Review

Imprinted polymers assisting protein crystallization

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Molecularly imprinted polymers (MIPs) are 'smart materials' polymerised in the presence of a template molecule, of which they retain a chemical 'memory'. When the template molecule is extracted from the polymer, it leaves behind cavities that are complementary to it, thus making the material capable of rebinding that molecule with high affinity and selectivity. Such materials, imprinted both with small molecule and with protein templates, have been used in chromatographic, chemical, and biological sensing applications. Here, we review a variety of uses for MIPs, focusing on their recently discovered role as nucleation inducing substances for protein crystals. This discovery makes them useful tailor-made 'nucleants' to be used both for optimisation of protein crystal growth and for discovering new crystallization conditions.

Molecularly imprinted polymers and their applications

X-ray crystallography is the most successful method for determining 3D protein structures at high resolution. Such structures are crucial to structure-guided drug design and to many other biotechnological and industrial applications. This method however requires well-diffracting protein crystals and the difficulty in obtaining such crystals is the principal bottleneck on the way to success [1]. Control of nucleation, that is, of the crystal conception stage, is a powerful means to obtain optimal crystals for structure determination [2] (Box 1). In the wide-ranging search for nucleation inducing substances, molecularly imprinted polymers were recently shown to be versatile, tunable, and amenable to manipulation [3].

MIPs are polymers formed in the presence of a template molecule that is afterwards removed, thus leaving behind cavities or 'ghost sites' that are geometrically and electrostatically complementary to the template molecule [4,5] (Figure 1A). Functional monomers are made to interact chemically with the template molecules, forming prepolymerisation complexes. Subsequent polymerisation in the presence of crosslinker 'freezes' these template–monomer complexes in place. Removal of the templates results in the formation of an imprinted polymer matrix. The overall shape and size of the cavities that are left behind create the geometrical complementarity, whereas the 'frozen' positioning of the preorganised monomers, thus of the distribution of charges, creates the electrostatic complementarity [6].

The basic method was described over 60 years ago. Many of the concepts and testing methods adumbrated in Frank Dickey's pioneering paper communicated to the Proceedings of the National Academy of Sciences USA in 1949 by Linus Pauling [7] are still with us when discussing present-day MIPs (or 'specific adsorbents' as Dickey called them) and their uses:

> This method consists in forming the structure of the adsorbent in the presence of the particular compound for which it is desired to prepare a specific adsorbent. The presumable explanation of the formation of a specifically attracting structure under these conditions is that the adsorbent in the process of formation has accessible to it a very great number of structures which differ only slightly in stability, and that in the presence of a foreign molecule those structures that are stabilized through attraction for the foreign molecule are preferentially assumed. The adsorbent is thus pictured as automatically forming pockets that fit closely enough to the foreign molecule to hold it by van der Waals' forces, hydrogen bonds, interionic attractions, and other types of intermolecular interaction. This mechanism is the same as that proposed by Pauling for the formation of antibodies with use of antigen molecules as a template, which formed the basis for the manufacture of artificial antibodies reported by Pauling and Campbell. [7]

The original specific adsorbents were made of silica gels in the presence of various dyes and they were compared against gels polymerised in the absence of dye. Cross-comparisons were also made, where gels made with different dyes were tested for adsorbance of their cognate and noncognate dyes. Optical isomer separation and artificial enzymatic action were proposed as possible applications of the method.

Actual applications, however, have been few and slow, and the method has always had to compete with other alternatives for each possible application. Only rather recently, since the 1990s, has there been a steady flow of proposals and advances in the field of MIPs. MIPs have worked at their best with imprinting of smaller molecules,

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Box 1. Nucleation and growth of protein crystals

Crystal growth requires a solution that is supersaturated with respect to the crystallised molecule. Supersaturation, that is, the amount of molecule present in solution in excess of the maximum amount that can be dissolved under the given conditions at thermodynamic equilibrium, is the driving force for crystallization.

