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Instantaneous fibrillation of egg white proteome with ionic liquid and macromolecular crowding

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The wide application of protein fibrils as functional materials has been restricted by the limited scalability of fibrillation methods, slow kinetics, and use of expensive purified proteins. Herein, inspired by the biological cooperativity of proteins in macro-molecularly crowded environments, these restrictions have been overcome. Using ionic liquid cholinium tosylate that acts as a fibrillation agent, instantaneous production of protein fibrils is shown directly from a real and low-cost matrix, i.e. egg white. The fibrillation of egg white proteome is confirmed by microscopy, whereas the fibrillation kinetics is monitored by fluorescence changes of the thioflavin T dye and secondary structural transitions. Spectroscopic and molecular docking studies are used to identify the proteins involved and to appraise the molecular-level mechanisms ruling the proteins structural changes upon fibrillation. The obtained fibrils have enhanced mechanical stiffness and cytocompatibility, demonstrating their potential to act as improved enzyme supports.

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Given the connection to amyloidogenic diseases, protein fibrillation is a hot topic of research^{1–3}. In addition to their relevance in neurological diseases, their potential as smart and/or functional materials in nano/biotechnology has recently attracted significant interest due to their bio-based origin, high mechanical strength, and stiffness^{4–7}. However, the low scalability of the production process, requirement of high purity and expensive proteins, and slow fibrillation kinetics, are still major hurdles to overcome when envisioning their use in large scale applications as functional biomaterials⁸.

Within a biological perspective, fibrils formation occurs due to multiple factors, such as environmental (heat and oxidative) stress, cellular mutations, translational errors, and aging. These factors cause partial or complete misfolding of folded intermediates due to dysfunctional chaperones (the folding regulators)⁹. The thermodynamically unfavorable misfolded intermediates then lead to the formation of either disordered aggregates or prefibrillar aggregates, which slowly change into stable amyloid fibrils via electrostatic, H-bonding, van der Waals, and aromatic stacking interactions^{9,10}. Serpell et al.¹¹ reported that plasma proteins that form amyloid fibrils share a common feature, namely a high β -sheet conformation, representing a clue of the proteins secondary structural requirements for fibrils formation.

The formation of amyloid fibrils has been investigated in vitro to better understand the initiation and propagation fibrillation mechanisms, applying changes on the physiological environment (pH, heat, and osmotic environment) of proteins^{12–14} and of synthesized peptides^{15–18}. It has been concluded that the rate and efficiency of proteins fibrillation are ruled by the proteins hydrophobic–hydrophilic nature, secondary structure changes, possibility of establishing electrostatic, $\pi\cdots\pi$ and hydrogen-bonding interactions, among others, which can be potentiated by fibrillation agents^{12–21}. Two major mechanisms of fibrils formation have been proposed: (i) seedling of fibrils in the form of misfolded monomeric intermediates, showing a lag phase in the kinetics curve of fibrils formation²²; and (ii) formation of misfolded oligomer intermediates as seeds, in which a lag phase is absent in the kinetics curve²³. Accordingly, when the goal is to induce proteins fibrillation, fibrillation agents exhibiting both polar and nonpolar domains are the best option since they can induce the formation of misfolded oligomer intermediates, while assisting in fast fibrillation by surpassing the lag phase.

Ionic liquids (ILs)²⁴, a class of low melting temperature organic salts, if properly designed may display the desired features to promote proteins fibrillation. ILs have been used in proteins fibrillation studies^{25–27}. Byrne et al.²⁵ reported the fibrillation of pure lysozyme using a protic IL, where the fibrillation was induced by heating for 2 h at 70 °C and keeping the mixture at room temperature for one week. Kalhor et al.²⁶ demonstrated the inhibition of lysozyme fibrillation using the IL tetramethylguanidinium acetate, highlighting the role played by the IL carboxyl group. Contrarily to the findings of Kalhor et al.²⁶ and Kang et al.²⁷ demonstrated the role of the carboxyl group of ester functionalized ILs in the fibrillation of bovine serum albumin. Albeit promising results have been reported, the following features are common to all works: (i) pure proteins (lysozyme or bovine serum albumin) were used; (ii) long periods are required for the production of protein fibrils; and (iii) all processes were developed at the laboratory scale (maximum of 1–8 mg of proteins used per batch).

An additional relevant aspect in proteins fibrillation is molecular crowding. Proteins in biological fluids are never found individually, but rather in the form of a proteome in a macromolecular crowded pool. In this complex scenario, every macromolecule affects (assists or inhibits) each other's physiological functions²⁸. The effects of macromolecular crowding (MC) on the

understanding of biochemical pathways (gene expression, protein dynamics, and folding) have faced significant advances in recent years²⁹. For instance, MC has been harnessed for modulating systems dynamics of in vitro circuits and programmable artificial cells^{30,31}. MC has been studied within the framework of proteins fibrillation as well, but these studies are limited either to single proteins at high concentration and/or use of some polymers as crowding media^{32–34}.

