Controlled Delivery of Proteins by Absorption into Hydrogels

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Summary

Investigations on protein deposition into hydrogel contact lenses have not only provided strategies to reduce associated problems, they have also triggered the idea of their use as protein delivery vehicles. In this work, pure pHEMA and its more hydrophilic copolymer with methacrylic acid (MAA) were used as carriers of lysozyme, insulin and albumin. The effect of protein concentration, size and compact structure on mechanism of absorption was investigated. Surface charge of hydrogel, isoelectric point of protein and ionic strength of solution were also examined. The results indicated that absorption of protein was a function of hydrophilic nature of contact lens material. It was shown that pure HEMA was poor carrier for proteins as compared to HEMA/MAA at pH 7.2. The positively charged lysozyme not only adsorbed but also absorbed into the matrix of MAA copolymer.

Keywords: 2-Hydroxyethyl methacrylate; methacrylic acid; protein adsorption; contact lenses spoliation; hydrogel.

Introduction

Hydrogels are hydrophilic polymer molecules which are cross-linked by water. They do not dissolve, but swell in water. The capacity of hydrogels to absorb water is enormous and can be as much as 1000 times the weight of the polymer. The amount of water adsorbed by a hydrogel is expressed as the equilibrium water content (EWC) and is defined as:

$$\text{EWC} = \frac{\text{Weight of water in the gel}}{\text{Weight of the hydrated gel}} \times 100\%$$

Water in polymers can exist in more than one state (1) and these states, as well as the EWC, affect the properties of the hydrogels (2). The water in a hydrogel network exists in a continual state between two extremes. The "bound" or non-freezing water which is strongly associated with the hydrogel network through hydrogen bonds, whereas the "free" or freezing water has a much greater mobility and is unaffected by the polymeric environment. Hydrogels have been used in many fields due to their ease of preparation, their capacity of absorbing and releasing water, and the excellent oxygen permeability. The most widely uses of hydrogels include drug delivery systems (3, 4), artificial tissues and organs (5), and some soft contact lenses (6). Many commercial soft contact lenses are based on poly-2-hydroxyethyl methacrylate (PHEMA) more commonly referred to as HEMA having EWC 38-80% (7). To increase the oxygen permeability of HEMA, some ionic monomers such as methacrylic acid MA are added to the HEMA hydogels (8).

One of the major problems with hydrophilic contact lenses is their spoilage from tear film. The spoilage of contact lenses is due to different factors such as calcium films, organic plaques and protein films. The most important of these surface coatings appear to consist of proteins. Hydrogel contact lenses with high water content tend to have an open matrix structure that may influence the uptake of proteins (9).

Protein absorption is the overall result of various types of interactions between the different components present in the system, i.e. the sorbent surface, the protein molecules, the solvent (water) and any other solutes such as low-molecular mass ions. The surface of a protein is often complex in nature, with different characteristics such as hydrophilicity and charge (Figure 1).

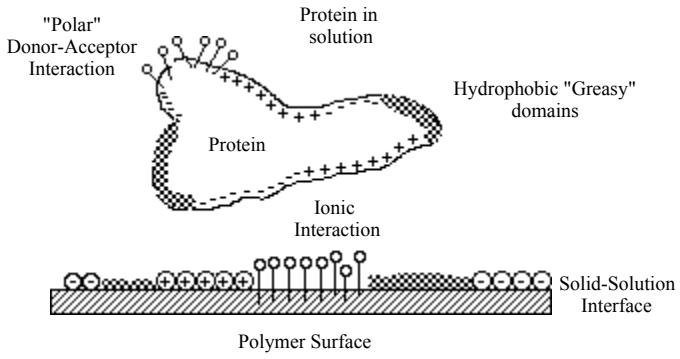
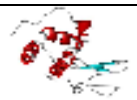


Figure 1. Possible interactions between the hydrogel surface and protein in the solution.

Protein adsorption may be affected by numerous factors including protein size, shape, the presence of charged groups (10-12), ionic strength of solution (13), pH, temperature (14), and surface treatment (15). The principle of protein absorption has frequently been used to design protein delivery devices. For example, contact lenses were used as vehicles of ophthalmic drug delivery (16).

