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Chemically modified poly(2-hydroxyethyl methacrylate) cryogel for the adsorption of heparin

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Abstract: Various clinical procedures, such as cardiovascular surgery or extracorporeal blood purification, involve systemic anticoagulation using heparin. High concentrations of circulating heparin require neutralization due to possible serious bleeding complications. The intravenous administration of the heparin antagonist protamine sulfate is routinely clinically performed, but is frequently associated with adverse reactions. Therefore, there is a need for a valid and safe alternative to achieve extracorporeal heparin removal from blood or plasma, such as a filter, a matrix, or an adsorbent. Here, we describe the development of a macroporous poly(2-hydroxyethyl methacrylate)-based monolithic cryogel functionalized with L-lysine (pHEMA-lys) and the characterization of its selective heparin adsorption. The maximum binding capacity was quantified *in vitro* using aqueous and serum solutions under static and dynamic conditions, and fresh human plasma under static conditions. The pHEMA-lys bound 40,500 IU and 32,500 IU heparin/g cryogel at the equilibrium in aqueous solution and 50% serum, respectively. In human plasma spiked with 100 IU/mL of heparin, the binding was still highly efficient (4330 IU/g cryogel after 30 min, i.e., 87% of the initial concentration). The cryogels showed good blood compatibility, as indicated by negligible adsorption of albumin, antithrombin III, and total protein, and may thus be suitable for extracorporeal heparin removal. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2014.

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INTRODUCTION

Heparin is a heterogeneous highly sulfated glycosaminoglycan with an average molecular weight of 15 kDa, and commonly used as an anticoagulant during surgical procedures and extracorporeal therapies, such as hemodialysis.[1–3] Systemic heparinization is often associated with bleeding risks, hemorrhagic complications, and possible mortal consequences for patients.[4–6] In addition to hemorrhagic complications, heparin has limitations based on its pharmacokinetic, biophysical, and nonanticoagulant biological properties, due to its binding to plasma proteins, to proteins released from platelets, and to endothelial cells. These limitations may result in a variable anticoagulant response to heparin and the phenomenon of heparin resistance, the induction of immune-mediated platelet activation, which can lead to heparin-induced thrombocytopenia, the activation of osteoclasts, and suppression of osteoblasts, which in turn leads to bone density reduction, and other effects on metabolism, such as hyperlipidemia.[7–10]

Clinically, an overdose of heparin is neutralized with the polypeptide protamine sulfate, a heparin antagonist administered intravenously at a dose of 1 mg per 100 IU of active heparin,[11] although, due to its short half-life, repeated administration may be required.[12] Protamine sulfate is a 5 kDa arginine-rich basic protein derived from fish sperm that binds to heparin to form a stable insoluble complex, mainly due to the strong electrostatic interaction between the sulfate groups of the heparin chain and the arginine residues of protamine.

It has been estimated that around 2 million patients undergo surgical procedures annually which include the mandatory use of heparin and protamine.[13] Their combined effect has been reported to produce severe adverse reactions.

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Among them are negative cardiovascular responses (hypotension, bradycardia, and postoperative stroke), organ failure, pulmonary capillary permeability, complement activation, and allergic reactions including anaphylaxis.[14–19]

An alternative approach to the administration of protamine is the development of biomaterials for the extracorporeal removal of excess heparin. Synthetic, medium sized peptides, polypeptides, low molecular weight protamine, low molecular weight helix peptides, polycationic molecules, such as poly(amido-amine), cationic derivatives of dextran and hydroxypropylcellulose, L-lysine, and bis-2-methyl-4-amino-quinolyl-6-carbamide, have been identified as suitable heparin antagonists.[20–27]

Attempts have been made to immobilize protamine or other heparin neutralizing ligands on biocompatible substrates in order to obtain extracorporeal heparin-removal devices to be used during surgical procedures or to implement commonly used extracorporeal hemodialysis purification systems,[28–32] but this goal has not been reached yet. Diethylaminoethyl modified cellulose is currently clinically used to remove remaining heparin from whole blood as a part of the Heparin-Induced Extracorporeal Lipoproteins Precipitation system.[33] This system uses heparin molecules to precipitate low-density lipoprotein cholesterol, triglycerides, lipoprotein a, and fibrinogen using a low reaction pH. Because a great amount of heparin is eliminated with those precipitates, the quantity of soluble free heparin to be removed from plasma is not large.

The objective of this work was the development and characterization of a macroporous cryogel suitable for the selective adsorption of heparin. Poly(2-hydroxyethyl methacrylate) (pHEMA) was chosen as a starting material due to its biocompatibility[34] and low adsorption of proteins[35] as well as was functionalized with L-lysine.

Macroporous cryogels are applied in biomedicine and biotechnology,[36–39] for example, as chromatographic materials, as carriers for the immobilization of molecules and cells, and as matrices for electrophoresis, due to their interconnected pore structure. The synthesis of macroporous materials may be performed using various techniques, such as porogen leaching, gas foaming, phase separation, templating methods like high internal phase emulsions templating, and cryogelation in aqueous media or in organic solvent.[40–46] The pHEMA polymeric materials used in this study were synthesized through a cryostructuration method where, at temperature below the solvent freezing point, the polymerization takes place around the frozen solvent crystal, forming a stable three-dimensional network. When the polymerization is complete, thawing of the frozen solvent produces cavities as a replica of the crystals. Therefore, cryogels typically have interconnected micro-, meso-, and macro-pores ($<$2 nm, 2–50 nm, $>$50 nm, respectively, according to the IUPAC classification) that allow unrestricted access of solutes with a broad size range, as well as mass transport of nano- and microparticles. The high porosity is associated with a large inner surface area, resulting in good adsorption capacity.

