New Generation Polymeric Nanospheres for Lysozyme Adsorption

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ABSTRACT: Novel hydrophobic nanospheres with an average size of 100 nm utilizing *N*-methacryloyl-(l)-tryptophan methyl ester (MAT) as a hydrophobic monomer were prepared by surfactant free emulsion polymerization of 2-hydroxyethyl methacrylate (HEMA) and MAT. MAT was synthesized using methacryloyl chloride and l-tryptophan methyl ester. Specific surface area of the nonporous nanospheres was found to be 1914 m^2/g. Poly(HEMA–MAT) nanospheres were characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). Average particle size, size distribution, and surface charge measurements were also performed. Elemental analysis of MAT for nitrogen was estimated as 1.95 mmol/g polymer. Then, poly (HEMA–MAT) nanospheres were used in the adsorption of lysozyme in batch system. Using an optimized adsorption protocol, a very high loading of 1075 mg lysozyme/g nanosphere was obtained. The adsorption phenomena appeared to follow a typical Langmuir isotherm. It was observed that enzyme could be repeatedly adsorbed and desorbed without significant loss in adsorption amount or enzyme activity. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1608–1615, 2010

Key words: nanobiotechnology; nanospheres; lysozyme; adsorption; hydrophobic ligand

INTRODUCTION

Nanotechnology[1] is enabling technology that deals with nano-meter sized objects. Nanobiosystem science and engineering is one of the most challenging and fastest growing components of nanotechnology. It is essential for better understanding of living systems and for developing new tools for medicine and solutions for health care (such as synthesis of new drugs and their targeted delivery, regenerative medicine, and neuromorphic engineering). One important challenge is understanding the processes inside cells and neural systems. Nanobiosystems are sources of inspiration and provide models for man-made nanosystems. Research may lead to better biocompatible materials and nanobiomaterials for industrial applications. The confluence of biology and nanoscience will contribute to unifying concepts of science, engineering, technology, medicine, and agriculture.[2] The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties.[3] Now they have entered a commercial exploration period.[4–6]

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Nanosized particles can produce larger specific surface areas and, therefore, may result in high immobilization capacity for biomolecules. Therefore, it may be useful to synthesize nanosized particles with large surface areas and utilize them as suitable carriers for the immobilization of enzymes.[7] Relatively small size of carrier materials can generally improve the efficiency of immobilized enzymes. Various nanostructures, generally providing a large surface area for the immobilization/separation of biomolecules, have been actively developed for enzyme stabilization. It is leading to higher enzyme loading per unit mass of particles.[8] In the case of enzyme immobilization into porous materials, much reduced mass-transfer resistance is expected for smaller porous particles owing to the shortened diffusional path of substrates when compared to large-sized porous materials. Overall, nanoparticles provide an ideal remedy to the usually contradictory issues encountered in the optimization of immobilized enzymes: minimum diffusional limitation, maximum surface area per unit mass, and high enzyme loading.[9]

There are many approaches to improve the enzyme stability, such as enzyme immobilization, enzyme modification, and protein engineering. Among these enzyme immobilization represents the attachment of enzyme molecules onto solid phases, via adsorption,[10–16] covalent attachment,[17,18] or encapsulation.[19,20] Adsorption would also be

advantageous in any case, improving the control of the reactor, permitting reuse of the enzyme, and avoiding protein contamination of the final product, which as a result of the high temperatures may promote some colored compounds that make processing of the final product difficult. However, noncovalent adsorption techniques, such as hydrophobic adsorption of the enzyme on hydrophobic adsorbent may be a good option because adsorption is very simple and produces very little work and time consumption. In addition to this, the supports may be reused after desorption of the inactivated enzyme, and, by this way, reduce the final price and generate less residues. However, scarce work is found referring to the reversible hydrophobic adsorption.

In this article, poly(HEMA)-based matrix containing a hydrophobic group as a support matrices have been prepared in the nanosphere form by polymerization of 2-hydroxyethyl methacrylate (HEMA) and *N*-methacryloyl-(l)-tryptophan methyl ester (MAT). Poly(HEMA–MAT) nanospheres were prepared by surfactant free emulsion polymerization. The nanospheres were used for the adsorption of lysozyme via adsorption. The system parameters, such as effect of the adsorption conditions (i.e. enzyme concentration, medium pH, and temperature) and the reusability of the support were studied on the lysozyme adsorption.

