Adsorption of Trypsin onto Magnetic Ion-Exchange Beads of Poly(glycidylmethacrylate-*co*-ethyleneglycoldimethacrylate)

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ABSTRACT: Magnetic beads were prepared from glycidyl methacrylate (GMA), and ethyleneglycol dimethylmethacrylate (EGDMA) in the presence of Fe_3O_4 nanopowder via suspension polymerization. The magnetic beads were characterized by surface area measurement, electron spin resonance (ESR), and scanning electron microscopy (SEM). ESR data revealed that the beads were highly super-paramagnetic. The effects of contact time, pH, ionic strength, and temperature on the adsorption process have been studied. Adsorption equilibrium was established in about 120 min. The maximum adsorption of trypsin on the magnetic beads was obtained as 84.96 mg g^{-1} at around pH 7.0. At increased ionic strength, the contribution of the electrostatic component to the overall binding decreased, and so the adsorption capacity. The experimental equilibrium data obtained trypsin adsorption onto magnetic beads fitted well to the Langmuir isotherm model. The result of kinetic analyzed for trypsin adsorption onto magnetic ion-exchange beads showed that the second order rate equation was favorable. It was observed that after six adsorption–elution cycle, magnetic beads can be used without significant loss in trypsin adsorption capacity. Finally, the magnetic beads were used for separation of bovine serum albumin (BSA) and trypsin from binary solution in a batch system. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 2810–2819, 2008

Key words: magnetic beads; adsorption; trypsin; BSA; kinetic characterization; thermodynamic parameters

INTRODUCTION

Chromatographic separations play a dominant role in the downstream processing of monoclonal antibodies, recombinant proteins, and therapeutic plasmids.[1–4] The development of protein purification techniques has been essential for many of the recent advancements in biotechnology research.[5,6] In the literatures, many kinds of chromatographic techniques have been reported such as gel filtration,[7] dye-ligand,[8] immobilized metal-affinity,[9] affinity chromatography,[10] hydrophobic interaction,[11] ion-exchange chromatography.[12–15] The later is ion-exchange, in which the separation can be achieved based on the electrostatic interaction between the surface charges of proteins and the charged groups on the adsorbents. In the most chromatographic applications, beaded adsorbents used in the purification of proteins involve affinity and ion exchange adsorption mechanisms.[16–18] Affinity interactions are characterized by a highly specific interaction between the ligand and the target molecule, whereas for an ion-exchange process the functional groups on the adsorbent and the target molecule should have opposite charge.[19–21]

The acrylic based support materials in the bead form are almost ideal ones to perform for adsorption of proteins, very stable in a range of buffers from pH 1.0 to 11.0 and was resistant to microbial degradation and several chemicals.[11,22] In addition, magnetic separation technique, using magnetic polymeric beads, is quick and easy method for sensitive and reliable capture of inorganic or organic pollutants.[23–25] This method is also nonlaborious, cheap and often highly scalable. Moreover, techniques employing magnetism are more amenable to automation and miniaturization.[26,27] The magnetic character implies that they respond to a magnet, making sampling and collection easier and faster, but their magnetization disappears once the magnetic field is removed. In addition, magnetic beads promises to solve many of the problems associated with chromatographic separations in packed bed and in conventional fluidized bed systems.[28,29] Magnetic separations is relatively rapid and easy, requiring a simple apparatus, composed to centrifugal separation. Recently, there has been increased interest in the use of magnetic carriers in protein purification.

Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system, where it breaks down proteins. Trypsin specifically hydrolyzes peptide bonds at the carboxyl side of lysine and arginine residues. It is

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used for numerous biotechnological processes such as protein primary structure analysis, to breakdown casein in milk for baby food and to re-suspend cells adherent to the cell culture dish wall during the process of harvesting cells.[30–32]

In this work, magnetic poly(GMA/EGDMA) beads were prepared via suspension polymerization and an amino acid ligand (i.e., glutamic acid) was grafted on the magnetic beads surfaces. The magnetic beads were characterized and their efficiency in trypsin adsorption was investigated under a wide range of conditions, such as contact time, initial pH of solution, ionic strength, and initial protein concentrations using a batch method. It is expected that the glutamic acid (GA) grafted magnetic beads should have better adsorption property for the model basic protein ''trypsin'' because there is high density of carboxyl groups on the on the magnetic bead surfaces. The experimental adsorption data are tested for a number of theoretical kinetic and isotherm models and evaluated of thermodynamic parameters on trypsin adsorption. The magnetic beads were used for the separation of trypsin and BSA from binary protein solutions.

MATERIAL AND METHOD

Materials

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), polyvinyl alcohol (PVA), GA, and toluene were supplied from Sigma (St Louis, MO), and the monomers distilled under reduced pressure in the presence of hydroquinone and stored at 4°C until use. α-α′-Azoisobisbutyronitrile (AIBN) was obtained from Fluka (Switzerland). Magnetite nanopowder (Fe_3O_4, diameter: 20–50 nm), bovine serum albumin (BSA) and trypsine (EC. 3.4.21.4; Type 1) were supplied from Sigma Chemical Co. (St Louis, MO) and used as received. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in the present work was purified using a Barnstead (Dubuque, IA) ROpure LP reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure organic/colloid removal and ion exchange packed-bed system.

