

Preparation of Micron-Sized Di-Functional Magnetic Composite Polymer Particles

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ABSTRACT

In this investigation micron-sized monodisperse magnetic composite polymer particles with amino and amide functional groups were prepared considering their applications in biotechnology. First, polystyrene/poly (acrylic acid-acrylamide-N-N-methylene-bis-acrylamide) [PS/P(AA-AAm-MBAAm)] composite polymer particles were prepared by seeded copolymerization. The carboxyl groups present on or near the particles surface were modified by amine-nucleophile, ethylene diamine (EDA), through pre-activation with dicyclohexyl carbodiimide as coupling agent. The aminated particles were then magnetically modified and named as aminated-Fe₃O₄ composite particles. Formation of such magnetic composite particles was confirmed by scanning electron micrographs, FTIR-spectra and magnetic susceptibility measurement. The produced composite particles were paramagnetic. To see the relative hydrophilic character of the particles surface the adsorption behavior of trypsin (TR) as biomolecule was studied on PS particles and aminated-Fe₃O₄ composite particles. The magnitude of adsorbed TR on PS particles was higher than that on aminated-Fe₃O₄ composite particles.

Keywords: Aminated Fe₃O₄ Composite, Amino and Amide Groups, Trypsin, Hydrophilic

1. Introduction

In recent years polymer particles with well defined particle size and various reactive functional groups on the surface are attracting much interest because of their applications in a broad range of fields, e.g. as binders in paints, adhesives, paper coating, textile and as a solid support in the biochemical and biological field as well as in catalysis and calibration standards [1-5]. The functional groups include hydroxyl [6-8], aldehyde [9-11], carboxyl [12-16], amino [17-19], epoxy [20-22], cyano [23], sulfhydryl [24] and chloromethyl groups [25-27]. A variety of methods are available to produce monodispersed particles in the micrometer-size range. Of these, dispersion polymerization in non-aqueous media allows successful preparation of micron-sized polymer particles with ease [28-33]. However, this methodology has suffered drawbacks when functional monomers were employed [34, 35]. These limitations include polydispersity, odd-shaped particles and in extreme case coagulation during polymerization. Moreover, the presence of functional comonomers in dispersion copolymerization produces polymer particles with limited number of functional groups on the surface. To overcome these limitations dispersion pol-

merization followed by seeded copolymerization is expected to be suitable to prepare functional polymer particles in the micrometer-size range.

The carboxyl group is one of the most widely studied functional groups for polymer particles intended for biomedical or diagnostic applications. This functional group can undergo easy derivatization using water/oil-soluble coupling agents. In a previous work H. Ahmad et al. reported the amination of carboxyl functionalized submicron-sized copolymer latex particles using amine-nucleophiles [36]. The present work is an extension of this early work, emphasizing the precipitation of magnetic nanoparticles on the surface of micron-sized aminated composite polymer particles to diversify their applications in magnetic support based separation in various fields of biotechnology and biomedicine, like cell separation [37], enzyme immobilization [38,39], drug delivery [40], and protein separation [41,42]. Most of the works available in the literature are concerned with preparation of nano to submicron-sized magnetic latex particles [43-50] and a few with micron-sized latex particles [51,52] bearing identical functional group. To prepare magnetic latex particles numerous routes have been investigated. They include the precipitation of iron oxide submicron particles

in presence of water soluble polymers [53-55], polymerization of suitable monomer mixtures in presence of iron oxide particles stabilized by surfactants [56-58] and layer-by-layer self-assembly of alternating layers of polyelectrolytes and magnetic nanoparticles onto colloidal templates [59]. In a recent work, preparation of submicron-sized sulfate- and carboxyl-functionalized magnetite/polystyrene spheres is reported for further deposition of gold nanoparticles [60]. Despite the success of these approaches little attention has been made for the preparation of di-functional micron-sized magnetic latex particles.

