Corrections

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that their conflict of interest statement was omitted during publication. The authors declare that a patent application for the use of MIPs for crystallization has been filed.

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IMMUNOLOGY

The authors note that, due to a printer’s error, the author name Kai Fen Cheng should instead appear as Kai Fan Cheng. The corrected author line appears below. The online version has been corrected.

Ona Bloom, Kai Fan Cheng, Mingzhu He, Angelos Papatheodorou, Bruce T. Volpe, Betty Diamond, and Yousef Al-Abed

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NEUROSCIENCE

The authors note that the author name Alfred Awander should instead appear as Alfred Anwander. The corrected author line appears below. The online version has been corrected.

Daniela Perani, Maria C. Saccuman, Paola Scifo, Alfred Anwander, Danilo Spada, Cristina Baldoli, Antonella Poloniato, Gabriele Lohmann, and Angela D. Friederici

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PLANT BIOLOGY

The authors note that Hauke Holtorf should be added to the author line between Daniel Lang and Colin Brownlee. Hauke Holtorf should be credited with designing research and analyzing data. The corrected author line, affiliation line, and author contributions footnote appear below. The online version has been corrected.

Enas Qudeimat, Alexander M. C. Faltusz, Glen Wheeler, Daniel Lang, Hauke Holtorf, Colin Brownlee, Ralf Reski, and Wolfgang Frank

*Plant Biotechnology, Institute of Biology II, Faculty of Biology, Freiburg Initiative for Systems Biology, Faculty of Biology, and Centre for Biological Signaling Studies, University of Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany; †Biotechnologisches Gymnasium, Albert-Schweitzer-Schule, 78048 Villingen-Schwenningen, Germany; and marine Biological Association of the United Kingdom, Citadel Hill, Plymouth PL1 2PB, United Kingdom


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Protein crystallization facilitated by molecularly imprinted polymers

Emmanuel Saridakis*, Sahir Khurshid, Lata Govada, Quan Phan, Daniel Hawkins, Gregg V. Crichtlow, Elias Lolis, Subrayal M. Reddy, and Naomi E. Chayen

Laboratory of Structural and Supramolecular Chemistry, Institute of Physical Chemistry, National Centre of Scientific Research "Demokritos," Aghia Paraskevi, Athens 15310, Greece; *Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, United Kingdom; †Chemical Sciences Division, Faculty of Health and Medical Science, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom; and ‡Department of Pharmacology, Yale University, New Haven, CT 06520-8066

Edited* by Ada Younath, Weizmann Institute, Rehovot, Israel, and approved May 4, 2011 (received for review November 11, 2010)

We present a previously undescribed initiative and its application, namely the design of molecularly imprinted polymers (MIPs) for producing protein crystals that are essential for determining high-resolution 3D structures of proteins. MIPs, also referred to as "smart materials," are made to contain cavities capable of rebinding protein; thus the fingerprint of the protein created on the polymer allows it to serve as an ideal template for crystal formation. We have shown that six different MIPs induced crystallization of nine proteins, yielding crystals in conditions that do not give crystals otherwise. The incorporation of MIPs in screening experiments gave rise to crystalline hits in 8–10% of the trials for three target proteins. These hits would have been missed using other known nucleants. MIPs also facilitated the formation of large single crystals at metastable conditions for seven proteins. Moreover, the presence of MIPs has led to faster formation of crystals in all cases where crystals would appear eventually and to major improvement in diffraction in some cases. The MIPs were effective for their cognate proteins and also for other proteins, with size compatibility being a likely criterion for efficacy. Atomic force microscopy (AFM) measurements demonstrated specific affinity between the MIP cavities and a protein-functionalized AFM tip, corroborating our hypothesis that due to the recognition of proteins by the cavities, MIPs can act as nucleation-inducing substrates (nucleants) by harnessing the proteins themselves as templates.

Molecularly imprinted polymers (MIPs) are polymers formed in the presence of a molecule that is extracted afterward, thus leaving complementary cavities (or ghost sites) behind. The molecular imprint remains as a memory effect in the gel after the molecule is removed, and the cavities exhibit highly selective rebinding of the given molecule (1, 2).

MIPs were initially used for separation and purification of small molecules such as enantio separation of racemic mixtures in chiral compounds (3), separation of carbohydrate derivatives (4), and in thin layer chromatography (5). More recently MIPs have become an important tool in the preparation of artificial recognition materials that are capable of mimicking natural systems (6, 7). In the context of proteins, MIPs have been used for protein purification/isolation applications (8), replacement of biological antibodies in immunoassays (9 and references therein), catalysis (10), and biosensors for medicine (7 and references therein). MIPs, however, have never before been used to facilitate protein crystallization.

This study presents a previously undescribed approach to the use of MIPs by harnessing them as surfaces for inducing the formation of protein crystals. Protein crystallization is vital to the success of structural biology as well as structural genomics/proteomics projects worldwide that have set out to determine the structures of more than 100,000 proteins. Obtaining useful crystals remains a major bottleneck to progress (11); thus it is crucial to design improved means of producing the desired crystals.