In the present research, *in vitro* adsorption of selected proteins onto different HEMA hydrogels were investigated. Selection of protein was based on their differences in size, shape, isoelectric point and compact structure. Two types of HEMA hydrogels were synthesized in our laboratory differing in their monomer ionic properties. Lysozyme is the most abundant protein in human tears, constituting one third of the total protein (17). It is a relatively small, compact protein positively charged at neutral pH (pI 10.5-11.1). We have found previously that majority of proteins causing contact lens spoliation is composed of lysozyme (18). Lactalbumin is the albumin found in milk with a molecular weight and compact structure similar to lysozyme, but possesses negative charges at the neutral pH values (pI 4.2-4.5). The small protein, insulin was selected because its molecular weight is less than half of the two proteins, however due to its tendency to form dimmers its molecular dimension may be varied in solution (19). However, its dimension at both forms is still lower than other lysozyme and lacalbumin (Table I). Besides, ophthalmic delivery of insulin may be achieved through its entrapment into hydrogels. Table I has gathered some features of used proteins determinant in their absorption into hydrogels.

Table I. Characteristics of proteins affecting their absorption into hydrogel matrix.

Protein (source)	Molecular weight (Da)	Dimensions (A°)	Isoelectric pH	Charge at pH 7.2	Compact structure
Lysozyme (hen egg)	14320	45×30×30	10.5-11.1	+++	
Insulin (bovine pancreas)	5808	Monomer 21×12×8 Dimmer 23×25×18	6.8	-	
Lactalbumin (human milk)	14178	37×32×25	4.2-4.5	--	

Materials and methods

Materials

Lactalbumin (human milk), lysozyme (hen egg white) and insulin (bovine pancreas) were obtained from Sigma Chemical Company. HEMA, MAA and azo-bis(iso-butyronitrile) were from Roche Molecular Biochemical Company, Germany. Mono and dibasic sodium phosphate, sodium acteate, hydroxymethyl aminomethane (tris), sodium tetraborate, *N*-methylpyrrolidone and acetic acid were purchased from Merck representative in Iran. The purity of proteins was >99.9% and all chemicals were reagent grade.

Synthesis of HEMA and HEMA-MAA hydrogels

Poly (HEMA-MAA) was prepared by free radical polymerization using a Co(III) complex as catalytic chain transfer agent to control the molecular weight of the polymer by limiting the occurrence of side reactions. Before polymerization, inhibitor was removed from both monomers using a column of basic alumina.

In a typical copolymerization mixture, 0.5 g, 5.81×10^{-3} mol MAA, 9.5 g, 7.3×10^{-2} mol HEMA, 7.1 mg, 4.35×10^{-5} mol azo-bis(iso-butyronitrile), 1.2 mg, 2.5×10^{-6} mol Co(III) complex and 10 ml *N*-methylpyrrolidone were combined in a round bottom flask. The reaction mixture was sealed accurately and nitrogen bubbled through for one hour.

To polymerize pure HEMA, the reaction mixture contained 10.0 g, 7.3×10^{-2} mol HEMA, 7.1 mg, 4.35×10^{-5} mol azo-bis(iso-butyronitrile), and 10 ml *N*-methylpyrrolidone were combined in a round bottom flask, sealed, nitrogen bubbled in for one hour.

Each reaction mixture was then injected into the space between two sealed glass plates, 2mm apart at room temperature. Bubble formation was carefully avoided, as the air could inhibit polymerization. The polymer formation was completed in about two hours confirmed by a sharp line observed at air interface. The gel was removed from the cast and cut into 1cm^2 squares, dried in 30°C oven and kept sealed at 4°C until use.

Preparation of protein solutions

Protein solutions (200 mg%) were prepared in phosphate buffer (pH 7.2) at room temperature just before use. 10 μM sodium benzoate was added to each solution to prevent possible contamination.

