) microspheres through an emulsification/solvent evaporation technique using poly[ethylene oxide]-poly[propylene oxide] (PEO-PPO) block copolymers as stabilizer, the PEO-PPO adsorbed to the PLG microsphere to provide a hydrophilic layer of pendent PEO chains, Mark E. Keegen et al. (12) have prepared poly(lactice-co-glicolic acid) (PLGA) microspheres with the same method, they used poly(ethylene alt-maleic acid) (PEMA) instead of conventional poly(vinyl alcohol) (PVA) as a stabilizer, resulting in a high density of carboxylic acid groups at the microsphere surface. Polyl(actide) microspheres with 38.3 mg/m^2 bovine serum albumin (BSA) at the surface were prepared using BSA as stabilizer by M. J. Montisci et al. (13).

Casein is an amphiphilic protein, it has excellent emulsifying properties when it is in a solution and dispersed form (14, 15). There are many hydroxyl, carboxyl and amino groups in the casein molecules. Owing to its good solubility, surface activity, heat resistance and water-holding property, casein is widely used as an emulsion stabilizing agent in

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foods such as ice-cream, coffee lightener, cream liqueurs and whipped toppings (16).

In this context, the aim of this work was to develop a new method to introduce functional (hydroxyl, carboxyl and amino) groups from casein molecules onto the surface of polystyrene microspheres that have potential for applications in life science and medicine. We employed suspension polymerization, only using casein to act as the emulsifier and stabilizer. Casein is protein with good amphiphathy like HAS (human serum albumin), casein molecules can strongly adhere to the oil-water interface (17) and form a protein multiplayer (18–20) to protect and stabilize the fine oil droplets against recoalescence. The polymerization takes place in the mini-container surrounded by a casein multilayer.

2 Experimental

2.1 Materials

Styrene (St), from the Damao Chemical Reagent Factory (Tianjin, P. R. China) was washed with 0.5 M NaOH and then purified by distillation under reduced pressure prior to use. Divinylbenzene (DVB) from Kelong Chemical Factory, Chengdu (P. R. China) was washed with 0.5 M NaOH to get rid of the inhibitor before use. Benzoyl peroxide (BPO) purchased from the Chemical Factory of Hubei University (P. R. China) was used as initiator and without any pre-treatment. Casein obtained from Kelong Chemical Factory, Chengdu (P. R. China) was treated with EDTA (1 wt%) for 48 h to remove metal ions before use. Glutaraldehyde (GA, 50%) was purchased from Shantou Chemical Reagent Factory (Guangdong, P. R. China). All chemical agents were of analytical grade and all the water in the experiment was de-ionized.

2.2 Preparation of Polystyrene Microspheres

Polystyrene microspheres were prepared by suspension polymerization. First, to obtain casein solution, 3.0 g pretreated casein and 0.1 g Na_2CO_3 were dissolved in 100 g de-ionized water at 50°C for 2 h under mechanical stirring. 0.12 g initiator (BPO) was dissolved in 11.4 g St. The pH of the casein solution was adjusted to pH 7.4. For the suspension polymerization, the monomer mixture (11.4 g St + 0.6 g DVB) with initiator dissolved was emulsified into 54 g 1.5 wt% casein solution by a homomixer BME-100LX (from Shanhai Weiyu Machinery and Electronics CO., LTD, P.R. China) in 8000 rpm for 20 sec.

The above emulsion was transferred to a four-necked glass flask equipped with a mechanical stirrer, a condenser, and a nitrogen inlet nozzle. The nozzle was lifted above the surface of the emulsion after the emulsion had been purged with nitrogen for 1 h, and the temperature was elevated to 70°C gradually for the polymerization. The polymerization was carried out continuously at 70°C for 8 h. The obtained microspheres were thoroughly washed by water three times, and then incubated overnight in a water solution containing 2 wt% of sodium dodecyl sulfate (SDS) under room temperature. The microspheres were washed by water three times after incubation and then dried at 50°C in a vacuum drying oven for 24 h to eliminate any volatile matter (water, monomers) from the surface of the particles.