The ultimate way to obtain high-quality crystals is to control their conception stage, namely their nucleation, which is the first step that determines the entire crystallization process (12). Once nucleated, crystal growth is optimal at metastable conditions, where crystals do not nucleate spontaneously but existing nuclei will grow in a controlled manner that will minimize structural defects. Crystallization at metastable conditions can be induced by inserting crystal seeds into the trials (e.g., ref. 13); however, this requires the availability of crystals of the given protein or at least some crystalline material to start with. In an ongoing search for alternative heterogeneous seeding materials, a variety of substances such as minerals (14), horse (15) and human (16) hair, thin films (17), charged surfaces (18, 19), mesoporous materials (20–22), and other materials (23) have been used as nucleants with varied success. The problem with such materials is that they are random substances, which have helpful properties such as porosity, nanostructure, or electrostatic attractive potential, but no designed specificity for proteins. Our hypothesis was that MIPs would be very likely to serve as ideal nucleants, because they are designed to specifically attract their template protein.

This paper reports crystallization experiments performed with various model and target proteins, which demonstrate the effectiveness of MIPs as nucleants for protein crystallization. The mechanism of action of MIPs, based on atomic force microscopy measurements and on recent work on protein crystal nucleation, is also discussed.

Results

Crystallography. The MIPs made for this work were referred to as HydroMIPs (hydrogel-based MIPs) because they are water-based and thus suitable for imprinting biological molecules (see Materials and Methods and SI Text). The HydroMIPs were imprinted with seven proteins, namely lysozyme, trypsin, catalase, haemoglobin, intracellular xylanase IXT6-R217W, alpha crustacyanin, and human macrophage migration inhibitory factor (MIF). These will be referred to here as L-MIP, T-MIP, C-MIP, BHb-MIP, IX-MIP, AC-MIP, and MIF-MIP, respectively. Nucleation-inducing properties of the MIPs were investigated on 10 proteins. Each MIP was tested for its nucleation-inducing capability on its own cognate protein as well as on others, as detailed in


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*This Direct Submission article had a prearranged editor.

†To whom correspondence may be addressed. E-mail: s.reddy@surrey.ac.uk or n.chayen@imperial.ac.uk.

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Tables 1 and 2 and below. For every MIP created, a nonimprinted polymer (NIP) was also produced using the same procedure but without the protein template, in order to serve as a control for the MIP. Additional controls without any polymer were also set up. Table 1 shows the results of experiments performed at metastable conditions. The crystallization conditions are detailed in SI Materials and Methods.

**Complex of HIV proteins.** Trials in the presence of L-MIP produced crystals that diffracted up to 4.2 Å. Previous attempts to crystallize this complex using conventional and unconventional methods, as well as known nucleating agents, had failed to produce crystals with diffraction beyond 9 Å. T-MIP also produced crystals but not as well diffracting as the L-MIP.

**Human MIF.** Crystals formed within 8 d at 1.15 M ammonium sulfate in the presence of MIF-MIP, L-MIP, and T-MIP (Fig. 1). All other trials remained clear for at least 2 mo. At 1.1 M ammonium sulfate and below, all drops remained clear. At 1.20 M ammonium sulfate, drops with NIPs also gave crystals but the controls remained clear. At 1.25 M ammonium sulfate and above, all trials produced crystals, with crystals appearing faster in the controls. Above 1.2 M ammonium sulfate and below, all drops remained clear. At 1.25 M ammonium sulfate and above, all drops remained clear for at least 6 wk after setup. At 2.9% (wt/vol) PEG 3350, all drops remained clear, whereas at 25% (wt/vol) PEG 3350 all drops yielded crystals. In the controls and the drops containing NIPS they appeared after 7 d. The diffraction resolution limit of crystals grown with MIPs was 2.8 Å compared with 3.2 Å of crystals grown without MIPs.

**Haemoglobin.** Crystals formed within 5 d at 22.5% (wt/vol) PEG 3350 only in the presence of the BHb-MIP. At 20% (wt/vol) PEG 3350, all drops remained clear, whereas at 25% (wt/vol) PEG 3350 all drops yielded crystals. In the controls and the drops containing NIPS they appeared after 7 d. The diffraction resolution limit of crystals grown with MIPs was 2.8 Å compared with 3.2 Å of crystals grown without MIPs.

**RECQ1.** Yielded crystals, the first appearing within 2 d, at 15% (wt/vol) PEG 3350, only in the presence of T-MIP. At 14% (wt/vol) PEG all trials remained clear for at least 3 wk. At 16% (wt/vol) PEG 3350, drops with NIPs also gave crystals after 4 d with the controls and drops containing L-MIP remaining clear. At 17% (wt/vol) PEG and above, all trials gave crystals. The ones with MIPs were obtained faster. The diffraction resolution limit of crystals grown in the presence of MIPs was 2.0 Å compared with 2.3 Å of crystals grown without MIPs.

**Lysozyme.** Crystals formed within 4 d at 2.8% (wt/vol) sodium chloride only in the presence of L-MIP and T-MIP, but not in the presence of the other MIPs, NIP or in the controls. Below the metastable conditions at 2.7% (wt/vol) sodium chloride, all trials remained clear for at least 6 wk after setup. At 2.9% (wt/vol), controls remained clear and drops with NIPs also yielded crystals a day after the ones with MIP. At 3% (wt/vol) sodium chloride and above, which are labile conditions, all drops gave crystals, albeit sooner with the MIPs. The diffraction resolution limit of crystals grown in the presence of MIPs was 1.5 Å.

