Monodispersed Carboxylated Composite Polymer Microspheres and Physical Immobilization of Biomolecules

Hasan AHMAD,[†] Md. Masem HOSSAIN, Md. Mahbubor RAHMAN, and M. A. Jalil MIAH

Department of Chemistry, Rajshahi University, Rajshahi-6205, Bangladesh

(Received December 27, 2006; Accepted February 6, 2007; Published March 15, 2007)

ABSTRACT: Carboxylated polymer microspheres were prepared by multi steps seeded polymerization using methacrylic acid as a comonomer. Ethylene glycol dimethacrylate was used as a crosslinking agent in the second step of seeded copolymerization to provide a crosslinked network in order to limit the penetration of carboxyl group to interior of the microspheres. In this research a series of polystyrene/poly(methyl methacrylate-ethylene glycol dimethacrylate)/poly(methyl methacrylate-methacrylic acid) composite polymer microspheres with varying proportion of methacrylic acid were prepared. Carboxyl group density increased with the increase of methacrylic acid content in the recipe. Physical immobilization of some biologically active macromolecules was studied at the respective isoelectric pH value under similar conditions. The specific activities of adsorbed trypsin on different composite polymer microspheres were also measured. [doi:10.1295/polymj.PJ2006204]

KEY WORDS Carboxylated Polymer / Biomolecules / Immobilization / Specific Activity / Hydrophilic /

Monodispersed functional polymer microspheres in the micrometer-size range have been widely studied and used in separation media, ion exchangers, support materials, calibration standards, information industries and as carrier for biomolecules.^{1–3} Since many of these application properties are influenced by the morphology and surface prosperities of particles attention has been focused on the control of particle size, its distribution and functional group density on the surface. A variety of methods are available to produce monodispersed microsphere in the micrometer-size range. Activated swelling method developed by Ugelstad,⁴ dynamic swelling method developed by Okubo⁵ are the most frequently quoted procedures although some other less known approaches are also available.^{6,7} All of these methods are complex and proved to be difficult when water-soluble monomers are involved. Dispersion polymerization is another process that allows the successful preparation of micron-sized polymer microspheres with ease.^{8–10} However this methodology has suffered drawbacks when functional comonomers were employed.^{11,12} These limitations include polydispersity, odd-shaped particles and in the extreme case coagulation during copolymerization. Moreover for dispersion copolymerization in presence of functional comonomer only a portion of the functional groups are on the particles surface and the remainder exist inside the particle or in the continuous phase. Dispersion polymerization followed by seeded polymerization technique can be used to overcome these problems.

Micron-sized microspheres with carboxyl groups

on/near the surface have been found to improve certain properties such as colloidal stability, mechanical and freeze thaw stability, rheology, adhesion etc.¹³ These microspheres can also find application in biomedical field especially for protein adsorption. A number of reports dealing with the preparation of functional microspheres containing carboxyl groups are available in the literature.^{14–16} C. Kan *et al.* reported the preparation of only submicron-sized poly(methyl methacrylate-ethyl acrylate-methacrylic acid) particles *via* seeded emulsion polymerization.¹⁴ He had noticed secondary nucleation during seeded polymerization when the amount of methacrylic acid exceeds only 8 mol %. WD. He used time consuming pre-swelling technique before interfacial-initiated seeded emulsion polymerization to prepare submicron-sized carboxylated particles.¹⁵ Zhang reported the preparation of micron-sized polystyrene/poly(styrene-methacrylic acid) microspheres by seeded dispersion polymerization.¹⁶ In this case the presence of steric stabilizer on the particles surface may interfere with the immobilization process of biomolecules as most such applications require clean latex surface.

In this research monodispersed polymer microspheres with varying amount of carboxyl groups on/near the surface were prepared by multi steps seeded copolymerization. First polystyrene (PS) seed particles were prepared by dispersion polymerization in ethanol medium and after purification introduced these PS particles into second step seeded copolymerization with methyl methacrylate (MMA) and ethylene glycol dimethacrylate (EGDM). We called these

[†]To whom correspondence should be addressed (Tel: +88-0721-750916, E-mail: samarhass@yahoo.com).

produced particles as PS/P(MMA-EGDM) composite seed. EGDM was used to provide a thin layer of cross-linked network around the PS seed particles. In the third step, a number of seeded copolymerizations were carried out with varying amounts of methacrylic acid (MAA) and MMA in presence of PS/P(MMA-EGDM) composite seed. Final PS/P(MMA-EGDM)/P(MMA-MAA) composite microspheres were characterized for carboxyl groups on/near the surface, adsorption behavior of biomolecules and specific activity of physically immobilized trypsin (TR).