2.3 Polystyrene Microspheres Treated by GA

A given amount of microspheres obtained was mixed with 100 ml of 1%(v/v) glutaraldehyde (GA) solution in water (pH 6.0). The mixture was magnetic stirred at room temperature overnight, then washed with water several times.

2.4 Investigation of Surface Hydrophilicity or Hydrophobicity

An amount of aquatic suspension of microspheres was dropped onto a glass slide, then the glass slide was dried at 50°C in a vacuum drying oven for 24 h to form a particle layer on the surface. Then, one drop of water (dyed black) was dropped onto the particle layer to examine whether it would spread or not. Photographs were taken to create records.

2.5 Characterization of Microspheres

The study of size and size-distribution of microcapsules were carried out on a laser analyzer for particle size (Mastersizer2000, Malvern). A scanning electron microscope (JEOL JSM 5900LV) was used to observe the surface morphology of the microspheres. With regard to SEM, one drop of the microspheres dispersion was placed on a nickel SEM stub and air-dried, then the dried sample was gold-coated.

The microspheres were characterized with a Fourier transform infrared spectroscopy (FT-IR) (Nicolet MX-1E). To confirm whether the casein molecules remained at the microsphere surface, X-ray photoelectron spectroscopy (XSAM 800) was used to determine the chemical composition of the surface.

3 Results and Discussion

3.1 Particle Morphology and Size Distribution

Figure 1(A) shows the electron scanning micrographs of microspheres with casein molecules at the surface, the shape was spherical and the surface was not very smooth, as if there were some nano-sized particles adsorbed on them. We think that the nano-sized particles were polymers that formed by emulsion polymerization that accompanied suspension polymerization. In Figure 1(B), the sample was hydrolyzed by NaOH of 6.0M at a temperature of 80°C for 24 h. The hydrolyzed microspheres were very coarse and there were many pores on the surface. The roughness may be caused by eliminating casein from the surface through hydrolysis.

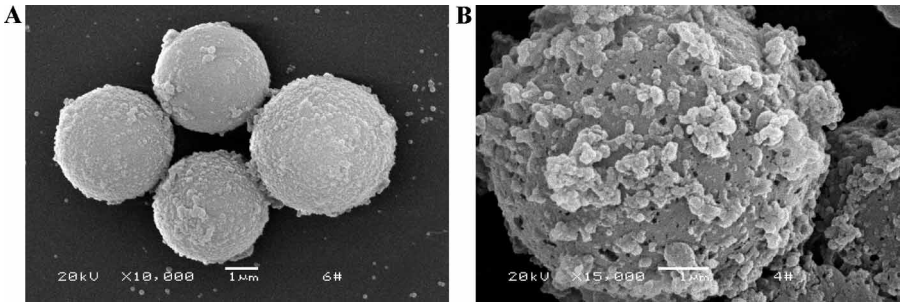

Fig. 1. SEM graphs of PSt microspheres (A) and PSt microspheres after hydrolysis (B).

Figure 2 shows the particle size distribution of prepared microspheres. The size of most of the resulting microspheres was below 6 μm, and the size distribution was narrow. The sample had an average diameter of 3.527 μm, and it was much smaller than that of particles obtained by most suspension polymerization. The main reason should be that the casein molecules had strongly adsorbed onto the oil droplets of monomer forming a multiplayer to protect the oil droplets. Furthermore, it is difficult to allow the multiplayer collapse within short time during polymerization. In addition, at pH 7.4, all the casein molecules were negatively charged which may enhance the detachment of particles through the polymerization. Thus, the size of particles remained unchanged through the polymerization.

With a sufficient amount of casein in solution during the emulsion process, the size of microspheres prepared was determined by the agitation speed of the homomixer in the emulsifying stage. Figure 3 describes the influence of agitation speed on the average particle size. The average diameter decreased when the agitation speed increased, but the effect became small and the average diameter remained almost constant when the agitation speed exceeded 7000 rpm.