The functionalization of this material with small cationic molecules, such as L-lysine or L-arginine, instead of protamine or polypeptide derivatives for instance, leads to a more convenient synthetic route and a higher stability in different chemical environments. Moreover, a great advantage is that L-lysine and L-arginine are nontoxic, harmless, natural amino acids that can be easily metabolized in case of leakage from the carrier.

Because deprotection is faster and easier for protected lysine than for arginine, we chose bis-Boc-L-lysine for functionalization of pHEMA cryogels and report on the characterization of the L-lysine-functionalized pHEMA cryogels with respect to their temperature-dependent adsorption capacity in saline and in serum solutions. The adsorbents were tested using fresh human plasma spiked with heparin to a specific heparin activity close to a likely clinical overdose, and their biocompatibility was assessed by monitoring adsorption of antithrombin III (ATIII), albumin, and total protein.

MATERIALS AND METHODS

Chemicals and plasma

2-Hydroxyethylmethacrylate (HEMA), *N,N′*-methylenebisacrylamide (MBAA), ammonium persulfate (APS), and 1,2-bis(dimethylamino)ethane (TEMED) were purchased from Sigma (St. Louis, MO).

N,N′-diisopropylcarbodiimide (DIC), 4-dimethylxamino pyridine (DMAP), trifluoroacetic acid (TFA), dimethylformamide anhydrous (DMF), deuterium oxide (D_2O), and dichloromethane were purchased from Sigma-Aldrich (Steinheim, Germany), whereas Boc-Lys(Boc)-OH DCHA was from Novabiochem (Hohenbrunn, Germany).

Unfractionated heparin with an activity of 150 IU/mg was purchased from Sigma-Aldrich (Steinheim, Germany). Unfractionated heparin used for experiments with human plasma was from Baxter (Vienna, Austria). Fetal bovine serum (FBS) was from Fisher Scientific (Göteborg, Sweden).

Human plasma was obtained by collecting fresh blood from healthy volunteer donors into tubes containing unfractionated heparin to a calculated concentration of 100 IU/mL (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). The blood was centrifuged at 3000*g* for 10 min at room temperature (RT) to obtain plasma.

Synthesis of pHEMA cryogel and functionalization with L-lysine (pHEMA-lys)

The pHEMA cryogel was obtained following the procedure previously described[35] using 10% w/w monomers (HEMA and MBAA) solution in water with a molar ratio of 6:1, HEMA:MBAA, and 1% w/w APS/TEMED of the total monomers. Each cryogel was synthesized in 0.5 mL solution in a glass tube of 7 mm diameter and frozen overnight at -12°C; the cryogel was then washed with water and ethanol and left to dry at RT. At this stage, the unmodified pHEMA cryogel represents the plain cryogel used as a reference. For functionalization, pHEMA cryogels (80 mg, 0.51 mmol as calculated on monomers) were placed into a round flask with 4 mL anhydrous DMF and 1.6 mL CH_2Cl_2 as solvents, then DIC (203 μL, 1.30 mmol), DMAP (157 mg, 1.28

mmol), and Boc-Lys(Boc)-OH DCHA (677 mg, 1.28 mmol) were added in an argon atmosphere.

The cryogels were left to react for 65 h at RT with gentle shaking. Thereafter, they were washed with DMF/CH_2Cl_2 at a ratio of 2:1, 1:2, and finally with pure CH_2Cl_2 to obtain pHEMA-*N,N′*-di-Boc-L-lysine. Deprotection of N-Boc to form free amino groups was carried out by reacting the cryogel in 6 mL TFA for 5 h with gentle shaking. After that, the solvent was discarded and the functionalized cryogel was washed with CH_2Cl_2 and dried under vacuum (Scheme 1). Finally, they were washed with 1*M* NaCl followed by solutions of water and ethanol with progressively increasing percentage of ethanol from 0 to 100% and left to dry at RT for 72 h under vacuum.

Physico-chemical characterization of the cryogels

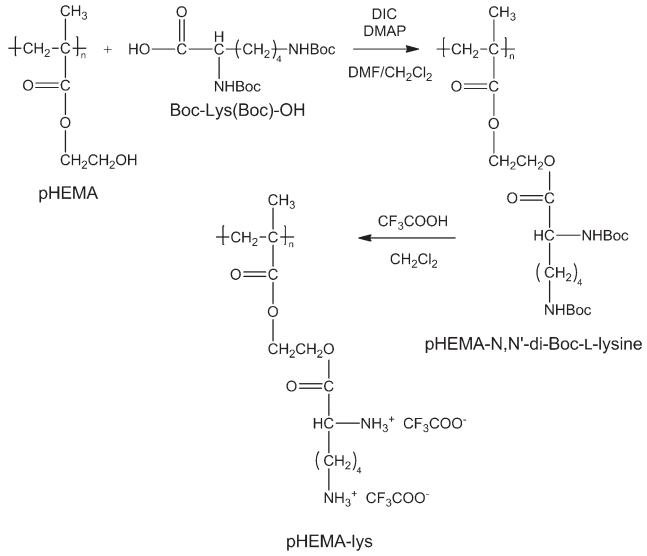
Microscopic analysis. Images of plain pHEMA and pHEMA-lys were obtained using an Inverted Microscope Zeiss Axiovert 100 (Carl Zeiss, Göttingen, Germany) equipped with CCD camera AxioCam ICc1.