However, even under conditions in which a crystalline phase is thermodynamically stable, an energy barrier has to be overcome in order to form the initial crystal nucleus (Figure I). This is because for a crystal to form, a crystal–solution interface (i.e., the crystal surface) has to form first, and this interface formation is energetically costly. This leads to the energy barrier, that is, to the need for a higher supersaturation for formation of the initial nucleus than for post-nucleation growth. Above a critical cluster size, the favourable free energy contribution from protein molecules being incorporated into the bulk of the crystal becomes greater than the unfavourable surface formation contribution and the cluster, then called a critical nucleus, can grow. Conditions under which crystal growth is favoured but the supersaturation is too low for spontaneous nucleation within a realistic time are commonly called metastable.

The higher the supersaturation of the solution, the lower the energy barrier, hence the easier the formation of critical nuclei. However, excessive supersaturation also increases the speed at which crystals grow, which in itself has undesired side effects, such as the build-up of structural defects leading to low crystal order and premature cessation of growth, and the fast creation of protein-depleted zones around the growing crystals. In addition, the occurrence of excessive nucleation can lead to the growth of thousands of small crystals instead of a few large ones ([2] and references therein). The ideal way around this problem is to introduce nucleation-inducing substrates, known as nucleants, into metastable solutions.

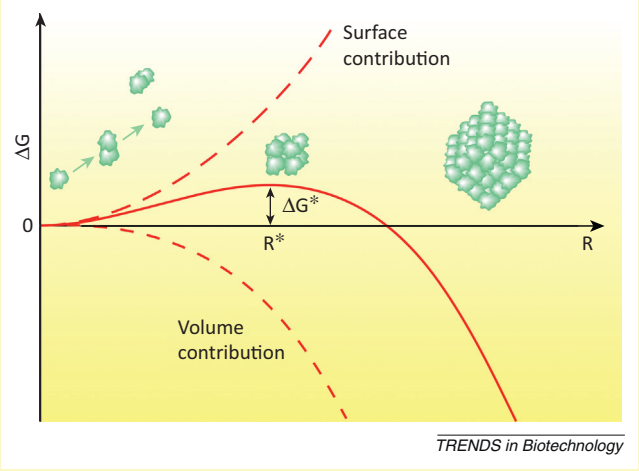

Figure I. The energy barrier for nucleation. Gibbs free energy for crystallization (ΔG) as a function of the aggregate size (R), shown as an unbroken line. Contributions of surface formation and bulk incorporation are represented by broken lines. R* and ΔG* represent the size of the critical nucleus and the activation free energy (i.e., the energy barrier) for nucleation, respectively. Reproduced, with permission, from [2].

such as amino acids, peptides, steroids, dyes, drugs, and sugars [6,8]. Proposed or actual applications include the detection and analysis of trace levels of compounds in complex mixtures, separation of peptide mixtures and of undesired compounds from foods (preparative separation) or biological fluids [8], or for slow and targeted drug delivery. An example of the latter are timolol-imprinted and -loaded therapeutic soft contact lenses, which allow the slow and sustained release of timolol (an anti-glaucoma drug) in the precorneal area [9]. Surface imprinting of viruses [10] as well as whole cells (microorganisms) has also been performed for sensing applications [11,12] and for tissue engineering [13].

Methods of fabrication – general

Imprinting can be either via noncovalent template–monomer linking (based on hydrogen bonds, van der Waals, and electrostatic interactions) or via reversibly formed covalent bonding. Most research nowadays is directed towards noncovalent attachment [6].

The most successful methods for noncovalent imprinting of small molecules are based on commodity acrylic or methacrylic monomers. The monomer–template interactions preassemble the monomers around the template. The mixture is then polymerised in a suitable solvent (a porogen) with a large excess of a crosslinker such as ethylene glycol dimethacrylate (EDMA). Methacrylic acid (MAA)