3.2 FT-IR Spectrum

FT-IR spectrum of the microspheres (b) and that of microspheres treated by GA (a) is presented in Figure 4. Both curves (a) and (b) exhibit distinct characteristic peaks of polystyrene at a band of 1599 cm^{-1}, 1581 cm^{-1}, 1491 cm^{-1}, and 1450 cm^{-1}, both curves had absorption peaks at or close to 1721 cm^{-1} and 923 cm^{-1} that belonged to the carboxyl group. Curve (b) had a strong absorption peak at a band of 3439.5 cm^{-1}, and hydroxyl and amino groups from casein macromolecules were believed to contribute. In curve (a), there were no adsorption peaks at or close to 3439.5 cm^{-1}, indicating that few hydroxyl or amino groups were present in the microspheres as a result of GA treating. The mechanism of the chemical reactions between casein and glutaraldehyde (GA) can be explained as Scheme 1 and Scheme 2 in Figure 5. GA treating should generate an absorption peak for C═N stretching in 1690∼1520 cm^{-1}. Because the phenyl ring stretching peaks were almost in the same area, it was difficult to identify the stretching peak of C═N in curve (a).

3.3 Analysis by X-ray Photoelectron Spectroscopy

The chemical composition of the surface was studied by X-ray photoelectron spectroscopy and the result showed that the elements of nitrogen and oxygen were present on the surface of microspheres. Figure 6 demonstrates the C1s high solution spectra, and Table 1 shows the results of surface analysis from XPS narrow scan spectra. There was no evidence of sulfur and phosphorus in their corresponding high solution spectra, indicating that the surface concentration of these two elements was lower than the XPS

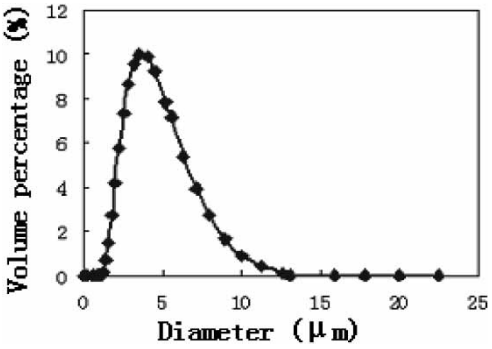

Fig. 2. Size distribution of microspheres.

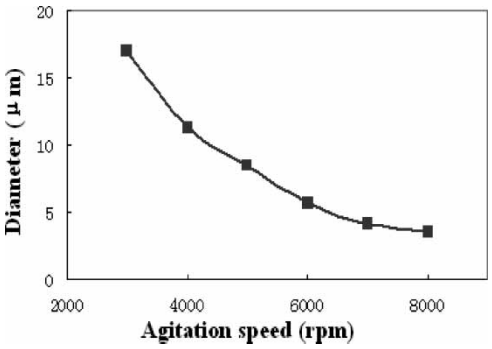

Fig. 3. Effect of agitation speed on particle size.

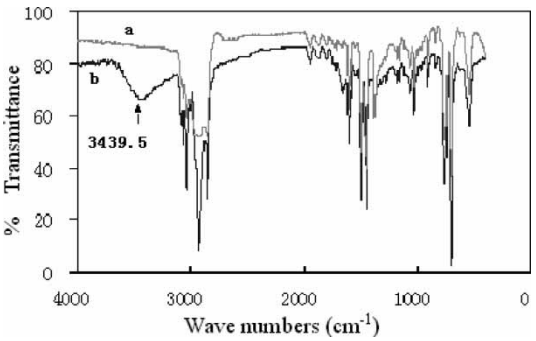

Fig. 4. FT-IR spectrum of the microspheres treated by GA (a) and that of not (b).

