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Synthesis and Characterization of Macroporous Poly(Acrylamide-Methacrylamido Histidine) Cryogels and Their Use in Antibody Purification

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Abstract

Poly(acrylamide-methacrylamidohistidine) [poly(AAm-MAH)] cryogel was prepared by bulk polymerization which proceeds in aqueous solution of monomers frozen inside a syringe column (cryo-polymerization). After thawing, the monolithic cryogel contains a continuous polymeric matrix having interconnected pores of 10-100 μm size. Poly(AAm-MAH) cryogel was characterized by swelling studies, FTIR and scanning electron microscopy. The equilibrium swelling degree of the poly(AAm-MAH) monolithic cryogel was 6.97 g H_2O/g cryogel. Poly(AAm-MAH) cryogel was used in the adsorption/desorption of immunoglobulin G (IgG) from aqueous solutions and human plasma. The non-specific adsorption of IgG was very low (0.22 mg/g). The maximum amount of IgG adsorption from aqueous solution in HEPES buffer was 32.879 mg/g at pH 7.4. Higher IgG adsorption value was obtained from human plasma (up to 100 mg/g). Desorption of IgG from poly(AAm-MAH) cryogel was obtained using 20 mM Acetate buffer containing 1.0 M NaCl at pH 4. It was observed that IgG could be repeatedly adsorbed and desorbed with poly(AAm-MAH) cryogel without significant loss in the adsorption capacity.

Key Words: Affinity chromatography, cryogel, protein purification, IgG

INTRODUCTION

Traditional packed-bed chromatography with immobile stationary phase, despite its elegance and high resolving power, has a major limitation: incapability of processing particulate-containing fluids, for example human blood. Blood cells are trapped between the beads of the chromatographic carrier resulting in increased flow resistance of the column and complete blockage of the flow. Expanded-bed chromatographic set-up overcomes the problem of handling particulate-containing fluids. However, the high shear stresses occurring in expanded bed chromatographic set-up could be detrimental for the integrity of blood cells. It is attractive to have a packed-bed chromatographic carrier with pores large enough to accommodate blood cells without being blocked. The high porosity of cryogels makes them appropriate candidates as the basis for such supermacroporous chromatographic materials [1-6]. Owing to supermacroporosity and interconnected pore-structure, such a chromatographic matrix has a very low flow resistance [7].

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or

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polymeric precursors. Cryogels typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano- and even microparticles. The unique structure of cryogels, in combination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography of biological nanoparticles (plasmids, viruses, cell organelles) and even whole cells. At present, polymeric gels have applications in many different areas of biotechnology including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immunodiffusion, and as a gel basis for solid cultural media. A variety of problems associated with using polymer gels, as well as the broad range of biological objects encountered, lead to new, often contradictory, requirements for the gels. These requirements stimulate the development and commercialization of new gel materials for biological applications. One of the new types of polymer gels with considerable potential in biotechnology is 'cryogels' (from the Greek krios (kryos) meaning frost or ice). Cryogels are formed as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting) of low- or high molecular-weight precursors, as well as colloid systems–all capable of gelling. Cryogels were first reported, 40 years ago and their properties, which are rather unusual for polymer gels, soon attracted attention. The biomedical and biotechnological potential of these materials has now been recognized [2].

Antibodies are a group of biologically active proteins produced by plasma cells in response to the presence of foreign substances. The growing role of monoclonal antibodies in biomedical research and development is widely acknowledged. In parallel, antibody-based therapeutics and in vivo diagnostics are gaining wider approval from regulatory agencies around the world [8]. For medical applications, specific immunoglobulins (IgGs) have been thoroughly purified using a combination of various physicochemical methods, mainly precipitation and chromatography [9]. Fractionation by ethanol precipitation is the most used process to purify IgG from human plasma on industrial scale (27 ton/year) [10]. At present, protein A sorbents shows high selectivity and can therefore be employed as a one-step procedure for the purification of antibodies in pilot scale [11-14]. However, in spite of its high selectivity, protein A sorbents has some drawbacks which are worth considering: (i) a considerable amount of protein A may leak from the matrix and such contamination cannot, of course, be tolerated in clinical applications; (ii) the cost of protein A sorbents remains high. In addition, it is difficult to immobilize protein A in the suitable orientation. It is also susceptible to degradation [15]. Compared to protein A, pseudo-specific ligands should display not only reduced production costs at large scale purification, but also increased resistance to chemical and biological actions. Among the pseudo-biospecific ligands, L-histidine can be used to purify a wide range of biomolecules [16-23]. L-Histidine interacts through its carboxyl, amino and imidazole groups with several proteins at around their isoelectric points and has shown particular efficacy in separating IgG subclasses from human plasma and purification of monoclonal antibodies from cell culture or ascites fluids [24].

This work reports on the purification of an IgG antibody from human plasma by a novel histidine containing supermacroporous cryogelic column. Poly(AAm-MAH) cryogel is a copolymer of Acrylamide (AAm) and N-methacryloyl-(L)-histidine-methylester (MAH) which was obtained by bulk polymerization. Poly(AAm-MAH) cryogel was characterized using Scanning Electron Microscope (SEM), FTIR and swelling test. IgG adsorption on the cryogels from aqueous solutions containing

different amounts of IgG, at different pH`s and ionic strengths, and also from human plasma was also performed. In the last part, desorption of IgG and stability of the material were tested.

EXPERIMENTAL

Materials

Immunoglobulin G (IgG) (Sigma Cat. No: 160101), L-histidine methylester, methacryloyl chloride were supplied from Sigma (St Louis, USA). Acrylamide (AAm, more than 99.9% pure, electrophoresis reagent), N,N'-methylene-bis(acrylamide) (MBAAm) and amonium persulfate (APS) were supplied by Sigma (St Louis, USA). N,N,N',N'-tetramethylene diamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption 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.

Production of cryogel

Synthesis of 2-methacryloylamidohistidine

The following experimental procedure was applied for the synthesis of MAH. 5.0 g of L-histidine methylester, methacryloyl chloride and 0.2 g of hydroquinone were dissolved in 100 ml of dichloromethane solution. This solution was cooled down to 0°C. 12.7 g triethylamine was added to the solution. 5.0 ml of methacryloyl chloride was poured slowly into this solution and then this solution was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, hydroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether-cyclohexane mixture and then dissolved in ethyl alcohol.

