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Influence of the Chirality of Short Peptide Supramolecular Hydrogels in Protein Crystallogenesis

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For the first time the influence of the chirality of the gel fibers in protein crystallogenesis has been studied. Enantiomeric hydrogels 1 and 2 were tested with model proteins lysozyme and glucose isomerase and a formamidase from B. cereus. Crystallization behaviour and crystal quality of these proteins in both hydrogels are presented and compared.

Chirality is ubiquitous in nature and has major implications in cell interactions and biological processes. Within this topic, chiral discrimination on chiral surfaces has significant effects in protein adsorption and cellular adhesion and proliferation. It would be expected that similar chiral discrimination were relevant in 3D-environments, such as in protein crystallogenesis. Remarkably, the expected influence of the chirality in protein crystallogenesis remains unexplored probably owing to the difficulty to obtain a pair of enantiomeric environments suitable for crystallization. In this sense, the use of stereochemically well-defined fiber hydrogels can be an ideal media to study the influence of the chirality in the process of crystal growth. Recently, peptide hydrogels have found useful applications in biology due to their biocompatible and biodegradable nature. It has been shown that these supramolecular fibrous aggregates interact selectively with proteins and also reduce denaturation. Considering this, we wondered if supramolecular hydrogels based on the self-assembly of short peptides could be compatible with the process of protein crystallization since gels are used as an excellent media for crystallization. It is known that peptides hydrogels are able to form stereochemically well defined 3D ordered structures and, therefore, the inherent chirality of peptides can interact diastereoisomerically with proteins, thus expecting different behaviors. It is worth noting that the structural simplicity of short peptides is essential for this purpose, allowing an easy access of their corresponding enantiomers. This property is not feasible with other common macromolecular hydrogels, which either have no stereocenters (polyacrylamide) or are constituted by the natural chiral product (agarose).

To test this hypothesis we selected two cysteine-based peptides, compound 1 (N,N'-di(benzoyl)-L-cysteine diamide) and its corresponding D enantiomer 2 (N,N'-di(benzoyl)-D-cysteine diamide) (Figure 1) due to their capacity to self-assembly in neat water to give the corresponding hydrogels. They were tested with two model proteins, chicken HEWL lysozyme and glucose isomerase, and one target protein, a formamidase from B. cereus being the first time that supramolecular peptide based hydrogels are used for this purpose. Additionally, the results were compared with crystal growth in agarose gel to investigate their potential in protein crystallization.

Figure 1. Characterization of hydrogels 1 and 2 by TEM microscopy, circular dichroism and rheology. TEM images of negatively stained dried hydrogels at 3 mM: A) 1; B) 2. Scale bar at 0.2 µm. C) CD spectra and D) rheology of hydrogels 1 and 2.
10 to 20 nm and lengths that go up to μm in size (Figure 1). Fibrous aggregates co-exist with regions where fibers are more dispersed. Hydrogel 2 presented fibers of the same aspect. To gain insight into the molecular arrangement of these hydrogels circular dichroism (CD) spectra were measured (Figure 1). Hydrogel 1 exhibited a negative band near 218 nm (π* transition), a positive band near 250 nm (nπ* disulfide band and/or ππ* of the aryl groups) and a negative band near 280 nm (π* of the aryl groups) in good agreement with the CD spectrum from other oligopeptides hydrogels identified as the β-sheet conformation. The CD spectrum of hydrogel 2 is the specular image of hydrogel 1 showing that the chirality of the peptides is conserved and transferred supramolecularly to the fibers. Hydrogels 1 and 2 were also analyzed using a regime of simple oscillatory shear stress through frequency (see Supporting Information) and amplitude sweep tests. Both hydrogels showed values of G’ (elastic modulus) higher than G” (viscous modulus) indicating the predominant elastic behavior of the gels (Figure 1). Based on the magnitude of the values of G’, G” and σc (G’ ≈ 30 Pa; G” ≈ 4 Pa; σc < 1 Pa) we can conclude that these hydrogels are extremely weak. The similar values of G’ and G” for hydrogels 1 and 2 confirm that both enantiomeric hydrogels have the same rheological properties. VT-NMR studies for both hydrogels showed that the signals of the aromatic region were sifted 0.5 ppm to downfield as the temperature increased from 25 °C to 70 °C. The small values of T2 (1.84 s for 7.74 ppm signal) and the negative values of nOe indicated that the 1H-NMR signals corresponded to aggregates of the di-peptide which are not higher enough to be NMR-silent.

Lysozyme crystals grown in hydrogel 2 were better than those obtained in hydrogel 1, reaching the resolution limit of the detector (0.95 Å) and having the best quality indicators in terms of I/σ(I), Rmerge and mosaicity (Figure 2B). It is also worth mentioning the excellent reproducibility of crystals grown in hydrogel 2 (Figure S1 and Table S2). Moreover crystal quality indicators of crystals grown in agarose (able to produce protein crystals of high quality) were similar than those obtained in hydrogel 1 but still inferior to hydrogel 2. Agarose gel has been proven to facilitate the soaking of different compounds including cryo-protectants. In order to evaluate the possible cryo-protection capacity of these new hydrogels we compared the crystal quality of cryo-protected and “naked” (flash frozen without cryo-protectant) lysozyme crystals grown in hydrogel 1 and agarose from two data sets collected at beam-lines ID23 and BM30 (ESRF). It is important to notice that the polymeric nature of both agarose and hydrogel 1 allowed us to collect full data sets without needing to use any additive (Figure S2, S3 and Table S3).