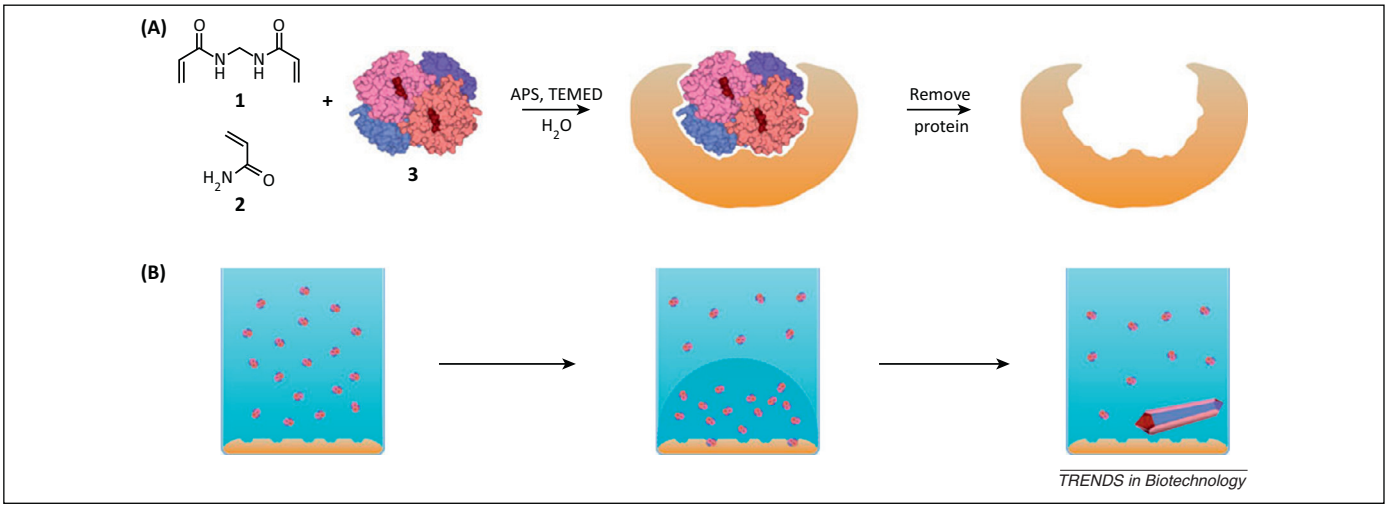

Figure 1. Molecularly imprinted polymers (MIPs) for protein crystallization. **(A)** An imprinted hydrogel is formed when acylamide (**2**) is polymerized with a crosslinker (methylene-*bis*-acylamide, **1**) in the presence of a protein template. Polymerisation in water with ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) gives rise to the hydrogel with embedded protein molecules. Removal of the template protein results in the formation of the imprints, cavities complementary in size and shape to the protein templates. **(B)** Stages of the protein crystallization process on a MIP. First, a piece of hydrogel with many protein cavities is brought into contact with a solution containing the target protein. A drop of protein-rich liquid then phase-separates from the bulk solution at the surface of the hydrogel and, following nucleation at the surface of the gel, a crystal begins to grow. Structure **3** © shutterstockphoto.com/Robert Sanchez. Reproduced, with permission, from [35].

remains one of the most widely used functional monomers. Styrene derivatives provide a different class of successfully used monomers [5]. The porogen is a compound that generates a pore structure in the polymerising material. That structure aids mass transfer of the template into the polymer during rebinding.

A MIP can be bulk- or surface-imprinted, referred to respectively as 3D and 2D (thin-film) imprinting. In the former, the template molecule diffuses into the prepolymerisation mixture and is then trapped upon polymerisation. In the latter, the template only forms its complementary cavities at a polymeric surface. Both types of MIP have been extensively studied and each has its own advantages and disadvantages. 3D MIPs can rebind much larger amounts of material but also need larger amounts of template for their fabrication compared with 2D, the latter being therefore best suited for use in sensors [14]. The 3D monolith also needs to undergo a stage of grinding or sieving, so that the cavities in the bulk of the polymer become accessible for template removal and for rebinding. Problems of slow diffusion of large molecules in the bulk of the polymer can be a problem for 3D MIPs, especially for biomacromolecules (see also below). By contrast, 3D imprinting is generally easier to perform, because it often relies on mixing all the components together, including the template molecule.

