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Adsorption of cellulase on poly(2-hydroxyethyl methacrylate) cryogels containing phenylalanine

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Abstract: The aim of this study was to prepare a supermacroporous cryogel that can be used for the adsorption of cellulase. The macroporous cryogel of poly(2-hydroxyethyl methacrylate-N-methacryloyl-L-phenylalanine) [p(HEMA-MAPA)] was prepared by copolymerization of 2-hydroxyethyl methacrylate (HEMA) with a functional monomer of N-methacryloyl-L-phenylalanine (MAPA). The cryogel was characterized by scanning electron microscopy, Fourier transform infrared spectroscopy, and swelling tests. The effects of several parameters such as medium pH, temperature, ionic strength, and flow rate on cellulase adsorption were also investigated. Maximum cellulase adsorption was observed at 25 °C and pH 4.0. Furthermore, adsorbed cellulase was desorbed from the cryogel by using 1.0 M NaCl. The p(HEMA-MAPA) cryogel could be used many times without the cellulase adsorption capacity decreasing significantly.

Key words: Cellulase, adsorption, cryogel, P(HEMA-MAPA)

1. Introduction

The use of industrial enzymes has incrementally increased in recent years in a variety of fields such as wine making and brewing; food, animal feed, and biofuel production; agriculture; and the paper, textile, and detergent industries; as well as in research and development. Cellulase has many potential industrial applications.[1,2] Cellulase (EC 3.2.1.4.) is a hydrolase group enzyme that hydrolyzes $\beta - 1 \rightarrow 4$ glycosidic bonds of cellulose to produce glucose.[3] In the food industry, it plays a major role in the conversion of renewable cellulosic biomass into commodity chemicals.[4] In most fermentation industries, cellulosic materials are first converted into glucose by either chemical or enzymatic processes, and then the product is fermented to ethanol.[5] Nowadays enormous amounts of industrial, agricultural, and municipal cellulosic wastes are accumulating in the environment or used inefficiently due to the high cost of their utilization processes. In nature, cellulose is used as a food source by a wide range of invertebrate animals as well as a wide variety of organisms including bacteria, fungi, and protists. Lignocelluloses are regarded as the most important renewable resource for bioconversion due to their being the most abundant and lowest-cost biomass in the world.[6] Many cellulosic substances were hydrolyzed to simple sugars for making single cell protein, sweeteners, etc. It has attracted considerable economic interest for developing processes for the effective treatment and utilization of cellulosic wastes as cheap carbon sources.[7]

Cryogelation techniques, discovered more than 30 years ago, are simple strategies that allow the preparation of macroporous gels of high toughness and superfast responsivity.[8–10] Cryogel technology has progressed

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rapidly over the last two decades and these materials have emerged as a novel generation of stationary phases in separation science.[11,12] Cryogels have a highly interconnected porous structure that is produced by solvent crystals during cryotropic gelation, which is performed at subzero temperatures. The solvent used is usually water because it is cheap and benign to biological systems.[12−14] After polymerization, ice crystals melt at room temperature and an elastic, macroporous, hydrophilic cryogel structure occurs.[15−17] Supermacroporous cryogels are a good alternative to traditional protein-binding matrices, having many advantages such as large pores, short diffusion property, and very short residence time for adsorption and elution. At the same time, supermacroporous cryogels are used for enzyme immobilization as a sponge-like matrix. For example, in situ entrapment of urease supermacroporous poly(*n*-isopropylacrylamide) (PNIPAAm) cryogels was developed as an effective strategy via noncovalent immobilization.[18] These unique features of cryogels make them suitable to be used in medical, biotechnological, and environmental applications.[19,20]

Poly(2-hydroxyethyl methacrylate) [p(HEMA)]-based support materials have been previously used for immobilization of various enzymes via covalent attachment or entrapment or physical adsorption due to their stability under a wide variety of reaction conditions.[21] Hydrophobic groups can be used with p(HEMA) and these groups could be introduced on the p(HEMA) either by copolymerization of 2-hydroxyethyl methacrylate (HEMA) with a hydrophobic group carrying comonomer or attachment of a hydrophobic group after activation of a functional –OH group. In this way, hydrophilic p(HEMA) supports can be used for immobilization of proteins and enzymes by hydrophobic interactions.[22]