Production of Poly(AAm-MAH) Cryogel

Briefly, monomers (0.397 g of AAm, 415 µl of MAH and 0.269 g MBAAm) were dissolved in 10 ml deionized water and the mixture was degassed under vacuum for about 5 min to eliminate soluble oxygen. Total concentration of monomers was 8% (w/v). The cryogel was produced by free radical polymerization initiated by TEMED (30 µl) and APS (24 mg). After adding APS (3% (w/v) of the total monomers) the solution was cooled in an ice bath for 2-3 min. TEMED (3% (w/v) of the total monomers) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into plastic syringe (5 ml, id. 0.8 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at -12 °C for 20 h and then thawed at room temperature. After washing with 200 ml of water, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

Characterization of cryogel

Swelling Tests

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed on porous filter until washing was clear. Then it was sucked dry and then transferred to pre-weighed vial and weighed ($m_{wet\ gel}$). After drying to constant mass in the oven at 60°C, the mass of dried sample was determined ($m_{dry\ gel}$). The swelling degree was calculated as:

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

Surface Morphology

The morphology of a cross section of the dried cryogel was investigated by scanning electron microscope. The sample was fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer overnight, post-fixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to +10 ^{o}C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40 ^{o}C and the pressure to ca. 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of +40 ^{o}C resulted in dried cryogel sample. Finally, it was coated with gold-palladium (40: 60) and examined using a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

FTIR Studies

FTIR spectra of the poly(AAm-MAH) cryogels were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogels (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a pellet and the FTIR spectrum was then recorded.

NMR Studies

The proton NMR spectrum of MAH monomer was taken in $CDCl_3$ on a JEOL GX-400-300 MHz instrument. The residual non-deuterated solvent (TMS) served as an internal reference. Chemical shifts were reported in ppm (δ) downfield relative to TMS.

IgG Adsorption-Desorption Studies

Adsorption of Human-IgG from Aqueous Solutions

IgG adsorption studies were carried out in a recirculating system. The cryogel was washed with 30 ml of water and then equilibrated with 20 mM HEPES buffer (pH 7.4). Then, the prepared IgG solution was pumped through the column under recirculation for 2 h. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. The amount of adsorbed human-IgG was calculated as:

$$Q= [(C_o – C) V] / m \quad (2)$$

Here, Q is the amount of human-IgG adsorbed onto unit mass of supermacroporous monoliths (mg/g); C_o and C are the concentrations of human-IgG in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the cryogel used (g).

Effects of flow rate, concentration of IgG, pH and temperature of the medium and ionic strength on the adsorption capacity were studied. The flow rate of the solution (i.e., 40 ml of the aqueous IgG solution) was changed in the range of 0.5 – 3.0 ml/min. To observe the effects of the initial concentration of IgG on adsorption, it was changed between 0.05 – 3.0 mg/mL. To determine the effect of pH and temperature on the adsorption, they were changed between 5.2 – 8.5 and 4-37 ^{o}C, respectively. To observe the effects of ionic strength, NaCl was used at ionic strength values of 0.01 and 0.1.

IgG-Adsorption from Human Plasma

Human blood was collected into EDTA-containing vacutainers and red blood cells were separated from

plasma by centrifugation at 4000 g for 30 min at room temperature, then filtered (3 μm Sartorius filter) and frozen at -20 °C. Before use, the plasma was thawed for 1 h at 37 °C. Before application, the viscous sample was diluted with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.4). Dilution ratios were 1/2 and 1/10. 50 ml of the human plasma with IgG content of 14.6 mg/ml was pumped through the cryogelic column at a flow rate of 1.0 ml/min for 1.5 h. The amount of IgG adsorbed on the cryogels was determined by a solid-phase-enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG (Sigma, I-9384) diluted 1/1000 in 50 mM $NaHCO_3$, pH 9.6, was adsorbed to PVC microtitre plates at 4°C for 12 h. The plates were washed with PBS containing 0.05% Tween 20 (wash buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% BSA, and 0.1% sodium azide (blocking buffer). Samples (2.5 ml, neutralized with 0.5 ml of 1.0 M trisodium citrate) or controls containing known amounts of IgG were added and incubated at 37°C for 1 h. Bound IgG was detected with the anti IgG labeled with biotin followed by peroxidase-conjugated streptavidin and o-phenylenediamine. The absorbance was measured at 492 nm.

Adsorptions of albumin and fibrinogen were also monitored. The cryogel was contacted with a human plasma containing albumin (37.2 mg/ml), fibrinogen (2.2 mg/ml) and γ-globulin (14.6 mg/ml) in a continuous system which described before at room temperature for 1.5 h. The flow-rate was kept contant at 1.0 ml/min. Total protein concentration was measured using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm which based on Biuret reaction. Chronometric determination of fibrinogen according to the Clauss method on plasma was performed using Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France). Human serum albumin concentration was determined using Ciba Corning Albumin Reagent (Catalog Ref. No: 229241) which based on bromocresol green (BCG) dye method. IgG concentration was determined by ELISA as described above.

Desorption and Repeated Use

In all cases adsorbed IgG molecules were desorbed using 20 mM Acetate buffer containing 1.0 M NaCl solution at pH 4. In a typical desorption experiment, 50 ml of the desorption agent was pumped through the cryogelic column at a flow rate of 1.0 ml/min for 1 h. The final IgG concentration in the desorption medium was spectroscopically determined by a solid-phase-enzyme-linked immunosorbent assay method (ELISA). When desorption was achieved, the cryogel was cleaned with 20 mM sodium hydroxide and then re-equilibrated with 20 mM HEPES buffer (pH 7.4). The desorption ratio was calculated from the amount of IgG adsorbed on the cryogel and the final IgG concentration in the desorption medium.

In order to test the repeated use of poly(AAm-MAH) cryogel, IgG adsorption-desorption cycle was repeated ten times using the same cryogelic column. In order to regenerate and sterilize, after the desorption, the cryogel was washed with 20 mM sodium hydroxide solution.

RESULTS AND DISCUSSION

Characterization of Poly(AAm-MAH) Cryogels

Swelling Properties

The poly(AAm-MAH) cryogels are crosslinked hydrophilic matrices, i.e., hydrogels, therefore, they do not dissolve in aqueous media, but do swell, depending on the degree of cross-linking and on the hydrophilicity of the matrix. The equilibrium swelling

degree of the poly(AAm-MAH) monolithic cryogel was 6.97 g H_2O/g cryogel. Poly(AAm-MAH) monolithic cryogel is opaque, sponge like and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1-2 s restored its original size and shape. Increasing of surface area may effect the swelling ratio of the matrix.

Surface Morphology

The cryogelic columns prepared in syringe tube were observed under scanning electron microscopy (SEM). The obtained photographs (Figure 1) first shows the typical structure of cryogelic materials. Poly(AAm-MAH) cryogels produced has non-porous and thin polymer walls, large continuous inter-connected pores (10-100 μm in diameter) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the immunoglobulin G molecules, allowing them to pass easily. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. This open structure allows liquid to be forced through the polymer without compression of the polymer using flow rates suitable for chromatographic purposes.

