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Boronic Acid Functionalized Adsorbents for Antibody Removal

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Abstract

In this study a novel approach was developed to obtain an efficient separation of human-IgG from human plasma. 4-Vinylphenylboronic acid (VPBA) and 2-hydroxyethylmethacrylate (HEMA) were chosen as monomers. Spherical microbeads with an average size of 100-140 µm were obtained by the radical suspension polymerization of VPBA and HEMA conducted in an aqueous dispersion medium. VPBA monomer was characterized by FTIR. p(HEMA-VPBA) beads were characterized by swelling studies, FTIR and SEM. The pHEMA and p(HEMA-VPBA) microbeads were contacted with blood in in-vitro systems. Loss of the blood cells and the clotting times were also followed. Good blood compatibility properties of both pHEMA and p(HEMA-VPBA) were clearly observed with blood coagulation experiments. Loss of cells in the blood contacting with pHEMA and p(HEMA-VPBA) microbeads were nearly the same. These VPBA containing p(HEMA-VPBA) microbeads with a swelling ratio of 57 % were used in the adsorption/desorption of human-IgG from aqueous solutions and human plasma. The maximum human-IgG adsorption (139 mg/g polymer) was observed at pH 8.5 for HEPES buffer. IgG adsorption from human plasma was 38.75 mg/g. It was also observed that human-IgG could be repeatedly adsorbed and desorbed with p(HEMA-VPBA) microbeads without significant loss in the adsorption capacity.

Key words: Boronic Acid, Antibody Removal, Boranate affinity

INTRODUCTION

Antibodies and antibody derivatives constitute a significant percentage of biopharmaceutical products and many new therapeutic products based on monoclonal antibodies (MAbs) are on the way of approval for use. The production of MAbs is not limited by fermentation, but by the purification of these molecules from the complex media in which they are produced [1,2]. Chromatographic separations play an important role in the downstream processing of MAbs, recombinant proteins and therapeutic plasmids. Chromatography is widely used at all stages of the purification process ranging from capture from cell free harvests to the polishing step needed to remove trace levels of products and process related impurities [3-7].

Although many different separation technologies are available, downstream processing of MAbs is still based on bead-based chromatography with an affinity capturing steps and one or more polishing steps. The purification of antibodies by affinity chromatography using different types of ligands has recently been reviewed [8]. Affinity chromatography on natural immunoglobulin-binding ligands such as Protein G and Protein A is by far the most widely used method for MAb capture from crude extracts

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[9-14]. Despite their extended use, natural immunoglobulin binding protein ligands suffer from a number of problems including the high cost, their biological origin (difficulties in isolation and purification from microbial extracts), the requirement for accurate analytical tests to ensure the absence of toxic contaminants, and the poor stability to cleaning and sanitizing agents [15-16]. Synthetic affinity ligands are low molecular weight compounds that can circumvent the drawbacks of natural antibody-binding ligands by imparting resistance to chemical and biochemical degradation, facile and low cost of production, and ready in situ sterilization. These ligands include synthetic matrices with different interaction modes with immunoglobulins [17-20], peptides obtained through the synthesis and screening of peptide combinatorial libraries [21-23], and non-peptidic artificial mimic ligands such as histidine ligand, thiophilic, hydrophobic and immobilized metal affinity chromatographic ligands [24-30].

Boronate-sugar interactions were also widely employed in affinity chromatography. Main interaction in boronate and analyte is the ester formation between boronate ligand and cis-diol compound, ideally 1,2-cis diol compounds [31]. Secondary interactions may also play important role in binding; hydrophobic interactions or aromatic π-π interactions participate when aromatic boronates are used. The negative charge of the active tetrahedral boronate can produce attractive or repulsive forces for ionic compounds. As was true for hydrophobic effects, there are some cases in which ionic interactions may provide additional selectivity to boronate columns. There is also several possible sites for hydrogen bonding, although the effect is small [31]. Considering its usefulness as a ligand, boronate affinity columns were first employed for the separation of sugars and nucleic acid components [32]. Since then this technique has been exploited for the separation of a wide variety of *cis*-diol compounds, including nucleosides, nucleotides nucleic acids [33-36], carbohydrates [37,38], glycoproteins and enzymes [39-41], and these interactions were used for the detection of saccharides, sensors for monitoring glucose, as well as synthesis of sugar-responsive materials [42-45].

This work reports on the purification of an IgG antibody from human plasma by boronate affinity chromatography with a boronic acid containing bioaffinity sorbent which was obtained by suspension polymerization of 2-hydroxyethyl methacrylate (HEMA) and 4-vinylphenylboronic acid (VPBA). p(HEMA-VPBA) microbeads were characterized using Scanning Electron Microscope (SEM), FTIR, and swelling test. IgG adsorption on the microbeads from aqueous solutions containing different amounts of IgG, at different pHs and ionic strengths, and also from human plasma was performed. In the last part, desorption of IgG and reusability of the material were tested.

