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Mannose Imprinted Affinity Cryogels for Immunoglobulin G Binding

Immünoglobülin G bağlanması için Mannoz Baskılı Afinite Kriyojeller

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ABSTRACT

The design, preparation and characterization of mannose imprinted cryogels for the detection of immunoglobulin G (IgG) in aqueous solutions were carried out. Methacryloylamido phenylboronic acid (MAPBA) was used as the functional monomer since its boronic acid moiety has ability to interact with mannose groups on the Fc region of IgG. Free radical cryopolymerization technique was used for the preparation of mannose imprinted affinity cryogels towards IgG. The effects of medium pH, IgG concentration, ionic strength and flow rate on the IgG binding were investigated. The maximum IgG binding was achieved as 61.7 mgg^{-1} at pH 8.0. The prepared mannose imprinted cryogels exhibited excellent selectivity towards IgG in the existence of albumin and hemoglobin. Also, the result showed that the prepared cryogels preserved their stability even after 10 binding-desorption cycles.

Keywords

Molecularly imprinted cryogels, IgG, mannose, antibody purification.

ÖZ

Bu çalışmada sulu çözeltilerde immunoglobulin G (IgG) bağlanması için mannoz baskılanmış kriyojelerin tasarımı, hazırlanması ve karakterizasyonu gerçekleştirilmiştir. Boronik asidin IgG'nin Fc bölgesinde bulunan mannoz grupları ile etkileşim yeteneğinden dolayı metakroilamido fenilboronik asit (MAPBA) fonksiyonel monomer olarak kullanılmıştır. IgG için mannoz baskılanmış afinite kriyojellerinın hazırlanmasında serbest radikal kriyo-polimerizasyon tekniği kullanılmıştır. Ortam pH'ı, IgG derişimi, iyonik şiddet ve akış hızının IgG bağlanmasına etkisi araştırılmıştır. Maksimum IgG bağlanması pH 8.0'de 61.7 mgg^{-1} olarak elde edilmiştir. Hazırlanan mannoz baskılanmış kriyojeller albumin ve hemoglobin varlığında da IgG'ye karşı oldukça yüksek seçicilik göstermiştir. Ayrıca, sonuçlar hazırlanan kriyojellerin 10 bağlanma-desorpsiyon döngüsünden sonra da kararlılığını koruduğu gösterilmiştir.

Anahtar Kelimeler

Moleküler baskılanmış kriyojeller, IgG, mannoz, antibadi bağlanması.

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INTRODUCTION

Immunoglobulin G (IgG) is the main and therapeutically crucial antibody exists in the human serum. The existence of Fc oligomannose groups of IgG molecule has gained great interest since the mannose receptors play a crucial role in the removal of pathogens having mannose on their surfaces as well [1]. IgG has been extensively used for the treatment of various diseases such as immunodeficiency [2] and chronic inflammatory disorders [3]. IgG is also commonly used in immuno assays such as ELISA, radioimmunoassays, immunosensor platforms and antibody-based microarray platforms [4-7]. Thus, the purification of IgG is very important. The purification of IgG using various affinity materials such as protein A immobilized-membranes, L-lysine imprinted cryogel columns, protein A immobilized-beads and protein A immobilized-cryogel column was carried out and the achieved IgG binding capacities were 9.8, 55.1, 56.1 and 83.2 mgg^{-1} [8-11].

Molecularly imprinted polymers (MIPs) are highly cross-linked tailor-made materials having selective binding regions for the desired compound. For the preparation MIPs towards the desired compound, appropriate functional monomers and a cross-linker are polymerized together in the existence of the desired compound which is called template. After removal of the template from the highly cross-linked polymeric network, the obtained MIPs display high affinity and selectivity towards the desired compound. MIPs can be successfully applied in different applications fields such as biosensor systems, biomimetic catalysis and solid phase extraction [12-14]. MIPs are cheap, reusable and stable affinity materials under extreme operational conditions such as high temperature, high pressure, high and low pH values [15-24].