FTIR Studies

2-methacryloyl amidohistidine (MAH) was selected as the comonomer and pseudospecific ligand for the selective separation of human-IgG from human plasma. In the first step, MAH was synthesized from histidine and methacrylchloride. Then, MAH was incorporated into the bulk structure of the pAAm cryogels by bulk polymerization. The molecular formula of newly synthesized poly(AAm-MAH) cryogel was given in Figure 2.

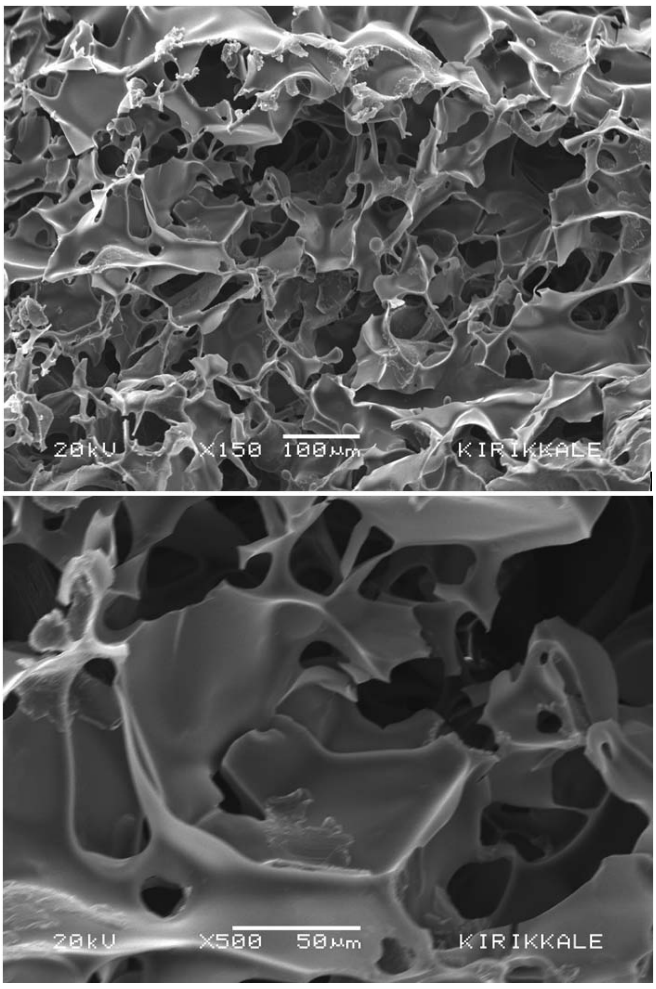

Figure 1. SEM micrographs of poly(AAm-MAH) cryogel.

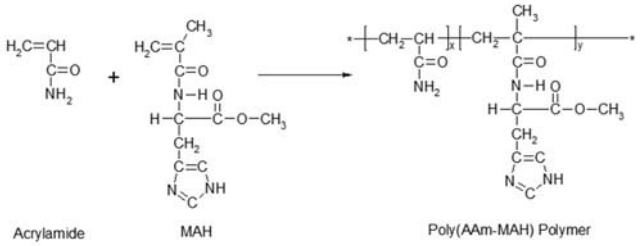

Figure 2. The molecular formula of poly(AAm – MAH) cryogels.

FTIR spectrum of MAH having the characteristic stretching vibration band amide I and amide II absorption bands at 1651 cm^{-1} and 1558 cm^{-1}, a carbonyl band at 1724 cm^{-1} was shown in Figure 3. FTIR spectrum of poly(AAm-MAH) having the characteristic stretching vibration band amide I and amide II absorption bands at 1651 cm^{-1} and 1558 cm^{-1}, a carbonyl band at 1724 cm^{-1} was shown in Figure 5.

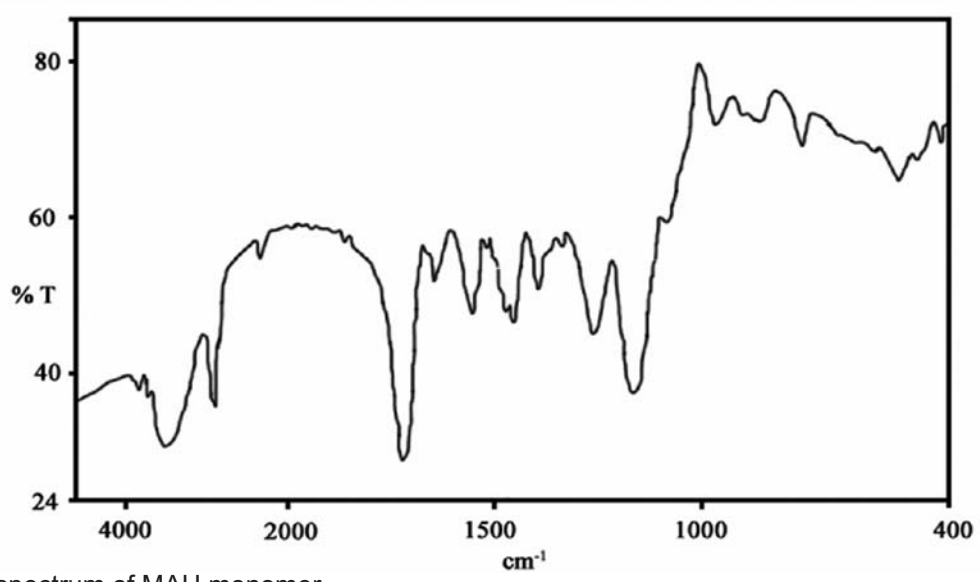

Figure 3. FTIR spectrum of MAH monomer.

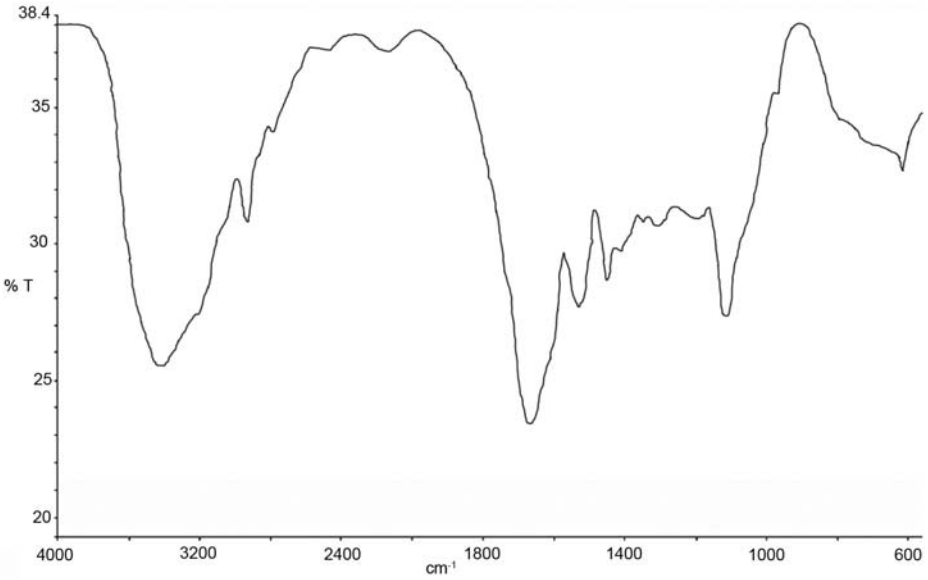

Figure 4. FTIR spectrum of AAm monomer.

