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Adsorption of BSA onto radiation-crosslinked poly(AAm/HPMA/MA) terpolymers

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Summary

The terpolymeric poly(acrylamide/2-hydroxypropylmethacrylate/maleic acid) (AAm/HPMA/MA) hydrogels in the rod form have been prepared by γ-radiation of quaternary mixtures of acrylamide, 2-hydroxy propylmethacrylate, maleic acid and water. The hydrogels were used in experiments on swelling, diffusion and adsorption of bovine serum albumin (BSA) from aqueous BSA solutions. In the experiment of BSA adsorp-tion, the effect of initial concentration of BSA, content of MA, irradiation doses and medium pH on the adsorption efficiency of the hydrogel were studied. The binding of BSA onto AAm/HPMA/MA hydrogel corresponds to L4-type (*Langmuir type with two layers*) adsorption isotherms in the Giles classification system for adsorption of a solute from its solution The maximum adsorption capacity of monolayer adsorption (Q_{mon}) and the binding constant were found to be 1.27 mg BSA (g gel)$^{-1}$ and 67.9 L (mg BSA)$^{-1}$, respectively. The adsorption of BSA within AAm/HPMA/MA hydrogels increased with the increase in MA content in the AAm/HPMA/MA hydrogels. When the irradiation doses of hydrogel increased the adsorption of BSA also increased. The maximum adsorption of BSA was observed at pH 3.7. Significant amount of the adsorbed BSA (up to 95%) was eluted in the elution medium containing 1.0 M NaSCN at pH 8.0.

Introduction

The removal and separation of proteins has many applications and hence the adsorption of proteins onto polymeric materials has been studied from all viewpoints for the last two decades. Adsorption methods are the most powerful tools for the isolation and puri-fication of natural physiologically active compounds. These methods are widely used for the immobilization of enzymes. Different types of the polymers are used as adsor-bent for adsorption purification and chromatographic separation of biomolecules (1-3).

Hydrogels are water-swollen macromolecular matrices consisting of crosslinked polymeric chains and insoluble in water and physiological temperature, pH and ionic strength. The water imbibing capacity of hydrogels is not only responsive to the pH, temperature and ionic strength of swelling reservoir, but also to the chemical architecture of the network such as chain flexibility, crosslinking density, hydrophilic functionals, osmotic potentials, and free volume. The high water content of the hydrogels not only imparts soft and rubbery texture to the material, but also develops antithrombogenicity in the polymer matrix because of the lowered free energy of the hydrated interface (4). These physicochemical properties of hydrogels enable them to serve as potential biomaterials in biomedical engineering and allied fields.

At present, a wide number and great variety of clinically important hydrogel are finding applications as short and long term materials in kidney dialyzers, blood oxygenators, heart valves, vascular grafts, contact lenses, etc. (5, 6). All of these implants and devices contain

materials that are recognized by blood as foreign; the result is a process of thrombosis often followed by formation of thromboemboli. This process generally involves a sequence of protein adsorption steps followed by cell interactions. In fact, protein adsorption onto biomaterial surfaces is believed to be the earliest event following implantation. This process determined by the nature of the protein, the component of biological fluid, and surface characteristics of implanted devices (7). Conditioning with the protein layer can induce series of consequential processes which may be beneficial or detrimental performance of the biomaterials (8).

Thus, looking to the significant consequences of protein adsorption on biomaterial surface, we are reporting in the present study results on the adsorption of bovine serum albumin (BSA) onto the surface of a novel terpolymeric hydrogels prepared with acrylamide (AAm), 2-hydroxypropyl methacrylate (HPMA) and maleic acid (MA) via radiation technique (9). The selection of AAm as a hydrophilic monomer for synthesizing hydrogel rests upon the fact that it is low cost, water soluble, neutral and biocompatible, and has been extensively employed in biotechnical and biomedical fields. On the other hand, HPMA is a hydrophobic monomer containing alkyl, hydroxyl and ester groups. Maleic acid is ionic in aqueous media. Thus, these monomers are selected for the preparation of the hydrogel and BSA uptake. The protein chosen for in vitro adsorption study was BSA that is among the most abundant proteins in vertebrates and in commercially available at low cost. BSA has also been widely used in biochemical work as a generic protein.