On the other hand, molecular imprinting of large biomolecules such as antibodies and proteins is a quite challenging process. Epitope imprinting is an efficient approach that was commonly used for the imprinting of this kind of molecules. In this technique, an epitope of the desired antibody or protein that is a short fragment is used as the template molecule. The desired antibody or protein can efficiently be captured via this fragment [25-28].

Cryogels are efficient affinity materials which have many advantages such as interconnected macropores that provide higher flow rates and lower back pressure. The macropores of the cryogel network have a crucial role during the binding process of the target compound and provide convective mass transfer throughout the cryogel and rapid binding kinetics [29-32].

The present study demonstrates the preparation of mannose imprinted cryogels for IgG binding in aqueous solutions. For this purpose, methacryloylamido phenylboronic acids (MAPBA) was chosen as the functional monomer since its boronic acid moiety has ability to interact with mannose groups on the Fc region of IgG. The design, preparation, and characterization of the imprinted cryogels are discussed as well as the selective recognition behavior of the synthesized cryogel-based materials towards IgG in aqueous solutions.

MATERIALS and METHODS

Chemicals

Mannose, IgG, albumin, hemoglobin, boric acid, benzyl bromide, thionyl chloride, sodium carbonate, ammonium persulfate (APS), N,N-methylenebisacrylamide (MBAAm), 2-Hydroxyethyl methacrylate (HEMA), N,N,N,N-tetramethyl ethylene diamine (TEMED) and all solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instruments

Bruker-UltraShield 500 MHz NMR spectrometer was used for the ^{1}H-NMR, ^{13}C-NMR and ^{11}B-NMR analyses. The scanning electron microscope (SEM) images of the mannose imprinted cryogels were obtained by using Zeiss Ultra Plus model SEM. UV spectroscopic studies for the IgG analyses were done by using Shimadzu UV-3600 model UV-Vis spectrophotometer.

Synthesis of the Functional Monomer Methacryloylamido Phenylboronic Acid (MAPBA)

The MAPBA synthesis was carried out in 5 steps as schematically shown in Figure 1. In the first step, the synthesis of triisopropyl borate (4) was carried out as briefly described in the following: 10.5 g $SOCl_2$ (8.8 mmol) was slowly mixed with the boric acid solution in isopropanol (IPA) (1.4 mmol, 0.9 g). After the reaction solution was refluxed at N_2 atmosphere, the distillation of the excess of IPA was performed at 90 ^{o}C. Then, the product triisopropyl borate was obtained (90 % yield).
In the second step, N,N-dibenzyl-4-bromoaniline (7) was synthesized. For this purpose, 0.46 g sodium carbonate (4.35 mmol) was added into the 1 g benzyl bromide (6.1 mmol) and 0.5 g 4-bromoaniline (2.9 mmol) in 20 mL DMF. This solution was allowed to stir for 8h