EXPERIMENTAL

Monomers and Chemicals

Styrene, methyl methacrylate (MMA) and methacrylic acid (MAA) of monomer grade, purchased from Fluka, Chemika, Switzerland, were distilled under reduced pressure and preserved in the refrigerator until use. 2,2'-Azobisisobutyronitrile (AIBN) was recrystallized from ethanol and preserved in the refrigerator before use. Potassium persulphate (KPS) of LOBA Chem., India, was recrystallized from distilled water and preserved in the refrigerator. Tricaprylylmethyl-ammonium chloride (aliquate³³⁶), ethylene glycol dimethacrylate (EGDM) and tween-80 of Fluka, Chemika, Switzerland, polyvinylpyrrolidone (PVP) and *N,N'*-methylene bis-acrylamide (MBAAM) of LOBA Chem., India, were used as received. The biomolecules used were trypsin (TR) from E. Merck, Germany, albumin (AL) from LOBA Chem., India and casein (CS) from Matheson Coleman and Bell, USA. L-Lysine monohydrochloride purchased from BDH Chem., England was used for the preparation of lysine methyl ester hydrochloride (LME). Other chemicals used were of reagent grade. Deionized water was distilled using a glass (Pyrex) distillation apparatus.

Transmission Electron Microscope, TEM (Zeiss EM 912 Omega); Scanning Electron Microscope, SEM (LEO Electron Microscopy Ltd. UK); Helios Gamma Single-Beam UV-Visible Spectrophotometer from Unicam, UK; HI 9321 Microprocessor pH-meter from HANNA instruments, Portugal; centrifuge machine from Kokuson Corporation Tokyo, Japan; were used in this study.

Preparation of PS Seed Particles

PS particles were prepared by dispersion polymerization of styrene (20 g) using AIBN (0.3 g) as oil soluble initiator. 0.8 g of PVP and 0.2 g of aliquate³³⁶ were used as steric stabilizer and co-stabilizer respectively. Solid content was fixed at 20% (w/w) in ethanol as continuous phase. Polymerization was carried out in a three-necked round bottom flask under a nitro-

gen atmosphere at 70 °C.

Percentage of conversion reached nearly 100% as measured gravimetrically. PS seed particles were transferred to distilled water by repeated centrifugation.

Preparation of Micron-Sized PS/P(MMA-EGDM) Composite Seed

Micron-sized PS/P(MMA-EGDM) composite seed was prepared by seeded copolymerization of MMA with EGDM in presence of micron-sized PS seed particles using KPS as water-soluble initiator under a nitrogen atmosphere in a three necked round bottom flask. The core/shell ratio was fixed at 1/0.8 and the crosslinking agent EGDM was 3% (w/w) relative to total monomer. The polymerization was continued until the monomer smell disappeared. The composite seed was washed by replacing serum with distilled deionized water.

Preparation of Micron-Sized PS/P(MMA-EGDM)/P(MMA-MAA) Composite Polymer Microspheres

Three different batches of micron-sized PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres were prepared by seeded copolymerization of MMA with MAA in presence of washed micron-sized PS/P(MMA-EGDM) composite seed under a nitrogen atmosphere in a three necked round bottom flask. Preparations were carried out at various MAA contents (w/w) in the shell layer with a constant core/shell ratio of 1/0.5. The reaction was continued until almost monomer disappeared. The preparation conditions are shown in Table I. Each composite microsphere was repeatedly washed by serum replacement with distilled deionized water before characterization.

Measurement of Carboxyl Value

PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with different amounts of MAA were washed repeatedly to remove any unreacted/adsorbed MAA. The concentration of carboxyl groups on the surface of composite micro-

Table I. Preparation of micron-sized PS/P(S-EGDM)/P(MMA-MAA)^a composite polymer microspheres^b

MAA contents % (w/w)	20	30	40
PS/P(MMA-EGDM) composite seed emulsion ^c (g)	28.25	28.25	28.25
MMA (g)	1.20	1.05	0.90
MAA (g)	0.30	0.45	0.60
KPS (g)	0.015	0.015	0.015
Water (g)	60	60	60

^aCore/shell ratio: 1/0.5. ^b70 °C, 12 h, N₂, 100 rpm. ^cSolid content: 106.02 gL⁻¹.

spheres was determined by conductometric titration technique. 0.1 g of each composite microsphere was diluted with distilled deionized water and titrated against 0.01 M KOH aqueous solution using a conductivity meter (model HI 8819N, Hanna Instrument, Portugal).