MATERIALS AND METHODS

Chemicals

HEMA (Sigma Chem., St. Louis, USA) and ethylene dimethacrylate (EDMA, Aldrich, Munich, Germany) were distilled under vacuum (100 mmHg). Lysozyme (chicken egg white, EC 3.2.1.7) was supplied from Sigma Chemical (St. Louis, USA) and used as received. All other chemicals were of the highest purity commercially available and were used without further purification. All water used in the experiments 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.

Synthesis of poly(HEMA–MAT) nanospheres

Preparation of *N*-methacryloyl-(l)-tryptophan methyl ester (MAT) had been reported in our previous report.[8] The poly(HEMA–MAT) nanospheres with an average particle size of 100 nm with a particle size distribution (as polydispersity index, PDI) of 1.158 were produced by surfactant free emulsion polymerization. In a typical polymerization procedure may be summarized as follows: The stabilizer, PVAL (0.5 g), was dissolved in 50 mL deionized water for the preparation of the continuous phase. Then, the comonomer mixture was added to this dispersion, which was mixed in an ultrasonic bath for about half an hour. Before polymerization, initiator was added to the solution and nitrogen gas blown through the medium for about 1–2 min to remove dissolved oxygen. Polymerization was carried out in a constant temperature shaking bath at 70°C, under nitrogen atmosphere for 24 h. After the polymerization, the polymeric nanospheres were cleaned by washing with methanol and water several times to remove the unreacted monomers. For this purpose, the nanospheres were precipitated at the rate of 18,000 g for 2 h in a centrifuge (Zentrifugen, Universal 32 R, Germany), the collected precipitate was resuspended in methanol and water several times. After that poly(HEMA–MAT) nanospheres were further washed with deionized water. Poly(HEMA) nanospheres were produced by same formulation without MAT comonomer. In this study poly(HEMA–MAT) nanospheres having the size range of 100 nm were used as a solid matrix for the immobilization of lysozyme. Figure 1 shows that the hypothetic structure of poly(HEMA–MAT) nanospheres.

Characterization of poly(HEMA–MAT) nanospheres

FTIR spectra of the nanospheres were obtained by using FTIR spectrophotometer (Varian FTS 7000, USA). The dry nanospheres (about 0,1 g) were thoroughly mixed with KBr (0,1 g, IR Grade, Merck, Germany), and pressed into a tablet form, and the spectrum was then recorded.

The particle size, the size distribution, and the surface charge were determined by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England).

The surface morphology of the poly(HEMA–MAT) nanospheres was examined using SEM. The samples were initially dried in air at 25°C for 7 days before being analyzed. A fragment of the dried bead was mounted on a SEM sample mount and was sputter coated for 2 min. The sample was then mounted in a scanning electron microscope (SEM, Phillips, XL-30S FEG, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the nanospheres.

The degree of MAT incorporation in the synthesized poly(HEMA–MAT) nanospheres was determined by elemental analyser (Leco, CHNS–932, USA).

The surface area of poly(HEMA–MAT) nanospheres was calculated using the following expression[21]:

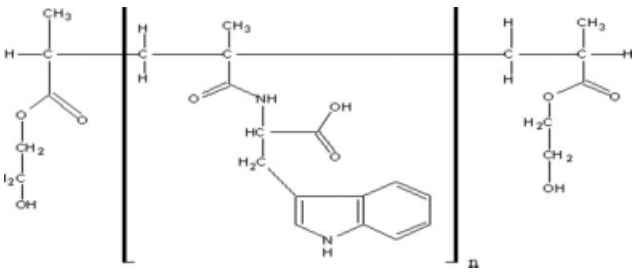

Figure 1 Hypothetic structure of poly(HEMA–MAT) nanospheres.

$$N = 6.10^{10}.S/100.\rho_s.d^3 \quad (1)$$

Here, N is the number of nanospheres per milliliter; S is the % solids; ρ_s is the density of bulk polymer (g/mL); d is the diameter (nm). The number of nanosphere in mL suspension was determined from mass-volume graph of nanospheres. From all these data, specific surface area of poly(HEMA–MAT) nanospheres were calculated by multiplying N and surface area of 1 nanosphere.

