Structural characterization of protein-imprinted gels using lattice Monte Carlo simulation

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Abstract

Molecular imprinting finds relevance and importance in a range of applications including the development of new chemical sensors, separation technologies, and selective binding particularly for catalytic and bioreactors. While molecular imprinting technology has found success when imprinting small molecules, progress in molecular imprinting of biomacromolecules, such as polypeptides and proteins, is hindered, in part due to their large size and complex functionality. Unlike with small molecules, imprinting of biomacromolecules results in poor performance of the imprinted gel, characterized by significant nonspecific binding of molecules of similar shape and size to the template. Our research in this field aims to characterize the structure of protein-imprinted gels in an effort clarify the effects that lead to poor protein imprinting.

The determination and study of the three dimensional molecular structure of such supramolecular assemblies is a challenging problem. To gain a general understanding of the process, we focus on simplified coarse-grained lattice models that are known to capture some generic and universal properties of polymer gels and biopolymers. The protein and imprinting matrix are modeled using coarse-grained lattice techniques, where gelation is simulated using a modified kinetic gelation algorithm. Structural and functional properties are probed using protein diffusion within the gel. Different gelation conditions are considered and analyzed for imprinting performance. The effects of protein size, hydrophobicity, charge, and flexibility on gel structure are studied.

In this work, we focus on a bulk imprinting method using radical polymerization of hydrogels, in an effort to reveal the problematic points in the polymerization process, which ultimately result in low imprinting efficiency. We relate to the work of Ou et al. [1] and Kimhi et al. [2], who studied molecular imprinting of small globular proteins (lysosyme and cytochrome c) in polyacrylamide gels with electrostatic functional groups. We will present the method and results of the structural, energetic and functional properties of these imprinted gels and compare them with experimental observations.

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Molecular imprinting is a process in which organic or inorganic polymeric materials that are able to recognize specific substrates are fabricated [3]. These molecularly imprinted polymers (MIPs) are formed by 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 [3-5].

Molecular imprinting of bio-macromolecules and proteins in particular, is a challenging area of research that is currently receiving a great deal of attention [6, 7]. 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 [8]. Selective protein binding is very useful in isolation, extraction, biosensors and other issues applied in laboratory practice [8], procedures that are almost entirely dependent on antibodies, which are extremely expensive [6, 8]. 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 [9]. 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 [9].

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 [6]. 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 [3, 10]), 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 monomer-template assembly, even for small imprint molecules [4, 5, 10, 11]. 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 [7, 8], 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 [8]. 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 [7, 8] .

The following experimental works exemplify the unimpressive protein selectivity of PIPs achieved so far in comparison with MIPs. Ou et al. [1] 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 [2], in their study of PIPs using cytochrome c and lysozyme on lysozyme-imprinted polymer. Although absorption in the imprinted gel showed preference towards the template protein, it is far less significant compared to MIPs. Takeuchi et al. [12] 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. [13] 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. [14] prepared a cholic acid-imprinted polymer exhibiting imprinting factor of 7.9.

In view of the difficulties associated with the production of efficiently selective PIP [6, 8], 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 absorbance? How does the number of recognition sites in the imprinted cavity affect the PIP specificity, and does 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, the fraction of functional monomers etc., 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 [3, 10]. The kinetic gelation model (KGM) describes the chemistry of irreversible polymer gelation and relates to the rules of initiation, propagation, termination and radical trapping [15-17]. KGM models generally describe the formation of a rigid network without relating to the flexibility of the resulting structure. Verdier et al. [18, 19] 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.

We use a three-dimensional lattice MC simulation to model the complexation between a template protein and charged functional monomers. This stage is followed by KGM

simulating radical reaction mechanism [20-22] of gelation in 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 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 [23], 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 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 computational 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 [24-29]. 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 [30].

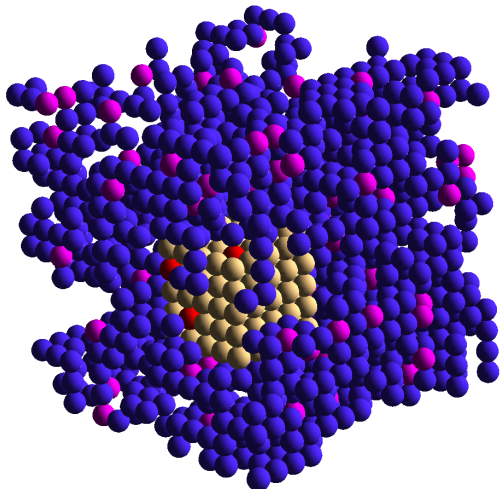

Figure 1. Illustration of protein imprinted polymer. Different colors indicate different particle types: monomers (blue), crosslinkers (pink), protein residues (beige), and protein residues that are linked to functional monomers (red). Simulation parameters correspond to $\phi = 0.4$, cross-linkers percent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

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 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. Lattice size L=15 was found to be sufficiently large so that lattice size effects are negligible [31].