Preparation of magnetic poly(GMA/EGDMA) beads

The magnetic beads were prepared by suspension polymerization. The aqueous dispersion medium comprised from NaCl solution (0.1*M*, 400 mL). The organic phase contained GMA (15 mL) and EGDMA (7.5 mL), 1.0 g magnetite (Fe_3O_4) nano-particles, and 5.0% polyvinyl alcohol (20 mL, as stabilizer) were mixed together with 0.2 g of AIBN as initiator in 20 mL of toluene. The resulting medium was transferred into the reactor and sonicated for about 5 min at 200 W within an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. The polymerization reactor was placed in a water bath and heated to 70°C. It was then equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The polymerization reaction was maintained under nitrogen atmosphere at 70°C for 2.0 h (stirring rate: 250 rpm) and then at 80°C for 1.0 h. After the reaction, the resultant beads were filtered under suction and washed with distilled water and ethanol. Finally, the synthesized magnetic beads were separated from the reaction medium, washed in ethanol solution (70%; 250 mL) for 30 min, and then washed with purified water. The magnetic beads were finally dried in a vacuum oven at 50°C and stored at room temperature until use.

To determine the amount of leached magnetite nanopowder from the poly(GMA/EGDMA) beads, a 0.5 g beads were placed in a glass containing acetic acid solution (0.1*M*, pH 4.0, 10 mL) and shaken on a rotary shaker at 150 rpm for 24 h. The amount of leached magnetite in the medium was determined by flame atomic absorption spectrophotometer (Shimadzu, Model AA-6800, Japan).

Grafting of magnetic beads with glutamic acid

To prepare GA grafted magnetic beads following procedure was applied. The magnetic poly(GMA-EGDMA) beads were incubated with GA solution (2.0%, pH 10) at 65°C in a reactor containing 20 g of dry magnetic beads and were shaken for 6 h. After this period, the GA immobilized magnetic beads were removed from the medium by applying external magnetic force and washed with 1.0*M* NaCl and then washed twice with purified water and dried in the oven at 55°C under vacuum for 24 h.

Characterization of magnetic beads

The amount of available surface functional epoxy groups content of the poly(GMA/EGDMA) beads was determined by pyridine-HCl method as described previously.[33] The amount of available carboxyl group contents of the poly(GMA/EGDMA)-GA beads was determined by potantiometric titration. Briefly, the magnetic beads (0.1 g) was allowed soak into water (10 mL) for 24 h. Then, NaOH solution (0.1*M*, 20 cm^3) was added to the mixture and it was then incubated in a shaking water-bath at 35°C for 6 h. After this reaction period, the beads filtered

and the final NaOH concentration in the solution was assayed by a potentiometric titration with 0.05*M* HCl solution.

The average size and size distribution of the magnetic beads were determined by screen analysis performed by using molecular sieves. The specific surface area of the beads was measured by a surface area apparatus and calculated using the BET (Brunauer, Emmett and Teller) method. Scanning electron micrographs (SEM) of the dried magnetic beads were obtained using a JEOL, JMS 5600 scanning electron microscope, after coating with gold under reduced pressure. The FTIR spectra of the magnetic poly(GMA/EGDMA) beads were obtained using an FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). Electron spin resonance (ESR) spectroscopy was carried out with a conventional X-band (ν = 9.75 Hz) Bruker ESP 300E spectrometer at 100 kHz magnetic field modulation frequency, 31.7G modulation amplitude and 0.1 mW microwave power. The magnetic beads about (50 mg) were placed into quartz tube and the measurements were performed at room temperature. The first derivative of the power absorption had been recorded as a function of the applied magnetic field.

Adsorption studies

Adsorption of trypsin on the magnetic beads from aqueous solutions was investigated in batch mode. The magnetic beads (50 mg) were incubated with 5 mL of the aqueous solutions of trypsin for 2 h, the cell was agitated magnetically at 150 rpm. Effects of the trypsin concentration, pH of the medium, temperature and ionic strength on the adsorption capacity were studied. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of the initial concentration of trypsin on adsorption, it was changed between 0.2 and 3.0 mg mL^{-1}. To observe the effects of the temperature on the adsorption, adsorption studies were carried out between 4 and 37°C. Each experiment was performed in triplicate for quality control and statistical purposes. The initial and final concentrations of trypsin in the medium were measured at 280 nm by using a double beam UV/Vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601). Calibration curves were prepared using trypsin as standard (0.025–2.0 mg mL^{-1}). The amount of adsorbed trypsin was calculated using mass balance as previously described.[5] It should be noted that the dry-stage of magnetic beads used as the mass unit in the adsorption experiments. Each set of experiments was carried out in triplicate, the arithmetic mean values and standard deviations were calculated and the margin of error for each data set was determined according to a confidence interval of 95% using the statistical package under Excel for Windows.

Desorption and repeated use

To determine the reusability of magnetic beads consecutive adsorption–desorption cycles were repeated six times by using the same magnetic poly(GMA/EGDMA) beads. The desorption of the adsorbed trypsin from the magnetic beads was studied in a batch mode. Trypsin adsorbed magnetic beads were placed in desorption medium, containing 0.5*M* GA solution at pH 4.0, and stirred at 150 rpm for 2 h at room temperature. The final trypsin concentration in desorption medium was determined by as described above.