Lysozyme adsorption studies

Chicken egg white lysozyme was selected as a model protein. Lysozyme adsorption of the poly (HEMA–MAT) nanospheres were studied at various pH. The pH of the adsorption medium changed between **4.0 and 11.0** by using different buffer system (0.1*M* acetate buffer for pH 4.0–6.0; 0.1*M* phosphate for pH 7.0–8.0; carbonate for pH 9.0–11.0). All pH measurements were made with a digital pH/mVmeter. Lysozyme initial concentration was 0.5 mg/mL in each corresponding buffer. Adsorption experiments were conducted for 120 min at 25°C with continuous stirring. The equilibrium adsorption time was determined to be 120 min with pre-experiments. The effect of temperature on lysozyme adsorption was carried out in phosphate buffer (20 mL, 0.1*M*, **pH: 9.0**) containing 0.5 mg/mL lysozyme at **4–45°C**. The amount of adsorbed lysozyme on the poly(HEMA–MAT) nanospheres was determined by measuring the initial and final concentrations of protein within the adsorption medium using Coomassie Brilliant Blue.[22] A calibration curve was constructed with lysozyme solution of known concentration (0.05–2.0 mg/mL) and was used in the calculation of protein amount. The experiments were performed in replicates of five and the samples were analyzed in replicates of five as well. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples to determine the margin of error.

The lysozyme elution experiments were performed in a buffer containing 1.0*M* NaSCN at pH 7.0. The lysozyme adsorbed poly(HEMA–MAT) nanospheres were placed in a elution medium and magnetically stirred for 1 h at 25°C, at a stirring rate of 100 rpm. The final lysozyme concentration within the elution medium was determined by spectrophotometry. The elution ratio was calculated from the amount of lysozyme adsorbed on the nanospheres and the amount of lysozyme desorbed.

RESULTS AND DISCUSSION

Poly(HEMA–MAT) nanospheres

Nanoparticles provide an ideal remedy to the usually contradictory issues, encountered in the optimization of adsorbed enzymes: minimum diffusional limitations, maximum surface area per unit mass, and high enzyme loading. In addition to the promising performance features, the unique solution behaviors of the nanoparticles also point to an interesting transitional region between heterogeneous and homogeneous catalysis. Theoretical and experimental studies demonstrated that particle mobility, which is governed by particle size and solution viscosity, could impact the intrinsic activity of the particle-attached enzymes.[23]

The enzyme supports are the most frequently obtained after chemical modification of natural and synthetic polymers, such as, chitin, chitosan, cellulose acetate, acrylic polymers, poly(vinyl alcohol), or inorganic glass hollow fibres.[24] In this article we suggest a novel method of introducing a hydrophobic group into the polymeric nanospheres via polymerization of HEMA and MAT. The distinctive feature of this method is the elimination of the activation and ligand coupling steps during the preparation of the affinity matrices. Some other advantages over other methods include the use of a known amount of ligand in the polymer preparation mixture and the good reproducibility of the affinity matrix.[24]

Figure 2 shows the FTIR spectra of poly(HEMA) and poly(HEMA–MAT) nanospheres. As seen in Figure 2, stretching vibration of hydroxyl groups of both poly(HEMA) and poly(HEMA–MAT) nanospheres was observed at 3531 cm^{-1}. The peak in this wavenumber of poly(HEMA–MAT) nanospheres is **sharper** than the peak of the poly(HEMA). It may be possible because the peak also contains N—H stretching vibrations in the poly(HEMA–MAT) nanospheres. The peak around 749.8 cm^{-1} indicates the aromatic characteristic in the MAT structure.These data confirmed that the poly(HEMA–MAT) nanospheres was formed with functional groups MAT.

