Reactive Red 120 and NI(II) Derived Poly(2-hydroxyethyl methacrylate) Nanoparticles for Urease Adsorption

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ABSTRACT: Non-porous poly(2-hydroxyethyl methacrylate) [p(HEMA)] nanoparticles were prepared by surfactant free emulsion polymerization. The p(HEMA) nanoparticles was about 200 nm diameter, spherical form, and non-porous. Reactive Red 120 (RR 120) was covalently attached to the p(HEMA) nanoparticles and Ni(II) ions were incorporated to attach dye molecules. Urease was immobilized onto RR120-Ni(II) attached p(HEMA) nanoparticles via adsorption. The maximum urease adsorption capacity of RR120-Ni(II) attached p(HEMA) nanoparticles was 480.01 mg g^{-1} nanoparticles at pH 7.0 in phosphate buffer. It was observed that urease could be repeatedly adsorbed and desorbed without significant loss in adsorption amount. K_m values were 21.50 and 34.06 mM for the free and adsorbed enzyme. The V_{max} values were 4 U for the free enzyme and 3.3 U for the adsorbed enzyme. The optimum pH was 25 mM pH 7 phosphate buffer for free and adsorbed enzyme. The optimum temperature was determined at 35°C and 55°C for the free and adsorbed enzyme, respectively. These findings show considerable promise for this material as an adsorption matrix in biotechnological applications. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 39757.

INTRODUCTION

Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. Urease is ubiquitously presents in plants, fungi, and bacteria.[1] In 1926, jack bean (*Canavalia ensiformis*) urease was crystallized by Sumner[2] and these were the first crystals of a characterized enzyme.[3] Jack bean urease has a homohexameric structure assembled as two trimers of α subunits (α_3)[4] each of them has 840 amino acids.[5] The molecular mass of hexamer is suggested to be 545.34 kDa, 12 nickel ion included.[1] The applications of urease in biotechnology are urea content analysis in blood, urine alcoholic beverages, natural water, and environmental wastewaters.[6]

Adsorption of an enzyme onto a solid support is probably the simplest way of preparing immobilized enzymes. However, adsorption is generally not very strong and some of the adsorbed protein will desorb during washing and operation. Thus, immobilization via adsorption requires a strong hydrophobic and/or electrostatic interaction between the enzyme and support. Combined application of dye ligand affinity chromatography and immobilized metal affinity chromatography (IMAC) meet these requirements. Dye ligands are commercially available and inexpensive. They can easily be immobilized, especially on matrices bearing hydroxyl groups. The interaction between dye ligand and proteins can be complex combination of electrostatic, hydrophobic, and hydrogen binding.[7–11] Reactive Red 120 is one of the commonly used dye ligand for protein adsorption. IMAC method exploits the chemical affinity of a target protein toward the metal ions immobilized onto a stationary phase.[12–16] These interactions derive mainly from the coordination of bonds formed between particular side chain groups on the protein surface and metal ions. The divalent metal ions such as Cu(II), Ni(II), and Zn(II) are considered soft Lewis acids and interact with soft Lewis-based such as nitrogen and sulfur as in histidine and cysteine residues.[17]

Interest in using non-porous nanoparticles as support for adsorption of proteins is progressively increasing nowadays.[18–21] These supports have very small size and despite being non-porous, they permit to immobilize over 100 mg protein/g wet particles. Additionally, the most important advantage of non-porous nanoparticles over porous ones is that they have no external diffusion problems.[22]

