A New Approach To Design Imprinted Polymer Gels without Using a Template

Ruth D'Oleo,[†,‡] Carmen Alvarez-Lorenzo,[*,†,§] and Gregory Sun[‡]

Department of Physics and Center for Materials Science and Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139; Department of Physics, University of Massachusetts Boston, 100 Morrisey Blvd., Boston, Massachusetts 02125; and Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15706-Santiago de Compostela, Spain

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ABSTRACT: With the aim of developing polymeric gels sensitive to external stimuli and able to reversibly adsorb and release divalent ions, we prepared copolymer gels of *N*-isopropylacrylamide (NIPA) and *N,N′*-cystaminebis(acrylamide) (BAC) weakly cross-linked with *N,N′*-methylenebis(acrylamide) (BIS). After polymerization, the −S−S− bonds of BAC mers were broken and oxidized to form pairs of sulfonic groups. The juxtaposition of two anionic groups favors the interaction with a divalent cation. Calcium adsorption experiments showed that the gels prepared in this way memorized the position of the pairs of sulfonic groups after swelling and reshrinking. This "imprinting" effect was tested for several concentrations of adsorbing groups and permanent cross-linkers. The control gels prepared with 2-acrylamido-2-methylpropanesulfonic acid (AMPS), where sulfonic groups were randomly distributed, showed difficulty in forming pairs. Their affinity for calcium ions decayed exponentially as a function of cross-linker concentration. In contrast, the affinity of the imprinted gels was much greater than that of random gels and did not decrease with BIS, showing that memorization has been achieved.

Introduction

The development of artificial systems able to mimic the molecular recognition carried out by proteins and enzymes is receiving increased attention, for such systems would have an enormous potential of applications.

One of the most common approaches to the synthesis of host molecules, which can recognize target guest species, is a polymerization technique in which the target molecules are used as template, known as "molecular imprinting".[1] To control the sequence and spatial arrangement of the monomers, the template molecule and the functional monomers (receptors) are allowed to associate before polymerization through reversible covalent bonds[2,3] or through noncovalent or metal coordination interactions.[4−6] In all cases, the imprinted gels incorporate a high proportion of functional monomers (to adsorb efficiently an important amount of target molecules) and an even higher proportion of cross-linker (to keep the spatial arrangement of the functional groups after the target is removed). The network obtained is robust and has good mechanical properties. The utility of these materials to prepare samples and to quantify substances for analytical purposes has been broadly proved.[7−10] However, since the conformation is not variable and the affinity for the target molecules cannot be switched on and off, their ability to respond to external stimuli, a particularly useful property from the point of view of biomedical and environmental applications, is limited. To overcome this limitation, weakly cross-linked imprinted polymer gels have been developed.[11,12] These gels consist of a major monomer component, which allows the gel to swell and shrink reversibly in response to environmental changes, and a minor monomer component able to capture target molecules via a multiple-point electrostatic interaction.[13] These minor functional components (receptors) develop affinity for the target when the adsorbing sites come close to each other, but when they are separated, the affinity diminishes. A theoretical physical approach to explain this behavior based on the memory of a polymer conformation was previously proposed.[14,15]

The success of the imprinting depends on the stability and solubility of the complexes template/functional monomers formed before polymerization. If the molar ratio in the complex is not appropriate[16] or if the complex dissociates to some extent during polymerization, the functional monomers would be far apart from both the template and each other, resulting in a small difference between imprinted and nonimprinted gels. The influence of the solvent used in the polymerization process is usually critical.[17−19] This effect has been observed in imprinted gels prepared with divalent salts of methacrylic monomers in different solvents.[20] Finally, in other cases, it is difficult to remove the template completely after polymerization to obtain the pure gel. The release of template from the polymer network during adsorption studies can subsequently interfere with the analysis.[21]

To circumvent some of these drawbacks, we propose to use functional monomers directly bonded to each other prior to polymerization. These can be separated after polymerization to obtain pairs of ionic groups with the same charge. Since the members of each pair are close together, they can capture target molecules through multiple-point ionic interactions. On the basis of this idea, we present a procedure of developing hydrogels that are able to adsorb calcium ions and simultaneously have switching capacities. The starting point is the poly(*N*-isopropylacrylamide) hydrogel, one of the most widely

[†] Massachusetts Institute of Technology.
[‡] University of Massachusetts Boston.
[§] Universidad de Santiago de Compostela.
* To whom correspondence should be addressed. Fax (34) 981-547148; E-mail anfalde@usc.es.