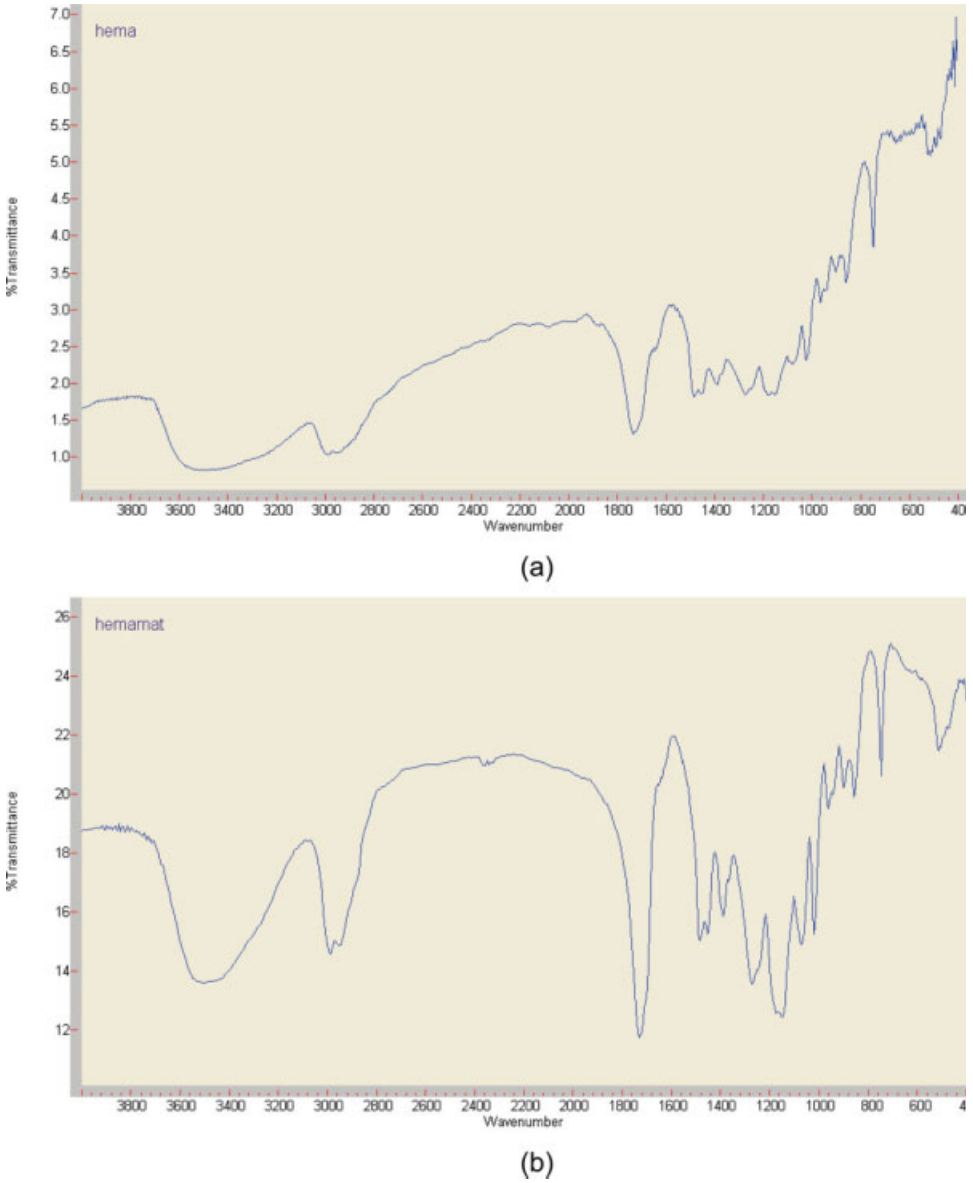

Figure 2 FTIR spectrum of; (a) poly(HEMA), (b) poly (HEMA–MAT) nanospheres. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

As seen in Figure 3, particle size of the poly (HEMA–MAT) nanospheres were measured by Zeta Sizer and about 100 nm with 1.189 polydispersity. The particle size was an average of minimum 30 measurements, and the size distribution was recorded automatically by the software of these repeated measurements.

To evaluate the degree of MAT incorporation into the polymeric structure, elemental analysis of the synthesized poly(HEMA–MAT) nanospheres was performed. The incorporation of the MAT was found to be 1.95 mmol/g polymer using nitrogen stoichiometry.

