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Hemoglobin recognition of molecularly imprinted hydrogels prepared at different pHs

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ABSTRACT

The hemoglobin-imprinted hydrogels were fabricated by using *N*-*t*-butylacrylamide (TBA) acrylamide (AAM) and itaconic acid (IA) monomers and hemoglobin (Hb, MW 65 kDa) imprinted molecule in pH buffer solutions (pH 4.0, 6.8 and 8.0). The nonimprinted hydrogels were also prepared at same conditions without Hb imprinting molecule. The effects of pH, initial concentration and adsorption time over the Hb adsorption capacity of both imprinted and nonimprinted hydrogels were analyzed and found to be strongly dependent on the preparation pH (pH_{prep}). The maximum Hb adsorption for the imprinted hydrogel prepared at pH 4.0 was found to be 12.4 mg protein g^{-1} dry gel in pH 4.0 buffer solution. This behavior was attributed to the formation of more accessible adsorption sites (imprints) because of the non-covalent interactions between the template and network during formation in pH 4.0 buffer solution which is below of the isoelectric point (pI 6.8) of Hb. Langmuir and Freundlich adsorption models were applied to describe the equilibrium isotherm. Langmuir analysis showed that an equal class of adsorption was formed in the hydrogels. Moreover, batch adsorption equilibrium and selectivity studies were also performed by using two reference molecules as fibrinogen (Fb, MW 340 kDa) and myoglobin (Mb, MW 17 kDa). The imprinted hydrogels have 1.5–2.2 times higher adsorption capacity for Hb than the non-imprinted hydrogels prepared at the same pHs, and also have 2.0–3.1 times higher selectivity for the imprinted molecule.

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1. Introduction

Hb is the iron-containing oxygen transport metalloprotein in the red cells of blood in mammals and other animals. Hb is a globular protein, and structural changes in any of the four molecular subunits that is comprised of, can result in the manifestation of hereditary diseases such as sickle cell anaemia, thalassaemia and hemoglobinopathies [1]. The laboratory diagnosis of such conditions requires a combination of complicated and time-consuming procedure [2], whereas molecularly imprinting technique may offer a rapid, sensitive and selective approach to the screening, diagnosis and monitoring of Hb disorders.

Generally, tests of detecting Hb are either indicative rapid tests, or they need to use expensive natural antibodies (i.e. clinical immunoassays). In addition, processes for detecting Hb immunoassay, in the clinical setting, are usually complicated as antibody activity is not easy to maintain. Hence, if molecularly imprinted hydrogels (MIHs) could be fabricated, such that they served as antibodies able to replace their natural counterparts, they have an application in diagnostic analysis.

A target molecule as a template is used in the molecular imprinting technique. Around this template functional monomers assemble in a prepolymerization complex and subsequently, after polymerization, they are maintained in

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position by crosslinking agents to form, after extracting the template, recognition cavities. Ideally, each of the polymer's recognition cavities will have a specific shape and contain complimentary functional groups spatially situated for subsequent recognition of the template. The interactions that mediate recognition are achieved by utilizing either non-covalent interactions such as hydrogen bonds, hydrophobic and electrostatic interactions, or by reversible covalent interactions. On the other hand, the imprinting of large biomolecules such as proteins, shows a variety of challenges. Proteins as well as being relatively labile, have conformations, which are changeable and sensitive to various factors, e.g. pH, temperature and solvent composition [3–5]. Therefore, during the polymerization reaction around the target protein, the shape of template is also affected by pH of medium.

Previous studies have been reported on MIHs with protein with varying chemical structures or techniques, these studies have been described mainly the MIHs adsorption capacity, their imprinting effect and the mechanism of recognition [6–10]. However, selectivity of imprinted hydrogels has not been demonstrated in many systems. Of course, this depends on how close the challenge molecules are to the template.

