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S. H. Mejias ; R. Mincigrucci ; A. Beratto-Ramos ; C. Svetina ; A. L. Cortajarena ; C. Masciovecchio

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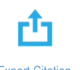
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S. H. Mejias,[1,2,a)] R. Mincigrucci,[3] A. Beratto-Ramos,[1,2] C. Svetina,[1,4] A. L. Cortajarena,[5,6,a)] and C. Masciovecchio[3,a)]

AFFILIATIONS

[1] IMDEA-Nanoscience, Campus de Cantoblanco, 28049 Madrid, Spain
[2] Centro Nacional de Biotecnología (CNB-CSIC)–IMDEA Nanociencia Associated Unit "Unidad de Nanobiotechnología," Cantoblanco, 28049 Madrid, Spain
[3] Elettra Sincrotrone Trieste, S.S. 14 km 163, Basovizza, 34149 Trieste, Italy
[4] European XFEL, Holzkoppel 4, 22869 Schenefeld, Germany
[5] Center for Cooperative Research in Biomaterials (CIC biomaGUNE), Basque Research and Technology Alliance (BRTA), Paseo de Miramón 194, Donostia-San Sebastián 20014, Spain
[6] Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain

[a)] **Authors to whom correspondence should be addressed:** sara.hernandez@imdea.org; alcortajarena@cicbiomagune.es; and claudio.masciovecchio@elettra.eu

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ABSTRACT

Understanding complex biological macromolecules, especially proteins, is vital for grasping their diverse chemical functions with direct impact on biology and pharmacology. Techniques such as x-ray crystallography and cryo-electron microscopy face limitations such as radiation damage and difficulties in crystallizing certain proteins. To address this, we present a strategic concept involving engineered protein scaffolds to create ordered arrays of proteins with controlled orientations, aiming at enhancing the signal at the detector. This innovative strategy opens avenues to solve the existing challenges for determining protein structures under physiological conditions. Moreover, it holds promise for studying conformational changes resulting from photoinduced changes, protein–drug, and/or protein–protein interactions. In the near future, our group will focus on showing the potential of the proposed strategy by combining molecular biology, protein self-assembly, and x-ray spectroscopy.

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The determination of protein and macromolecular structures has traditionally relied on x-ray crystallography. This technique necessitates the growth of high-quality crystals, which must be both large enough to effectively diffract x rays and withstand radiation damage.[1] Indeed, protein crystallization is challenging due to the diverse properties of proteins, including poor solubility, conformational flexibility, and sensitivity to experimental conditions. Over the past few years, the technical advancements in cryo-electron microscopy (cryo-EM) methodologies have triggered a resolution revolution in single-particle structural determination.[2] This method involves imaging samples at extremely low temperatures to reduce radiation damage and preserve the structural integrity of the biological specimens. One of the primary limitations of cryo-EM lies in the resolution attainable for smaller proteins or complexes, where achieving atomic-level details becomes increasingly difficult. Another critical concern revolves around sample heterogeneity. This challenge becomes particularly pronounced when dealing with complexes that exhibit flexibility or multiple conformational states. It is important to underline that both x-ray crystallography and cryo-EM are inherently unable to provide a real-time depiction of dynamic processes, which prevents studying rapid conformational changes or ultrafast interactions. Moreover, crystallization or cryogenic treatment of protein brings the specimen into environments that may be far from the physiological one.[3] The recent development of artificial

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intelligence-based platforms as AlphaFold is allowing the determination of protein structures starting from their amino acid sequence and is tremendously helping in the prediction of protein structures.[4] While AlphaFold represents a groundbreaking advancement, its current limitations include dependencies on existing structures, challenges with flexible regions, dynamic proteins and complexes, limited information on modifications, difficulties with membrane proteins, and constraints when dealing with large molecular assemblies. AI platforms can generate a single structure consistent with the learned patterns, but currently, they lack the ability to provide a range of alternative conformations influenced by factors such as pH, temperature, or the binding of ions, ligands, or other proteins. Experiments are still essential for assessing these effects, for which the proposed approach will allow overcoming current limitations.[5]

