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Structural Characterization of Protein-Imprinted Gels Using Lattice Monte Carlo Simulation

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Summary: Molecular imprinting is a technique for the creation of artificial recognition sites in synthetic organic or inorganic polymers, formed by cross-linking in the presence of a template molecule. Removal of the templates leaves cavities that fit the template molecules in size, shape and functionality. Although this technique has been shown to be effective when targeting small rigid molecules, attempts to extend it to larger templates, such as proteins, have failed to show similar success. As opposed to small molecules, proteins are characterized by large size, flexible structure, and large number of functional groups available for recognition. These characteristics, among others, make it impossible to use imprinting procedures of small molecules for protein imprinting. In order to plan new imprinting strategies for proteins, one must reveal the problematic points in the polymerization process of the current methods, which result in low imprinting efficiency. In our research we focus on a bulk imprinting method using radical polymerization of hydrogels, applied to small globular proteins (e.g., lysosyme and cytochrome c). We model the system using lattice Monte Carlo (MC) simulation, using a modified kinetic gelation model (KGM) to simulate the polymerization process, which results in an inflexible protein-imprinted gel. The effect of initiator molar ratio, polymer volume fraction (VF), cross-linker density, and protein charge density and distribution on gelation, gel structure, and on the imprinted pore structure and functionality is investigated in the dry state of the gel. It is found that the imprinted pores wrap the template protein more fully and with larger number of linking sites than the non-imprinted pores. The insertion of non-template proteins with various charge densities and distributions into the imprinted pores indicates that charge density of the adsorbing protein is the dominant factor influencing the number of linked recognition sites.

Keywords: molecular imprinting; monte Carlo; proteins; polymer gel; simulation

Introduction

Molecular imprinting is a process in which organic or inorganic polymeric materials that are able to recognize specific substrates are fabricated.[1] These molecularly imprinted polymers (MIPs) are formed by the cross-linking of functional monomers in the presence of a template molecule. When the template is removed, it leaves cavities that are complementary to the target molecule in size, shape, and functional group orientation. These cavities will preferentially rebind in specific locations to the template molecule and not to similar molecules on account of the matching location of the functional groups.[1–3]

MIPs have considerable potential for applications in the areas of clinical analysis, medical diagnostics, environmental monitoring and drug delivery. MIPs have been vastly employed as stationary phases in liquid column chromatography for analytical or preparative separations of various compounds.[2–5] Capillary electro-chromatography, in which the mobile phase is

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transported through a capillary by means of electro-osmosis is another separation procedure containing MIPs.[1,2] The most advanced application amongst MIPs is the molecular-imprinted based solid-phase extraction, a process that is usually used to clean up a sample before using a chromatographic or other analytical method of quantification.[5,6] Another promising potential application area for MIP development is as a replacement of biological receptors such as antibodies in analogues of immunoassays.[1] Additionally, the specificity, robustness and low costs of MIPs provide opportunities for integration of such materials into sensing applications.[2,3,5] Molecularly imprinted polymer membranes were also developed and are used to act as a barrier between two liquid phases for purification or selective detection of compounds.[2,5] In addition, MIPs are used for synthesis as the supporting reagents and protecting groups or by providing spatial definition and directionality of chemical groupings within the imprinted cavities, which support selective and asymmetric synthesis.[7] MIPs have also operated as catalysts that stabilize the intermediate state and lower the activation energy of the reaction. These catalytic MIPs are mostly fabricated by using a transition state analogue as template.[7] Over the past few years, MIPs have revealed an important potential serving as vehicles for controlled release of drugs that are able to control the rate, activation, and feedback regulation of drug release.[8–11]

Molecular imprinting of bio-macromolecules and proteins, in particular, is a challenging area of research that is currently receiving a great deal of attention.[12,13] As a well-established technology in the field of synthetic molecular recognition, it offers a generic, robust and cost-effective alternative to existing biological recognition techniques such as monoclonal antibodies.[14] Selective protein binding is very useful in isolation, extraction, biosensors and other issues applied in laboratory practice,[14] procedures that are almost entirely dependent on antibodies, which are extremely expensive.[12,14] Protein imprinted polymers (PIPs) can be used as the solid phase for chromatographic analysis and purification of proteins, and can also provide researchers with a low-cost easily obtainable method for studying the fundamental interactions occurring during biological recognition processes.[15] The potential application of such materials could also extend beyond the laboratory by serving as a tool to control biological systems. For example, artificial receptors, which are capable of recognizing proteins in aqueous media, would allow for creating materials and devices capable of mimicking natural processes and could potentially be used in medical diagnostic applications and clinical analysis.[15]