Inspired by the proteins in vivo cooperativity and MC, we mimicked these conditions to prepare protein fibrils from protein-rich pools. Herein, we explored the production of protein fibrils directly from chicken egg white (EW), selected as a low-cost MC media (rich in different proteins at concentrations >100 mg/mL)³⁵, using ILs as fibrillation agents at room temperature. The EW proteome (EWP) provides the heterogeneity of proteins required in crowded environments. Several ILs, namely cholinium methylsulfonate, cholinium chloride, benzylcholinium chloride, and cholinium tosylate ([Cho][Tos]), whose chemical structures are depicted in Fig. 1a, were screened according to their ability to establish electrostatic, $\pi\cdots\pi$ and hydrogen-bonding interactions with proteins and to induce protein–protein interactions and further fibrillation.

A schematic representation of the work here developed to produce protein fibrils is displayed in Fig. 1b. The fibril formation was confirmed by UV–vis, Fourier transform-infrared (FT-IR), circular dichroism (CD), and thioflavin T fluorescence spectroscopy, and their morphology was appraised by optical, atomic force (AFM) and transmission electron (TEM) microscopy. To better understand the role of MC in inducing fast fibrillation we also carried out fibrillation experiments of individual proteins, namely ovalbumin, lysozyme, and their mixture. Furthermore, to gather insights on the fibrillation mechanisms at the molecular level, molecular docking studies of different ILs ions with the different proteins present in the EWP were carried out. The properties of the prepared fibrils, namely thermal stability, mechanical strength/stiffness, and functional nature were analyzed by thermogravimetry analysis (TGA), dynamic mechanical analysis (DMA), and zeta potential assays. The fibrils cyto-compatibility was appraised with mouse fibroblasts (L929) cell lines. The prepared fibrils were finally investigated as support materials in biocatalysis.

Results and discussion

Preparation of protein fibrils. EW was collected from chicken eggs (purchased at a local supermarket), and diluted (1:10, v:v) in water. This solution was stirred and filtered twice to remove the insoluble lipids fraction. The aqueous filtrate (pH 8.82) containing the EWP was used to prepare protein fibrils. Since it is known that the denaturation of proteins promotes fibrillation due to the formation of unfolded intermediates³⁶, the EWP was treated with 1 M HCl at pH 2.0 to denature the proteome. Different ILs, namely cholinium methylsulfonate, cholinium chloride, benzylcholinium chloride, and cholinium tosylate ([Cho][Tos]), whose chemical structures are shown in Fig. 1a, were then investigated as fibrillating agents at low (0.1 M) and high (1 M) concentrations, which were added to the EWP treated with acid. Among the ILs investigated, only [Cho][Tos] showed an immediate/instantaneous (within seconds) protein fibrillation at both concentrations. No fibrillation was observed with the remaining ILs. Although the presence of an aromatic ring in the fibrillation agent is one of the identified criteria to promote fibrillation, it is not a sufficient criterion per se. One relevant additional issue is to carry out the fibrillation at pH 2.0, with all proteins present in their cationic state. Although benzylcholinium chloride also has an aromatic ring at the cation, charge repulsion may dominate while

Fig. 1 Fibrillation agents and schematic representation of the work carried out. a Tested fibrillation agents to induce the fibrillation of the egg white proteome. **b** Schematic representation of the protein fibrils formation directly from egg white using the IL [Cho][Tos] as a fibrillation agent. The protein crowding effect responsible for fast fibrillation and the application of the prepared fibrils as supports for enzymes are also highlighted.

Fig. 2 Morphological characterization of the prepared fibrils. a Optical microscopy image showing instant fibrillation of egg white proteome with [Cho][Tos] (scale bar = 10 μm). **b, c** 2-D AFM (scale bars = 2.8 μm and 800 nm) and **d** 3-D AFM images of freshly prepared fibrils showing entangles in the protein fibrils and fibril oligomers in the background. **d, e** TEM images of the lyophilized protein fibrils showing one-dimensional nanorods (scale bars = 500 and 100 nm).

avoiding hydrophobic interactions and instant fibrillation. By being an anion, tosylate can establish electrostatic interactions with the cationic side chains of different proteins, in addition to H-bonding, hydrophobic, and π -stacking interactions. Overall, the set of ILs investigated allow to conclude that a synergic effect and various interaction forces play a role in fibrillation. The detailed procedure to prepare the proteins fibrils is provided in

the Supplementary Note 1 and Supplementary Figs. 1–3. The macroscopic visual turbidity of the solution and optical, AFM and TEM images of the prepared fibrils are shown in Fig. 2 (additional images are provided as Supplementary Fig. 2).

Highly branched fibril networks are observed at low [Cho][Tos] concentrations, as shown by optical microscopy images given in Fig. 2a (additional images are given in Supplementary

Fig. 3 Kinetics of fibrillation. **a** Time dependence of the photoluminescence (P L) intensity of the Thioflavin T dye upon binding to the cross-beta structure formed due to EWP fibrils formation and **b** comparative ThT, P L intensity vs. time plots for ThT binding to ovalbumin, ovalbumin + lysozyme, and EWP fibrils.

Fig. 2b), which turn thicker with time and at higher IL concentrations (cf. Supplementary Fig. 2c, d). AFM images show the presence of entangled protein fibrils in parallel/antiparallel arrangements (Fig. 2b–d). TEM images (Fig. 2e, f) reveal a thick bundle of fibrils with an average aspect ratio varying from 7 to 10.