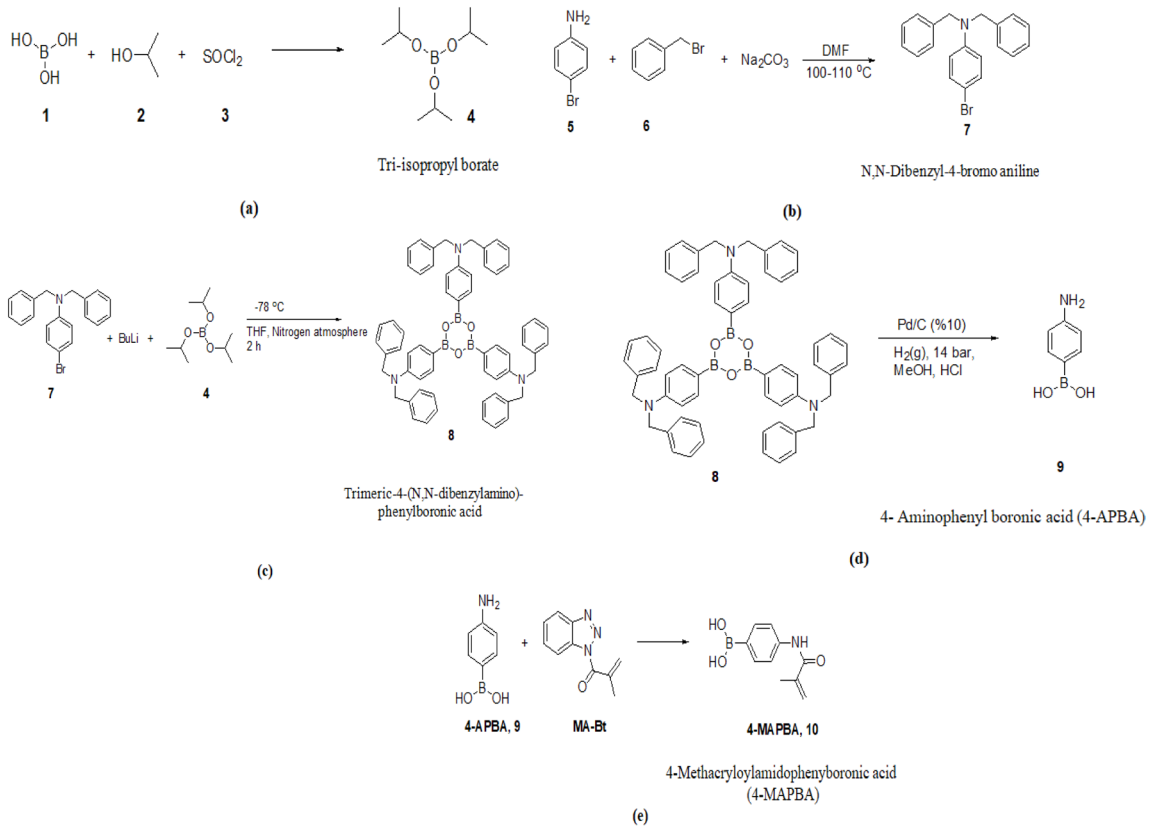

Figure 1. Synthesis of tri-isopropyl borate (a), N,N-dibenzyl-4-bromoaniline (b) trimeric-4-(N,N-dibenzylamino)phenylboronic acid (c) 4-aminophenylboronic acid (d) and 4-methacryloylaminophenyboronic acid (e).

at 100°C. The obtained precipitate of N,N-dibenzyl-4-bromoaniline was separated from the reaction mixture, washed with deionized water and dried.

In the following step, the trimeric 4-(N,N-dibenzylamino) phenylboronic acid (8) synthesis was carried out. For this purpose, 13 mL of 2.5 M BuLi in hexane was slowly added to the solution of 7.5 g of N,N-dibenzyl-4-bromoaniline in 100 mL tetrahydrofuran. Then, 8.2 mg tri-isopropyl borate (4) was added to this solution and allowed to stirr for 45 min at room temperature. After the solvent evaporation, the synthesized solid product was dissolved in 100 mL EtOAc. In the following step, H_2O content of the organic layer was removed using magnesium sulfate. The suspension of the product in hexane was prepared and then filtration was carried out. Than, the obtained solid was dried and finally, the trimeric 4-(N,N-dibenzylamino) phenylboronic acid (8) was obtained (78 % yield, 12 g) (Figure 1c).

In the next step, the sythesis of 4-aminophenyl boronic acid (4-APBA) (9) was performed. For this purpose, small amount of Pd/C and concentrated hydrochloric acid were mixed with the previously synthesized 5 g of trimeric-4-(N,N-dibenzylamino)phenylboronic acid in MeOH. This solution was allowed to stir for 4 h. After the reaction is completed, the catalyst was removed from the reaction mixture and solvent was evaporated. Finally, the product 4-APBA was obtained (2.3 g, 97% yield) (Figure 1d).