Experimental

The three monomers used in this work, namely, acrylamide (AAm), 2-hydroxypropyl methacrylate (HPMA) and maleic acid (MA) were obtained from BDH (Poole, UK). Bovine serum albumin (BSA) and Coomassie Brilliant Blue G250 were purchased from Sigma (St Louis, USA).

Preparation of radiation induced hydrogels

Aqueous solutions of monomers of 1 g AAm, 1 g HPMA and 0, 20, 40, 60, 80, 100 and 120 mg MA were prepared in 2 mL of distilled water. The solutions were placed in PVC straws of 3 mm diameter and irradiated to 2, 3, 4, 5, and 6 kGy in air at ambient temperature in a ^{60}Co Gammacell 220 type • irradiator at a fixed dose rate of 0.45 kGy h^{-1}. Freshly obtained long cylindrical shaped hydrogels were cut into pieces of 3-4 mm in length. They were washed, thoroughly rinsed with distilled water, blot dried with filter paper, dried in air and in vacuum (9), and stored for swelling and adsorption studies.

Swelling experiments

To correlate the swelling tendency of AAm/HPMA/MA hydrogel containing 100 mg maleic acid and irradiated to 5 kGy with their BSA adsorption capacity, the swelling behavior of the gel was studied in distilled water, in physiological saline (NaCl 0.9% (w/v)), in 10 mg L^{-1} BSA solution in distilled water, and in 10 mg L^{-1} BSA solution in physiological saline at 25 °C. Swollen gels removed from the water bath at regular intervals were dried superficially with filter paper, weighed and placed in the same bath. The radii of cylindrical swollen gels were measured by a micrometer.

The effect of initial BSA concentration on BSA adsorption

About 0.1 g AAm/HPMA/MA hydrogel containing 100 mg MA and irradiated to 5 kGy were transferred into 20 mL of solutions containing 1-20 mg BSA L^{-1}. This solution was incubated in a rotary shaker (J. P. Selecta, s. a. Spain) for 24 h at 25 °C. An aliquot of protein solution was then removed and its concentration was determined by the method of Bradford using a Shimadzu A160 double beam spectrophotometer at ambient temperature (10).

The effect of MA content and irradiation dose on BSA adsorption

The influence of MA content and irradiation dose was investigated for adsorption of BSA on AAm/HPMA/MA hydrogels. Hydrogel samples (0.1 g) prepared with different MA content and irradiation doses were added to 20 mL of 10 mg L^{-1} of BSA solutions. The samples were left in the solution for one day at 25 °C in a rotary shaker. To determine the BSA concentration spectrophotometric method was applied to these solutions.

The effect of pH on BSA adsorption

To investigate the effect of pH on the adsorption of BSA, 100 mg MA containing and 5 kGy irradiated AAm/HPMA/MA hydrogel samples weighing 0.1 g were added to 20 mL of 10 mg L^{-1} of BSA solutions at various pH. The pH of the experiment medium was changed between 3.7 and 7.4 by using buffer system. (0.2 M CH_3COONa – CH_3COOH for pH 3.7 - 5.6, 0.2 M Na_2HPO_4 - NaH_2PO_4 for pH 6.0 - 7.4). The samples were left in the solution for one day at 25 °C in a rotary shaker, and BSA concentrations were measured.

Desorption of BSA

Hydrogels loaded with BSA were left for 3 days in water, and 1 M NaSCN solution (pH 8.0) at 25 °C to investigate of desorption. Then BSA analysis in this solution was done.

