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SHORT COMMUNICATION

Composite polymer particles with stimuli-responsive surface properties and specific activity of adsorbed/released trypsin

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Abstract Micron-sized monodispersed stimuli-responsive polys' (PS)/poly(2-dimethylaminoethyl methacrylate-*N*-isopropyl acrylamide-ethylene glycol dimethacrylate) [P(DM-NIPAM-EGDM)] composite polymer particles were prepared by seeded copolymerisation of DM, NIPAM and EGDM with PS seed particles. Adsorption behaviour of trypsin suggested that composite particles surface has both temperature- and pH-responsive swelling–deswelling characteristics. The performance of composite polymer particles as a carrier for biomolecules in adsorption/release experiments was evaluated by measuring the specific activities of adsorbed trypsin as a function of temperature and pH.

Keywords Stimuli-responsive · Composite polymer · Trypsin · Specific activity

Introduction

Composite polymer particles exhibiting reversible phase transition due to small variation in physical or chemical stimuli such as temperature, pH, electric current, ions or chemical species are most commonly known as stimuli-responsive composite microspheres. These composite microspheres would find application in drug delivery systems, separation operations in biotechnology, sensors and diagnostic reagent. Recently, many researchers are concentrating their effort on the preparation of multi-responsive hydrogel microspheres, that is they exhibit reversible volume phase transition due to variation of more than one stimuli [1–3]. However the application of these hydrogel microspheres is mostly limited particularly in industrial field because of their low mechanical strength, poor dimensional stability and slow response to stimuli [4]. To overcome these disadvantages relatively rigid composite polymer particles with narrow size distribution are taken into choice.

Both poly(*N*-isopropyl acrylamide) [PNIPAM] and poly (2-dimethylaminoethyl methacrylate) [DM] have been extensively studied regarding temperature-responsive phase transition, defined by the phenomenon of lower critical solution temperature (LCST) in aqueous solution [5–10]. In a recent work, we reported the preparation of composite polymer particles consisting of polystyrene as inert core and poly(2-dimethylaminoethyl methacrylate-*N*-isopropyl acrylamide-ethylene glycol dimethacrylate) [P(DM-NIPAM-EGDM)] as temperature- and pH-responsive shell [11]. The ionization of tertiary amine group in PDM at pH below 7 produced pH-responsive phase transition. EGDM was used as a cross-linking agent to prevent the dissolution of soluble PDM and PNIPAM from composite particles surface.

One of the important criteria for all composite particles to be used as carrier for biomolecules is that the biomolecule adsorbed or released from the particles surface should retain their activity during adsorption/desorption experiments. When a biomolecule adsorbs from an aqueous solution onto a solid surface, it affects the three-dimensional structure, structural stability and activity [12]. This behaviour is associated with conformational change of adsorbed biomolecule and is affected by the properties of the solid surface, nature of protein molecule, temperature and pH of the environment [13–16].

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In this paper, usefulness of our previously reported stimuli-responsive PS/P(NIPAM-DM-EGDM) composite polymer particles [11] is evaluated by measuring specific activity of adsorbed/released trypsin (TR).

Experimental

Materials

Styrene of monomer grade was distilled under reduced pressure and preserved in the refrigerator until use. DM and EGDM of monomer grade, purchased from Fluka Chemika, Switzerland, were preserved in the refrigerator and used without any purification. NIPAM of monomer grade purchased from Tokyo Kasei kogyo was recrystallised from a mixture of 90% hexane and 10% acetone, dried at a low temperature under vacuum and preserved in the refrigerator. 2,2′Azoisobutyro nitrile (AIBN) was recrystallised from ethanol and preserved in the refrigerator until use. 2,2′-Azobis(2-amidinopropane)hydrochloride (V-50) of reagent grade was recrystallised from water before use. Polyvinyl pyrrolidone (PVP) from LOBA Chem., India and tricaprylylmethyl ammonium chloride (aliquate^{336}) 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 and used without any purification. 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; 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.

Preparation of PS seed particles

PS seed particles were prepared by dispersion polymerisation of 20 g of styrene dissolving in a mixture of 80 ml of ethanol and 5 ml of water in presence of AIBN as oil soluble initiator. PVP and aliquate^{336} were used as stabiliser and co-stabiliser, respectively. Polymerisation was carried out in a three-necked round flask under a nitrogen atmosphere at 70 °C for 12 h. The conversion was almost 100%, measured gravimetrically. PS particles were washed repeatedly by replacing the continuous phase with distilled deionized water.