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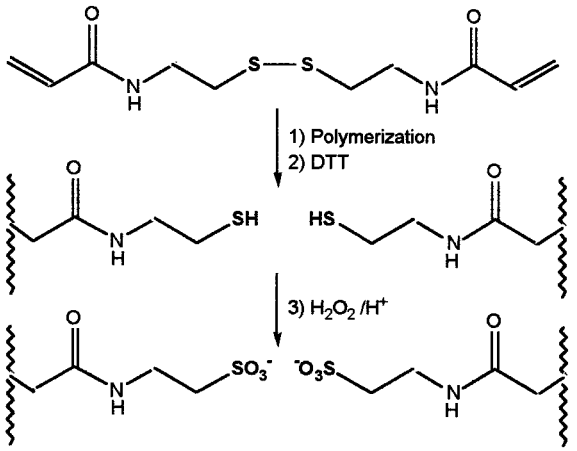

Figure 1. Reaction mechanism used to break the −S−S− bonds in the NIPA/BAC gel and oxidize them to obtain imprinted sulfonic groups (DTT = HS−CH_2−CHOH−CHOH−CH_2−SH).

studied examples of gel system undergoing a temperature-controlled volume phase transition.[22,23] The gel becomes swollen at temperatures lower than 33 °C and collapses when the temperature is raised. To obtain a system with the capacity to adsorb divalent ions, we use monomers with disulfide (−S−S−) groups, which can be broken and oxidized to sulfonic groups after polymerization. We compared this gel with a reference gel made of randomly distributed sulfonic groups.

Experimental Section

Preparation of Imprinted Gels. The gels were prepared by free radical polymerization using *N*-isopropylacrylamide (NIPA, 6 M (Kohjin Co., Japan)) and *N,N′*-cystaminebis-(acrylamide) (BAC (Polysciences Inc., PA)), with cross-linker *N,N′*-methylenebis(acrylamide) (BIS (Polysciences Inc., PA)). To study the influence of the proportion of functional groups, we fixed the cross-linker proportion (40 mM BIS) and used BAC concentration ranging from 4 to 40 mM. For the cross-linker dependence study, the cross-linker BIS ranged from 10 to 200 mM, while BAC concentration was fixed at 16 mM. After the monomers were dissolved in dimethyl sulfoxide (DMSO), 2,2′-azobis(isobutyronitrile) (AIBN, 10 mM, initiator) was added, and the solutions were immediately transferred to test tubes in which micropipets of inner diameter of approximately 0.5 mm were placed. The solutions filled the micropipets and were then degassed under vacuum for a few seconds. The polymerization was carried out at 60 °C for 24 h. After gelation was completed, the micropipets were crushed, and the gels were washed with deionized water for 10 days.

The gels containing BAC were treated to break the −S−S− bonds and oxidize them to obtain sulfonic groups (Figure 1). First, the gels were immersed in a 0.1 M solution of dithioerythritol (DTT) for 24 h to break the disulfide bond into two thiol groups (−SH).[24] Second, the gels were transferred to a 50:50 solution of hydrogen peroxide 30% and acetic acid glacial for 12 h to oxidize the −SH groups to SO_3^-.[25−27] Finally, the gels were washed in a 10 mM NaOH aqueous solution for 2 days, replacing the medium every 12 h. Then, the gels were immersed in deionized water and collapsed at 60 °C to reduce the amount of water bonded. The gels were removed from the solution and dried under vacuum for 1 week.

Preparation of Nonimprinted Gels. The nonimprinted gels were synthesized as described above, using 2-acrylamido-2-methylpropanesulfonic acid (AMPS (Polysciences Inc., PA)) instead of BAC monomers. Since each BAC can give two sulfonic groups, for comparative purposes the amount of AMPS used was double that of BAC. The oxidation step was omitted, and the final washing and drying steps were the same for all gels.

Characterization of the Gels. *IR Spectroscopy Analysis.* IR analysis (FTIR, ATR mode, Magna-air 860 spectrometer, Nicolet) was carried out to verify the cleavage of the −S−S− bonds and the formation of $-SO_3^-$ groups.[28] For this analysis, the gels were prepared in a slab shape, between glass plates separated by 1 mm, with a slightly higher concentration of BAC (70 mM) or AMPS (140 mM) than the cylindrical gels, and they were treated in the same manner as explained above.

