Effect of Reversible Cross-linker, *N*,*N*′-Bis(acryloyl)cystamine, on Calcium Ion Adsorption by Imprinted Gels

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Received January 9, 2001. In Final Form: April 3, 2001

Imprinted gels incorporating two different breakable cross-linkers, a $PbMAA_2$ complex and a disulfide (S−S) bond, were prepared by radical polymerization. After the lead ions were removed by washing, these gels showed a high affinity for calcium ions. Breakage and subsequent reconnection of the S−S bonds in the absence of Ca^{2+} decreases the Ca^{2+} binding affinity of the gel. This indicates that random reconnection of the S−S bonds produces a frustration in the adsorption of Ca^{2+} by the carboxyl groups. However, if the S−S bonds were reconnected in the presence of Ca^{2+} and the Ca^{2+} was subsequently removed (the post-imprinting technique), the resulting gels showed a higher binding affinity for Ca^{2+}. This indicates that the post-imprinting technique creates a more favorable conformation for Ca^{2+} binding in the polymer network. We interpret our data to mean that "memory" of target-binding sites was encoded effectively into the polymer network by the initial imprinting technique and then enhanced by the post-imprinting technique.

Introduction

Proteins have the ability to reversibly fold into specific conformations that are thermodynamically stable and to recognize specific target molecules. Because a protein almost always folds into the same low-energy conformation, it is said that it has "memorized" this conformation. Furthermore, proteins can reversibly capture target molecules and release them by obeying certain "molecular signals". It is an exciting challenge to mimic these protein-like abilities in synthetic polymers.

Recently, efforts were made to achieve this goal, or at least to make a step in this direction, by using weakly cross-linked polymer gels that can reversibly swell and shrink in response to environmental changes.[1−4] In some ways, this thrust follows the lead of the pioneering works by Wulff and Mosbach in the 1970s, as well as the works of their numerous followers.[5−8] These authors worked out the so-called method of molecular imprinting that allows one to design dense polymeric substances, such as strongly cross-linked gels, with prefabricated "active sites", i.e., molecular-scale regions whose structure is sufficiently rigid and provides for a high affinity toward desirable "target molecules". The preparation of molecule-imprinted polymers involves the preformation of complexes of functional monomers and target molecules, followed by polymerization with a large number of cross-links. Both the affinity and the selectivity of the active sites fabricated using these methods can be made quite high, although not quite as high as those of proteins.

For most structures created by this imprinting method, the affinity is not variable. The structure of the polymer matrix is molded once and does not change. However, natural proteins offer a fundamentally different ability from the typical polymers used for imprinting. The protein chain is flexible and responds to external stimuli. Therefore, the affinity of its active site can be altered. This motivated us to reexamine the imprinting method using weakly cross-linked polymer gels that can reversibly swell and shrink in response to environmental changes. The other inspiration for our experimental efforts came from the recent advances in the statistical mechanics of heteropolymers.[9−12] These theories provide insight into the physical principle behind the "memory of conformation", which, inter alia, allows proteins to memorize their active sites despite their flexibility and responsiveness to

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§ This paper is dedicated to Professor Toyoichi Tanaka who passed away on May 20, 2000. He had inspired, planned, and guided our research. All other co-authers are responsible for the way in which the results are presented.

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10.1021/la010056m CCC: $20.00
Published on Web 06/08/2001

external stimuli. One of these newly revealed principles is that of frustration, and the other is variably called "minimization of frustration", "design of sequence", or, to use the same word as refs 5−8, "imprinting". It is worth stressing that, despite the strong overlap in terminology, the underlying physics in refs 5−8 and 9−12 is dramatically different. Experimentally, the very flexibility and responsiveness of these weakly crosslinked gels might make it harder to imprint them than the classical systems originated by Wulff.[5−8] One has to be prepared for a sacrifice in terms of both affinity and selectivity, although one expects that these problems will eventually be overcome. In our previous works on weakly cross-linked gels using chemically different polymers, such as *N*-isopropylacrylamide (NIPA) methacrylamidopropyl−trimethylammonium chloride (MAPTAC) copolymers,[1] acrylamidopropyl sulfonic acid (AMPS)−MAPTAC copolymers,[13] and NIPA−methacrylic acid (MAA) copolymers,[2] we were able to demonstrate the validity of such concepts as the occurrence of multiple-point adsorption in shrinkable gels,[1] the existence of frustrations,[13] and the ability to minimize frustrations through proper gel design.[2,3]