The present work aims to describe the preparation of monodispersed micron-sized magnetic composite particles bearing amino and amide groups on or near the particles surface. The produced di-functional magnetic latexes, named as aminated-Fe₃O₄ latexes, would offer advantages in biomedical applications as amino functional group can be utilized for coupling of affinity ligands or binding of biomolecules and the remaining amide functional group can simultaneously be used to modify the particles surface or even can act as spacer among the immobilized biomolecules preventing self reaction.

2. Experimental Details

2.1 Materials

Styrene (S) and acrylic acid (AA) purchased from Fluka, Chemika, Switzerland, were distilled under reduced pressure to remove inhibitors and preserved in the refrigerator until use. Acrylamide (AAm) from LOBA Chem. India and N,N'-methylene-bis-acrylamide (MBAAm) from Sigma, Chemical Company, USA, a crosslinking agent were used without any purification. 2,2'-Azobisisobutyronitrile (AIBN) and potassium persulfate (KPS) both from LOBA Chem. India, were recrystallized from ethanol and distilled water respectively and preserved in the refrigerator before use. Poly(N-vinylpyrrolidone) (PVP) from Fluka, Chemika, Switzerland of molecular weight 3.6×10^5 g/mol was used as a polymeric stabilizer. Tri-caprylylmethylammonium chloride (aliquate³³⁶) from Fluka, Chemika, Switzerland was used as a cationic co-stabilizer. The biomolecule used was trypsin (TR) from E. Merck, Germany, used as adsorbent on the surface of polymer particles without any purification. Dicyclohexyl carbodiimide (DCC), ethylene diamine (EDA) all from LOBA Chem. India, were used as supplied. Ferric chloride hexahydrate (FeCl₃·6H₂O), ferrous sulphate (FeSO₄), sodium hydroxide (NaOH), oleic acid and other chemicals were of analytical grade. Deionized water was distilled using a glass (Pyrex) distillation apparatus.

Scanning Electron Microscope, SEM (LEO Electron Microscopy Ltd., UK); Burker Proton NMR, 250 MHz; FTIR (8044 Simadzu, Japan); Centrifuge machine from Kokuson Corporation, Tokyo, Japan; Helios gamma single-beam UV-Visible spectrophotometer from Unicam,

UK; HI 9321 Microprocessor pH-meter and conductivity meter both from HANNA instruments, Portugal were used in this study. NICOMP 380 (USA) particles sizer was used to measure hydrodynamic diameter of the polymer particles. Sherwood Scientific Magnetic Susceptibility Balance was used for susceptibility measurement. Thermal analysis was carried out using thermogravimetry (TG) analyser from Seiko Instruments Inc EXSTAR 6000 TG/DTA 6300.

2.2 Preparation of Polystyrene (PS) Seed Particles

Polystyrene (PS) seed particles were prepared by dispersion polymerization of 10 g styrene in presence of PVP (0.432 g) and aliquate³³⁶ (0.123 g) using 0.162 g AIBN as oil soluble initiator. The dispersion media used was ethanol (65 mL) and the polymerization was carried out in a 250 mL three necked round bottom flask under a nitrogen atmosphere at 70°C for 24 h. The conversion was nearly 100% as measured gravimetrically.

2.3 Preparation of Micron-Sized Composite Polymer Particles

Micron-sized PS/P(AA-AAm-MBAAm) composite polymer particles were prepared by seeded copolymerization of AA, AAm and MBAAm in presence PS seed particles. Prior to the seeded copolymerization PS seed particles were transferred into water and magnetically stirred with AA, AAm and MBAAm comonomers at room temperature for 12 h in order to minimize the formation of soluble copolymer. The reaction flask was then transferred to a preheated thermostat water bath maintained at 70°C. The polymerization reaction was carried out under a nitrogen atmosphere for 12 h using KPS as an initiator. Polymerization conditions are shown in **Table 1**.