Recently, HEMA-based cryogels containing N-methacryloyl-L-phenylalanine (MAPA) as a hydrophobic ligand were prepared by both conventional and modified cryogelation methods. These cryogels were then used for plasmid DNA (pDNA) purification from *E. coli* lysate.[23] In another study it was reported that beads-embedded composite cryogel of the poly(2-hydroxyethyl methacrylate-N-methacryloyl-L-phenylalanine) [p(HEMA-MAPA)] was synthesized to purify lysozyme from chicken egg white.[24]

In the present study, the performance of cryogel containing a hydrophobic monomer was investigated for cellulase adsorption. As mentioned above, there are many studies about both p(HEMA)-based support materials and p(HEMA-MAPA) cryogels. However, to the best of our knowledge, there is no reported study on a model of enzyme cellulase adsorption on the supermacroporous monolithic cryogels and their evaluation in terms of some physicochemical properties. For this aim, p(HEMA-MAPA) cryogel was prepared by copolymerization of HEMA monomer with a hydrophobic comonomer MAPA. The prepared p(HEMA-MAPA) cryogel was characterized using swelling ratio measurements, scanning electron microscope (SEM), and Fourier transform infrared (FTIR) spectroscopy. Several adsorption conditions, such as pH, temperature, ionic strength, and flow rate were studied in appropriate ranges. The reusability of cryogel was also tested.

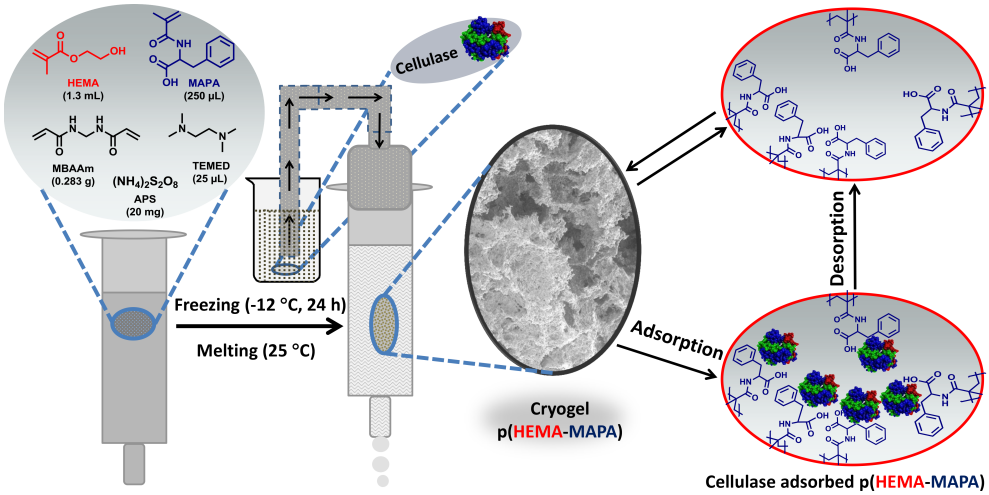
2. Results and discussion

2.1. Characterization of cryogel

A macroporous p(HEMA-MAPA) cryogel was prepared using copolymerization of HEMA monomer with a hydrophobic amino acid conjugated comonomer MAPA. This cryogel was used for adsorption of cellulase. A schematic illustration of p(HEMA-MAPA) cryogel with cellulase enzyme is given in the Scheme.

The chemical structure of p(HEMA-MAPA) cryogel was established by FTIR spectroscopy. The cryogel has a characteristic broad peak at 3316 cm^{-1} that indicates O–H stretching vibrations. The band at 2943 cm^{-1} was attributed to asymmetric C–H stretching vibrations. The presence of a band at about 1708 cm^{-1} is

related to C=O stretching vibration. The bands at around 1652 cm^{-1} and 1450 cm^{-1} show C=O (amide I) and N–H bending (amide II), respectively. The absorption bands appearing at 1070 cm^{-1} and 749 cm^{-1} arise from the vibrations of the benzene ring of MAPA monomer (Figure 1).



Scheme. Schematic representation of p(HEMA-MAPA) cryogel with cellulase.