EXPERIMENTAL

Materials

Human-immunoglobulin G (human-IgG), 4-vinylphenylboronic acid (VPBA) and HEPES were supplied by Kedrion S.p.A (Barga, Italy), Aldrich (St Louis, USA) and Sigma (St Louis, USA), respectively. The monomers, 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4°C until use. Poly(vinylalcohol) (PVAL; MW: 100.000, 98% hydrolyzed) was supplied from Aldrich Chem. Co. (USA). 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 NANO-

pure® organic/colloid removal and ion exchange packed-bed system. The resulting purified water (deionized water) has a specific conductivity of 18 megaohm/cm^{-1}.

Preparation of pHEMA microbeads

PHEMA microbeads were prepared by a radical suspension polymerization technique [46]. Polymerization was carried out in an aqueous dispersion medium containing polyvinylalcohol (PVAL) as stabilizer. Toluene and EGDMA was included in the recipe as the diluent (as a pore former) and cross-linker, respectively. The monomer phase containing HEMA, EGDMA, toluene and α,α´-azobisisobutyronitrile (AIBN) was added to the dispersion medium within a laboratory type reactor (i.e., a two neck flask with a volume of 500 ml) provided with a blade type stirrer. In order to produce spherical particles of about 100-150 μm in diameter and with narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1:3 (v:v), 1:10 (v/v), 0.33 (mol EGDMA/mol HEMA), 0.0015 (mol AIBN/mol HEMA), 600 rpm, respectively. Polymerization was carried out at 65°C for 4 h and then at 90°C for 2 h. After cooling, the particles were separated from the polymerization medium by filtration, and the residuals (e.g., monomer, etc.) were removed by a cleaning procedure [47]. Briefly, particles were transferred into a reservoir, and washing solutions (i.e., a dilute HCl solution and a water-ethanol mixture) were recirculated through the system which includes also an activated carbon column, until to be assured that the particles are clean.

Preparation of p(HEMA-VPBA) Microbeads

The monomers, 2-hydroxyethyl methacrylate (HEMA) and VPBA, were copolymerized in suspension, as well. Table 1 gives the recipe and polymerization conditions to obtain copolymer microbeads. At the end of the polymerization reaction, soluble residuals were removed from the polymer and the same cleaning procedure was applied as above.

Table 1. Recipe and polymerization conditions for the preparation of p(HEMA-VPBA) beads.

Aqueous Dispersion Phase		Organic Phase	
Distilled water	: 50 ml	VPBA	: 250 mg
PVAL	: 0,5 g	HEMA	: 4,0 ml
		EGDMA	: 8,0 ml
		Toluene	: 12 ml
		AIBN	: 10 mg
Polymerization Conditions			
Reactor volume	: 100 ml		
Stirring Rate	: 600 rpm		
Temperature and Time	: first at 65°C for 4 h and then at 90°C for 2 h		

When not in use, the resulting particles were kept under refrigeration in 0.02% NaN_3 solution for preventing of microbial contamination.

Characterization of Microbeads

Size Analysis

The average size and size distribution of the p(HEMA-VPBA) beads were determined by screen analysis performed by using Standard Test Sieves (Retsch GmbH & Co., Germany).

Surface Area Measurements

Pore volumes and average pore diameter greater than 20Å were determined by mercury porosimeter up to 2000 kg/cm^2 using a Carlo Erba model 200 (Italy). The surface area of the polymer sample was measured with a surface area apparatus (BET method).

Swelling Test

Water uptake ratios of the pHEMA and p(HEMA-VPBA) microbeads were determined in distilled

water. The experiment was conducted as follows: initially dry microbeads were carefully weighed before being placed in a 50 mL vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25±0.5°C) for 2 h. The bead sample was taken out from the water, wiped using a filter paper, and weighed. The weight ratio of dry and wet samples was recorded. The water content of the pHEMA and p(HEMA VPBA) microbeads was calculated by using the following expression:

$$\text{Water uptake ratio } \% = [(W_s - W_o)/ W_o] \times 100 \quad (1)$$

where W_o and W_s are the weights of microbeads before and after uptake of water, respectively.

Surface Morphology

The surface morphology of the polymeric microbeads was examined using scanning electron microscopy (SEM). The samples were initially dried in air at 25°C for seven days before being analyzed. A fragment of the dried microbead was mounted on a SEM sample mount and was sputter coated for 2 minutes. The sample was then mounted in a scanning electron microscope (Model: Raster Electronen Microscopy Leitz-AMR-1000, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the p(HEMA-VPBA) microbeads.

FTIR Studies

FTIR spectra of VPBA, pHEMA and the p(HEMA-VPBA) microbeads were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry microbeads (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.

Blood Compatibility Studies

Activated Partial Thromboplastin Time (APTT)

pHEMA and p(HEMA-VPBA) microbeads were incubated in 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed on a glass filter with 0.5 M NaCl solution and distilled water. Fresh frozen pooled human plasma (0.1 mL) was preheated to 37°C for 2 min. The partial thromboplastin (0.3 mL, bioMerieux, Marcyl'Etoile, France) was also preheated to 37°C for 2 min and was added to preheated human plasma. Then, 10 mg of microbead were added into this medium. Thirty seconds later, $CaCl_2$ (0.1 mL, 0.025 M) was added, then, the active partial thromboplastin time (APTT) was determined by using the fibrometer method [48].