2.4 Measurement of Hydrodynamic Particle Size

PS/P(AA-AAm-MBAAm) composite polymer particles were washed by serum replacement to remove any unreacted monomer and initiator fragments. A dilute dispersion of approximately 0.01% polymer solid was prepared,

Table 1. Preparation of PS/P(AA-AAm-MBAAm)^{a)} composite polymer particles^{b)}

Ingredients		
PS dispersion ^{c)}	(g)	29.5130
AA	(g)	0.8881
AAm	(g)	0.8759
MBAAm	(g)	0.036
KPS	(g)	0.29
Water	(g)	60.0

^{a)}Core/shell ratio: 1/0.6; ^{b)}70°C, N₂, 120 rpm, 24 h; ^{c)}Polymer solid, 4.89 g/L

pH was adjusted using acid/alkali solution and intensity weighted average hydrodynamic size was measured by NICOMP particle size. Each sample was measured twice and the average value is reported. The deviation between successive measurements was less than $\pm 5\%$.

2.5 Amination of PS/P (AA-AA-MBAAm) Composite Particles

PS/P(AA-AA-MBAAm) latex particles were transferred from water to ethanol medium by ultra-centrifugal washing at 12,000 rpm and the solid content of the latex was adjusted to about 2%. EDA was covalently bonded to the carboxyl groups of composite particles by the following method [36].

Pre-activation method: 50 g of latex dispersion was mixed with 2.1166 g coupling agent (DCC) in a reagent bottle, magnetically stirred at 25°C for 1 h. The activated carboxyl group was then reacted with 0.6154 g EDA at 25°C for 3 h and then again at 0°C for 24 h.

2.6 Preparation of Aminated-Fe₃O₄ Composite Particles

2.5 g of modified aminated composite particles were taken in a round bottom flask containing 70 mL water. The dispersion was cooled (0-5°C) in an ice-water bath under a nitrogen gas bubbling. 0.5 g (3 mmol) of FeCl₃·6H₂O and 0.2558 g (2.7 mmol) of FeSO₄ were dissolved in 25 mL water. Then the iron solution was added to the flask containing the aminated composite dispersion. A light brown color mixture was formed. The ice bath was removed and the flask was immersed in a preheated water bath at 85°C. Immediately, 2.9123 mL of 5 M aqueous NaOH solution was added. The reaction mixture gradually turned to black. The mixture was kept stirring at 85°C for 2 h. Oleic acid (0.01456 g, 2.26 mmol) was slowly added towards the end of the process to stabilize the aminated-Fe₃O₄ dispersion. Then the dispersion was cooled to room temperature. The resultant magnetic particles were centrifuged and thoroughly washed with deionized distilled water, followed by 0.1 M HCl and again by deionized distilled water.

2.7 Measurement of Magnetic Susceptibility

Aminated-Fe₃O₄ composite particles were washed, separated from their dispersion by centrifugation and dried in oven at 80°C for 2 h. The dried powders were placed in a pre-weighed sample tube and measured the magnetic susceptibility (χ_g) using the following equation:

$$\chi_g = \frac{C \times L \times (R - R_0)}{m \times 10^9}$$

where, C is calibration constant of the balance, L is length of the sample, R_0 and R are reading of the empty and sample tubes and M is weight of the sample (dry

basis) in C.G.S unit.

2.8 Thermogravimetric Analysis (TGA) of Aminated-Fe₃O₄ Composite Polymer Particles

Thermal properties of the dried aminated-Fe₃O₄ powder was measured by heating samples under flowing nitrogen atmosphere from 40°C to 600°C at a heating rate of 20°C/min and the weight loss was recorded.

2.9 Adsorption of Trypsin (TR) on PS Seed and Aminated-Fe₃O₄ Composite Polymer Particles

A mixture of 20 mL was prepared from purified polymer dispersion (polymer solid 0.15 g) and TR (200 mg/g). The pH value of the mixture was immediately adjusted at the isoelectric point (pH 10.0) using a buffer solution. In each measurement the dispersion-TR mixture was allowed to stand at 25°C for 45 min. Polymer particles were then separated by centrifugation at 10,000 rpm for 10 min. In order to remove wafting particles completely, the supernatant was centrifuged once more. The concentration of TR in the supernatant was determined by a UV-visible spectrophotometer at the wavelength of 280 nm. The magnitude of TR adsorbed was calculated by subtracting the concentration of TR in the medium from that of the initial concentration. Calibration curve was used for this purpose.