In this respect, we prepared Hb-imprinted hydrogels by using TBA, AAm and IA monomers and Hb template molecule at three different pH buffers, namely 4.0, 6.8 and 8.0, aiming to create stereospecific 3D cavities for the binding of Hb based on hydrogen bonding between the hydrogel and the template. The same hydrogels were later prepared in non-imprinted manner to compare their adsorption capacities for Hb molecules. As will be shown below, the increase of affinity, capacity, and selectivity for a relatively small protein was determined depending on the pH_{prep} . This was achieved by maximizing the non-covalent interactions between the template and the network during formation at various pHs.

2. Experimental

2.1. Materials

TBA, AAm, IA monomers, *N,N*-methylenebis(acrylamide) (MBAAm), ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and used as received. Hb (MW 65 kDa, pI 6.8–7.0), Fb (MW 340 kDa, pI 5.8) and Mb (MW 17 kDa, pI 6.8–7.2) biomolecules were purchased from Sigma Company (St Louis, MO, USA). Glacial acetic acid, phosphoric acid, boric acid, and standardized sodium hydroxide were used to prepare Britton–Robinson (B–R) buffers. The stock B–R buffer solution was prepared by using 2.5 mL of glacial acetic acid, 2.7 mL of phosphoric acid, 2.47 g of boric acid and its dilution with deionized water to 1000 mL. 50-mL portions of this stock solution were taken, and their pHs were adjusted between 2.0 and 9.0 by addition of an appropriate amount of 2.0 M NaOH or HCl solutions.

2.2. Preparation of hydrogels

The TBA (0.8 g), AAm (0.2) and IA (0.15 g) were dissolved in 4.0 mL pH buffer solution (pH_{prep} 4.0, 5.8 and 8.0). Then,

MBAAm (0.05 g, crosslinker), APS (0.06 M, 1.0 mL, initiator), and TEMED (0.064 M, 0.5 mL, accelerator) and Hb (0.04 g) were added to the solution. After nitrogen bubbling for 5 min, these solutions were placed in poly(vinyl chloride) straws 4 mm in diameters and about 20 cm long. The poly(vinyl chloride) straws were sealed and placed in a thermostated water bath at 22 °C. The polymerization was conducted for 24 h. The non-imprinted hydrogels was prepared simultaneously under the same condition without Hb. After polymerization, the resulting hydrogels were purified by immersing in deionized water for 1 week to remove Hb and unreacted chemicals. The water was replaced three to four times every day. The removal of Hb was confirmed by UV spectrometer at 406 nm. All the purified hydrogels were cut into disc-like pieces approximately 10 mm in length and freeze-dried in a Virtis freeze drier (Lobconco, USA) for 2 days to completely remove water for further studies.

2.3. Adsorption kinetics of hydrogels

To investigate the adsorption kinetics of imprinted and non-imprinted hydrogels, ~0.1 g of freeze-dried hydrogels was first swollen to equilibrium and then immersed into 10 mL of 0.32 mg mL^{-1} Hb solution at 22 °C. The concentration of Hb in the solution was determined for different times using a spectrophotometer at 406 nm.

2.4. Adsorption experiments

The swollen hydrogels were placed in 25 mL conical flask and mixed with 10 mL of a known concentration of Hb solution at 22 °C for 24 h. The concentration of Hb in the solution was determined using a spectrophotometer at 406 nm. Hb adsorption from aqueous solution containing 4.0 mg mL^{-1} was also studied at different pH values. The pH of adsorption medium was changed between 2.0 and 9.0 by using B–R buffers. For selectivity experiments, Fb and Mb were used and their absorption measurements were measured at 585 and 409 nm, respectively.

3. Results and discussion

3.1. Adsorption kinetics of hydrogels

Adsorption kinetics experiments were carried out for the imprinted and nonimprinted hydrogels prepared at various pH_{preps} by using 4.0 mg mL^{-1} Hb solution. The adsorption curves are given in Fig. 1. It can be seen that the hydrogels have a rapid increase in 10 h, and then it increases slowly with the time extension. 24 h later, the adsorption process reach equilibrium. Obviously, the first parts of the kinetic plots represent the Hb molecule first adsorbed on the surface of hydrogels, the second parts of the plots with smaller slopes indicate slow adsorption rates. In other words, the Hb adsorption is fast at the beginning, but after the adsorption onto the surface, the penetration of Hb molecule into the hydrogels becomes much more difficult.