Preparation of Lysine Methyl Ester Hydrochloride (LME)

LME was prepared by following the conventional method of ester preparation as reported elsewhere.¹⁷ Product obtained was dried in a vacuum desiccator over anhydrous calcium chloride. LME was finally confirmed from its sharp melting point (196.5 °C), thin layer chromatography (TLC) measurement and ¹H NMR.

Adsorption of Biomolecules on PS/P(MMA-EGDM) Composite Seed and PS/P(MMA-EGDM)/P(MMA-MAA) Composite Polymer Microspheres

A mixture was prepared from each purified polymer emulsion (polymer solid 0.1 g), biomolecules (20 mg) and phosphate buffer. The phosphate buffer was used to adjust the pH value of the mixture at the respective isoelectric point (TR, pH 10; AL, pH 6; CS, pH 7.5). The mixture was allowed to stand at 20 °C for 45 min, and then centrifuged at 20,000 rpm for 10 min. In order to remove wafting particles completely, the supernatant was centrifuged once more at 20,000 rpm. The concentration of the biomolecules in the supernatant was determined by UV-visible spectrophotometer at 280 nm. The magnitude of adsorption was calculated by subtracting concentration of biomolecules in the medium from that of the initial concentration. Calibration curves were used for this purpose.

Measurement of Specific Activity of Adsorbed Trypsin (TR) on PS/P(MMA-EGDM) Composite Seed and PS/P(MMA-EGDM)/P(MMA-MAA) Composite Polymer Microspheres

The enzyme activities of adsorbed TR were determined at 20 °C by the pH state method,¹⁸ using LME as a substrate according to the following procedure.

Purified polymer emulsions were mixed with a known amount of TR aqueous solution and pH was immediately adjusted at 10 using dilute KOH aqueous solution. In each case, TR concentration (30 mg g⁻¹ of microsphere) was kept constant and maintained below the minimum amount (33.9 mg g⁻¹) adsorbed by composite polymer microsphere prepared with 40% (w/w) MAA. After maintaining the emulsion-TR mixture at 20 °C for specific time, the emulsion-TR mixture was added to 100 mL of 10⁻³ mole L⁻¹ LME aqueous solution. Then the pH was immediately adjusted at 10

Figure 1. Carboxyl group distribution on/near the surface of washed PS/P(S-MAA), PS/P(S-MAA-MBAAm) and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with various MAA contents.

and maintained using a pH-meter with 0.02N KOH under constant stirring at the 20 °C for 3 min. The activity was calculated from the amount of KOH consumed to neutralize the acid liberated from the hydrolysis of LME. The unit of specific activity was expressed as $\mu\text{mole min}^{-1} \text{mg}^{-1}$ of TR adsorbed.

The specific activity of free TR was also measured by following the above procedure.

RESULTS AND DISCUSSION

First different types of composite polymer microspheres were prepared using various synthetic routes. In order to control the carboxyl group density on/near the surface, MAA content was varied in each type of composite microsphere. Figure 1 shows the carboxyl group density on/near the surface of washed PS/P(S-MAA), PS/P(S-MAA-MBAAm) and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with varying amounts of MAA. In PS/P(S-MAA) and PS/P(S-MAA-MBAAm) composite microspheres carboxyl group density decreased with the increase of MAA content in the recipe after reaching a peak value. However in PS/P(MMA-EGDM)/P(MMA-MAA) composite microsphere this carboxyl density increased continuously with the increase of MAA. From this behavior it can be predicted that in the cases of PS/P(S-MAA) and PS/P(S-MAA-MBAAm) composite microspheres seeded copolymerizations are accompanied by secondary nucleation and hence copolymers/homopolymers are by-produced. TEM photographs not shown here, also confirmed the byproduction of many small copolymer particles. Whereas an increase in carboxyl value with

Figure 2. TEM photographs of unwashed (a) PS seed, (b) PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with different MAA contents (w/w) (c) 20% (d) 30% and (e) 40%.

the increase of MAA content in the recipe for PS/P(MMA-EGDM)/P(MMA-MAA) composite microspheres suggests that seeded copolymerization of MMA and MAA proceeded mostly within PS/P(MMA-EGDM) composite seeds with little or no secondary nucleation. Since this measurement is generally carried out in the dispersion state so it is expected that obtained carboxyl value always include certain fraction of the carboxyl groups buried inside the particle due to swelling effect. However in this case the introduction of crosslinked P(MMA-EGDM) network around PS particles reduced the swelling during such measurement and hence the measured value is predominantly related to the carboxyl group on the particles surface. Though not measured separately, the presence of crosslinked network is also expected to reduce the penetration of carboxyl groups inside the particles during seeded copolymerization.