While imprinting of small molecules has advanced significantly over the years and a number of companies now sell tailor-made imprints, efforts to generate imprints of protein targets have been far less successful.[12] The reason for this can be attributed to several factors related to the properties of the protein templates. A key contribution to poor specificity is inherent to the non-covalent approach (the most widely used method for MIP fabrication due to its simplicity[1,16]), where heterogeneity of the binding sites cannot be avoided mainly as a result of the relative instability of template-monomer complex and the limited choice of polymerization conditions that will favor the monomer-template assembly, even for small imprint molecules.[2,3,5,16] Furthermore, while the majority of molecular imprinting relies on hydrogen and ionic bonding for recognition and, therefore, takes place in apolar, organic solvents that maximize electrostatic interactions,[13,14] proteins obligate the use of water as the reaction solvent, which further diminishes the power of molecular recognition. Moreover, water limits the choice of monomers, since many monomer types commonly used in MIP preparations are insoluble in water.[14] The large number of potential recognition sites on the protein also favor nonspecific binding. An additional drawback of the large size of the protein is poor mass transport, and permanent entrapment within traditionally highly cross-linked MIP

gels. Reducing the degree of cross-linking results in highly porous and thus less selective gels, in addition to poor mechanical properties.[13,14]

The relatively flexible structure and conformation of proteins, that is easily affected by changes in the environment (e.g., pH, temperature, or ionic strength), also contributes to the difficulty of forming well-defined recognition sites within the gel.[13,14] In addition, protein denaturation and aggregation can occur during polymerization due to the inherently non-physiological conditions. Moreover, flexible proteins may change conformation in order to fit an imprint formed for a different protein and, as a result, PIP selectivity is impaired. This last factor, however, can be potentially used for the fabrication of catalysts that force a protein into a known conformation.[14]

There are a number of different strategies for creating polymeric receptors targeting proteins, which can be subdivided into either two-dimensional (2D) or three-dimensional (3D) platforms. Extensive work has been made preparing PIPs using 3D acrylate chemistries by bulk free-radical polymerization.[14] Special attention has been devoted to polyacrylamide (PAA) gels due to their biocompatibility, neutrality, and inert nature, which minimizes non-specific interactions with proteins.[13,17–19] An alternative to the 3D strategy is the 2D approach, in which imprinted polymers are produced in thin films or fixed to a supporting surface.[20,21] In this process, the recognition sites of PIPs are confined to surfaces and, therefore, enable to overcome the steric and diffusion problems associated with the 3D imprinting of biological macromolecules. However, the 2D imprinting may not offer high specificity since only a small portion of the surface of the protein would be recognized. In the epitope approach, a small sequence of amino acids from the large protein target molecule is used to create the imprint.[13–15] As a result, the actual imprinted part is based on a well-defined rigid molecular structure through which the entire protein can be recognized and bound. While this approach may lead to the formation of more stable recognition sites, it requires directional adsorption and the above mentioned diffusion limitations are not alleviated.

The following experimental works exemplify the unimpressive protein selectivity of PIPs achieved so far in comparison with MIPs. Ou et al.[17] created acrylate-based lysosyme-imprinted polymer by free-radical bulk polymerization using charged functional monomers, with imprinting factor (binding of protein to imprinted polymer / binding of protein to non-imprinted polymer (NIP)) of 1.83–3.38. However, rebinding was not strictly template-specific as the polymer also bound albumin. Furthermore, more than 25% of the original template remained in the polymer, a situation that impairs selectivity as it reduces diffusion efficiency, diminishes the number of free recognition sites within the polymer, and may lead to protein aggregation during rebinding. Similar results were obtained by Kimhi and Bianco-Peled,[18] in their study of PIPs using cytochrome c and lysozyme on lysozyme-imprinted polymer. Although adsorption in the imprinted gel showed preference towards the template protein, it was far less significant compared to MIPs. Takeuchi et al.[22] created an array of imprints, which together were used to determine unique binding profiles for a set of analyte proteins. Though every imprint bound its own template protein with the highest affinity, the selectivity was modest (at most 2.5-fold). By using acidic or basic proteins and monomers, it was anticipated to find differences in the strength of interactions accordingly. However, no clear trend was observed. In comparison, Yavuz et al.[23] performed non-covalent imprinting of cholesterol in polymeric particles using bulk polymerization and showed that adsorption capacity of cholesterol by MIP particles was 8-fold higher than by the control NIP particles, while Wang et al.[24] prepared a cholic acid-imprinted polymer exhibiting an imprinting factor of 7.9.