Adsorption isotherms and kinetic models

Two important physico-chemical aspects for evaluation of the adsorption process as a unit operation are the kinetics and the equilibrium of adsorption. Modeling of the equilibrium data has been done using the Langmuir and Freundlich isotherms. The Langmuir and Freundlich isotherms are represented as follows eqs. (1) and (2), respectively.

$$1/q_e = (1/q_{\max}) + (1/q_{\max}b)(1/C_e) \quad (1)$$

$$ln\ q_e = 1/n(ln\ C_e) + ln\ K_F \quad (2)$$

where b is the Langmuir isotherm constant, K_F is the Freundlich constant, and n is the Freundlich exponent. $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The value of q_e gives the theoretical monolayer saturation capacity of the poly(GMA/EGDMA)-GA beads. Some model parameters were determined by nonlinear regression with commercially available software and are shown in Table I.

The kinetics of trypsin adsorption on the GA grafted magnetic beads was determined with different kinetic models i.e. the first- and second-order. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a solution. The model has the following form:

$$\log\ (q_e/q_e - q_t) = (K_1\ t)/2.303 \quad (3)$$

where K_1 is the rate constant of first-order adsorption (min^{-1}) and q_e and q_t denote the amounts of adsorption at equilibrium and at time t (mg g^{-1}), respectively. In a true first order process log q_e should be equal to the intercept of a plot of log ($q_e - q_t$) against t.

TABLE I
The Isotherm Model Constants and Correlation Coefficients of Tyripsin Adsorption on the Magnetic Beads

	Experimental	Langmuir constant			Freundlich constant			
Temperature (K)	q_{exp} (mg g^{-1})	q_m (mg g^{-1})	$K_d \times 10^{-6}$ (M)	R^2	K_F	n	R^2	ΔG (kcal mol^{-1})
277	67.01	71.43	5.82	0.997	85.99	2.31	0.899	−6.63
288	78.01	83.33	4.19	0.998	85.30	2.42	0.862	−7.09
298	84.96	90.91	3.23	0.996	89.91	2.81	0.807	−7.49
310	96.67	98.04	6.61	0.999	128.96	3.03	0.893	−8.76

Ritchie proposed a method for the kinetic adsorption of gases on solids.[34] If protein adsorption medium is considered to be a second-order reaction, Ritchie equation is:

$$(1/q_t) = (1/K_2\ q_e\ t) + (1/q_e) \quad (4)$$

where K_2 (g $mmol^{-1}$ min^{-1}) is the rate constant of the second order adsorption. The rate constant (K_2) and adsorption at equilibrium (q_e) can be obtained from the intercept and slope, respectively, and there is no need to know any parameter beforehand.

Theoretical treatments of intra-particle diffusion yield rather complex mathematical relationships which differ in forms as functions of the geometry of the sorbent particle. A functional relationship common to most treatments of intra-particle diffusion is that uptake varies almost proportionately with the half–power of time $t^{0.5}$, rather than t; a nearly linear variation in the quantity adsorbed with $t^{0.5}$ is predicted for a large initial fraction of reactions controlled by rates of intra-particle diffusion. According to Weber and Morris[35] if the rate limiting step is intra-particle diffusion, a plot of solute adsorbed against the square root of the contact time should yield a straight line passing through the origin. The most-widely applied intraparticle diffusion equation for sorption system is given by Weber and Morris:[35]

$$q_t = k_i\ t^{0.5} \quad (5)$$

where k_i is the intraparticle diffusion rate constant (mg g^{-1} $min^{0.5}$) The k_i values under different conditions were calculated from the slopes of the straight-line portions of the respective plots.

The dispersion coefficient, defining the equilibrium partitioning of solute between the aqueous medium and the chromatographic support due to the adsorption, were measured by means of conventional and modified batch test.[36,37] There are different ways of representing the dependence of the dispersion coefficient as a function of one or more parameters. In general, mass transfer effects, i.e., convective dispersion, fluid to particle mass transfer, and intra-particle diffusion will have a significant influence on the dynamics of the process.[38,39]

The amount of protein absorbed on the sold phase and the respective dispersion coefficient (D) were computed from the measured activities. The resulting dispersion coefficient characterizes the process of adsorption on solid particles, without distinguishing the size of particles;

$$D = (V/m)(C_0 - C)/C \quad (6)$$

V is the volume of the adsorption medium (mL), m is the amount of dry solid particles in the batch system (g) and (C_0) and (C) are initial and final concentration of the protein, respectively.

Thermodynamic parameters

The adsorption isotherms obtained at various temperatures were used to gain a better understanding of the adsorption mechanism. The dependency of the equilibrium association constant ($K_a = b$) versus $1/T$ for the binding of trypsin on the magnetic beads was analyzed in terms of van't Hoff plots. From the van't Hoff plot for the adsorption process, the thermodynamic parameters such as free energy changes (ΔG°), enthalpy change (ΔH°), and entropy change (ΔS°) were then extracted. The value of the change in enthalpy during the adsorption process was determined from the gradient of the plots between $ln\ K_a$ versus $1/T$ ($lnK_a = (\Delta S^\circ/R) - (\Delta H^\circ/RT)$). The value of ΔG° and ΔS° can be estimated from the relationships $\Delta G^\circ = -RT\ lnK_a$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$.