Results and Discussion

Preparation of hydrogels

Hydrogels are synthesized using either chemical methods or irradiation technique. Hydrogels can also be synthesized by γ-irradiation (11-12). It is very well known that the presence of an initiator and a crosslinking agent affects the macromolecular structure and phase behavior of hydrophilic polymers in solution and contributes to inhomogeneity of the network structure. It is important to note that more homogeneous network structures can be synthesized, if crosslinking is accomplished with γ-irradiation in the absence of an initiator and a crosslinking agent. The structural homogeneity of the network affects the swelling behavior and mechanical properties (13). Gamma radiation was used to prepare AAm/HPMA/MA copolymers. When monomers of AAm, HPMA and MA were irradiated with ionization rays such as γ-rays, weak π-bonds of –C=C– on the monomers were broken by ionization irradiation and free radicals are generated. These free radicals react with each other, and a copolymer is produced. When AAm, HPMA, MA and water quaternary mixtures were irradiated, polymerization and crosslinking occur simultaneously. It is reported that complete gelation of AAm is obtained with 2 kGy of γ-rays irradiation doses at ambient temperature (14). Thus, a minimum dose of 2 kGy of γ-rays is used to prepare AAm/HPMA/MA hydrogels (9). In dry state, AAm/HPMA/MA hydrogels were hard and glassy, but in swollen state, gels were soft, tender and easy to handle. The hydrogels are obtained in the form of cylinders. Upon swelling the hydrogels retained their integrity (9).

Swelling and Diffusion

Swelling behaviors of AAm/HPMA/MA hydrogel containing 100 mg MA, and irradiated at 5 kGy were followed gravimetrically. Swelling degree (S%) of the hydrogels was calculated from the following relation. $S\%=[(m_t-m_o)/m_o]x100$ where m_t is the mass of swollen gel at time t and m_o is the initial mass of the dry gel.

The swelling curves of AAm/HPMA/MA hydrogels for distilled water (DW), physiological saline (PS), albumin solution (BSA) and physiological saline plus albumin solution (PS+BSA) are shown in Figure 1a. The values of S % increased with time but reached a constant value (Fig 1). This value of swelling was called equilibrium swelling degree (S_{eq} %). The values of S_{eq} % of the AAm/HPMA/MA hydrogels are presented in Table I. The swelling of the hydrogels in the solutions was in following order; DW > PS > BSA > PS+BSA.

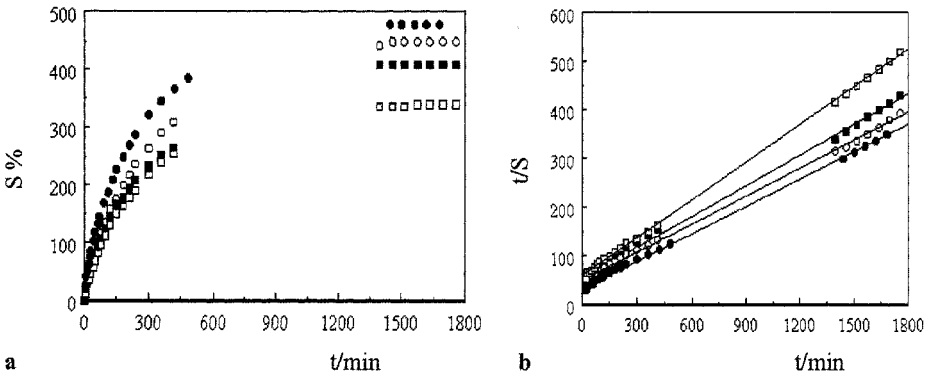

Fig. 1. Swelling (**a**) and second order swelling kinetics (**b**) curves of AAm/HPMA/MA hydrogel in the solutions, ●; DW, ○; PS, ■; BSA, □; BSA+PS.

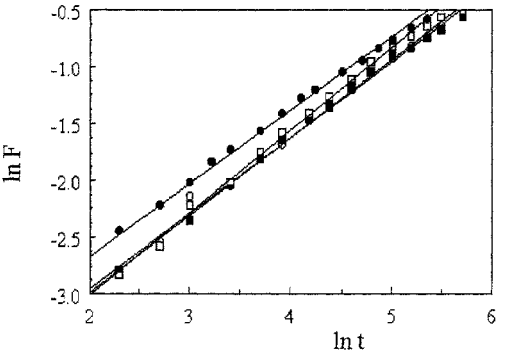

Fig. 2. Plots of ln F vs. ln t for AAm/HPMA/MA hydrogel in the solutions, ●; DW, ○; PS, ■; BSA, □; BSA+PS.

In order to examine the mechanism controlling the swelling processes, several kinetic models were used to test experimental data. The large number and array of different chemical groups on the AAm chains (e.g., amine, amide, carbonyl, carboxyl or hydroxyl) imply that there are many types of polymer-solvent interactions. It is probable that any kinetic is likely to be global. From a system design viewpoint, a lumped analysis of adsorption rates is thus sufficient to the practical operation.