In particular, the work of Enoki et al.[3] describes the so-called "post-imprinting technique". In their study, the heteropolymer gels incorporated, in addition to the adsorbing monomers and NIPA monomers, a small amount of permanent cross-linkers [*N*,*N*′-methylenebis(acrylamide), BIS] and monomers with thiol groups (SH). The frustration in this random gel decreased if disulfide (S−S) bonds were formed while all adsorbing monomers were complexed together with target molecules. Frustration was reduced because the SH groups were given the freedom to find the best partners before disulfide bond formation. However, this post-imprinting technique has a fundamental drawback. The sequence of the component monomers had already been predetermined and randomly quenched. For this reason, the imprinting of conformational memory was only partially successful.

Based on these ideas, this paper investigates the possibility that breaking and re-forming reversible cross-links in the presence of quenched ones can distort an imprinted polymer network and create frustrations for the imprinted groups to adsorb the target. The gels used here are composed of different amounts of *N*,*N*′-bis(acryloyl)cystamine (BAC) as a reversible cross-linker. BIS was used as the permanent cross-linker (0.65 mol %), NIPA monomer was used as the major component that allowed swelling and shrinking of the gels in response to temperature changes, and a $PbMAA_2$ complex was chosen as the imprinting monomer to create recognition sites for divalent ions. It is worth repeating that our goal here is not to achieve better results in terms of affinity and/or selectivity compared to what can be done by classical imprinting.[5−8] Instead, we are checking the very possibility that "active sites" can reversibly self-organize themselves upon gel swelling and reshrinking.

After polymerization, removal of lead ions, and adsorption of calcium ions, we can consider that these gels have two different breakable cross-linkers. One is BAC, which has an S−S bond, and the other is the complex between two methacrylic acids and a calcium ion. Breaking both types of imprinted cross-links and then reconnecting one of them might create a frustration to re-form the other one. The repercussions of the mutual frustration system on the adsorption of calcium ions using imprinted gels is demonstrated in this work.

Experimental Section

Materials. *N*-Isopropylacrylamide (NIPA) was provided by Kojin Co., Ltd, Japan. Lead methacrylate ($PbMAA_2$) was purchased from Monomer-Polymer & Dajac Laboratories, Inc. (Feastersville, PA). *N*,*N*′-Methylenebisacrylamide (BIS; J. T. Baker, Phillipsburg, NJ), *N*,*N*′-bis(acryloyl)cystamine (BAC), 2,2′-azobis(isobutyronitrile) (AIBN), dithiothreitol (DDT), and sodium bromate ($NaBrO_3$) (Aldrich Chemical Company, Milwaukee, WI) were used as received.

Polymerization of Imprinted Gel (Initial Gel). The procedure for the synthesis of divalent-ion-imprinted gel was as follows. The gels were prepared in dioxane by free radical polymerization of 6 M NIPA (main component); 40 mM BIS (permanent cross-linker); 40, 80, or 160 mM BAC (reversible cross-linker); and 40 mM $PbMAA_2$ (complex to imprint the polymer for a divalent cation). Lead ions were used as targets instead of calcium ions because $PbMAA_2$ does not dissociate in dioxane.[4] The solution, which had been previously heated to 60 °C and to which AIBN (10 mM, initiator) had been added, was transferred into a test tube into which cylindrical micropipets (∼0.5 mm i.d.) were then placed. The reaction mixture was degassed under reduced pressure and allowed to polymerize at 60 °C for 24 h. After polymerization, all of the gels were washed successively with deionized water and 0.1 M HCl, 0.1 M NaOH, and 1 mM NaCl solutions. Each type of washing lasted for 2 days, during which the respective washing solution was replaced every 12 h, encompassing a complete washing period of 8 days. This procedure served to remove the unreacted molecules and template ions,[14] resulting in the initial gel.

Preparation of Modified Gels. After preparation of the initial gel, the batch of gels formed in the micropipets was divided into four equal groups. One group was set aside to serve as the control *initial gel*. The other three groups were treated to produce samples of the *reduced gel*, *reoxidized gel*, and *post-imprinted gel*. The preparation methods for each of these gel types are as follows.

(a) Reduced Gel. The initial gel was treated with degassed 0.1 M DDT aqueous solution for 24 h at room temperature to reduce the disulfide (S−S bond) into thiol (SH) groups. After the reaction, the gel was washed with degassed 1 mM NaCl solution for 48 h.