Swelling behavior and porosity. The amount of water retained by the cryogel monoliths at the equilibrium was calculated as an average of three cryogel cylinders after the last washing and drying step. The equilibrium water content (EWC) was obtained after 4 h of incubation in ultrapure water.

Modulus of elasticity. The modulus of elasticity of the pHEMA and pHEMA-lys was calculated using a TA-XT2 texture analyzer from Stable Micro Systems (Godalming, Surrey, UK) at RT with a 25 mm diameter plunger. Samples of 7 mm diameter and 10 mm length were compressed to 70% of deformation and the elastic modulus calculated at 40% of deformation.

Nuclear magnetic resonance. Magic-angle spinning nuclear magnetic resonance (^{1}H-MAS NMR) was used to determine the structure and the degree of functionalization of the pHEMA cryogel with L-lysine. The ^{1}H-MAS NMR spectrum of pHEMA-*N,N′*-di-Boc-L-lysine cryogel was taken using a NMR Bruker Avance TM 400 spectrometer (400.13 MHz) from Bruker Biospin (Rheinstetten, Germany) equipped with a high-resolution Magic Angle Spinning probe (hr-MAS 400). Chemical shifts were reported in ppm (δ) downfield relative to the solvent, DMF.

^{1}H-NMR spectroscopy was also used to initially estimate the amount of heparin adsorbed per mg of pHEMA-lys cryogel. Ten milligrams of powdered pHEMA-lys cryogel was added to 500 μL of D_2O and aliquots of 25 mg/mL of heparin solution were added.



SCHEME 1. Schematic representation of pHEMA cryogel funtionalization with Boc-Lys(Boc)-OH followed by trifluoroacetic acid deprotection.

Adsorption of heparin in phosphate-buffered saline and fetal bovine serum

Static conditions. Batch experiments in buffer were carried out at RT and at 37°C with gentle shaking. Aliquots of 3 mg of dried plain pHEMA and pHEMA-lys cryogel were added to 0.9 mL of 10 m*M* phosphate-buffered saline (PBS, 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.4) containing heparin in different concentrations ranging from 140 mg/L to 4 g/L. To assess the adsorption of heparin in a more complex matrix, half of the PBS buffer was replaced with FBS and the adsorption experiments were carried out at 37°C.

Experiments on adsorption kinetics established the time of incubation that is required for these systems to be at the equilibrium of adsorption for a known heparin concentration. These experiments were carried out at RT and 37°C in PBS and at 37°C in FBS:PBS (1:1) solution, by observing the amount of heparin adsorbed as a function of the time; the initial heparin concentration was in the order of 2 g/L. All experiments were conducted in triplicates.

Flow conditions. Experiments under flow were performed at RT and at 37°C, in PBS and in FBS:PBS (1:1) at a flow rate of 0.08 mL/min. Fractions of 50 μL were collected and further analyzed.

Adsorption of heparin from fresh human plasma

Before each experiment, plain pHEMA and pHEMA-lys cryogels were incubated four times for 1 h with pyrogen free 0.9% NaCl solution with gentle shaking. After the swelling procedure, cryogels were gently wiped on a blotting paper. Aliquots of 1.8 mL of heparinized (100 IU/mL) human plasma were incubated with cryogels (∼35 mg dry weight) at 37°C with gentle shaking. Spiked plasma without adsorbents served as a control. After 30, 60, and 120 min, samples were centrifuged for 5 min at 4600*g*, and supernatants were collected and stored at −70°C until further analysis. Experiments were conducted in triplicates.

Quantification of heparin

The amount of adsorbed heparin was calculated from the colorimetric analysis of heparin remaining in the supernatant using methylene blue as chromogenic reactant.[47] The absorbance was measured spectrophotometrically at 665

nm (Biowave II WPA lab vision, Cambridge, UK). Heparin concentration in fresh human plasma was quantified using a Sysmex CA-560 coagulation analyzer (Siemens Healthcare Diagnostics Products, Marburg, Germany), using the Coamatic Heparin test (Coachrom, Vienna, Austria) as described.[48]

Quantification of ATIII, albumin, and blood proteins

ATIII remaining in plasma after incubation was quantified using a Sysmex CA-560 coagulation analyzer (Siemens Healthcare Diagnostics Products, Marburg, Germany), with the Dade Berichrom ATIII test-kit (Siemens Healthcare Diagnostics) according to the manufacturer's instructions. Adsorption of albumin and total blood protein was monitored by quantifying the amount remaining using a Roche/Hitachi 902 automatic analyzer and the ALB Plus and TP reagents, respectively (all from Roche Diagnostics GmbH, Mannheim, Germany).

RESULTS AND DISCUSSION

Physico-chemical characterization of pHEMA-lys

Microscopic analysis. In Figure 1, images of pHEMA and pHEMA-lys cross sections are shown. A difference in the size of the macropores for plain and L-lysine modified cryogels (40 and 70 μm, respectively) is visible at this scale. The overall pHEMA-lys inner morphology appears smoother and more compact as compared with the nonfunctionalized cryogel.