Significant technical efforts pursued at x-ray free electron lasers (XFELs) have made it possible to recently carry out experiments of serial crystallography (SC).[6] SC allows obtaining high-resolution structural information of proteins by collecting the diffraction patterns of randomly oriented small protein crystals, which are usually impossible to characterize with synchrotron radiation. SC was initially envisioned as a stepping stone toward single particle imaging: one of the major science drivers for the creation of XFEs, based largely on the promise of coherent diffractive imaging methods and the concept of *diffract-before-destroy*.[7] Atomic-scale structure determination of individual particles via coherent diffractive imaging holds significant scientific promise for biology by revealing the structure (and possibly dynamics) of complexes under near-physiological conditions. The concept proposed in Ref. 7 is currently hindered by technical limitations, such as the very low number of photons diffracted and collected by the detector.

It is crucial to emphasize that none of the aforementioned methods enable the determination of a protein's structure under physiological conditions, particularly during interactions with other molecules such as drugs or other proteins. Of paramount importance is also understanding how external stimuli can affect the structure and dynamics of protein, for example, the study of the dynamic behavior of photosystems for photoprotection.[8] A deep understanding of these mechanisms is key for the photosystems behavior and their manipulation.

In this paper, we show a new concept for a sample delivery approach, based on the use of engineered scaffolds, involving the fabrication of sample supports designed to host the target protein in a near-native or dry environment. The concept revolves around achieving an array of aligned proteins enhancing the emitted signal well above the detectors sensitivity. This would allow measurements of macrosystems in a hydrated environment maintaining conditions close to physiological settings during data acquisition similar to the idea previously proposed by our group.[9] In that work, the proteins were presumed to be tethered to a substrate using techniques such as split-intein mediated ligation. However, that method does not guarantee the complete absence of angular jitter among the deposited proteins. The ideal chemical binding method employed for protein attachment to the surface must ensure a 2D-ordered layer on the generated arrays, preserving the protein in its biologically active state. Nano-patterning and nano-deposition with nanoscale precision is nowadays possible with current technologies but, in general, requires expensive lithography methods and can only be done in relatively small sizes, which makes this possibility quite impractical. On the other hand, the scaffolding approach introduced here is expected to reduce the conformational entropy of the arranged target proteins or complexes, thereby promoting conformational homogeneity in flexible proteins and complexes. By constraining degrees of freedom, scaffolded structures facilitate the study of dynamic proteins, allowing for easier characterization and analysis of structural dynamics. Moreover, this approach will also enable *in situ* studies of protein responses to binding events or interactions with drugs, providing valuable insights into biological processes at room temperature.

To generate the proposed arrays, we suggest a new methodology that uses a bottom-up approach combining site-specific protein modification and encoded protein self-assembly. Based on this proposal, first, template protein scaffolds are modified with peptide binding sites to anchor the target protein of interest with site and orientation specificity.[10,11] This anchoring strategy will generate linear nanostructures of the target protein that expand through different length scales (from ~3–20 nm at the molecular level to hundreds of nm (μm) at the supramolecular level), where the distance and orientation are controlled by the linking position engineered in the scaffold. For transferring the nanostructure to the macroscale, we will encode self-assembly properties to the template scaffolds, introducing interaction interfaces between them that generate ordered 2D or 3D-dimensional arrays. In these arrays, the nanostructure is expected to be ordered by peptide anchoring at the nanoscale. It has been demonstrated, through the assembly of efficient functional multi-enzymatic pathways, that this scaffolding strategy is compatible with the preservation of the structure and function of the scaffolded proteins.[10]