In the final step of the synthesis of the functional monomer methacryloylamido phenylboronic acid (MAPBA), 0.3 g of methacryloylbenzotriazole (MA-Bt) was added to the solution of 0.2 g 4-ABPA in 25 mL MeOH. The final solution was refluxed for 8 h. After evaporation of the solvent, the obtained solid was dissolved in 50 mL EtOAc. Then, sodium carbonate (20%) was used for the extraction. Then, organic layer was dried using magnesiim sulfate, filtered and the recrystallization of the obtained product was conducted (0.28 g, 87% yield) (Figure 1e).

Preparation of Mannose Imprinted Cryogels Towards IgG

In the first step, the pre-polymerization solution composed of mannose and MAPBA was prepared. For this purpose, 0.1 mmol MAPBA and 0.1 mmol mannose in 20 mL dimethyl sulfoxide were mixed and allowed to stir for 2h. Then, 2 mL of this solution was added into the 2.6 mL HEMA (Phase 1).

On the other hand, phase 2 containing 0.46 g MBAAm in 15 mL H_2O was mixed with phase 1 and allowed to stir for 1 h. Then, 30 mL TEMED and 25 mg APS were slowly added to this solution. The polymerization solution was then transferred to a disposable plastic syringe. Finally, the polymerization was carried out at -18°C for 18h. After completion of the polymerization, the frozen mannose imprinted cryogel was allowed to thaw at room temperature. Then, the prepared mannose imprinted cryogel was washed with EtOH and H_2O for the removal of undesired impurities (e.g. unreacted monomers, initiator etc). The removal of the template (mannose) from the polymeric cryogel structure was performed by using glycine-HCl buffer pH 3.0. The non-imprinted cryogels were prepared by using the same procedure without considering the template compound.

Characterization Studies

^{1}H-NMR, ^{13}C-NMR and ^{11}B-NMR analyses performed the characterization studies of the synthesized functional monomer MAPBA. TMS was used as the reference standard for ^{1}H-NMR and ^{13}C-NMR analyses. BF_3.OEt_2 was used as the reference standard for ^{11}B-NMR analyses.

On the other hand, swelling tests and SEM analyses of the prepared mannose imprinted cryogels were also carried. To perform the SEM analyses, a gold layer (ca. 25 nm) was deposited on the surface of a piece of cryogel to provide conductivity. Then, SEM images were acquired.

In the experiments for the investigation of the swelling behavior of the mannose imprinted cryogels (MIPs) and the non-imprinted cryogels (NIPs), the cryogels were placed into the 50 mL of distilled water and allowed to swell for 2h. After this incubation time, the mass of the cryogels in swollen state was measured.

The following formula was used for the determination of the swelling ratio of the cryogels.

Swelling ratio= $(m_{swollen-mdry})/(m_{dry})$ (1)

m_{dry} is mass of the dry cryogels and $V_{swollen}$ is mass of the cryogels after swelling.

The binding of IgG to the Prepared Mannose Imprinted Cryogels

A cryogel-based continuous column system was used to conduct the IgG binding experiments. For this purpose, an aqueous solution of 1.0 $mgmL^{-1}$ of IgG was passed through the mannose imprinted cryogel column with a flow rate of 0.5 $mLmin^{-1}$ for 1h. The amount of the IgG that was bound to the cryogel was calculated by the determination of absorbance at 280 nm.

The desorption of the IgG from the cryogels was performed by using glycine-HCl buffer pH 3.0. To determine the optimum conditions for the binding of IgG to the imprinted cryogels, the effects of various parameters such as pH, IgG concentration, ionic strength and flow rate on the IgG binding were also carried out.

Reusability of the Mannose Imprinted Cryogels

IgG binding and desorption experiments were performed 10 times using the same cryogel column for the evaluation of the reusability of the prepared mannose imprinted cryogels. After each step, cryogels were washed with glycine-HCl buffer pH 3.0 for the regeneration.

Selectivity Studies Towards IgG

The selectivity experiments of the mannose imprinted cryogels for IgG was also conducted. For this purpose, hemoglobin and albumin were chosen as potential competing proteins. For the selectivity experiments, aqueous solutions of the proteins (1.0 $mgmL^{-1}$) was passed through the mannose imprinted cryogel column for 1h at 0.5 $mLmin^{-1}$ flow rate. The amounts of IgG, hemoglobin and albumin that were bound to the mannose imprinted cryogel were calculated by the determination of absorbance at 280 nm.