Figure 2 shows TEM photographs of unwashed PS seed, PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with different amounts of

MAA. The average diameters and coefficients of variations are $1.77\text{ }\mu\text{m}$ and 1.75% for PS seed, $2.98\text{ }\mu\text{m}$ and 1.87% for PS/P(MMA-EGDM) composite seed, $3.09\text{ }\mu\text{m}$ and 1.54% for composite microsphere prepared with MAA content of 20% (w/w), $3.12\text{ }\mu\text{m}$ and 1.29% for composite microsphere prepared with MAA content of 30% (w/w) and $3.0\text{ }\mu\text{m}$ and 2.69% for composite microsphere prepared with MAA content of 40% (w/w) respectively. The composite polymer microspheres are spherical but with dented surface. By-produced particles are visible but in fewer number. It is generally believed that during seeded polymerization the seed polymer is swelled with monomer and polymerization starts with the diffusion of radical from the dispersion media. It may also be possible that during the process some soluble oligomeric radical is formed and later captured by the growing polymer particle. The partitioning of monomers between polymer seed and dispersion media depends on their solubility. The dented surface in composite seed and composite microspheres is possibly attributed to non-homogeneous swelling of PS seed

Figure 3. SEM photographs of washed (a) PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with different MAA contents (w/w) (b) 20% (c) 30% and (d) 40%.

particles during second step seeded copolymerization, which is effected by the presence of cross linking agent in the composite seed. This behavior is common in crosslinked microspheres.¹⁹ Figure 3 shows the scanning electron micrographs of composite seed and composite microspheres to show the surface morphology. It is clearly evident that the surface of PS/P(MMA-EGDM) composite seed is not smooth whereas in various PS/P (MMA-EGDM)/P(MMA-MAA) composite microsphere the surface became relatively smooth.

Figure 4 shows the physical adsorption behaviors of TR, CS and AL on PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite microspheres prepared with different MAA contents. The adsorption of the biomolecule was carried out at the isoelectric point of the respective biomolecule to eliminate ionic interaction between the particle surface and the biomolecule. The magnitude of adsorption decreased with the increase of carboxyl density on composite microsphere. It is expected that the increase in polar functional group on/near the surface increased the surface hydrophilicity and thereby decreased the magnitude of adsorption through hydrophobic attraction. The adsorption magnitude of TR and CS almost overlapped with each other whereas AL exhibited a relatively less adsorption magnitude. This adsorption behavior can be explained by considering the difference in molecular structures among the biomolecules.²⁰

Figure 5 shows the variation of specific activities of

Figure 4. Magnitude of trypsin (TR), casein (CS) and albumin (AL) adsorbed on PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with various MAA contents, measured under the constant concentration against the total solid content at 20 °C. Biomolecules immobilized: 200 mg g⁻¹ of microsphere; polymer solid: 0.1 g; immobilization time: 45 min; pH: TR, pH 10; CS, pH 7.5; AL, pH 6.

adsorbed TR on PS/P(MMA-EGDM) composite seed and PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres prepared with different MAA content. In each experiment the concentration of TR was kept below the minimum amount (33.9 mg g⁻¹ of microsphere) adsorbed by the PS/P(MMA-

Figure 5. Specific activities of adsorbed trypsin (TR) on PS/P(MMA-EGDM) composite seed and different PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres measured under the constant concentration against the total solid content at 20 °C. TR immobilized: 30 mg g⁻¹ of microsphere; polymer solid: 0.1 g; immobilization time: 45 min.; pH 10.

EGDM)/P(MMA-MAA) composite polymer microsphere prepared with 40% (w/w) MAA contents. This was done to ensure almost complete adsorption of TR on each type of composite microsphere. Specific activities of adsorbed TR on composite polymer microspheres improved appreciably as compared to PS/P(MMA-EGDM) composite seed. Composite polymer microsphere having MAA content of 40% has the maximum specific activity for adsorbed TR. This result indicates that the localization of carboxyl group on/near the surface of composite microsphere derived from MAA comonomer makes the surface relatively hydrophilic and thereby decreases the hydrophobic interaction of adsorbed TR with the microspheres surface. Hence the native conformation of adsorbed TR retained more and more with the increase of carboxyl value.