(b) Reoxidized Gel. The initial gel was reduced as in the procedure for the reduced gel. This reduced gel was subsequently treated with degassed 0.1 M $NaBrO_3$ solution at 60 °C for 18 h to reoxidize the SH groups into S−S bonds. After washing with 1 mM NaCl solution for 48 h, a gel having re-formed S−S bonds was obtained.

(c) Post-imprinted Gel. As an alternative to procedure b, the initial gel was reduced and then immersed in 500 μM $CaCl_2$ solution at 60 °C for 5 h, to allow Ca^{2+} adsorption by MAA pairs. The gel was subsequently immersed in 0.1 M $NaBrO_3$ solution at 60 °C for 18 h to create S−S bonds. After reoxidation, the calcium ions were removed from the gel by washing consecutively with 0.1 M HCl, 0.1 M NaOH, and 1 mM NaCl solutions.

The methods of preparation for the four gels are schematically depicted in Figure 1. These four gels were immersed and maintained throughout all subsequent experiments in a 1 mM NaCl solution to ensure complete dissociation of the carboxyl groups.[15]

Degree of Swelling of the Gels. The degree of swelling, d/d_0, of each gel in 1 mM NaCl was measured under a microscope (Nikon, TMS-F) at 25 °C (d denotes the gel diameter in equilibrium for the swollen state and d_0 is the gel diameter upon synthesis).

Adsorption of Ca^{2+} by the Gels. Pieces of each type of gel, 10 mg in dry weight, were placed in 10 mL of aqueous solutions of $CaCl_2$ of various concentrations ranging from 8 to 256 μM. These solutions also contained 1 mM NaCl to provide monovalent sodium ions to replace the calcium ions. Samples were allowed

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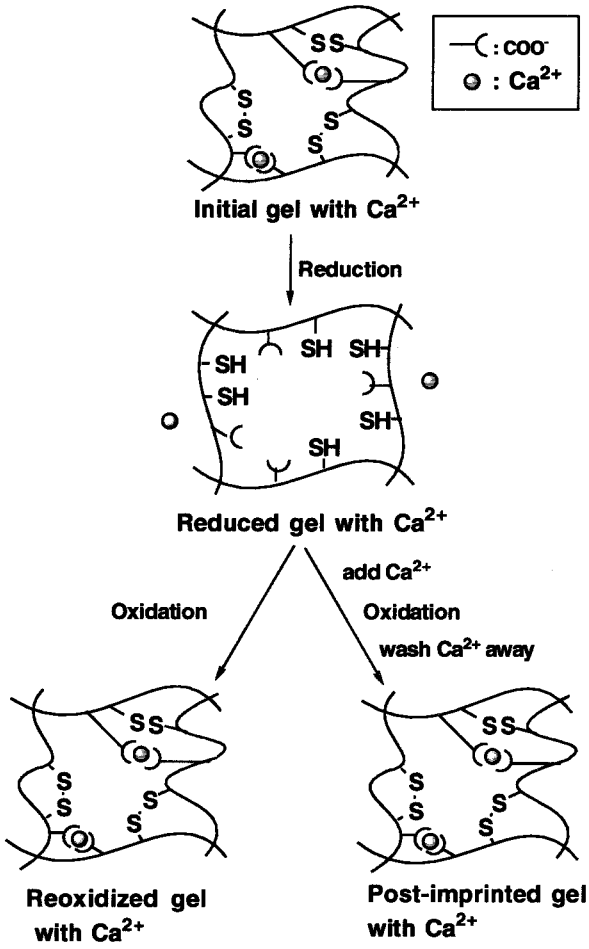

Figure 1. Schematic illustration of the procedure for preparing the modified imprinted gels, showing the expected S−S bond formation. The initial gels, reoxidized gels, and post-imprinted gels are expected to have the same conformation, even after re-formation of the S−S bonds. Calcium ions are present in the drawings, as they are present under the measurement conditions for the gels. The initial gels, reoxidized gels, and post-imprinted gels are expected to show the same calcium absorption affinity.