Protein-imprinted polymers

Historically, the molecular imprinting of biological macromolecules such as proteins has been a lot more problematic. The methods, although based on the same broad principles, must be different because of the great flexibility of protein molecules and of their instability at harsh, nonphysiological polymerisation conditions such as the ones normally used for the fabrication of small-molecule MIPs. At the very least, the monomer/solvent system used must be one in which the protein molecule is soluble, stable, and in a more or less physiological state (to preserve its spatial conformation), that is, aqueous rather than organic solvent based.

Other problems with imprinting biological macromolecules are: (i) poor site accessibility due to the impeded diffusion of macromolecules into and out of monolithic imprints (for the case of 3D MIPs), leading to low yield of specific binding sites; and (ii) the fact that macromolecules have a large number of varied functional sites at their surface. Both these features lead to low specific binding, that is, poor rebinding selectivity by comparison with small-molecule MIPs.

Proposed and actual applications for protein-imprinted MIPs are more varied and potentially more interesting than for small-molecule MIPs. They include the original and more well-established applications in solid phase extraction and affinity chromatography of proteins ([15] and references therein), for example, for downstream processing in biotechnology, but also drug delivery (proteins are currently one of the most important classes of biopharmaceuticals), biosensors and chemo/biosensors ([6] and references therein), including electrical and electrochemical sensors [16,17] and optical sensors [18,19], and in diagnostic protocols, for example, replacing the more expensive, perishable, and difficult to handle antibodies in immunoassays ([5] and references therein). This last example recalls one of the applications hinted at in the original Dickey 1949 paper (see last sentence of quote above) [7].

That the imprinting of macromolecules, despite its specific problems, quickly became a prime focus for MIP research is hardly surprising. The archetypal examples of specific molecular recognition and tight binding in nature, cases such as the biotin–avidin antibody antigen binding fragment Fab D1.3–lysozyme complexes, are after all proteins. They rely on complex networks of residues ideally positioned for multiple interactions between the two elements of the pair, analogous to the artificially built complex networks of ideally positioned functional monomers in the cavity that subsists in the MIP after removal of the imprinting template.

Some protein imprinting methods

Water-based gels can be formed from chemically inert, water-soluble biocompatible polymers, under mild conditions. Their porosity can be tuned by the degree of crosslinking. Reduced crosslinker content leads to easier diffusion of macromolecules into and out of imprinted complexes. The paradigmatic work began with imprinted proteins within polyacrylamide gels (3D), which were later sieved and the resulting particles packed into chromatography columns [20]. Polyacrylamide was selected due to its biocompatibility and chemical inertia towards protein. High rebinding selectivity was observed for four proteins.

Sol-gels are formed by the hydrolysis of silane derivatives under mild aqueous conditions and are thus another possible medium for protein imprinting. Pretreated PQC gold electrodes have been directly coated with a film of sol-gel, prepared using methyl- and phenyl-derivatised silanes as monomers, imprinted with human serum albumin [21]. The silanes presumably interact hydrophobically with the protein. The electrodes were then used as detectors of template rebinding (analysed by PQC impedance) and showed good selectivity, which may be further improved by using silanes with hydrogen-bonding functionality [5].

An alternative approach to 2D protein imprinting is not strictly a MIP but rather a protein-imprinted lipid monolayer [22]. An aqueous solution of ferritin was added to a Langmuir–Blodgett trough and the monolayer was formed at the air/water interface. Ferritin being an acidic protein, the lipids in the monolayer contained uncharged and cationic head groups. The dynamic nature of a lipid monolayer means that the lipids can reorder to form complementary interactions with the template.

Atomic force microscopy (AFM) has been used to directly probe imprinted cavities on the polymer with an AFM tip functionalised with covalently bound cognate protein [23]. Specific tip-cavity binding events were recorded as single, well-defined peaks on adhesion force curves recorded as the cantilever approached and then was retracted from the MIP.