In our work, on-lattice Monte Carlo (MC) simulations of protein imprinting are

performed according to the basic principles of kinetic gelation models (described below). The simulated process is composed of protein-monomer complexation followed by gelation via free-radical polymerization, and results in a rigid structure of imprinted polymer. The relation between the degree of recognition and the fraction of linked recognition sites is studied. The effect of concentration, cross-linkers, and fraction of charged monomers on the degree of recognition is examined. Template proteins of various sizes, charge density and distribution are used to investigate the effects of these parameters on selectivity and performance. Our qualitative results are compared with experimental works. For instance, imprinting factors of polymers made from various monomer concentrations and different cross-linker ratios could be qualitatively compared to our results.[17,18,22] Our results are anticipated to reveal the problematic issues which result in low PIP efficiency, and may help guide processes that would result in higher imprinting selectivity and performance.

Model

In view of the difficulties associated with the production of efficiently selective PIPs,[12,14] the need to plan new imprinting strategies for proteins arises. For this purpose, one must first reveal the problematic points in the polymerization process of current methods that result in low imprinting efficiency. For example, does the protein-imprinted, fairly loose, gel retain the form of the imprinted protein pore following protein extraction, and, if not, does it have a ''memory'' of the template protein such that it reconstructs the pore upon template adsorption? How does the number of recognition sites in the imprinted cavity affect the PIP specificity, and does a higher number of linked sites necessarily result in higher recognition and better selectivity? Moreover, could imprinting particular sites on the protein surface promote or inhibit specific recognition? What is the effect of protein flexibility on PIP recognition and selectivity? Does the lack of molecular rigidity play an important part in lowering the efficiency of the imprinted polymer? What is the effect of protein size and shape on imprinting efficiency? How does charge density and charge distribution on the protein shell affect recognition? How do gel properties such as chain flexibility, cross-linker concentration, volume fraction, and fraction of functional monomers, affect recognition and PIP selectivity?.

The most common polymer system for PIP production is free-radical initiated vinyl polymerization, which is easy to prepare using a broad range of commercially available monomers and cross-linkers.[1,16] The kinetic gelation model (KGM) describes the chemistry of irreversible polymer gelation and relates to the rules of initiation, propagation, termination and radical trapping.[25–27] KGMs generally describe the formation of a rigid network without relating to the flexibility of the resulting structure. Verdier et al.[28,29] present a MC method for studying polymer chain dynamics and equilibrium configurations. In their model, the polymer is represented by a self-avoiding walk on a simple cubic lattice. Trial repositioning of chain particles is performed by several distinct types of motion (end rotation, crankshaft, kink jump, etc.), while obliging a fixed bond length.

A three-dimensional cubic lattice MC simulation is used to model the complexation between a template protein and charged functional monomers. This stage is followed by KGM simulating radical reaction mechanism[30–32] of gelation in the presence of the template protein. The occupied lattice sites in the model represent protein residues and polymer particles. The monomers are bifunctional and either neutral, negatively or positively charged, and the cross-linkers are all tetrafunctional and neutral. In the current model, the protein is modeled as a rigid cube composed of a fixed number of lattice sites, each representing one amino acid unit. Charged

protein residues are randomly distributed on the protein shell according to a charge density parameter. The electrostatic functionality for charged monomers and charged protein residues is taken to be unity. Reversible electrostatic bonds are allowed to form between two oppositely charged functional monomers, or between a functional monomer and a protein residue with opposite charges. Both covalent and electrostatic links can only form between two nearest lattice neighbors. No statistical condition limits the formation of covalent or electrostatic links; however, the covalent bonding is of first priority. Hence, a particle or a cluster will first implement all covalent bonding opportunities and only then will have the option to link electrostatically. However, a pair of covalently linked monomers cannot link electrostatically as well.

Initially, particles (monomers, cross-linkers and protein) are randomly distributed amongst lattice sites achieving a chosen volume fraction, ϕ. Each simulation is divided into two main stages: The first is a prepolymerization stage, during which energetic equilibrium is achieved via particle movement and electrostatic interactions. The energy of the system is given by, $E = b\varepsilon$, where ε is the interaction energy of a single electrostatic bond, and b is the number of electrostatic bonds in the system. It was our aim to first isolate the effect of protein-monomer complexation, which is determined by electrostatic *attraction* and is presumed to dictate the functionality of the imprinted pore. For completeness, electrostatic repulsion between similarly charged elements should also be included. However, since the system reaches equilibrium before polymerization, this addition is not expected to affect the number of recognition sites but should diminish the number of unfavorable interactions within the pore. The second simulation stage begins with fast initiation, as radical ''signatures'' are randomly distributed amongst polymer particles,[33] and continues with propagation, as covalent bonding occurs. The process ends either when all polymer particles are connected to the main cluster (gelation is completed) or when the number of clusters does not change during a sufficient number of iterations, which means that all radicals are either terminated or trapped. Since this simulated process is fairly rapid, we can be certain that the last situation assures the ending of the polymerization.