Preparation of PS/P(DM-NIPAM-EGDM) composite polymer particles

PS/P(DM-NIPAM-EGDM) composite polymer particles were prepared by seeded copolymerisation of DM, NIPAM and EGDM with micron-sized PS seed particles using V-50 as initiator under a nitrogen atmosphere in a three-necked round flask. The preparation conditions are detailed in Table 1.

Adsorption of TR on PS/P(DM-NIPAM-EGDM) composite polymer particles

For each measurement, a mixture of 20 ml was prepared from purified emulsion (polymer solid, 0.1 g) and biomolecule (500 mg g^{-1} of particles) aqueous solution. For temperature-dependent adsorption studies, the pH value of the mixture was immediately adjusted at the isoelectric point of TR (pH 10) using a phosphate buffer solution. The mixture was allowed to stand at the respective temperature for 45 min. Whereas, for pH-dependent adsorption measurements, the mixture was adjusted at different pH values using KOH/HCl aqueous solution and was allowed to stand at 20 °C for 45 min. Polymer particles were then separated by centrifugation at 7,000 rpm. To remove dust particles completely, the supernatant was centrifuged once more at 8,000 rpm. The concentration of the biomolecule in the supernatant was determined by a UV-Vis spectrophotometer at the wavelength of 280 nm. The magnitude of biomolecule adsorbed was calculated by subtracting the biomolecule concentration in the medium from that of the initial concentration. A calibration curve was used for this purpose.

Preparation of lysine methyl ester hydrochloride

LME was prepared from lysine monohydrochloride by following the conventional method of ester preparation as reported elsewhere [17]. The ester obtained was recrystallised from ethyl acetate solution by adding methanol and finally dried in a vacuum desiccator over anhydrous calcium chloride. LME was confirmed from its sharp

Table 1 Preparation PS/P(DM-NIPAM-EGDM) composite polymer particles by seeded copolymerisation (60 °C, 100 rpm, N_2, 8 h)

Ingredients	Grams
PS emulsion[a]	29.1010
DM	0.987
NIPAM	1.05
EGDM	0.063
V-50	0.21
Water	119

[a] Solid content; 103.39 gl^{-1}

melting point (196.5 °C), thin layer chromatography measurement and ^{1}HNMR (chemical shift appeared at 3.9 ppm indicated the methyl protons, -COOCH$_3$, also no chemical shift is observed for the proton of -COOH).

Specific activity of TR

Specific activities of free TR (control experiment) and adsorbed/released TR were measured at different temperatures of above and below the LCST (32 °C) at pH 10 by a pH stat method using LME as a substrate. The same procedure was applied to measure specific activity at different pH values. 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 the specific pH value using 0.02N KOH/0.02N HCl aqueous solution. In each measurement, TR concentration (250 mg g^{-1} of particles) was kept constant. After maintaining the emulsion–TR mixture at certain temperature (variable 20 to 40 °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 LME aqueous solution. Then the pH of the mixture was adjusted at the pH value of specific measurement 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 neutralise the acid liberated from the hydrolysis of LME.

Results and discussion

Figure 1a,b shows the transmission electron micrographs (TEM) of PS seed and PS/P(DM-NIPAM-EGDM) composite polymer particles. Both seed and composite polymer particles are monodispersed and spherical. P(DM-NIPAM-EGDM) copolymer particle was not by-produced during seeded copolymerisation as submicron-sized copolymer particle is not visible in the TEM photograph of composite polymer particles. The diameters and coefficients of variation are, respectively, 2.025 μm and 2.50% for PS seed and 2.1 μm and 2.38% for PS/P(DM-NIPAM-EGDM) composite polymer particles. These results indicate that seeded copolymerisation of DM, NIPAM and EGDM proceeds mainly within PS seed particles without any secondary nucleation.