Studying of urease immobilization is very important, because immobilized urease can be used in biomedical applications for
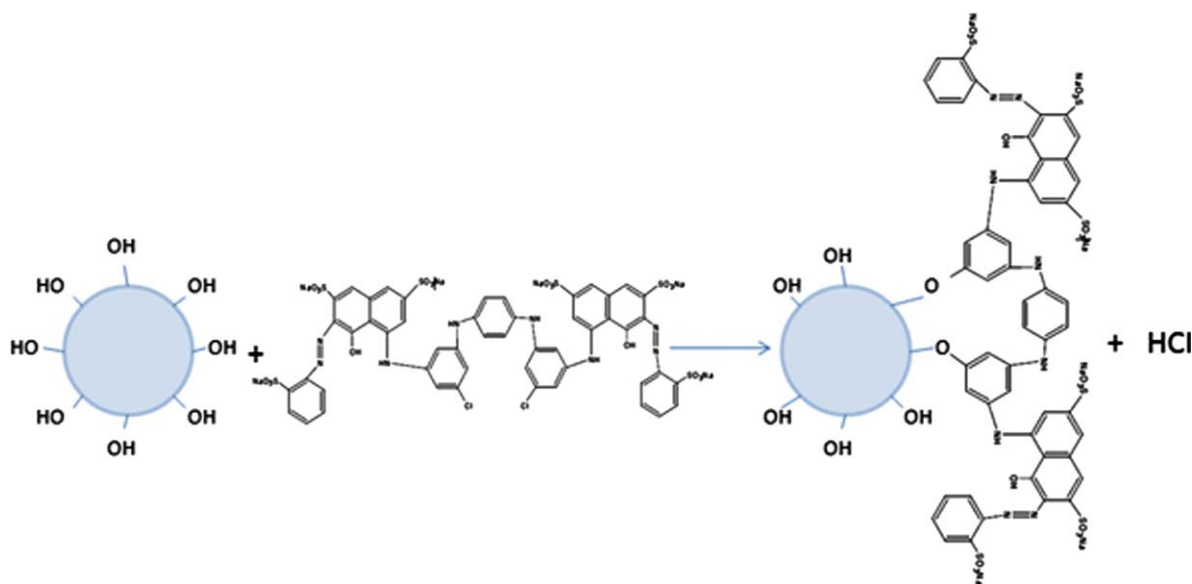
Figure 1. Chemical reaction between Reactive Red 120 and p(HEMA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the removal of urea from blood in artificial kidneys, in food industry, in analytical applications as urea sensor.[23] In this purposes, urease has been widely immobilized to different solid support materials. For example, chitosan-poly(glycidyl methacrylate) copolymer,[24] poly(N-isopropylacrylamide-co-N-acryloxy-succinimide-co-2-hydroxyethyl methacrylate) composite hydrogel membrane,[25] clay matrixes (Laponite and Zn-Al layered double hydroxides),[26] polyamide hollow-fiber membranes,[17] amorphous SiO_2,[27] poly(acrylonitrile) chitosan composite membranes,[23] nanocomposite matrix,[28] nanoporous TiO_2 film,[29] modified acrylonitrile copolymer membranes,[30] supermacroporous poly(N-isopropylacrylamide) cryogels,[31] nanostructured polymer membrane,[32] phosphonate grafted iron oxide nanoparticles,[6] alkyl modified nano-porous silica,[1] functionalized gold nanoparticles,[33] and cellulosic cotton fibers[34] have been used for urease immobilization a support materials.

In this study, RR 120 was covalently attached to the poly(2-hydroxyethyl methacrylate) p(HEMA) nanoparticles. Ni(II) ions were then incorporated to attach dye molecules. The RR120-Ni(II) attached p(HEMA) nanoparticles were used for the immobilization of urease via adsorption. Urease adsorption properties of RR120-Ni(II) attached p(HEMA) nanoparticles from aqueous solutions were investigated at different experimental conditions.

EXPERIMENTAL

Materials
Urease (from Jack beans, Type III, EC 3.5.1.5), HEMA and Reactive Red 120 (RR120) was purchased from Sigma (St. Louis, MO). Ethyleneglycoldimethacrylate (EGDMA) was obtained from Merck (Darmstadt, Germany). Potassium persulfate and poly(vinyl alcohol) (molecular weight: 100,000, 98% hydrolyzed) were supplied by Aldrich (Munich, Germany). All other reagents were of analytical grade and used without further purification. All solutions were prepared with deionized ultra-pure Millipore Simplicity® (18.2 MΩcm) water.

Synthesis of p(HEMA) Nanoparticles
Nanoparticles were synthesized by surfactant free emulsion polymerization. Briefly, the stabilizer, poly(vinyl alcohol) (0.275 g) was dissolved in 25 mL deionized water and added to the glass sealed polymerization reactor. Then, 0.6 mL of HEMA and 0.3 mL of EGDMA were added to this solution. Then 1.98 mg 45 mL potassium persulfate was added to the solution and nitrogen gas blown through the medium for about 1–2 min to remove the dissolved oxygen. Polymerization was carried out in shaking bath at 70°C for 5 h. After the polymerization, the nanoparticles were washed with ethanol and water several times. For this purpose, the nanoparticles were precipitated and collected with the help of a centrifuge at 14,100 × g for 1 h. After that, the p(HEMA) nanoparticles were resuspended in deionized water. The surface morphology of nanoparticles was examined using scanning electron microscopy (SEM, Philips XL-305 FEG, Almelo, The Netherlands). The average nanoparticle size distribution was determined by Zeta Sizer (Malvern Instruments, Model 3000, UK).