Swelling Degree. The equilibrium diameter *d* of the cylindrical gels, after 24 h, in water or 5 mM NaCl was measured using a microscope equipped with a color video camera. The degree of swelling was expressed as

$$\text{swelling ratio:} \quad V/V_0 = (d/d_0)^3 \tag{1}$$

where d_0 was the gel diameter upon polymerization.

Adsorption of Target Molecules. Pieces of cylindrical gel of dry weight 20−40 mg were placed in 10 mL $CaCl_2$ aqueous solutions (8 μM to 0.5 mM). The solutions also contained 5 mM NaCl to provide monovalent sodium ions to replace calcium ions. The samples were kept swollen at 20 °C or shrunken at 60 °C for 48 h while being stirred. Equilibrium calcium concentration in the medium was measured using a calcium electrode (97−20 Ionplus, Orion, MA). The amount of calcium adsorbed by the samples was then evaluated by the difference between the initial and the final concentrations.

The adsorption isotherms were analyzed in terms of the Langmuir equation:

$$A = SKC_{eq}/(1 + KC_{eq}) \quad \text{or} \quad C_{eq}/A = 1/SK + C_{eq}/S \tag{2}$$

where *A* is the amount of calcium adsorbed per unit volume of gel in the collapsed state, C_{eq} is the final equilibrium concentration in the solvent, *S* is the number of adsorbing sites per unit volume of gel or the amount of calcium necessary to saturate the adsorbing sites, and *K* is the affinity of one adsorption site by a calcium ion. From the slope and the intercept at zero C_{eq} we can deduce both *S* and *K* and the overall affinity *SK*.

Results and Discussion

In an initial screening of components to prepare a gel with perfect imprinted pairs of ionic groups, we tried several monomers with cleavable bonds. In particular, methacrylic anhydride and similar compounds, which can provide two carboxylic groups after hydration, failed to incorporate into the polymer network due to their low reactivity rate compared to that of the NIPA monomer. In the case of *N,N′*-cystaminebis(acrylamide) (BAC) monomer, as we will show below, the reactivity was good enough for the polymerization process.

Monomers containing disulfide linkages are commonly used to synthesize hydrogels with reversible cross-links. The reduction and reoxidation of the disulfide bonds allows the development of cleavable and cross-linkable environment-sensitive gels.[24,29−31] In contrast, in our case, once the disulfide bonds are broken, they will be irreversibly oxidized to pairs of sulfonic groups. The gels described in this article were prepared by incorporating also another monomer that can act as permanent cross-linker. It has been previously established that the oxidation of thiol groups with hydrogen peroxide in acidic media transforms these groups into sulfonic acids under mild conditions in a few hours.[25−27] The efficiency of the oxidation process was evaluated by IR analysis and by measurements of the degree of swelling of the gels before and after treatment. Finally, the adsorption behavior of the gel obtained by this procedure was compared with the adsorption ability of the nonimprinted gel made directly with sulfonic monomers.

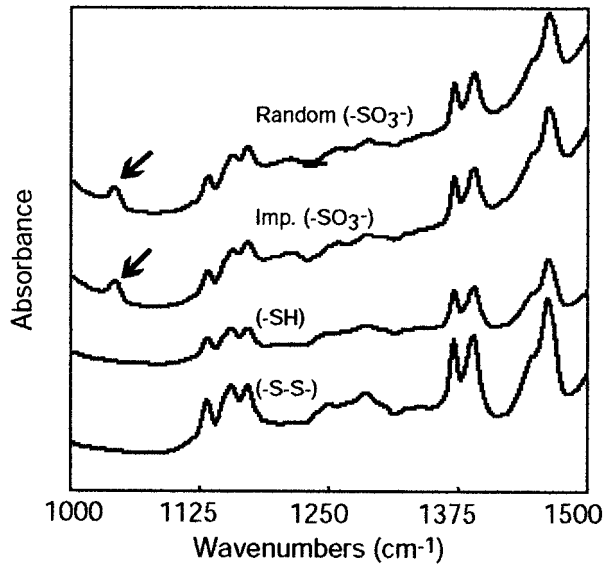

Figure 2. IR spectra of slab gels taken after each step of the procedure outlined in Figure 1. The presence of the sulfonic groups in the nonimprinted and imprinted gels is evident by the characteristic adsorption peak at 1040 cm^{-1}.