The imprinted hydrogel prepared at pH 8.0 (above pI) adsorbed about 11 mg Hb g^{-1} dry gel within 5 h, or 15 mg Hb g^{-1} dry gel within 24 h, whereas the imprinted hydrogel prepared

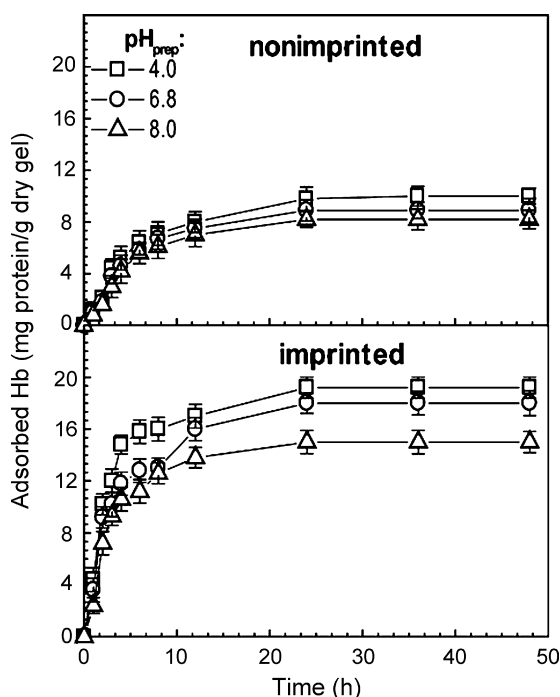


Fig. 1 – Adsorption kinetics of Hb on nonimprinted and imprinted hydrogels: initial concentration of Hb, 4.0 mg mL⁻¹; pH 4.0; ionic strength 0.02 (adjusted with NaCl); temperature 22 °C; total solution volume, 10 mL.

at pH 4.0 (below *pI*) adsorbed about 15 and 19, respectively, within the same time frames. These results showed that the amount of adsorption is strongly dependent on the pH_{prep} . Moreover, all the imprinted hydrogels adsorbed Hb more quickly than the nonimprinted hydrogels because their macroporous structures and cavities make transfer of Hb molecules easier between the hydrogel matrix and the external aqueous phase.

3.2. Effect of pH on Hb adsorption

Proteins are highly charged and folded structures with many differing areas of negative positive charges that are evident due to the charged functional groups throughout the protein structure [11]. However, they do not have a net electrical charge at their *pI*s. Therefore, in pH buffer solutions, the maximum protein adsorption onto hydrogels is usually shown at their *pI*s [12]. Fig. 2 shows the effect of pH on the Hb adsorption of the hydrogels prepared at various pHs. As shown this figure, for the hydrogels prepared at pH 6.8 and 8.0, the maximum Hb adsorption was observed at pH 7.0 which is close to the *pI* of Hb (6.8), but, it was shown at pH 4.0 for the hydrogels prepared at pH 4.0. This behavior was attributed to the formation of accessible adsorption sites (imprints) due to the ionic interactions between the carboxylic acid groups of IA and amino acid groups with slight cationic character of Hb during the polymerization at pH 4.0 which is below of *pI* for Hb. In this case, the dominant effect during whole adsorption process in pH buffer solutions is molecular recognition which is affected by pH_{prep} . The recognition sites were tailor-

made by the copolymerization of monomers and crosslinker in the presence of Hb template protein in pH 4.0, 6.8 and 8.0 buffer solutions. The template Hb was subsequently removed from the hydrogel, leaving pore canals and recognition cavities complementary to the protein in shape and in the positioning of polar groups. It is believed that the side chains of Hb interact via electrostatic interactions and hydrogen bonds with the positioned carboxylic acid and amide groups of the polymer chain. Distribution of charge and polar on Hb surface is very complicated in polymerization solutions with different pH. Electrostatic distribution of cavity is adjusted according to the protein surface, so that the electrostatic information from the template Hb that is essential recognition can be transferred to the imprinted cavity. In this case, the special adsorption involves molecular recognition process, which depends on tailored stereo-cavity and combined points via multiple-point hydrogen bonding or ionic interactions. In the imprinted hydrogels, self-assembly process results in exist of stereo-cavity and combined points after polymerization and removal of target molecule Hb. Therefore, in all pH buffer solutions studied, the imprinted hydrogels exhibited higher Hb adsorption capacity than the nonimprinted hydrogels prepared at same conditions.