Reactive Red 120 Attachment to p(HEMA) Nanoparticles
The hydroxyl groups of the p(HEMA) nanoparticles were derivatized by reaction with RR120. For this purpose, the following experimental procedure was applied: 2.5 mL of 3.0 mg mL^{-1} RR 120 solution in $1M$ NaOH was added onto 7.5 mg p(HEMA) nanoparticles. The medium was heated 80°C in a sealed reactor and vigorously stirred at 400 rpm for 8 h. The dye-attached nanoparticles were washed sequentially with distilled water and ethanol several times until all the unbound dye was removed. Figure 1 shows chemical reaction between Reactive Red 120 and p(HEMA) nanoparticles.

Incorporation of Nickel(II) Ions to Dye Attached p(HEMA) Nanoparticles
Incorporation of Ni(II) Ions to dye attached p(HEMA) nanoparticles were studied with different buffer systems (25 mM, pH 7.0 phosphate and 25 mM, pH 7.0 MES). Then, the effect of
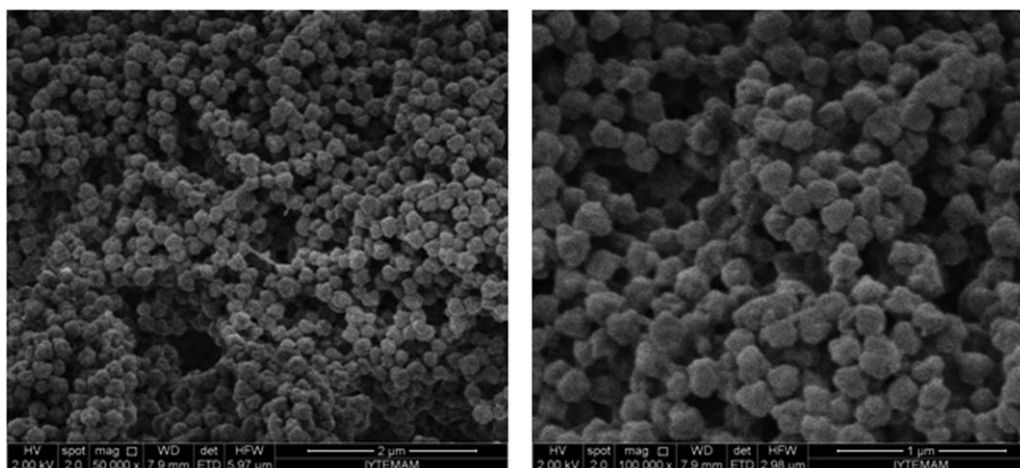
Figure 2. SEM photographs of p(HEMA) nanoparticles.

the Ni(II) concentration on the adsorption of Ni(II) was investigated with appropriate buffer system. For this purposes, Ni(II) concentration was changed between 3.0 and 16.0 mg mL^{-1}. Adsorbed Ni(II) concentration was determined using a spectrophotometric method.[35] In this method, Ni(II) ions were reacted with 4-(2-pyridylaze-resorcinol) to form colored hydrophobic chelates and the absorbance of this complex was read spectrophotometrically at 494 nm.

Immobilization of Urease on the RR120-Ni(II) Attached p(HEMA) Nanoparticles

Immobilization of urease was performed by adsorption. Adsorption of urease was carried out in a batch experimental set up. The adsorption experiments were performed for 2.0 h at 25°C a stirring rate of 120 rpm.