During propagation, each radical molecule randomly links to one of its nearest neighbors, thus starting the formation of a chain of covalently linked particles. Only a radical molecule can covalently bond to another molecule, and by doing so transfers the radical signature to it. Once no options of further linking are left, cluster displacement moves take place. At the end of each movement, another series of covalent bonding is performed, as well as an update of electrostatic bonding around the moved cluster. This order of events is significant only computationally and does not affect the final gel structure.

In each attempted move, one particle or cluster is randomly selected as a candidate for displacement. Clusters move as unities similar to single particles and currently there are no independent movements of their inner chains. The probability P that a cluster will attempt to move depends on its size such that $P = 1/M^{\gamma}$, where M is the number of particles composing the cluster and γ is a parameter that correlates the degree to which particle size affects its diffusion.[34–39] Once a particle has been selected, the direction of the movement is randomly chosen out of the six nearest neighbor lattice directions and an overlap check is performed. If no overlap is found, the displacement takes place with probability P' that depends on energetic calculations as follows. The energy difference between the current and proposed conformation is calculated according to $\Delta U = \varepsilon (b_2 - b_1)$, where ε is the interaction energy of a single electrostatic bond, b_1 and b_2 are the number of electrostatic bonds in the current and proposed configurations, respectively. Moves are accepted according to the Metropolis criterion.[40]

Results and Discussion

Gel Structure

In order to study PIP selectivity, it is necessary to simulate the processes of protein extraction and re-adsorption, which require simulations of protein diffusion into and out of a swollen gel, and is the subject of our continuing research. In this work, we focus on the properties of the dry gel, which facilitates the investigation of the imprinted pore structure and functionality, and provides an indication on the optimal potential of protein recognition. A sample configuration of the protein-imprinted gel is illustrated in Figure 1, for a cubic lattice containing 15^3 sites. The effect of lattice size on gel composition is shown in Figure 2. It can be seen that the deviation in the properties of the system are not significant for $L \geq 15$. That is, both the length of (Figure 2a) and the average fraction of monomers belonging to (Figure 2b) the gel backbone, free ends, and loops, shows minor changes. Hence, the remainder of our simulations were carried out on $L = 15$.

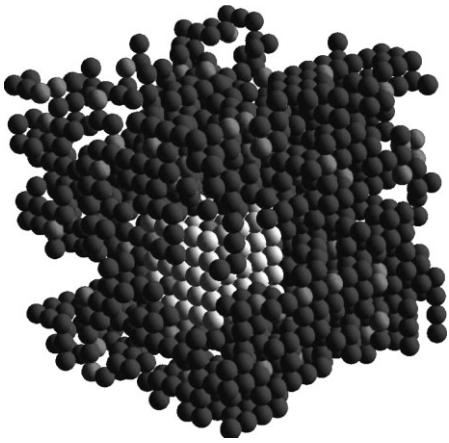

Figure 1.
Illustration of a protein-imprinted polymer. Different colors indicate different particle types: monomers (dark grey), cross-linkers (grey), protein residues (light grey), and protein residues that are linked to functional monomers (between grey and light grey). Simulation parameters correspond to $\phi = 0.4$, cross-linkers percent is 7.6%, protein charge density is 0.6, fraction of charged monomers is 0.6.

Figure 3 shows that the initiator molar ratio (number of initiators / total number of monomers) influences gelation by changing the final gel fraction (GF) and gel composition; GF represents the fraction of particles (monomers and cross-linkers) that belong to the main cluster. For each particle volume fraction, ϕ, studied, an optimal range of initiator concentration exists in which the GF value is maximal (Figure 3a) and the resulting gel is mostly composed of backbone chains (Figure 3b). It is striking that the initiator molar ratios used in experimental works of protein imprinting via acrylate-based bulk radical polymerization[17,18] correspond to the optimal range found in the current results.