Swelling behavior. The EWC was determined as EWC = $(W_w - W_d)/W_w \times 100$ (where W_w = wet weight and W_d = dry weight). Average water content was larger than 90% for plain and L-lysine modified cryogels.

Modulus of elasticity. The pHEMA-lys cryogels were elastic, sponge-like structures and retained their original shape after releasing stress from compression. The elastic modules of pHEMA and pHEMA-lys cryogel were calculated as 32 ± 3 kPa and 28 ± 2 kPa, respectively.

Nuclear magnetic resonance. The functionalization of the pHEMA cryogel with the Boc-Lys(Boc)-OH DCHA was confirmed by ^{1}H-MAS NMR. In Figure 2, the ^{1}H-MAS NMR spectra of the pHEMA[49] and pHEMA-*N,N′*-di-Boc-L-lysine are shown. The characteristic peaks of *N,N′*-di-Boc-L-lysine are at 1.43 ppm [$C(CH_3)_3$], 1.77 ppm (CH_2), 3.07 ppm (CH_2N), 4.13 ppm (OOCCHN), and 6.59 and 6.95 ppm (NH-Boc).

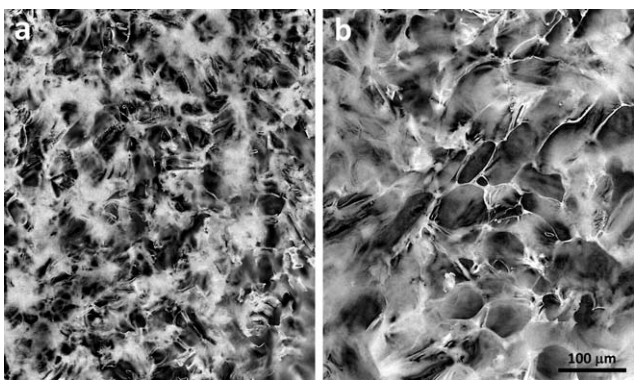


FIGURE 1. Optical microscopy of pHEMA (a) and pHEMA-lys (b). The scale bar of 100 μm is applicable to both images.

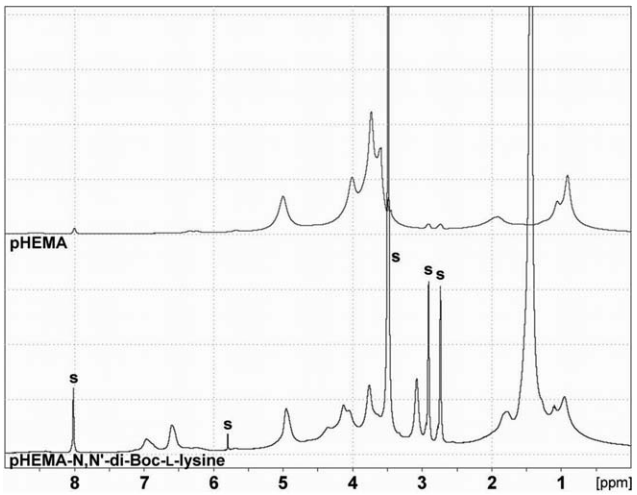


FIGURE 2. ^{1}H-MAS MR spectra of pHEMA and pHEMA-*N,N′*-di-Boc-L-lysine cryogels in deuterated DMF. The peaks labeled with "s" are related to the solvent.

The degree of functionalization of the pHEMA cryogel with L-lysine was assessed through ^{1}H-MAS NMR. The amount of L-lysine binding to the cryogel was calculated by the ratio of the peak at 3.07 ppm (CH_2N) related to pHEMA-lys and 0.91 ppm (CH_3) related to the pHEMA and it was between 57% and 60%.

Preliminary data regarding the amount of heparin adsorbed per mg of pHEMA-lys cryogel were calculated through ^{1}H-NMR titration. Powdered pHEMA-lys in D_2O was titrated with heparin solution as described in "Materials and Methods" until the heparin peaks appeared, using *t*-butanol as internal standard for NMR area calculation [Figure 3(a)]. Using this approach, 10 mg of cryogel bound a maximum of 3.2 mg of heparin (corresponding to 48,000 IU/g cryogel).

The binding between pHEMA-lys cryogel and heparin was reversible as seen through NMR titration, in which aliquots of NaCl were added to the complex of pHEMA-lys with heparin. Desorption of 95% heparin in D_2O solution was reached at a concentration of 3*M* NaCl [Figure 3(b)].

Adsorption of heparin in phosphate-buffered saline and fetal bovine serum

Static conditions. Equilibrium studies were performed to evaluate the maximum amount of heparin adsorbed by the pHEMA-lys cryogels. The maximum adsorption capacity of the cryogel q (mg/g adsorbent) was calculated with the following equation[50]:

$$q = \frac{V(C_0 - C_{eq})}{M} \quad (1)$$

where C_0 is the initial concentration of heparin in solution, C_{eq} is the equilibrium concentration in solution, V (mL) is the volume of solution, and M (g) is the mass of the cryogel.