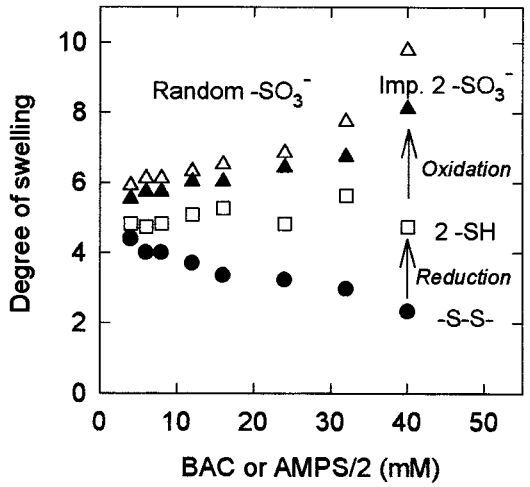

Figure 3. Degree of swelling of the imprinted gels prepared with different BAC concentration, before and after treatment, compared to the degree of swelling of the nonimprinted gel.

IR Analysis. The intensity of the characteristic adsorption peak of sulfonic groups at 1040 cm^{-1} was used to quantify the presence of AMPS units in the nonimprinted gel[28] as well as the transformation of −S−S− bonds in sulfonic groups in the case of the imprinted gel. Figure 2 shows the spectra of slab gels before, during and after the treatment. The appearance of a band at 1040 cm^{-1} after the final step of the preparation of imprinted gels, with an intensity similar to that showed by the nonimprinted gels, corroborates the validity of the oxidation procedure.

Degree of Swelling. The degree of swelling was significantly affected by the concentrations of charged monomers and cross-linking agents in the gels (Figures 3 and 4). At room temperature, water is considered a good solvent for NIPA gels.[22,23] According to the Flory−Huggins theory,[32] the equilibrium swelling degree in a good solvent is controlled by the competition between the gel's rubberlike elasticity and the volume interaction of monomers. This theory predicts an exponential decay of the degree of swelling as the cross-linking density increases.[33] Since the gels prepared with BAC (imprinted) have both −S−S− cross-linkers and permanent cross-linkers (BIS), these gels showed the lowest degree of swelling. This is an indication of the good reactivity rate of BAC monomers to incorporate in the NIPA network, which can be attributed to the fact that both monomers have an identical monosubstituted acrylamide polymerizable moiety.

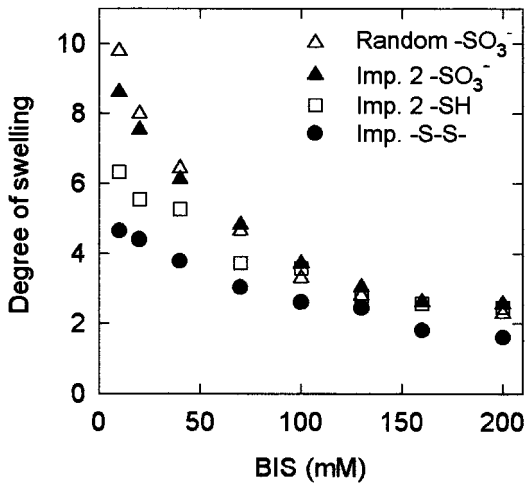

Figure 4. Influence of the degree of cross-linking on the degree of swelling of the nonimprinted and the imprinted gels at room temperature (at 60 °C, in the shrunken state, the degree of swelling was approximately equal to 1 for all gels).

To reduce −S−S− bonds into −SH groups, DTT was used.[24] Figure 3 shows that after this treatment the diameter of the gels increases. All imprinted gels reached the same degree of swelling independent of the initial concentration of BAC. This is attributed to the fact that all gels have the same concentration of permanent cross-linking (40 mM, BIS), and no ionic groups are yet present. When, in the next step, −SH groups are oxidized to sulfonic groups, there is an increase in hydrophilicity and charge repulsion and, consequently, an increase in water uptake. The degree of swelling of the imprinted gels now reaches the values obtained for the gels prepared with AMPS, which indicates a complete transformation of the initial −S−S− bonds into sulfonic groups. The sulfonic group is strongly ionizable and dissociates completely over the whole pH range. In this case, the equilibrium degree of swelling increases exponentially with the concentration of sulfonic groups.[28,33]

Adsorption Studies. The adsorption of calcium ions by both imprinted and nonimprinted gels was well described by the Langmuir's isotherm as formulated in eq 2. The adsorption of calcium ions by the imprinted gels in the shrunken state was proportional to the amount of BAC used in the preparation (Figure 5). This confirms that the BAC monomer was well incorporated and that pairs of sulfonic groups can adsorb calcium ions.

