Correlation and Cross-Linking Effects in Imprinting Sites for Divalent Adsorption in Gels[†]

Kimani A. Stancil, Michael S. Feld,[‡] and Mehran Kardar*

Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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We examine a method to mimic active sites in proteins by chemical imprinting of *p*-valent templates in heteropolymer gels. Previous studies have confirmed successful formation of sites by adsorption of targets with $p \geq 2$ contacts. We investigate the recovery of sites with $p = 2$ imprinted by lead methacrylate Pb(MAAc)$_2$ (placing two carboxyl groups in close proximity). The improved binding ability of gels with more cross-links, and the relative insensitivity to changes in gel volume contradict simple theory. We conclude that adsorber pairs are predominantly located on the same polymer chain, posing a challenge to mimicking protein-like function.

Proteins are long chain heteropolymers that adapt specific shapes in their native state. This native form enables the protein to perform its task, such as the recognition and binding of a target molecule by active sites. The native form is lost and function is disabled when a protein is denatured due to changes in temperature or chemical composition. Similarly, we make long-chain heteropolymer gels that through large volume expansions/contractions change shape in response to temperature variations. Macromolecular conformations in gels are established by random spatial arrangements, whereas the protein's native form is defined by the precise sequence of amino acids along its backbone. By exploiting similarities between heteropolymer gels and proteins, can we make gels that mimic some functions of proteins?

To this end, we follow the vision of Toyoichi Tanaka for engineering a unique configuration in heteropolymer gels analogous to a protein's native shape.[1,2] Using molecular imprinting,[3] we design active sites in gels that collectively establish a gel's native configuration at synthesis. To confirm imprinting, we use adsorption experiments to estimate the number of active sites from the number of molecules captured by the gel in aqueous salt solutions. We view the polymer gel network as an adsorbing substrate, similar to a lattice gas (where lattice sites adsorb molecules).[4,5] Our assumption is that an active adsorption site can only be formed through multiple contacts, $p \geq 2$. In this work, we highlight our study of divalent molecular capture and show that adsorber correlations are well established by imprinting but are not necessarily an advantage for creating protein mimics.

We synthesize gel configurations that are weakly cross-linked, consisting mainly of a hydrophobic polymer NIPAm (*N*-isopropylacrylamide) backbone with 0.2%−1.7% molar amount of ionizable carboxyl (COO−) groups. After synthesizing the gel in its collapsed (dense) state, we use temperature changes to swell and re-collapse the gel to see if it recovers its original conformation. By imprinting, we attempt to incorporate the template Pb(MAAc)$_2$ (lead methacrylate) that consists of two adsorbers (COO−, carboxyl groups), and a guest Pb^{2+}, a divalent metal ion; subsequently, the latter is removed from the gel. If successful after removing the guest, the remaining adsorbers are correlated in location and/or spatial proximity in the network. Nonimprinted gels are similarly prepared with the same number of adsorbers but without use of a template, so that adsorbers are not correlated (unless by chance). For gels made with our recipe, Alvarez-Lorenzo et. al.[6,7] used divalent calcium (Ca^{2+}) as a target to demonstrate that imprinted gels adsorb more strongly (improved binding affinity) and in greater numbers (higher target saturation) than nonimprinted gels. To check if the native conformation is reestablished in the collapsed state, we use the original guest molecule (Pb^{2+}) as a target. While demonstrating the structural differences between *imprinted* and *nonimprinted* gels, we also observe (in the swollen state) unexpected cross-linker dependences originating from adsorber correlations in imprinted gels.

It should be noted that we confirmed the role of carboxyl groups as adsorbers by measuring the adsorption performance of gels that we prepared using our same recipe but without carboxyl groups. Gels without carboxyl groups were measured to have no ability to adsorb lead (Pb^{2+}) and calcium (Ca^{2+}) target for our concentration range.

