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Activity of Trypsin Adsorbed on Temperature and pH-Responsive Micron-Sized PS/P(NIPAM-MAA-MBAAm) Composite Polymer Particles

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Abstract: Micron-sized polystyrene (PS) seed particles were prepared by dispersion polymerization of styrene in ethanol-water media. Seeded copolymerization of N-isopropyl acrylamide (NIPAM), methacrylic acid (MAA) and N,N'-methylene-bis-acrylamide (MBAAm) was then carried out in presence of PS seed particles. The produced PS/P(NIPAM-MAA-MBAAm) composite polymer particles exhibited volume phase transition to small changes in temperature and pH, respectively. Specific activities of trypsin aqueous solution (free trypsin) and trypsin in presence of PS seed and composite polymer particles were measured under identical conditions. Relative study suggested that trypsin adsorbed on PS/P(NIPAM-MAA-MBAAm) composite polymer particles retained their native conformation.

Key words: Temperature and pH-responsive, composite, trypsin, specific activity

INTRODUCTION

Poly(N-isopropyl acrylamide) (PNIPAM) is a typical temperature-sensitive polymer gel which exhibits volume phase transition at approximately 34°C, which is frequently known as lower critical solution temperature (Pichot, 1995). Below this temperature, the polymer gel is swollen and it shrinks as the temperature is raised. These materials are useful for drug delivery systems, separation operations in biotechnology, processing of agricultural products, sensors and diagnostic reagent (Ugelstad *et al*., 1992; Snowden, 1992; Okubo and Ahmad, 1996; Elaissari *et al*., 1999; Pelton, 2000; Kawaguchi, 2000; Lee *et al*., 2004). Meanwhile, according to Brondsted and Kopecek (1992a) conventional pH-sensitive polymers are mostly based on those containing ionizable groups of a weak acid (carboxylic acid) or a weak base (amino group). Following the carboxylic acid based pH-sensitive hydrogels in mid 1950s (Kachalsky an Michaeli, 1955) numerous pH-sensitive polymeric systems, including linear polymers (Branham *et al*., 1996), grafted polymers (Konturi *et al*., 1996), hydrogels (Jabbari and Nozari, 2000) and interpenetrating networks (Xiao *et al*., 2006) have been investigated for applications in pharmaceutical areas, such as enteric coating materials (Schacht *et al*., 1996), site-specific targeting (Brondsted and Kopecek, 1992b), tumor-specific delivery (Kitano *et al*., 1986), self-regulating insulin delivery systems (Horbett *et al*., 1984) and sensors (Liebert and Walt, 1995).

Recently, many researchers are showing interest on the preparation of hydrogel microspheres having both temperature- and pH-responsive volume phase transition (Yoo *et al*., 1997; Jones and Lyon, 2000; Bokias *et al*., 2000; Yin *et al*., 2006). However, these gel microspheres have certain limitations particularly in industrial field due to poor mechanical strength, low dimensional stability and poor response to temperature and pH variations (Zhang and Wu, 2004). Polymer particles with comparatively rigid chain network may be useful to overcome those limitations. In the last few years we reported the preparation of a series of composite polymer particles having both temperature and pH-responsive swelling/deswelling characteristics (Alam *et al*., 2003; Alam *et al*., 2005; Alam *et al*., 2007a, b).

One of the important requirements for any composite polymer particles to be used as biological carrier is that biomolecules adsorbed or released should retain most of their native conformation. In this research specific activity of trypsin (TR) enzyme in presence of one of such temperature and pH-responsive composite polymer particles is taken into consideration assuming that specific activity has a direct relationship with its conformation. Most of the added TR to the polymer particles has been considered as adsorbed on the particles surface under the measured conditions. This study was done to evaluate the performance of the prepared composite polymer particles as a carrier for biomolecules in the biomedical field.