Influence of Sulfonic Groups Content. The analysis of the dependence of the parameters *S*, *K*, and *SK* on the concentration of sulfonic groups in the gels (Figure 6) showed the following:

(a) Both imprinted and nonimprinted gels exhibit a linear relationship between the amount of ions needed to saturate the adsorption sites, *S*, and the concentration of the sulfonic groups in the polymer. In the swollen and in the shrunken state, the number of adsorption

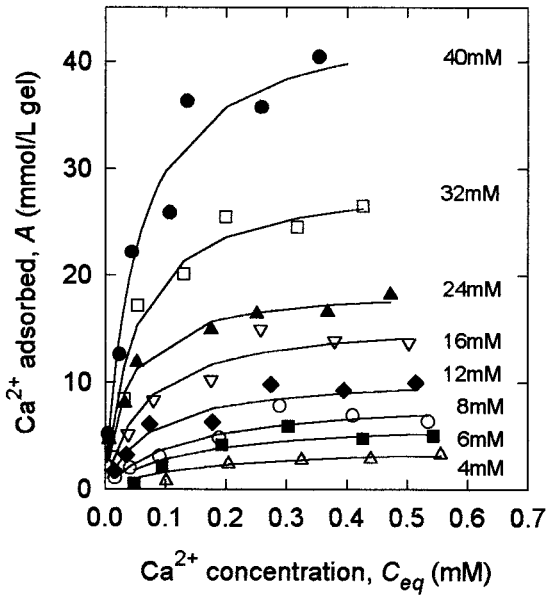

Figure 5. Calcium adsorption isotherms of the imprinted gels prepared with the concentration of BAC indicated in the plot, in the shrunken state.

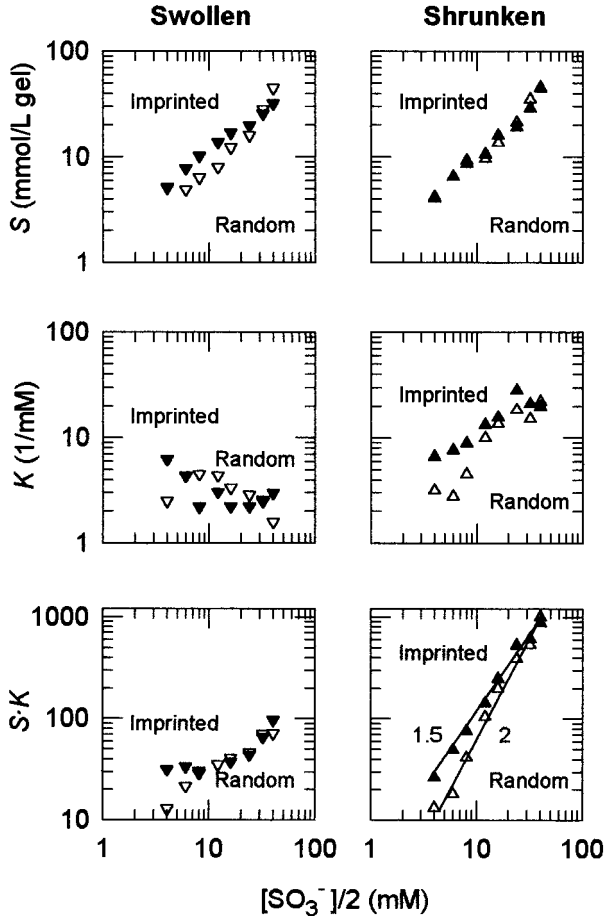

Figure 6. Dependence of the saturation value, *S*, the affinity per adsorption site, *K*, and the overall affinity, *SK*, on the concentration of adsorbing monomer, for the imprinted and nonimprinted gels.

sites is approximately half the number of sulfonic groups that were incorporated during the synthetic procedure: $S = [SO_3^-]/2$. This indicates that all sulfonic groups participate in forming adsorption sites for calcium ions when the concentration of calcium ions is high enough.