***In vitro* protein absorption**

Individual hydrogel disks were soaked in 50ml of each protein solution for five days at 22±0.1°C with frequent shaking. This period was required for the protein adsorption process to be completed and to reach an equilibrium state (18). The absorbance was measured at 280 nm using UV-vis spectrophotometer, M350 Double Beam (20). The amount of absorbed protein was obtained by subtracting the remained protein in the solution from its initial concentration. Due to a dynamic process, the adhesion of these proteins to the walls of the vials was assumed negligible.

Release of proteins

The rate of release was measured by inserting the protein loaded hydrogels individually in buffer solutions. In practice, 2.0 ml samples of buffer solution was withdrawn and the 280 nm absorption measured using UV-visible spectrophotometer at 9.0 am for 10 days until the value remained constant for two days.

Results and discussion

Figs. 2 and 3 show the time dependent absorption of lysozyme, insulin and lactalbumin on pure HEMA and HEMA/MAA hydrogels respectively. It can be seen that the order of protein absorption in both types of hydrogels is lysozyme >lactalbumin > insulin. The maximum absorption occurred in 3 days and almost no increase was observed during the following days. It is known that addition of MAA into HEMA polymerization mixture, introduces negative charges to the polymer obtained (10). The resulting copolymer not only is negatively charged on its surface, it also possesses some negative charges within its matrix. This is due to the presence of a carboxylate groups in HEMA/MAA in contrast to non-charged HEMA surface. On the other hand, at pH 7.2 the charge on lactalbumin (pI 4.9) and insulin (pI 6.8) is negative, while lysozyme (pI 10.7) possesses positive charges. The greater lysozyme adsorption onto the HEMA-MAA surface can, therefore, be attributed to its positive charge causing the electrostatic interactions between HEMA-MAA and lysozyme. The low tendency of lactalbumin and insulin for adsorption onto MAA surface, on the other hand, is due to the presence of a negative surface charge on their surface at pH 7.2 leading to very low electrostatic interactions between copolymer surface and protein.

Considering that sequence similarity of α-lactalbumin with lysozyme, it can be explained that surface charge of protein plays a more important part for its absortion into hydrogels than its size (21). On the other hand, insulin monomers bind one another through the folding of an interchain β sheet by hydrogen-bonding. The insulin structure is expected to vary if the protein adsorbed to the surface was either monomer or a combination of monomer and dimmer. However, the molecular size and dimensions of dimeric form is smaller than the other two proteins. Using a model for insulin fibrillation at hydrophobic interfaces, it has been proposed that monomer, dimer and hexamer of insulin can adsorb to the surface (22). As insulin monomer is less stable than its dimmer or other possible conformations, it most likely changes conformation upon adsorption to a hydrophobic surface, and thereby possibly initiates the fibrillation process (23). On the other hand, a recent study has revealed that the same range of insulin species could be adsorbed on Teflon surfaces (24). However, the absorption behavior of the charged insulin into hydrophilic surfaces such as HEMA/MAA is expected to be different. The present study showed that at pH 7.2, insulin absorbed to amount of about 20 mg% into HEMA as well as HEMA/MAA copolymers. Therefore, it is expected that fibrillation is less likely occurred (Figs 2 and 3).