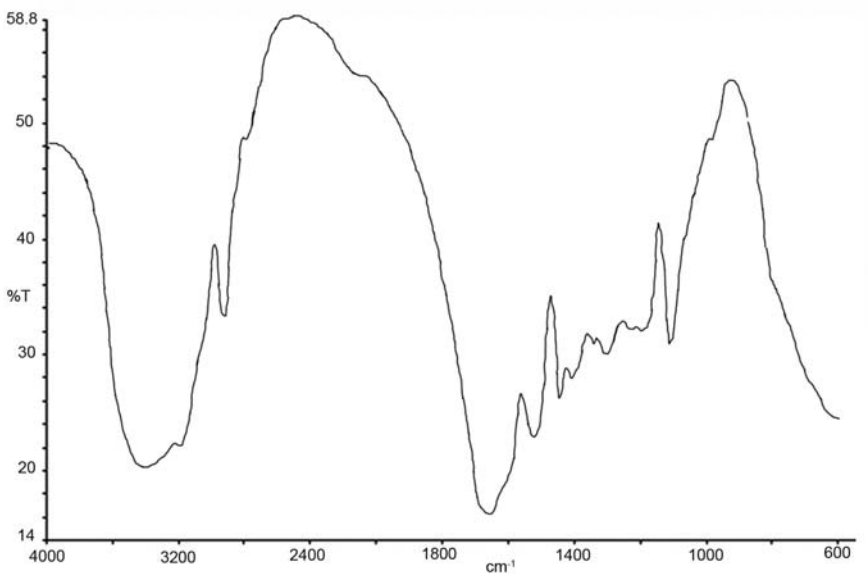

Figure 5. FTIR spectrum of poly(AAm-MAH) cryogel.

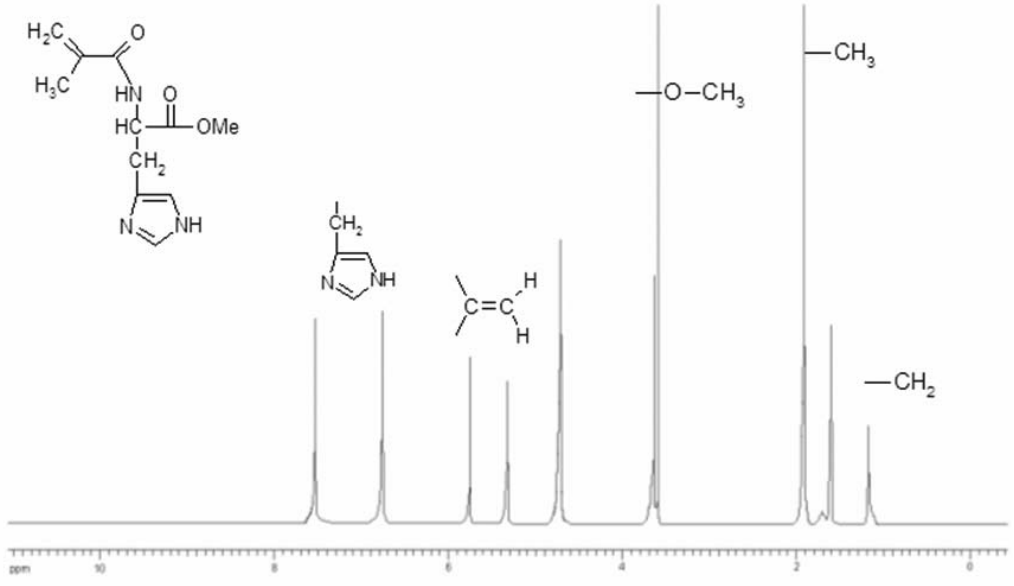

Figure 6. ^{1}H-NMR Spectrum of MAH Monomer.

^{1}H-NMR Studies

^{1}H-NMR was used to determine the MAH structure. Figure 6 shows the ^{1}H -NMR spectrum of MAH. ^{1}H -NMR spectrum is shown to indicate the characteristic peaks from the groups in MAH monomer. These characteristic peaks are as follows: ^{1}H -NMR ($CDCl_3$): δ 1.99 (t; 3H, J=7.08 Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (t; 3H, -OCH_3) 4.82-4.87 (m; 1H, methin), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86 (δ; 1H, J=7.4 Hz, NH), 7.82 (δ; 1H, J=8.4 Hz, NH), 6.86-7.52 (m; 5H, aromatic),

Adsorption of IgG from Aqueous Solutions

Effect of Flow – Rate

The adsorption capacity at different flow-rates are given in Figure 7. The adsorption capacity decreased significantly from 19.67 mg/g to 7.58 mg/g for the poly(AAm-MAH) cryogel with the increase of the flow-rate from 0.5 ml/min to 3.0 ml/min. One of the explanation for such phenomenon would be a faster ligand-protein (i.e., MAH) dissociation rate compared to the association rate. Hence, the adsorbate (i.e., protein molecules) would pass through the cryogel column without adsorption at high flow-rate.

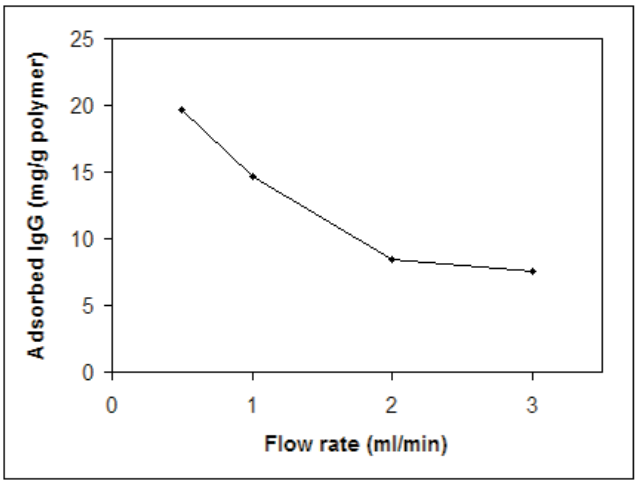

Figure 7. Effect of flow-rate on IgG adsorption: IgG concentration: 0.5 mg/ml; adsorbing buffer HEPES (pH 7.4); T: 25°C.