Nanospheres can produce larger specific surface area and therefore may result in high enzyme loading. Therefore, it may be useful to synthesize

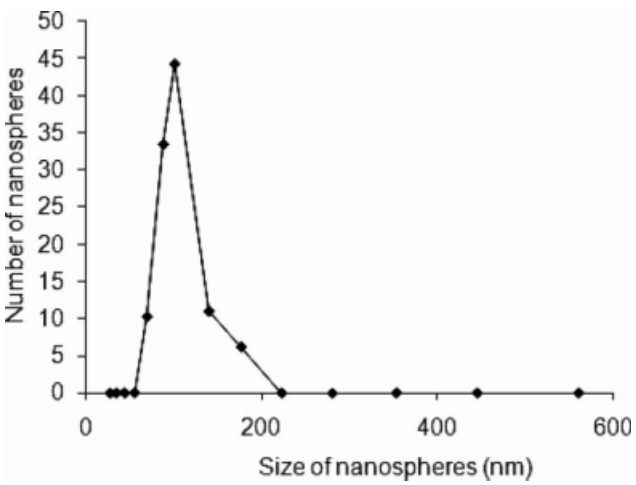

Figure 3 Size analysis of poly(HEMA–MAT) nanospheres.

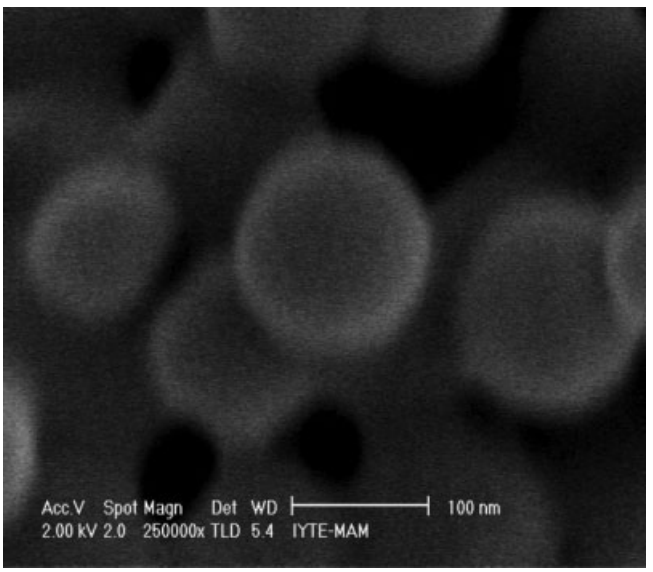

Figure 4 Microscopic observations; SEM micrographs of poly(HEMA–MAT) nanospheres.

nanoparticles with large surface area and utilize them as suitable carriers for the adsorption of enzymes. The specific surface area was calculated as 1856 m^2/g for poly(HEMA) and 1914 m^2/g for poly(HEMA–MAT) nanospheres. As seen in Figure 4, the SEM clearly show the spherical character of poly(HEMA–MAT) nanospheres. In addition, the total monomer conversion was determined as 98.6% (w/w) for poly (HEMA–MAT) nanospheres.

Lysozyme adsorption desorption

Effect of initial concentration of lysozyme

Figure 5 shows the effect of initial lysozyme concentration on adsorption: with increasing lysozyme concentration in solution, the amount of lysozyme adsorbed by the nanosphere increases and approaches saturation. This adsorption isotherm represents a high affinity between lysozyme and MAT groups. It becomes constant when the lysozyme concentration is greater than 1.0 mg/mL. A negligible amount of lysozyme is adsorbed nonspecifically on the poly(HEMA) nanospheres (5.2 mg/mL). Incorporation of MAT groups significantly increases lysozyme adsorption capacity of the nanospheres (upto 1075 mg/mL). This is due to a strong specific interaction between lysozyme and MAT groups.

Langmuir adsorption model

An adsorption isotherm is used to characterize the interaction of each protein with the adsorbents. This provides a relationship between the concentration of protein in the solution and amount of protein adsorbed on the solid phase when the two phases are at equilibrium. The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined, each of which is capable of holding only one molecule. These sites are also assumed to be energetically equivalent, and distant from each other so that there no interactions between molecules adsorbed on adjacents sites.