Effects of pH of the medium, urease concentration, and temperature on the adsorption capacity were studied. The adsorption was followed by monitoring the decrease in urease concentration by UV absorbance at 280 nm. The amount of adsorbed urease was calculated by using the following equation:

$$Q = (C_0 - C) \times V/m \tag{1}$$

where Q is the amount of protein adsorbed onto unit mass of the nanoparticles (mg g^{-1}), C_0 and C are the concentrations of the protein solution in the initial solution and in the aqueous phase after adsorption, respectively (mg mL^{-1}), V is the volume of the aqueous solution (mL), and m is the mass of nanoparticles (g). Urease adsorption on the RR120-Ni(II) attached p(HEMA) nanoparticles was investigated at MES buffer (pH 5.0–6.0) and phosphate buffer (pH 6.0–8.0) with 25 mM ionic strength. To evaluate the effects of the initial concentration of urease, urease concentration was changed between 0.2 and 2.0 mg mL^{-1}. To observe the effects of the temperature on the adsorption, adsorption studies were performed at 4, 25, 37, 55°C.

Urease Activity Measurement

After the urease activity reaction, ammonia was obtained in the medium. Ammonium sulfate standards were prepared for activity measurement. Nessler reagent was added to the above standards.

$$NH_2CONH_2 + 2H_2O \xrightarrow{urease} 2NH_4^+ + CO_3^{2-} \tag{2}$$
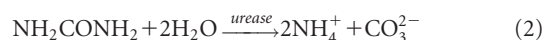
Urease activity reaction observed ammonia was determined by spectrophotometric method in 436 nm with Nessler reagent.[36,37]

Free Enzyme and Adsorbed Enzyme Activity Measurement

0.8 mL urea solution (5–500 mM in phosphate buffer pH 7.0) and 0.2 mL urease 0.076 mg mL^{-1} (in phosphate buffer pH 7.0) were incubated at 25°C for 30 min. Same procedure was used for adsorbed enzyme activity measurement. RR120-Ni(II) attached p(HEMA) nanoparticles were added 0.2 mL above the 0.8 mL urea solution and incubated at 25°C for 30 min. After the enzymatic reaction 250 μL was transferred to another test tube, and added Nessler Reagent and ammonia amounts were determined spectrophotometrically in 436 nm.

Effect of pH and Temperature on Free and Adsorbed Enzyme Activity

The effect of pH on free and adsorbed enzyme activity measurement was investigated in an batch system at 25°C. Urea concentration (50 mM) was prepared in sodium acetate buffer (25 mM) in the pH range 4.0–5.0 and in phosphate buffer (25 mM) in the pH range 6.0–8.0. The effect of temperature was investigated in the range 25–65°C with urea concentration was 50 mM in phosphate buffer (25 mM pH 7.0).

Desorption of Urease from RR120-Ni(II) Attached p(HEMA) Nanoparticles and Reusability of the Nanoparticles

To determine the reusability of the RR120-Ni(II) attached p(HEMA) nanoparticles, the urease adsorption and desorption cycle was repeated five times using the same nanoparticles. Urease desorption from these nanoparticles was performed with 1.0M NaOH solution and stirred magnetically for 2 h at room temperature. The nanoparticles were removed and washed several times with phosphate buffer (25 mM, pH 7.0) and reused in urease adsorption.

RESULTS AND DISCUSSIONS

Characterization of p(HEMA) Nanoparticles

The surface morphology and structure of the synthesized p(HEMA) nanoparticles were investigated by SEM. As seen in Figure 2, the particles were mono sized, spherical form,
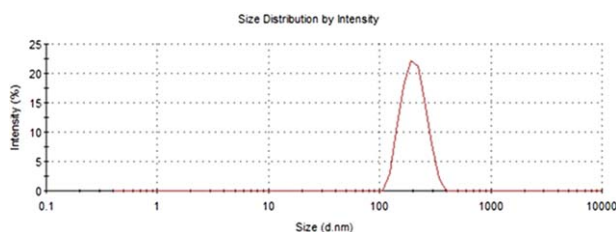
Figure 3. Size analysis of p(HEMA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