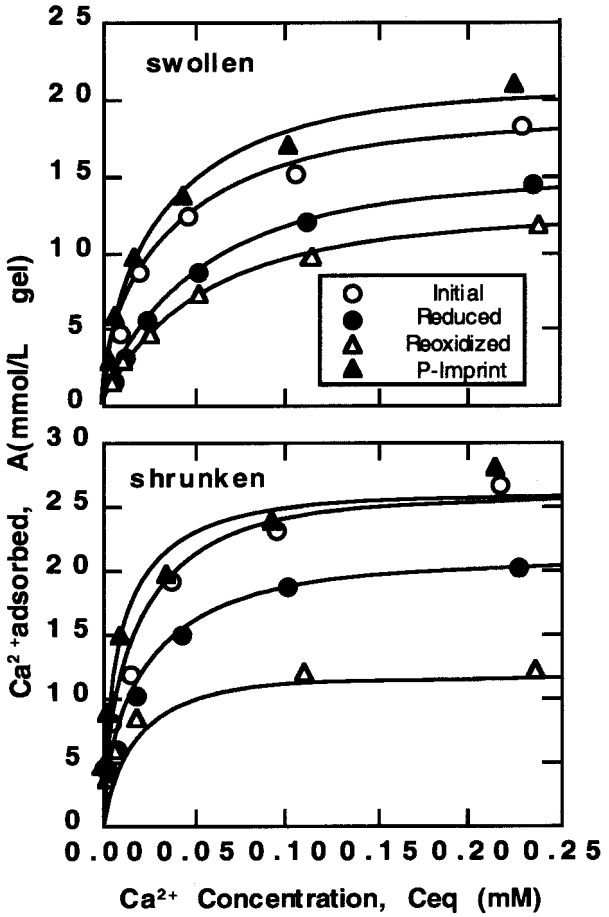

Figure 2. Adsorption of calcium ions by the various types of imprinted gels, each containing 40 mM BAC. The swollen state was measured at 25 C° and the shrunken state at 60 C°. In each case, the adsorption curves closely follow a Langmuir isotherm.

to equilibrate at 25 °C for the swollen state or at 60 °C for the shrunken state for 48 h. The equilibrium calcium concentration in the outer solution was measured using a calcium electrode (Orion Research Inc., model 97-20 ionplus). After the calcium adsorption was measured for a gel sample, the sample was discarded.

The calcium electrode was calibrated prior to the gel experiments by testing the calcium concentration of a set of standard calcium solutions. These solutions were prepared at concentrations of 8, 16, 32, 64, 128, and 256 μM $CaCl_2$. A linear voltage vs concentration curve was found, indicating the effective calibration of the electrode. These solutions were later used for testing the absorption of calcium ions by the gels.

The sodium ions were not expected to interfere with the calcium concentration measurements significantly. Errors related to the presence of sodium should be less than 1%, according to sodium interference test data provided by the equipment manufacturer.

The amount of Ca^{2+} adsorbed by each gel was calculated as the difference between the initial and final concentrations in the surrounding solution. The adsorption isotherms were analyzed in terms of the Langmuir equation[16]

$$A = SKC_{eq}/(1 + KC_{eq}) \quad (1)$$

where A is the amount of Ca^{2+} adsorbed per unit volume of gel, S is the saturated adsorption capacity, K is the affinity of one adsorption site, and C_{eq} is the residual calcium concentration in the solution at equilibrium. To facilitate the fitting of experimental data for parameter evaluation, eq 1 can be rearranged to the more convenient form

$$C_{eq}/A = (1/SK) + (1/S)C_{eq} \quad (2)$$

From the slope and intercept of a plot of C_{eq}/A vs C_{eq}, S, K, and SK (the overall affinity for Ca^{2+}) could thus be calculated.

Results and Discussion

As shown in Figure 2, the adsorption isotherms of Ca^{2+} show a good fit to the Langmuir equation. Table 1 shows the Ca^{2+} binding affinity of the gels. The affinity for Ca^{2+} is significantly higher in the shrunken state than in the swollen state. In the swollen state, the carboxyl groups are far apart, and the recognition sites for Ca^{2+} are broken. However, in the shrunken state, MAA pairs can come into proximity to reconstruct the active site for capturing Ca^{2+} more efficiently. The proximity is controlled by the reversible phase transition of the gel, provided by NIPA. Consequently, these imprinted gels can reversibly change their affinity for the target in response to temperature changes.