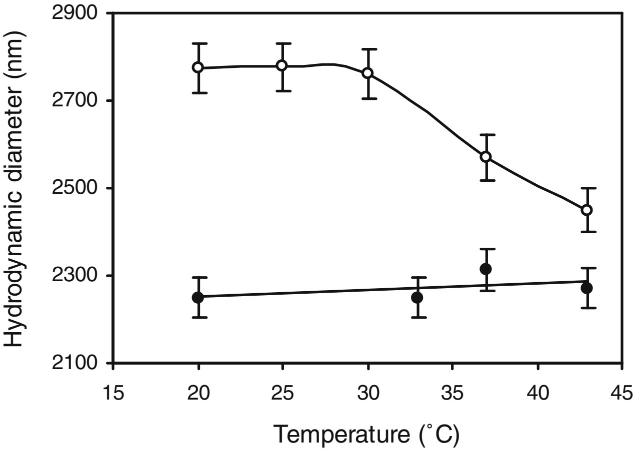

Fig. 2 Variation of hydrodynamic diameter of PS seed (*filled circle*) and PS/P(DM-NIPAM-EGDM) composite polymer (*open circle*) particles with temperature at pH 7

Figure 2 shows the variation of hydrodynamic diameter of PS seed and PS/P(DM-NIPAM-EGDM) composite polymer particles. The hydrodynamic diameter of composite polymer particles remained almost identical until 30 °C but then decreased sharply with increasing temperature. This result indicates that the copolymer shell layer on composite polymer particles deswells and becomes hydrophobic at temperature above LCST. On the other hand, the average size of PS core particles remained almost same with temperature.

Figure 3 shows the magnitude of adsorption of TR on PS/P(DM-NIPAM-EGDM) composite polymer particles at temperature above and below the LCST, which is close to 32 °C. TR is a kind of globular proteins in aqueous solution and hence folded in such a way that the hydrophobic parts (alkyl and aromatic parts) of different amino acids are turned inward and away from the water, whereas the hydrophilic parts (amine and carboxyl groups) lie on the surface of globules. During this self-orientation in aqueous

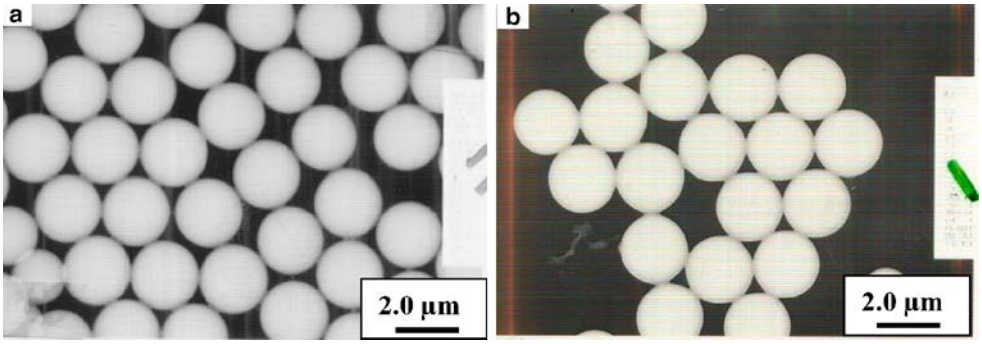

Fig. 1 Transmission electron microscopic photographs of unwashed PS seed (**a**) and PS/P(DM-NIPAM-EGDM) (**b**) composite polymer particles