3.3. Adsorption isotherm

Fig. 3 shows the dependence of the adsorbed amount of Hb on the initial concentration. As seen this figure, the adsorption values increased with increasing concentration of Hb and a saturation value was achieved at Hb concentration of 0.032 mg mL⁻¹, which represents saturation of the active

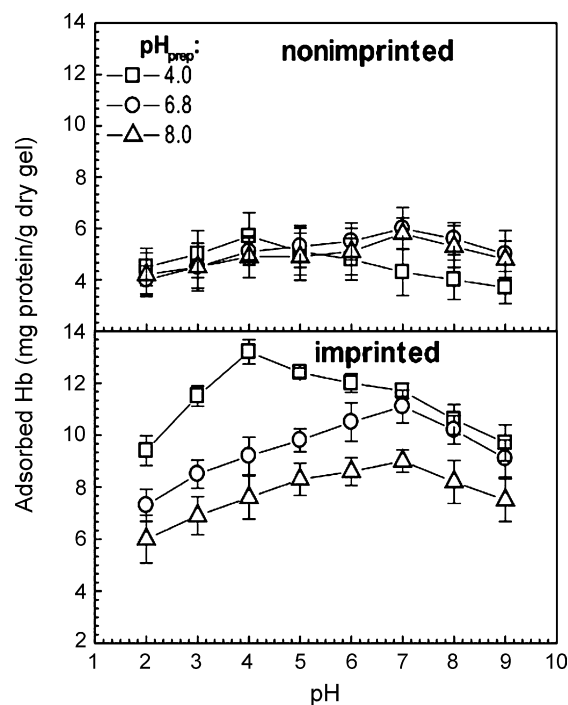


Fig. 2 – Effect of pH on Hb adsorption by nonimprinted and imprinted hydrogels: initial concentration of Hb, 4.0 mg mL⁻¹; ionic strength 0.02 (adjusted with NaCl); temperature 22 °C; total solution volume, 10 mL.

Table 1 – Langmuir and Freundlich isotherm constants of the hydrogels

Type of hydrogel	pH _{prep}	Langmuir			Freundlich		
		q _m (mg g ⁻¹)	K _m (mg mL ⁻¹)	r _L	K _F (mg g ⁻¹)	n	r _F
Nonimprinted	4.0	5.27	40,994	0.983	39.2	4.0	0.900
Imprinted	4.0	18.46	14,275	0.973	170	3.3	0.973
Nonimprinted	6.8	5.29	23,734	0.941	30.2	4.3	0.822
Imprinted	6.8	13.03	19,710	0.994	64.0	4.6	0.945
Nonimprinted	8.0	6.37	6,932	0.981	290	2.0	0.824
Imprinted	8.0	12.04	12,104	0.993	101	3.4	0.942

adsorption and recognition sites of hydrogels. It was observed that there is almost no effect of pH_{prep} on the adsorption capacity of nonimprinted hydrogels. However, the adsorption capacity of imprinted hydrogels was found to be strongly dependent on the pH_{prep}. Moreover, when compared to the hydrogels prepared at pH 5.8 and 8.0, the imprinted hydrogels prepared at pH 4.0 showed the maximum adsorption capacity due to their slight anionic character during the polymerization process. Therefore, the dominant effect during whole adsorption is molecular recognition, but it is affected by pH_{prep}. Adequate adsorption cavities and combined points for Hb can be fabricated in the hydrogel matrix during polymerization in the presence of Hb as template. In this case, the crosslinker chains do not disturb the formation of the complex of Hb–IA. Thus, the excess of adsorption can be reached through the imprinting effect. As for nonimprinted hydrogels, physical adsorption is dominant effect due to the lack of self-assembly process. Hence, in the absence of target molecule Hb, the IA units of hydrogel are distributed randomly in the net-

work structure and isolated each other by crosslinker chain. The crosslinker from the multiple points prevents interactions between carboxyl groups of IA and Hb, resulting in smaller adsorption of Hb.