Effect of Buffer Type

Figure 8 shows IgG adsorption capacity in different buffer systems at different pH's. IgG adsorption onto cryogel seemed to depend on the pH. Buffer ranges are 5.2 – 7.0 for MES, 6.0 – 8.0 for phosphate, 6.5 – 8.0 for HEPES, 6.5 – 8.0 for MOPS and 7.0 – 8.0 for Tris-HCl. In HEPES buffer, adsorption capacity is higher than other buffers. Maximum adsorption capacities are observed at pH 7.4 for Phosphate (11.98 mg/g), at pH 7.4 for HEPES (15.71 mg/g), at pH 7.0 for MES (10.08 mg/g), at pH 7.0 for MOPS

(9.57 mg/g) and at pH 7.4 for Tris-HCl (9.74 mg/g). Below and over the maximum adsorption pH's, adsorption capacities decreased significantly. pKa values for MOPS, HEPES and MES are pH 6.5, 7.0 and 5.4, respectively. From the structure of the buffer ions used, it is obvious that tris-HCl and phosphate carries one or more charge of the same sign - only (positive) or only (negative) whereas the zwitter ionic buffers MES, MOPS and HEPES carry two charges of opposite sign below their pKa. It can be clearly observed that the maximum adsorption was obtained with HEPES.

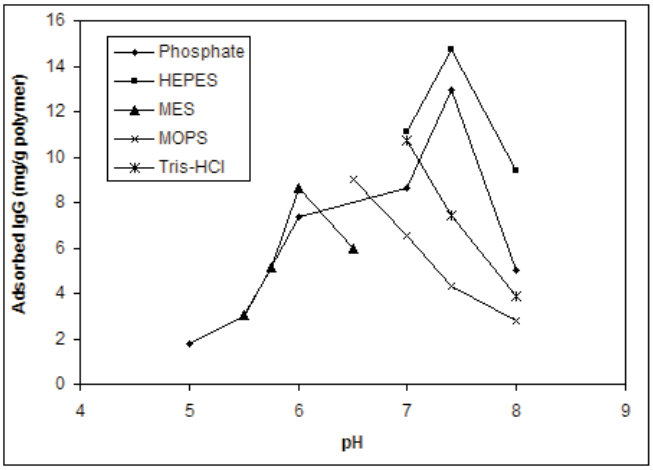

Figure 8. Effect of buffer type on IgG adsorption: IgG concentration: 0.5 mg/ml; Flow-rate: 1.0 ml/min; T: 25 °C.

Effect of Concentration of IgG

Figure 9 shows the effect of IgG concentration on adsorption. IgG adsorption onto the poly(AAm) cryogel was low (0.22 mg/g), while adsorption of IgG molecules onto poly(AAm-MAH) cryogel through MAH monomer was significant (up to 32.73 mg/g). As expected, the amount of IgG coupled to cryogels reached almost a plateau value around 2.0 mg/ml, due to the saturation of active binding sites.

Effect of Ionic Strength

IgG adsorption to the poly(AAm-MAH) cryogels was performed at different NaCl concentrations. The effect of ionic strength on IgG adsorption is shown in Figure 10. As seen here, IgG adsorption capacity decreased with the increasing salt concentration. The decrease in the adsorption capacity as the ionic strength increases can be attributed to the repulsive electrostatic forces between the poly(AAm-MAH) cryogel and protein molecules. When the salt concentration increased in the adsorption medium, this can lead to coordination of the deprotonated amino groups of the histidine with cations of the salts, which leads to low protein adsorption. The distortion of existing salt bridges between protein molecules and pseudo-specific affinity ligand in the presence of salt also contributed to this low protein adsorption at high ionic strength.

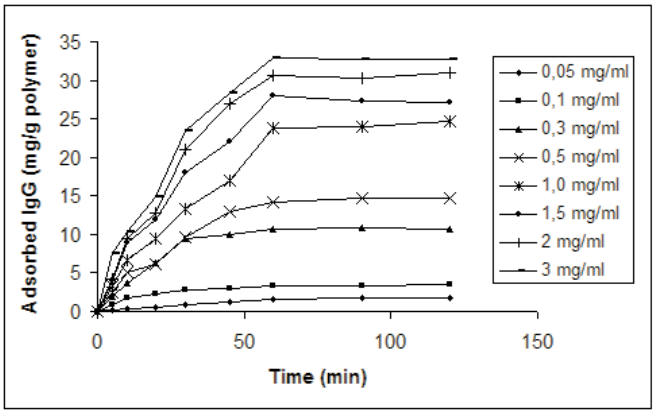

Figure 9. Effect of IgG concentration on adsorption capacity; Adsorbing buffer HEPES (pH 7.4); flow rate: 1.0 ml/min; T: 25°C.

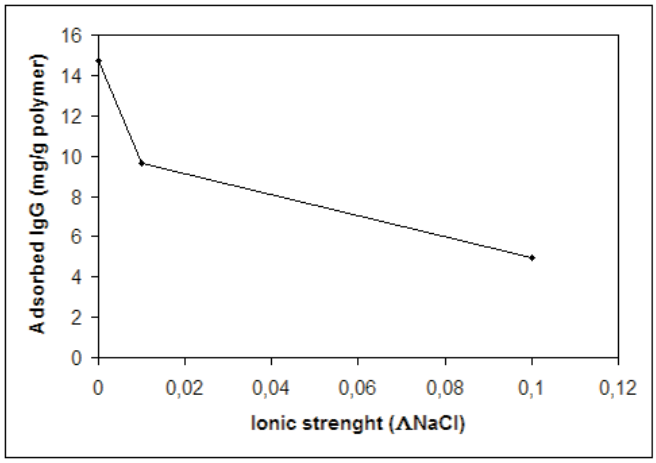

Figure 10. Effect of ionic strength on IgG adsorption; IgG concentration 0.5 mg/mL; flow rate: 1.0 ml/min; adsorbing buffer HEPES (pH 7.4); T: 25 °C.

Effect of Temperature

The effect of temperature on IgG adsorption were studied in the range of 4-37°C. The equilibrium adsorption of IgG onto the poly(AAm-MAH) cryogel significantly decreased with increasing temperature and the maximum adsorption was achieved at 4°C (Figure 11). From 4°C to 37°C, the adsorption capacity of the cryogels decreased about 57% for the poly(AAm-MAH) cryogel. A possible explanation for this behavior is the exothermic nature of the adsorption process.