3. Results and Discussion

Figures 1(a) and (b) shows the SEM photographs of PS and PS/P(AA-AAm-MBAAm) composite polymer particles. The average diameters and coefficient of variations are 1.47 μm and 5.87% for PS seed and 1.51 μm and 2.43% for PS/P(AA-AAm-MBAAm) composite particles. The average size of PS/P(AA-AAm-MBAAm) composite particles increased slightly after seeded copolymerization. Both the PS seed particles and the composite polymer particles are fairly monodispersed and spherical, having a smooth homogeneous surface. Submicron-sized P(AA-AAm-MBAAm) copolymer particles are not observed. This suggests that seeded copolymerization of AA, AAm and MBAAm proceeds smoothly on PS seed particles, with

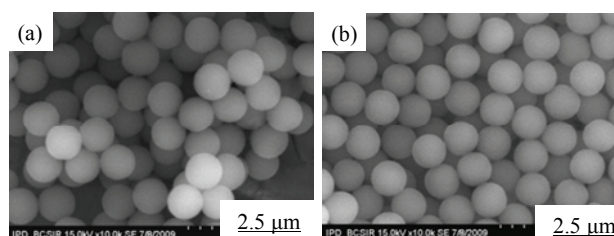


Figure 1. SEM photographs of (a) PS seed; (b) PS/P(AA-AAm-MBAAm) composite polymer particles

out any secondary nucleation.

Figure 2 shows the FTIR spectra of PS seed particles and PS/P(AA-AAm-MBAAm) composite polymer particles. Both polymer particles were washed and dried at 70°C prior to the recording of FTIR spectra. In the spectrum of PS/P(AA-AAm-MBAAm) composite particles a broad O-H stretching of carboxyl group appeared at 2400-3500 cm^{-1} . The signal due to N-H stretching of primary amide expected to appear at 3200-3500 cm^{-1} is possibly been overlapped with O-H stretching signal. The inclusion of moisture may also affect the appearance of N-H stretching. Distinct but weak signals due to C=O stretching vibration for -COOH and -CONH₂ groups in composite particles are appeared at around 1718 and 1690 cm^{-1} , respectively. The combination band due to phenyl ring of PS largely affected the appearance of those stretching signals. The weak stretching signals of -COOH and -CONH₂ groups are attributed to the smaller total surface area of micron-sized composite particles.

Figure 3 shows the ¹H NMR spectrum of PS/P(AA-AAm-MBAAm) composite polymer particles. The chemical shifts of the aliphatic groups -CH₂ and -CH appeared at 1.4 ppm and 1.86 ppm, respectively. The intense signal due to aromatic protons from PS ring is observed at 6.6 ppm. The characteristic NMR signals due to acidic (-COOH) and amidic (-CONH₂) protons are expected to be at 10.0-12.0 ppm and 5.0-9.0 ppm, respectively. But it is known that the chemical shifts of these groups are variable, depend not only on the chemical environment, but also on concentration, temperature, and solvent [61]. In the magnified spectra of composite particles, signals appeared at 8.1 ppm and 4.68 ppm, indicated the presence of -COOH and -CONH₂ groups. The deviation of both signals to upfield is due to possible hydrogen bonding between amide and carboxyl groups, hence

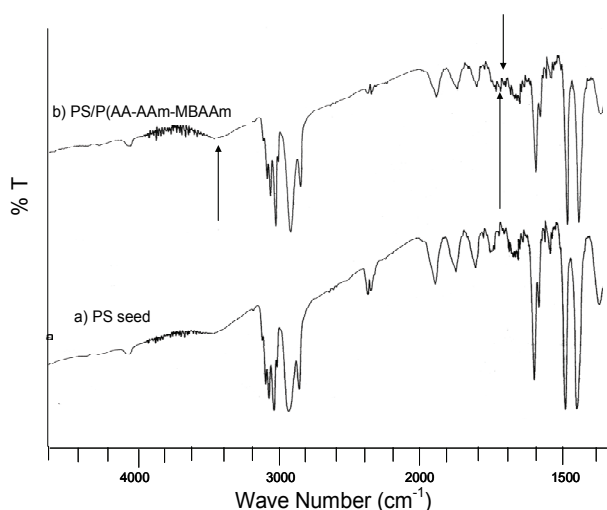


Figure 2. FTIR spectra of a) PS and b) PS/P(AA-AAm-MBAAm) composite polymer particles