RESULTS and DISCUSSION

Characterization Studies

The obtained results from the ^{1}H-NMR spectra of MAPBA is given in the following:

Singlet peaks observed at 5.5 ppm (1H) and 5.9 ppm (1H) belong to protons of $=CH_2$ exists on the methacryloyl functional groups. The peak observed at 9.6 ppm (2H) belongs to the –OH group of boronic acid. The peak

observed approximately 2.0 ppm (3H) indicates the CH_3 of methacryloyl functional groups. On the other hand, aromatic protons were observed at 7.3 ppm and 7.7 ppm (4H).

The obtained ^{13}C-NMR results confirmed that the peak observed at 165 ppm indicates the carbonyl group. The peaks which indicate the olefinic carbons of methacryloyl functional groups were observed at 118.9 ppm and 121.2 ppm. The peak observed at 18.39 ppm indicates the methyl group of methacryloyl functional groups.

In the ^{11}B-NMR spectra, the peak obtained around 12.9 ppm confirmed the boron nucleus that belongs to the functional monomer MAPBA. Figure 2 shows the obtained SEM images of the mannose imprinted cryogels. The SEM images confirmed that the prepared cryogels have macropores which provide the easy and efficient migration of the IgG molecules throughout the cryogel structure. Also, the monomer HEMA having hydrophilic feature enhances the hydrophilicity of the mannose imprinted cryogels. Therefore, the prepared cryogels swell in water. The obtained swelling ratios (g H_2O g^{-1} cryogel) of the mannose imprinted cryogels (MIPs) and their corresponding non-imprinted cryogels (NIPs) were determined as 11.68 and 8.79, respectively. These swelling ratios indicated that the prepared MIPs and NIPs have supermacropores and display hydrophilic feature. The obtained higher swelling ratios of the MIPs than NIPs can be explained by the 3-D cavities for IgG molecules formed in the polymeric cryogel network which provide the high adsorption of H_2O molecules.

IgG Binding from Aqueous Solutions

The Effect of pH on the IgG Binding

The effect of pH on the IgG binding to the mannose imprinted cryogels was also evaluated changing the medium pH from 4.0 to 9.0. The obtained outcomes from these experiments are given in Figure 3. As seen, the maximum IgG binding was achieved at pH 8.0. This result can be explained by the pKa value of boronic acid (~ 9.0) . At pH 8.0, boronic acid group of the functional

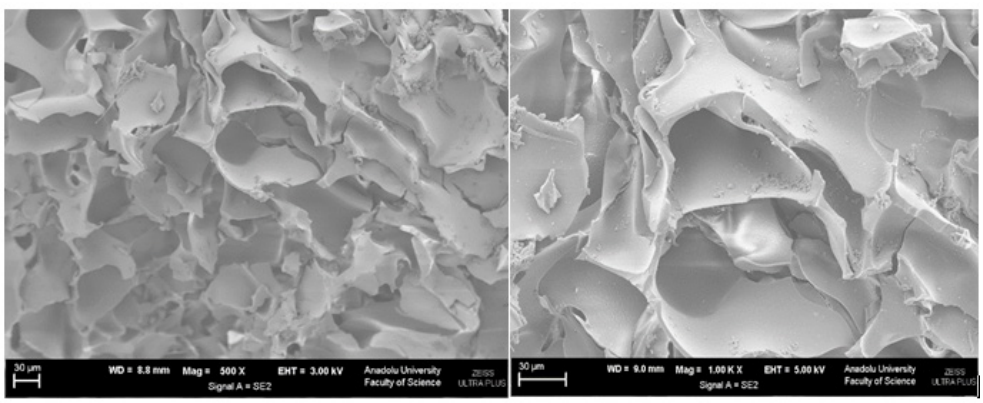

Figure 2. SEM images of the prepared cryogels for IgG.