The adsorption behaviors of hydrogels can be described with the Langmuir adsorption equation as

$$\frac{C_e}{q_e} = \frac{1}{K_m q_m} + \frac{C_e}{q_m}$$

where C_e is the equilibrium or final concentration of Hb in solution (mg mL⁻¹), q_e is the adsorption capacity of Hb adsorbed per unit mass of hydrogel at equilibrium concentration (mg Hb g⁻¹ dry gel), q_m is the theoretical maximum adsorption capacity (mg g⁻¹ dry gel) and K_m is the Langmuir adsorption equilibrium constant (mg mL⁻¹).

A linearized plot of C_e/q_e versus C_e gives q_m and K_m . Table 1 lists the calculated results. It can be seen from the data of Table 1 that the Langmuir equation fits well for Hb adsorption on the imprinted and nonimprinted hydrogels prepared at different pH_{prep} under the concentration range studied (correlation coefficient, $r_L > 0.94$). The maximum adsorption and the Langmuir adsorption equilibrium constant of the imprinted hydrogels were higher than those of the nonimprinted hydrogels prepared at the same pH_{preps} according to the adsorption isotherms.

It has been reported that the effect of isotherm shape with a view to predicting if an adsorption system is “favorable” or “unfavorable” [13]. The essential features of a Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, E_L , which defined by

$$E_L = \frac{1}{1 + K_m C_0}$$

where C_0 is the initial Hb concentration (mg mL⁻¹). The parameter $E_L > 1$, $E_L = 1$, $0 < E_L < 1$, $E_L = 0$ indicates the isotherm shape according to unfavorable, linear, favorable and irreversible, respectively.

The values of E_L calculated for different initial Hb concentration of imprinted and nonimprinted hydrogels are given in Table 2. The E_L values show that favorable adsorption of Hb on both the imprinted and nonimprinted hydrogels takes place; therefore the hydrogels are favorable adsorbers.

The other well known isotherm is Freundlich adsorption isotherm, which is a special case for heterogeneous surface energy in which the energy term in the Langmuir equation varies as a function of surface coverage strictly due to variation

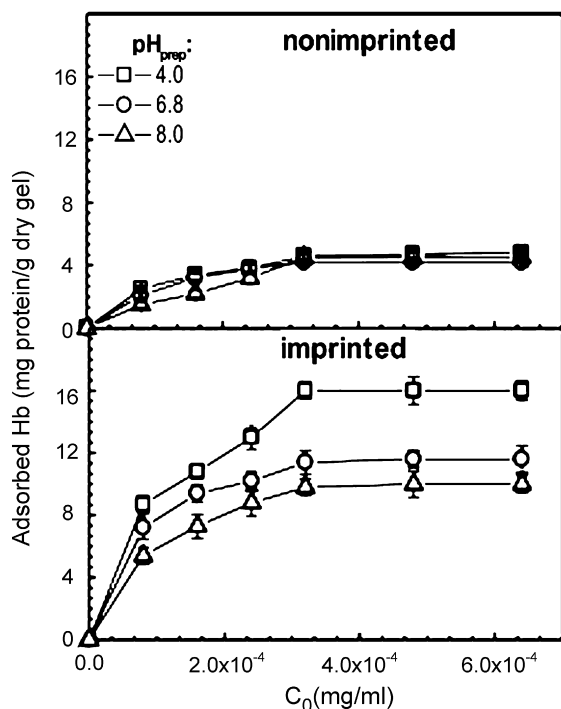


Fig. 3 – The adsorption isotherms of Hb on nonimprinted and imprinted hydrogels. pH 4.0; ionic strength 0.02 (adjusted with NaCl); temperature 22 °C; total solution volume, 10 mL.