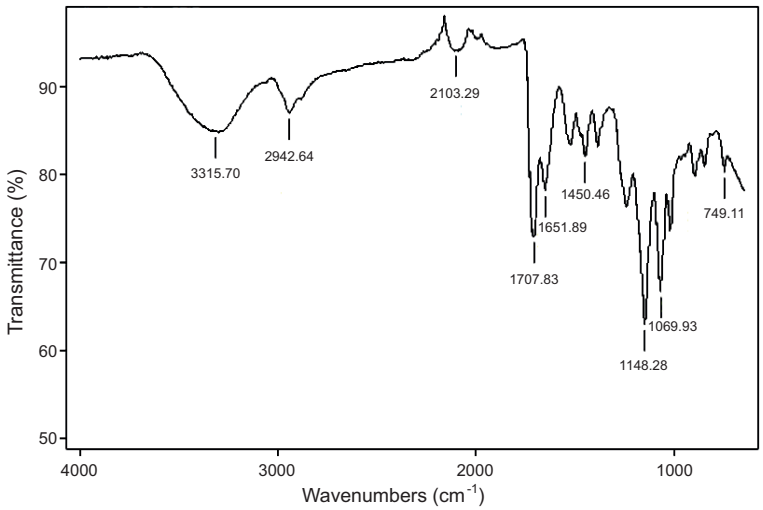


Figure 1. FTIR spectrum of p(HEMA-MAPA) cryogel.

The SEM images with different magnification ratios of the p(HEMA-MAPA) cryogel are presented in Figures 2A and 2B. As seen in the figures, cryogel has a sponge-like structure and is supermacroporous (10 to 200 μm in diameter), providing a size that can result in less fouling of pores with bioparticles and easy cleaning of the cryogel.[17] SEM analysis revealed that scaffolds possess large and interconnected macropores generated by the process of cryogelation. The interconnected macropores allow cellulase enzyme molecules to flow easily from the cryogel. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible.[25] Smaller pores within the walls of p(HEMA-MAPA) cryogel also help to increase the available surface area for cellulase binding.[26] The swelling degree and macroporosity of the p(HEMA-MAPA) cryogel were found to be 7.44 g H_2O/g cryogel and 70.3%, respectively. When the compressed piece of cryogel

was submerged in water, it soaked in water and within 1–2 s regained its original size and shape due to its shape memory.

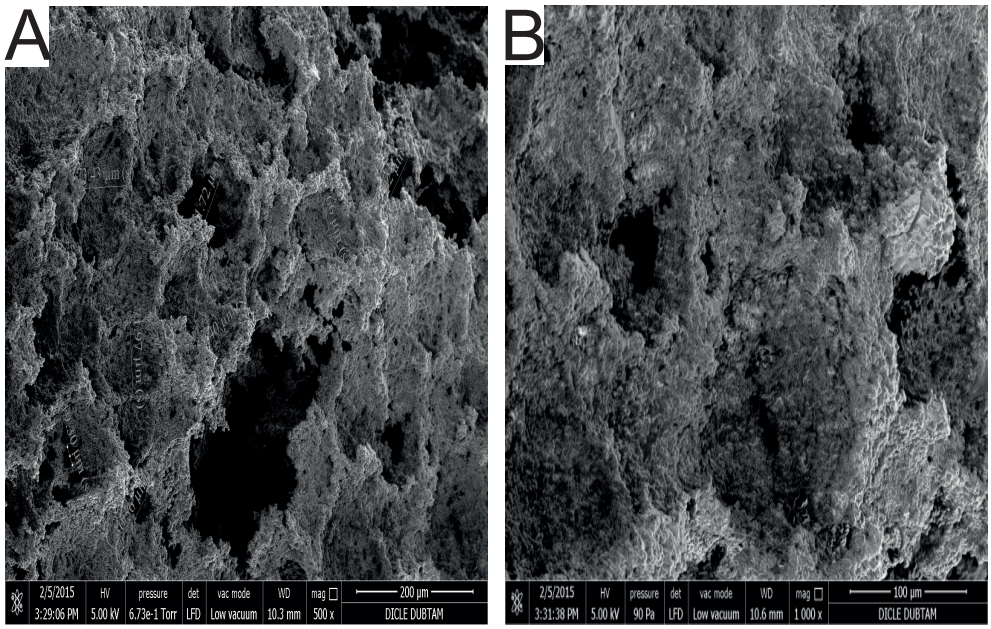


Figure 2. SEM photographs of p(HEMA-MAPA) cryogel at various magnifications, A) 500× magnification and B) 1000× magnification.