Kinetics of proteins fibrillation and evidences of cross-beta structure. The kinetics of fibrils formation was appraised by fluorescence changes of the Thioflavin T (ThT) dye—results depicted in Fig. 3 and Supplementary Figs. 5 and 6. The ThT dye exhibits specific binding with the cross-β structure of proteins, leading to an enhancement in its fluorescence intensity^{37,38}. Changes in the fluorescence intensity of ThT were recorded at different time intervals, up to 7 h. Before fluorescence, the absorption spectra of the dye were acquired, showing that ThT absorbs at 412 nm with and without fibrils (results given in Supplementary Fig. 4).

At the studied concentration (30 μM), ThT forms micelles in aqueous media, which specifically bind, mainly via hydrophobic interactions, along the length of the proteins cross-β structure, leading to an increase in the fluorescence relative intensity³⁷. When excited at 412 nm, ThT shows emission at 482 nm, whose emission relative intensity increases with time (Fig. 3). The increase in the fluorescence intensity with time is due to the growth of protein fibrils, in agreement with the formation of cross-β structures^{37,38}. The fluorescence spectra for samples containing protein fibrils are depicted in the Supplementary Fig. 5. To better understand the effect of MC in the EWP fibrillation, we studied the ThT binding curves with individual ovalbumin and its binary mixture with lysozyme (Fig. 3b, Supplementary Figs. 5 and 6), whose results are discussed below.

Unlike the commonly reported sigmoidal curves of protein fibrils growth using the ThT assay^{22,39,40}, no lag phase was observed, supporting the claims of the instantaneous (within seconds) fibrillation visually observed. The absence of the lag phase indicates that the unfolded intermediates of the various proteins formed may be directly intercalated with [Cho][Tos] to form oligomer intermediates. The formation of oligomer intermediates of EWP is confirmed by AFM images of the fresh sample dried overnight along with fibrils (as shown in the Supplementary Fig. 7). The oligomer intermediates are usually

circular in shape, and then intercalate with each other to grow into fibrils along time^{23,41}. In the current work, the formed oligomer intermediates could be directly formed via H-bonding and π-stacking interactions with [Cho][Tos]. The tosylate anion has an aromatic anion similar to phenylalanine, which has been reported to induce oligomers formation before the formation of fibrils⁴¹.

Secondary structural changes and proteins involved in fibrillation. To gain additional insights on the fibrils structure at the microscopic level we analyzed the changes in the secondary structure of the EWP by CD (Fig. 4a–e, Supplementary Fig. 8)^{42,43} and FT-IR in the amide I region (1600–1700 cm^{−1}) using a deconvolution method (Fig. 4f, g)^{44–47}. Since the EWP is a complex matrix, to gather evidences on the proteins involved in the fibrillation process we carried out sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4h) and UV-vis spectroscopic analysis (Supplementary Fig. 9) of the EWP before and after fibrillation. The CD spectrum of the acidic denatured EWP shows characteristic all-α secondary structure of proteins, in agreement with literature (Fig. 4a)⁴². However, upon fibrillation, a conformational transition of all-α secondary structure to antiparallel β-sheet or cross-β type occurs (Fig. 4e, Supplementary Fig. 8). This transition is a direct evidence of fibrils formation. Moreover, the time dependent CD analysis showed fast conformational transition wherein complete transition from all-α secondary structure to antiparallel β-sheet or cross-β type occurs within 20 min (Fig. 4b–d).

The formation of the cross-β structure due to fibrillation is further supported by FT-IR results (Fig. 4f, g). The spectrum of the acid denatured EWP shows seven characteristic bands, corresponding to different secondary structural contents, i.e., α-helix (1650 cm^{−1}), unordered (1660 cm^{−1}), β-sheet (1634 and 1680 cm^{−1}), β-turn (1667 and 1696 cm^{−1}), and cross-β (1626 cm^{−1})⁴⁵. Upon addition of [Cho][Tos], there is a shift in the absorption bands at 1626 to 1620 cm^{−1} and increase in the peak area, and at 1650 (α-helix) to 1647 cm^{−1}, thus confirming the formation of cross-β structures leading to fibrillation^{46,47}.

The participation of various proteins of the EWP in fibrils formation was appraised by SDS-PAGE and UV-vis spectroscopy. The SDS-PAGE results (Fig. 4h) reveal the participation of

Fig. 4 Proteins secondary structural changes and proteins involved in fibrillation. **a** Circular dichroism (CD) spectrum of EWP showing all- α conformation. **b–e** CD spectrum showing transition in all- α conformation of EWP upon fibrillation at different time interval to antiparallel- β or cross- β in fibrils. **f, g** Deconvoluted FT-IR spectra showing changes in secondary structure of EWP **f** before fibrillation and **g** after fibrillation. **h** SDS-PAGE results of the protein markers (PM), egg white proteome (EWP) solution, and protein fibrils (PF) solution.