Table 2 – E_L values based on the Langmuir equation

Hb initial concentration (mg mL ⁻¹)	pH _{prep} 4.0		pH _{prep} 6.8		pH _{prep} 8.0	
	Nonimprinted	Imprinted	Nonimprinted	Imprinted	Nonimprinted	Imprinted
8.0×10^{-5}	0.234	0.467	0.345	0.388	0.643	0.508
1.6×10^{-4}	0.132	0.305	0.208	0.241	0.474	0.341
2.4×10^{-4}	0.092	0.226	0.149	0.175	0.375	0.256
3.2×10^{-4}	0.071	0.180	0.116	0.137	0.311	0.205
4.8×10^{-4}	0.048	0.127	0.081	0.096	0.231	0.147
6.4×10^{-4}	0.037	0.099	0.062	0.073	0.184	0.114

of the sorption. The Freundlich equation is given as

$$q_e = K_F C_e^{1/n}$$

where K_F is roughly an indicator of the adsorption capacity and $1/n$ is the adsorption intensity. K_F and $1/n$ can be determined from the linear plot of $\ln q_e$ versus $\ln C_e$, Table 1 listed the calculated results. The magnitude of the exponent $1/n$ gives an indication of the favorability of the adsorption. The values, $n > 1$ represent a favorable adsorption condition for Hb adsorption on the hydrogels, and the high correlation coefficients ($r_F > 0.82$) showed that the Freundlich isotherm agrees well with experimental data also.

3.4. Selectivity of hydrogels

The special selectivity tests of imprinted and nonimprinted hydrogels prepared at different pHs were carried out using different substrates as Fb and Mb in pH 4.0 buffer solutions. Their amounts of adsorption to the imprinted and nonimprinted hydrogels were determined with the equilibrium adsorption method and illustrated in Fig. 4. The selectivity ($\alpha_1 = \text{Hb}/\text{Fb}$ and

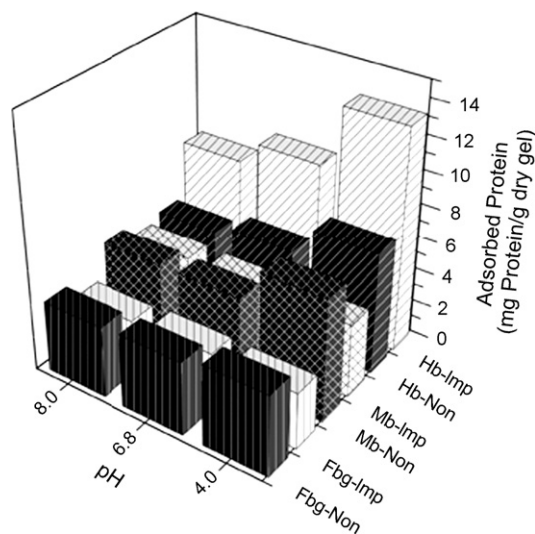


Fig. 4 – Adsorption capacities of Hb, Fb and Mb on nonimprinted and imprinted hydrogels prepared at pH 4.0, 5.8 and 8.0: initial concentration of proteins, 4.0 mg mL⁻¹; pH 4.0; ionic strength 0.02 (adjusted with NaCl); temperature 22 °C; total solution volume, 10 mL. Each adsorption capacity reported in this part is average of at least two separate measurements. Standard derivations are smaller than the symbols themselves for those without error bar.