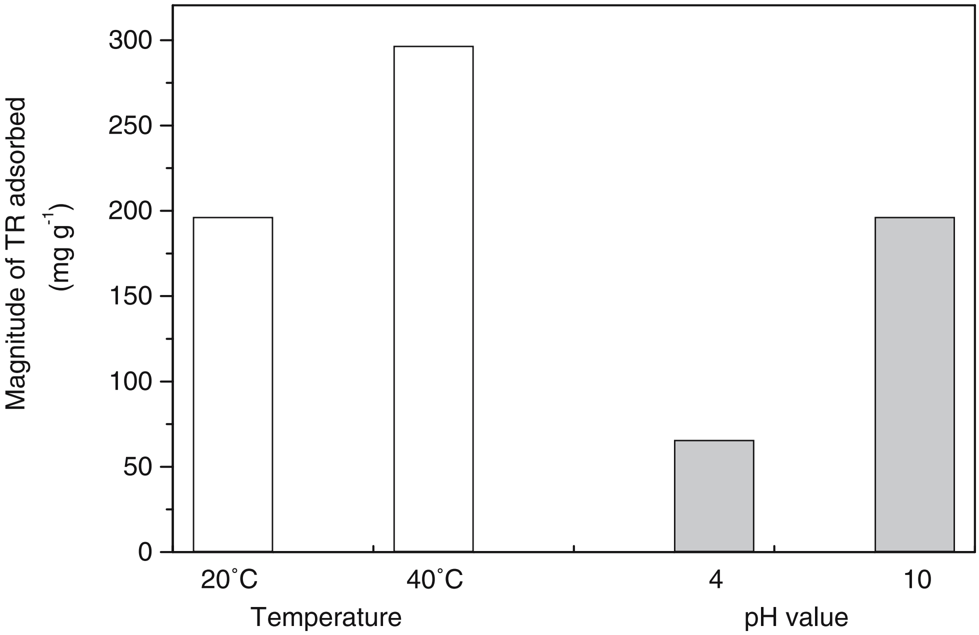

Fig. 3 Magnitude of TR adsorbed on PS/P(DM-NIPAM-EGDM) composite polymer particles at temperatures 40 and 20 °C and at pH values 4 and 10, respectively. Temperature-dependent measurement is carried out at pH 10 and pH-dependent measurement is carried out at 20 °C. TR, 500 mg g^{-1} of particle; polymer solid, 0.1 g; immobilization time, 45 min

solution, a few of the hydrophobic parts generally remain as pockets on the surface of mostly hydrophilic TR globules, and as these hydrophobic pockets will always have the tendency to move away from water, they are likely to be attracted towards the hydrophobic substrate due to hydrophobic interaction. Therefore, the lower magnitude of adsorption at 20 °C, which is below the LCST, implies that the composite particles surface is comparatively hydrophilic due to the formation of hydrogen bond with surrounding water molecules. On the other hand, the higher magnitude of adsorption at temperature above the LCST suggests that the composite particles surface becomes hydrophobic accompanied by the rupture of hydrogen bonds with surrounding water molecules. In comparison with composite polymer particles, the surface of PS particles is hydrophobic as observed by the higher magnitude of adsorption (data not shown) measured under similar conditions. The magnitudes of adsorption at 20 and 40 °C on PS particles were, respectively, 338.44 and 342.45 mg g^{-1} of particles. The pH-dependent adsorption measurement shown in Fig. 3 indicates that the magnitude of adsorption of TR at pH 4 is much lower than that at pH 10. This adsorption behaviour is attributed to two possible reasons. One is that the surfaces of PS/P(DM-NIPAM-EGDM) composite polymer particles are positively charged at pH below 7 due to the protonation of tertiary amine group in PDM, and the other is that TR is positively charged at below the isoelectric point (pH 10). Therefore, repulsion among the like charges on the particles surface and biomolecules reduced the magnitude of adsorption to relatively lower value at pH 4 than that at pH 10. The adsorption maximum at the isoelectric point is related to the absence of any electro-repulsive force between neighbouring protein molecules [18]. Previously, it is reported that on further increasing the pH beyond the isoelectric point, the magnitude of adsorption decreased due to the ionization of TR [11].

Figure 4 shows the variation of specific activities of free TR and adsorbed TR on PS seed and PS/P(NIPAM-DM-EGDM) composite polymer particles against temperature measured under the constant concentration of TR (250 mg g^{-1} of particles) at pH 10. Specific activities of free TR increased steadily until 40 °C as the hydrolysis rate of LME as a substrate is accelerated with increasing temperature. This is simply the general characteristic of any enzyme as catalyst. Specific activities of adsorbed TR on PS seed are relatively high as compared to free TR but interestingly remained almost linear with increasing tem-

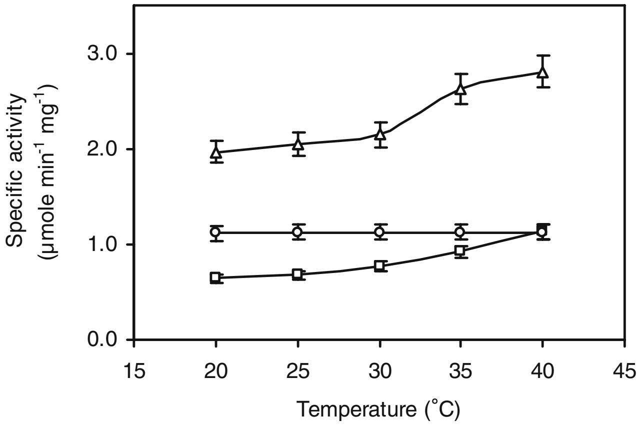

Fig. 4 Variation of specific activities of free TR (*square*), adsorbed TR on PS seed (*circle*) and PS/P(DM-NIPAM-EGDM) composite (*triangle*) polymer particles with temperature at pH 10 measured under a constant concentration of TR. TR, 250 mg g^{-1} of particle; immobilization time, 45 min