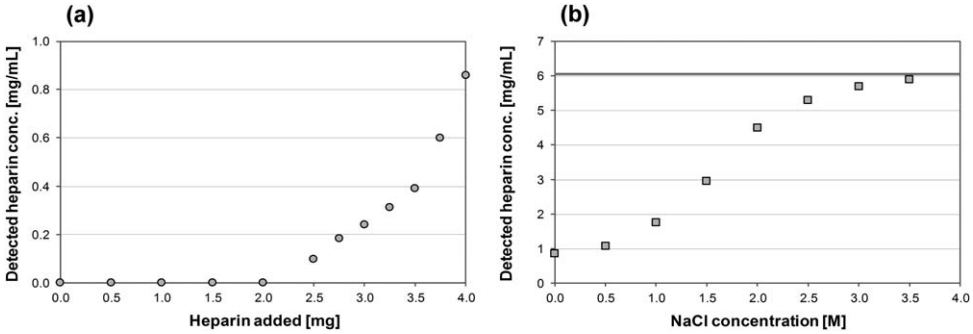


FIGURE 3. Heparin desorption for HEMA-lys detected by ^{1}H-NMR titration. The continuous gray line represents the concentration of heparin added.

The largest binding capacity under different conditions was calculated using the Langmuir isotherm theory,[50] which assumes that the maximum adsorption occurs when the surface is covered by adsorbate forming a monolayer

$$\frac{C_e}{q_e} = \frac{C_e}{q_{max}} + \frac{1}{K_1 q_{max}} \quad (2)$$

where C_e is the concentration of heparin in solution at equilibrium (mg/L), q_e is the amount of heparin adsorbed onto the cryogel at the equilibrium (mg/g), q_{max} (mg/g) and K_1 (L/mg) are Langmuir constants related to the maximum capacity of heparin adsorbed per unit of dried cryogel and its affinity to the binding sites. The linear plot of C_e/q_e versus C_e describes the adsorption through the Langmuir isotherm equation and allows the extrapolation of q_{max} and K_1, listed in Table I.

The static equilibrium adsorption of heparin onto pHEMA-lys cryogel was evaluated in PBS and in a 50% FBS solution at RT and at 37°C (Figure 4). These studies were carried out with an initial concentration of heparin ranging from 140 mg/L to 4 g/L. The adsorption of heparin onto plain pHEMA cryogels was negligible in the concentration range studied.

As shown in Figure 4, the pHEMA-lys cryogel exhibited a greater ability to bind heparin in PBS at RT. The interaction between heparin and L-lysine is mainly due to charge recognition[26]; at pH 7.4, the amino groups of the L-lysine are positively charged, producing a strong interaction with the highly negatively charged heparin. The maximum adsorption capacity of the cryogel decreased by 20% at 37°C is due to the increase in temperature, which clearly impaired the physical adsorption process.[51] The maximum adsorption capacity of pHEMA-lys in FBS:PBS (1:1) solutions at 37°C showed a further decrease of 25% in comparison with the studies performed in PBS at the same temperature. This is likely due to nonspecific adsorption of proteins from serum, which results in reduced heparin adsorption. However, under the experimental conditions, the pHEMA-lys cryogel showed the ability to bind heparin even in the complex matrix of serum solutions.

In Table I, the maximum amount of heparin adsorbed per gram of cryogel in buffer at RT and 37°C and in FBS:PBS (1:1) at 37°C is summarized. The q_{max} adsorbed in heparin buffered solution corresponds to 40,500 IU at RT and 32,550 at 37°C per gram of cryogel. These amounts are higher compared with other values reported in literature,[28,32] when similar conditions (temperature or solvent) were applied.

The same data were fitted using the empirical Freundlich equation; this model describes the adsorption on a heterogeneous surface.

$$q_e = K_F C_e^{1/n} \quad (3)$$

where K_F and $1/n$ are the Freundlich constant for the system corresponding to the adsorption capacity and adsorption intensity, respectively. By comparing the linear regression for the Freundlich and Langmuir model, it was found that the Langmuir model is able to fit more accurately the experimental data for the adsorption of heparin by the pHEMA-lys. This suggests that the adsorption mechanism is mainly due to a monomolecular layer adsorption.[51]

Adsorption kinetics. Kinetic studies were performed at RT and 37°C in PBS and at 37°C in FBS buffered solution to estimate the time for each system to reach the equilibrium; the heparin initial concentration was in the order of 2 g/L.

TABLE I. q_{max} (mg/g) and K_1 (L/mg) Values Obtained With the Langmuir Equation for the Adsorption of Heparin onto the pHEMA-lys Cryogel in PBS (RT and 37°C) and in FBS:PBS (1:1)

Sample	Temperature (°C)	q_{max} (mg/g)	K_1 (L/mg)	R^2
PBS	RT	270 ± 14	$5.3 \times 10^{-4} \pm 2 \times 10^{-5}$	0.99
PBS	37	217 ± 21	$7.6 \times 10^{-4} \pm 4 \times 10^{-5}$	0.99
FBS:PBS (1:1)	37	164 ± 16	$6.3 \times 10^{-4} \pm 5 \times 10^{-5}$	0.99