MATERIALS AND METHODS

Styrene and MAA of monomer grade, purchased from Fluka Chemika, Switzerland, were distilled under reduced pressure and preserved in the refrigerator until

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use. NIPAM of monomer grade purchased from Acros Organics of USA. was recrystallized from a mixture of 90% hexane and 10% acetone, dried at a low temperature under vacuum and was preserved in the refrigerator. MBAAm from Sigma Chemical Co. of USA was used without any purification. 2,2'Azoisobutyro nitrile (AIBN) was recrystallized from methanol and preserved in the refrigerator until use. Potassium persulfate (KPS) of reagent grade was recrystallized from water before use. Polyvinyl pyrrolidone (PVP) from LOBA Chem., India, tricaprylylmethyl ammonium chloride (aliquate^{336}), Sodium dodecyl sulfate (SDS) and hexadecyl trimethyl ammonium bromide (HTABr) all from Fluka, Chemika, Switzerland, were used as received. Enzyme, TR from E Merck Germany was used without any purification. 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. Distilled deionized water of conductivity less than 5 μs cm^{-1} was used for all the measurements.

Hitachi H-7100 transmission electron microscope (TEM); Helios gamma single-beam UV-visible spectrometer from Unicam, UK; HI 9321; Bruker proton NMR 250 MHZ spectrometer; Microprocessor pH and conductivity meters from HANNA Instruments, Portugal were used in this study. Hydrodynamic diameters of composite polymer particles were measured by NICOMP 380 Particle Sizer.

The experiment was carried out in the Organic Chemistry Research Laboratory of the Department of Chemistry in Rajshahi University, Bangladesh, during the period of May to September, 2006.

Preparation of PS seed particles: Styrene (40 g) was taken in a mixture of ethanol-water media to form a homogeneous mixture and transferred to a three-necked round flask. 1.6 g of PVP and 0.46 g of aliquate^{336} were used as stabilizer and co-stabilizer, respectively. The flask was dipped in a thermostat water bath and temperature was raised to 70°C. Then 0.6 g of AIBN dissolving in 10 mL of ethanol was added and polymerization was continued for 12 h under a nitrogen atmosphere. The conversion was nearly 100%, measured gravimetrically. PS seed particles were washed repeatedly with distilled deionized water by centrifugation.

Preparation of PS/P (NIPAM-MAA-MBAAm) composite particles: Seeded copolymerization of NIPAM, MAA and MBAAm was carried out in presence of micron-sized PS seed particles using KPS initiator in water media under a nitrogen atmosphere. Reaction conditions are shown in Table 1.

Table 1: Preparation of PS/P(NIPAM-MAA-MBAAm) composite polymer particles [a]

Ingredients	Weight (g)
PS emulsion[b]	23.28
NIPAM	0.77
MAA	0.20
MBAAm	0.03
KPS	0.0192
Water	75.00

[a]: 70°C, 100 rpm, N_2, 12 h, [b]: Solid content, 85.9 g L^{-1}

Preparation of lysine methyl ester hydrochloride (LME): LME was prepared from lysine monohydrochloride by following the conventional method of ester preparation as reported elsewhere (Alam *et al*., 2007b). The ester obtained was recrystallized from ethyl acetate solution by adding methanol and finally dried in a vacuum desiccator over anhydrous calcium chloride. LME was confirmed from its sharp melting point (196.5°C), Thin Layer Chromatography (TLC) measurement and ^{1}HNMR (chemical shift due to methyl protons, -$COOCH_3$, appeared at 3.9 ppm while no chemical shift is observed for the carboxylic proton). TLC measurement of LME and lysine was carried out in ethanol solvent and the calculated value R_f was much higher for LME (0.57) than lysine (<0.1).

Measurement of specific activity of trypsin (TR): Specific activities of free TR and TR in presence of seed and composite particles were measured at different temperatures at pH 7 by a pH stat method using LME as a substrate. The procedure is outlined below:

Seed or composite emulsion was mixed with a known amount of TR aqueous solution and pH was immediately adjusted at pH 7 using 0.02N KOH aqueous solution. In each measurement, TR concentration (70 mg g^{-1} of particles) was kept constant. After maintaining the emulsion-TR mixture at certain temperature (variable 20 to 45°C) for 45 min, a known amount of the seed or composite emulsion with free or adsorbed TR was added to 100 mL of 10^{-3} mol L^{-1} LME aqueous solution. Then the pH of the mixture was adjusted at pH 7 and maintained with 0.02N KOH under magnetic stirring at the respective temperatures for 3 min. The activity was calculated from the amount of KOH consumed to neutralize the acid liberated from the hydrolysis of LME.