Prothrombin Time (PT)

In order to determine prothrombin time (PT), one-stage prothrombin method was used. pHEMA and p(HEMA-VPBA) microbeads were incubated in 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature. Fresh frozen pooled human plasma (0.1 M) was preheated to 37°C for 2 min. The thromboplastin (0.2 mL, bioMerieux, Marcy l'Etoile, France) was also preheated to 37°C for 2 min and was added to preheated human plasma. Then, 10 mg of microbeads were added into this medium. Thirty seconds later, $CaCl_2$ (0.1 mL, 0.025 M) was transferred into the medium. After these operations, the prothrombin time was measured by using fibrometer method [49].

Coagulation Time (CT)

pHEMA and p(HEMA-VPBA) microbeads were incubated in 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed on a glass filter with 0.5 M NaCl solution and distilled water.

Fresh frozen pooled human plasma (0.1 mL) was preheated to 37°C for 2 min and then 10 mg of microbeads were added into this medium and mixed immediately. The clotting time was measured by using fibrometer method [49].

IgG Adsorption-Desorption Studies

Adsorption of Human-IgG From Aqueous Solutions

Adsorption of human-IgG on the p(HEMA-VPBA) microbeads was studied in batchwise. The polymeric microbeads (100 mg) were incubated with 5 mL of human-IgG for 2 h (i.e., equilibrium time), in flasks agitated magnetically at 130 rpm. Effects of initial concentration of human-IgG, buffer type and pH, temperature and ionic strength on the adsorption rate and capacity were studied. To observe the influence of buffer type on the adsorption capacity, the adsorption studies were carried out in respective buffering ranges of each buffer system, i.e., 25 mM morpholinoethanesulfonic acid (MES), 25 mM morpholinopropanesulfonic acid (MOPS), 25 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES), 25 mM acetate, phosphate and carbonate buffers (contains 0.15 M NaCl). The initial concentration of human IgG was 1.0 mg/mL. To observe the effects of the initial concentration of human-IgG on adsorption, it was changed between 0.5-5.0 mg/mL (at pH: 8.5 HEPES buffer). Human-IgG concentration was determined by measuring the protein absorbance at 280 nm. The amount of adsorbed human-IgG was calculated as:

$$q = [(C_o - C) V] / m \qquad (2)$$

Here, q is the amount of human-IgG adsorbed onto unit mass of microbeads (mg/g); C_0 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 microbeads used (g).

Adsorption of Human-IgG From Human Plasma

Adsorption of human-IgG from human plasma on the pHEMA and p(HEMA-VPBA) microbeads was studied batch-wise. Fresh human plasma obtained from a healty donor was used in all experiments. Blood samples were centrifuged at 500 g for 30 min at room temperature. The pHEMA and p(HEMA-VPBA) microbeads were incubated with 10 mL plasma at 20ºC for 2 h. Plasma samples were diluted with isotonic solution when needed. The amounts of human-IgG adsorbed by p(HEMA-VPBA) microbeads were determined by a solid-phase-enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG (Sigma, 1-9384) 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 (washing buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% BSA, and 0.1% sodium azide (microorganism blocking buffer). Samples *(2.5* ml, neutralized with 0.5 mL of 1.0 M trisodium citrate) or controls containing known amounts of human-IgG were added and incubated at 37°C for 1 h. Bound human-IgG was detected with the anti human-IgG labeled with biotin (Sigma, B-3773) followed by peroxidase-conjugated streptavidin (Sigma) and o-phenylenediamine. The absorbance was measured at 492 nm.

Desorption and Repeated Use

Desorption of human-IgG was studied with 1 M NaCl in 25 mM, pH 4.0 acetate buffer. The p(HEMA-VPBA) microbeads-adsorbed human-IgG were placed in this desorption medium and stirred continuously (at a stirring rate of 600 rpm) for 2 h at room temperature. The final human-IgG concentration in desorption medium was determined by a

solid-phase-enzyme linked immunosorbent assay method (ELISA). Desorption ratio was calculated from the amount of human-IgG adsorbed on the microbeads and the final human-IgG concentration in desorption medium by the following equation: In order to test the reusability of p(HEMA VPBA) microbeads, human-IgG adsorption-desorption procedure was repeated five times by using the same polymeric sorbent. In order to regenerate and sterilize, after the desorption procedure, the microbeads were washed with 50 mM NaOH solution.

RESULTS AND DISCUSSION

Boronate affinity chromatography has long been used to purify glycoproteins. Separation is accomplished due to the unique capacity of the boronate ligand to form complexes with substances containing hydroxyl groups [32]. In this study, VPBA containing bioaffinity sorbent was prepared for the selective and efficient separation of human-immunoglobulin G (human-IgG) from human plasma and aqueous solutions in batch system.