Epitope-imprinted MIPs

It was soon realised that instead of imprinting whole proteins, which can be problematic when faced with lack of sufficient quantities of reasonably pure and concentrated material or with conformational stability problems, epitope technology can be used. Linear epitopes are short

continuous stretches of peptide chain, parts of antigenic proteins, which are specifically recognised and bound by antibodies. Thus, the MIP can be imprinted with an epitope rather than with the whole protein. This will make the cavities complementary to the epitope of the target protein and induce rebinding of the whole protein. The method was pioneered for peptide hormone oxytocin [24]. The method was adapted so that whole dengue virus protein could bind to the epitope-imprinted sites [25]. An additional, welcome property of that system, which is not the case with conventional whole protein imprinting, is that protein molecules orient themselves, because they can only bind via their epitopes. In this particular application, the correct orientation afforded advantages in using the system as a highly specific diagnostic system [25]. It is however easy to imagine how such a feature could be harnessed for other uses, such as protein crystallization. For example, one group [26] used synthetic peptides identical in sequence to the C termini of the imprinted proteins and tethered them covalently on a substrate to which the acrylamide-based polymerisation cocktail was added [26]. Photochemical polymerisation at 4°C resulted in an epitope-imprinted thin film.

Recently, particles epitope-imprinted with a fibronectin peptide sequence were shown to increase cell proliferation on a polymer film, because of enhanced fibronectin binding [27]. These results suggest possible use of such MIPs as bioactive scaffolds for tissue regeneration.

Imprinting of crystals for protein binding

Polyacrylamide polymer can be imprinted with lysozyme crystals rather than molecules in solution, producing crystal imprinted polymer (CIP) thin films [28]. Protein molecules are regularly oriented, virtually immobile and better able to retain their native (solution) conformation in the crystal and allow for better imprinting. The crystals are deposited on a cellulose membrane upon which is layered the prepolymerisation mixture. After polymerisation, the cellulose membrane is dissolved, leaving behind the crystal imprint. Surface plasmon resonance measurements show good selectivity for lysozyme compared with noncognate proteins and better selectivity than that of conventional lysozyme-imprinted MIPs.

Polymer has also been imprinted with protein-coated inorganic microcrystals, using a solvent used for small molecule imprinting but which would normally not be compatible with protein molecules in solution, namely acetonitrile [29]. Nonaqueous suspensions of haemoglobin- and myoglobin-coated potassium sulfate microcrystal templates generate highly selective imprints.

Both these unconventional imprinting methods are used for conventional protein binding and not to produce nucleation-inducing templates for further crystallization.

Imprinting of crystals for enhanced or controlled nucleation

Imprinting inorganic crystals results in polymeric templates capable of speeding up crystal formation and, in addition, of directing the process into forming crystal structures different from those that would form in solution at the tested conditions [30]. Divinylbenzene (DVB, the crosslinker) is polymerised with 6-methacrylamidohexanoic acid (the monomer) in the presence of calcite crystals of ~4 μm. The polymer monolith is ground to 100×100 μm particles and the template crystals washed out with methanol. Acrylic and methacrylic acids are less successful as functional monomers, possibly because of their smaller flexibility, making the matching of the spacing of ions on the crystal surface less easy.

Nucleation experiments were performed by mixing sodium carbonate with calcium chloride. The surface of the imprinted polymer contained more and larger calcite crystals than the control polymers. The difference in size was merely ascribed to the quicker nucleation, giving them more time to grow in the course of the experiment. Their morphology resembled that of the original template rather than that of spontaneously nucleating crystals in the solution at those conditions, that is, even at conditions favouring the formation of aragonite crystals, calcite was still formed at the imprinted sites. The reverse was however not observed.

Crystallization of the RNA base uracil induced by the imprinting effect was observed [31] when a poly(acrylonitrile-co-methacrylic acid) uracil-imprinted membrane was prepared in supercritical CO_2 (a highly tunable porogen) using phase-inversion imprinting technology [32]. The uracil-imprinted membrane formed flower-like crystals of uracil on the surface, but neither a nonimprinted membrane nor the membrane left in the uracil aqueous solution displayed crystal formation.