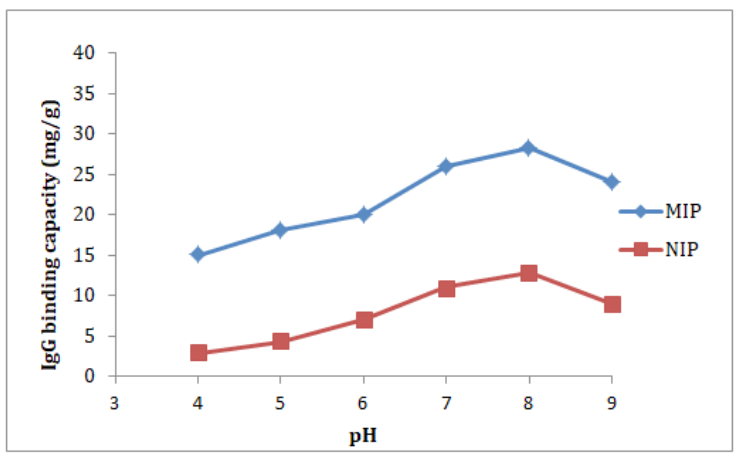

Figure 3. The effect of pH on IgG binding. IgG concentration: 1.0 $mgmL^{-1}$, t: 1h. flow rate: 0.5 $mLmin^{-1}$.

monomer interacts and forms a tetrahedral boronate ester structure with the mannose groups on the Fc region of IgG. The formation of tetrahedral boronate ester is not favored at pH 7.0 and cleaved at lower pH values.

The effect of IgG Concentration on Binding

To investigate effect of IgG concentration on binding, initial IgG concentration was changed from 0.5 to 3.5 $mgmL^{-1}$. Figure 4 shows the effect of IgG concentration on the binding of IgG molecules to the mannose imprinted cryogels. The results indicated that the binding of IgG molecules increased at higher concentrations of IgG. At 2.5 $mgmL^{-1}$ IgG, all binding regions of the imprinted cryogel were occupied with IgG molecules. After 2.5 $mgmL^{-1}$ concentration value, no significant change was observed for the binding capacity of the imprinted cryogels towards IgG at higher concentration values. The highest IgG binding was obtained as 61.7 $mgg^{-1.}$

The effect of ionic strenght on the IgG binding

The ionic strength effect on the binding of IgG was also evaluated. For this purpose, the salt concentration was changed from 0 to 1.0 M. The results from these experiments are given in Figure 5. The binding of IgG molecules to the mannose imprinted cryogels considerably decreased at higher concentrations of NaCl. This could be explained by the interactions of the counter NaCl ions with the target IgG molecules. This ionic interaction may lead to masking of the binding sites of the imprinted cryogels for IgG. The repulsive electrostatic interactions between the IgG molecules and the imprinted cryogels at these concentration values may also lead to a decrease in IgG binding to the cryogels.

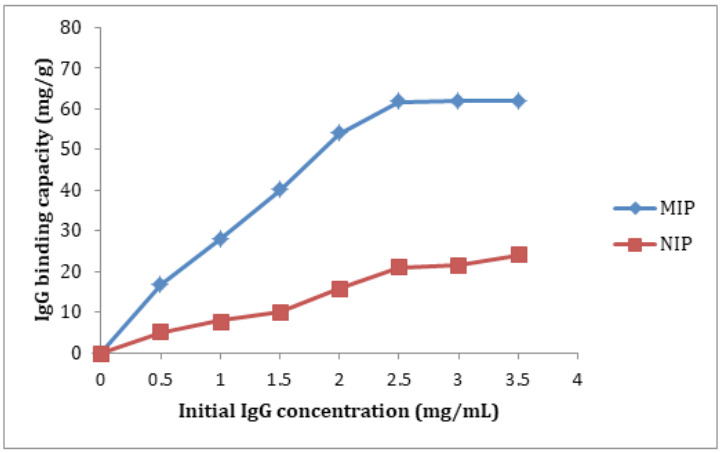

Figure 4. The effect of initial IgG concentration on binding. pH: 8.0, t: 1h, flow rate: 0.5 $mLmin^{-1}$.