In the reduced gel, the SH groups fluctuate around some strongly correlated locations. At low concentrations, they should be located in pairs, because they were obtained by reducing S−S bonds. In this sense, the gel has imprinted sites for the S−S bonds. Thus, during subsequent reoxidation, every SH group might react with its original

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Table 1. Langmuir Parameters for the Gels with 40 mM BAC[a]

	S (mmol/L)		*K* (L/mmol)		*SK*	
	swollen	shrunken	swollen	shrunken	swollen	shrunken
initial gel	21	28	31	69	646	1937
reduced gel	18	22	18	60	322	1312
reoxidized gel	14	13	20	82	273	1060
post-imprinted gel	23	29	35	86	802	2487

[a] The affinity *SK* for calcium ions is significantly higher in the shrunken state than in the swollen state. For reference, the saturated adsorption capacity *S* and single site adsorption affinity *K* are also shown.

partner, thus re-forming all of the original S−S bonds. As long as the concentration of SH groups in the initial gel remains low enough, it should be unlikely that they will reconnect in any pairs other than original ones. Because the carboxyl groups of MAA were also introduced as pairs in the gel, we expect that reconnecting the SH groups would not create any frustration for making the Ca^{2+}/MAA complex in either the reoxidized gel or the post-imprinted gel. Therefore, for this situation in the initial gel, the initial, reoxidized, and post-imprinted gels were all expected to have the same polymer conformation and to exhibit the same binding affinity for Ca^{2+}.

However, it is difficult to access experimentally this regime of very low SH group concentrations. In our experiments, the re-formation of S−S bonds different from the original pairs was not supressed, and the behavior was also different. The affinity of the reoxidized gel to Ca^{2+} was lower than that of the initial gel. In contrast, the post-imprinted gel showed a slightly higher affinity than the initial gel did.

Reoxidized Gel. The efficiency of the oxidation of SH groups into S−S bonds was studied previously by C. Wang, using weakly cross-linked gels of the same composition as in the present work.[17] Wang measured the concentrations of SH groups in the gels quantitatively before and after reoxidation using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid). In that experiment, it was shown that the fraction of SH groups reoxidizing into S−S bonds was at least 95% for all BAC concentrations of at least 4 mM, including 4, 8, 16, 32, and 64 mM. This confirms that virtually all pairs of SH groups in the gels reoxidized into S−S bonds.

In other related experiments, e.g., the work of Wulff et al.,[18] the reoxidation of SH groups was found to be less efficient. However, Wulff also showed that, as the cross-linker concentration decreases, the reoxidation efficiency improves. The high reoxidation efficiency of our system can be explained by the weak cross-linking of our gel. Qualitatively speaking, the polymer network in our weakly cross-linked gels should be flexible enough to allow the SH groups to come together for reoxidation. In the present work and in the work of Wang, gels with 0.65 and 0.60 mol % cross-linking, respectively, were used. In contrast, in the Wulff experiments with low reoxidation efficiencies (<40%), the mole percent of cross-linker used was approximately 100 times that of our experiment.

According to the hypothesis described in Figure 1, the d/d_0 values for the initial gel, the reoxidized gel, and the post-imprinted gel should have been similar. Table 2 shows the degree of swelling, d/d_0, of the gels in swollen state. However, d/d_0 of the reoxidized gel was lower than that of the initial gel. The difference increased at the lower BAC concentration (40 mM).

Table 2. Degree of Swelling, d/d_0, of the Imprinted Gels in 1 mM NaCl in the Swollen State[a]

[BAC] (mM)	initial gel	reduced gel	reoxidized gel	post-imprinted gel
40	2.00	2.17	1.62	1.95
80	1.67	2.07	1.53	1.85
160	1.61	2.12	1.56	1.86

[a] The degrees of swelling, d/d_0, for the reoxidized gels are lower than those for the initial gels. The difference increases at lower BAC concentrations.

The BAC dependence can be understood as follows. After the cleavage of the initial S−S bonds and the swelling of the gel, the SH groups should be far apart from each other. Upon reoxidation, the probability of finding the original partner is lower for smaller amounts of BAC incorporated into the gel. Indeed, the volume explored by an SH group in its thermal motion increases when the number of acting cross-links, including BAC, decreases. This, of course, increases the chances for every SH group to reoxidize and form an S−S bond with a randomly found partner different from the original one. The SH groups might not react with their original partners, instead reacting with other SH groups that are physically closer. This occurs even though the pairs of SH groups were initially imprinted into the polymer network. Because of the high reactivity of the SH groups, the remaining SH group would then seek out another free SH group with which to react, even one that might not be close by. This phenomenon would cause a greater distortion of the polymer network, and therefore, the degree of swelling of the gel could be lower than that of the initial gel.