A simple kinetic analysis is the second order equation in the form of $\frac{dS}{dt} = k_S (S_{max} - S)^2$ where k_S is the rate constant of swelling and S_{max} denotes the theoretical maximum or equilibrium of swelling degree. After definite integration by applying the initial conditions S=0 at t=0 and S=S at t=t, this equation becomes $\frac{t}{S} = \alpha + \beta t$ where α is reciprocal of initial swelling rate r_i or $1/k_S S_{max}^2$ and β is inverse of the degree of swelling at equilibrium.

To test the kinetics models, t/S vs. t graphs were plotted (Fig. 1b). The calculated kinetic parameters are tabulated in Table I. As depicted from Table I, the results of kinetic model are in agreement with swelling experiment.

Table 1. The swelling and diffusion parameters of AAm/HPMA/MA terpolymer containing 100 mg MA (Total given doses were 5 kGy)

Solution	S_{eq} %	S_{max} g (g gel)$^{-1}$	$r_i \times 10^2$ g (g gel)$^{-1}$ min^{-1}	n	k x 10^2	D x 10^6 cm sec^{-1}
DW	480	5.31	3.05	0.64	1.91	5.3
PS	450	4.92	2.21	0.67	1.40	6.1
BSA	410	4.40	1.88	0.68	1.30	6.6
PS+BSA	340	3.69	1.88	0.72	1.17	11.3

The values of maximum swelling of the hydrogels suggest similar swelling behavior (Table I). Swelling processes of the hydrogels in water are quicker than the swelling rate of the hydrogels in the other solutions.

The following equation is used to determine the nature of diffusion: $F = k\,t^n$ where F is the fractional uptake at time t, k is a constant incorporating characteristic of the macromolecular network system and the penetrant, and n is the diffusional exponent, which is indicative of the transport mechanism. Thisequation is valid for the first 60% of the fractional uptake. Fickian diffusion and Case II transport are defined by n valuing to 1/2 and 1, respectively. Anomalous transport behavior (non-Fickian diffusion) is intermediate between Fickian and Case II. That is reflected on n between 1/2 and 1 (15).

For radiation crosslinked terpolymers, ln F vs. ln t graphs were plotted and are shown in Fig. 2a. n exponents and k parameters are calculated from the slopes and intercepts of the lines, respectively, and are listed in Table I.

Table I shows the number determining type of diffusion (n) is over 0.50. Hence, the diffusion of water into the super water-retainer hydrogels is generally found as a *non-Fickian* character. When diffusion type is anomalous behavior, the relaxation and diffusion time are of the same order of magnitude. As the solvent diffuses into the hydrogel, rearrangement of chains does not occur immediately. The n values of the hydrogels in the solutions were in following order; DW > PS > BSA > PS+BSA and the k parameters of the hydrogels in the solutions the reverse in order.

The diffusion coefficients D of the penetrants were calculated from the following relations (16): $D^n=(k/4)(\pi l^2)^n$ where D is in $cm^2\ s^{-1}$, l is the radius of the gel. The values of the diffusion coefficient of the hydrogels are shown in Table 1. The diffusion coefficients of the hydrogels were in the following order; DW > PS > BSA > PS+BSA.

Adsorption of BSA

In this stage, it was purposed that investigation of uptake of BSA via the radiation crosslinked terpolymeric hydrogels and usability as a sorbent for BSA.

In an adsorption system at equilibrium, total solute concentration (C_o, g L^{-1}) is (17) $C_0 = C_B + C$ where, C_B is the equilibrium concentration of the solute on the adsorbent in g per liter (bound solute concentration) and C is the equilibrium concentrations of the solute in the solution in g L^{-1} (free solute concentration). The binding ratio, Q, defined by $Q=[(C_o-C)/m]xV$. Where V is volume of the protein solution, and m is mass of the dry terpolymer i.e. the absorbent.

A plot of the binding isotherm of BSA is shown in Fig. 3b. The binding of BSA onto AAm/HPMA/MA hydrogel corresponds to L4-type (*Langmuir type with two layers*) adsorption isotherms in the Giles classification system for adsorption of a solute from its solution (17) (Fig. 3b).