Characterization of p(HEMA-VPBA) Microbeads

Size Analysis of p(HEMA-VPBA) Microbeads

p(HEMA-VPBA) microbeads are synthesized by suspension polymerization method having a wide range of size distribution. As a result of screen analysis, microbeads with various size distributions were obtained. Figure 1 shows the size distribution of the p(HEMA-VPBA) microbeads obtained with the polymerization recipe given in Table 1. As seen in the figure, the size of the polymeric microbeads obtained with this recipe mainly falls between 63-250 μm and the highest portion has the size range between100-140 μm. In this study p(HEMA-VPBA) microbeads having the size range of 100-140 μm were used as a solid matrix for HIgG adsorption.

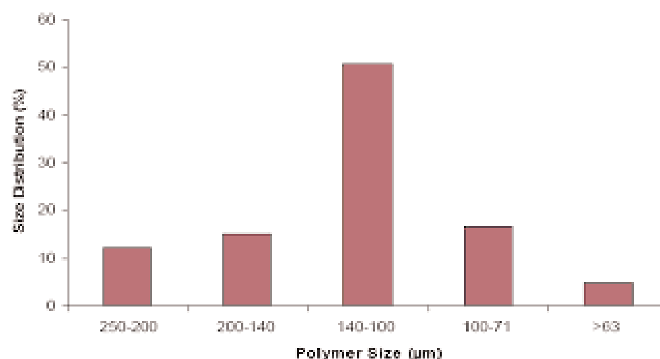

Figure 1. Size and percent size distribution of p(HEMA-VPBA) microbeads.

Surface Area Measurements

The radical suspension polymerization procedure provided porous and cross-linked p(HEMA VPBA) microbeads in the spherical form mostly in the size range of 100-140 μm. Specific surface area of the p(HEMA-VPBA) microbeads was found to be 80.1 m^2/g.

Swelling Properties

P(HEMA-VPBA) microbeads prepared in this study are hydrophilic structures, i.e., hydrogels. Hydrogels are water swollen, cross-linked polymeric structures produced by the simple reaction of one or more monomers or by association bonds such as hydrogen bonds and strong van der Waals interactions between chains. An integral part of the physical behavior in water, since upon preparation they must be brought in contact with water to yield the final, solved network structure. A dry, hydrophilic crosslinked network is placed in water. Then, the macromolecular chains interact with the solvent molecules owing to the relatively good thermodynamic compatibility. Thus, the network expands to the solvated state. Hydrogels have received significant attention, especially in the past 30 years, because of their exceptional promise in biomedical applications [50]. P(HEMA-VPBA) microbeads do swell in aqueous solutions, but do not dissolve. As seen in the Figure 2, swelling occurs rapidly and equilibrium swelling ratio (i.e. 57%) is reached approximately in 30 min

for p(HEMA-VPBA) beads and in 60 min for pHEMA beads (60 %). It should be also mentioned that the swelling properties of microbeads nearly the same after VPBA attachment. Equilibrium swelling ratio was not significantly effected with VPBA inclusion into the structure.

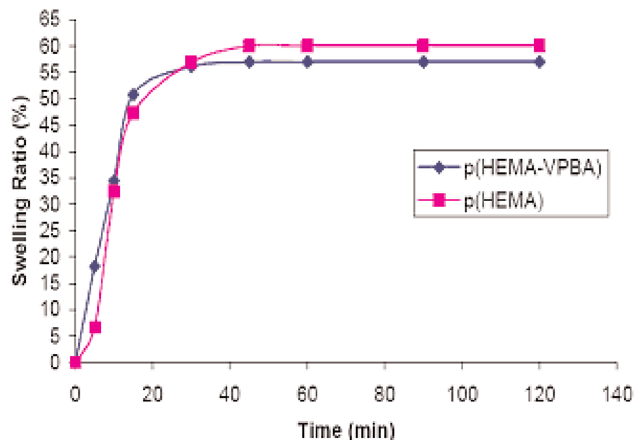

Figure 2. Swelling behavior of p(HEMA) and p(HEMA-VPBA) microbeads.

Surface Morphology

The surface morphology of the p(HEMA-VPBA) microbeads is exemplified by the scanning electron micrographs. As clearly seen in Figure 3, the polymeric microbeads have a spherical form and rough surface due to the pores which formed during the polymerization procedure. The presence of mesopores within the microbead surface is clearly seen in this photograph. The roughness of the microbead surface should be considered as a factor providing an increase in the surface area. In addition, these mesopores reduce diffusional resistance and facilitate mass transfer because of high internal surface area. This also provides higher human-IgG adsorption capacity.