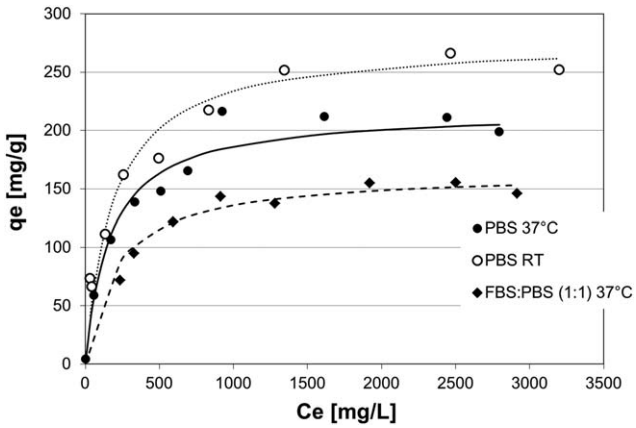


FIGURE 4. Heparin adsorption in 0.9 mL of solution with increasing heparin concentrations, under different conditions, after 4 h of incubation (n = 3). q_e (mg/g) = mg of heparin uptake at the equilibrium per g of dry cryogel; C_e (mg/L) = concentration of nonadsorbed heparin at the equilibrium.

The first-order kinetics model was not able to fit the data. A pseudo-second-order adsorption equation was used to fit the data over the range of adsorption time studied[52]

$$\frac{t}{q}=\frac{1}{K_2 q_e^2}+\frac{t}{q_e} \tag{4}$$

where t is the time (min), q is the amount of heparin adsorbed at time t, q_e (mg/g) is the amount of heparin adsorbed per gram of cryogel at equilibrium, and K_2 is the rate constant of pseudo-second-order adsorption.

Under all the conditions applied, the curve showed a steep increase in adsorption over time until the system reached the saturation point. Specifically, a sharper increase is observed for the adsorption at RT in PBS than at 37°C in PBS and FBS:PBS solution. This different behavior can be explained by the phenomena of adsorption/desorption happening at 37°C and the competition with serum proteins, which disrupts the diffusion of the heparin onto the cryogel. The heparin adsorption kinetics for a known amount of pHEMA-lys cryogel are shown in Figure 5.

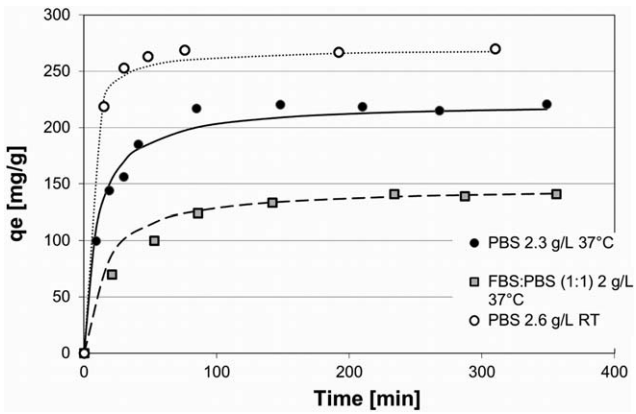


FIGURE 5. Adsorption kinetics of heparin (initial concentration ~2 g/L) onto pHEMA-lys cryogel in PBS at RT and at 37°C as well as in FBS:PBS (1:1) solution at 37°C (n = 3).

The linear plot of t/q versus t describes the pseudo-second-order adsorption and allows the extrapolation of q_e and K_2 for that specific heparin concentration, listed in Table II.

The values of q_e obtained at a heparin concentration of 2 g/L are in agreement with the maximum capacity calculated with the Langmuir model confirming that the two models are valid for this system.

Flow conditions

The ability of the column to bind heparin under continuous flow condition was deduced from the shape and the area of a breakthrough curve, shown in Figure 6. The curve was obtained by plotting the normalized effluent heparin concentration at the time t (C_t) with the initial heparin concentration (C_0) versus time (t). The breakthrough point (q_b) and the exhaustion point (q_{total}) are set at 10% and 90% of the ratio of outlet solute concentration to inlet solute concentration, respectively.[53]

The following equations were used to calculate the amount in mg of heparin adsorbed by the column, q_b

$$q_b=\frac{QB}{1000}\times\frac{1}{X} \tag{5}$$

where Q is the flow (mL/min), B is the integrated adsorbed concentration (mg/L) as a function of time t (min) at the breakthrough point; X is g of dried adsorbent. The total amount of heparin adsorbed is given by[54]

$$q_{total}=\frac{QA}{1000} \tag{6}$$

where Q is the flow rate (mL/min) and A is the integrated adsorbed concentration as a function of time (min) at the exhaustion point.

The total amount of heparin passed through to the columns m_{total} is calculated from:

$$m_{total}=\frac{C_0 Q t_{total}}{1000} \tag{7}$$

C_0 is the initial heparin concentration (mg/L) and t_{total} is the total time (min) needed to reach the saturation of the column.The equilibrium uptake of heparin (maximum capacity of column), q_{eq} is given by

$$q_{eq}=\frac{q_{total}}{X} \tag{8}$$

The total removal percent of heparin is calculated by the ratio of the total amount of heparin adsorbed (q_{total}) to the total amount of heparin passed through the column (m_{total}) using[55]

$$\text{total removal}\%=\frac{q_{total}}{m_{total}}\times 100 \tag{9}$$

Column performance. The effect of the initial inlet heparin concentration on the shape of breakthrough curve and

TABLE II. q_e (mg/g) and K_2 (g/mg × min) Values Obtained With the Pseudo-Second-Order Kinetics Model

Sample	Temperature (°C)	q_e (mg/g)	K_2 (g/mg × min)	R^2
PBS	RT	270 ± 7	$12.7 \times 10^{-4} \pm 3 \times 10^{-5}$	0.99
PBS	37	213 ± 6	$5.2 \times 10^{-4} \pm 4 \times 10^{-5}$	0.99
FBS:PBS (1:1)	37	147 ± 8	$4.71 \times 10^{-4} \pm 1 \times 10^{-6}$	0.99

column performance at constant flow rate (0.08 mL/min) was investigated.