Even if our strategy is still at the conceptual level, our plan is to use engineered proteins, and in particular Consensus Tetratricopeptide Repeat Proteins (CTPRs) [Fig. 1(a)], to validate the proposed approach. These proteins are composed of tandem arrays of 3–20 modular repeats that form superhelical structures of different lengths. The secondary and tertiary structures of these modular proteins are determined by a few amino acids. Thus, the rest of the amino acids can be modified without disrupting the protein structure. This structural control has made it possible to genetically encode protein functionalities to link nanoparticles or chromophores at specific protein positions, using engineered CTPR proteins as biomolecular templates.[12–14] Moreover, these scaffolds show "head-to-tail" and "side-to-side" interactions that have been used to form nanostructured supramolecular materials.[12–15] These inherent self-assembly properties have been engineered to encode tailored hierarchical assemblies, such as linear fibers,[16] 3D nanotubes,[17] tightly packed monolayers,[18] or anisotropic films.[15–18] For the film formation, the most used fabrication strategy is by drop-casting, where a drop is deposited on a substrate dried under controlled conditions, allowing the proteins to self-assemble. A more accurate approach combines drop casting and spin coating to achieve a complete control over the thickness and homogeneity of the film, from monolayers to multilayers of μm-thickness.[19] The methodology was optimized on silicon and SiO_2 substrates but can be translated to a substrate of choice upon optimization of depositing conditions, as we have demonstrated, for example, by depositing materials on a gold substrate.[12] In addition, we have previously shown that CTPRs can be used to generate well-organized 2D thin

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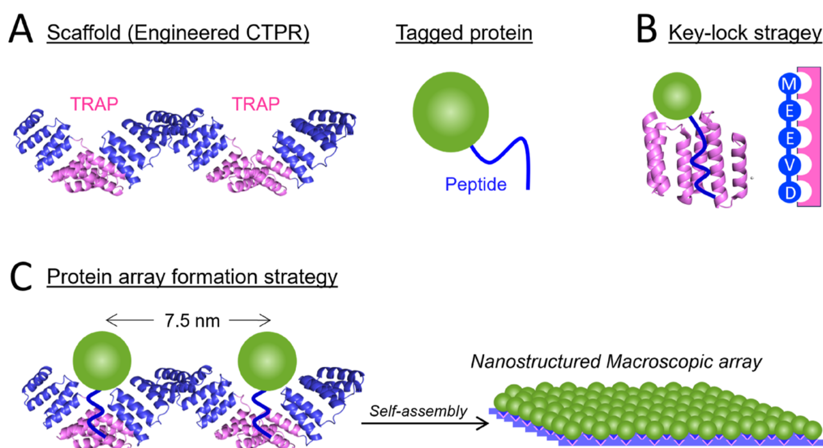

FIG. 1. (a) Designed CTPR scaffold with TRAPs peptide binding sites are indicated by pink. Tagged targeted protein with peptide is indicated by blue. (b) TRAP scaffold-based key-lock strategy with the engineered MEEVD peptide sequence for TRAPs binding. (c) Targeted protein nanostructure ordered by engineered CTPR protein. Macroscopic array formation led by CTPR protein self-assembly.

films through co-assembly with amphiphilic peptides, resulting in highly ordered peptide–protein architectures with tunable dimensionality.[20] These results provide proof of principle for the new methodology proposed in this paper, highlighting CTPRs as ideal scaffold substrates to generate hierarchical assemblies. Building on this knowledge, we will take advantage of the functional flexibility and self-assembly properties of CTPR scaffolds to encode protein functional arrays with precise spatial order (Fig. 1). A CTPR16, with 16 TPR repeats, will be decorated with tetratricopeptide affinity repeat proteins (TRAPs), creating a selective scaffold for peptide-tagged target proteins[10,11] [Fig. 1(a)]. TRAPs are composed of the same TPR motif as CTPRs and include a binding pocket optimized for peptide recognition.[11] Previous studies have shown that TRAPs bind peptides with micromolar (μM) affinity and have been successfully used to generate ordered enzyme assemblies.[10,11] In our proposal, we leverage two key properties within a single scaffold [Fig. 1(a)]: (1) the self-assembling capability of CTPRs and (2) the peptide-binding functionality of TRAPs. This chimeric protein is the foundation of our design. The target protein is linked to the CTPR16 scaffold selectively by following a key lock strategy [Fig. 1(b)]. The size of the CTPR16 allows us to control the distance and orientation of the target protein at the nanoscale to optimize the nanostructure having at least 7.5 nm between proteins on an initial design leaving enough space for protein conformational freedom [Fig. 1(c)]. We will use the self-assembly properties of the CTPR to scale the order from the nanoscale to the macroscale [Fig. 1(c)]. The proposed approach allows us to generate 2D and potentially 3D arrays of well-ordered proteins for the x-ray experiments. While 2D arrays are attractive for studying protein structure and dynamics as all proteins face an external layer that is free to interact, 3D structures can be attractive to further enhance the x-ray signal following the same interference process as in Bragg diffraction generated in bulk crystals. Finally, CTPR scaffolds could be additionally engineered to modify their assembly properties by engineering protein–protein contacts, achieving scaffolds that assemble selectively in one or two dimensions, which could be useful to decrease the conformational entropy of flexible proteins.