RESULTS AND DISCUSSION

Figure 1a-b shows the Transmission Electron Micrographs (TEM) of polystyrene (PS) seed and PS/poly(N-isopropyl acrylamide-methacrylic acid-N,N'-methylene-bis-acrylamide) [P(NIPAM-MAA-MBAAm)] composite polymer particles. Both seed and composite polymer particles are spherical and

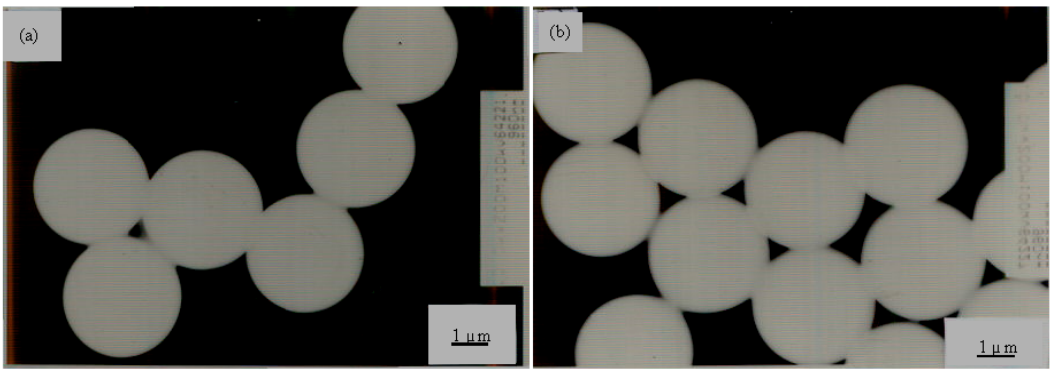

Fig. 1: TEM photographs of unwashed PS seed (a) and PS/P(NIPAM-MAA-MBAAm) composite polymer (b) particles prepared by dispersion polymerization and seeded copolymerization, respectively

monodispersed. The average diameters and coefficients of variations are 1.7 µm and 2.1% for PS seed, 1.8 µm and 0.42% for PS/P(NIPAM-MAA-MBAAm) composite polymer particles, respectively. The average diameter of composite particles increased slightly from the seed particles and submicron-sized P(NIPAM-MAA-MBAAm) copolymer particle is not visible in the TEM photograph of composite particles. The monodispersity and particle size distribution suggest that seeded copolymerization of NIPAM, DM and MBAAm proceeds mostly in PS seed particles without secondary nucleation.

Figure 2 and 3 show the variations of hydrodynamic diameter of PS/P(NIPAM-MAA-MBAAm) composite polymer particles against pH and temperature. The pH dependent measurement shows that the hydrodynamic diameter increases with the increase of pH value i.e., at higher pH the surface of composite polymer particles becomes hydrophilic. This surface hydrophilicity is resulted from the ionization of carboxyl group derived from PMAA component of the shell layer. Temperature dependent variation measured at pH 7.1 (Fig. 3) indicates that hydrodynamic diameter decreases sharply at temperature around the LCST of PNIPAM with the increase of temperature. This result suggests that above the LCST (about 32°C) the shell layer consisting of P(NIPAM-MAA-MBAAm) copolymer is hydrophobic and shrinks and below the LCST it is hydrophilic and swell. The slight deviation in LCST from that of NIPAM homopolymer (Pichot, 1995) is possibly due to the partial ionization of carboxyl group at pH 7.1 and incorporation of rigid crosslinked network.