the protons are shielded. The weak signal intensity of -COOH and -CONH₂ groups are attributed to the poor solubility of cross-linked hydrophilic composite particles in non-polar CDCl₃. Additionally, the presence of -COOH and -CONH₂ was also confirmed by elemental and functional group analyses.

Figure 4 shows the variation of hydrodynamic diameter of washed PS/P(AA-AAm-MBAAm) composite polymer particles with different pH values at 20°C. Hydrodynamic diameter increased with the increase of pH value. This behavior indicates that at lower pH value the composite polymer particles are relatively hydrophobic as the majority of carboxyl groups derived from AA comonomer is protonated. Whereas with increasing pH value more and more carboxyl groups are deprotonated, which facilitated the swelling of composite polymer particles through hydrogen bonding with water.

Figure 5 shows the SEM photographs of aminated PS/P(AA-AAm-MBAAm) composite, magnetite (Fe₃O₄),

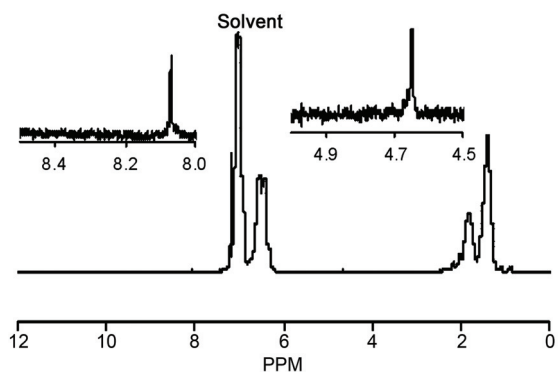


Figure 3. ¹H NMR spectrum of composite polymer particles

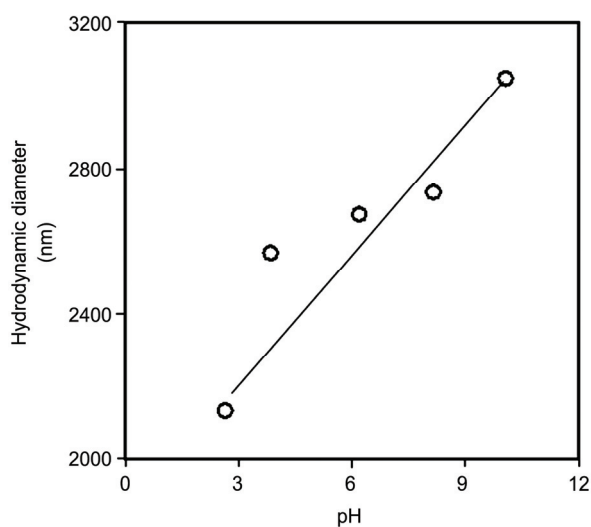


Figure 4. Variation of average hydrodynamic diameter of washed PS/P(AA-AAm-MBAAm) composite polymer particles with pH values measured at 20°C