2.2. Effect of flow rate

The adsorption capacities at different flow rates are shown in Figure 3. The results show that the amount of cellulase adsorption onto the p(HEMA-MAPA) cryogel decreased (about 65.5%) when the flow rate was increased from 1.0 to 4.0 mL/min. This is due to the decrease in contact time between the cellulase and the cryogel at higher flow rates. These results are in agreement with previously published reports for lysozyme, β-casein, and catalase.[27–29] Contact time in the column is longer when the flow rate decreases. At lower flow rates, the cellulase molecules have more time to move through interconnected pores and diffuse into the smaller pore walls of the cryogel and bind to the molecular cavities containing hydrophobic MAPA domains, and hence a higher cellulase adsorption amount is obtained.[30]

2.3. Effect of pH

The cellulase adsorption capacity of p(HEMA-MAPA) cryogel was studied in the pH range of 3.5 to 8.0, using 0.1 M of acetate (pH 3.5–5.8) and 0.1 M of phosphate (pH 6.5–8.0) buffer solutions. As shown in Figure 4, maximum cellulase adsorption was observed as 106.13 mg/g cryogel at pH 4.0. At below the maximum adsorption pH value, the adsorption capacity of cellulase slightly decreased (6.9%). However, above pH 4.0, the adsorption capacity of cellulase decreased significantly (127.6%) when the pH was increased from 4.0 to 4.8. This result can be explained in the following way: the cellulase enzyme obtained from a commercial cellulase preparation form *Aspergillus niger* was an acidic protein with an isoelectric point at pH 3.67 and pK values between 4.2 and 5.3.[31,32] It is a protein-based amphoteric molecule containing both basic and acidic functional groups, and the electronegativity depends on the system pH.[33] Proteins have no net charge at their isoelectric

points and total ionic charge must be equal to zero. Thus, the specific interactions between cellulase and MAPA groups of the cryogel can be attributed to an increase in the conformational size because of zero net charge. Therefore, the maximum cellulase adsorption from aqueous solution was observed at pH 4.0. At pH values above 4.0, the decrease in the value of adsorption capacity can be explained by the fact that buffer ions play an important role in adsorption, interacting with the cellulase via charge–charge interactions and masking the binding sites.

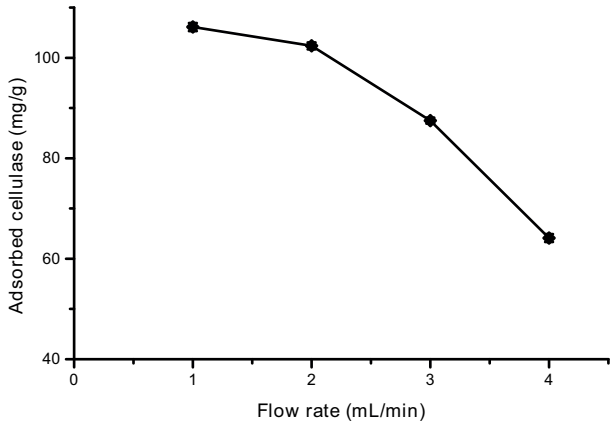


Figure 3. Cellulase adsorption at different flow rates onto the p(HEMA-MAPA) cryogel at 0.5 mg/mL initial concentration of cellulase and 25 °C, pH 4.0. Values are the mean ± SD of the data ($n = 3$).

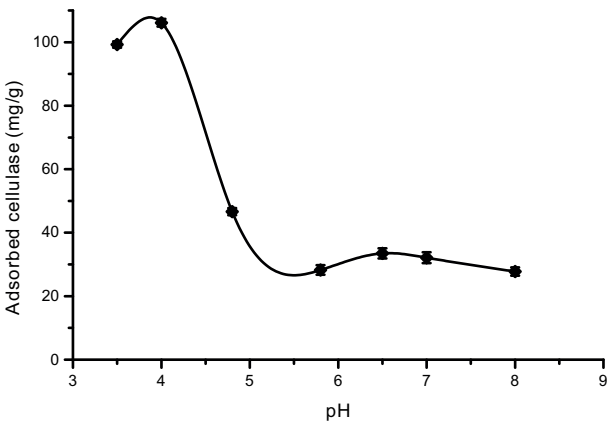


Figure 4. Effect of pH on cellulase adsorption onto the p(HEMA-MAPA) cryogel at 0.5 mg/mL initial concentration of cellulase and 25 °C, 1 mL/min flow rate. Values are the mean ± SD of the data ($n = 3$).