the most abundant proteins, namely ovomucin, ovotransferrin, ovalbumin, avidin, and flavoprotein, in fibrils formation. The formation of fibrils was also confirmed by the disappearance of UV-vis absorption bands corresponding to flavoprotein (at 365 nm) and ovotransferrin (at 450 nm) and blue shifting of the bands at 280 nm (shown in the Supplementary Fig. 9). The blue shift at 280 nm (corresponding to the tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) aromatic amino acids) is accompanied by its pentafluorination to 280 nm (Trp), 225 nm, 261 nm (Phe), 267 nm, and 271 nm (Tyr). The possible association of the tosylate aromatic anion with the aromatic amino acid

residues at the proteins surface via hydrophobic or π -stacking interactions during fibrillation thus contrasted their respective vibronic bands. This observation is supported by previously reported hydrophobic and π -stacking interactions of Phe and Tyr residues as playing a key role in fibrillation^{19,41}. The relevant role of Phe23 (owing to its conformational flexibility) in fibrillation by inducing the association between sheets to cement macromolecular assemblies through interactions with other aliphatic residues has been demonstrated by simulation²¹ and experimental⁴¹ studies. The relevance of π -stacking interactions in proteins fibrillation occurring with cholinium tosylate is

Table 1 Interaction energies of the IL ions with the proteins present in egg white.

Ion	Energy (kcal mol ⁻¹)				
	Ovalbumin	Ovotransferrin	Lysozyme	Ovomucoid	Flavoprotein
[Cho] ⁺	-2.1	-3.0	-2.2	-2.6	-2.5
[BzCho] ⁺	-3.2	-4.3	-3.6	-3.4	-3.8
Cl ⁻	-0.8	-0.9	-0.6	-0.8	-0.8
[Tos] ⁻	-3.7	-4.8	-3.8	-4.0	-4.5
[CH ₃ SO ₃] ⁻	-2.1	-2.5	-2.4	-2.1	-2.6

additionally confirmed by the results obtained with the remaining cholinium-based ILs that do not comprise an aromatic anion and do not induce protein fibrillation.

To address the role of individual ions or of the IL as a specific cation-anion pair on fibrillation, studies with Na[Tos] and [Cho]Cl were additionally carried out. Aqueous solutions of 1 M of [Cho]Cl, Na[Tos], [Cho]Cl + Na[Tos] (1:1, mol:mol) and [Cho][Tos] were added to the EWP at pH 2.0. The results obtained are shown in the Supplementary Fig. 10. No fibrillation was observed with [Cho]Cl, even after 24 h (Supplementary Fig. 11). Nevertheless, instant fibrillation was observed with Na[Tos], demonstrating the major role played by the tosylate anion that bears sites for H-bonding and π -stacking interactions to drive fibrillation. The relevance of π -stacking interactions is also reflected in the UV-vis spectra, with a maximum increase in the absorbance with the pentafluorination of the respective band. It should be however remarked that the fibrils formed with [Cho][Tos] are thinner than those obtained with Na[Tos]. On the other hand, no instant fibrillation was observed with [Cho]Cl + Na[Tos] mixtures; it only occurs when higher amounts of salts are added. This suggests a relevant role also played by the cholinium cation, probably by balancing the strength of the interactions of the tosylate anion with the proteins. Overall, [Cho][Tos] induces the fibrillation of the EWP via the secondary structural transition of proteins to the cross- β structure, mainly due to H-bonding and π -stacking interactions established with amino acids, while assisting side chain interactions between different proteins as discussed below. Once nucleated the fibril seeds, propagation occurs like prion mechanism along with time⁴⁸.

Molecular docking and spectroscopic studies. For a better understanding of the protein's fibrillation mechanism, we investigated the protein-protein and IL-protein interactions by molecular docking. The proteins studied correspond to ovalbumin, ovotransferrin, flavoprotein, ovomucoid, and lysozyme, and were selected based on the previous evidences of their participation in fibrillation. Details of the computational procedure are given in Supplementary Note 2. In addition to [Cho][Tos], the interactions of EWP with [BzCho][Cl] (IL with an aromatic ring) and [Cho][CH₃SO₃] (a nonaromatic IL) that do not induce instant fibrillation were investigated by molecular docking. The interaction energies of the IL ions with the various proteins are given in Table 1. Additional data (docking potential) are provided in Supplementary Tables 1–5. The interaction energies of the IL ions with the different proteins follow a similar trend, decreasing according to the following rank: [Tos]⁻ > [BzCho]⁺ > [Cho]⁺ ~ [CH₃SO₃]⁻ > Cl⁻. These results are in agreement with experimental findings, where IL aromatic ions establish stronger interactions with proteins, being favorable for protein fibrillation, and where the [Tos]⁻ anion plays a major role.

Docking studies (Fig. 5a–c, Supplementary Figs. 12–35) reveal that hydrogen bonding and hydrophobic interactions are the major interactions occurring between the tosylate anion and amino acids. Preferential hydrogen-bonding occurs with alanine,

asparagine, tyrosine, histidine, lysine, glycine, and serine, whereas preferential hydrophobic interactions occur with leucine, phenylalanine, valine, histidine, arginine, alanine, and tyrosine. Accordingly, the success of [Cho][Tos] in fibrillation is due to strong hydrogen-bonding and hydrophobic interactions established with the amino acids proteins. This trend is further supported by the fact that the [BzCh]⁺ and [CH₃SO₃]⁻ ions, which interact with amino acids mainly via electrostatic interactions (Supplementary Tables S5), are not able to induce the EWP fibrillation. [Tos]⁻ also exhibits π -stacking interactions with Phe645 of ovotransferrin, being these interactions additionally favorable to promote fast fibrillation.