**Trypsin.** Crystals formed within 7 d at 13% (wt/vol) PEG 8000, 0.2 M ammonium sulphate, and 0.1 M sodium cacodylate at pH 6.5 only in the presence of T-MIP. Crystals also formed at 14% in the presence of T-MIP and L-MIP, but not in the presence of the other MIPs, NIP, or in the controls. At 12% (wt/vol) PEG all drops remained clear and at 15% and above all trials gave crystals with crystals appearing faster in the drops containing the MIPs. The diffraction resolution limit of crystals grown in the presence of MIPs was 1.5 Å compared with 2.3 Å of crystals grown without MIPs.

**Thaumatin.** Crystals formed within 1 to 5 d at 0.3 M and 0.4 M sodium/potassium tartrate only in the presence of L-MIP and T-MIP At 0.2 M Na/K tartrate, all trials remained clear for at least 4 wk. All trials gave crystals at 0.5 M Na/K tartrate and above, albeit later in the controls. The diffraction resolution limit of crystals grown with MIPs was 1.5 Å compared with 1.9 Å of crystals grown without MIPs.

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<th>T-MIP</th>
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✓ represent hits. The hit conditions are listed in SI Materials and Methods.
excessively depleting the catalase solution. This has been corroborated by spectrophotometric measurements at 280 nm of the concentration of protein a few hours after setup at metastable conditions, in drops containing T-MIP, C-MIP, and in controls. The concentration of protein was highest in controls (3.56 mg/mL), marginally lower in drops containing T-MIP (3.38 mg/mL), and appreciably lower in the presence of C-MIP (2.99 mg/mL). At higher supersaturations, C-MIP allows crystal growth when precipitation is already too heavy for crystallization without its presence. Other MIPs do not seem to promote catalase nucleation. This may be due to the much higher size of the catalase molecule, making it a far worse binder to noncognate MIPs, which thus neither promote nor inhibit its nucleation.

In order to compare the nucleation-inducing capability of MIPs with other known nucleants, experiments were repeated at the same conditions in the presence of human hair, horse hair, zeolites, and bioglass powder. No crystals were obtained in any of the trials containing these nucleants other than for lysozyme and trypsin, which at these conditions produced small crystals in the presence of human hair, horse hair, and the bioglass powder. The crystals obtained, however, were multiple and small compared to large single crystals that appeared in the drops containing MIPs.

Fig. 2 shows how the crystals often evolve from the MIP. Initially the drops with MIP are clear, after which there is a sequence of events: (i) first a separation of liquid phases occurs, forming protein-rich droplets on the MIP, which can reach a diameter of ca. 100 μm (Fig. 2A); (ii) after 6 d, crystalline aggregation is observed in these droplets (Fig. 2B); (iii) after 24 h single, large, and well-diffracting crystals appear from these protein-rich areas (Fig. 2B). The time of observing crystalline aggregation depends on the protein; for lysozyme and RECQ1, for example, the equivalent times were 3 d and 1 d respectively.

The Application of MIPs for Screening Experiments. In order to test whether MIPs would also be effective in finding new hits during initial screening, four proteins were screened in the presence and in the absence of their cognate MIPs. These encompassed three target proteins (alpha crustacyanin, MIF, and intracellular xylanase IXT6-R217W) and one model (trypsin). The index screen was chosen for this investigation because it is a popular diverse reagent crystallization screen that is widely used. The above-mentioned target proteins were selected because two of them have not produced useful crystals to date and the third (MIF) requires higher resolution crystals. The fourth, trypsin, which crystallizes with relative ease, was included to act as a comparison.

Experiments using solutions 1–48 of the index screen gave four to five hits for each of the three target proteins when their cognate MIPS were present (Table 2), whereas no hits were obtained in their absence. Four hits were obtained in the case of alpha crustacyanin, and five hits were attained for intracellular xylanase IXT6-R217W and MIF. In the case of trypsin the same two solutions produced hits with and without MIP. Only conditions that had crystals or crystallites were considered as hits (Fig. 3A and B). The hits appeared between 24 h and 4 d after setting up the trials. Control drops (i.e., without MIPs and drops containing NIPs) did not produce any hits after 4 wk and beyond. Other known nucleants such as horse hair (Fig. S1A), zeolites (Fig. S1B), human hair, and bioglass powder were tested at the conditions that gave hits with MIPs. Except in the case of trypsin these did not produce any hits after 4 wk either.

The results demonstrate that in the presence of MIPs, 8–10% of the screening trials of the target proteins produced hits that would have been missed even when other nucleants were applied.