2.4. Effect of temperature

The efficiency of the adsorption processes depends on the characteristics of the adsorbent such as particle size, structure, and specific area. Furthermore, the types of adsorption mechanism that can happen between the phases, the conditions of the process, and changing forces during the control process are the main factors that influence the adsorption process. Protein adsorption is very complicated process driven by different protein–surface forces, such as hydrophobic, electrostatic, and ionic interactions; van der Waals forces; or hydrogen bonds; and the temperature dependency of these forces is different from each other.[34,35] Effect of temperature on the adsorption process was studied at five different temperatures (5–40 °C). As seen in Figure 5, maximum cellulase adsorption was observed at 25 °C. It was observed that the maximum cellulase adsorption onto p(HEMA-MAPA) cryogel was increased slightly (8.9%) by increasing temperature from 5 to 25 °C. This slight increase in adsorption probably promotes hydrophobic interactions with hydrophobic amino acid residues like phenylalanine, tyrosine, tryptophane of cellulase, the hydrophobic group of the p(HEMA-MAPA), and van der Waals attraction forces. Consequentially, increasing of cellulase binding capacity with increasing temperature is an anticipated result. However, the amount of adsorbed cellulase decreased slightly (12.5%) when the temperature increased from 25 to 40 °C. The main reason for the decrease in adsorption capacity at temperatures of above 25 °C is the three-dimensional changes in the temperature-sensitive enzyme structure.[36] Furthermore, this observed decrease may be due to a reduction in interactions between cellulase molecules and cryogel or steric hindrance.[27]

2.5. Effect of ionic strength

The effect of ionic strength on the cellulase adsorption onto p(HEMA-MAPA) cryogel was studied at pH 4.0 using different NaCl concentrations. Figure 6 shows the effect of ionic strength on the cellulase adsorption onto p(HEMA-MAPA) cryogel. The data presented in Figure 6 clearly indicate that adsorption capacity decreased with increasing medium ionic strength. Cellulase adsorption capacity decreased 138% with the increasing NaCl concentration (0.01–1.0 M). This observation could be attributed to the repulsive electrostatic interactions between p(HEMA-MAPA) cryogel and cellulase molecules.[3]

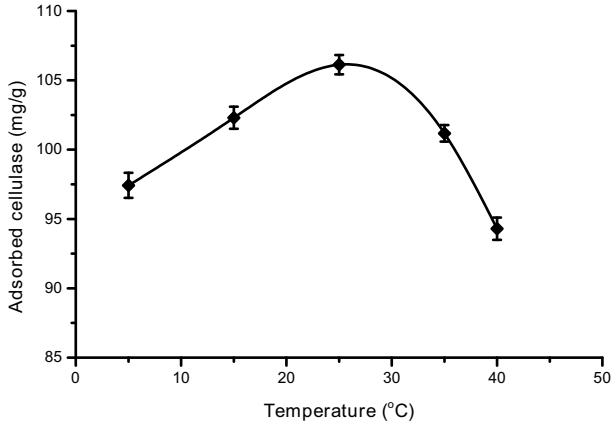


Figure 5. Effect of temperature on cellulase adsorption onto the p(HEMA-MAPA) cryogel at 0.5 mg/mL initial concentration of cellulase and pH 4.0, 1 mL/min flow rate. Values are the mean ± SD of the data ($n = 3$).

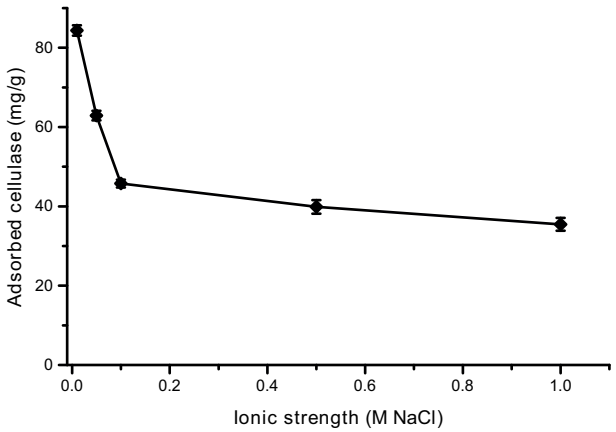


Figure 6. Effect of ionic strength on cellulase adsorption onto the p(HEMA-MAPA) cryogel at 0.5 mg/mL initial concentration of cellulase and 25 °C, pH 4.0, 1 mL/min flow rate. Values are the mean ± SD of the data ($n = 3$).