The Ca^{2+} binding affinity, as well as the *S* value, of the reoxidized gel is smaller than that of the initial gel in the case of 40 mM BAC. When the BAC concentration was increased, the difference in d/d_0 between the reoxidized gel and initial gel was smaller. This is because, for higher BAC concentrations, there is a stronger constraint on the positions of the SH groups. The SH groups are thus more likely to react with their initial SH partners. In this case, re-formation of the S−S bonds might not be able to distort the gel in an appreciable way, which also explains why they did not cause much frustration in the Ca^{2+} adsorption by the gel.

As a further test of this influence of the BAC concentration on Ca^{2+} adsorption, similar gels with lower concentrations of BAC (0, 10, and 20 mM) were prepared by the same method as mentioned above. The S−S bonds in the gels were reduced and subsequently reoxidized, as with all of the reoxidized gels. Figure 3 shows the dependence of the *S* values obtained in the Ca^{2+} adsorption experiments on the amount of BAC incorporated into the gel. The *S* value of the reoxidized gels decreased with decreasing BAC concentration. In the case of the gel having no BAC, there is no frustration in the Ca^{2+} adsorption, and the *S* value is higher than those of the other reoxidized gels. This fact strongly supports our hypothesis that breaking and re-forming S−S bonds can modify the

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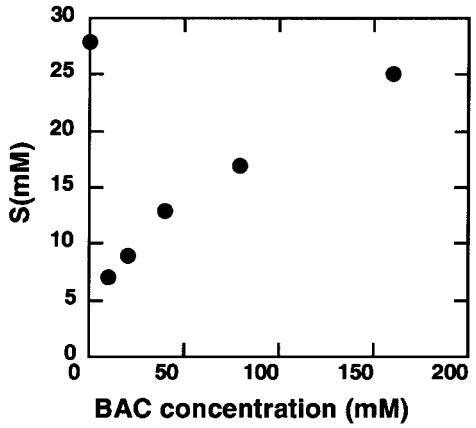

Figure 3. Saturated adsorption capacity, *S*, as a function of the BAC concentration in the reoxidized gels in the shrunken state. *S* increases with the BAC concentration. In the case of the gel having no BAC, there is no frustration in the Ca^{2+} adsorption, and *S* is higher than for the other cases.

binding sites for Ca^{2+} in an initially imprinted gel. These results suggest that the "memory" of the S−S bonds is destroyed as soon as they are broken into SH groups, even though the conformational memory of the Ca^{2+}−carboxyl group complex remains stored in the polymer network.[2,19] This can be explained as follows. The interaction between a MAA pair and one divalent ion is an ionic exchange in which Ca^{2+} is bound and released repeatedly by the two carboxyl groups. In this process, an equilibrium state is eventually attained. The reoxidation of the SH groups into the S−S bonds, on the other hand, is a covalent reaction. Once two SH groups meet, they react promptly and irreversibly through the formation of a stable covalent S−S bond. The structure of the re-formed S−S bonds is embedded in the polymer network. If one SH group reacts with a partner other than the original one, the resulting gel should have a conformation different from that of the initial gel, and therefore, it should show a different degree of swelling. Our results confirmed that reconnecting the S−S bonds produced frustration for Ca^{2+}, because of the disturbance of the conformation of the initial gel. This conclusion can be drawn from the fact that the affinity of the reoxidized gel to Ca^{2+} was lower than that of the initial gel (Figure 4).

Post-imprinted Gel. As shown in Table 1, the Ca^{2+} binding affinity of the post-imprinted gel is close to, but slightly higher than, that of the initial gel. In the post-imprinting procedure, first, the reduced gels adsorbed Ca^{2+}, and after equilibration, the S−S bonds were re-formed. The Ca^{2+}−MAA_2 complexes can act as cross-linked points in the gel. It can be expected that each pair of SH groups would be closer to each other than the pairs in the reoxidized gel. If the MAA pairs were the same as in the initially imprinted gel, then the SH groups would be forced to react with their original partner or with a closer SH group after Ca^{2+} adsorption by the carboxyl groups. In the post-imprinted gel, as Ca^{2+} ions are bound by the carboxyl groups prior to reconnection of the SH groups, re-formation of the S−S bonds cannot give any frustration for formation of the complex of Ca^{2+}−carboxyl groups. The higher affinity of the post-imprinted gels also suggests that a small frustration might exist in the initial gels. Subsequent S−S cross-linking in the presence of Ca^{2+} could create a more stable conformation for the binding sites in the polymer network, thereby increasing the affinity for Ca^{2+} (Figure 4).