RESULTS AND DISCUSSION

Characterization of magnetic beads

The beads were sieved and 75–150 μm size of fraction was used for adsorption of trypsin. The specific surface area (75–150 μ size fractions) of the magnetic beads was measured by the BET method and was found to be 13.2 m^2 g^{-1} beads. The amount of available epoxy groups on the magnetic beads surface was determined by the HCl-pyridine method and was found to be 2.05 mmol g^{-1} beads. GMA monomer was used initially to incorporate epoxy groups on the polymer surface for further modification of

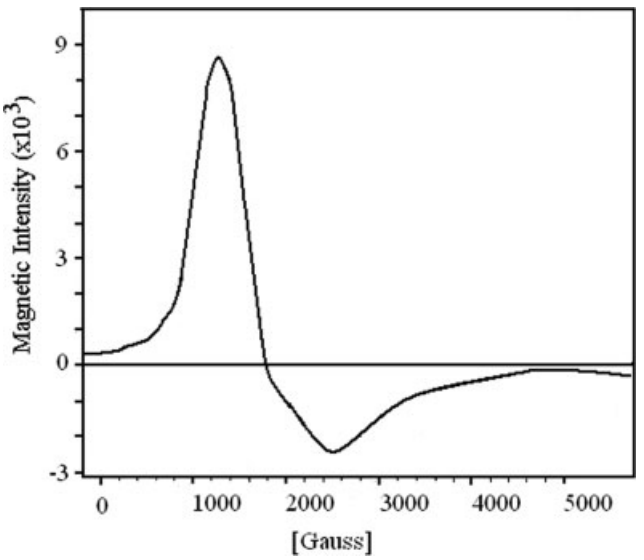

Figure 1 Electron spin resonance (ESR) spectrum of magnetic poly(GMA/EGDMA)-GA beads at room temperature.

the beads. The obtained value for epoxy groups was lower than that of the theoretical value (about 5.2 mmol g^{-1}) because some of the epoxy groups remain inside of the magnetic beads and are not accessible for subsequent reactions or analytical determinations. The amount of available carboxyl groups after GA grafting on the magnetic poly(GMA/EGDMA)-GA beads surface was determined by potansiometric method and was found to be 96.2 μmol g^{-1} beads. The studies of entrapped magnetite nanopowder leakage from the poly(GMA/EGDMA) beads showed that there was no magnetite nanopowder leakage in the medium used throughout this study, even in long storage period of time (more than 10 weeks).

The magnetic properties of the ion-exchange bead were confirmed with electron spin resonance spectroscopy (ESR) at room temperature and the intensity versus the magnetic field (Gauss) is presented in Figure 1. The spectrum has two components: (i) a typical low field high intensity ferromagnetic resonance signal (below 2000 Gauss), and (ii) a broad line pick extended up to 5000 Gauss. In this ESR spectrum, about 1350-Gauss magnetic field was found to be sufficient to excite all of the dipole moments of the sample beads that consist of magnetite. The value of this magnetic field is a function of the flow velocity, particle size and magnetic susceptibility of the beads to be displaced. In the literature, the value was reported between 1000 and 20,000 Gauss,[1] so the magnetic beads developed in this study will need less magnetic intensity for various reactor configurations. So, the magnetic beads can be easily separated within a few second by a conventional permanent magnet. When the applied magnetic force is removed, the magnetic beads can easily be dispersed by simple shaking. Thus, the magnetic beads can be removed or recycled in the adsorption medium.

FTIR spectra of magnetic poly(GMA/EGDMA) bead are presented in Figure 2. The FTIR spectra of magnetic beads have the characteristic stretching vibration band of hydrogen-bounded alcohol at $\sim$ 3200 cm^{-1}. Among the characteristic vibrations of both GMA and EGDMA is the methylene vibration at $\sim$ 2952 cm^{-1}. The vibration at around 1720 cm^{-1} represents the ester configuration of GMA. On the other hand, several bands appear in the finger print region for magnetic beads between 1600 and 1200 cm^{-1}. These peaks are assigned to the —CH_2 scissoring band of both GMA and EGDMA at 1452 cm^{-1}, anti-symmetric and symmetric stretching band of carbonyl groups of GMA and EGDM at 1388 cm^{-1} and 1265 cm^{-1}, respectively. The most important adsorption bands at 1550 cm^{-1} was representing —N—H bending which is due to grafted GA on the magnetic beads surfaces. The Fe_3O_4 molecule has the characteristic band at around 600 cm^{-1} and also this indicates that Fe_3O_4 molecules are successfully formed within the beads structure. This was also confirmed by gravimetric analysis and the amount of precipitated iron oxide crystal in the polymer structure was 116 mg g^{-1} beads.

Scanning electron microscopy (SEM) micrographs presented in Figure 3 shows the porous surfaces structure of the magnetic beads. The beads have a spherical form and rough surface due to the pores, which formed during the polymerization process. The porous surface structure should be considered as a factor providing an increase surface area. In addition, these pores reduce the mass transfer resistance and facilitate the diffusion of metal ions because of high internal surface area with low diffusional resistance in the magnetic beads (imply high adsorption capacity and rate).