During the experiments, adsorption isotherms were used to evaluate adsorption properties. For the system considered, the Langmuir model was found to be applicable in interpreting lysozyme adsorption by poly(HEMA–MAT) nanospheres. The Langmuir adsorption isotherm is expressed by equation (2). The corresponding transformations of the equilibrium data for lysozyme gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{max}.b.C_{eq}/(1 + b.C_{eq}) \quad (2)$$

where Q is concentration of bound lysozyme in the adsorbents (mg/g), C_{eq} is the equilibrium lysozyme concentration in solution (mg/mL), b is the Langmuir constant **(0.228)** (1/mg), and Q_{max} **(3087 mg/g)** is the adsorption capacity (mg/g).

Figure 6 shows the linear representation of Langmuir equation. The correlation coefficient (R^2) was 0.991 for poly(HEMA–MAT) nanospheres, indicating that the Langmuir adsorption model can be applied in this affinity adsorbent system.

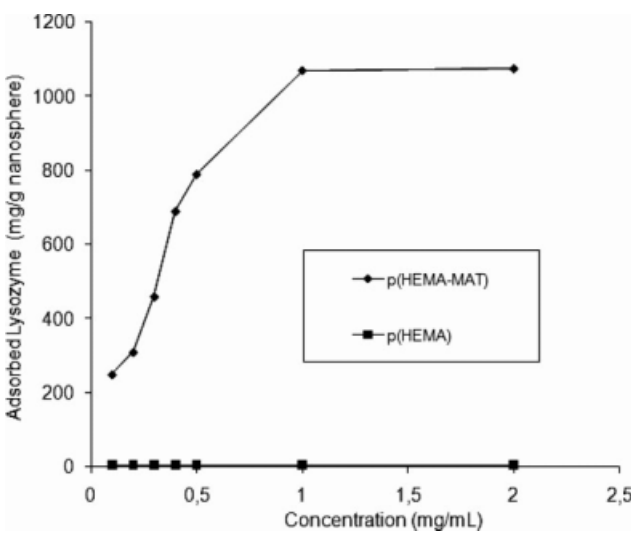

Figure 5 Lysozyme adsorption on poly(HEMA–MAT) nanospheres as a function of lysozyme concentration; MAT loading: 1.95 mmol/g, pH: 9.0, T: 25°C, Each data point represents the average of five runs.

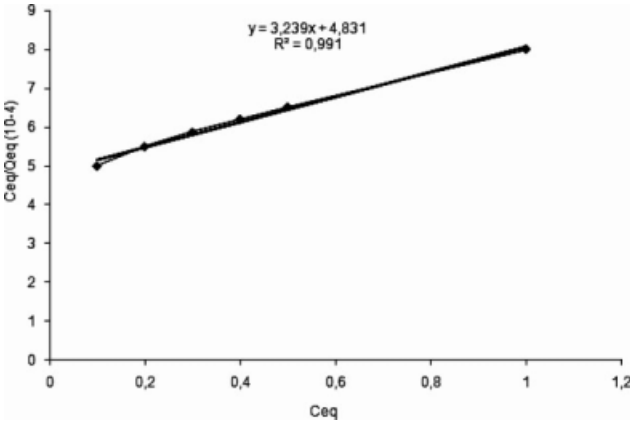

Figure 6 Linear representation of Langmuir equation of lysozyme with poly(HEMA–MAT) nanospheres; MAT loading: 1.95 mmol/g polymer, *T*: 25°C.

Effect of pH

The effect of pH on the adsorption of lysozyme onto poly(HEMA–MAT) nanospheres was studied in the pH range **4.0–11.0** and the effects of pH on adsorption are presented in Figure 7. The decrease in the protein adsorption capacity in more acidic and more alkaline regions can be attributed to electrostatic repulsion effects between the opposite charged groups. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points. The isoelectric pH of lysozyme is 11. In this study, the maximum adsorption pH is shifted to 9.0. Notably the affinity between lysozyme and MAT group containing nanospheres stems primarily form hydrophobic interaction. The conformational changes of lysozyme molecules due to specific interactions at this pH may also contribute to the specific interactions.