and aminated- Fe_3O_4 composite particles. All the particles were washed by serum replacement prior to the observation by SEM. It is to be mentioned that after three washings the supernatant obtained from the aminated- Fe_3O_4 composite particles was clear indicating no leaching of Fe_3O_4 particle. The observed magnetite (Fe_3O_4) particles were prepared by co-precipitation of Fe^{2+} (aq) and Fe^{3+} (aq) in presence of NaOH and aminated- Fe_3O_4 composite particles were prepared by the impregnation of iron ions and precipitation of Fe_3O_4 on aminated composite polymer particles. The mean diameter of aminated- Fe_3O_4 composite particles is $1.60\ \mu\text{m}$ which is greater than the mean diameter of aminated composite polymer particles ($1.54\ \mu\text{m}$). It is also observed that after amination the average size of composite particles slightly increased as compared to that before amination ($1.51\ \mu\text{m}$). Similar increase in average size after amination was also observed in our previous work [36]. The surface of the aminated composite polymer particles is smooth but the surface of the aminated- Fe_3O_4 composite particles is not smooth with many small Fe_3O_4 particles attached to the surface of aminated composite particles. Additionally, the precipitation of Fe_3O_4 particles on aminated composite surface was confirmed by the measurement of magnetic susceptibility and the value was recorded as positive (2.4768×10^{-4}). This result suggests that the prepared aminated- Fe_3O_4 composite particles are paramagnetic. Prior to this measurement the dispersion was centrifuged and dried at low temperature (80°C) to prevent any crystal deformation of the impregnated Fe_3O_4 particles.

Figure 6 shows the FTIR spectra of magnetite (Fe_3O_4) and aminated- Fe_3O_4 composite particles. In the FTIR spectra of Fe_3O_4 , the characteristic stretching vibrations due to Fe-O bonds of Fe_3O_4 particles appeared at 582.5 and $439.7\ \text{cm}^{-1}$ respectively, as also reported elsewhere [62,

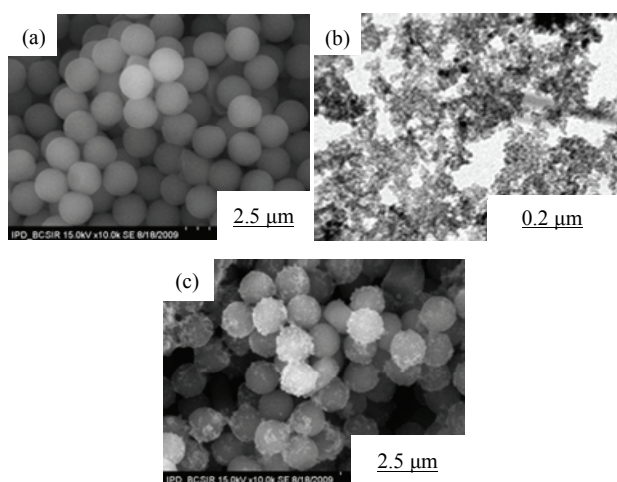


Figure 5. SEM photographs of (a) aminated PS/P(AA-AAm-MBAAm) composite polymer; (b) magnetite (Fe_3O_4); (c) aminated- Fe_3O_4 composite particles

63]. The broad band at $3330.8\ \text{cm}^{-1}$ can be assigned to the stretching vibration of surface water molecules or the envelope of hydrogen bonded surface OH groups. In aminated- Fe_3O_4 composite particles a combined strong signal for N-H and -OH stretching derived from amide, carboxyl and surface water molecules is appeared at $3404.1\ \text{cm}^{-1}$. The stretching signal due to Fe-O is also observed in aminated- Fe_3O_4 composite particles at around $439\ \text{cm}^{-1}$ and the signal appeared at $540\ \text{cm}^{-1}$ broadened due to the out of plane C-H bending vibration for substituted phenyl ring of PS. The above results also confirmed that the Fe_3O_4 particles have successfully been precipitated on the aminated composite polymer particles. TGA was used to determine the total inorganic material *i.e.* iron oxide content in aminated- Fe_3O_4 composite particles. TGA curve is illustrated in **Figure 7**. It is expected that as the aminated- Fe_3O_4 composite particles are heated from ambient temperature to 600°C , the organic part of the