Protein crystal nucleation facilitated by MIPs

Accelerated growth of lysozyme crystals has been observed to occur upon exposure of a protein-imprinted polymer surface to a 20 mg/ml lysozyme solution [11]. The protein was imprinted on prereacted oligomeric mixtures by stamping. The mixture consisted of MAA, styrene, DVB, and azo-isobutyronitrile as a starter. Lysozyme is however a protein that can nucleate easily and rather generically on various substrates and coatings [33]. That intriguing observation was to the best of our knowledge not pursued further until 2011.

The first systematic and large investigation of the effect of MIPs imprinted with various proteins on a range of cognate and noncognate proteins was reported by the Chayen and Reddy groups and collaborators [3,34,35]. Water-based MIPs, prepared according to a protocol that had already been developed by the Reddy group [36] were used in that study. Acrylamide was the functional monomer, and *N*,*N*′-methylenebisacrylamide the crosslinker. These were dissolved in water in the presence of protein and polymerised with ammonium persulfate and tetramethylethylenediamine (TEMED). The resulting polymeric monoliths were crushed through a 75-μm sieve and the template protein was extracted with acetic acid:sodium dodecyl sulfate [3,36].

The MIPs, bulk (3D) imprinted with six different proteins, induced nucleation of nine different proteins at metastable conditions. Two sets of crystallization experiments were performed. The first set tested nucleation of model and target proteins at known metastable conditions, that is, at supersaturations just below those of known

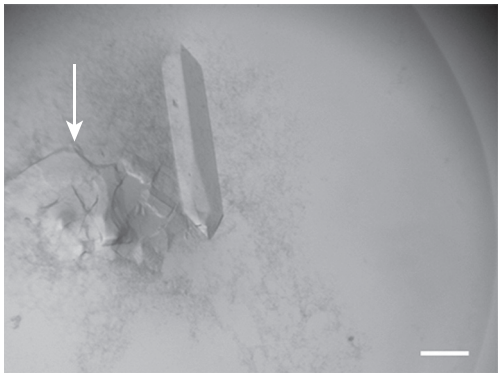

Figure 2. Human macrophage migration inhibitory factor crystal grown in a drop containing trypsin-imprinted molecularly imprinted polymer (MIP). The MIP is indicated by the arrow. Scale bar corresponds to 0.1 mm. Reproduced, with permission, from [3].

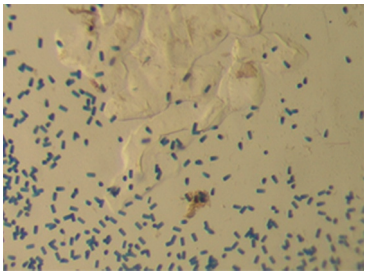

Figure 3. Crystals of α-crustacyanin obtained in the presence of cognate molecularly imprinted polymer (MIP) in screening trials. Adapted, with permission, from [3].

crystallisation conditions. The second set tested nucleation induction in a popular commercial crystallization screen, that is, against an indiscriminate series of sparse-matrix conditions.

In the first set of experiments, five different MIPs were tested against eight proteins. Lysozyme-imprinted MIP (L-MIP) induced nucleation not only of crystals of lysozyme but also of thaumatin, human macrophage migration inhibitory factor (MIF), and a complex of HIV proteins. Trypsin-imprinted MIP (T-MIP) induced nucleation of trypsin, lysozyme, thaumatin, MIF (Figure 2), the HIV complex, and DNA helicase RECQ1. MIF-imprinted and bovine haemoglobin-imprinted MIPs only induced nucleation of their cognate proteins. Only the catalase-imprinted MIP was unsuccessful against any protein including its cognate catalase, nucleation of which could not be induced by any MIP. Under the conditions that produced the crystals mentioned above, crystallization drops into which nonimprinted polymer (NIP) was introduced did not result in crystal formation.

In the second set of experiments, testing MIPs in screening, trypsin and MIF were again used, complemented by two additional target proteins, the intracellular xylanase IXT6-R217W and α-crustacyanin. The three target proteins yielded crystalline material under four to five conditions (out of a set of 48) in the presence of their cognate MIPs (Figure 3). L-MIP also gave two hits for MIF, and T-MIP gave three hits for MIF and one for the xylanase. In order to test whether the crystals would have been obtained in any case had the protein concentration been higher, the authors repeated the experiments without MIPs but with 15–30% higher concentrations of MIF and α-crustacyanin. These led to heavy precipitation, demonstrating that the successful conditions revealed by the MIPs would not have been discovered conventionally.