As a strategy to validate our method of aligned protein sample, we propose to use two complementary diffraction techniques that have been employed to study 2D crystals: (1) Grazing Incidence X-ray Diffraction (GIXD, Fig. 2(a)[21] and XFEL diffraction [Fig. 2(b)].[22]

GIXD has already been used to measure 2D arrays of membrane proteins obtained by adsorbing proteins to ligand–lipid monolayers at the surface of water.[21] However, the latter process can be applied only to a few membrane proteins, and while strategies have been proposed to ameliorate it,[21] such as the use of cross-linkers, the achieved resolution stops at 10 Å due to the dynamic disorder. The use of chemical cross-linkers, which suppress thermally activated structural fluctuations, one of the primary sources

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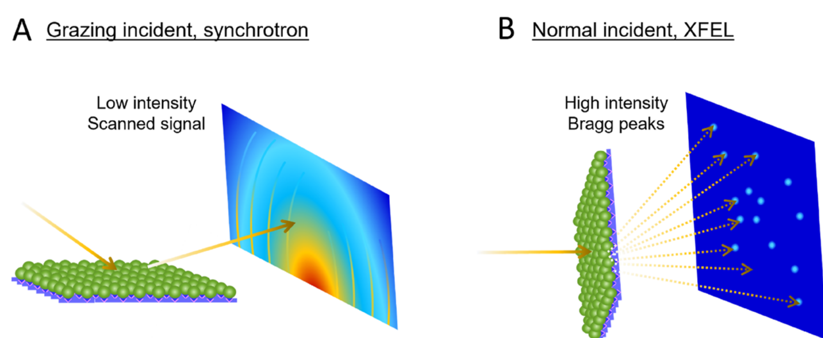

FIG. 2. (a) X-ray diffraction experiment based on grazing incidence experiments using synchrotron radiation. (b) Bragg peaks obtained in XFEL experiments based on a high-energy single shot.

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of dynamic disorder, can be readily incorporated into our protein assembly strategy. For example, our proteins can be functionalized with cysteines and cross-linked using BMB-type linkers to stabilize the assembled arrays and fix the protein structure. The linker length can be tuned to balance coherent protein fluctuations, allowing time-resolved diffraction with structural disorder. Alternatively, non-specific cross-linkers such as Di-(N-succinimidyl) glutarate (DSG) or similar cross-linkers can also be used, although they result in less controlled labeling. The conceptual extension of Ref. 22 to our engineered arrays supports the feasibility of our proposed approach when using GIXD at synchrotrons.