The snapshots of the molecular docking of the various proteins with [Tos]⁻ are shown in Fig. 5a–c and Supplementary Figs. 16, 21, 26, and 31. The interaction energies of [Cho][Tos] with the different proteins decrease in the order: Ovotransferrin > Flavoprotein > Ovomucoid > Lysozyme > Ovalbumin. Among all proteins investigated, the strongest interaction occurs between [Tos]⁻ and ovotransferrin, with an interaction energy of -4.8 kcal/mol (Table 1 and Fig. 5a–c). This order of the [Cho][Tos] interaction strength with the various proteins is related with their secondary structure, where β -sheet containing proteins are more prone to fibrillation¹¹. Along with the [Cho][Tos] interactions, which correspond to the initiation step of the fibrillation mechanism, protein-protein interactions and the respective aggregation correspond to the key propagation step for a fast fibrillation. Evidences of π -stacking interactions established between the proteins were obtained by UV-vis assays (as discussed earlier), supporting this perspective.

Prediction of amyloid structure aggregation (PASTA)⁴⁹ was used to determine the aggregation free energy in protein-protein, protein disorder and secondary structure (Fig. 5d, Supplementary Tables 6–8). From the data provided in Supplementary Tables 6–8, it is shown that ovalbumin has the highest self-aggregation free energy (-11.07 kcal mol⁻¹), followed by ovotransferrin and flavoprotein. On the other hand, the ovotransferrin-flavoprotein aggregate has the highest co-aggregation free energy (-7.64 kcal mol⁻¹) in a crowded media. From the analysis of Fig. 5d, there is a significant increase in β -strands of most proteins. This increase in the β -strands estimated by PASTA is in agreement with the increase in the cross- β structure observed by CD and FT-IR spectra upon fibrillation.

Effect of molecular crowding and possibility of cross-seeding.

After addressing the major interactions occurring between proteins and the fibrillation agent, the relevance of molecular crowding in EWP fibrillation was analyzed. Fibrillation experiments with pure ovalbumin, pure lysozyme, and their mixtures were carried out. The concentration of ovalbumin and lysozyme were kept as equivalent to their amount in EWP. Upon addition of [Cho][Tos] at pH 2.0, ovalbumin, lysozyme, and their mixtures show fibrillation as indicated by visual turbidity. These solutions were centrifuged after 24 h of incubation and the obtained solid samples were redispersed in distilled water. The CD spectra of the

Fig. 5 Molecular docking and PASTA. Snapshots illustrating **a** hydrogen-bonding ability and **b** hydrophobicity of amino acids residues on docking pose with the lowest absolute value of affinity (kcal/mol) for Ovotransferrin with [Tos][−]. **c** Molecular interaction diagrams of [Tos][−] and amino acids residues of Ovotransferrin. **d** Changes in the secondary structural conformation of various proteins of EWP: Ovalbumin (OVA), Ovotransferrin (OVT), Flavoprotein (FLA), Ovamucoid (OVD), and Lysozyme (LYS) after fibrillation with [Cho][Tos].

redispersed fibrils show the proteins conformational transition to cross- β type secondary structure only for ovalbumin and ovalbumin/lysozyme mixtures, and not for pure lysozyme (Supplementary Fig. 36a–c). These results demonstrate that [Cho][Tos] just forms a charged complex with lysozyme at pH 2.0, which is dissolved upon going back to pH 7.0.

To confirm whether the lysozyme participates in the fibrillation in the ovalbumin/lysozyme mixture we further analyzed the CD spectra of the supernatants of all samples. The lysozyme supernatant spectrum reveals the presence of proteins with a disordered structure. For pure ovalbumin and the mixture of two proteins, there are evidences for $\alpha + \beta$ conformational state, indicating that proteins are on their way to cross- β conformation. These CD spectra, together with the SDS-PAGE results of the supernatants (Fig. 4), indicate that although lysozyme does not fibrillate individually, it does when in proteins mixtures. It should be however highlighted that the cross- β structure of ovalbumin and ovalbumin/lysozyme mixture is not as organized as in EWP (Supplementary Fig. 36d). These observations demonstrate the role played by molecular crowding in the fibrillation process via cross seeding.

We further confirmed the previous observations by ThT assays of individual proteins and ovalbumin/lysozyme mixture, whose results are given in Fig. 3b and Supplementary Figs. 5 and 6. When moving from a single protein (ovalbumin) to the molecularly crowded EWP proteome, kinetics curves change drastically. The stationary phase is attained faster in EWP with lower fluorescence intensity of ThT as compared to the individual protein assays. Although we cannot assure that cross-seeding is happening in EWP with the accommodation of all proteins into a single fibril, the ThT binding results (Fig. 3b) seem to support this possibility.