Additional trials were set up to see if noncognate MIPs would also give rise to the hits (Table 2). T-MIP and L-MIP were added to screening trials at the conditions that gave hits for the three target proteins, and in the case of alpha crustacyanin, C-MIP was applied in addition to T-MIP and L-MIP due to the high molecular weight (320 kDa) of this protein. In the case of MIF, two hits were obtained with L-MIP and three hits with T-MIP. Intra- cellular xylanase IXT6-R217W produced one hit with T-MIP; and, as expected, no hits were obtained for alpha crustacyanin with these MIPs. The screening results are commensurate with those at metastable conditions in that MIPs of compatible size

![Fig. 1. MIF crystallization trials in the presence of T-MIP and NIP. The MIP and NIP have a translucent gel-like appearance. When added to crystallization drops, they spread out and can fragment. (A) A single MIF crystal grown in a drop containing T-MIP. The MIP is indicated by the arrow. Scale bar corresponds to 0.1 mm. (B) Drop containing NIP at identical conditions; no crystals are formed. Scale bar corresponds to 0.15 mm.](image1)

![Fig. 2. Progression of the formation of trypsin crystals on trypsin-imprinted MIP. (A) Phase separation, (B) crystalline aggregation at the protein-rich droplets (Bottom Left), and large single crystal. Scale bars correspond to 0.05 mm.](image2)
to the protein also give hits, albeit not as many as the cognate MIPs.

In order to test whether raising the concentration of the proteins would produce the hits without MIPs, all the trials that yielded hits were set up using 15–30% higher concentrations of MIF and alpha crustacycin (The intracellular xylanase IXT6-R217W could not be concentrated above 8 mg/mL, the concentration that was applied for the experiments.) Screening with the higher concentrations of the two former proteins led to heavy precipitation meaning that the MIPs were not only revealing hits that would have been otherwise missed, but also achieving this while consuming significantly lower concentrations of the proteins.

Atomic Force Microscopy (AFM) Binding Measurements. The results above demonstrate that our hypothesis that MIPs would work as nucleants has materialized. To test this beyond the practical evidence of crystallization, atomic force measurements were performed to assess affinity of protein to the MIPs and compare it with affinity to NIP and also to a polylysine control surface.

In a study totally unrelated to protein crystallization, El Kirat et al. (24) have recently shown that atomic force spectroscopy could be used to probe polyacrylamide based MIPs used for cytochrome c imprinting of thin film MIPs attached to a mica surface. The atomic force measurements of the MIPs in our study were on the bulk gel MIPs that were used for the crystallization experiments (described in SI Materials and Methods). NIP and bovine haemoglobin (BHb) imprinted polymer were tested and a polylysine-coated coverslip acted as a control.

Fig. 4 details representative force curves that were generated as a result of interactions that occurred between the AFM probes with BHb attached and the MIP sample. This is given as an exemplar figure of the hysteresis observed during the approach and retraction of the protein-modified AFM tip onto the MIP surface. Force curves for NIP exhibited similar profiles and differed only in the force value. The distinctive, single peaked retraction curve displayed suggests that a single type of host-guest binding event is occurring.

One of the most powerful ways in which a MIP effect can be defined is in relation to a NIP prepared in an identical manner to that of the MIP, but in the absence of the template molecule. For a given polymer surface, the repeat adhesion events were found to have a narrow force distribution about the mean force measured. We were able to discriminate between each polymer surface based on the force distributions recorded. A distinctive trend was observed. The polylysine control exhibited the smallest force, with a (mean) value of 13.51 nN (standard deviation ± 0.38) required to remove the AFM probe from the surface. A somewhat greater force of 18.90 nN (±0.31) was required to withdraw the probe from the NIP surface. The increase in attractive forces exhibited between the two samples can be attributed to the BHb showing a greater affinity to the polyacrylamide than to the polylysine. Most significantly, a force of 23.08 nN (±0.31) was required to withdraw the template-modified AFM tip from the cavity-containing MIP sample. This indicates that binding between these sites and the BHb molecule was occurring, which in turn resulted in a greater force being required to withdraw the tip from the sample.

Literature values for single protein-pulling experiments typically show force values of 400–600 pN (25). Our values are significantly greater due to the cyrogenic mode of preparation of the MIP and control samples. This was required in order to stabilize the hydrogel surfaces. The cryogenic preparation allows the surface to be frozen and the difference between the MIP and control surface remains the presence of cavities in the former and their absence in the latter. The results show that there is a stronger force of attraction between cognate protein-modified tip and MIP surface compared with control surfaces. The narrow standard deviation about the mean value measured for each surface adds further assurance that MIPs are behaving differently to control surfaces. It can therefore be concluded that

Fig. 4. A typical force-distance graph detailing the interrogation of MIP with a BHb-conjugated 10 nm (radius of curvature) silicon nitride AFM probe. The gray line shows the tip descending, initially without contact with the surface. At some point, the tip jumps into contact with the surface and indents into it. The black line shows the tip retracting: The adhesion/bonding between tip and sample causes the cantilever to adhere to the sample. As the retraction continues, the adhesion breaks. The cycle can then be repeated.
highly specific interactions were reproducibly occurring with each sample investigated using this technique.

**Discussion**

It has been shown that crystal nucleation may proceed in two steps, namely aggregation of molecules into a dense fluid droplet and then ordering. This lowers the height of the energy barrier for nucleation: Instead of a single, steep energy barrier that would occur if ordering of the molecules happened at the same time as their aggregation (the classical nucleation model), we would have two lower barriers if the two processes happened separately and in succession. We now have direct evidence of this mechanism, which ten Wolde and Frenkel (26) showed by simulation studies, Lutsko and Nicolaus (27) by theoretical considerations, and Vekilov (28, 29) by a variety of experimental and theoretical approaches. It seems that the MIPs, soon after their insertion (overnight for the cases of lysozyme, trypsin, and RECO1), promote aggregation of protein molecules, forming a protein-rich phase, which at a later stage becomes crystalline (Fig. 2).