The idealized scenario for imprinting is depicted in Figure 1, which contrasts imprinted and nonimprinted gels in their swollen and collapsed states. When swollen, both gel types are not expected to fully adsorb target molecules, because adsorbers are too distant to effect capture (assuming that adsorbers are a small fraction of all monomers in the polymer backbone). Upon collapse, by recovering the native conformation, pre-correlated adsorbers form adsorption sites in imprinted gels. In nonimprinted gels, adsorption sites are randomly determined during collapse; hence fewer sites are expected. When adsorber density is increased such that any two single adsorbers are well correlated, we expect little difference in adsorption between gel types. Multicontact adsorption has been observed in *collapsed nonimprinted* gels.[8] Further work has shown that by increasing cross-linking density, adsorption affinity diminishes, indicating frustration effects in nonimprinted gels.[6,7,9]

In our experiments, monovalent salt cations of concentration [Re] replace targets and form complexes with adsorbers. In a generalized picture of *p*-valent adsorption (*p* = 2 for capture of divalent metal ions), $S = [A]/p$ sites are formed from clusters of *p* adsorbers [A]. By considering the per-site affinity K_{eq}, or the equilibrium constant for target capture in nonimprinted gels

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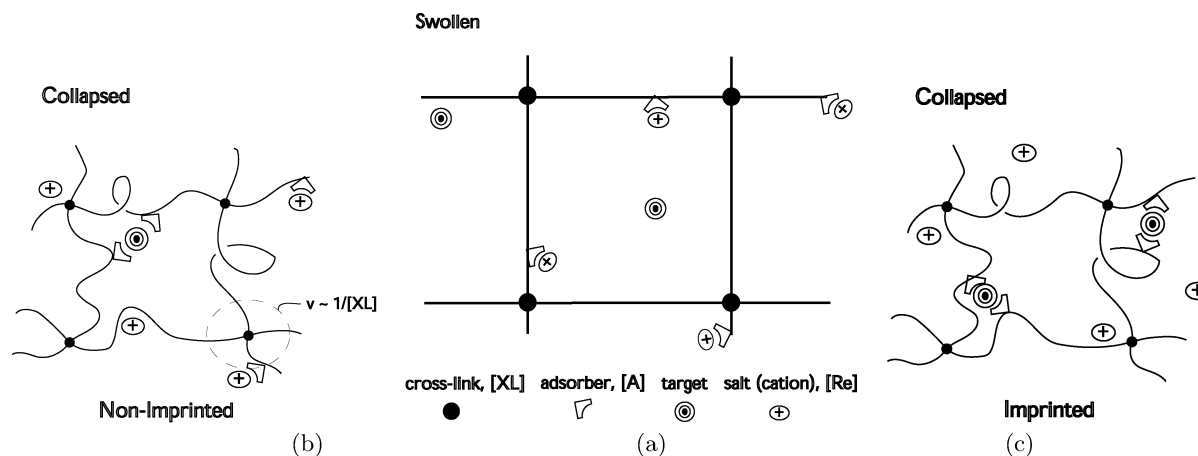*J. Phys. Chem. B, Vol. 109, No. 14, 2005* **6637**

Figure 1. Ideal scenario for divalent site formation in nonimprinted and imprinted gels. (a) Both gel types are equivalent when swollen, with no sites formed by the distant adsorbers. (b) Collapsing nonimprinted gels may create sites due to closer overall proximity. Network frustration restricts an adsorber's freedom due to cross-linking to a volume v, inversely related to the cross-linker density. (c) Collapsing imprinted gels is expected to enable pre-correlated adsorbers to form sites.

(Figure 1b), the gel affinity is estimated by the sum of all local contributions as

$$SK_{eq} = \frac{[A]^p}{p[Re]^p} \exp\left[-\left((p-1)C_o \frac{[XL]}{[A]^{2/3}}\right)\right] e^{-\beta(p\Delta\epsilon)} \quad (1)$$

Features of the first two factors of eq 1, which we refer to as the Tanaka equation, have been confirmed by experiments.[6,7,9,10] The first factor is simply the relative probability of bringing p-adsorbers together, replacing the cations, given their relative concentrations. The second factor highlights network frustration, i.e., the entropy loss of the polymer (assumed to be a Gaussian chain) due to forced contacts. The latter is related to the cross-linker density, [XL], which limits the adsorber's range of motion to a volume v, which scales as 1/[XL], (Figure 1b). C_o is an intrinsic property of the gel related to the polymer's persistence length, and the concentration of the majority monomer (NIPAm in this case). The third factor estimates the relative energy cost $\Delta\epsilon$, of target molecules to bind with either gel adsorbers [A] or with replacement molecules (salt anions) [Re].