The phenomenon of cross seeding of misfolded proteins of different kinds, mainly focused on neurodegenerative diseases, has been previously reported^{50–56}. In these studies it has been

concluded that cross-seeding is directly proportional to the sequence homology of misfolded intermediates; however, proteins with heterogenous sequences also aggregate together⁵⁰. Juhl et al.⁵³ studied the co-aggregation of plasma and cerebrospinal proteins in presence of preformed amyloid fibrils, concluding that fibrils with diverse primary sequences and ultrastructural morphologies only differ slightly in the composition of the co-aggregated proteins, whereas exposure to a fibril surface can push aggregation-prone proteins to accumulate. The behavior of our ThT binding curves shown in Fig. 3b is in agreement with these results. When moving from an individual protein fibril to EWP fibrils containing proteins of heterogenous sequences, the ThT fluorescence intensity decreases, indicating diverse primary sequences in EWP fibrils with specific monomer packing compared to ovalbumin fibrils⁵³.

Other proteins show significant co- and self-aggregation tendency in the crowded medium, as appraised by the data shown in Supplementary Tables 6 and 7. This phenomenon is supported by the beginning of co-/self-aggregation of different proteins from the EWP in the absence of IL as a fibrillation agent, occurring yet after three days as evidenced from the CD spectra of aggregates shown in Fig. 6a.

The overall picture of the possible molecular-level mechanism behind fibrils formation is summarized in Fig. 6, highlighting the essential role of protein crowding in balancing various interactions among the side chains of different proteins (cross seeding) for efficient and fast fibrillation directly from the EWP, which occurs at room temperature in presence of a suitable fibrillation agent such as [Cho][Tos].

Thermal and mechanical properties. The thermal and mechanical properties of the obtained protein fibrils were determined by TGA and DMA. TGA profiles shown in Supplementary Fig. 37a reveal a high thermal stability of protein fibrils, with a glass

Fig. 6 Schematic overview of the fibrillation mechanism. **a** Cartoons summarizing the proposed fibrillation mechanism in the egg white proteome with and without the fibrillation agent ([Cho][Tos]) along with the circular dichroism spectra (double sided arrows ($\leftrightarrow$) in the acid denatured proteins indicate the interactions between different proteins). **b** Schematic representation of the formation of protein fibrils from the egg white proteome using [Cho][Tos], which interacts via hydrogen bonding, hydrophobic, and π -stacking interactions with different proteins to form oligomer intermediates and protein fibrils.

transition temperature (T_g) occurring at ca. 95 °C, and thermal degradation temperatures above 307 °C, being comparable with that of amyloid fibrils formed by single proteins⁵⁷ or peptides⁵⁸. The stiffness of the fibrils corresponds to a 2-order of magnitude difference in bulk (E') and loss modulus (E'') at 25 °C (Supplementary Fig. 37b)⁵⁹. A high value of E' (42 GPa) was obtained for the fibrils at 25 °C, revealing high stiffness. This value is significantly higher than that obtained for fibrils prepared from β -casein (4 GPa)³⁸ and stacked β -sheet amyloid of other proteins (0.67–5 GPa)⁵⁹, and comparable with typical A β 40 amyloid fibril (4.5–54.3 GPa) with varying monomers units⁶⁰. The fibrils strength usually depends on the packing density of the side chains of neighboring β -sheet, whereas the lack of significant side chains in a protein can give rise to nano-mechanical traits which are potential low mechanical junctions⁵⁹. The proteome heterogeneity in the EWP avoids such junctions, resulting in a better intercalation of fibrils with better packing density and higher mechanical strength. The decrease in E' with an increase in temperature occurs due to the fact that different chains of fibrils begin to move freely under thermal motions, causing voids in the fibril matrix.

We also analyzed the mechanical loss factor or damping parameter, $\tan\delta$ (the ratio of loss and bulk modulus), of the prepared fibrils. Changes in temperature reveal the transition between glassy and rubbery regions of materials from two sides of the maxima in a curve⁵⁹. The prepared fibrils display two maxima

of low and high magnitude in the $\tan\delta$ vs. temperature profile, as shown in Supplementary Fig. 37c. The first low magnitude maximum occurring at ca. 80 °C might not be a true glassy-rubbery transition, but rather a relaxation of the cooperative motions of cross-linked chains due to elastic deformations. The second maximum observed at around 170 °C indicates a true glassy-rubbery transition. Furthermore, it should be remarked that the overall magnitude of $\tan\delta$ is low, suggesting an improved interfacial adhesion which is further reflected in the fibrils high mechanical strength.

The nature of the prepared fibrils was further addressed by zeta potential (ζ) after dispersing them in water at various pH values, ranging from 2.0 to 12.0, whose results are given in Supplementary Figs. 38 and 39. The prepared fibrils have enhanced colloidal stability and charge tunable nature, with their zeta potential values varying from +31 mV at pH 2.0 to –21 mV at pH 10.0. Taking into account their colloidal stability and charged nature they could be applied as dispersants^{61,62}, in biotechnology applications^{63,64} and in biocatalysis^{65–67}.