It therefore appears that in these cases, MIPs may function by facilitating the nucleation and stabilization of droplets of the protein-rich liquid phase, at conditions that would be quite far from the liquid–liquid phase separation conditions in the absence of nucleant (i.e., in the bulk).

Lysozyme is one of very few proteins for which quantitative liquid–liquid demixing data has been obtained (30). From that data, it appears that liquid–liquid phase separation at 20 mg/mL lysozyme in sodium acetate buffer at pH 4.5 and 20°C (our conditions) requires at least 7% (wt/vol) NaCl, instead of the 2.8% (wt/vol) at which nucleation of protein-rich droplets and subsequently of crystals occurs in this study in the presence of MIP. The liquid–liquid demixing curve obtained at a concentration of 3% (wt/vol) NaCl by Muschol and Rosenberger (30) gets very close to 0°C for 20 mg/mL lysozyme.

No quantitative liquid–liquid demixing data exists for the other proteins in this study. However, no visible droplets or clouding of the drops could be observed under the microscope at any time during the experiments in the absence of MIP, not only at metastable conditions but also well within the spontaneous nucleation zone of conditions. This qualitatively supports the general structure of a globular protein phase diagram proposed by Muschol and Rosenberger (30) and by Asherie (31), who place the liquid–liquid demixing curve in the bulk well beyond (at much higher supersaturations than) the solubility curve.

The MIP cavities, although they have a well-defined shape, are randomly dispersed through the gel. They therefore cannot induce the protein molecules to orient themselves in a specific pattern, i.e., providing a surface for epitaxial growth. It seems however, that the MIP can pull together a sufficient number of those molecules in order to overcome the energy barrier for the first step of forming a (yet disordered) precursor. The fabrication method is such that we expect a very high density of cavities due to the abundance of protein mixed with the monomer, making isolation of the cavities unlikely. The second step, ordering of the nucleus, may be aided by immobilized protein molecules in the cavities attracting further protein molecules. If the attractive forces between immobilized protein and protein in solution are similar in magnitude to the protein–polymer forces, then rearrangement of the assembled molecules to form ordered nuclei becomes possible in spite of the disorder in the cavities’ orientations.

Another explanation of the effectiveness of the cavities that are not oriented in a way directly conducive to proper crystal packing is the possibility of a statistical effect with only a few of the cavities in favorable mutual orientations. Various studies (32, 33) have shown that a very small number (less than 12) of macromolecules can be sufficient for the formation of a critical nucleus. This explanation is supported by the results, showing crystals forming on some parts of the MIP and not throughout it. This is actually an advantage, because we desire one or few crystals, rather than many. It may well be that there is a combination of the statistical effect with the lowering of the energy barrier.

An issue that may arise is that the imprints will be single isolated receptor sites, many of which will be partially or wholly buried in the polymer structure, thus not allowing the protein molecules to access them and also preventing the crystals from growing due to lack of space. Indeed, some of the pores will be buried due to the nature of the imprinting procedure. But, for the purpose of protein nucleation it does not matter that some are buried because only a few pores are needed at the surface for nucleation to occur. In order to ensure that some of the pores are on the surface, the imprinting hydrogels are broken into smaller particles thereby exposing cavities on the particle surface.

In summary, AFM results demonstrate that there is a definite binding of protein to the cavities of the MIP and less so to the NIPs. The crystallization results follow this pattern, showing that in the presence of MIPs (i) crystals are formed in conditions that do not give crystals otherwise and (ii) crystals form faster in conditions that will produce crystals eventually. For crystals to grow in the presence of NIPs, the crystallization conditions need to be at a higher supersaturation than in the presence of MIPs, thereby yielding poorer quality crystals.

We have demonstrated that MIP nucleants can be used in twoways: (i) as a heterogeneous seed for growing crystals in the metastable zone of the crystallization phase diagram, where crystals do not spontaneously nucleate but can often grow to higher quality, and (ii) as an addition to standard screening conditions, where they can help to produce hits that would have been missed in their absence.

When embarking on the MIP experiments we expected that each MIP would work exclusively on its cognate protein and that it would be necessary to make a MIP for each protein to be crystallized. In practice, our results demonstrate that MIPs such as those imprinted with lysozyme and trypsin also induced the crystallization of other proteins with a molecular weight of the same order of magnitude. These observations promise further possibilities than initially envisaged, meaning that a MIP of one protein may be successfully used for other, size-compatible proteins. This is very important in the case of difficult to crystallize proteins, which are usually too scarce in supply for imprinting and would therefore benefit from the use of a related MIP.

The findings of this study open up a previously undescribed scope for protein crystallization corroborating our hypothesis that by harnessing the proteins themselves as templates, MIPs are effective nucleation-inducing substrates for both the screening and optimization stages of crystallization.

**Materials and Methods**

The materials required for the fabrication of the HydroMIP samples, the reagents utilized for the crystallization trials, and the information pertaining to the preparation of the proteins tested are all documented within SI Materials and Methods.