In a previous article (Alam *et al*., 2007c) on the basis of these sharp temperature- and pH-responsive volume phase transition of PS/P(NIPAM-MAA-MBAAm) composite polymer particles, a detail study is reported on the adsorption of different macromolecules. However, the development and usefulness of any such biological carrier

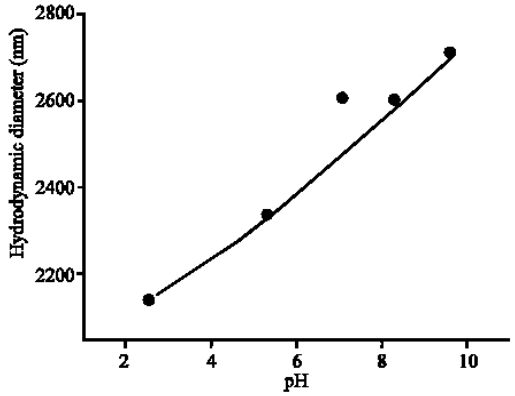

Fig. 2: Variation of hydrodynamic diameter of washed PS/P (NIPAM-MAA-MBAAm) composite polymer particles with pH measured at 20°C

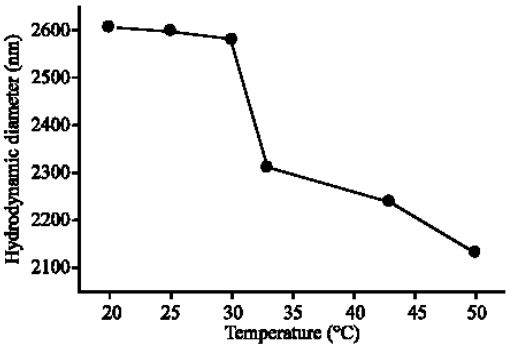

Fig. 3: Variation of hydrodynamic diameter of washed PS/P (NIPAM-MAA-MBAAm) composite polymer particles with temperature measured at pH 7.1

is based on the fact that the biomolecules adsorbed or released from the particles retain their native conformation or activity. The following measurements were therefore carried out with this in mind.

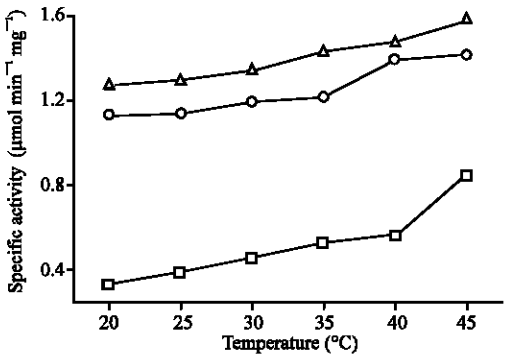

Fig. 4: Variations of specific activities of free trypsin (TR) (□) and TR in presence of PS seed (○) and PS/P(NIPAM-MAA-MBAAm) composite polymer (Δ) with temperature measured under identical conditions. Immobilization time: 45 min; pH: 7; polymer solid: 0.1 g; TR: 70 mg g^{-1} of particles

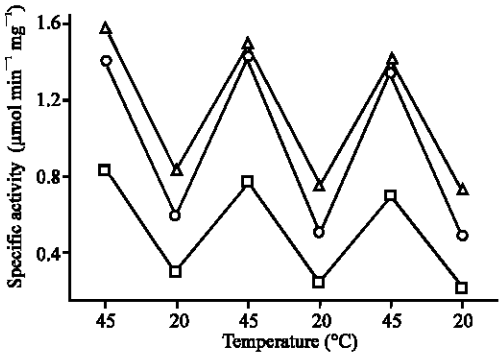

Fig. 5: Specific activities of free trypsin (TR) (□) and TR in presence of PS seed (○) and PS/P(NIPAM-MAA-MBAAm) composite polymer (Δ) particles measured alternatively at 45 and 20°C, respectively under identical conditions. Immobilization time: 45 min; pH: 7; polymer solid: 0.1 g; TR: 70 mg g^{-1} of particles