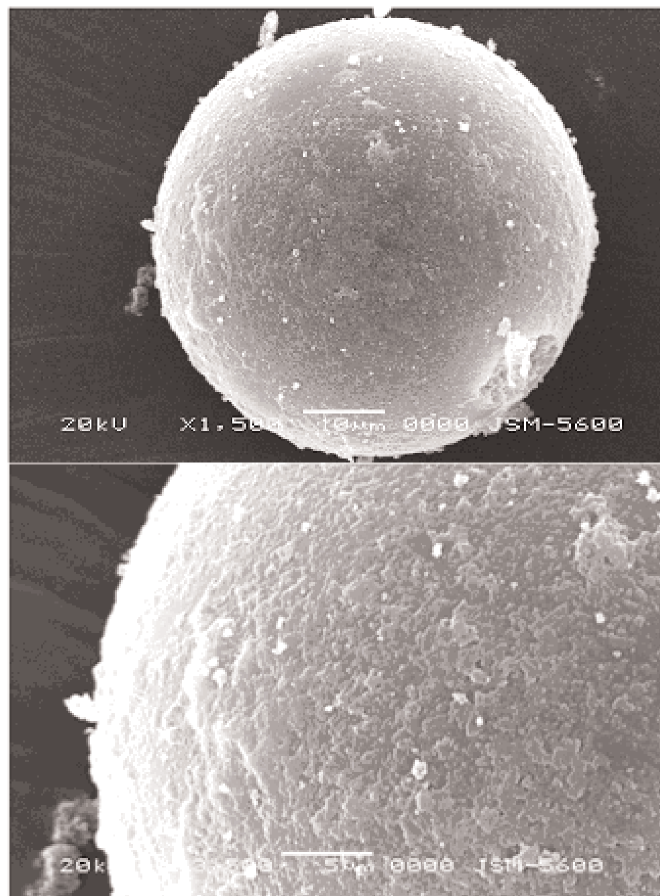

Figure 3. SEM micrographs of p(HEMA-VPBA) microbeads.

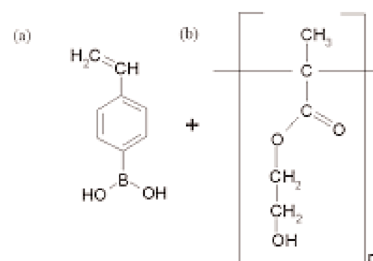

Figure 4. The molecular formulas of (a) VPBA and (b) p(HEMA).

FTIR Studies

4-Vinylphenylboronic acid (VPBA) was selected as the comonomer for the selective separation of human-IgG from human plasma. VPBA is included in the polymerization mixture as a comonomer. Themolecular formulas of VPBA and p(HEMA) are given in Figure 4. FTIR spectra of the p(HEMA), VPBA monomer and p(HEMA-VPBA) are shown in Figure 5, Figure 6 and Figure 7 respectively.

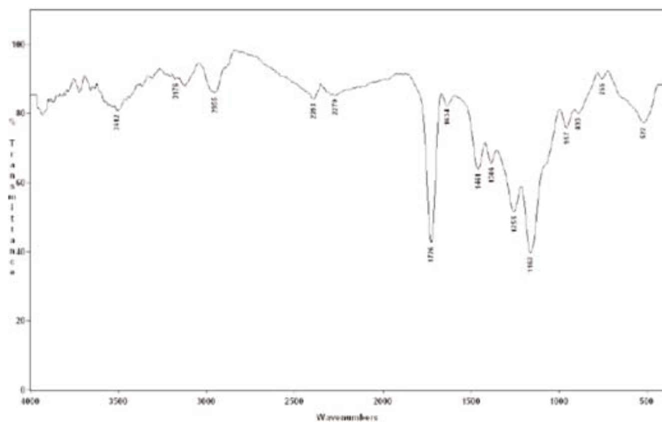

Figure 5. FTIR spectrum of p(HEMA).

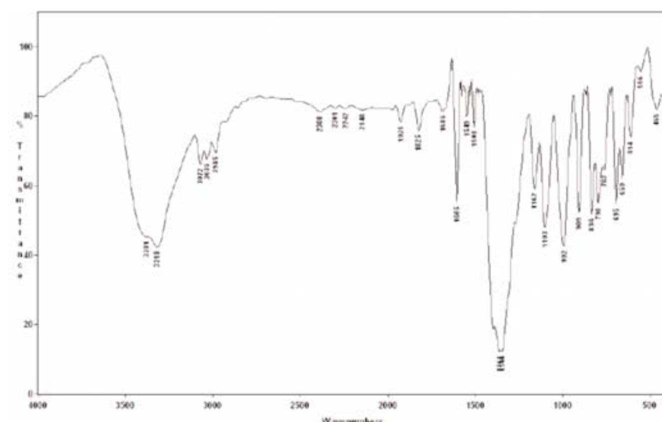

Figure 6. FTIR spectrum of VPBA monomer.

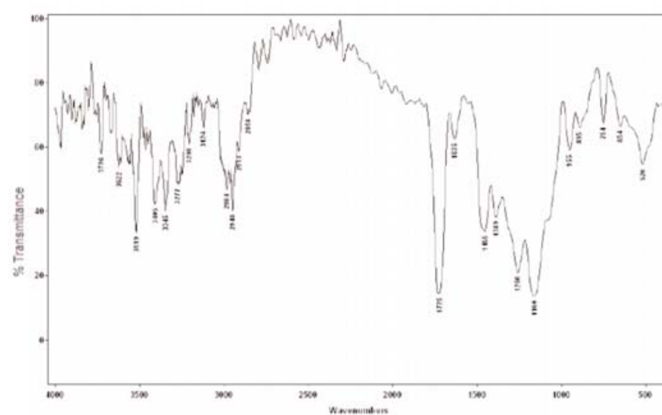

Figure 7. FTIR spectrum of p(HEMA-VPBA).