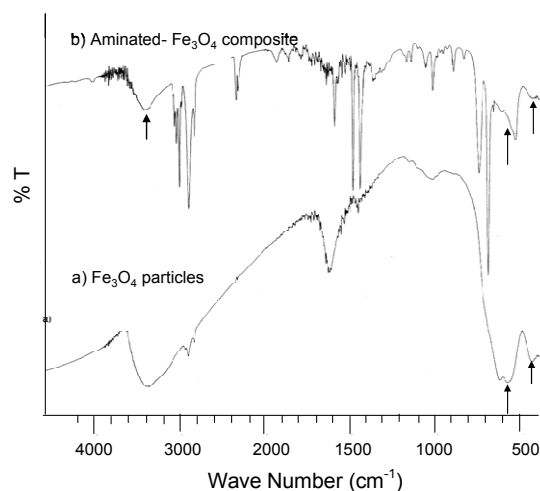


Figure 6. FTIR spectra of a) magnetite (Fe_3O_4); b) aminated- Fe_3O_4 composite polymer particles

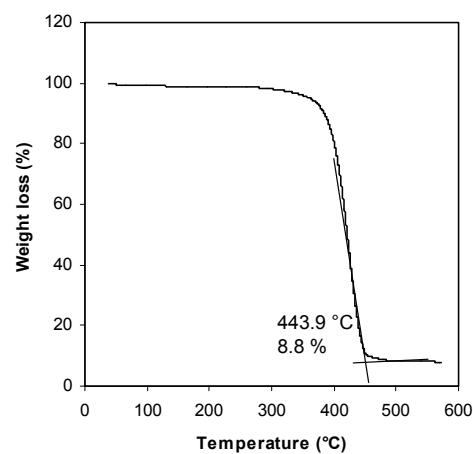


Figure 7. TGA thermogram of aminated- Fe_3O_4 composite polymer particles

composite would be burned and the percentage of the remaining part after calcination would give the iron oxide content. As such TGA thermogram indicates that about 8.8% (w/w) magnetic Fe_3O_4 nano-particles are precipitated on the aminated composite polymer particles. Considering the larger average size of the composite particles, the percentage of magnetic iron oxide is pretty high to modify the particles surface [64].

Figure 8 shows the adsorption behaviors of TR on the PS seed and aminated- Fe_3O_4 composite particles. It is well known that magnitude of adsorption is largely dependent on the degree of hydrophobic interaction between the particle surface and biomolecules and also on the total particle surface area. Since the sizes of the seed and aminated- Fe_3O_4 composite particles are not the same as measured from the SEM photographs, so the adsorption behavior of TR has been presented per unit area of the particles. The magnitude of adsorption on the aminated- Fe_3O_4 composite particles is lower than that on PS seed particles. As already mentioned, in order to use polymer particles, as a carrier for biomolecules, the particle surface should be sufficiently hydrophilic with limited amount of hydrophobicity. It is well known that the strong hydrophobic interaction of biomolecules with solid surface results in the loss of native conformation as well as the activity of adsorbed biomolecule.

4. Conclusions

A synthetic route for the preparation of monodisperse micron-sized magnetic composite polymer particles with amine and amide functional groups on or near the surface has been developed. The process involved the preparation of micron-sized PS latex particle by dispersion polymerization followed by seeded copolymerization with AA, AAm and MBAAm, surface modification with EDA and subsequent precipitation of iron ions to form Fe_3O_4 .

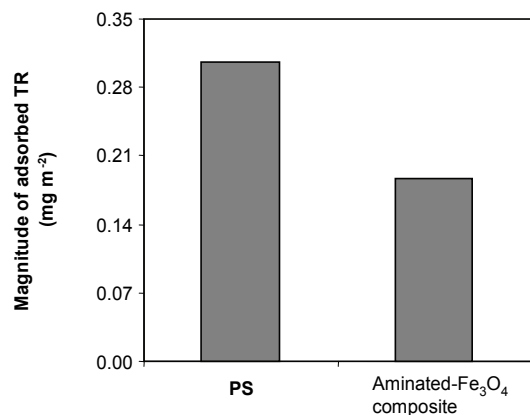


Figure 8. Magnitude of trypsin (TR) adsorbed on PS seed and aminated- Fe_3O_4 composite particles measured under the constant concentration against the total solid content at 25°C. pH: 10.0; particles: 0.15 g; TR: 200 mg/g of particles

The presence of amino and amide groups improved hydrophilicity and hence could prevent nonspecific adsorption of proteins.

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