non-porous and about 200 nm diameter. Average particle size and size distribution of p(HEMA) nanoparticles were measured by Zeta Sizer and was found about 195.6 nm with 0.043 polydispersity (Figure 3). Nonporous materials, to which enzymes are attached to the surface, are subject to minimum diffusion limitation while enzyme loading per unit mass of support is usually low.[38] On the other hand, in the case of surface attachment, smaller particles provide a longer surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles.[18] As seen in Figure 4, there were FTIR spectrum about p(HEMA), Reactive Red 120 and Reactive Red 120 bonded p(HEMA) nanoparticles. Red line was spectrum of Reactive Red 120, black line was spectrum of p(HEMA), blue line was spectrum of Reactive Red bonded p(HEMA) nanoparticles. Vibration of aryl C═C bonds were from 1324 cm^{-1} to1617 cm^{-1}, 3406 cm^{-1} indicated N─H and phenolic O─H groups. Adsorption band at 1158 cm^{-1} and 1041 cm^{-1} indicated SO_3 asymmetric stretching and S═O vibration. Adsorption band at 3411 cm^{-1} and 2951 cm^{-1} were due to stretching of alcoholic O─H and aliphatic CH_2/CH_3, respectively. Adsorption band at 1158 cm^{-1} and 1250 cm^{-1} were C─O─C band and C─H band, respectively. Major differences were the disappearance of C─Cl adsorption band, decreased intensity of 3411 cm^{-1} band. The other differences were the ratio of 3406 cm^{-1} and 2952 cm^{-1} (blue line) were bigger than 3411 cm^{-1} and 2951 cm^{-1} (black line).[39,40]
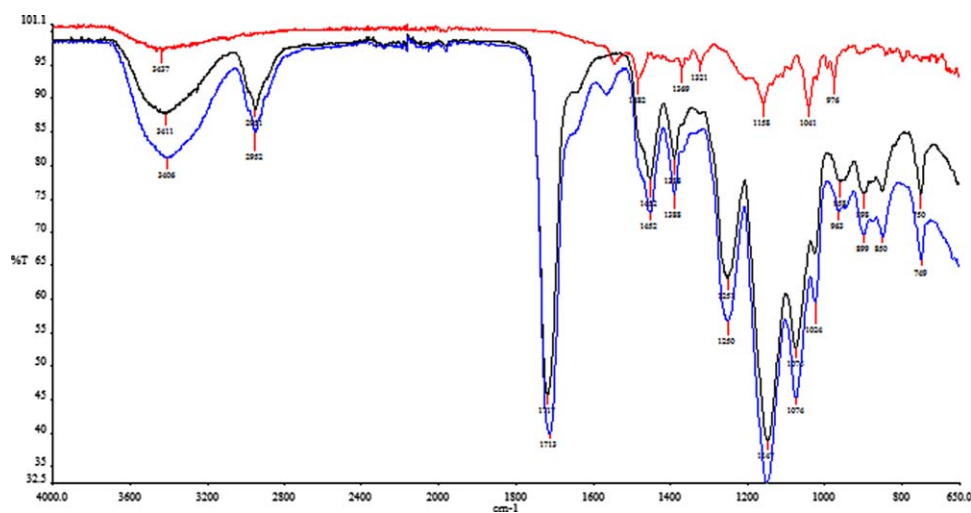
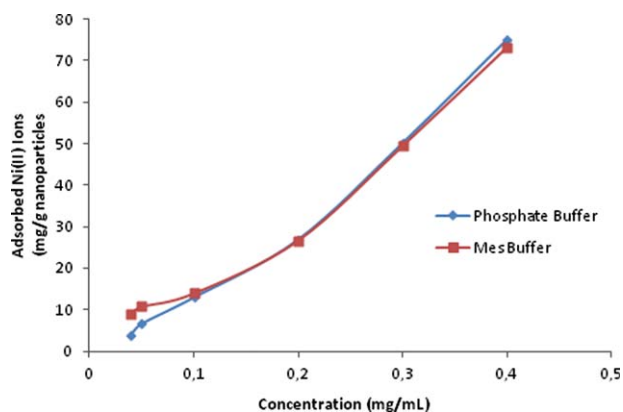## Optimization of Ni(II) Incorporation to Dye Attached Nanoparticles

RR120 is a dichlorotriazine dye and this dye has six sulfonate groups, four secondary amino groups, and two hydroxyl groups. Metal ions bind to this dye ligand by ion-exchange interactions. RR120 molecules were covalently attached into the p(HEMA) nanoparticles and this attachment was macroscopically detected by the color change of nanoparticles from white to red. It is accepted that, ether linkages are formed between reactive triazine ring of the dye and the hydroxyl groups of the p(HEMA) nanoparticles.[40] The RR120 attached nanoparticles were extensively washed to ensure that there was no dye leakage from any of the dye attached nanoparticles and in any media used at adsorption or desorption studies.