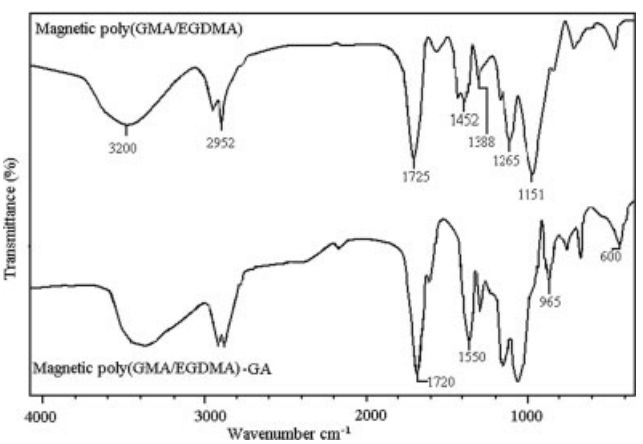

Figure 2 FTIR spectrum of the magnetic poly(GMA/EGDMA) and poly(GMA/EGDMA)-GA beads.

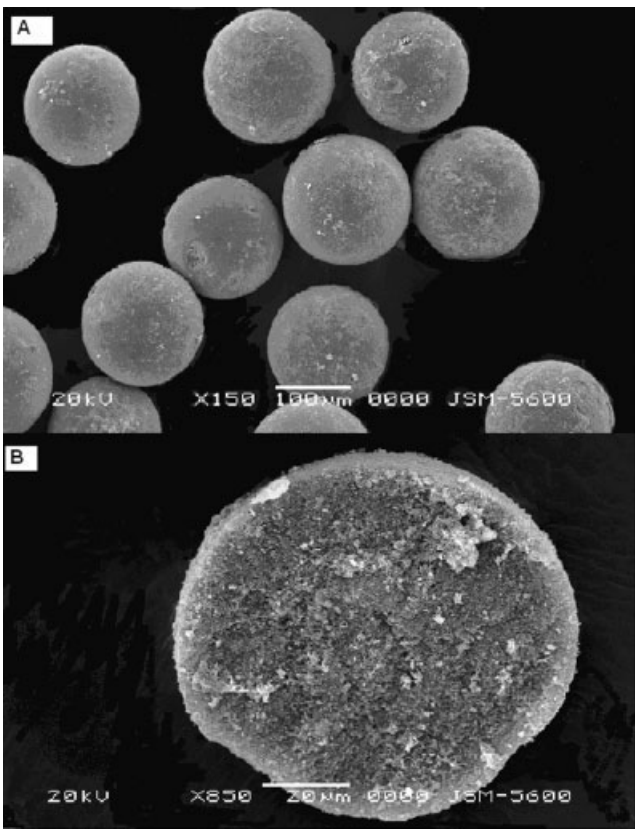

Figure 3 SEM micrograph of the magnetic poly(GMA/EGDMA)-GA beads: A: surface picture: magnification ×150; B: the intersection picture for inside pores of magnetic beads: ×850 magnifications.

The water content is very important when use of the support material in chromatographic application is contemplated. The magnetic poly(GMA/EGDMA) beads are hydrophilic polymer networks capable of imbibing large amounts of water yet remain insoluble and preserve their three dimensional shape. The equilibrium-swelling ratio of the magnetic beads was 39.2%.

Effect of medium pH and ionic strength on adsorption

To pH effects of the trypsin adsorption efficiency and capacity on the magnetic poly(GMA/EGDMA) were investigated between pH 4.0–8.0 and presented in Figure 4. As seen in this figure, pH is an important parameter for the adsorption of trypsin on the ion-exchange magnetic beads. Trypsin adsorption on the magnetic ion-exchange beads is greater at neutral pH and decreases with increasing and/or decreasing pH. The functional carboxyl groups of the GA on the magnetic beads are considered active sites for the adsorption of trypsin. The pI value of trypsin is 10, therefore, trypsin molecules have net positive charges when medium pH is less than 10.0. Whereas, the carboxylic groups of the GA has net negative charge above its pI value of 2.82. At around pH 7.0 the electrostatic interaction between protein and ion-exchange adsorbent should be predominant. Thus, depending on the solution pH, the amine groups of the trypsin molecules can undergo protonation to NH_3^+ and the extent of protonation will be dependent on the solution pH. The protonated amino groups on the surface of the trypsin will determine the type of electrostatic interaction between the magnetic beads and the trypsin molecules.[40–42]

It appears that as the amount of salt increases, the adsorption efficiency of trypsin decrease with increasing ionic strength though the decrease is insignificant at lower ionic strength (Fig. 5). It is a typical feature of ion exchange adsorption. Under different ionic strength conditions, significant conformational changes that occur within trypsin itself. Thus, the addition of salt should screen these favorable interactions and destabilize the native state. These destabilizations may cause hindrance of ionic binding sites between the carboxylic groups of GA and the enzyme, which may lead to lower adsorption of the trypsin to the magnetic beads. In addition, the ionic interactions between the GA ligand and the enzyme decrease with increasing ionic strength due to the Debye-Hückel screening effect.[43] At different salt concentration, the differences in the binding behavior may be attributed more than one option, for example ion-exchange effect, ligand/enzyme conformation or solvation can all play a role. Several researchers have reported similar phenomenon for different protein/adsorbent pairs.[44–48]