**HydroMIP Fabrication.** Traditional MIPs demonstrate their selectivity only when they rebind template in the organic solvent in which they were synthesized. These methods are therefore unsuitable for imprinting of biomolecules such as proteins, as they are denatured under such organic solvent conditions. The MIPs made for this work were therefore water-based MIPs, also referred to as HydroMIPs. They offer a compromise between the polymerization required for cavity formation and the need to keep protein structural integrity during imprinting. HydroMIPs are made of polyacrylamide, a nitrogen-containing member of the acrylate family of polymers, which is a suitable imprinting matrix for biological molecules, as it is water compatible, cheap, easily produced, and can be derivatized to introduce functional groups (namely hydroxyl, carboxylate, and amino groups) to better engineer the complementary interactions between the template molecule and the polymer (8).
HydroMIPs were prepared following a previously reported procedure (35) and with the intention of using as little protein sample as possible and at significantly smaller final volumes. For every MIP created, a NIP (non-imprinted polymer) was also created using the same material concentration as the MIP but without the protein template (SI Materials and Methods). The HydroMIPs and NIPs are translucent and have a gel-like appearance and texture.

Crystallization Experiments. The MIPs and other nucleants (human hair, horse hair, zeolites VPI-5 and MCM-41, crushed glass, and bioglass powder) were inserted into crystallization trials set up in hanging drops in either EasyXtal tools™ (Qiagen) or Linbro plates. These drops consisted of 1 μL protein solution mixed with 1 μL reservoir solution. Then 0.2 μL of polymer (as a viscous gel) was dispensed into these drops using a standard micropipette. The same polymer but not imprinted with protein (NIP) was also dispensed at the same conditions, as a control. An additional control without any polymer was also set up.

A simple “working phase diagram” was constructed for each protein (except for IXT6-R217W and alpha crustacycin, which were used only for the screening experiments) in order to find metastable conditions. Protein concentrations and buffers were kept fixed, and supersaturation was varied by spanning a range of precipitating agent concentrations. It was ensured that a suitable range of conditions, spanning from undersaturation to spontaneous nucleation, was being searched in each case.

For each protein and each tested condition, trials were set up at the same time from the same batch of protein at identical crystallization conditions with L-MIP, T-MIP, C-MIP, BHb-MIP, MIF-MIP, as well as with NIP and without polymer. Each combination was repeated in at least six different drops. Detailed methodology and the precise range of conditions for each of the proteins are documented in SI Materials and Methods.

Solutions 1–48 of the index screen (catalog no. HR2-144, Hampton Research) were used for screening experiments of trypsin, alpha crustacycin, MIF, and intracellular xylanase IXT6-R217W (details of the stock protein solutions are given in SI Materials and Methods). The experiments were incubated at room temperature (ca. 22 °C), and all trials that gave hits were repeated in at least duplicate to ensure reproducibility.

The HIV complex, REQ1, thymatin, and trypsin crystals were X-rayed at the Diamond Light Source on the MX beamline I04. MIF, lysosome, and haemoglobin crystals were X-rayed on the Rigaku 007HF-M X-ray generator at Imperial College London, operating at 40 kV and 30 mA, with very high frequency optics producing a spot size of less than 100 μm, Saturn 944+ CCD detector and Oxford Cryosystems 700 liquid nitrogen cryostream.

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Supporting Information

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SI Materials and Methods.

Materials. Thermaxon® coverslips and poly-L-lysine (0.1%) (polylysine) were purchased from Electron Microscopy Sciences. Unmodified silicon nitride atomic force microscopy (AFM) probes with an average spring constant of 0.03 N/m and a radius of curvature of 10 nm were purchased from Novascan Technologies. Silicon nitride probes of the same dimension and manufacturer were modified (using a polyethylene glycol cross-linker, PEG) with lyophilized bovine haemoglobin purchased from Sigma.

Acrylamide, ammonium persulfate (APS), N,N’-methylenebisacrylamide, SDS, and N,N’,N”,N”-tetramethylenediamine (TEMED) were purchased from Sigma. Acetic acid (AcOH) was purchased from Fisher Scientific.

Hen egg-white lysozyme (L 7651), thaumatin from Thaumato- coccus danielli (T 7638), bovine pancreatic trypsin (T 4665), bovine liver catalase (C 9322), and bovine haemoglobin (H 2500) were obtained from Sigma-Aldrich. RECO1 was provided by Opher Gileadi of the Structural Genomics Consortium, Oxford, UK. The production of the protein is detailed in ref. 1. Human macrophage migration inhibitor factor (MIF) was produced as described in ref. 2. Alpha crustacyanin was provided by Peter Zagalsky of Royal Holloway University of London (3) and intracellular xylanase IXT6-R217W from Geobacillus steathermophilus T-6 was prepared and provided by Vered Solomon from the Hebrew University of Jerusalem and Orli Tabachnikov and Yuval Shoham of the Technion, Haifa. HIV complex was prepared and provided by Dennis A. Veselkov, Julien Bergeron, Michael Malim, and Mark R. Sanderson of Kings College London, London, UK.