perature. Immobilisation of TR definitely reduced the self-digestion as normally happened in case of free TR, but once the TR adsorbed on PS particles surface due to relatively strong hydrophobic interaction, the native conformation of adsorbed TR may have partially been distorted. On the contrary, specific activities of adsorbed TR on composite particles are much higher than those of both free TR and adsorbed TR on seed particles. Moreover, specific activities increased sharply at around 33 °C, which is close to the LCST of composite polymer particles. It is expected that at temperature below the LCST, a part of the added TR remained free in solution as the composite particles surface is relatively hydrophilic, and as the temperature reached the LCST point, the composite particles surface turned hydrophobic and most of the added TR adsorbed on the particle. Therefore, this sharp transition in specific activity around the LCST is attributed to the phase transition of composite particles surface from hydrophilic to hydrophobic. The comparative specific activity profile of adsorbed TR on composite particles and free TR suggests that the adsorbed TR did not suffer major conformational change during immobilisation. The higher specific activity of adsorbed TR on composite polymer particles at temperature above the LCST than that on PS seed particles suggests that the composite particles surface is relatively less hydrophobic even at temperature above the LCST than that of PS seed particles.

Figure 5 shows the variation of specific activities of free TR and adsorbed TR on PS seed and PS/P(DM-NIPAM-EGDM) composite polymer particles as a function of pH value at 40 °C measured under identical conditions. Specific activities of free TR increased steadily with pH and reached the maximum at pH 10, which is close to isoelectric point. It is already known that at the isoelectric point, the biomolecules are uncharged and retain their native conformation although with time self-digestion may reduce the overall specific activity [19, 20]. Specific activities of adsorbed TR on PS seed remained almost same with the increase of pH. In this case, immobilisation of TR reduced the self-digestion, but strong hydrophobic interaction of adsorbed TR with the PS particles surface possibly imparted some conformational change. Importantly, the immobilisation of TR on composite polymer particles increased the specific activity, and the rate of enhancement with pH is higher than that of free TR. At lower pH value, a part of the added TR remained free in solution as both composite particles surface and TR are positively charged, whereas as the pH increased, composite particles surface turned hydrophobic and thereby increasing magnitude of the added TR is adsorbed. In other words, self-digestion is prevented at higher pH value as most of the added TR molecules are adsorbed on the particles surface.

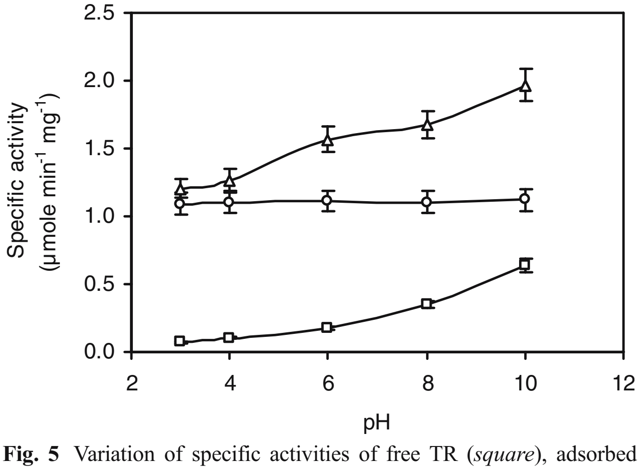

Fig. 5 Variation of specific activities of free TR (*square*), adsorbed TR on PS seed (*circle*) and PS/P(DM-NIPAM-EGDM) composite (*triangle*) polymer particles with pH value at 20 °C measured under a constant concentration of TR. TR, 250 mg g^{-1} of particle; immobilization time, 45 min

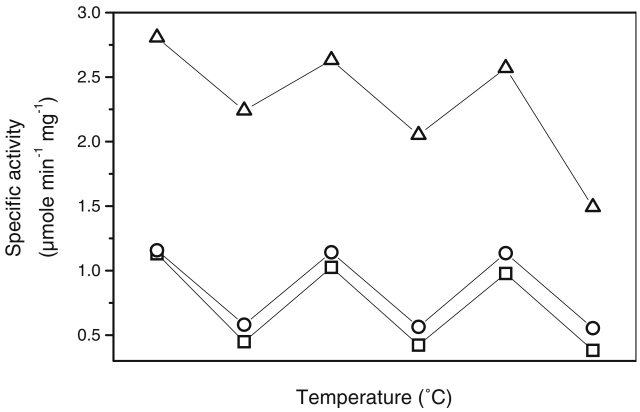

Fig. 6 Specific activities of free TR (*square*), adsorbed TR on PS seed (*circle*) and PS/P(DM-NIPAM-EGDM) composite (*triangle*) polymer particles measured alternatively at 40 and 20 °C under a constant concentration of TR. TR, 250 mg g^{-1} of particle; pH 10; immobilization time, 45 min