Cylindrical gels were prepared with concentrations of 6 M NIPAm, 20−200 mM BIS (bisacrylamide), and 12 {6}−100 {50} mM MAAc (methacrylic acid) {Pb(MAAc)$_2$}, for nonimprinted {imprinted} gels. Our adsorber concentration range overlaps with the range (8−64 mM) used by Alvarez-Lorenzo et. al.[7] (note that frustration was observed in nonimprinted gels with 32 mM MAAc). Radical polymerization was used with the aid of initiator AIBN (azoisobutylnitrile) at 60 °C in micropipet molds (diameter $d \simeq 300$ μm). The swollen state experiments are conducted at $T = 20$ °C, and the collapsed state experiments at $T = 60$ °C for our gels ($T_{transition} \simeq 34$ °C).[11] After guest removal by flushing with 0.1 M HCl, and later with 0.1 M NaOH to neutralize ionized carboxyl groups, gels are dried using vacuum. Both gel types are washed with the same procedure to maintain equivalent access to a similar conformational landscape.

Examination of the sizes of the gels (after drying) revealed that both imprinted and nonimprinted gels have similar swelling characteristics[12] (measured by the ratio $\alpha \equiv d/d_o$ of the gel to micropipet diameters) as a function of the cross-linker density ([XL] $\equiv$ [BIS]). In fact, we fitted the results for the expansion factor using a modified Flory−Huggin's theory[13] for gels with ionizable groups, in which the free energy is estimated as

$$F_{gel/chain} = F_{elastic} + F_{counterions} \approx \alpha^x + Nf \ln\left(\frac{n_o f}{\alpha^3}\right) \quad (2)$$

In the first term, a phenomenological exponent x is introduced to account for non-Gaussian elasticity (it is believed that entanglements result in anharmonic effects with $x > 2$). The second term is the entropy of counterions, with f as the mole fraction of ionizable groups, and $n_o \sim N/R_o^3$ where $N \sim$ [NIPAm]/[BIS] is the average chain length between cross-links, and $R_o \sim N^{1/2}$ is the average end-to-end chain distance. Minimizing the above free energy results in the swelling factor $\alpha \approx (Nf)^{1/x} \sim (f/[BIS])^{1/x}$. From such fits, we estimate $x_{imprinted} = 6.44 \pm 1.20$ and $x_{nonimprinted} = 6.67 \pm 1.08$. The similarity of the exponent for both gel types is consistent with our assertion that they possess essentially the same swelling behavior. [By way of comparison, we note that prior experiments for poly-(N,N-dimethylacrylamide) based gels resulted in an estimate of $x = 5$, for the exponent of anharmonic elasticity.[13]]

Adsorption is estimated by measuring the concentration of free target in the gel's outer solution at equilibrium, C_{eq} (μM). An aqueous solution sample of lead is extracted. Using a Hitachi U-2000 spectrophotometer, the sample's absorbance is measured with the help of chemical indicator, pyridylazo-resorcinol, which forms a stable complex with lead[14] at pH $\simeq 10$. We calculate the target moles adsorbed relative to the gel's estimated volume (obtained from the dry gel weight and recipe) after synthesis X (mM), on the basis of changes in the number of targets in the gel's outer solution. Gels adsorb target from solution in polypropylene vials. Each prepared solution contains 0.1 M NaClO$_4$ (salt), and a range of initial target concentrations spanning 4−192 μM.