Figure 4 shows the effects of temperature on specific activities of free trypsin (TR) i.e., TR aqueous solution and TR in presence of PS seed and PS/P(NIPAM-MAA-MBAAm) composite polymer particles measured under identical conditions. These measurements were carried out at pH 7, lower than the isoelectric pH 10 as sharp phase transition was not observed for the composite particles at higher pH due to the ionization of carboxyl group of PMAA unit. Specific activity of free TR is increased with the increase of temperature. This is attributed to the increased hydrolysis rate of LME with increasing temperature and this is the general phenomenon of all enzymes. Specific activities of TR in presence of PS seed and PS/P(NIPAM-MAA-MBAAm) composite polymer particles also show similar trend with increasing temperature. However the interesting point is that specific activities of TR in presence of seed and composite particles are much higher than those of free TR. It is expected that most of the added TR in presence of seed and composite polymer particles are adsorbed due to hydrophobic interaction and thereby self-digestion is reduced as occurred in the case of free TR. Here the polymer matrix prevented the active part of adsorbed TR to come into contact with each other. In a previous article we observed that specific activity of TR in presence of PS seed particles at the isoelectric point (pH 10) remained almost identical with increasing immobilization temperature. However in this case since the immobilization is carried out at relatively lower pH than the isoelectric pH value of TR the ionization of TR and consequently the lower hydrophobic interaction with the particle surface is expected to reduce the conformational change of adsorbed TR. The specific activity of TR in presence of composite polymer particles is enhanced further indicating that native conformation and activity remained sufficient for application as a biological carrier.

Figure 5 shows the specific activities of free TR and TR in presence of PS seed and PS/P(NIPAM-MAA-MBAAm) composite polymer particles measured alternatively at 45 and 20°C. It is believed, as the temperature-responsive shell is hydrophilic at 20°C most of the added TR remained free in solution whereas as the temperature is increased to 45°C the temperature-responsive shell turned hydrophobic and most of the added TR is adsorbed. So this measurement would give idea about the activity and conformation of TR molecules during repeated adsorption-desorption process. Specific activities of free TR and TR in presence of seed and composite polymer particles followed the same profile i.e., at 45°C specific activities are always higher than those at 20°C. In all cases specific activities decreased slightly from the starting value indicating limited conformational change either due to self-digestion of free TR or due to strong hydrophobic interaction with PS particles surface or due to denaturation during alternative adsorption and release of biomolecules from composite particles surface. Despite this limitation, the improved specific activity of TR in presence of composite particles suggests that TR is relatively stable and retain its native conformation during repeated alternative measurement.

Figure 6 shows the variations of specific activities of free TR and TR in presence of PS seed and PS/P(NIPAM-MAA-MBAAm) composite polymer particles against time measured at 45°C. In all three cases specific activities decreased with time. However for free TR specific activity is much lower and reduced to almost zero after 6 h and in presence of polymer particles specific activities of TR are

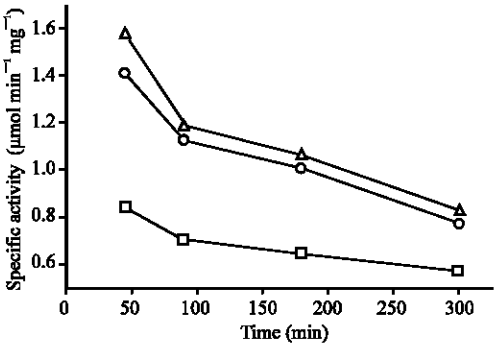

Fig. 6: Variations of specific activities of free trypsin (TR) (□) and TR in presence of PS seed (○) and PS/P(NIPAM-MAA-MBAAm) composite polymer (Δ) particles with time measured at 45°C under identical conditions. pH: 7; polymer solid: 0.1 g; TR: 70 mg g^{-1} of particles

much higher, retained at least 50% of the initial activity. This indicates that in presence of polymer particles self-digestion is reduced as polymer matrix prevented the active part from being coming into frequent contact. Relatively higher specific activity of TR in presence of PS/P(NIPAM-MAA-MBAAm) composite particles suggests that conformational change effected by the hydrophobic interaction between adsorbed TR and composite particles surface is lower than that in presence of PS seed particles. The decreasing tendency of specific activity with prolonged immobilization time can be minimized by carefully controlling the time factor.

These results suggest that monodispersed micron-sized PS/P(NIPAM-MAA-MBAAm) composite polymer particles having stimuli-responsive surface properties are hydrophilic enough at pH 7 and at temperature as high as 45°C, to prevent conformational change of biomolecules. Hence the prepared stimuli-responsive composite polymer particles may find application as biological carrier for biomolecules.

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