Hydrogel-Based Molecularly Imprinted Polymer (HydroMIP) Fabrication. In order to use as little protein sample as possible and at significantly smaller final volumes, 54 mg of functional monomer (acrylamide) and 6 mg of cross-linker (N,N’-methylenebisacrylamide) were dissolved in 1 mL of deionized water. Then 90 μL of this solution was added to 100 μL of a 5% (vol/vol) TEMED solution to create a 100 μL pre-MIP monomer solution containing 0.06 mg of protein. Two microliters of a 10% (wt/vol) PEG solution was added to the pre-MIP monomer solution, and the solution was purged with nitrogen for 5 min. Once the solution was degassed, 2 μL of a 5% (vol/vol) TEMED solution was added and the solution was left to polymerize (18 h) at room temperature.

For every MIP created, a NIP (nonimprinted polymer) was also created using the same material concentration as the MIP but without the protein template. After polymerization, the gels were put through a 75-μm sieve. The crushed gels were transferred into 1.5-mL centrifuge Eppendorf tubes and washed using five 0.5-mL volumes of reverse osmosis (RO) purified water and five 0.5-mL volumes of 10% AcOH:SDS solution to extract the protein. Eighty-five percent of the original MIP was recovered using this procedure. Each wash step was followed by centrifugation for 3 min at 2030 x g and the supernatants were discarded. The gels were then centrifuged for a further 3 min at 2030 x g in order to extract further supernatants. The remaining MIP was stored at 4°C.

Crystallization Experiments. The hanging drop crystallization trials were set up in either EasyXtal Tools™ provided by Qiagen or LINBRO plates with siliconized glass coverslips provided by Molecular Dimensions Ltd.

Conditions tested for the proteins were as follows:

- Human MIF at 11 mg/mL: from 1.0 to 1.6 M ammonium sulphate in 3% (vol/vol) isopropanol and 0.1 M Tris buffer, pH 7.5. The metastable conditions referred to in Table 1 correspond to 1.15 M ammonium sulphate.
- Lysozyme at 20 mg/mL: from 2% (wt/vol) to 6% (wt/vol) sodium chloride, all in 0.1 M sodium acetate buffer pH 4.5. The metastable conditions referred to in Table 1 correspond to 2.25% (wt/vol) NaCl.
- Thaumatin at 30 mg/mL: from 0.2 to 1.5 M sodium potassium tartrate in 0.1 M piperazine-N,N’-bis(2-ethanesulfonic acid) buffer, pH 6.8. The metastable conditions referred to in Table 1 correspond to 0.3 M NaK tartrate.
- Trypsin at 60 mg/mL: from 8–16% PEG of average molecular weight 8,000, 0.2 M ammonium sulphate, and 0.1 M Na cacodylate buffer pH 6.5. The metastable conditions referred to in Table 1 correspond to 14% (wt/vol) PEG 8 K.
- Haemoglobin at 60 mg/mL: from 20%–25% (wt/vol) PEG 3350 in 0.2 M MgCl₂ and 0.1 M Bis-Tris buffer, pH 5.5. The metastable conditions referred to in Table 1 correspond to 22.5% (wt/vol) PEG 3350.
- RECO1 at 9.5 mg/mL mixed with ADP at a 1:3 protein:ADP molar ratio: from 12% (wt/vol) to 20% (wt/vol) PEG 3350 in 0.2 M sodium bromide and 0.1 M bis tris propane buffer, pH 7.5. The metastable conditions referred to in Table 1 correspond to 15% (wt/vol) PEG 3350.
- Catalase at 12 mg/mL: from 4%–11% (wt/vol) PEG 6000, 5% (vol/vol) 2-methyl-2,4-pentanediol in 100 mM Tris buffer, pH 7.5. The metastable conditions referred to in Table 1 correspond to 6.5% (wt/vol) PEG 6 K.
- HIV complex. The conditions are confidential.

Solutions 1–48 of the index screen (catalog no. HR2-144) from Hampton Research were used for screening experiments of trypsin in 10 mM calcium chloride, 10 mg/mL benzamidine hydrochloride, and 25 mM Hepes pH 7.0 at 20 mg/mL, alpha crustacyanin in 0.1 M Tris-HCl pH 7.0, 1 mM EDTA, 10 mM NaCl at 10 mg/mL and 13 mg/mL, MIF in 20 mM TRIS pH 7.5, and 20 mM NaCl at 12 mg/mL and 16 mg/mL and intracellular xylanase IXT6-R217W in 50 mM Tris–HCl pH 7.0 and 0.02% sodium azide at 8 mg/mL.