The Donnan effect in gels results from the exclusion of salt from the gel region due to the presence of ionizable groups. With added salt, this effect diminishes.[15] We keep in mind that the bulk concentration of ionizable groups is at most equal to its preparation value when the gel is collapsed (because we synthesize the gel in its collapsed state). For our recipes and salt concentrations, we assume that the concentration of unbound target inside and outside the gel is approximately the same. However, we realize that as the fraction of ionizable groups is increased, the Donnan effect should play a more significant role.

Adsorption experiments begin by immersing dry gels in solution, which then reach swelling equilibrium at 20 °C. The equilibrium target concentration, C_{eq}, is measured. The gel is
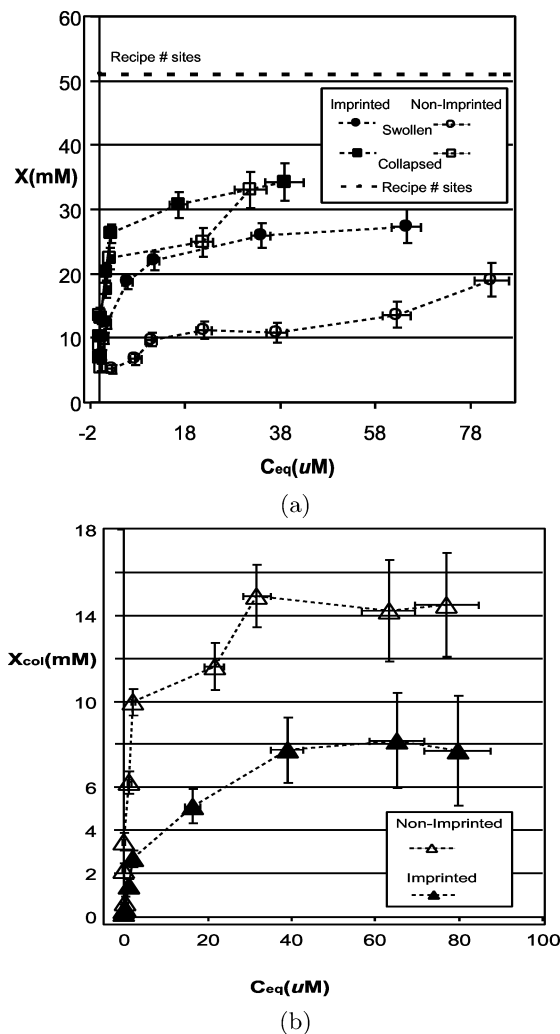
(a)

(b)

Figure 2. Adsorption performance of imprinted and nonimprinted gels. (a) Imprinted gels show greater adsorption in both collapsed and swollen states. (b) Nonimprinted gels form more additional sites when collapsed.

then collapsed using dry heat (glass bead baths), reaching equilibrium at 60 °C, where C_{eq} is measured again. The resulting data (see sample in Figure 2a) of X (mM) vs C_{eq} (μM) are analyzed using the Langmuir isotherm[16]

$$X = S\frac{K_{eq}C_{eq}}{1 + K_{eq}C_{eq}} \qquad \frac{C_{eq}}{X} = \frac{1}{S}C_{eq} + \frac{1}{SK_{eq}} \qquad (3)$$

The coverage $\theta = X/S$, the ratio of adsorbed target concentration X (mM) to the number of estimated sites S (mM), reflects the fraction of occupied sites.