(b) In the swollen state (room temperature), the affinity per site, *K*, was almost independent of the concentration of sulfonic groups for all gels. When the gel is swollen, the distance between the nearest sulfonic groups increases and the probability for pair formation decreases. In the shrunken state, the adsorption by the gels increased, especially in the case of the imprinted gels. For both gels, *K* was significantly larger than in the swollen state and proportional to $[SO_3^-]$. Therefore, we have succeeded, using the phase transition of the gel, to switch on and off the adsorption process.

(c) In the shrunken state, the overall affinity of the gels to calcium ions, *SK*, was found to be higher in the imprinted gels than in the nonimprinted gels, except for the highest concentration of sulfonic groups. The imprinting effect is maximum when the concentration of adsorbing monomers (sulfonic groups) is lower than that of the cross-linker (40 mM). This is because, if the concentration of adsorbing monomers is more than that of the cross-links, there will be no obstacle to forming pairs and thus no imprinting effect.

In the shrunken state, *SK* was proportional to $[SO_3^-]^{1.5}$ for the imprinted gels, whereas it was proportional to $[SO_3^-]^2$ in the nonimprinted gels. This latter dependency occurs because the probability that randomly distributed monomers come into proximity with one another is proportional to the square of the adsorbing monomer concentration. In contrast, if the gel is synthesized nonrandomly, the probability of a monomer to find a partner in its vicinity becomes more likely than in the random case.

In the swollen state, simple power relationships between *K* or *SK* and $[SO_3^-]$ are not expected. As $[SO_3^-]$ increases, the pair formation and, in effect, *K* should increase, but at the same time, the volume of the gel rises due to the osmotic pressure of counterions from the ionic groups (e.g., V/V_0 changes from 6 to 10 when AMPS increases from 8 to 80 mM; see Figure 3). This means that the gel is stretched and becomes less flexible, preventing pair formation. When the gels are shrunken again, the strong adsorption is recovered. This shows the destruction and re-formation of calcium adsorption sites made of a pair of sulfonic groups.

Influence of Cross-Linking Degree. Finally, the effect of the degree of cross-linking on the adsorption behavior of the nonimprinted and imprinted gels was investigated in more detail (Figure 7). When the degree of cross-linking was low (Figure 7A, 10 mM BIS), a very small distinction exists between the imprinted and nonimprinted gels in either shrunken or swollen states. The cross-linkers were in lower proportion than the sulfonic groups, and therefore, the cross-links do not hinder effectively the formation of the binding sites. At room temperature, the gels were highly swollen, the binding sites were destroyed, and the affinity for calcium ions almost disappeared. In the shrunken state, imprinted and nonimprinted gels both presented high adsorption capability. The ability to switch the adsorption process on and off by temperature changes was at a maximum. In contrast, for a higher degree of cross-linking (Figure 7B, 200 mM BIS), the behavior of imprinted and nonimprinted gels was dramatically different. In the swollen and shrunken states, imprinted gels presented a 50% higher binding capacity than the nonimprinted gels over the entire range of calcium concentrations essayed.

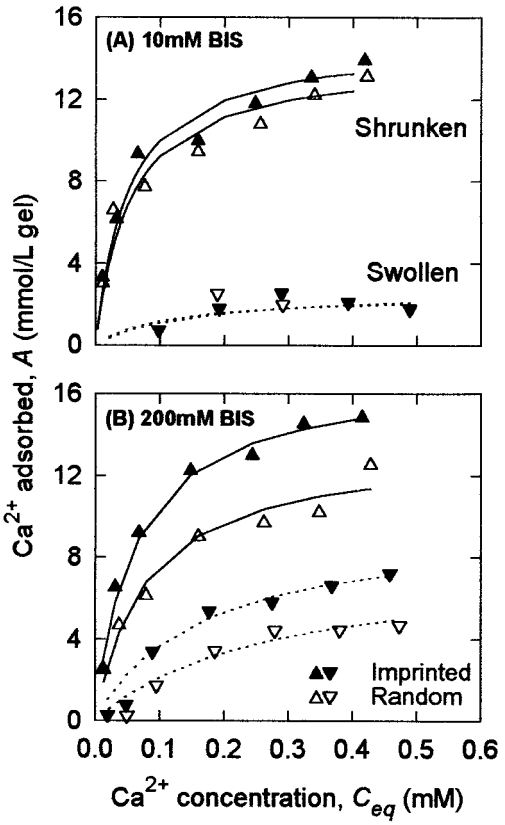

Figure 7. Calcium adsorption isotherms of the imprinted and nonimprinted gels, in the swollen (dotted line) and shrunken (continuous line) states, prepared with two different concentrations of permanent cross-linker (BIS).