Effect of the buffer type on the Ni(II) adsorption capacity was demonstrated in Figure 5. As shown in Figure 5, adsorbed Ni(II) amount did not change significantly when MES or phosphate were used as buffer medium. For this reason, phosphate buffer was selected for Ni(II) adsorption studies.

Figure 6 shows the adsorption capacities of Ni(II) ions on the RR120 attached p(HEMA) nanoparticles as a function of the initial concentration of Ni(II) ions within the aqueous phase. The Ni(II) ion adsorption capacity of RR120 attached p(HEMA) nanoparticles increased with the increasing initial concentration of Ni(II) ion. Above the 14 mg mL^{-1} of Ni(II) concentration Ni(II) adsorption capacity reached a saturation level. The maximum adsorption capacity of the RR120 attached p(HEMA) nanoparticles was 2700 mg g^{-1} for Ni(II) ions. This capacity level may have been due to the presence of functional chelating groups on the dye-attached nanoparticles. The specificity of the metal-chelating ligand (i.e., RR120 molecules) may also contribute to this high adsorption capacity.[41]

## Adsorption of Urease from Aqueous Solutions

Urease adsorption onto RR120-Ni(II) attached p(HEMA) nanoparticles was investigated depending on the buffer system and pH. For this purpose, adsorption studies were carried out using

Figure 4. FTIR analysis of p(HEMA) nanoparticles and Reactive red 120. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
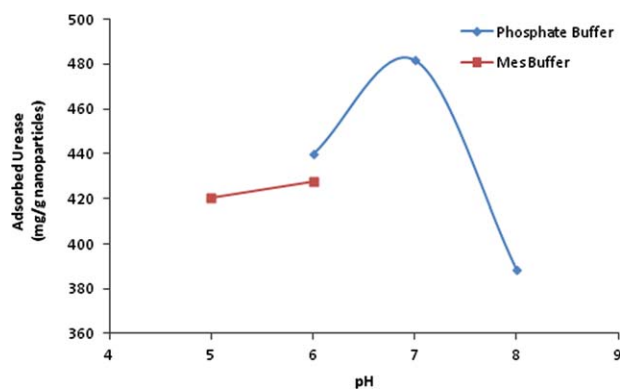
Figure 5. Effect of buffer type on adsorption Ni(II) ions onto RR120 attached p(HEMA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MES (pH 5.0–6.0) and phosphate (pH 6.0–8.0) buffers. Figure 7 shows urease adsorption capacity in these buffer systems at different pH values. The highest urease adsorption capacity was observed at pH 7.0 for phosphate buffer and was found to be 480.01 mg g^{-1} nanoparticles. Proteins have not net charge at their isoelectric points, and, therefore, the maximum adsorption from aqueous solution is usually observed at their isoelectric points. The isoelectric point of Jack bean urease is 4.9.[17] The pH of maximum adsorption was shifted to neutral pH value. Significantly, a lower urease adsorption was found for the RR120-Ni(II) attached nanoparticles in the entire tested pH region. The maximum adsorption at pH 7.0 may results from the formation of coordination complex between deprotonated amino acid side chain groups (i.e., especially imidazole and primary amino groups of histidine and lysine residues, respectively) of urease and incorporated Ni(II) ions onto attached dye molecules.