Equation 3 is used to estimate the saturation S, and affinity SK_{eq}. Isotherms in Figure 2 for gels with recipe 100 mM [A], and 100 mM [BIS], display the predominant trends in our results. Imprinted gels adsorb significantly better when swollen (with higher affinity and saturation values than nonimprinted gels) (Figure 2a). After collapse, the net adsorption due to swollen and collapsed states remains higher in imprinted gels (Figure 2a). Note that within the estimated error, net saturation values are similar for both gel types. Collapsing the gel adds more sites or improves adsorption more in nonimprinted gels (Figure 2b). For our range of target concentrations, we do not observe full recovery (i.e., saturation values S fall short of the recipe number of sites indicated by the dashed horizontal line in Figure 2a).
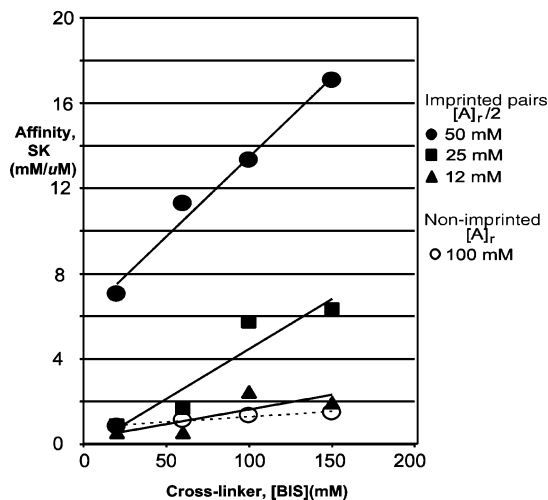

Figure 3. Improvement of swollen state affinity with cross-linker and adsorber recipe (highlighted by the increasing slopes). This effect is more pronounced in imprinted gels.

Unexpectedly, in the swollen state, affinity improves with increasing cross-linker ([XL] = [BIS], the intended after synthesis cross-linker density) and adsorber density (with the recipe concentration [A]$_r$) in gels prepared by imprinting. In Figure 3, results for imprinted gels with 12–50 mM adsorber pairs, and for a nonimprinted gel with the most single adsorbers (100 mM) can be summarized by the *empirical* relation

$$\ln SK_{swollen} \propto \ln[XL] + \ln([A]_r * G_i) \qquad (4)$$

which differs dramatically from eq 1 for nonimprinted gels in which $\ln SK_{swollen} \propto \ln[A]_r - ([XL]/[A]_r^{2/3})$. In contrast to the above, the first term in eq 4 highlights the affinity's positive cross-linking density ($+$[XL]) dependence, whereas the second term captures the difference between gel types. Using linear regression plots (best-fit) of all the recipe results, we obtain a *gel structure factor* G_i, from a secondary plot of fitted slopes against (half) the prepared adsorber density [A]$_r$. The resulting G_i is 17 times larger in imprinted gels ($G_i = 0.0017$ for imprinted gels and $G_i = 0.0001$ for nonimprinted gels).

We believe that the explanation of the above results lies in the microstructure of the gels, i.e., in relative locations of imprinted pairs and cross-linkers in the gel network. Figure 4 depicts four possible placements of pairs on the gel backbone. The improvement in adsorption of nonimprinted gels (cf. the idealized scenario of Figure 1b), can be attributed to site formation from formerly distant adsorbers (pairs (3) and (4) in Figure 4a). By contrast, the large adsorption of imprinted gels in their swollen state suggests that the corresponding adsorbers remain in close proximity, even as the polymer strands are stretched, pointing to pairs such as (1) and (2) in Figure 4a. Because of excluded volume constraints, adsorber pairs near a cross-link are likely to experience difficulty in molecular capture, and we thus conclude that imprinted adsorption sites are predominantly located on the same polymer chain, such as in pair (2) of Figure 4a.