Effect of temperature and contact time

The effect of temperature on trypsin adsorption was studied in the range of 4–37°C. The equilibrium

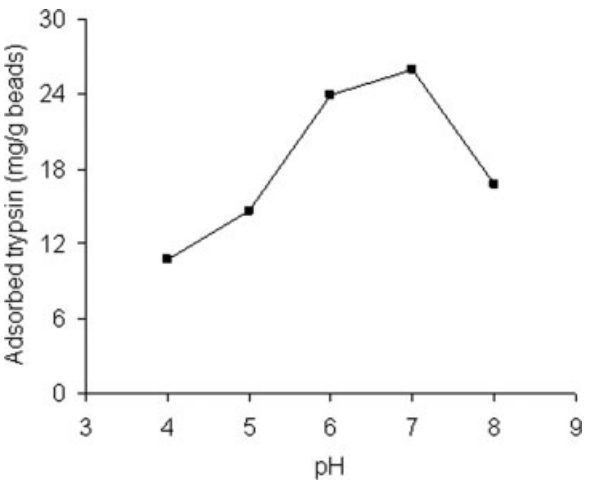

Figure 4 Effect of pH on the trypsin adsorption onto magnetic beads; Initial concentration of trypsin: 0.5 mg mL^{-1}; Volume of the medium: 5 mL; Temperature: 25°C.

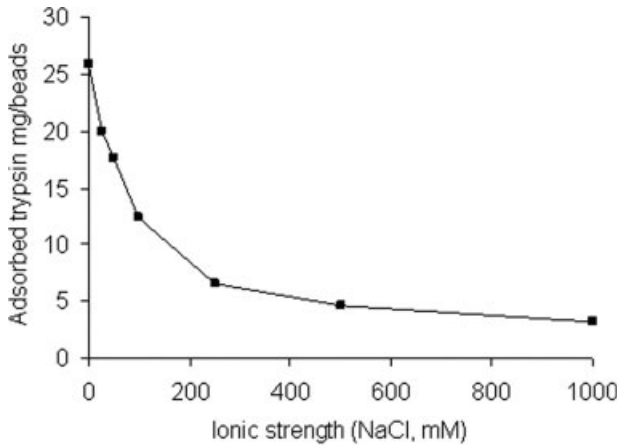

Figure 5 Effect of ionic strength on the adsorption system; Initial concentration of trypsin: 0.5 mg mL^{-1}; Volume of the medium: 5 ml; Amount of ion-exchange beads: 50 mg.

adsorption of trypsin onto the magnetic poly(GMA/EGDMA)-GA significantly increased with increasing temperature and the maximum adsorption was achieved at 37°C (Fig. 6). From 4°C to 37°C, the adsorption capacity of the magnetic beads increased for about 77% for poly(GMA/EGDMA)-GA. This indicated that the increase in the temperature was favorable to trypsin adsorption on GA grafted magnetic beads. In trypsin adsorption experiments contact time was determined for magnetic poly(GMA/EGDMA)-GA at different temperature. Enhancement of adsorption capacity at higher temperatures may be attributed to enlargement of pore size and/or activation of the adsorbent surface.[43] As seen from the Figure 6, trypsin adsorption rate is high at the beginning of adsorption at different temperatures and saturation levels were completely reached at about 120 min. After this period, the amount of adsorbed trypsin did not change significantly with time.

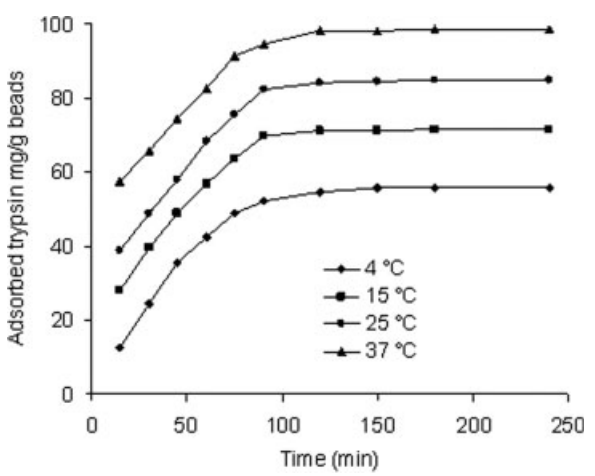

Figure 6 Effect of temperature and contact time for adsorption of trypsin on the magnetic poly(GMA/EGDMA)-GA beads. Initial concentration of trypsin: 0.5 mg mL^{-1}; Volume of the medium: 5 mL; pH: 7.0; Amount of ion-exchange beads: 50 mg.