A clear dependency of GF on the average length of the polymer backbone is seen in Figure 4. The decrease in kinetic chain lengths with increased initiator concentration was previously indicated by Burdick et al.,[41] in an experimental study on chain length distributions formed during the photoinitiated polymerization of divinyl monomers. By using the maximal initiator ratio, the current KGM is similar to the diffusion-limited cluster aggregation (DLCA) model in which each particle is able to polymerize at any given time. When maximal initiator ratio is used for cases of $\phi \geq 0.2$, several local clusters appear. However, gelation does not take place. Indeed, the original DLCA models have only considered volume fractions that are at least one order of magnitude lower than the volume fractions used in this research, and thus they enabled the formation of a single cluster.[36–39] Indeed, reversible aggregation models[42] and KGMs[26] are used for simulating the formation of dense networks.

The effect of polymer volume fraction and cross-linker molar ratio (number of cross-linkers / total number of monomers) on the resulting gel structure, shown in Figure 5, exemplify a similar trend with both parameters such that when increasing ϕ or cross-linker ratio the number of chains increases and chain length decreases. Finally, we note that while at the dilute

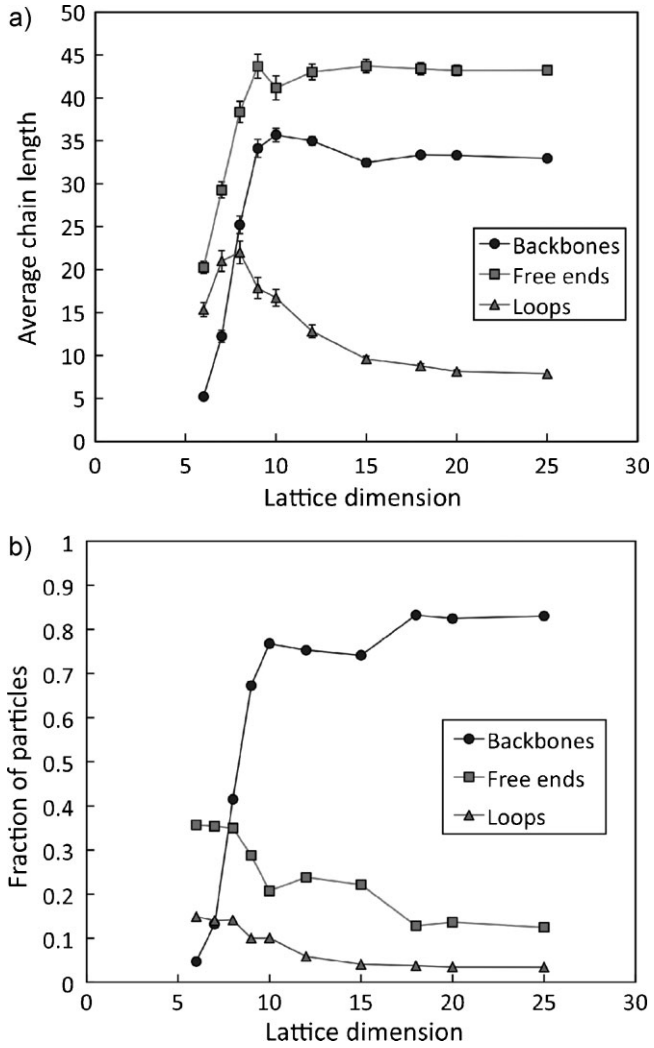

Figure 2.
Effect of the lattice size on gel (a) structure and (b) composition.

protein concentrations studied, our results indicate that gelation in the presence of the protein does not affect the final gel structure, the presence of the protein creates cavities in the dry gel that are of the order of protein size in dense gels (high ϕ), that are unlikely to form otherwise.

The remainder of our results focuses on analysis of the functionality and binding of the imprinted cavities in their collapsed state under various imprinting conditions. Since swelling of the gel was not considered at this point in our study, we found that the cross-linker ratio has no effect on pore size and protein binding (results not shown).

Clearly, pore size, and thus protein binding, is expected to be intimately correlated with the amount of swelling of the gel, which in turn depends on the cross-linker ratio. This will be the focus of a future study.