The proximity of imprinted adsorber pairs (i.e., pair on a single chain (2) of Figure 4a) can also explain the positive role of cross-linkers. In the original Tanaka scenario for nonimprinted gels (Figure 1b), cross-linking reduces the flexibility of adsorbers and thus diminishes (or frustrates) random formation of divalent sites in the collapsed state. With imprinted pairs already close-by prior to collapse, at low cross-linking densities, adsorber pairs may compete for the same target as depicted in Figure 4c. Here
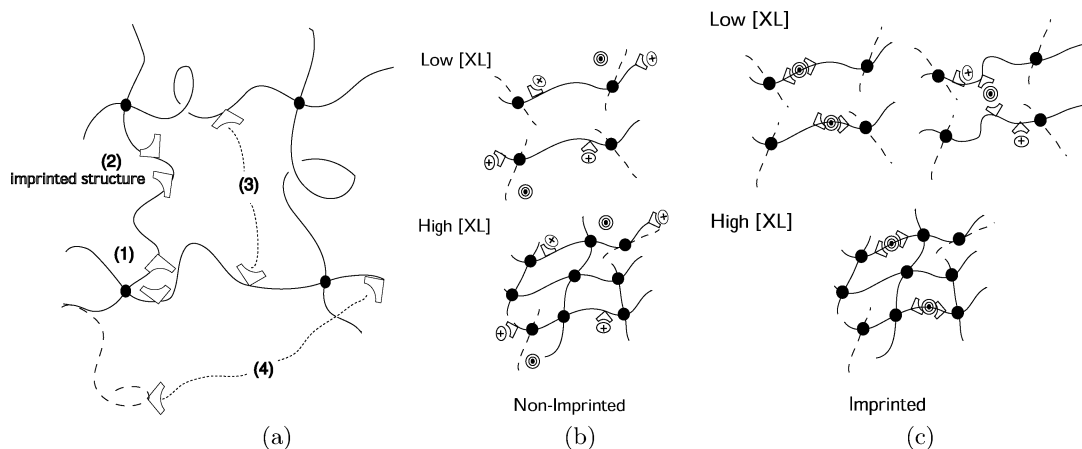
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Figure 4. Site formation and gel microstructure: (a) Candidate adsorber pairs may be located near a cross-link (1), on a single chain (2), on distant chains with (4) or without (3) intervening entanglements. (b) With increasing cross-linking density [XL], adsorbers are frustrated in nonimprinted gels. (c) In imprinted gels flexibility at low [XL] leads to competition for targets with possible topological consequences (mispairings) which diminish at high [XL].

the flexibility of the backbone allows for two adsorber pairs to come close together, in such a way that a target is captured by one adsorber from each pair. Topological restrictions may then prevent the lone (unbound) adsorbers from capturing an additional metal ion. As cross-linking density is increased, the pairs are less flexible in their motion and less likely to form mismatched sites. Cross-linking thus enhances the probability of maintaining pairs along a single chain and has a positive role in adsorption by imprinted gels. This is almost the reverse of the frustration effect predicted for nonimprinted gels. In the limit of high adsorber density, we expect to observe a diminished effect of frustration with a consequence that nonimprinted gels will behave similarly to imprinted gels (evidenced by nonimprinted gels with the highest adsorber density with affinity that also depends on [XL] and $[A]_r$; see Figure 3).

Our results highlight the potential and limitation of using imprinted gels for mimicking protein function. We do not observe conformational recovery in dense gels, in that the number of captured lead Pb^{2+} molecules is less than intended by imprinting (i.e., values of saturation are such that S indicates that $[A] < [A]_r$). In view of the significant adsorption when swollen coupled with a lesser benefit of collapse by imprinted gels, the volume phase transition does not strongly disable or enable function (i.e., molecular capture). In short, we do not observe simulated protein "renaturation". Whether we establish a native conformation in dense imprinted gels is unclear, but we do observe a structural bias of imprinting. In fact, adsorber pairs on a single chain present a challenge to observing more pronounced adsorption differences between swollen and collapsed imprinted gels. On the other hand, we are encouraged by the potential of molecular imprinting as a viable technique for designing heteropolymer gels. The advantages of designed or imprinted gels for molecular capture by multicontact adsorption are tangible and intriguing. Improved affinity is a desirable quality for use in potential "smart" sponges or low impact resins (chemo-sensors[17]) designed to capture divalent metals. More broadly, with this work, we would like to bring attention to the

need for an expanded view of the Tanaka equation, eq 1, which includes additional considerations of energetics and binding properties of heteropolymers (in addition to reexamining the impact of preparation-synthesis conditions, solvent, salt, etc., on gel microstructure). With improved understanding of molecular capture by heteropolymer gels, we hope that one small but no less important step in realizing Toyoichi Tanaka's vision to use gels as protein mimics can be achieved.

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