The binding data for first BSA adsorption layer were interpreted based on the uniform site-binding model (u.s.b.), which in statistical-thermodynamic terms corresponds to the formation of an ideal localised one-dimensional monolayer of solute on the polymer chains (18). This leads to the hyperbolic (*Langmuir*) form of binding isotherm, which

applies to many polymer/solute binding system: $Q=(Q_{mon}KC)/(1+KC)$ where K is the binding constant, that is, the equilibrium constant and, Q_{mon} is the site density (i. e. the limiting value of Q for "monolayer" coverage, which is thus a measure of the density of the sites along the polymer chain). To get the best values of the binding parameters from the experimental data, a linearization method have been developed by Klotz (18).

$$\frac{1}{Q} = \frac{1}{Q_{mon}} + \frac{1}{Q_{mon}K}\frac{1}{C} \quad (1)$$

The plot of polymer-protein binding system using Klotz method is shown in Fig. 3b. The binding parameters of protein/hydrogel binding system were calculated from the intercepts and slopes of the plots. The derived values of the binding parameters, Q_{mon} and K, for the protein/hydrogel binding system were found to be 1.27 mg BSA (g gel)$^{-1}$ and 67.9 L (mg BSA)$^{-1}$, respectively.

The effect of MA content and irradiation dose on BSA adsorption

The changes of BSA adsorption with MA content were illustrated in Fig. 4a. The adsorption of BSA within AAm/HPMA/MA hydrogels increased with the increase in MA content in the AAm/HPMA/MA hydrogels. In our previous study, it was observed that the addition of MA to AAm increased the swelling of AAm hydrogels (18). At the early stage of adsorption, the AAm/HPMA/MA hydrogels swelled with the solution of BSA as much as possible. The higher swelling of the hydrogel permitted the presence of more BSA molecules with the water inside the hydrogel. Some BSA molecules adsorbed onto the surface of the hydrogel via the electrostatical effects and some of BSA molecules infiltrated into the hydrogel.

The changes of adsorption with irradiation doses were illustrated in Fig. 4b. When the irradiation doses of hydrogel increased the adsorption of BSA also increased. The crosslinks of the hydrogels increased with the increase of the irradiation dose and reduced the size of the pores. In this way, it is difficult for the big BSA molecules to get into small pores and to be held on there.

On the other hand, in our previous study (19), the amount of absorbed BSA onto AAm/MA hydrogel containing 60 mg MA and irradiated to 5.2 kGy was found to be 0.56 mg BSA (g gel)$^{-1}$. The adsorption of BSA onto the terpolymer was increased with the incorporation of HPMA to the AAm/MA hydrogel approximately 1.5 fold.

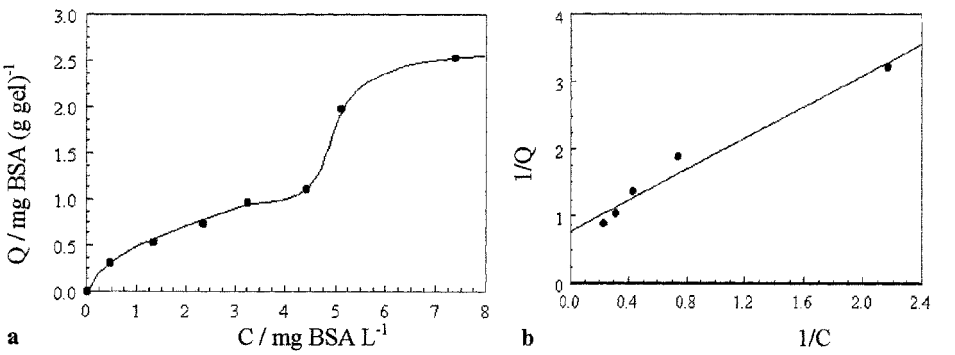

Fig. 3. The isotherm of adsorption (**a**) and Klotz graph (**b**) of adsorption system.