The index screen reagent numbers listed in Table 2 correspond to the following conditions:

- 4: 2 M ammonium sulfate and 0.1 M BisTris pH 6.5
- 5: 2 M ammonium sulfate and 0.1 M Hepes pH 7.5
- 6: 2 M ammonium sulfate and 0.1 M Tris-HCl pH 8.5
- 8: 3 M sodium chloride and 0.1 M sodium acetate tri-hydrate pH 4.5
- 19: 0.056 M sodium phosphate and 1.344 potassium phosphate pH 8.2
- 23: 2.1 M DL malic acid pH 7.0
- 26: 1.6 M ammonium tartrate dibasic pH 7.0
- 27: 2.4 M sodium malonate pH 7.0
- 30: 1.5 M ammonium sulfate, 0.1 M BisTris pH 8.5, and 0.1 M NaCl
- 31: 0.5% wt/vol PEG 5 k MME, 0.1 M Tris-HCl pH 8.5, and 0.1 M sodium potassium tartrate
- 41: 25% wt/vol PEG 3350, 0.1 M sodium acetate pH 4.5
- 43: 25% wt/vol PEG 3350, 0.1 M BisTris pH 6.5
- 44: 25% wt/vol PEG 3350, 0.1 M Hepes pH 7.5
- 46: 20% wt/vol PEG MME 5000, 0.1 M BisTris pH 6.5

All experiments were carried out at room temperature (ca. 22°C) with the exception of RECO1, which was crystallized
at 4 °C. Spectrophotometric protein concentration measurements were performed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific Inc.) with 2-μL drops.

**HydroMIP Preparation Prior to AFM Analysis.**

HydroMIP imprinted with haemoglobin (BHb) and HydroNIP (nonimprinted polymer) control gels were made as described above under HydroMIP fabrication but without scaling down the final volumes.

Following the sieving, the MIP gels were washed with five 2-mL volumes of RO water followed by five 2-mL volumes of SDS/acetic acid eluant. Each wash/elution step was performed by centrifugation at 1,613 × g for 5 min using a Centaur II centrifuge (Fisher Scientific). All gels were diluted 1:1 with RO water. Fifty microliters of each gel sample was pipetted into an Eppendorf tube to which 50 μL of a 5% (vol/vol) acrolein solution was added, and the samples were placed in a Pelco Biowave microwave (Ted Pella Inc.) and treated under vacuum at 20 °C (plate temperature) and 250 Watts for 2 min (on), 2 min (off), and 2 min (on). A 100-μL volume of RO water was added to the samples, vortex mixed, and microcentrifuged for 5 min before being treated under vacuum at 20 °C and 250 Watts for 1 min in the microwave. The supernatant was discarded. The RO water treatments were repeated in triplicate. The samples were then dehydrated using a series of 100-μL methanol washes that increased in concentration sequentially from 5% (vol/vol) through to 95% (vol/vol) (at 5% increments) in an identical manner as the RO washes. Three 100-μL volumes of 100% methanol were finally employed in an identical manner to the previous dehydration stages, which were followed by the addition of three drops of propylene oxide. The samples were treated with three 100-μL volumes of hexamethyldisilazane (HMDS), (mixed, centrifuged for 5 min, and supernatant removed after each HMDS addition) with the final treatment leaving a small dry sample at the base of the Eppendorf tube.

Thermanox coverslips were dipped in 0.1% polylysine and allowed to air dry. A spatula was used to apply a small measure (ca. 0.1 g) of each HydroMIP and HydroNIP sample to a polylysine-coated Thermanox® coverslip, with the hydrogel spread homogenously across the surface of the coverslip. Each sample was then cryogenically treated as follows and stored in a dry chamber prior to analysis. A 1-μL aliquot of each gel suspension was pipetted onto 400 mesh, carbon stabilized. Formvar coated glow discharged copper grids. The grids were plunged into liquid nitrogen. Following the constant agitation of the sample in the liquid nitrogen for approximately 30 s, the grids were transferred to 100% methanol and agitated for approximately 20 s. The grids were then transferred to HMDS and again agitated for approximately 20 s.

**Force-Microscopy Measurements.**

As the hydrogel is a soft material, attempts to approach the gel with the AFM tip resulted in the gel being “pushed away” from the tip and leading to erroneous force information. A cryogenic preparation technique that had been successfully used for the immobilisation and subsequent electron microscopic imaging of soft biological tissues (4) was employed in this study. It did not afford visualization of the imprinting on the hydrogel with AFM, but presented itself as a suitable method to fix the hydrogel, thereby enabling reproducible measurements of force values that represent the interactions of protein with cavities in the cryoimmobilized MIPs. The AFM analyses were performed as described below.

An AFM Bioscope System (Nanoscope 3A, Digital Instruments) AFM mounted on an Axiovert 100 TV inverted microscope (Zeiss) was used in contact mode operation. The Axiovert light microscope was used to focus upon a sample region that was homogenous in appearance and devoid of any topographic features of extreme height that would impede the free movement of the cantilever across the sample surface. The probe was advanced toward the sample surface using the automated approach function. The tip was allowed to repeatedly touch and retract from the sample surface for 3 min, resulting in approximately 90 force curves. The process was repeated on the same sample in three different sample areas. For each experiment, 30 force curves were randomly selected (10 from each repeat). The binding events were quantified using a proprietary software package (NForceR) to determine the adhesion force between AFM probe and hydrogel sample and analyzed using Matlab software (Math Works). Each of the HydroMIP and HydroNIP samples, plus a polylysine-coated control coverslip, were interrogated in an identical fashion using protein (BHb) modified probes operating in the force measurement mode.


Fig. S1. Trials containing alpha crustacyanin with nucleants in solution 43 of the index screen, demonstrating clear drops. (a) Trial in the presence of horse hair. Scale bar corresponds to 0.1 mm. (b) Trial in the presence of zeolite. Scale bar corresponds to 0.2 mm.