Strong broad bands at 3405 and 3277 cm^{-1} are associated with H-bonded OH in - $B(OH)_2$ in Figure 7. 659 cm^{-1} band corresponds to aromatic groups from the VPBA monomer. These findings show the incorporation of VPBA into the polymer structure.

Blood Compatibility Studies

A biomaterial is a substance that is used in medical devices or in prostheses designed for contact with the living body for an intended method of application and for an intended period. Synthetic polymers are the most diverse class of biomaterials. Polymeric biomaterials are widely used in both medical and pharmaceutical applications. These applications include a variety of implants or other supporting materials (e.g. vascular grafts, artificial hearts, intraocular lenses, joints, mammary prostheses and sutures), extracorporeal therapeutic and other supporting devices (e.g. hemodialysis, hemo perfusion, blood oxygenation and bags), controlled release systems and clinical diagnostic assays (mainly as carriers). All biomaterials must meet certain criteria and regulatory requirements before they can be qualified for use in medical applications. Depending on the intended end-use, a biomaterial may be subjected to a set of tests, such as blood-compatibility, tissue-compatibility, carcinogenecity, mutagenicity, biodegradation and mechanical stability. When biomaterials in use come into contact with blood, first small molecules (e.g. water and ions) reach to the surface which may or may not be adsorbed. This is followed by plasma protein adsorption. The first protein layer adsorbed on the biomaterial surface determines the subsequent events of the coagulation cascade (via the intrinsic pathway), and the complement activation (via the intrinsic-extrinsic pathways) [51-52].

Coagulation Times

In order to estimate the blood-compatibility of p(HEMA) and p(HEMA-VPBA) microbeads, in vitro coagulation times (CT), activated partial thromboplastin time (APTT) and prothrombin time (PT) tests were carried out. It should be mentioned that APTT tests exhibit the bioactivity of intrinsic blood coagulation factors and PT test relates to extrinsic blood

coagulation factors on biomaterial surface. CT test shows in-vitro coagulation time. Table 2 summarizes the coagulation data obtained in these tests. As can be seen from, all the clotting times for p(HEMA-VPBA) microbeads increased when compared with pHEMA microbeads and control plasma. Therefore, we concluded that the blood-compatibility of newly synthesized p(HEMA-VPBA) microbeads was rather good, and all the clotting times were quite reproducible. Consequently, for this pseudospecific affinity adsorption study, the incorporation of VPBA as a comonomer may exert beneficial effects in two ways. First, improved blood-compatibility of the microbeads will reduce adverse body reactions to possible biomedical treatment applications (i.e., cancer therapy), and second, reduction in non-specific adsorption will reduce undesirable losses of beneficial proteins from treated blood .

Adsorption of Human-IgG from Aqueous Solutions

Effect of pH and Buffer Type

IgG adsorption onto p(HEMA-VPBA) microbeads depends on the buffer system used [53]. To clarify this point, adsorption studies were carried out in the effective buffer ranges of each buffer system, i.e., MES (5.5-6.5), MOPS (6.5-8.0), HEPES (7.0-8.5), acetate-phosphate (4.0-8.0) buffers and carbonate (9.0-10.0) buffer. Figure 8 shows maximum IgG adsorption capacity in these buffer systems. Adsorption of purified IgG from aqueous solutions is possible between 4 and 10.0, but the optimum pH of adsorption is 8.5 in HEPES buffer (85 mg/g support). Maximum adsorption capacity is observed at pH 6.5 for MES (59 mg/g), pH 8 for MOPS (59 mg/g), pH 8 for acetate and phosphate (75 mg/g), and pH 9 for carbonate (78 mg/g). From that point of the study, adsorption experiments were carried out in HEPES buffer system at pH 8.5.

Effect of Time

Figure 9 gives the adsorption rate curve which was obtained by following the decrease of the concentration of human-IgG with time. As seen here, relatively faster adsorption rates were observed at the beginning of adsorption process, and then adsorption equilibrium was achieved gradually in about 120 min. A point worth noting that, there was a negligible non specific human-IgG adsorption (i.e., the adsorption onto the plain pHEMA microbeads) which