2.6. Desorption and reusability

The main advantages of MAPA involving p(HEMA-MAPA) cryogel are its low cost and simplicity, and stability of the adsorbent. In order to show the reusability of the p(HEMA-MAPA) cryogel the adsorption/desorption cycle of cellulase was repeated eight times using the same cryogel. To ensure sterility, the cryogel was washed with 50 mL of distilled water for 30 min after completing one full adsorption/desorption cycle. At the end of eight adsorption/desorption cycles, there was no remarkable decrease in cellulase adsorption capacity. As seen in Figure 7, the cryogel is very stable, and maintains its adsorption capacity at an almost constant value of about 86%.

In summary, cryogels are well known as new generations of stationary phases in bioseparation science. Cryogels have several advantages such as short diffusion, low cost, large pore sizes, and short residence time for both adsorption and elution. Cryogels can be easily functionalized with a variety of ligands and used for the adsorption of definite types of biomolecules. This paper reports the performance of p(HEMA-MAPA) cryogel for cellulase adsorption, which is a very important enzyme used widely in industrial applications. The effects of pH, medium temperature, ionic strength, and flow rate on cellulase adsorption were also investigated. Maximum adsorption capacity was determined as 106.13 mg/g at pH 4.0, 25 °C, and 1.0 mL/min flow rate. Furthermore, the adsorption–elution cycle was repeated eight times using the same p(HEMA-MAPA) cryogel. No remarkable reduction was detected in the cellulase adsorption capacity. The results obtained from this study clearly indicate that p(HEMA-MAPA) cryogels can be used for the adsorption of cellulase and other enzymes showing effectiveness towards the p(HEMA-MAPA) cryogel.

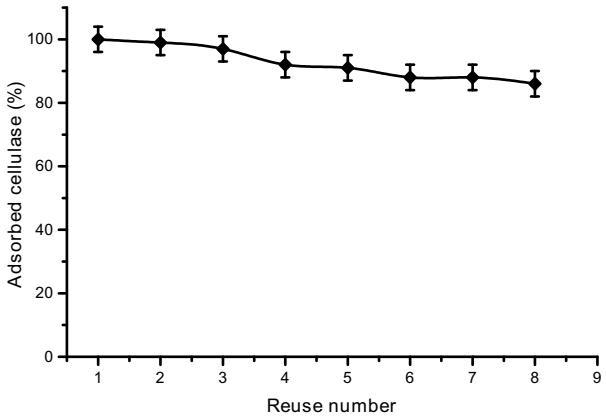


Figure 7. Reusability of p(HEMA-MAPA) cryogel for cellulase adsorption at 0.5 mg/mL initial concentration of cellulase and 25 °C, pH 4.0, 1 mL/min flow rate.

3. Experimental

3.1. Materials

Cellulase (from *Aspergillus niger*, 0.8 U/mg; EC 3.2.1.4.), N, N, N', N'-tetramethylethylene diamine (TEMED), N, N'-methylene bisacrylamide (MBAAm), 2-hydroxyethyl methacrylate (HEMA, 97% purity containing $\leq$250 ppm monomethyl ether hydroquinone (MEHQ) inhibitor), and ammonium persulfate (APS) were purchased from Sigma and used as received. All other chemicals were supplied by Merck AG (Darmstadt, Germany) and were of reagent grade. N-methacryloyl-L-phenylalanine (MAPA) was kindly supplied by Professor Adil Denizli at Hacettepe University (Ankara, Turkey).