Effect of initial urease concentration, that is, the amount of urease adsorbed on the RR120-Ni(II) attached p(HEMA) nanoparticles versus the initial concentration of urease in the solution, was shown in Figure 8 As can be seen, urease binding increases with its concentration in the adsorption medium until it
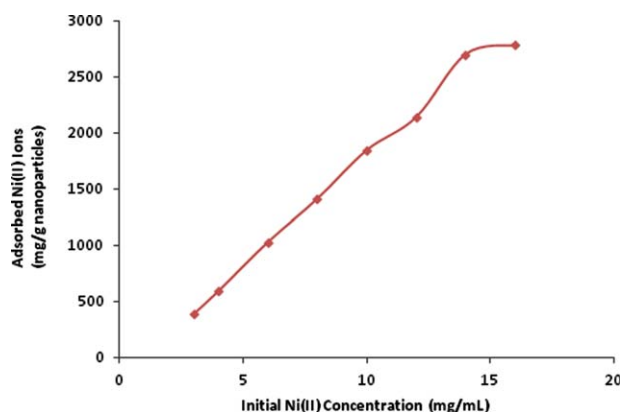
Figure 6. Effect of initial Ni(II) ions concentration on Ni(II) ions adsorption onto RR120 attached p(HEMA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
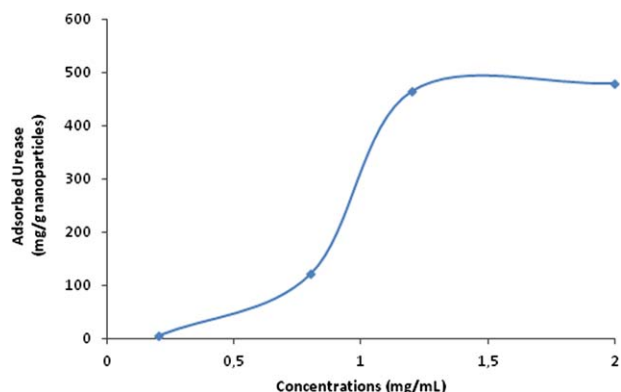

Figure 7. The effect of pH on urease adsorption. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

approaches at concentration 1.5 mg mL^{-1}. This is attributed to the binding sites of RR120-Ni(II) attached p(HEMA) nanoparticles becoming saturated. The steep slope of the initial part of the adsorption isotherm represents a high affinity between urease and incorporated RR120-Ni(II) groups. As seen in Figure 8, a maximum urease adsorption of RR120-Ni(II) attached p(HEMA) nanoparticles were found to be 480.01 mg g^{-1} nanoparticles.
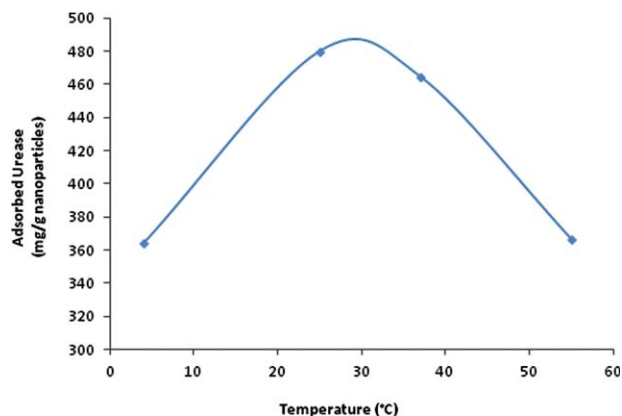
Effect of temperature on the adsorption of urease onto RR120-Ni(II) attached p(HEMA) nanoparticles was also presented in Figure 9. An increase in temperature (form 4 to 25°C) causes increasing the adsorption amount of urease. An increase in the adsorption capacity of the nanoparticles for urease adsorption at a higher temperature indicated that the binding between the proteins and RR120-Ni(II) attached p(HEMA) nanoparticles is mainly hydrophobic. But after 30°C, a decrease in urease adsorption could be explained by the creation of conformational changes of urease.

Effect of pH and Temperature on Enzyme Activity
Optimum pH was determined 7.0 (25 mM phosphate buffer) for free and adsorbed enzyme. As seen in Figure 10, there is a graph about effect of pH on free and adsorbed enzyme. Adsorbed and free enzyme had same character on different pH medium. Active site of enzyme may be effected by same
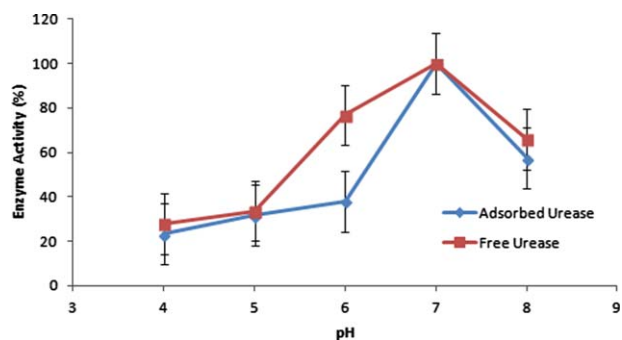