The effect of varying initial heparin concentration was studied using concentrations of heparin of 558 and 1804 mg/L at RT in PBS. Increasing the heparin concentration produced a sharpened breakthrough curve and the saturation was achieved in a shorter time. As shown in Figure 6 and summarized in Table III, an increase in the heparin concentration affected the column performance with a slight decrease in the total amount of heparin removed. However, the adsorption capacity of this cryogel at the breakthrough point (q_b) and the exhaustion point (q_{eq}) increased from 119 to 190 mg/g and from 160 to 247 mg/g, respectively. These data suggested that the mechanism of adsorption was mass transfer controlled. The driving force was the concentration gradient between the free and adsorbed heparin. This may explain the higher capacity of the cryogel at increasing heparin concentrations.[53,55]

When the experiment was performed at 37°C using the highest heparin concentration, the increase of temperature caused a sharp breakthrough curve and the saturation point was reached more quickly than at RT, indicating that the adsorption process is exothermic. The same effect was observed in the batch system and was in agreement with similar systems.[51]

Thomas model. The Thomas model is widely used to describe the column performance and to predict the maximum capacity of a column in a continuous system.[54] This model is based on second-order reaction kinetics[57] and assumes no axial dispersion and a constant separation factor in the columns.

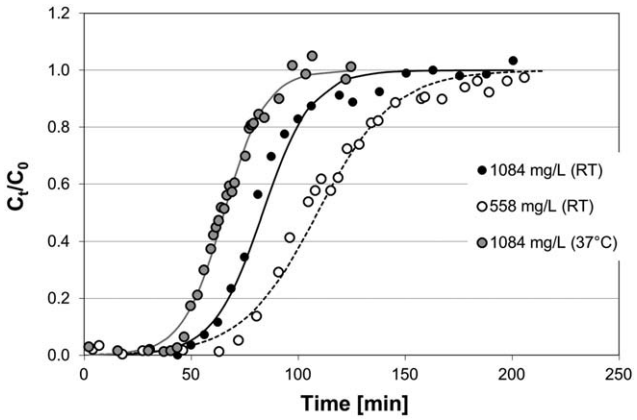


FIGURE 6. Breakthrough curves of heparin in PBS at different inlet concentrations and temperatures at the constant flow rate of 0.08 mL/min.

$$\frac{C}{C_0} = \frac{1}{1+\exp\left(K_{th}\left(\frac{q_0 X}{Q} - C_0 \times t\right)\right)} \quad (10)$$

where Q is the flow rate (mL/min), X represents the mass of dried cryogel (g), K_{th} is Thomas rate constant (mL/min × mg), and q_o is the maximum solid-phase concentration of the solute (mg/g).

This equation can be linearized[15] as follows:

$$\ln\left(\frac{C}{C_0} - 1\right) = \frac{K_{th} q_0 X}{Q} - K_{th} C_0 \times t \quad (11)$$

the plot of $\ln((C_t/C_0) - 1)$ versus t determines q_0 and K_{th}. Table IV summarizes the Thomas model parameters for heparin adsorption onto pHEMA-lys at different concentration and temperature (flow rate = 0.08 mL/min).

The data were fitted in the interval range of breakthrough between 10% and 90%. The correlation coefficient ranging from 0.94 to 0.98 indicates a good agreement between the experimental data and Thomas model.

Heparin adsorption in fresh human plasma

The efficiency of pHEMA-lys cryogels was tested in fresh human heparinized plasma by incubating the cryogels at 37°C with an initial concentration of 100 IU/mL (~5000 IU/g of dry cryogel).

Table V shows that the L-lysine modified cryogel adsorbed a higher amount of heparin than the plain cryogel

TABLE III. t_{total}, q_b, and q_{eq} for pHEMA-lys Adsorbent at the Heparin Concentration of 558 and 1084 mg/L in PBS at RT and 1084 mg/L in PBS at 37°C

Heparin Concentration (mg/L)	Temperature (°C)	t_{total} (min)	q_b (mg/g)	q_{eq} (mg/g)
558	RT	157	119	160
1084	RT	125	190	247
1084	37	91	142	199

TABLE IV. q_0 (mg/g) and K_{th} (mL/min × mg) Values Obtained With the Thomas Kinetics Model

Heparin Concentration (mg/L)	Temperature (°C)	q_0 (mg/g)	K_{th} (mL/min × mg)	R^2
558	RT	162	0.101	0.96
1084	RT	258	0.080	0.98
1084	37	209	0.095	0.94

TABLE V. Amount of Heparin Removed per Gram of pHEMA and pHEMA-Lys Cryogels in Human Plasma, at 37°C ($n = 3$)