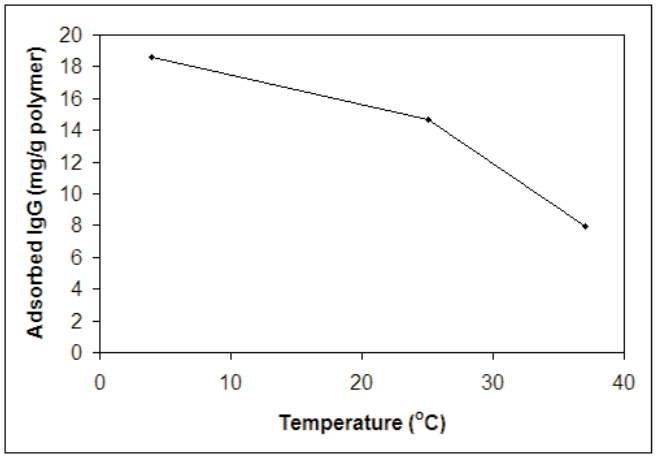

Figure 11. Effect of temperature on IgG adsorption; IgG concentration 0.5 mg/mL; flow rate: 1.0 ml/min; adsorbing buffer HEPES (pH 7.4).

Adsorption Isotherms

An adsorption isotherm is used to characterize the interactions of each protein molecule with the adsorbent. This provides a relationship between the concentration of the protein in the solution and the 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 sites, 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 are no interactions between molecules adsorbed on adjacent sites.

Adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm is expressed by Equation 3. The corresponding transformations of the equilibrium data for IgG gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{max}.\ b\ .\ C_{eq} / (1 + b\ C_{eq}) \quad (3)$$

where Q is the adsorbed amount of IgG (mg/g), C_{eq} is the equilibrium IgG concentration (mg/mL), b is the Langmuir constant (mL/mg) and, Q_{max} is the maximum adsorption capacity (mg/g). This equation can be linearized so that

$$C_{eq}/Q = 1/(Q_{max}.\ b) + C_{eq} / Q_{max} \quad (4)$$

The plot of C_{eq} versus C_{eq}/Q was employed to generate the intercept of $1/Q_{max}.b$ and the slope of $1/Q_{max}$ (Figure 12).

The maximum adsorption capacities (Q_{max}) data for the adsorption of IgG were obtained from the experimental data (Table 1). The correlation coefficients (R^2) were high for poly(AAm-MAH) cryogel. The Langmuir adsorption model can be applied in this affinity adsorbent system.

The other well-known isotherm, which is frequently used to describe adsorption behaviour, is the Freundlich isotherm. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed protein is the summation of adsorption on all binding sites. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. This emprical equation takes the form:

Table 1. Langmuir and Freundlich adsorption constants and correlation coefficients for IgG.

Type of polymer	Experimental	Langmuir constants			Freundlich constants		
	Q_{exp} (mg/g)	Q_m (mg/g)	b (mL/mg)	R^2	K_F	n	R^2
Poly(AAm-MAH)	32.73	43.67	1.199	0.9867	21.65	0.71	0.9528

$$Q_{eq} = K_F (C_{eq})^n \quad (5)$$

where, K_F and n are the Freundlich constants.

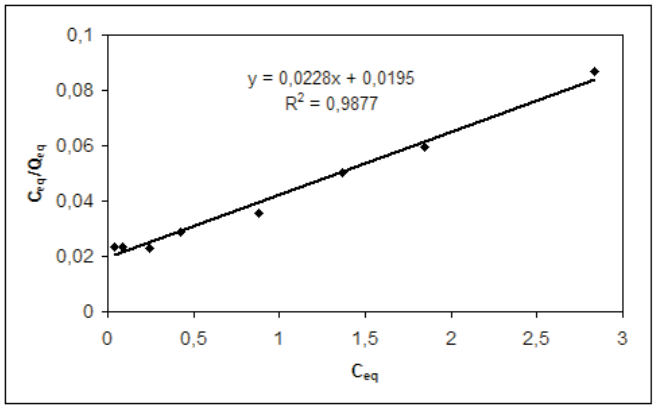

Figure 12. Langmuir adsorption isotherm of the poly(AAm-MAH) cryogels.

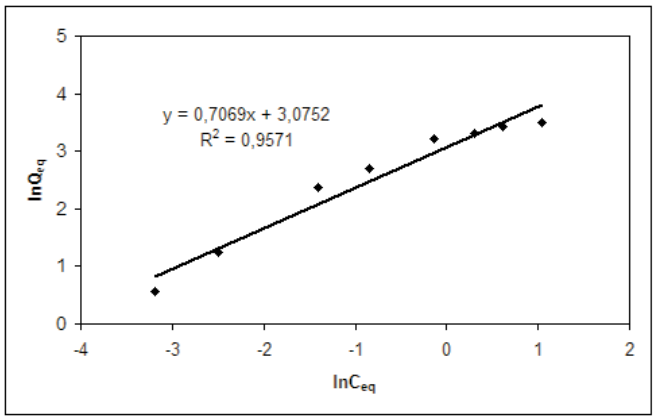

Figure 13. Freundlich adsorption isotherm of the poly(AAm-MAH) cryogels.

The adsorption isotherms of IgG were found to be linear over the whole concentration range studies and the correlation coefficients were high (Figure 13). According to the correlation coefficients of isotherms, Langmuir adsorption model is favorable. Table 1 shows the Freundlich adsorption isotherm constants, n and K_F and the correlation coefficients. The magnitude of K_F and n values of Freundlich model showed easy uptake of IgG from aqueus medium with a high adsorption capacity of the cryogel.

Adsorption Kinetics Modelling

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data. The kinetic models (Pseudo-first- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$dq_t / d_t = k_1(q_{eq}-q_t) \quad (6)$$

where k_1 is the rate constant of pseudo-first order adsorption (1/min) and q_{eq} and q_t denote the amounts of adsorbed protein at equilibrium and at time t (mg/g), respectively. After integration by applying boundary conditions, q_t=0 at t=0 and $q_t = q_t$ at t=t, gives

$$\log[q_{eq}/(q_{eq}-q_t)]= (k_1 t)/2.303 \quad (7)$$

Equation 7 can be rearranged to obtain a linear form

$$\log(q_{eq}-q_t)= \log(q_{eq}) - (k_1 t)/2.303 \quad (8)$$

a plot of $\log(q_{eq})$ versus t should give a straight line to confirm the applicability of the kinetic model. In a