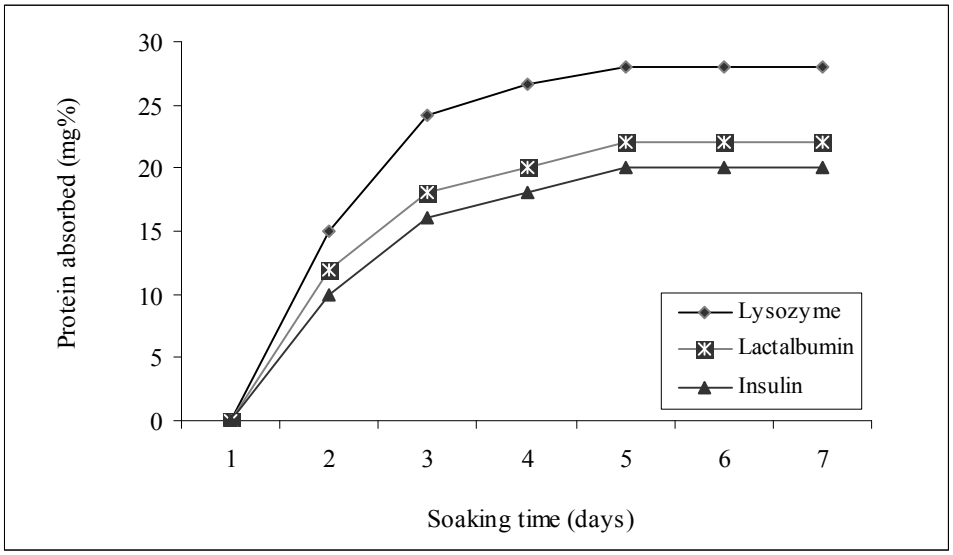


Fig. 2. The effect of soaking duration on adsorption of proteins on pure HEMA. Accuracy ±0. 005mg, temperature 22±0.1°C and pH 7.2.

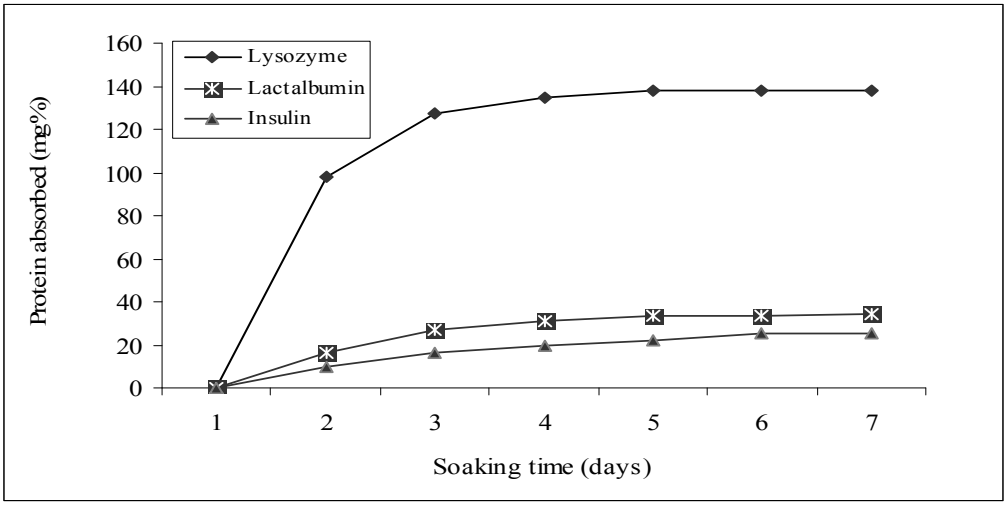


Fig. 3. Time dependent absorption of proteins on HEMA/MAA. Accuracy ±0.005 mg, temperature 22±0.1°C and pH 7.2.

Figure 4 shows compares the absortion into polymers of the three selected proteins. The data used are total amount of protein accumulated on hydrogels in 5 days. It was found that, within experimental errors, lysozyme was abosorbed to both hydrogels more than the other two proteins. This type of behavior is not expected for pure HEMA hydrogels as they should not be charged at the experimental conditions. However, the observed absorption suggests the presence of MAA impurities in HEMA which is formed during polymerization. We had previously obtained similar results using commercial hydrophilic soft contact lenses as hydrogels (18).