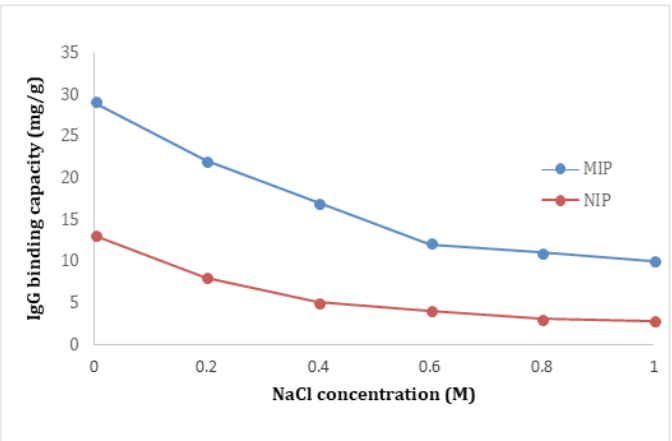

Figure 5. The effect of ionic strength on IgG binding. IgG concentration: 1.0 $mgmL^{-1}$, pH: 8.0, t: 1h, flow rate: 0.5 $mLmin^{-1}$.

The Flow Rate Effect on the IgG Binding

Various flow rates (0.5 mLmin^{-1} to 2.0 mLmin^{-1}) were applied for the evaluation of the flow rate effect on the IgG binding (Figure 6). The results confirmed that the IgG binding to the mannose imprinted cryogels decreased by the increasing flow rate. When the flow rate increases, the interaction time between IgG molecules and the binding regions of the imprinted cryogels decreases which lead to lower IgG binding. On the other hand, IgG can effectively diffuse into the supermacropores of the cryogel network and reach to the binding sites at low flow rate values.

Selectivity and Reusability Studies

The selectivity of the mannose imprinted cryogels for IgG was tested in the existence of hemoglobin and albumin which were chosen as potential competing proteins. The outcomes of these experiments are given in Figure 7. The prepared mannose imprinted cryogels displayed excellent selectivity towards the target IgG with a high imprinting effect. On the other hand, isoelectric points of IgG (6.9) and hemoglobin (7.2) are close each other. But, the molecular weights of IgG and hemoglobin are 150 kDa and 65 kDa, respectively. The molecular weight of albumin is 66 kDa which is very close the molecular weight of hemoglobin. Since the 3-D molecular cavities exists in cryogel network are matched to the size and shape of the target IgG, the binding of other protein molecules which have different shapes and molecular weights to these 3-D molecular cavities is quite difficult.

To test the reusability of the mannose imprinted cryogels towards IgG , IgG binding-desorption cycle was performed 10 times using the same cryogel (Figure 8). The results confirmed that the prepared mannose imprinted cryogels for IgG preserved their stability after 10 binding-desorption cycles.

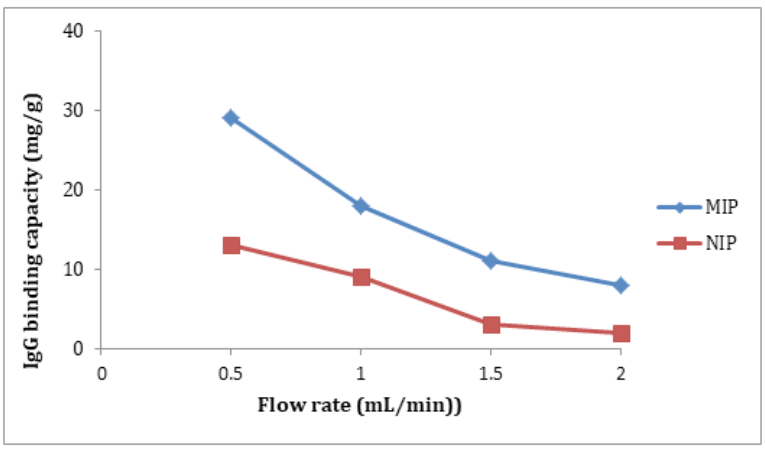

Figure 6. The effect of flow rate on the IgG binding. IgG concentration 1.0 mgmL^{-1}, pH: 8.0, t: 1h, flow rate: 0.5 mLmin^{-1}.