Cytocompatibility. The cytocompatibility of the prepared fibrils was assessed by appraising the viability of the mouse fibroblast L929 cell line via Live-Dead assays, metabolic activity and morphology upon incubation with fibrils (Fig. 7). No impair in cellular membrane integrity (Fig. 7a, b) and morphology (Fig. 7c–f)

Fig. 7 Cytocompatibility assays of protein fibrils. Live-Dead assay of **a** control cells and **b** in presence of 25% of protein fibrils (scale bar: 50 μ m). Green channel: live cells–Calcein–AM, Red channel: necrotic cells–PI. Fluorescent micrographs of the morphology of **c** control cells and **d** cells incubated with 25% of protein fibrils (scale bar: 20 μ m). Green channel: F-actin–fluorescein phalloidin, Blue channel: nucleus–DAPI. Optical micrographs of **e** control cells with no fibrils and **f** with cells incubated with 25% of the EWP fibrils stock solution (scale bar: 50 μ m). **g** Viability plot of L929 cells in presence of different concentrations of protein fibrils (5–25%). Data are presented as average $\pm$ standard deviation (NS = no significant difference compared to the control (no fibrils)).

was observed after 24 h in presence of up to 25% of protein fibrils, supporting their cytocompatibility (uncropped images are shown in Supplementary Fig. 40). The wide-fluorescent images of the Live-Dead assay in the presence of 15 and 20% fibrils are shown in Supplementary Fig. 41.

The green channels depicted in Fig. 7a, b show the cells stained with Calcein–AM, indicating a viable plasma membrane integrity, whereas the red channels are indicative of dead cell which could not be stained with Calcein–AM due to the membrane rupture. The cytocompatibility of fibrils toward L929 cells was further ascertained by analyzing the cells morphology by the f-actin and nucleus staining (Fig. 7c, d, Supplementary Fig. 42) and optical microscopy (Fig. 7e, f). Cells do not show changes in morphology in the prepared protein fibrils solutions (up to 25%), reinforcing the fibrils cytocompatibility. The ability of live cells to reduce bluish resazurin to a pinkish highly fluorescent resorufin molecule demonstrates that $\sim$ 100% of viable cells exist after the incubation with 5–25% of protein fibrils (Fig. 7g). A slight decrease in viability of $\sim$ 6% (94% viable cells) was observed with 25% of protein fibrils, being however negligible (Fig. 7g). The cytocompatibility of the prepared fibrils, and among other applications, may open the path to their application as low-cost and bio-based materials for natural extracellular matrix for 3-D cell culture and regenerative medicine^{68,69}.

Immobilization of cytochrome c on protein fibrils. Enzymes immobilization is among the most relevant biotechnological applications of protein fibrils, allowing an easy separation of products from the insoluble fibrils with the attached biocatalyst that can be recycled^{65,66}. As a proof of concept on the possible applications of the prepared protein fibrils, we studied the ability of the fibrils to immobilize Cytochrome c (Cyt c) and evaluated their impact upon its activity (Fig. 8). Overall, EWP fibrils display a good tendency to attach Cyt c (25.7 μ g/14 mg). The enzyme immobilization was confirmed by comparative AFM images of fibrils without and with Cyt c, as shown in Fig. 8a, b. Since Cyt c is an oxidoreductase enzyme we analyzed the change in its oxidation state by UV–vis spectra, whose results are depicted in Fig. 8c. The activity assays results using ABTS as substrate in presence of H_2O_2 are given in Fig. 8d.

Fibrils cause the reduction of Cyt c, from ferri-Cyt c (Fe^{3+}) to ferro-Cyt c (Fe^{2+}), as indicated by the increase in the absorbance of the peak at 550 nm and color shift from brown to pink-orange⁶⁷. The activity of immobilized Cyt c in the fibrils is 2.4537 units μ mol E^{-1} , which is 2.5-fold higher than that observed in water (0.976 units μ mol E^{-1}). Therefore, the fibrils act not only as a support but also allow increasing the activity of Cyt c, revealing their beneficial role in biotechnological applications. Although only one example of application is shown, the low-cost protein

Fig. 8 Enzyme immobilization and activity assay. 3-D AFM images of EWP fibrils **a** without Cyt c and **b** with Cyt c attached to EWP fibrils. **c** UV-vis spectra of Cyt c before and after immobilization with fibrils. **d** Comparative activity of Cyt c in water and protein fibrils solution according to the formation of $\text{ABTS}^{+\bullet}$ with time upon oxidation of ABTS as substrate.

fibrils here prepared can be used as smart and/or functional materials in a plethora of nano/biotechnology applications^{4–7}.

In summary, we report instantaneous (within seconds) preparation of proteins fibrils directly from a low-cost matrix, namely EW, driven by an IL that acts as a fibrillation agent and heterogeneous molecular crowding. By experimental and computational studies, it was found that H-bonding and hydrophobic interactions occurring between the IL and proteins are the driving forces to induce fibrillation, with additional beneficial effects afforded by ILs with aromatic anions by allowing the establishment of π - π interactions. The obtained fibrils have high mechanical stiffness and are cytocompatible. A case study on their application was shown, namely by acting as enzymes supports and where an enhancement in the activity of Cyt c was found. In summary, a cost-effective strategy to prepare protein fibrils is here reported, where 2 g of protein fibrils could be prepared from a single egg for less than 5€, i.e., at a cost of 2.5€ per g of protein fibrils (cf. Supplementary note 3). These values were obtained at a lab-scale, being estimated that significantly higher amounts of proteins fibrils per batch at a lower cost can be obtained by addressing and optimizing the process scale-up. The low-cost protein fibrils here prepared can open the path to their large-scale application as smart and/or functional materials in a wide range of nano/biotechnology applications.