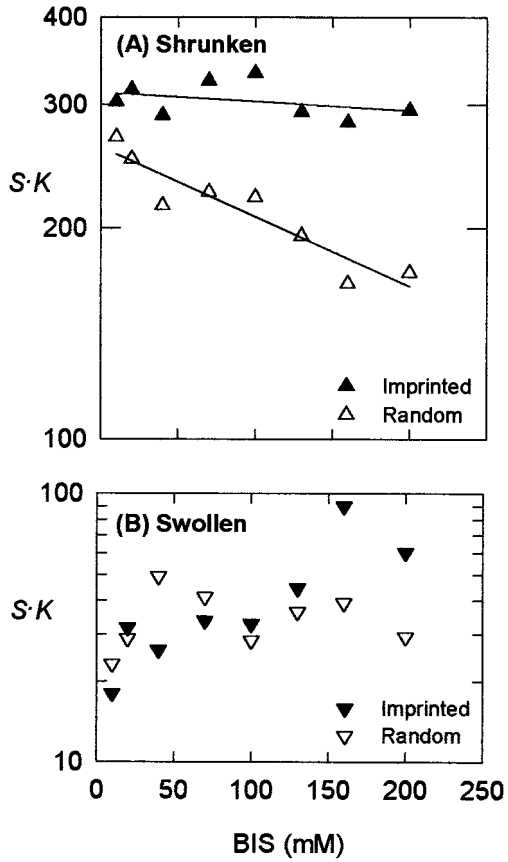

Figure 8. Dependence of the overall affinity, *SK*, of the imprinted and nonimprinted gels on the cross-linking density (BIS) in the shrunken (A) and swollen (B) states. The amount of adsorbing monomers was fixed at $[SO_3^-] = 32$ mM.

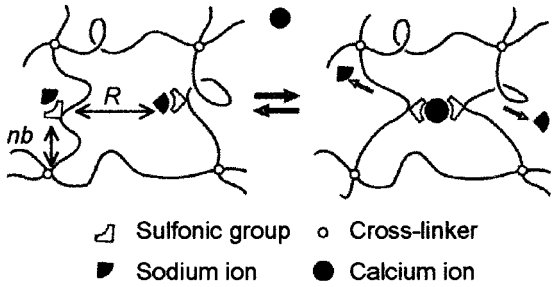

Figure 9. Schematic representation of the adsorption process by the polymeric network.

Figure 8A shows how in the shrunken state the overall affinity, *SK*, of the nonimprinted gels decayed exponentially as a function of cross-linker (BIS) concentration. The affinity of the imprinted gels was much larger than that of nonimprinted gels and did not decrease with BIS. These observations prove that cross-links prevent neighboring sulfonic groups from coming close to each other in the nonimprinted gels, and in consequence, the formation of some energetically favorable pairs of monomers prevents or hinders the formation of other such pairs. In the case of the imprinted gels, this hindrance is minimized by synthesizing the gels while sulfonic groups were paired prior to polymerization.[12] In the swollen state (Figure 8B), there was a slight increase in the affinity of the imprinted gels for the highest BIS concentrations, since a high cross-linking degree reduces the swelling of the gels (see Figure 4).

The exponential decrease of *SK* with the cross-linking degree of the nonimprinted gels in the shrunken state is in agreement with the adsorption behavior observed for other cross-linked polymers.[34,35] Hsein and Rorrer[34] showed an exponential decrease in cadmium adsorption by chitosan as the extent of cross-linking increases. Eichenbaum et al.[35] found that, on alkali earth metal binding by nonimprinted (methacrylic acid-*co*-acrylic acid) microgels, the cross-links prevent the carboxylic groups from achieving the same proximity as in a linear polymer, which affects the binding properties of the metals.