Effect of initial trypsin concentration

As shown in Figure 7, the adsorption capacity increased with increasing initial trypsin concentration in the medium. As expected, an increase in the adsorption capacity of the magnetic beads after GA grafting was observed for trypsin. The maximum adsorption capacities of trypsin on the magnetic poly (GMA/EGDMA) and poly(GMA/EGDMA)-GA beads were 1.82 and 84.96 mg g^{-1} dry beads, respectively. GA grafting caused in increase in the adsorption capacity of the magnetic beads about 46.7 folds compared to the plain poly(GMA/EGDMA) beads. As seen in figure, the experimental trypsin adsorption isotherm is very steep at low trypsin concentration and reached at a plateau about 2.0 mg mL^{-1} initial trypsin concentrations.

Evaluation of adsorption isotherm models and thermodynamic parameters

Two important physico-chemical aspects for evaluation of the adsorption process as a unit operation are the kinetics and the equilibrium of adsorption. Some model parameters were determined by nonlinear regression with commercially available software and are shown in Table I. From the comparison of correlation coefficients, it was found that the data were fitted better by Langmuir equation than by Freundlich equation for trypsin adsorption on the magnetic beads (Figure 8). The maximum adsorption capacity (q_m) was calculated to be 90.91 mg g^{-1} at 25°C.

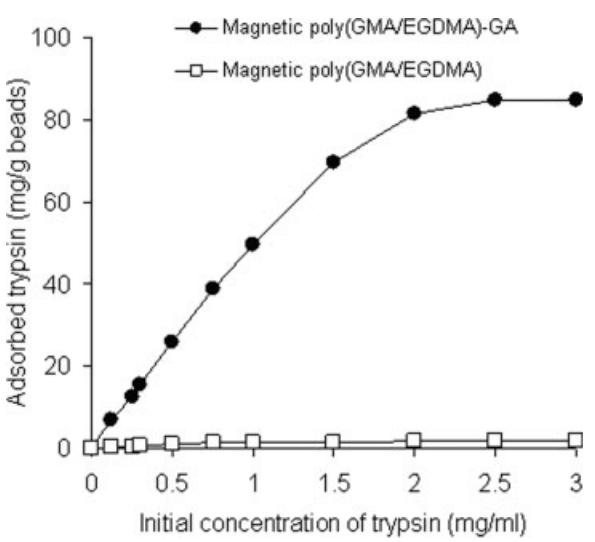

Figure 7 Effect of initial trypsin concentration on the trypsin adsorption; Volume of the medium: 5 mL; Temperature: 25°C; pH: 7.0; Amount of ion-exchange beads: 50 mg.

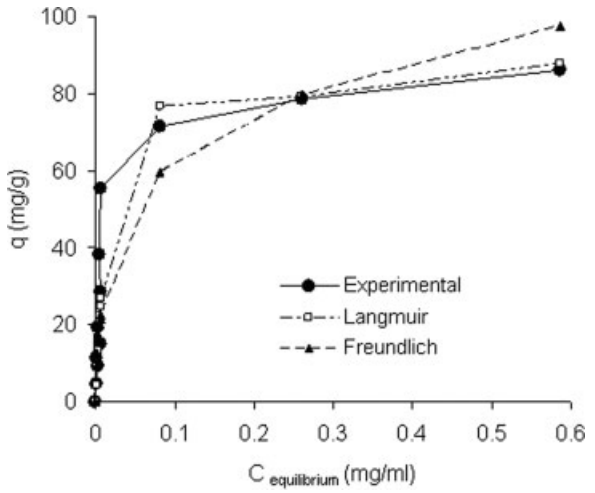

Figure 8 Comparison of the equilibrium experimental and the adsorption isotherms obtained from the Langmuir and the Freundlich models for trypsin adsorption on the magnetic poly(GMA/EGDMA)-GA beads.

The thermodynamic parameters for the adsorption system at different temperatures are tabulated in Table I. The negative values of ΔG° indicate that the adsorption of trypsin on the magnetic beads is feasible and spontaneous. The Gibbs energy of the interactions demonstrated that the processes are favorable for the formation of electrostatic interaction and/or protein–adsorbent complexes. The magnitude of ΔG^{o} for the adsorption of trypsin on adsorbent increased with the ascend temperature. ΔH^{o} for the adsorption of trypsin onto GA grafted magnetic beads was found to be 10.64 kcal mol^{-1} while ΔS^{o} was 61.84 cal mol^{-1} K. The positive value of ΔH^{o} shows that the trypsin adsorption is an endothermic process. The positive value of ΔS^{o} indicates that the randomness increases at the solid/solution interface during the adsorption of trypsin on the magnetic poly(GMA/EGDMA)-GA beads.

Evaluation of kinetic models

The comparison of experimental adsorption capacity and the theoretical value estimated from the first and second order equations are presented in Table II. The theoretical q_{eq} value estimated from the first order kinetic model gave significantly different value compared to experimental value, and the correlation coefficient was also found to be lower. These results showed that the first order kinetic model is improper for poly(GMA/EGDMA)-GA beads. On the other hand, the correlation coefficients for the pseudo-second order kinetic model are higher than the pseudo-first order kinetic model for all cases. These results suggest that the second order mechanism is predominant and that chemisorption might be the rate-limiting step that controls the adsorption process. The rate-controlling mechanism may vary during the course of the adsorption process three possible mechanisms may be occurring.[44] There is an external surface mass transfer or film diffusion process that controls the early stages of the adsorption process. This may be followed by a reaction or constant rate stage and finally by a diffusion stage where the adsorption process slows down considerably.[43]

Intra-particle diffusion is characterized by dependence between the adsorption capacities at any reaction time, q_t, and the square root of time, with the slope of the relationship being the intra-particle diffusion rate parameter expressed in Eq. (5). The value of the intra-particle diffusion rate constant was found to be 3.18 mg g^{-1} $min^{0.5}$. The relationships are not linear for the entire range of reaction time (data not shown). This nonlinearity has been reported previously[44,45] and has been explained in terms of both diffusion processes having an effect on the adsorption. The relationships yield a straight line passing through the origin for the beginning part, and this was more likely to occur at lower initial trypsin concentrations. It may be concluded that the rate limiting step is intra-particle diffusion in the initial period of the reaction.