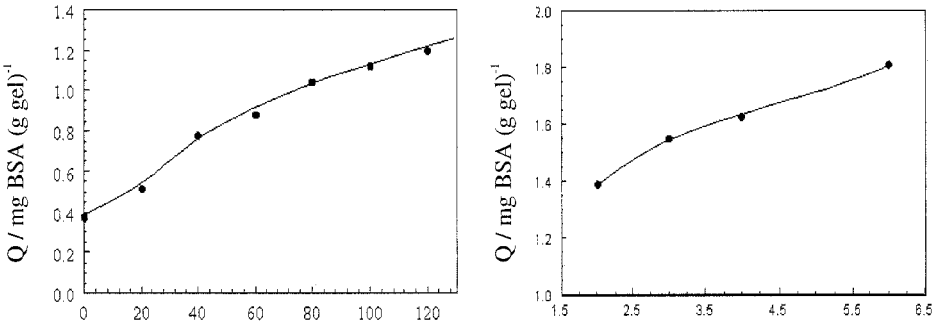

Fig. 4. The effect of MA content (**a**) and irradiation dose (**b**) on the adsorption of BSA within AAm/HPMA/MA hydrogel.

The effect of pH on BSA adsorption

Figure 5 shows the effect of pH on BSA adsorption. The maximum adsorption of BSA was observed at pH 3.7. At this pH, BSA adsorption performs via positively charged sites of amino acids of BSA. Anionic ligands bind in a hydrophobic pocket that is adaptable to the ligand, with its negative charge matched in a salt bond by the positive charge of a nearby lysyl or arginyl residue of BSA (20). Poly(AAm) is a nonionic polymer. Hydrophobic groups on the polymer were increased by the addition of HPMA to the AAm monomer and ionizable groups on the polymer were increased by adding of MA to AAm monomer. Therefore, these hydrogels have many carboxyl groups hence it can be causes to increase of interaction between BSA and carboxyl groups of hydrogels in the hydrophobic pocket of BSA. The decrease in the BSA adsorption capacity with increase in the pH can be attributed to electrostatic repulsion effects between the oppositely charged groups that are the carboxyl groups of BSA and AAm/HPMA/MA hydrogel.

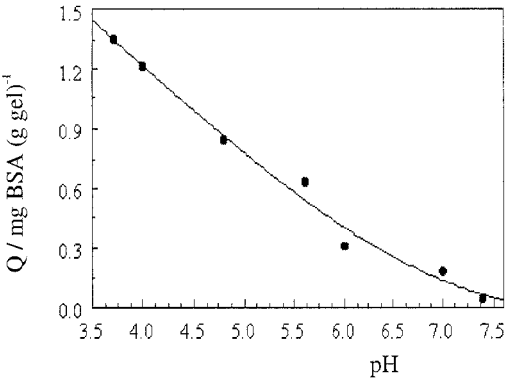

Figure 5. The effect of pH on the adsorption of BSA within AAm/HPMA/MA hydrogel.

Desorption

Hydrogels loaded BSA were left for three days, in distilled water and 1 M NaSCN solution (pH 8.0) at 25 ^{0}C to investigate the desorption. Desorption of BSA did not occurred in distilled water. More than 90 % of the adsorbed BSA was desorbed when NaSCN was used for elution. SCN^- is a chaotropic anion. Chaotropic ions prevent non-ionic interaction

by ordering the structure of water (21). So that BSA molecules adsorbed on hydrophobic groups of the hydrogel were eluted with NaSCN.

Conclusion

In this study, AAm/HPMA/MA hydrogel have been prepared via radiation technique. The highest swelling value of the hydrogel was found in the DW and the smallest swelling value was found in the aqueous solution of BSA+PS. The surface of the hydrogel exhibited affinity for adsorption of BSA molecules that varies in degree with varying composition of the MA, irradiation doses and pH. It is found that the adsorption of BSA follows *Langmuirian* nature. The absorbed amount of BSA increases with increasing amount of MA content in the terpolymer and irradiation dose. The adsorption is found to be sensitive to pH of the protein solution and becomes the highest at pH 3.7. The adsorption decreases with increasing of pH. The adsorbed BSA molecules were eluted with the solution of 1.0 M NaSCN.

The utilization of these types of hydrogels, in biomedicine, controlled drug delivery, pharmaceuticals, separation, purification and enrichment of some species makes hydrogel more popular. Furthermore, the use of irradiation method for hydrogel synthesis is viable and very promising especially protein adsorption and biomaterial application studies due to their innate sterility.

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