Figure 6 shows the specific activities of free TR (aqueous solution of TR) and adsorbed TR on PS/P(MMA-EGDM) composite seed and different PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres as a function of immobilization time. This has been measured to observe the stability of the adsorbed TR with immobilization time. The specific activities of adsorbed TR on various composite polymer microspheres decreased slowly with time as compared to free TR. This is because, in the case of free TR self digestion occurred rapidly due to the frequent contact among themselves whereas in the case of the adsorbed TR such frequent contact among the active sites of TR was inhibited by the shielding effect of the polymer microspheres.

Figure 6. Specific activities of free trypsin (TR) and adsorbed TR on PS/P(MMA-EGDM) composite seed and different PS/P(MMA-EGDM)/P(MMA-MAA) composite polymer microspheres as a function of immobilization time measured under the constant concentration against the total solid content at 20 °C. TR immobilized: 30 mg g⁻¹ of microsphere; polymer solid: 0.1 g; pH 10.

CONCLUSIONS

A variety of monodisperse micron-sized PS/P(MMA-EGDM)/P(MMA-MAA) composite microspheres were produced *via* multi steps seeded copolymerization. The number of carboxyl group on/near the surface of composite microspheres could be controlled by incorporating various amounts of MAA comonomer in the recipe during seeded copolymerization. Measurement of adsorption behavior of different biomolecules and specific activity of adsorbed TR suggest that surface of composite microsphere became relatively hydrophilic due to the introduction of polar functional group.

Acknowledgment. We would like to express our thanks to Professor M. Okubo of Kobe University, Japan for providing with electron microscope facility. Thanks are also due to MOSICT, Dhaka for financial support.

REFERENCES

1. M. S. Rosa and F. Jacqueline, *J. Polym. Sci., Part A: Polym. Chem.*, **35**, 1605 (1997).
2. K. Kang, C. Y. Kan, Y. Du, and D. S. Liu, *J. Appl. Polym. Sci.*, **92**, 433 (2004).
3. A. Tuncel and E. Serpen, *Colloid Polym. Sci.*, **279**, 240 (2001).
4. J. Ugelstad, A. Berge, T. Ellingsen, R. Schmid, T. N. Nilsen, P. C. Mørk, P. Stenstad, E. Hornes, and Ø. Osvik, *Prog.*

- Polym. Sci.*, **17**, 87 (1992).
5. M. Okubo, E. Ese, and T. Yamashita, *J. Polym. Sci., Part A: Polym. Chem.*, **36**, 2513 (1998).
6. S. Omi, K. I. Katami, T. Taguchi, K. Kaneko, and M. Iso, *J. Appl. Polym. Sci.*, **57**, 1013 (1995).
7. S. Gu, S. Inukai, and M. Konno, *J. Chem. Eng.*, **36**, 1231 (2003).
8. Y. Almog, S. Reich, and P. Levy, *Br. Polym. J.*, **14**, 131 (1982).
9. K. E. J. Barette, "Dispersion polymerization in Organic Media," Wiley, London, 1975.
10. H. Ahmad, M. A. J. Miah, S. Pervin, and M. M. Rahman, *Colloid Polym. Sci.*, **281**, 897 (2003).
11. J. S. Song, F. Tronc, and M. A. Winnik, *J. Am. Chem. Soc.*, **126**, 6562 (2004).
12. M. Yasuda, H. Seki, H. Yokoyama, H. Ogino, K. Ishimi, and H. Ishikawa, *Macromolecules*, **34**, 3261 (2001).
13. D. Y. Lee and J. H. Kim, *J. Appl. Polym. Sci.*, **69**, 543 (1998).
14. K. Kang, C. Kan, Y. Du, and D. Liu, *Eur. Polym. J.*, **41**, 439 (2005).
15. W. J. Liu, W. D. He, Y. M. Wang, D. Wang, and Z. C. Zhang, *Polym. Int.*, **55**, 520 (2006).
16. H. T. Zhang, H. Huang, R. Sun, and J. X. Huang, *J. Appl. Polym. Sci.*, **99**, 3586 (2006).
17. H. Ahmad, A. M. I. Ali, and M. A. J. Miah, *J. Polym. Mater.*, **18**, 287 (2001).
18. M. Laskowski, *Methods Enzymol.*, **2**, 26 (1955).
19. J. S. Song and M. A. Winnik, *Macromolecules*, **38**, 8300 (2005).
20. M. Okubo and H. Ahmad, *Colloid Polym. Sci.*, **274**, 112 (1996).