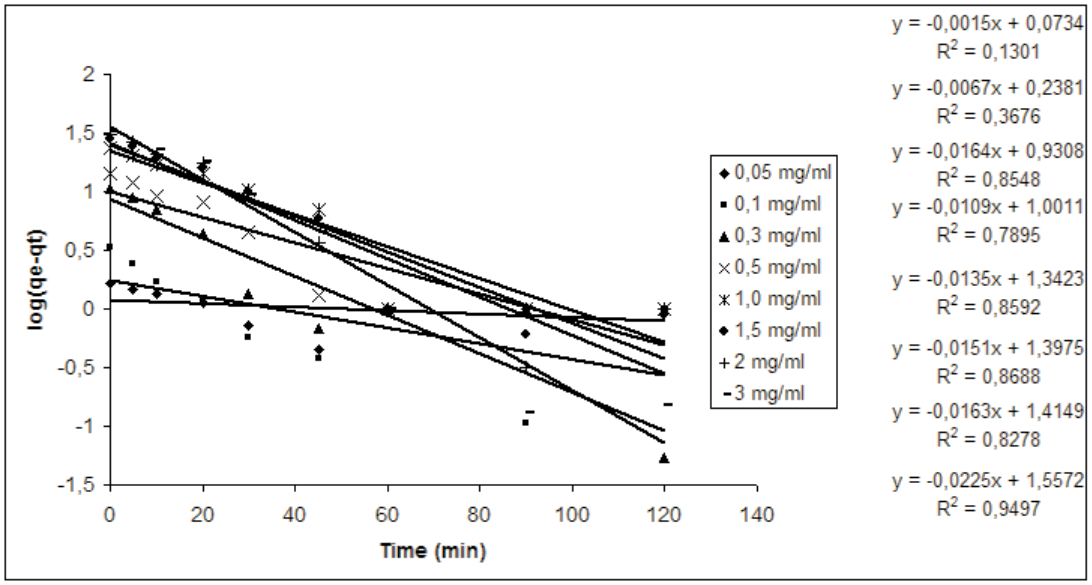

Figure 14. Pseudo-first order kinetic of the experimental data for the poly(AAm-MAH) cryogels.

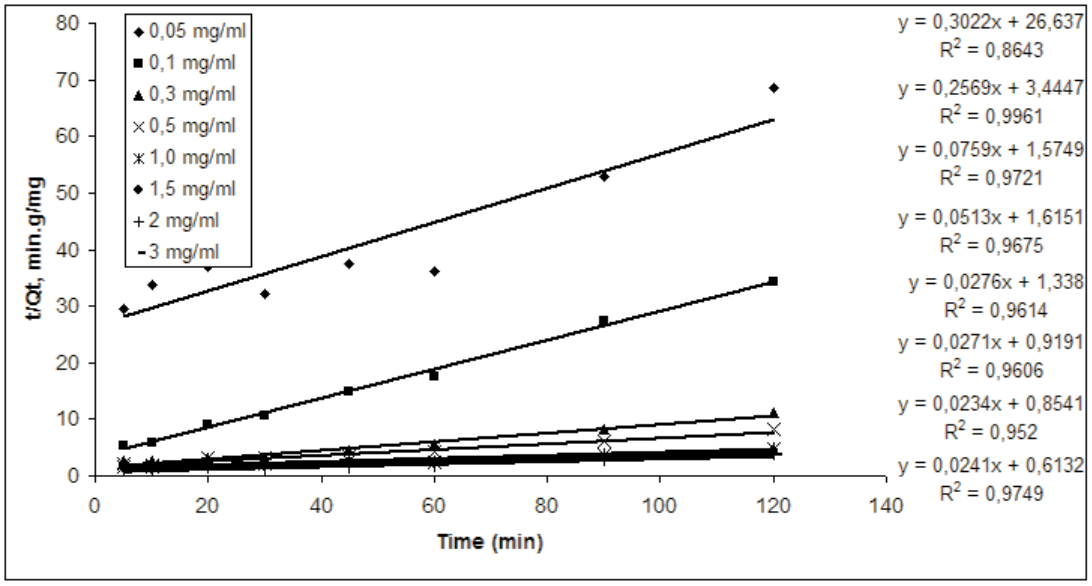

Figure 15. Pseudo-second order kinetic of the experimental data for the poly(AAm-MAH) cryogel.

true first-order process logq_{eq} should be equal to the interception point of a plot of log(q_{eq} - q_t) via t.

In addition, a pseudo-second order equation based on equilibrium adsorption capacity may be expressed in the form

$$dq_t / d_t = k_2 (q_{eq} - q_t)^2 \quad (9)$$

Where k_2 (g/mg·min) is the rate constant of pseudo-first order adsorption process. Integrating Equation 9 and applying the boundary conditions, q_t=0 at t=0 and q_t= q_t at t=t, leads to,

$$[1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2 t \quad (10)$$

or equivalently for linear form

$$(t/q_t)= (1/k_2 q_{eq}^{\ 2}) + (1/q_{eq})\, t \quad (11)$$

a plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively. A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figures 14 and 15 are presented in Table 2. The theoretical q_{eq} values for second order kinetic models were higher to that of pseudo-first kinetic models and the correlation coefficients were

Table 2. The first and second order kinetic constants for poly(AAm-MAH) cryogel.

Initial Conc.	Experimental	First-order kinetic			Second-order kinetic		
(mg/ml)	q_{eq} (mg/g)	k_1 (1/min)	Q_{eq} (mg/g)	R^2	k_2 (1/min)	q_{eq} (mg/g)	R^2
0.05	1.66	0.0035	1.18	0.1301	0.0035	3.31	0.8643
0.1	3.40	0.015	1.73	0.3676	0.019	3.89	0.9961
0.3	10.70	0.038	8.53	0.8548	0.0037	13.18	0.9721
0.5	15.71	0.025	10.03	0.7895	0.0014	20.83	0.9734
1.0	24.67	0.031	21.99	0.8592	0.0006	36.23	0.9614
1.5	27.07	0.035	24.97	0.8688	0.0008	36.90	0.9606
2.0	30.60	0.038	25.99	0.8278	0.0006	42.74	0.952
3.0	32.73	0.052	36.07	0.9497	0.0009	41.49	0.9749

high. Results indicate that this cryogel obeyed second order kinetic model.

IgG Adsorption from Human Plasma

Table 3 gives the adsorption data. As seen here, there was a pronounced adsorption of IgG (up to 100.0 mg/g) onto the poly(AAm-MAH) cryogel for plasma diluted with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.4). It is worth to point that the adsorption of IgG onto the poly(AAm-MAH) cryogel was higher than those obtained in the studies in which aqueous solutions were used. This is due to the high initial concentration of IgG in the plasma. IgG, has a molecular mass of 150 000 and consists of four peptide chains; two identical light chains. These chains are linked by strong disulphide bonds into a Y- or T-shaped structure with hinge-like flexible arms. Thus an IgG molecule would expand and contract significantly with the variation of the ionizable groups in the molecule. This high IgG adsorption may also be due to suitable conformation of IgG molecules within their native medium (i.e., human plasma pH 7.4) for interaction with histidine groups onto the poly(AAm-MAH) cryogel.