This exponential dependence of *SK* on BIS can be quantitatively explained as follows. The sulfonic groups in the gel can move rather freely within a certain volume determined by the cross-link density. Indeed, it is established that, below a certain length scale associated with the cross-link density, the gel behaves like a liquid, allowing the sulfonic groups to diffuse virtually freely.[36] Beyond that length scale, the gel behaves as an elastic solid body, and in particular, the sulfonic groups cannot diffuse beyond a given distance. To make a simple estimate of this distance, *l*, we may assume that each sulfonic group is at one end of a fictitious Gaussian chain with a length half that of the average polymer length between the nearest BIS cross-links (Figure 9),[12]

$$l = nb \tag{3}$$

Here *n* is the number of monomer segments of persistent length *b*. This fictitious Gaussian chain represents the restricted ability of the sulfonic groups to diffuse within a certain volume in the gel. It has been previ-

ously established that the affinity is proportional to the probability for two adsorbing groups to meet, which is proportional to the Boltzmann factor of the entropy loss associated with the formation of one pair of adsorbing groups[12,37]

$$P = P_0 \exp(-R^2/nb^2) \quad (4)$$

where R represents the average distance in space between two sulfonic groups

$$R = 0.1/([SO_3^-]N_A)^{1/3} \quad (5)$$

in which N_A is the Avogadro number. To come together, each group has to move the half of the distance. If there are $N = ([NIPA]/[BIS])/2$ monomers between the cross-link and the sulfonic group, then

$$n = N/m \quad (6)$$

$$b = ma \quad (7)$$

where m is the number of monomers involved in the persistent length and a is the length of each monomer. Therefore,

$$P = P_0 \exp(-c[BIS]/[SO_3^-]^{2/3}) \quad (8)$$

The parameter c can be roughly estimated considering that for flexible synthetic polymers the persistent length is around 1−2 nm,[36,38,39] e.g., 10 monomers ($m = 10$) of 2 Å length ($a = 2 \times 10^{-10}$ m). The theoretical value of c is then 0.29. The experimental value of the parameter c obtained from the values shown in Figure 8 was 0.23. Therefore, the theory predicts and explains well the exponential decay with [BIS]. Thus, we were able to create topological constraints using cross-links and polymerization so that the sulfonic groups could not lower the energy of the polymer by forming pairs to capture calcium ions.

In contrast, for the imprinted gels there was no dependence of the affinity on BIS concentration (Figure 8A). This is because the gel was synthesized using monomers that are precursors of the pairs of sulfonic groups. The imprinted gels have a much larger adsorption than that of nonimprinted gels because of the minimization of the topological constrains. If the imprinted gel did not memorize the position of the pairs of SO_3^- after swelling and reshrinking, a sulfonic group would have to find a new partner to form a pair, and the probability of forming such a pair would be the same as that in a randomly made gel. There would be no difference, then, between the calcium adsorption by the imprinted and the nonimprinted gels. We can therefore conclude that the excess calcium adsorption by the imprinted gel comes from the successfully memorized pairs. The adsorption by imprinted gels extrapolated to zero concentration of BIS was larger than that of the nonimprinted gels. This relatively small effect may reflect imprinting within linear portions of the polymers without the involvement of cross-links.

Conclusions

Imprinted positions of pairs of sulfonic groups were created in a polymer gel by the use of a monomer precursor, avoiding the use of template ions during polymerization. After gelation, −S−S− bonds were chemically broken in the precursors to yield the imprinted pairs of sulfonic groups. The members of each pair were not directly connected to each other, and their relative distance could be controlled by the volume phase transition of the gel. Pairs of sulfonic groups adsorb calcium ions upon shrinking and release them upon swelling. The affinity for calcium ions was much higher in the imprinted gel than in the gel prepared with randomly distributed sulfonic groups. This suggests that the cross-links and the polymer connections can prevent neighboring sulfonic groups to come close to each other to form a receptor site in the nonimprinted gels and that this obstruction is removed by imprinting. The calcium adsorption results obtained with the imprinted gel prove that, even in a weakly cross-linked polymer network that shows phase transition, a certain arrangement of functional groups can be memorized. Our experiments demonstrate the memory of the relative positions of one type of functional groups within a polymer network. While this pairing is far from the memory of an entire polymer conformation as achieved in proteins, we believe that the present work constitutes an important step toward the emulation of the folding behavior of proteins in a synthetic polymer.

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