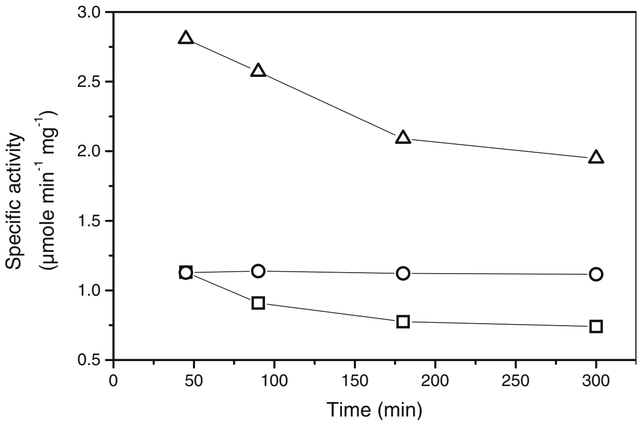

Fig. 7 Variation of specific activities of free TR (*square*), adsorbed TR on PS seed (*circle*) and PS/P(DM-NIPAM-EGDM) composite (*triangle*) polymer particles with immobilization time at 40 °C measured under a constant concentration of TR. TR, 250 mg g^{-1} of particle; pH 10

The comparatively rapid increase of specific activity also suggests that the native conformation of TR remained almost unchanged even the composite particle surface becomes relatively hydrophobic at higher pH values.

Figure 6 shows the specific activities of free TR and adsorbed TR on PS seed and PS/P(DM-NIPAM-EGDM) composite polymer particles during repeated alternative measurements at 40 and 20 °C, respectively. Specific activities at 40 °C are always higher than that at 20 °C. This result is based on the effect of temperature on the hydrolysis rate of LME rather than on the temperature-responsive P(DM-NIPAM-EGDM) shell. During repeated alternative measurements, specific activities of free TR and adsorbed TR on PS seed are close to each other and specific activities of free TR decreased at a slightly rapid rate, which is possibly caused by self-digestion. Low specific activities of adsorbed TR on PS seed particles are attributed to the combined effect of relatively strong hydrophobic interaction and fluctuation of temperature. On the other hand, specific activities of adsorbed/released TR from composite particles also decreased during repeated measurements but remained much higher. The continuous adsorption and release of TR from composite particles surface during repeated alternative measurements at 40 and 20 °C possibly imparted limited conformational change.

To check the stability of TR in the immobilised condition, specific activities of TR were measured. Figure 7 shows a comparative study for the variation of specific activities of free TR and immobilised TR on seed and composite particles with time at 40 °C measured under identical conditions. Specific activities of free TR decreased rapidly with time indicating rapid conformational change due to enhanced effect of higher temperature on self-digestion. Specific activities of adsorbed TR on PS seed remained steady with time but observed much lower than those of adsorbed TR on composite particles. This may suggest that in case of PS particles, relatively strong initial hydrophobic interaction with TR molecules produces either so much conformational change that after adsorption specific activity is not decreased with time, and/or polymer matrix prevented the active part of TR from self-digestion. On the contrary, specific activities of adsorbed TR on composite particles decreased with time but still remained much higher than those of both free TR and adsorbed TR on PS seed particles, indicating a limited conformational change if immobilisation time is prolonged at 40 °C. However, this effect can be minimised by carefully controlling the immobilisation time.

Conclusions

Specific activities of TR adsorbed on PS/P(DM-NIPAM-EGDM) composite polymer particles improved as compared to free TR and adsorbed TR on PS seed particles. This indicates that the composite polymer particles surface though turned relatively hydrophobic at higher temperature and higher pH, respectively, but the surface still remained sufficiently hydrophilic to retain native conformation of adsorbed TR. The TR adsorbed and released during repeated alternative measurements between temperatures above and below the LCST also retained native conformation. Optimisation of time at the immobilisation temperature (40 °C) would make our stimuli-responsive composite polymer particles suitable for application as carrier for biomolecules.

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