Protein Imprinting

Figure 6 illustrates the effect of monomer volume fraction on the number of recognition sites present on pore walls (normalized

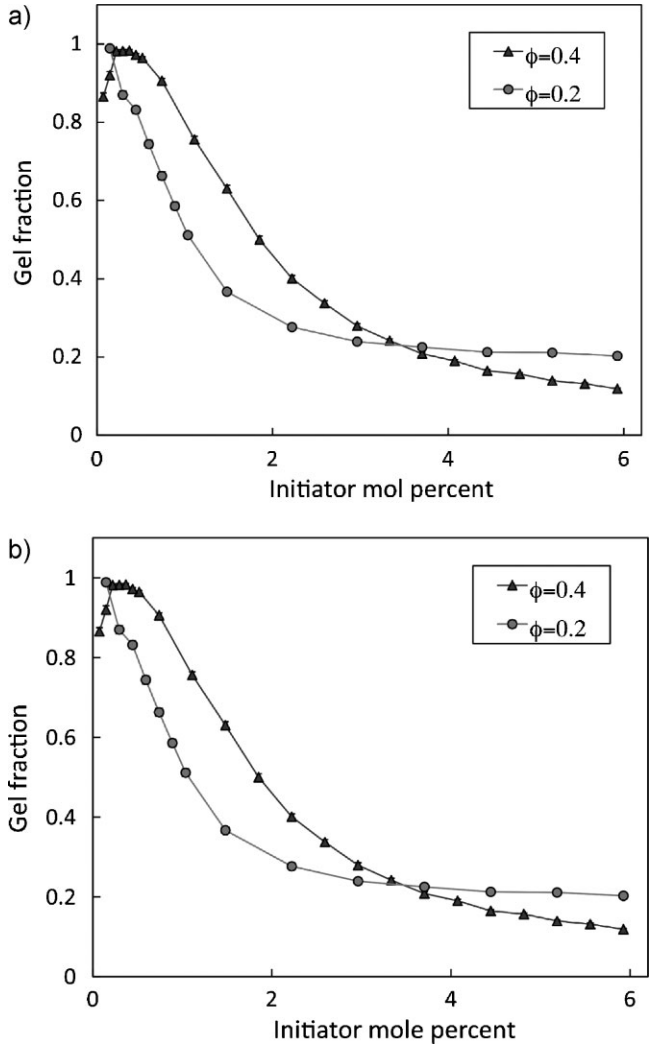

Figure 3.
Effect of initiator concentration on (a) gelation at $\phi = 0.2$ and 0.4 and (b) gel structure for $\phi = 0.4$.

by pore size) and on the average fraction of linked protein residues during diffusion of the protein within the cavity. Both these factors are linearly correlated with the ϕ, while the fraction of average links out of all potential recognition sites increases rapidly with ϕ and reaches a plateau at $\phi > 0.5$ when the cavity approaches protein size. The average size of the imprinted cavity obtained from diffusion simulations as a function of ϕ is shown in Figure 7. The large values of pore size are due to percolation of pores in the system that occurs in low particle densities.[43,44] Clearly, for $\phi \geq 0.3$, the average size of the imprinted pore approaches the size of the protein template. Ou et al. showed in their experimental work that increasing ϕ leads to increase in the imprinting efficiency. This trend was explained by elevated stability of the protein-monomers complex at high polymer concentrations,[17] and is directly related to the enveloping effect of the imprinted pore at higher values of ϕ.

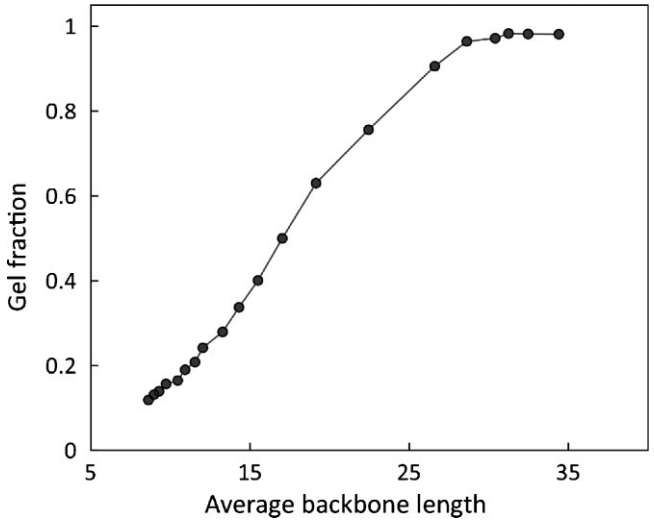

Figure 4.
Relation between the average backbone length and the average fraction of monomers in the main cluster ($\phi = 0.4$).