In discussing these results, it was pointed out that the success of MIPs with noncognate proteins is strongly correlated to similarities in size between template and crystallised protein. Another point is the liquid–liquid phase separation that is often observed on the MIP before the appearance of the first crystals. In these cases, the crystals first appear in the protein-rich phase-separated droplets [3].

In subsequent work [37], *N*-hydroxymethylacrylamide (NHMA) and *N*-isopropylacrylamide (NiPAm) were tested as functional monomers instead of the original acrylamide. Trypsin-imprinted poly-NHMA and polyacrylamide (but not poly-NiPAm) were successful in inducing nucleation of MIF and of thaumatin crystals, whereas all three trypsin-imprinted polymers could induce nucleation of trypsin and lysozyme crystals. All three haemoglobin-imprinted polymers induced nucleation of haemoglobin crystals, but not of the other proteins. In all cases in which crystals were obtained, those nucleated in the presence of poly-NHMA were larger than for the other two types of polymer, with the exception of lysozyme crystals, which were identical in size [37]. Thus, NHMA generally appears to be a superior functional monomer for protein crystal nucleation.

The above observations contribute to a complex picture of the binding of a protein to an imprinted cavity, a picture in which size, shape and specific electrostatic functionalities all play their distinctive roles.

This paper has covered various interesting uses of MIPs, with emphasis on the recent discovery that MIPs can be used to induce formation of protein crystals. MIPs are effective in yielding crystals where none are obtained in their absence, as well as producing higher quality crystals than those obtained with other techniques. The use of MIPs opens up a new scope for protein crystallization corroborating the hypothesis that by harnessing proteins as templates, MIPs become effective nucleation-inducing substrates.

Box 2. Outstanding questions

- Which is the precise mechanism by which MIPs induce nucleus formation?
- Is 2D imprinting more suitable than 3D imprinting for protein crystal nucleation?
- Can epitope (rather than whole protein) imprinting, provide in some cases an answer both to the scarcity of target protein material and to the need for better alignment of the protein molecules?
- What is the more effective strategy: preparing a different MIP for each target protein imprinted with that protein, or having a standard selection of MIPs imprinted with proteins of various sizes, shapes and properties and use them to nucleate crystals of non-cognate but similar proteins?
- Will MIPs prove useful nucleants for particularly problematic targets, such as integral membrane proteins, large protein complexes or unusually flexible proteins?

Concluding remarks

Questions and issues concerning MIPs as nucleants for protein crystallization are highlighted in Box 2.

An obvious possibility for future use of MIPs in aiding protein crystallization would be epitope imprinting, for those proteins for which such a possibility is available. Apart from dealing with the usual scarcity of protein samples, this method would also induce some ordering of the protein molecules, which might prove crucial for the formation of optimal nuclei.

Additionally, MIPs offer a yet untapped range of possibilities of functionalisation with, for example, hydroxyl, carboxylate [37] or amino groups. Furthermore, the functional monomer, the degree of crosslinking of the polymer, and the crosslinker itself can be widely varied [6]. These will obviously have a major effect on the porosity, the swelling properties and the protein binding capabilities of these gels. The already tested materials are therefore not the last word in terms of MIP possibilities and the scope for research into optimising these materials is open. For instance, chitosan/glutaraldehyde [38] and chitosan/*N,N′*-methylenebisacrylamide [39] systems, and a wide variety of crosslinking monomers such as tetraethyleneglycoldimethacrylate and polyethylene glycol 400 dimethacrylate [40] have been used for protein imprinting in other contexts and it will be fruitful to test such systems for nucleation.

Finally, protein crystal imprinting [28] or protein-coated microcrystal imprinting [29] as described above for conventional uses, could be used as a means for crystal optimisation of cognate protein, or (for the latter) to induce crystal nucleation of as yet uncrystallised cognate and noncognate proteins.

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