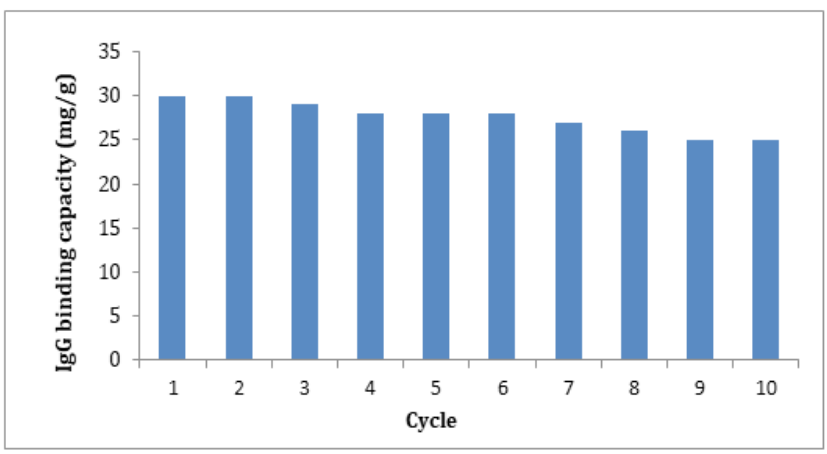

Figure 7. Reusability of the mannose imprinted cryogels towards IgG. IgG concentration: 1.0 mgmL^{-1}, pH: 8.0, t: 1h, flow rate: 0.5 mLmin^{-1}.

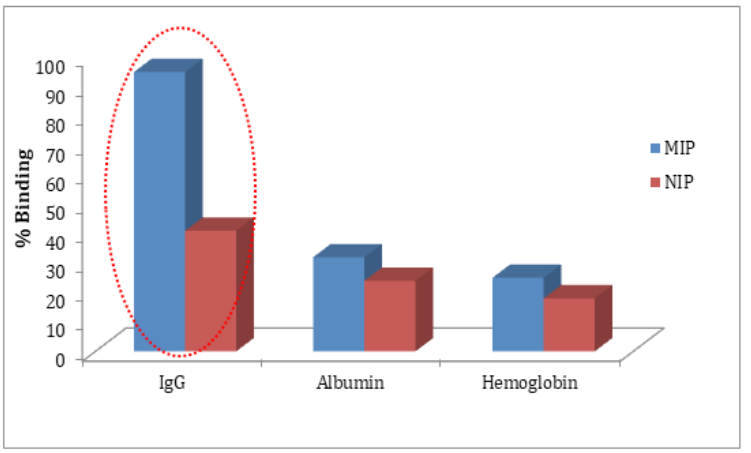

Figure 8. Selectivity of the prepared cryogels towards IgG. IgG and protein concentration: 1.0 mgmL^{-1}, pH: 8.0, t: 1h, flow rate: 0.5 mLmin^{-1}.

CONCLUSIONS

This research demonstrates the design, preparation and characterization of mannose imprinted cryogels as efficient separation materials for the selective detection of IgG in aqueous solutions. For this purpose, methacryloylamido phenylboronic acid (MAPBA) was chosen as the functional monomer because it has ability to interact with mannose groups on the Fc region of IgG. The highest IgG binding was obtained as 61.7 mgg^{-1} under optimum conditions (pH 8.0, flow rate: 0.5 mLmin^{-1}). The prepared cryogels displayed excellent selectivity towards IgG in the existence of other competing proteins such as albumin and hemoglobin.

In conclusion, this research contributes new insights into the design and synthesis of cheap, environmentally-friendly and efficient affinity materials. The outcomes of this research also demonstrate the application of these affinity materials for the selective binding of therapeutically crucial antibodies such as IgG.

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