Free-electron laser diffraction in the Laue configuration has already been demonstrated experimentally on two-dimensional bacteriorhodopsin crystals[22] mounted on a solid support and kept at room temperature. Bacteriorhodopsin is notable for its natural assembly into two-dimensional crystalline patches, providing a well-characterized platform for high-resolution structural studies.[23] While this technique permits the use of standard Bragg diffraction setups, the high pulse intensities required to generate signals in the detector will inevitably destroy the sample upon irradiation, following the concept of diffract-before-destroy.[7] This is not the case for GIXD at synchrotrons, where longer acquisition times are feasible without detrimental effects on the proteins. The result reported in Ref. 22 suggests that the technique is applicable to other artificially engineered two-dimensional protein assemblies. In that study, the attainable resolution was limited to ~7 Å, primarily due to the weak scattering signal from submicron-sized bacteriorhodopsin crystals, which led to insufficient intensity at higher-order Bragg reflections. This limited signal strength resulted in negligible intensities at large-angle Bragg reflections, constraining the structural information that could be extracted. We emphasize that the approach proposed here involves the use of significantly larger two-dimensional crystals, which enables the collection of diffraction data with markedly improved statistical quality compared to Ref. 22, where only 11 of 334 collected images met the required quality threshold. This improvement arises from the fact that larger crystals enable the collection of a significantly greater number of high-quality scattering events, while the use of uniformly oriented crystalline arrays ensures that diffraction images are acquired from identically aligned domains, thereby enhancing data consistency and signal strength. In addition, raster scanning of the sample further enhances data acquisition by systematically sampling different regions of the crystal. For instance, a sample area of 100 mm^2 is expected to yield at least a 10^3-fold increase in integrated scattering signal relative to the conditions in Ref. 22, thereby substantially improving the overall data quality and resolution potential. An additional improvement is anticipated through the use of attosecond-duration pulses, which are now achievable at x-ray free-electron lasers (XFELs).[24] Radiation damage to protein samples begins to occur within ~500 attoseconds after exposure,[25] implying that photon arrivals beyond this timescale contribute disproportionately to noise. In Ref. 22, the XFEL pulse duration was ~50 femtoseconds, suggesting that a significant fraction of the incident photons did not contribute to useful signal. Based on this, two-order-of-magnitude improvement in signal efficiency is expected by reducing pulse duration. Recent XFEL advancements have demonstrated the routine generation of 200 attosecond pulses with energies of ~100 μJ at 9 keV,[26,27] further supporting the experimental viability of this approach. Compared to the 2 mJ pulses used in Ref. 22, this represents an opportunity to collect a greater number of useful photons while mitigating damage. Moreover, employing beam spot sizes greater than 100 μm could further reduce local radiation damage by lowering the electric field strength at the sample surface. In the previous configuration,[22] tightly focused 2 mJ pulses over 0.1 μm^2 areas likely induced plasma formation within less than 1 fs,[28] compromising structural integrity. The combination of larger beam areas, lower pulse energies, and attosecond pulse durations provides a promising direction for minimally invasive, high-resolution structural studies of large-area 2D protein crystals.

In these proposed methodologies, both the scaffold and the protein/complex of interest contribute to the generation of Bragg peaks. While this may introduce some complexity in the structural determination of the specimens, the ability to measure the signals separately from the scaffold layer could aid in distinguishing signals from the two systems. Furthermore, it could serve as a reference field to assist in solving the phase problem during reconstruction similarly to holography where a known spherical wave is artificially generated and used as Ref. 29.

With this paper, our aim is to establish the basis of an innovative methodology for delivering two-dimensional, aligned arrays of biomolecules. Our approach integrates advances in protein design and x-ray spectroscopy into a unified conceptual framework, showing a novel approach that could enable a breakthrough in the structural characterization of proteins. In the near future, our group will focus on showing its potential and the applicability of structural biology techniques to macromolecules, especially proteins, which are traditionally challenging to crystallize. It potentially enables the utilization of synchrotron and x-ray free-electron laser to reconstruct protein structure under physiological conditions and study its function while interacting with ligands in real time. Finally, our approach requires only a moderate sample quantity, making it advantageous for challenging or expensive-to-produce proteins.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

S. H. Mejias: Conceptualization (equal); Methodology (equal); Writing – original draft (equal). **R. Mincigrucci**: Conceptualization (equal); Methodology (equal). **A. Beratto-Ramos**: Conceptualization (supporting); Validation (equal). **C. Svetina**: Conceptualization (supporting); Methodology (supporting); Writing – review & editing (supporting). **A. L. Cortajarena**: Conceptualization (equal); Writing – original draft (equal). **C. Masciovecchio**: Conceptualization (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal).

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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