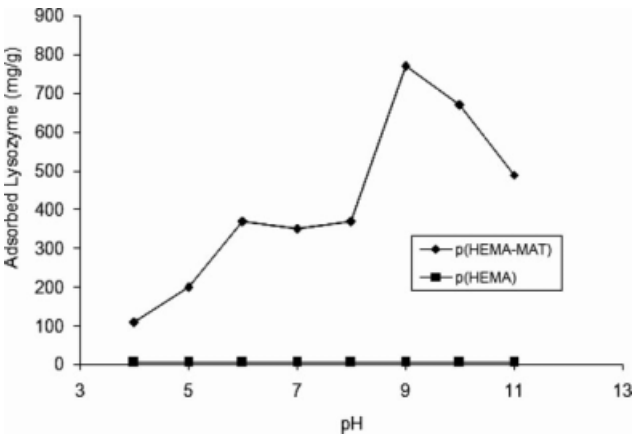

Figure 7 Variation of lysozyme adsorption capacities of the poly(HEMA–MAT) nanospheres as a function of pH; MAT loading:,1.95 mmol/g polymer, Initial lysozyme concentration: 0.5 mg/mL, *T:* 25°C, Each data point represents the average of five runs.

A point worth noting that there was a low non-specific lysozyme adsorption (5.2 mg/g). There are no reactive binding groups or binding sites onto poly(HEMA) nanospheres, which interact with lysozyme molecules. Hence, this non-specific adsorption may be due to weak interactions (van der Waals interaction and hydrogen binding) between lysozyme and hydroxyl groups on the surface of poly (HEMA) nanospheres. On the other hand, much higher adsorptions were observed when the poly (HEMA–MAT) nanospheres were used (1075 mg/g) due to the MAT reactive groups on the nanospheres. MAT is a hydrophobic comonomer and aromatic–hydrophobic (π–π) interactions are possible.

Effect of temperature

The effects of temperature in hydrophobic interaction may also be important especially. At higher temperatures during the unfolding process, the proteins expose buried hydrophobic amino acid residues on the surface. Thus, the contact area between the protein and the hydrophobic groups of the matrix should increase, resulting in an increase in the hydrophobic interaction of proteins for the adsorbent at a higher temperature. The effect of temperature on the lysozyme adsorption amount was carried out with poly(HEMA–MAT) nanospheres, and the results are presented in Figure 8. An increase in the temperature (from 4 to **45°C**) causes increasing the adsorption amount of lysozyme. An increase in the adsorption amount of the nanosphere for lysozyme adsorption at a higher temperature indicates that the binding between the protein and the MAT group of the nanosphere is mainly hydrophobic. During the adsorption process at 45°C, the lysozyme lost about 25.0% of its initial specific activity, and the remainder of the adsorption study was therefore carried out at 25°C and pH 9.0.

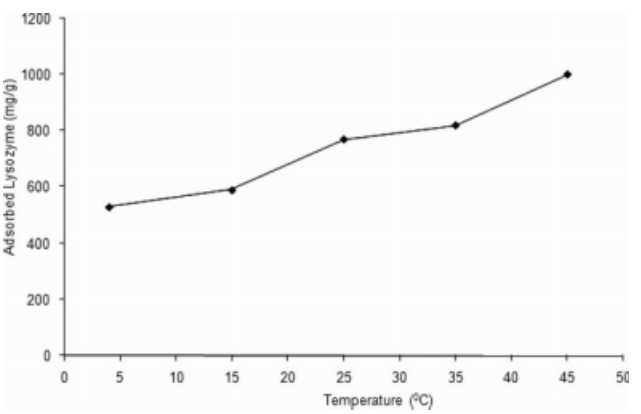

Figure 8 The effect of the temperature on poly(HEMA–MAT) nanospheres; MAT content: 1.95 mmol/g; lysozyme concentration: 0.5 mg/mL; pH 9.0. Each data point represents the average of five runs.