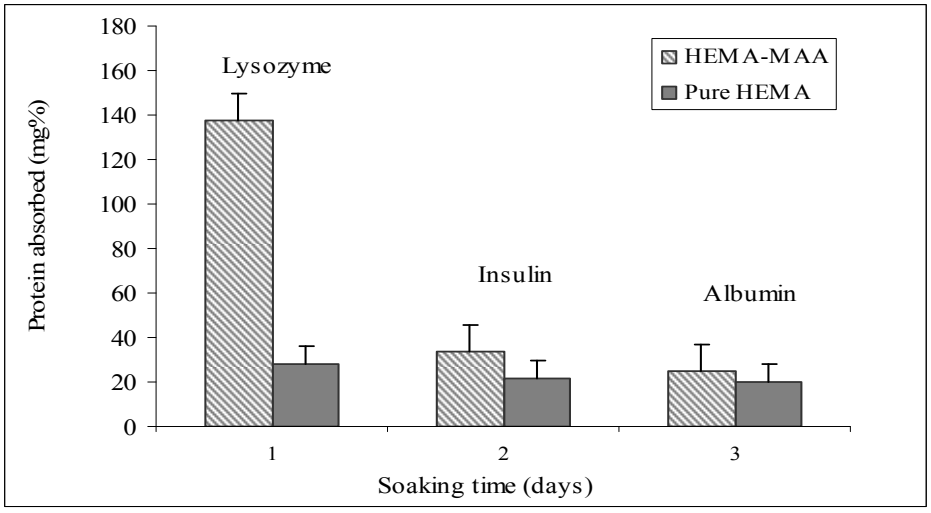


Fig. 4. Quantity of proteins accumulated into HEMA and HEMA/MAA hydrogels during 5 days. Accuracy ±0.005mg, temperature 22±0.1°C and pH 7.2.

Fig. 5 illustrates the effect of pH on adsorption of albumin, insulin and lysozyme into negatively charged HEMA/MAA hydrogels. The concentration of each individual protein solution was 200 mg%. It can be seen that absorption of protein into negatively charged hydrogel not only is a function of its size and compact structure, it also varies with pH of the solution. Depending to their pI, proteins carry different charges at various pH. It is known that at pH<pI all proteins carry positive charges, while at pH>pI they are negatively charged.

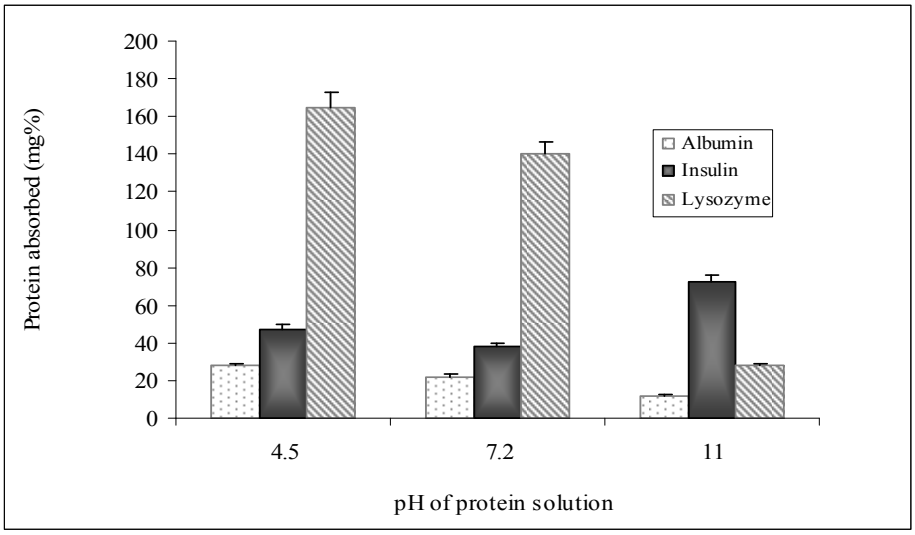


Fig. 5. Effect of pH on uptake of proteins by HEMA-MAA copolymer. Accuracy ±0.005 mg, temperature 22±0.1 °C and concentration of proteins 200 mg%.

Conclusions

The interaction of protein molecules with hydrogels is affected by many factors including pH, concentration of protein, soaking time, size and impact structure of the protein. Depending on its isoelectric point, a protein carries charges at different pH values. The concentration of protein solution used for in vitro spoliation studies also affects its adsorption onto the hydrogel. Addition of electrolytes with various concentrations caused an increase in the protein adsorption, as it may alter the electrostatic charge difference between the polymer and protein.

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