In the following, we compare the functionality and binding capacity of the simulated imprinted pores with those of non-imprinted gels and gels imprinted with different ''proteins''. Along with the linked fraction of charged residues, we also calculated the average protein coverage fraction (PCF), which represents the number of spatial directions through which the protein is linked according to $PCF = \frac{n_f+n_s+n_c}{T_f+T_s+T_c} = \frac{n_f+n_s+n_c}{26}$, where n_f, n_s, and n_c are the numbers of linked faces, sides and corners of the protein, respectively, and T_f, T_s, and T_c are the total numbers of faces, sides, and corners on the protein cube respectively. Figure 8 compares the imprinted

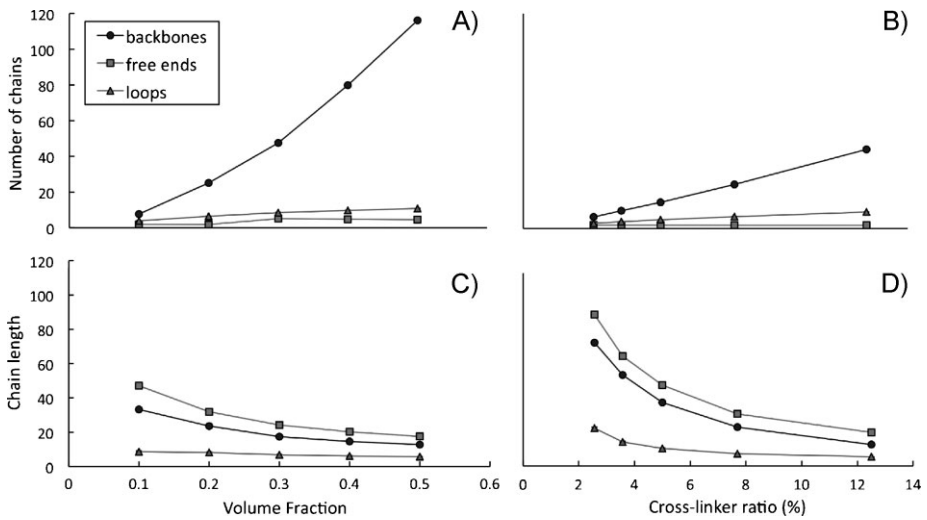

Figure 5.
Effect of the volume fraction and the cross-linker molar fraction on the gel structure. Cross-linker ratio in A and C is 1/13; ϕ in B and D is 0.2.

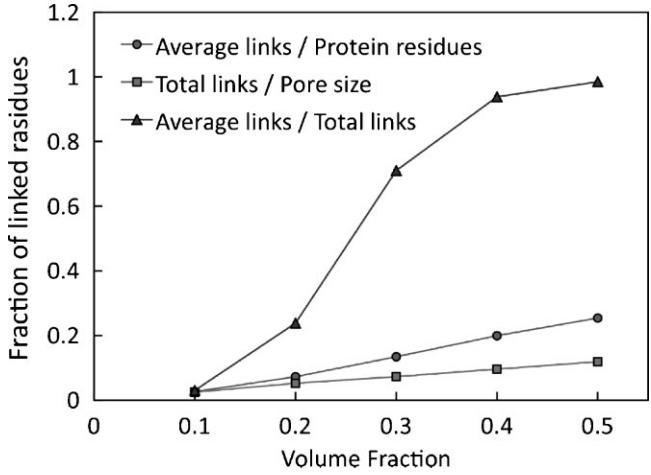

Figure 6.
Effect of ϕ on the linking characteristics of the template protein to the imprinted pore.

pore functionality to the functionality of a randomly formed pore within NIP. It can be clearly seen that the imprinted pores recognize the protein via more directions and by a larger number of recognition sites than the non-imprinted ones. In another simulated experiment that gives an indication on PIP selectivity, the template protein is removed from the gel following polymerization and replaced by other proteins of the same size but which differ from the template protein either by charge distribution alone or by charge density as well. Figure 9 shows a comparison of the fraction of linked residues of template and non-template proteins with various charge densities in such diffusion experiments. As expected, we find that when considering an identical charge density on the protein shell, the template protein has a higher

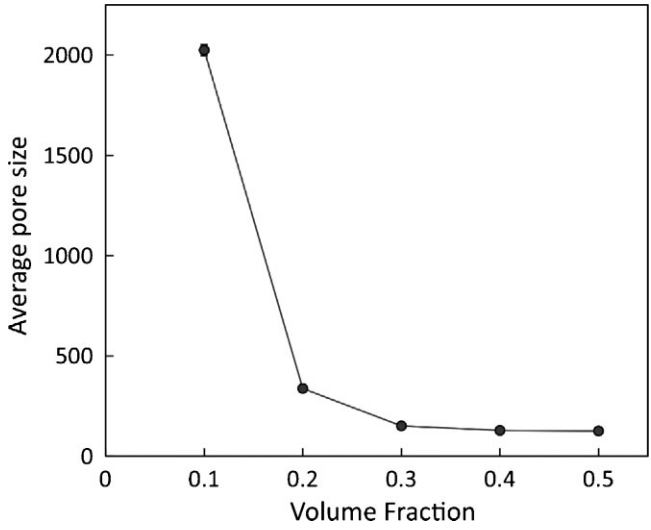

Figure 7.
Effect of ϕ on the average size of the imprinted pore.