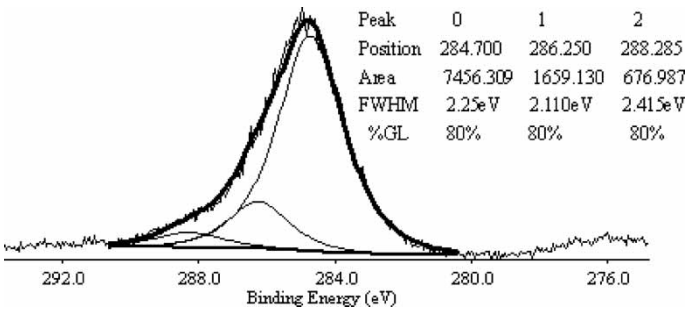

Fig. 6. XPS narrow scan spectra of C1s with the sample not hydrolyzed.

detection limit (0.1 atom %). In fact, casein macromolecules contain scarce sulfur and phosphorus. It was expected that, in the microspheres surface, C1s XPS spectra should be fitted to four carbon functionalities in this case: hydrocarbon [C-C/C-H at 285.0eV], carbon singly bonded to one oxygen atom [C-O at 286.5eV], carbon singly bonded to two oxygen atoms [-(C═O)-O at 289.0eV], carbon singly bonded to nitrogen atom [C-N at 286.0eV]. However, in this study, the C1s spectra only revealed three carbon functionalities (three peaks) at 284.70eV, 286.25eV, and 288.285eV, respectively. We think that the peak 1 (binding energy 286.250eV) in Figure 5 should contribute to the two kinds of carbon atoms from C-O and C-N.

Analysis of X-ray photoelectron spectra confirmed the existence of nitrogen and oxygen on a microsphere surface. Samples of hydrolyzed microspheres, microspheres washed by SDS and microspheres not washed by SDS, were investigated to compare the effect of hydrolysis and SDS washing to the surface retention of casein macromolecules. From the XPS narrow scan spectra (C1s, N1s, and O1s), the surface elemental compositions were calculated. Table 1 reveals that a very small concentration (0.85 at.%) of nitrogen still remaining on the surface even after hydrolysis by NaOH, and the exposure to SDS had very little impact on the surface nitrogen concentration. It is known that the adsorbed protein can be washed off the surface by surfactants and washing microspheres by 2% SDS aquatic solution leads to total removal of adsorbed protein within 2 h (2). The result strongly suggested an entrapment or entanglement of casein macromolecules by or with polystyrene rather than physical adsorption. Due to their good amphiphathy, casein macromolecules can strongly adhere to the oil-water interface with their hydrophobic parts penetrating into the oil phase and hydrophilic parts staying in the aquatic side. When microspheres were formed, the casein macromolecules were entrapped or entangled in the microspheres surface.

3.4 Study of Surface Hydrophilicity or Hydrophobicity

Hydrophilicity or hydrophobicity is one of the most important characteristics of functional microspheres. The most often used method to determine the hydropilicity (or hydrophobicity) is to measure the contact angle. In this study, we performed an easy experiment. We investigated the spreading of water (dyed black) on the surface of a layer formed by microspheres. In Figure 7, photographs A–E (each photograph was taken at a different time) show the spreading process of water on the layer of microspheres that had not been hydrolyzed. Figure 7 (F) exhibits that the water drop did not spread on the layer of hydrolyzed microspheres, remaining as a perfect spherical shape. In addition, seeing Figure 7 (F), microspheres could not form a smooth layer on the glass surface because of hydrophobicity. From the information above, conclusions can be made that because of the existence of casein macromolecules, the microspheres

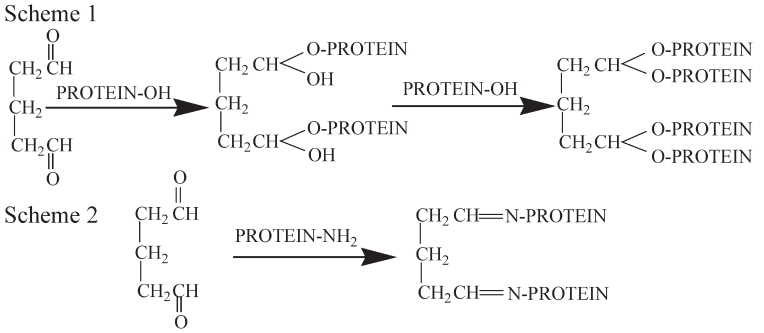

Fig. 5. Reaction of -OH and -NH_2 from casein with aldehyde groups from glutaraldehyde.