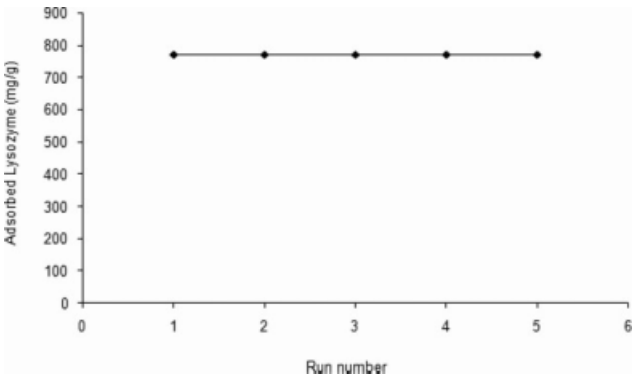

Figure 9 Repeated use of poly(HEMA–MAT) nanospheres; MAT content: 1.95 mmol/g; lysozyme concentration: 0.5 mg/mL; pH 7.0; *T:* 25°C. After washing with 2.0*M* NaCl and elution with 1.0*M* NaSCN, the nanospheres re-equilibrated and used again. Each data point represents the average of five runs.

Elution and stability of poly(HEMA–MAT) nanospheres

From the lyotropic series, SCN^- is a chaotropic anion, which could enhance protein elution. About 90% desorption by NaSCN were observed. With the elution data given earlier we concluded that NaSCN is a suitable elution agent and allows repeated use of the affinity adsorbents prepared in this study.

For practical applications, the stability of the prepared hydrophobic nanospheres is very important. To show the reusability of the poly(HEMA–MAT) nanospheres, we repeated adsorption–desorption cycle of lysozyme five times with the same nanospheres. As shown in Figure 9, the adsorption capacities for the nanospheres did not noticeably change during the repeated adsorption–desorption operations.

To evaluate the effects of the adsorption conditions on the lysozyme structure, fluorescence spectrophotometry was used. The fluorescence spectra of lysozyme sample obtained from the desorption step was recorded. The fluorescence spectra of native and heat-denaturated lysozyme (at 72°C) were also recorded. A clear difference was observed between the fluorescence spectra of native lysozyme and heat-denaturated lysozyme. An appreciable shift was seen in the maximum wavelength of denaturated lysozyme with respect to the native one. However, the fluorescence spectra of the sample withdrawn from the desorption step was very close to those of native lysozyme and no significant shift of the maximum wavelength was detected in the spectra of these samples in comparison with that of native lysozyme. It may be concluded that poly (HEMA–MAT) nanospheres can be applied to lysozyme adsorption without any conformational changes or denaturation.

CONCLUSION

Various nanostructures, generally providing a large surface area for the adsorption of enzyme molecules, have been actively developed for enzyme stabilization. Only limited work has been published on the application of nanosized particles in the adsorption of enzymes. Nanosized particles can produce larger specific surface area and, therefore, may result in high adsorption capacity for enzymes. Therefore, it may be useful to synthesize nanosized particles with large surface area and utilize them as suitable carriers for the adsorption of enzymes. Adsorption of enzyme via hydrophobic interaction is based on the association of hydrophobic groups on the surface of proteins with hydrophobic groups on the support surface. The type of hydrophobic group incorporated into the support is an important variable that would influence the adsorption of protein on the support. One of the most important aims of the enzyme technology is to enhance the conformational stability of the enzyme. In this study, hydrophobic tryptophan group containing nanospheres were prepared by polymerization of HEMA and MAT. The adsorption of lysozyme on hydrophobic nanospheres was investigated using various reaction conditions. Higher adsorption amounts were occurred due to the relatively larger surface area of the nanospheres. It has been shown that pH and temperature have important effects on the adsorption equilibrium. Desorption of lysozyme by NaSCN was carried out. To examine the effect of adsorption/desorption conditions on conformational changes of lysozyme molecules, fluorescence spectrophotometry was employed. The properties of the affinity nanosphere seem to provide an adequate approach to lysozyme adsorption based on their hydrophobic properties without causing any denaturation. This suggests that hydrophobic nanospheres are effective carriers for the adsorption of lysozyme, with high adsorbed amounts, almost complete desorption, and good preservation of bioactivity. The tryptophan containing nanospheres revealed good adsorption properties as a nano-support and will be useful in the enzyme adsorption technology.

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