A similar analysis was performed with glucose isomerase crystals grown in hydrogels 1, 2 and agarose. In this case crystals obtained in hydrogel 1 and agarose were more homogeneous and of better quality than those grown in hydrogel 2 (Figure 2C, Table S4 and Figure S4). However, a second crystal form (P212121) was identified only in the experiments run with hydrogel 2 that co-exist with the other polymorph (I222), the form normally found under similar crystallization conditions. Dauter and co-workers have described a new crystal form (P212121) of glucose isomerase and have solved its structure (PDB ID. 1OAD). Remarkably, the polymorph found in hydrogel 2 presents different unit cell parameters (Å): 86.00, 93.68, 99.22 versus 98.45, 129.59, 78.33 for a, b and c respectively (Figure S4). To date there is only
protein crystal lattice occurs with agarose, silica and PEG. The quality of this primitive orthorhombic crystal was the highest of all glucose isomerase crystals tested, with the structure determined at 1.20 Å and refined to R/Rfree values of 10.73/13.22 (%) (Table S5). When compared with the model used for molecular replacement (PDB: 1OAD) the deviation of the main chain is minimal (rmsd of 0.3). It is worth mentioning that this new polymorph has only been obtained in free solution using other precipitant, as in the case of the PDB ID: 1O1H that crystallized using MPD, or in the presence of high agarose concentration. In our case, under identical crystallization condition, the P2₁2₁₂₂ polymorph was obtained only with hydrogel 2 and not in agarose at 0.5% (w/v), our control, suggesting a novel interaction between the chiral gel fibers and the protein at the nucleation stage. It has been previously demonstrated that the nature of the gel fibers, such as agarose and silica, affect protein nucleation density (promoted and inhibited respectively) with no effect on the final crystal packing i.e. formation of new polymorph. Up to date, the correlation between the protein-fiber interaction and polymorphism is unknown. Taking into account that both, chemical composition and the physical properties of the media are identical (hydrogels 1 and 2), we hypothesized that the formation of a different polymorph has to be an effect of the fibers’ chirality in the crystallization process. The formation of the orthorhombic polymorph of glucose isomerase and the growth of the highest quality crystals of formamidase exclusively in hydrogel 2, support this hypothesis. This fact highlights the relevance that chirality may have in protein crystallogenesis for X-ray structural determination. Moreover these supramolecular peptide based hydrogels are excellent mediators for protein crystallization producing lysozyme and glucose isomerase and formamidase crystals with excellent quality indicators. Peptide based hydrogels also acted as a cryo-protectant avoiding the use of additives. Hence, these novel chiral gels would expand the field of protein crystallogenesis.

Notes and references

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Figure 3. Dissolution sequence of a lysozyme crystal grown in hydrogel 1. Crystal extracted from the gel was placed in a water drop and allowed to dissolve (t = 0 sec). A red square is drawn around the remaining hydrogel. The last picture was taken after partially drying of the remaining gel framework.

Finally, formamidase from B. cereus, was selected as a target protein to test its crystallizability using hydrogel 1, 2, and agarose as a control. Good diffracting crystal was already obtained by capillary counter diffusion method diffracting to a maximum resolution of 1.78 Å (Table S6). From previous experiments we knew that agarose used in combination with capillary counterdiffusion did not improve or even fully impeded crystallization. Crystals grown in three different crystallization conditions (Table S7) were evaluated. Only cryo-protected crystals obtained in hydrogel 2 using the condition C18 of the GSK crystallization screening kit (Triana S&T, Granada, Spain) produced the best crystals to date (1.4 Å resolution limit, Table S6). On the other hand hydrogel 1 inhibited nucleation while crystal grown in agarose were of low quality proving the different behavior of these hydrogels.

To sum up, we present the novel use of supramolecular hydrogels based on short-peptides in neat water for protein crystallization. These hydrogels, made of symmetrical L and D di-cysteine derivatives, were used to grow protein crystals of high quality. Differences in crystal quality and packing for the three proteins have been observed using enantiomeric hydrogels 1 and 2, suggesting a significant influence of the fibers’ chirality in the crystallization process. The formation of the orthorhombic polymorph of glucose isomerase and the growth of the highest quality crystals of formamidase exclusively in hydrogel 2, support this hypothesis. This fact highlights the relevance that chirality may have in protein crystallogenesis for X-ray structural determination. Moreover these supramolecular peptide based hydrogels are excellent mediators for protein crystallization producing lysozyme and glucose isomerase and formamidase crystals with excellent quality indicators. Peptide based hydrogels also acted as a cryo-protectant avoiding the use of additives. Hence, these novel chiral gels would expand the field of protein crystallogenesis.

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