The result of the conventional batch study showed that more than 50% of tripsin had been adsorbed on the solid support within 30 min. Dispersion coefficient, D, was determined to be 9.07×10^{-3} cm^3 g^{-1} s^{-1} at 120 min with an initial concentration of trypsin 3.0 mg mL^{-1} in the adsorption test.

Adsorption performance of ion-exchange beads for BSA

BSA adsorption was measured at two different pH values (i.e. 7.0 and 4.0). At pH 7.0, the surface

TABLE II
The First-Order and Second-Order Kinetics Constants for Adsorption of Tyripsin on the Glutamic Acid Grafted Magnetic Beads

	Experimental	First-order kinetic			Second-order kinetic		
Temperature (K)	q_{exp} (mg g^{-1})	$K_1 \times 10^2$ (min^{-1})	q_e (mg g^{-1})	R^2	$K_2 \times 10^4$ (g mg^{-1} min^{-1})	q_e (mg g^{-1})	R^2
277	67.01	6.55	158.6	0.926	5.21	76.93	0.995
288	78.01	5.93	136.0	0.889	7.91	81.97	0.989
298	84.96	3.20	174.5	0.905	8.89	90.91	0.993
310	96.67	8.75	156.3	0.961	15.10	101.73	0.991

carboxyl groups of the magnetic beads have been deprotonated, and the magnetic beads have net negative charges. Since the medium pH also affects the charging state of the protein, the ion-exchange interactions between the protein and the magnetic beads must be change with the medium pH. BSA has an isoelectric point (pI) 4.9. At neutral pH, BSA has net negative charges on the other hand, at acidic pH 4.0, BSA has net positive charges on its surface. Therefore, BSA was effectively adsorbed (23.13 mg g^{-1}) at pH 4.0 below its pI value. On the other hand, BSA was not significantly adsorbed (1.34 mg g^{-1} beads) at pH 7.0. From these observations, there should be an ion-exchange interaction between the surface interactions groups of BSA and the negatively charged magnetic beads. It should be noted that, the negatively charged carboxyl groups on the magnetic beads surface reduced the adsorption capacity of the acidic protein BSA compared to the basic protein trypsin (84.96 mg g^{-1} beads) under same experimental conditions. The difference in adsorption capacities of magnetic beads for trypsin and BSA should be due to the difference in molecular size and the orientation of the surface amino acid residues of these model proteins.

Elution and regeneration

The use of an adsorbent in the chromatographic area depends not only on the adsorptive capacity, but also on how well the adsorbent can be regenerated and used again. For repeated use of an adsorbent, adsorbed protein should be easily desorbed under suitable conditions. The desorption of adsorbed trypsin from the poly(GMA/EGDMA)-GA beads was studied in a batch system. The trypsin adsorbed magnetic beads were placed within the desorption medium containing 0.5*M* GA solution at pH 4.0, and the amount of trypsin released in 120 min was determined. For all the tested adsorbents more than 97% of the adsorbed trypsin was desorbed. To show the reusability of the poly(GMA/EGDMA)-GA beads, adsorption-desorption cycle of trypsin was repeated six times by using the same poly(GMA/EGDMA)-GA beads. The adsorption capacity of the ion exchange beads decreased only 5% after six times use in the repeated adsorption–desorption operations.

CONCLUSIONS

Magnetic separation technologies have been applied in diverse field of the separation technology. In this study, the magnetic poly(GMA/EGDMA) beads were prepared via suspension polymerization. GA was then grafted onto poly(GMA/EGDMA) beads via epoxy ring opening reaction. One of the objectives for immobilization of GA on the magnetic beads was to expose carboxylic groups on the surface in order to cerate ion-exchange binding sites for basic protein molecules. Furthermore, it is expected that the covalently grafted GA can be allowed regeneration and repeated use in batch and/or a column process. The magnetic analysis showed a high magnetic responsiveness in magnetic field, and no aggregation of the particles was observed after the particles had been treated in the magnetic field so it's called superparamagnetic. The magnetic beads were evaluated for a model protein ''trypsin'' by obtaining equilibrium adsorption data at different pH, ionic strength and temperatures. Results of batch equilibrium tests indicated that Langmuir isotherm describes well the trypsin adsorption process. The adsorption process has been found to be endothermic for trypsin (enthalpy change, ΔH^o = 10.64 kcal mol^{-1}). Finally, the GA grafted magnetic beads can be useful candidate as an ion-exchange adsorbent in the separation of basic protein from biological fluids.

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