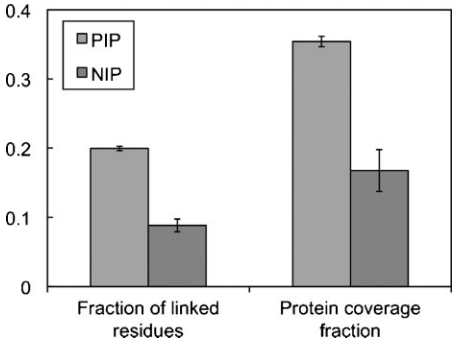

Figure 8.
Effect of imprinting on the linked fraction of charged protein residues and on the fraction of protein coverage by the imprinted pore walls ($\phi = 0.4$).

number of linked recognition sites than a non-template protein, suggesting that charge distribution on the protein shell affects the imprinting process. However, at varying charge densities it seems that the main influencing factor on the fraction of linked residues is the charge density on the protein shell. Irrespective of the charge density on the template protein, a highly charged protein will have more recognition sites on the imprinted pore walls than a poorly charged one, even if the latter is the template protein itself. This result corroborates the suggestion that multiplicity of recognition sites may impair selectivity[14] and provides an explanation for the differences in PIP recognition of lysosyme and cytochrome c, as was indicated by experiments.[18]

Conclusion

The structural properties of model imprinted polymer gels and the structure and functionality of the imprinted pores are studied immediately following polymerization within the collapsed (dry) gel. It is shown that there is an optimal initiator concentration which achieves maximal gel fraction and high fraction of backbone chains, which is similar to concentrations used in experimental works. At the low template concentrations considered, the presence of the protein does not affect the gelation process or the average gel structure. However, although both ϕ and cross-linker concentration affect the average gel structure, only ϕ influences the structural and functional properties of the imprinted pore in the dry gel. It is found that raising ϕ increases the number of potential recognition sites on pore walls, and, up to a certain ϕ, the fraction of linked recognition sites increases as well. When considering the fraction of linked protein residues and the PCF, an advantage is found for imprinted pores compared with non-imprinted ones. According to this result the imprinted pores envelope the template protein in more directions and with a larger number of linked (imprinted) sites than the randomly formed pores. The insertion of non-template proteins with various charge densities and distributions into the imprinted pores indicates that in

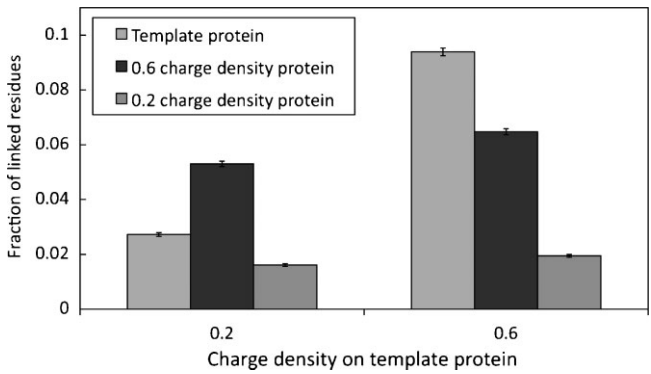

Figure 9.
Linking characteristics of template and non-template proteins with different charge densities ($\phi = 0.4$).

the current form of the gel, the charge density of the inserted protein is the dominant factor influencing the number of linked recognition sites. In fact, a highly charged protein will preferentially adsorb in a templated pore formed by a protein with lower surface charge.

Our preliminary results on the structure and functionality of the imprinted pore appear to provide an indication on the potential recognition properties of the PIP, even in its collapsed state. In view of experimental data, it can be postulated that at least some of the trends observed by our presented pore structure analysis will be corroborated by the results of more rigorous diffusion simulations. Future stages of the proposed research will include simulations of swelling, protein extraction and absorption (via diffusion within the gel), and polymer and protein flexibility. Such simulations will indicate whether it is possible to improve PIP performance and selectivity and will shed some light on the prominent issues on which one should focus in order to enable these improvements for practical applications.

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