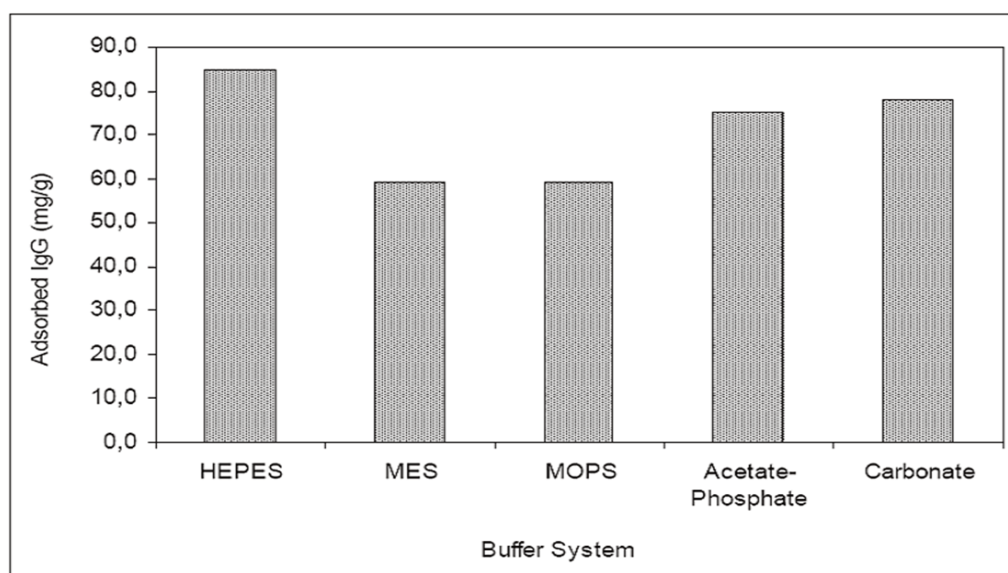

Figure 8. IgG adsorption capacity in different buffer systems Human-IgG concentration 1.0 mg/mL; Size: 100-140 µm; Incubation time: 2 h; T: 25ºC.

was about 3.48 mg/g for HEPES (pH 8.5). There is no reactive binding groups or sites onto pHEMA which interact with human- IgG molecules, hence, this non-specific adsorption may be due to diffusion of human- IgG molecules into the swollen pHEMA microbeads matrix and weak interactions (van der Waals interaction and hydrogen bonding) between human-IgG and hydroxyl groups on the surface of pHEMA microbeads. On the other hand, much higher adsorption rates were observed when the p(HEMA-VPBA) microbeads were used.

Table 2. Coagulation times of human plasma (reported in sec)*.

Material	Activated Partial Thromboplastin Time (APTT)	SD ±
Control Plasma	91.1	1.9
p(HEMA)	95.3	1.5
p(HEMA-VPBA)	97.5	2.7
	Prothrombin Time (PT)	**SD ±**
Control Plasma	26.6	0.2
p(HEMA)	26.3	0.3
p(HEMA-VPBA)	26.0	0.4
	Coagulation Time (CT)	**SD ±**
Control Plasma	33.7	0.8
p(HEMA)	34.7	0.1
p(HEMA-VPBA)	34.9	0.2

* Each result is the average of five parallel studies.

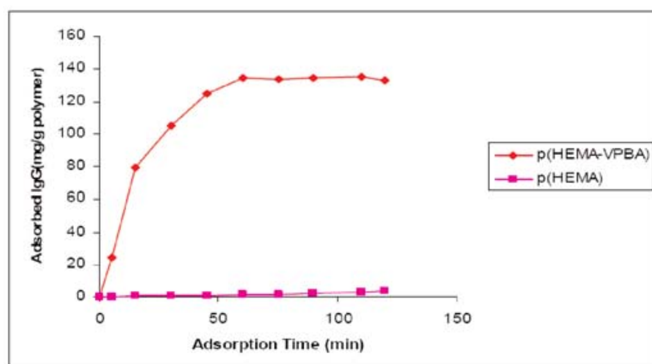

Figure 9. Effect of time on human-IgG adsorption Human IgG concentration: 3.5 mg/mL; pH: 8.5 in HEPES buffer; Size: 100-140 μm; T: 25ºC.

Effect of Initial Concentration of Human-IgG

Figure 10 shows the effect of initial human-IgG concentration on adsorption. As presented in this figure, with increasing human-IgG concentration in solution, the amount of human-IgG adsorbed per unit mass by the microbeads increases almost linearly at low concentrations, below about 3.50 mg/ml, then increases less rapidly and approaches saturation. The steep slope of the initial part of the adsorption isotherm represents a high affinity between human IgG and incorporated VPBA groups. It becomes constant when the protein concentration is greater than 3.50 mg/ml. Negligible amount of human-IgG molecules adsorbed non specifically on the plain pHEMA microbeads which were about 3.9 mg/g for HEPES buffer at pH 8.5. VPBA incorporation significantly increased the human-IgG adsorption capacity of the microbeads up to 139 mg/g. It is clear that this increase is due to specific interaction between VPBA and human-IgG molecules.