Table 3. IgG adsorption from human plasma. IgG concentration before dilution: 14.6 mg/mL; flow rate: 1.0 ml/min; T: 25 °C.

Dilution Agent	Adsorption Capacity (mg/g)
Plasma (undiluted)	100.0 ± 2.11
1/2 diluted plasma (phosphate pH: 7.4)	52.9 ± 2.60
1/10 diluted plasma (phosphate pH: 7.4)	34.3 ± 2.52

Adsorptions of albumin and fibrinogen were also determined. There was a pronounced adsorption of IgG onto poly(AAm-MAH) cryogel for undiluted plasma (100.0 mg/g). Adsorption capacities were found to be 9.6 mg/g for fibrinogen and 9.1 mg/g for albumin. The total protein adsorption was determined as 118.7 mg/g. IgG adsorption ratio is around 84.2% (100.0 mg IgG per gram adsorbent: 118.7 mg total proteins per gram adsorbent). Fibrinogen and albumin adsorption ratios are 8.1% (9.6 mg fibrinogen per gram sorbent: 118.7 mg total proteins per gram sorbent) and 7.7% (9.1 mg albumin per gram sorbent: 118.7 mg total proteins per gram sorbent). IgG adsorbed more than HSA under physiological condition, although the initial

concentration of IgG to HSA was in the ratio (14.6 mg/ml: 37.2 mg/ml). Most of described methods for IgG capture resulted in co-adsorption of few other proteins among them significant of albumin. Compared to albumin and fibrinogen, adsorption of IgG is significant.

Comparison with Related Literature

Different adsorption capacities were reported in literature for IgG adsorption. Füglistaller was interested in determining the dynamic binding capacities of different commercial protein A affinity chromatography matrices including Affi-Gel, Eupergit, Ultrogel, Sepharose series and Prosep A [14]. He presented adsorption capacities of 0.7-20 mg IgG_3/g. Bueno et al used Poly(ethylene vinyl alcohol) hollow fiber cartridge carrying L-histidine and they reported dynamic adsorption values up to 77.7 mg IgG per gram polymer [25]. Klein et al used microporous poly(caprolactam) hollow fibers and flat sheet membranes as the carrier matrix, and immobilised recombinant protein A as specific bioligand [26]. They reported IgG adsorption capacities around 12.4-28.3 mg/cm^3. Kim et al used hydrophobic amino acids (e.g., phenylalanine and tryptophan) containing membrane based on polyethylene and they obtained 50 mg per gram polymer for bovine gamma globulin [27]. Muller-Shulte et al used several polymeric carriers made of different polymers including Biograft, Sepharose 4B, Superose and Spherosil and histidine as the pseudo-specific ligand [28]. Their maximum IgG_1 adsorption values were in the range of 0.05-0.23 mg IgG_1 per millilitre sorbent. Denizli et al reported 24 mg IgG/g adsorption capacity with protein A-immobilised-PHEMA beads [29]. Langotz and Kroner reported 0.5 mg per mL rabbit IgG adsorption capacity with commercially available Sartobind Epoxy sorbents [30]. Dancette et al studied the performance of recombinant protein A/G affinity membranes which based on polymethyl methacrylate, and polyacrylonitrile for human and mouse IgG purification and they obtained the static binding capacity as 6.6 mg IgG per ml membrane [31]. Teng et al described a fully characterized IgG binding ligand comprising a triazine scaffold substituted with 3-aminophenol and 4-amino-1-naphthol [32]. They showed that this synthetic protein A mimic ligand interacted with HIgG and to be able to purify IgG selectively from diluted human plasma. They achieved adsorption capacities in the range of 52 mg/g moist weight gel. Özkara et.al., immobilized L-histidine covalently onto PHEMA beads and they obtained 3.5 mg IgG adsorption amount per gram polymer in batch system [33]. Protein A mimetic synthetic ligands carrying Sepharose 4B and Sepharose CL-6B sorbents were used in the purification of IgG from diluted human plasma and maximum adsorption capacity was found to be in the range of 7-25 mg/g [34-35]. The IgG adsorption capacity obtained in this study (up to 100 mg IgG/g) would seem to be sufficient to propose these poly(AAm-MAH) cryogels as affinity supports. Differences of IgG adsorption are due to the properties of each adsorbent such as structure, functional groups, ligand loading and surface area.

Desorption and Repeated Use

In the last step of the affinity separation, the main concern was to desorp the adsorbed protein in the shorthest time and at the highest amount possible. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. In this study, more than 95% of the adsorbed IgG molecules was removed easily from the cryogel in all cases when acetate buffer containing 1 M NaCl, at pH 4 was used as desorption agent. It should be noted that elution of IgG is achieved under relatively mild conditions employed during affinity chromatography on protein A sorbents.

In order to show reusability of the cryogels, the

adsorption-desorption cycle was repeated ten times using the same cryogel (Figure 16). For sterilization, after one adsorption-desorption cycle, cryogel was washed with 20 mM NaOH solution for 30 min. After this procedure, cryogel was washed with distilled water for 30 minutes, then re-equilibrated with HEPES buffer for the next adsorption-desorption cycle. It is observed that the adsorption behavior of IgG to poly(AAm-MAH) cryogels was little changed over ten cycles. These results demonstrated the stability of the present poly(AAm-MAH) cryogel as an affinity adsorbent.

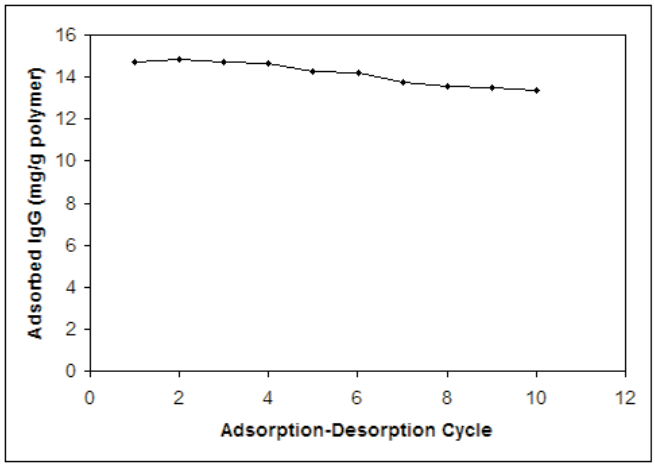

Figure 16. Repeated use of the poly(AAm-MAH) cryogel. IgG concentration 0.5 mg/mL; flow rate: 1.0 ml/min; adsorbing buffer HEPES (pH 7.4); T: 25 °C.

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