$\alpha_2 = \text{Hb}/\text{Mb}$) and separation ($\beta = \text{imprinted}/\text{nonimprinted}$) factors were calculated and given in Table 3. The data in both Table 3 and Fig. 4 show that the imprinted hydrogels exhibited high selectivity for the imprinting Hb molecule compared to the Fb and Mb molecules. However, the nonimprinted hydrogels exhibited low α values at same conditions. The molecular masses of Hb, Fb and Mb are 65, 340 and 17 kDa, respectively. The molecular sizes of these reference compounds are also different from the Hb molecule. Since the cavities formed of imprinted hydrogels are matched to the size of Hb, it is very difficult for the molecules with different dimension or molecular mass to enter the cavities; therefore the selectivity factors, α , of imprinted hydrogels prepared at every pH are higher correspondingly. However, the imprinted hydrogels prepared at pH 5.8 showed the lowest α values due to the pI formation of Hb during polymerization or crosslinking reactions. On the other hand, the separation factors, β , showed that the imprinting procedure produced approximately threefold enhancement in Hb adsorption.

Consistent with what one would expect, the adsorption data for the three proteins also reflected the electrostatic interactions between the protein molecules and the hydrogel dictated by the solution pH. For the species involved, the reported pIs were: 6.8–7.0 for Hb; 5.8 for Fb and 6.8–7.2 for Mb. However, pK_{a1} and pK_{a2} of IA are 3.85 and 5.44, respectively. Upon an examination of these isoelectric data and pK_a values, one can predict the charge of hydrogel or protein upon exposure to a buffer solution of a certain pH value. At pH 4.0, the hydrogel would be negatively charged, while all three proteins would be positively charged. This was the reason why electrostatic interactions predominated at the pH 4.0 where the hydrogel and the protein molecules were oppositely charged. In this case, the higher Hb affinity of the imprinted hydrogels over the control nonimprinted hydrogels may thus be attributed to the generation of Hb selective, high affinity adsorption sites in the hydrogel matrix, during the

Table 3 – Selectivity (α_1, α_2) and separation (β) factors of the hydrogels

Type of hydrogel	pH _{prep}	α_1	α_2	β
Nonimprinted	4.0	0.99	1.50	–
Imprinted	4.0	3.05	3.99	1.84
Nonimprinted	6.8	1.04	1.26	–
Imprinted	6.8	2.19	2.83	1.75
Nonimprinted	8.0	0.92	1.15	–
Imprinted	8.0	1.85	2.50	1.69

polymerization and/or crosslinking reactions at different pHs. Besides electrostatic attractions, shape selective fitting of Hb into the complementary cavities created into the imprinted hydrogel matrix during the imprinting procedure may be the cause of the improved selectivity and affinity for Hb for the imprinted hydrogels prepared at the same conditions.

Our experiments have shown that the imprinted hydrogels prepared at different pHs have overcome the main problem of biomolecule recognition in swollen state, i.e. the effect of flexibility of the polymer sequence between two crosslinking points. It is clear that the molecular imprinting procedure has produced recognizable cavities in a swollen state with an affinity for the imprinted Hb molecule. The shape and functional groups of these cavities give rise to the observed variation in adsorption capacities and specificities. A very key article in MIHs is the conformational relaxation of the adsorption pocket that is left exposed when the template is dissociated from the hydrogel. Although it is expected that the imprinted cavities will be distorted due to the swelling of the hydrogel in pH buffer solutions, our experimental results show that even the swollen hydrogels show remarkable recognition for Hb.

4. Conclusions

The experimental results presented in this study demonstrated that the molecular imprinting procedure of the imprinted hydrogels prepared at different pHs produced recognizable cavities in swollen state with an affinity for the Hb imprint. The study presented here showed that the adsorption capacity of Hb in the MIHs prepared at pH 4.0 using Hb as a template was as high as 16 mg g^{-1} dry gel. The adsorption capacities in the nonimprinted hydrogels prepared at the same pHs were significantly lower in all cases. In addition, significant specificity for Hb was also exhibited by the MIHs

prepared at pH 4.0, and this was demonstrated by threefold selectivity factors. The origin of the polymer specificity lies in the formation of the specific adsorption sites (imprints). The spatial orientation of the polymer chains at this pH_{prep} and template functional groups are major factors affecting the template recognition. Easy preparation of the MIHs, their high stability and their ability to recognize small and large proteins as well as to discriminate the molecules with small variations in charge make this approach attractive and broadly applicable in biotechnology, assays, and sensors.

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