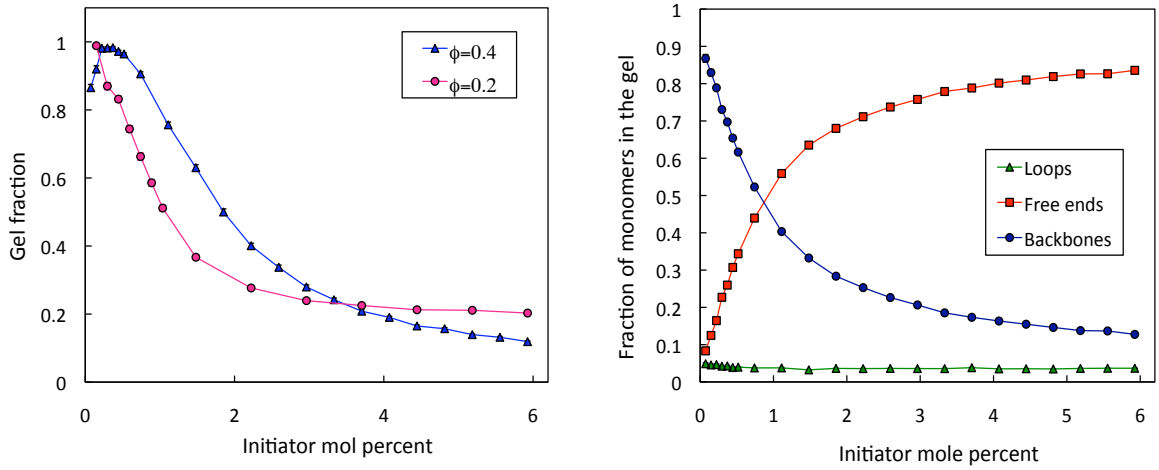

Figure 2. Effect of initiator concentration on gelation at ϕ = 0.2 and 0.4 (left) and gel structure for ϕ = 0.4 (right).

It is shown in Figure 2 that the initiator molar ratio influences gelation by changing the final gel fraction (GF) and gel composition; GF represents the fraction of particles (monomers and crosslinkers) that belong to the main cluster. For each particle volume fraction, ϕ, studied an optimal range of initiator concentration in which the GF value is maximal (Figure 2a) and the resulting gel is mostly composed of backbone chains (Figure 2b). It is striking that the initiator molar ratios used in experimental works of protein imprinting via acrylate-based bulk radical polymerization [1, 2] correspond to the optimal range found in the current results.

The effect of polymer volume fraction and cross-linker molar ratio on the resulting gel structure, shown in Figure 3, 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 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.

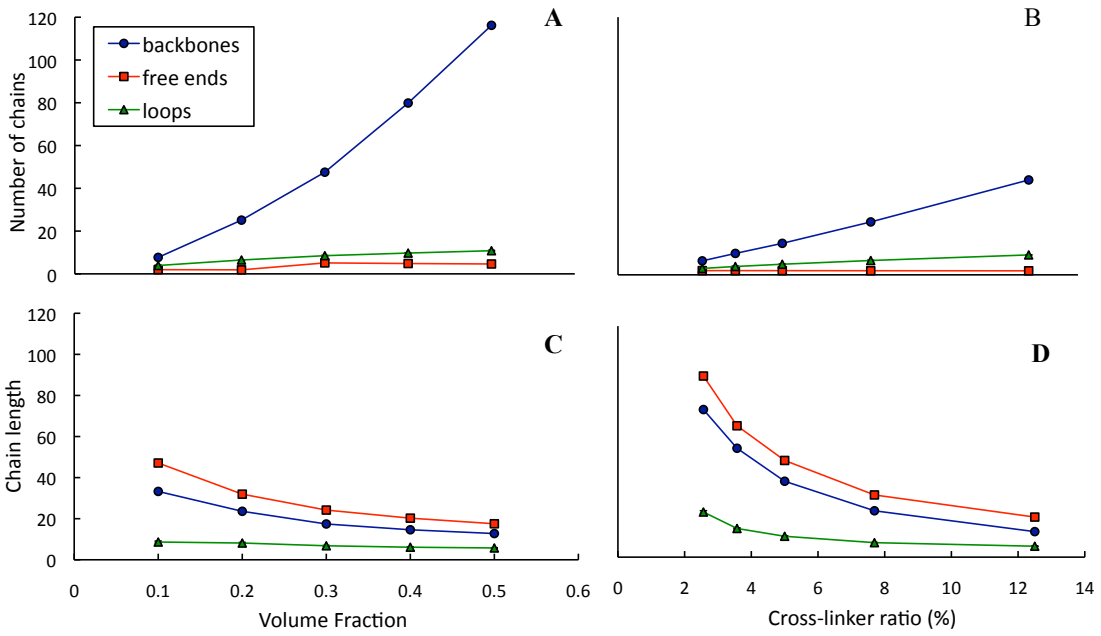

Figure 3. Effect of volume fraction and cross-linker molar ratio on gel structure. Cross-linker ratio in A and C is 1/13; ϕ in B and D is 0.2.