3.2. Preparation of p(HEMA-MAPA) cryogel

The synthesis of MAPA monomer was described in a previously reported article.[37] P(HEMA-MAPA) cryogel was prepared by free-radical cryopolymerization of HEMA monomer with MAPA as a hydrophobic comonomer, at –12 °C. APS and TEMED were used as redox initiators. Typically, 0.283 g of MBAAm was dissolved in water (10 mL) and the monomer phase was prepared by dissolving HEMA (1.3 mL) in water (3.7 mL). Then 250 μL of MAPA was added and the mixture was magnetically stirred until a homogeneous solution obtained. Next 20 mg of APS and 25 μL of TEMED were added to a solution consisting of HEMA and MAPA. The polymerization solution was poured into a plastic syringe (5 mL, i.d. 0.8 cm) with a closed outlet at the bottom. The polymerization in the syringe was frozen at –12 °C for 24 h and then thawed at room temperature. The cryogel was washed with 50 mL of distilled water and stored in distilled water containing 0.02% sodium azide (NaN_3) at +4 °C until use.

3.3. Characterization of cryogel

To determine the swelling ratio of the cryogel (S), it was washed with water until the washing solution became clear. Then it was sucked dry and transferred to a preweighed vial and weighed ($m_{wet\,gel}$). After drying at 60 °C in an oven, the mass of the cryogel was determined ($m_{dry\,gel}$). The swelling ratio was calculated using the following equation:

$$S = \frac{m_{\text{wet gel}} - m_{\text{dry gel}}}{m_{\text{dry gel}}} \quad (1)$$

The percentage porosity of macropores (%P) was calculated by measuring the content of volume and free water of a sample. A piece of the cryogel was saturated with deionized water. The mass of wet cryogel saturated with water ($m_{swollen\,gel}$) was weighed. The cryogel was weighed once again followed by squeezing the cryogel sample to remove the free water within the large pores ($m_{squeezed\,gel}$). The percentage of porosity for micropores was calculated according to the following equation:

$$\%\,P = \left(\frac{m_{\text{swollen gel}} - m_{\text{squeezed gel}}}{m_{\text{swollen gel}}}\right) \times 100 \tag{2}$$

The surface morphology of the p(HEMA-MAPA) cryogel was studied by SEM (FEI Quanta 250 FEG, USA). The spectroscopic characterization of the cryogel was investigated using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). About 0.1 g of the dried cryogel was thoroughly mixed with 0.1 g of IR grade KBr and pressed into a pellet. The spectrum was recorded in the wavenumber range from 4000 to 400 cm^{-1}.

3.4. Adsorption studies

The adsorption experiments were performed with 20 mL of cellulase solution (0.5 mg/mL) in a continuous system for 60 min, which was the equilibrium period for the adsorption of cellulase at 25 °C. For adsorption parameters, the cryogel was equilibrated with acetate buffer (0.1 M, pH 4.0) after washed with 20 mL of distilled water for 30 min. Then the cellulase solution (in 0.1 M acetate buffer, pH 4.0) was pumped through the cryogel column by a peristaltic pump (Watson-Marlow Bredel, UK). The adsorption studies were monitored by following the depletion in cellulase concentration by measuring UV absorbance at 280 nm (Shimadzu, UV mini, Japan). Cellulase adsorption on the p(HEMA-MAPA) cryogel was studied over a range of pHs (0.1 M sodium acetate buffer was used for pH 3.5–5.8; 0.1 M sodium phosphate buffer was used for pH 6.5–8.0), ionic strengths (0.01–1.0 M NaCl), and temperatures (5–40 °C). The effect of flow rate was also studied by varying it from 1.0 to 4.0 mL/min. The cellulase adsorption experiments were repeated at least three times ($n = 3$) with three different cryogels. All data were expressed as mean ± standard deviation (SD) unless otherwise outlined. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error.

3.5. Desorption and reusability studies

Adsorbed cellulase was desorbed by using 0.1 M of phosphate buffer containing 1.0 M NaCl at pH 9.0, and 20 mL of desorption reagent was pumped through the cryogel column for 2 h. The final concentration of cellulase in the solution was determined at 280 nm. Desorption rate was calculated from the amount of cellulase adsorbed on the cryogel and the final enzyme concentration in the desorption medium.

In order to determine the reusability of the p(HEMA-MAPA) cryogel, the cellulase adsorption/desorption cycle was repeated eight times using the same monolithic cryogel column. The cryogel was washed with deionized water for 1 h and after each adsorption/desorption cycle reequilibrated with working buffer solution (0.1 M acetate buffer, pH 4.0).

Acknowledgments

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