Table 1. Surface composition of the microspheres determined by XPS

Sample	Peak Area					Concentration of N (at. %)	Elemental ratio (n_N/n_C)
	C	N	S	P	O		
Hydrolyzed microspheres	7727.43	114.93	—	—	823.05	0.85%	0.89%
Microsphere washed by SDS	8075.79	827.537	—	—	3170.18	5.04%	6.10%
Microspheres not washed	9792.42	1016.47	—	—	2471.77	5.34%	6.18%

n_N/n_C = The atomic ratio of nitrogen to carbon.
C = Carbon, N = nitrogen, O = oxygen, S = sulfur, P = phosphorus.

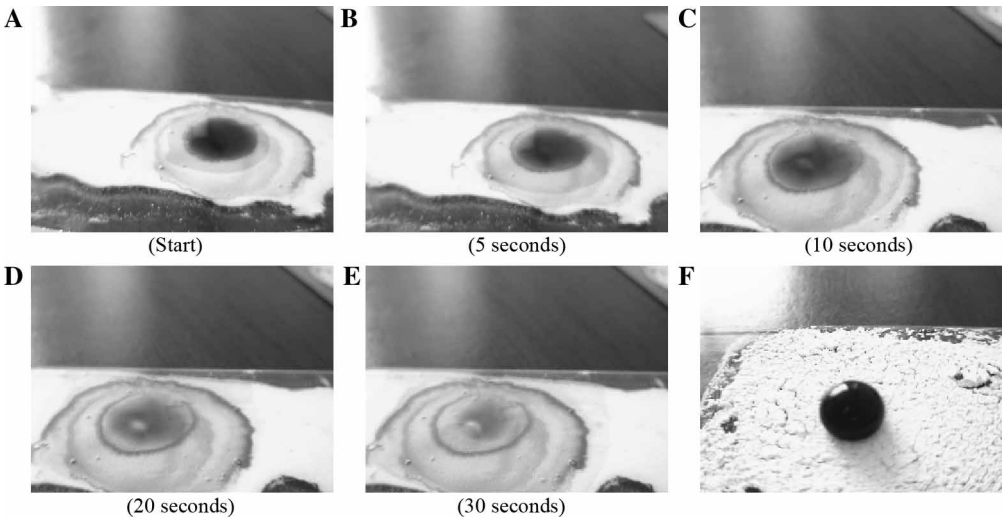

Fig. 7. Photographs of water dropped onto the glass covered with a layer of microsphere: A–E, microspheres not hydrolyzed; F, hydrolyzed microspheres.

prepared in this study were surface hydrophilic, and they turned into surface hydrophobic due to the loss of surface casein macromolecules by hydrolysis.

4 Conclusions

Hydrophilic polystyrene microspheres with a concentration of casein macromolecules on the surface were prepared by suspension polymerization only using casein as emulsifier and stabilizer, instead of any other type of surfactants. The microspheres had a spherical shape and the size distribution was narrow. Casein macromolecules were entrapped or entangled on the surface of microspheres. The nitrogen concentration was 5.04% on the surface of microspheres, and it remained almost unchanged when the particles were washed by 2% SDS. Because of the casein molecules, the microspheres prepared were hydrophilic and may have good bio-compatibility. In this study, hydroxyl, carboxyl and amino groups were introduced onto the microspheres surface, such microspheres can be potential support for many subjects such as enzyme, antigen and antibody. However, the microspheres made in this study should be activated prior to using, for example, in adequate conditions, one of the two aldehyde groups of GA reacts with casein molecules and the other one still remains active for further use. These microspheres are expected to have many applications in life science and medicine. Further work on these microspheres should focus on the protein (enzyme, antigen and antibody) immobilization and its various applications in scientific and industrial areas.

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