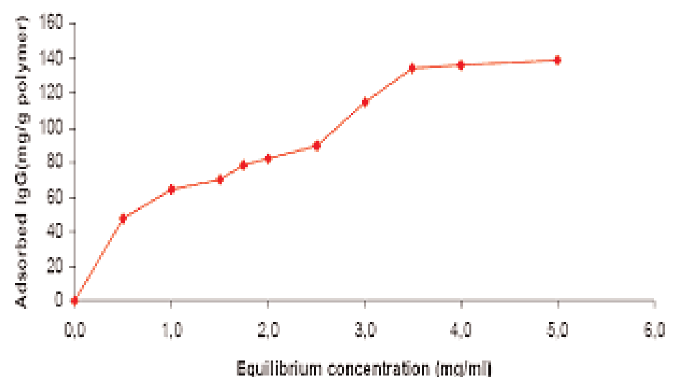

Figure 10. Effect of initial human-IgG concentration on adsorption capacity; pH: 8.5 (HEPES buffer); Size: 100-140 μm; Incubation time: 2 h; T: 25ºC.

Effect of Ionic Strength

In order to investigate the effect of ionic strength, the ionic strength of the medium was adjusted using different NaCl concentrations. Figure 11 shows the effect of ionic strength on the adsorption capacity of p(HEMA-VPBA) microbeads. As seen in the figure,

when the ionic strength of the buffer (HEPES buffer, pH 8.5) increases from 0.001M to 0.5 M, the adsorption capacity increases until 0.2 M NaCl. This can be explained by the increasing of the salt concentration in operating conditions suppresses the non-specific ionic interactions and increases the electrostatic attractions between the p(HEMA-VPBA) microbeads and the IgG molecules since the negative charge on the boronate anion in its active tetrahedral form can give rise to ionic interactions. After that point, adsorption capacity decreases probably due to the salting out effect at high ionic strength.

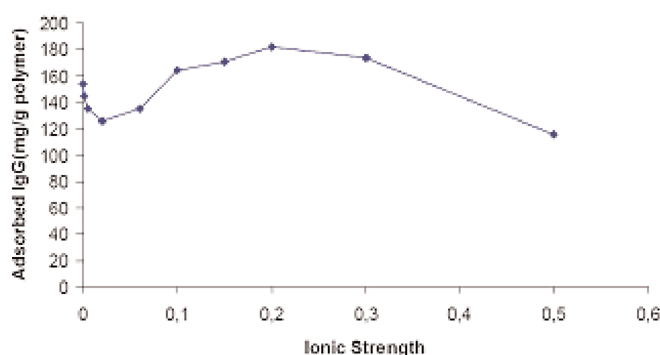

Figure 11. Effect of ionic strength on IgG adsorption; Salt:(NaCl); IgG concentration: 3.5 mg/mL; pH: 8.5 (HEPES); Size: 100-140 μm; Incubation time: 2 h; T: 25ºC.

Desorption and Repeated Use

Desorption of IgG from p(HEMA-VPBA) microbeads was also carried out in batch experiments using 1.0 M NaCl in acetate buffer as a desorption medium. The microbeads adsorbed the different amounts of IgG were placed within the desorption medium and the amount of IgG desorbed in 2 h was determined. Desorption ratios are given in Table 3.

In order to show reusability of the p(HEMA-VPBA) microbeads, the adsorptiondesorption cycle was repeated five times using the same polymeric microbeads. For sterilization, after one adsorption-desorption cycle, microbeads were washed with 50 mM NaOH solution for 30 min. After this procedure, microbeads were washed with distilled water for 30 minutes, then equilibrated within the HEPES buffer for the next adsorption-desorption cycle. Note that no VPBA release was observed after sterilization procedure. At the end of five adsorption desorption cycles, there was no remarkable decrease in the adsorption capacity. The human-IgG adsorption capacity decreased 4%, 9%, 13%, 19% by using 1 M NaCl as a desorption agent, whereas the adsorption media was HEPES buffer at pH 8.5.

Table 3. Desorption ratios.

Desorption medium (1 M NaCl in Acetate buffer, pH 4.0)	Desorption ratio (%)
1	81.46
2	80.65
3	75.23
4	76.43
5	72.75

Adsorption of Human-IgG From Plasma

The plasma proteins can be separated into three major groups as fibrinogen, albumin, and globulins and only albumin is not a glycoprotein. As seen from the given adsorption data in Table 4 for the capture of human-IgG directly from healthy human plasma with isotonic solution, human-IgG adsorption is lower than compared with the adsorption data in batch experiments. The adsorption of other main plasma proteins (i.e, albumin and fibrinogen) and blood glucose adsorption were also investigated; albumin, fibrinogen and glucose adsorption capacities were 20 mg/g, 1.45 mg/g and 3.2 mg/g respectively. This low human-IgG adsorption may because of to the competitive adsorption of the other serum proteins, i.e albumin, and human-IgG molecules for binding VPBA onto the pHEMA adsorbents.

Table 4. Human-IgG adsorption from human plasma: IgG concentration before dilution: 9.69 mg/mL; T: 25°C.1/10 diluted plasma (isotonic solution)

Dilution	Adsorption Capacity (mg/g)
Plasma (undiluted)	38.75
1/4 diluted plasma (isotonic solution)	13.75
1/10 diluted plasma (isotonic solution)	8.50

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