Figure 4 illustrates the effect of monomer volume fraction on pore size and the number of recognition sites present on pore walls (normalized 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 large values of pore size are due to percolation of pores in the system that occurs in low particle densities [32, 33]. 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 [1], and is directly related to the enveloping effect of the imprinted pore at higher values of ϕ.

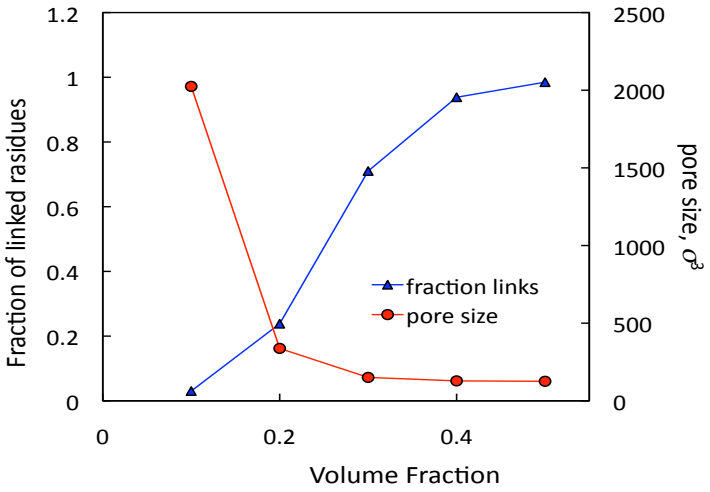

Figure 4. Effect of ϕ on pore size (circles) and linking characteristics of the template protein to the imprinted pore (triangles).

Along with the linked fraction of charged residues, we also calculated the average fraction of protein surface that interacts with the gel. **Figure 5** compares these values for an imprinted pore with that of a randomly formed pore within a nonimprinted polymer. It can be clearly seen that the imprinted pores recognize the protein via more directions and by 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.

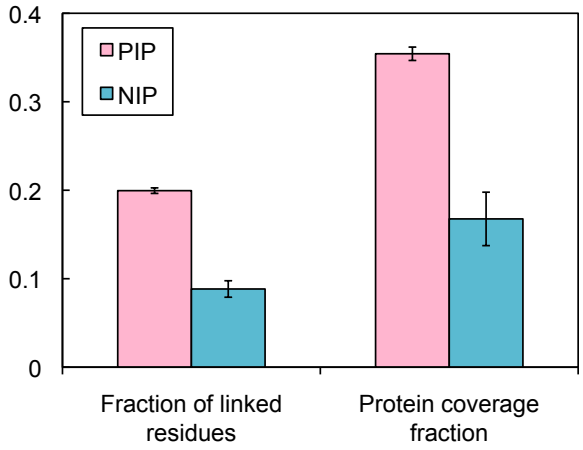

Figure 5. Effect of imprinting on the linked fraction of charged protein residues and on the fraction of protein coverage by the imprinted pore walls ($\phi = 0.4$).

Figure 6 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 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 [8] and provides an explanation for the differences in PIP recognition of lysosyme and cytochrome c, as was indicated by experiments [2].

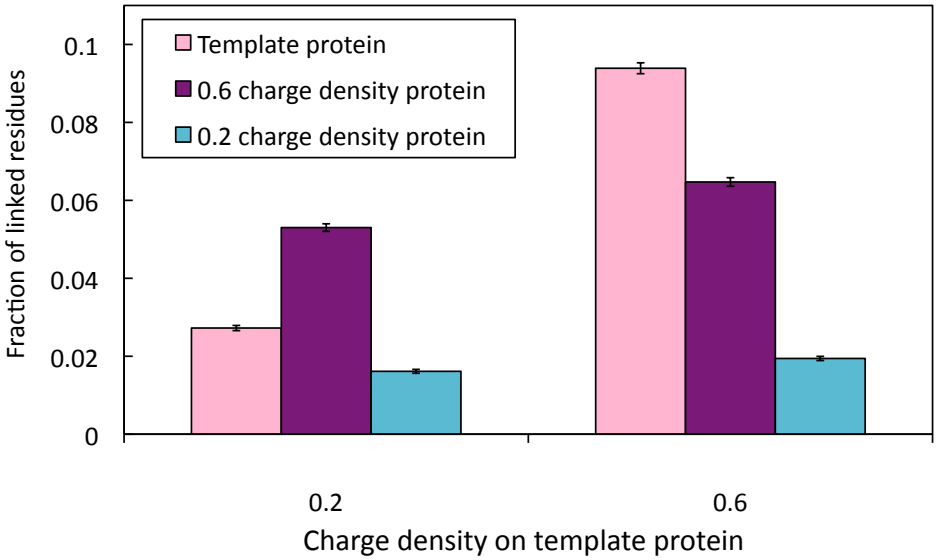

Figure 6. Linking characteristics of template and non-template proteins with different charge densities ($\phi = 0.4$).

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 dry (non-swollen) 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 PIPs 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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