Figure 8. Effect of concentration of urease on the urease adsorption. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
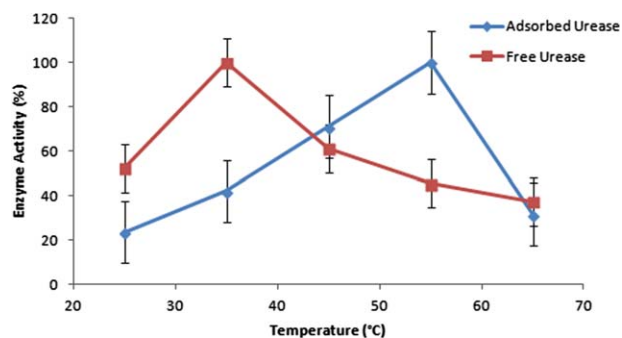
Figure 9. Effect of temperature on urease adsorption. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acidic character, especially. Amino acids in active site may be reacted by same optimum medium for free and adsorbed enzyme.

As seen in Figure 11, optimum temperatures were different for free and adsorbed enzyme. 35°C was optimum temperature for free enzyme. 55°C was optimum temperature for adsorbed enzyme. Adsorbed enzyme had steric barrier, so it may need to be obtained same conditions for temperature. When the tem-

Figure 10. Effect of pH on free and adsorbed enzyme activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 11. Effect of temperature for free and adsorbed enzyme activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
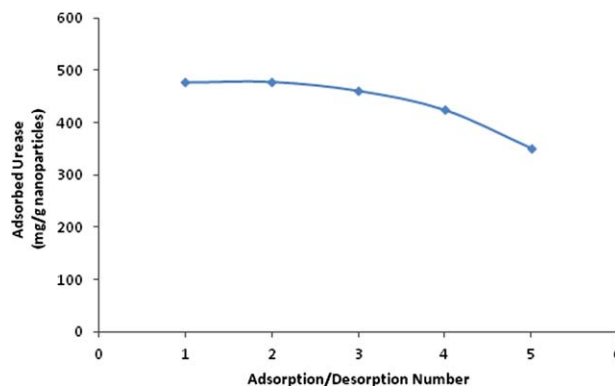
Figure 12. Reusability of RR120-Ni(II) attached p(HEMA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

perature was increased molecules mobility was increased for the adsorbed enzyme and free enzyme. Therefore, at 55°C, it had same condition with free enzyme for optimum temperature.

Kinetic Constants

Michelis constant K_m and V_{max} for the free and adsorbed enzyme were determined using urea as a substrate. K_m and V_{max} values were obtained from Lineweaver Burk plots. Values were found to be 21.50 mM and 34.06 mM for the free and adsorbed enzyme. The increase in K_m may be result from structural changes of enzyme or active site. On the other hand, the V_{max} values were 4 U for the free enzyme and 3.3 U for the adsorbed enzyme. Several researches reported that V_{max} values were decreased upon adsorption or immobilization.[17]

Desorption and Reusability of RR120-Ni(II) Attached p(HEMA) Nanoparticles

One of the most important properties of a support material is it provide repeated uses. In order to show the reusability of RR120-Ni(II) attached p(HEMA) nanoparticles, urease adsorption-desorption procedure was repeated five times by using the same nanoparticles. The urease adsorbed onto nanoparticles was desorbed with 1.0M NaOH and desorption rate was found to be 99.58%. As seen in Figure 12, at the end of five adsorption-desorption cycles, there was no remarkable reduction in the adsorption capacity. These results showed that RR120-Ni(II) attached p(HEMA) nanoparticles can be repeatedly used in enzyme immobilization, without detectable losses in their initial adsorption capacities.

CONCLUSIONS

The most effective way of removing urea from aqueous solutions is the utilization of immobilized urease as no efficient adsorbent is available for urea. Nanoparticles provide an ideal remedy to the conflicting issues usually encountered in the optimization of adsorbed enzymes: minimum diffusional limitation, maximum surface area per unit mass and high enzyme loading. In this article, a new type of adsorbent matrix with combined properties of dye ligand and immobilized metal ions was developed by the attachment of RR120 on p(HEMA) nanoparticles. The maximum urease capacity of the RR120-Ni(II) attached

nanoparticles was 480.01 mg g^{-1} nanoparticles at optimized conditions. Additionally, adsorbed urease was desorbed with 99.58% recovery. This results showed that RR120-Ni(II) attached nanoparticles could successfully be used urease immobilization.

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