	Heparin Adsorbed		
	IU/g Cryogel		
Adsorbent	30 min	60 min	120 min
pHEMA	540.1 ± 57	508.9 ± 67	491.0 ± 26
pHEMA-lys	4329.6 ± 346	4232.7 ± 410	3922.0 ± 292

TABLE VI. Activity of ATIII After Incubation of Heparin-Plasma With pHEMA and pHEMA-lys

	ATIII (%)			
Sample	0 min	30 min	60 min	120 min
Control	105.2 ± 0.6	105.2 ± 2.4	107.9 ± 1.1	105.5 ± 0.1
pHEMA		95.6 ± 1.8	94.6 ± 3.4	95.5 ± 0.6
pHEMA-lys		87.8 ± 2.8	84.1 ± 2.5	82.3 ± 3.1

The normal range is between 79% and 119% ($n = 3$).

and that the level of removed heparin slightly decreased in a time-dependent manner. From the kinetic studies performed in PBS and 50% FBS-PBS solution, described above, the saturation point was reached shortly before 2 h under the conditions applied. The greater amount of components in fresh plasma not only increases the possibility of competition and nonspecific adsorption thus interfering with the removal efficiency but also leads to earlier saturation. The amount of heparin depleted by the plain pHEMA cryogel was lower and not time-dependent, as values for 30 min and 2 h were similar. After 30 min of incubation, 1 g of plain pHEMA removed 540 IU of heparin, whereas 1 g of pHEMA-lys cryogels adsorbed 4330 IU. Values of heparin depleted by pHEMA-lys cryogels are corrected for nonspecific binding on unmodified gel.

In literature, there are reports on the development of heparin removal devices tested *in vivo*, but the heparin concentration in plasma or blood before extracorporeal blood purification was much lower than the value used here.[29,31] In other reports, the developed devices were tested under conditions far from clinical application (i.e., using aqueous solutions rather than undiluted biological fluids).[28,30,32,58]

Adsorption of other plasma components. Heparin acts by binding the enzyme inhibitor ATIII, causing a conformational change of ATIII which results in its activation through an increase in the flexibility of its reactive site loop. When activated, ATIII inhibits the activity of thrombin and other proteases involved in blood coagulation, most notably factor Xa, by forming an inactive complex with these enzymes. The removal of ATIII is unfavorable as its reduction would lead to increased coagulation and clot formation. Therefore, we investigated the ATIII adsorption as a measure of biocompatibility. As shown in Table VI, no time-dependent reduction of ATIII was observed when heparinized plasma was incubated with plain pHEMA cryogels, while a weak reduction of ATIII over time occurred upon incubation with pHEMA-lys. The value of ATIII after 2 h of incubation was still within the normal range.

The binding of albumin and blood proteins to pHEMA cryogels was investigated as well (Table VII). Albumin is an acidic protein that has binding sites for a wide variety of plasma components. There was no specific removal of albumin, because modified and unmodified pHEMA cryogels adsorbed similar amounts. Overall, a slightly higher adsorption of total blood proteins to pHEMA-lys was observed.

CONCLUSIONS

In this study, pHEMA macroporous hydrogels modified with L-lysine were developed to efficiently bind heparin. The positively charged amino groups of L-lysine adsorbed heparin under both static systems and under flow conditions. Such cryogel monoliths had a maximum capacity of heparin adsorption in PBS of 270 ± 14 mg (40,500 IU) at RT and 217 ± 21 mg (32,550 IU) at 37°C, per gram of cryogel (dry weight). The same trend was also observed under flow conditions, suggesting that temperature affected the total amount of heparin removed.

In diluted bovine serum at 37°C, the adsorption capacity decreased to 164 ± 16 mg (24,600 IU) of heparin per gram of dry cryogel. The comparison of maximum capacity in PBS and in serum solution at the same temperature highlights the competition between the serum components and heparin. Therefore, heparin adsorption was also evaluated in human plasma. Although human plasma represents a very complex matrix, the pHEMA-lys cryogel still bound 4330 IU/gram heparin after 30 min of incubation, that is, 87% of the initial amount of heparin. Under the same conditions, the nonspecific adsorption of heparin onto the plain pHEMA was 8% of the initial amount. Further tests on plain pHEMA and pHEMA-lys showed a slight reduction of ATIII, albumin, and total proteins, but they remained within the normal range. No difference between plain and functionalized cryogels with respect to albumin adsorption was observed.

Regeneration of pHEMA-lys was successfully achieved with 3*M* NaCl, which lead to the release of 95% of the previously adsorbed heparin.

TABLE VII. Albumin and Total Protein Removed per mg of pHEMA and pHEMA-lys Cryogels ($n = 3$)

	Albumin Adsorbed			Total Protein Adsorbed		
	mg/mg Cryogel					
Adsorbent	30 min	60 min	120 min	30 min	60 min	120 min
pHEMA	0.26 ± 0.04	0.26 ± 0.08	0.30 ± 0.03	0.49 ± 0.08	0.51 ± 0.07	0.51 ± 0.01
pHEMA-lys	0.23 ± 0.05	0.25 ± 0.05	0.26 ± 0.03	0.53 ± 0.11	0.58 ± 0.06	0.59 ± 0.04

All in all, the highly porous cryogels characterized in this study have clinical and pharmaceutical potential application to remove heparin, especially in cases where protamine injection is contra-indicated.

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