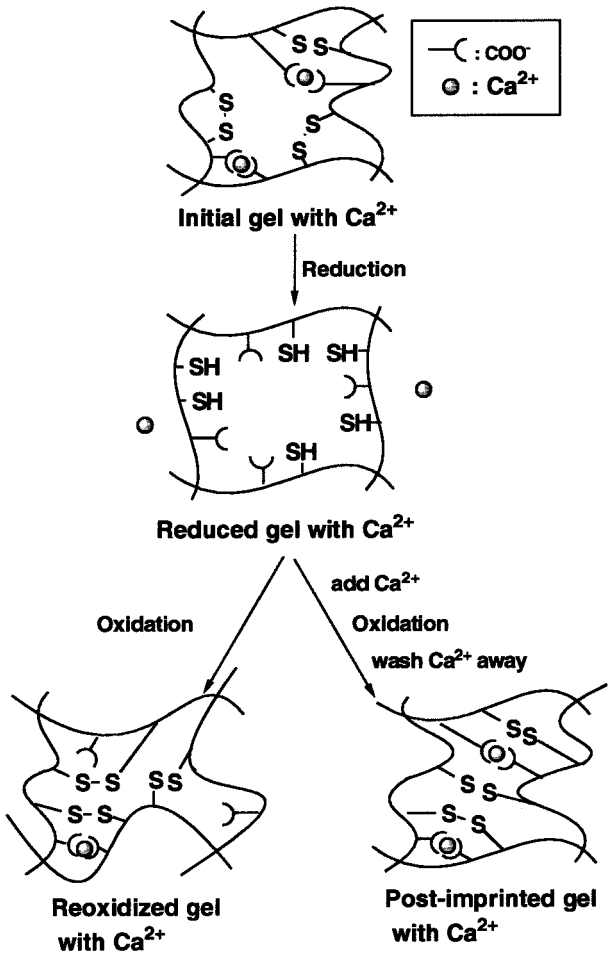

Figure 4. Schematic illustration of S−S bond re-formation based on Ca^{2+} adsorption data. Calcium ions are present in the drawings, as they are present under the measurement conditions for the gels. The initial gel and the reduced gel are identical to those in Figure 1. S−S bond re-formation in the reoxidized gel produces frustration for Ca^{2+} adsorption. In contrast, S−S bond re-formation diminishes the frustration that existed in the initial gel and creates a more favorable conformation for Ca^{2+} binding when reoxidation occurs in the manner of the post-imprinted gel.

Conclusion

The imprinted gels incorporated two different breakable cross-linkers, the first being a complex of binding sites and target and the second being an S−S bond. The gels were prepared by the imprinting method. Breaking and subsequently reconnecting the S−S bonds distorts the polymer network and produces a frustration against formation of the complex of the Ca^{2+} and the carboxyl groups. The result is that the Ca^{2+} binding affinity of a reoxidized gel is lower than that of an initial imprinted gel.

Making cross-links by forming complexes of Ca^{2+} and two carboxyl groups before reconnecting the S−S bonds, i.e., post-imprinting, increases the Ca^{2+} binding affinity of the gel. The frustration that existed in the initially imprinted gel is diminished. The subsequent S−S cross-linking modifies the polymer network, resulting in a more favorable conformation for Ca^{2+} binding in the polymer network.

Returning to the main theme of the Introduction, it is worth repeating that this work, as a continuation of the series of works reported in refs 1−4, is based on the combination of ideas of classical imprinting[5−8] and minimal frustration and the sequence design concepts of het-

eropolymer physics.[9−12] In this context, the major contribution of this particular paper is the improvement of the post-imprinting approach first suggested by Enoki et al.[3]

We expect that the combination of the imprinting technique and the post-imprinting technique will become a method for creating an ideal imprinted gel.

Acknowledgment. This work was supported by Menicon Co., Ltd. (Japan). C. A.-L. acknowledges the support of Fundacion Ramon Areces (Spain). We thank Kojin Co., Ltd. (Japan), for the generous gift of *N*-isopropylacrylamide.

LA010056M