Methods

Materials. Chicken eggs were purchased in a local supermarket. Bovine serum albumin (fatty acid free) was purchased from Fischer Scientific. Lysozyme from chicken EW and cytochrome c from equine heart (>95%) were acquired from Sigma Aldrich. Cholinium bicarbonate (80% in water with purity >98%), p-toluenesulfonic acid monohydrate (>98.5%), methanesulfonic acid (>99.0%), benzyltrimethyl-(2-hydroxyethyl)ammonium chloride (>97%), and cholinium chloride (98%) were purchased from Sigma Aldrich. Cholinium tosylate and cholinium methanesulfonate were synthesized by us using an acid base reaction as described elsewhere⁷⁰. The synthesized ILs were dried for 24 h under high vacuum at 70 °C for 48 h. A white solid was obtained for cholinium tosylate and a viscous liquid was obtained for cholinium methanesulfonate, which were collected and stored in a vacuum desiccator. The purity of these ILs was confirmed by ¹H and ¹³C NMR and elemental analysis, and found to be >98%. Thioflavin T (>99%) was purchased from ACROS Organics. Concentrated hydrochloric acid (37%) was purchased from Sigma Aldrich. All the cell culture media and supplements, namely GIBCO® Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS; E. U. approved, South America origin), Dulbecco's modified Eagle's medium low

glucose (DMEM-low Glc), TrypLETM Express, and GIBCO® antibiotic/anti-mycotic solution (ATB) containing 10,000 units/mL of penicillin and 10,000 mg/mL of streptomycin were purchased from ThermoFisher Scientific (Alfagene, PT). Adherent 96-well plates were purchased from In VitroCell (Sarstedt, PT), non-treated clear bottom 96-well black plates from Corning (Corning, USA) and 13 mm round-treated coverslip from Sarstedt (Sarstedt, PT). Calcein-AM, Propidium Iodide (PI), DAPI, and fluorescein phalloidin were acquired from Thermo Fisher Scientific Inc (Alfagene, PT).

Microscopy. Optical microscopic images of protein fibrils, at $\times 5$ magnification, were acquired using an Olympus BX51 light polarized microscope (Olympus Co., Tokyo, Japan). Optical microscopic images of cells were acquired using an inverted microscope (Zeiss Primovert, Carl Zeiss, Germany), equipped with Plan-ACHROMAT $\times 20/0.30$. All images were acquired and processed with the Zeiss Zen SP2 Software (Carl Zeiss, Germany). Atomic Force Microscopy (AFM) imaging was performed using the Veeco AFM Multimode Nanoscope (IV) MMAFM-2, USA, in semi contact mode. Samples were prepared by placing a drop of fibrils samples on a mica sheet and dried overnight. Transmission electron microscopy (TEM) images were acquired using a Hitachi H-9000 microscope operating at 300 kV. An aliquot of an aqueous dispersion of the protein fibrils was deposited onto a carbon-coated copper grid and the solvent was allowed to evaporate. Fluorescence micrographs were acquired in an upright Widefield fluorescence microscope (Zeiss Imager M2, Carl Zeiss, Germany), equipped with an EC Plan-Neofluar $\times 20/0.16$ objective lens and a 3MPix monochromatic camera. All micrographs were acquired and processed in the Zeiss Zen SP2 Software (Carl Zeiss, Germany).

Thermogravimetric analysis (TGA). TGA assays were carried out with a Setsys Evolution 1750 in TGA mode (sensor type S) from Setaram. Samples were heated at a rate of 10 °C min⁻¹ from 25 to 800 °C, under a 20 mL min⁻¹ N₂ flow.

Dynamic mechanical analysis (DMA). DMA measurements were performed on a Tritec 2000 DMA Triton Technologies equipment operating in a single cantilever bending mode. Analyses were performed in the temperature range from 20 to 200 °C (3 °C step) at 1 and 10 Hz, with the displacement amplitude set to 0.020 mm.

Thioflavin T (ThT) assays. Photoluminescence spectra of thioflavin T in the visible region were recorded at room temperature with a modular double grating excitation spectrofluorimeter with a TRIAX 320 emission monochromator (Fluorolog-3, Horiba Scientific) coupled to a R928 Hamamatsu photomultiplier, using a right angle acquisition mode. For the ThT assays, 30 μM ThT was dissolved in 0.5 mL EWP at pH 2.0 in a 700 μL quartz cell. The spectrum of this sample was acquired after the addition of 50 μL of 0.1 M [Cho][Tos]. For ovalbumin and ovalbumin/lysozyme mixture, 30 μM ThT was dissolved in 0.5 mL of their aqueous solutions at pH 2.0, followed by resting for 30 min and addition of 50 μL of 0.1 M [Cho][Tos]. Fluorescence spectra were recorded of each sample for a period of 7.1 h. The excitation source was a 450 W Xe arc lamp. The emission spectra were

corrected for detection and optical spectral response of the spectrofluorimeter and the excitation spectra were corrected for the spectral distribution of the lamp intensity using a photodiode reference detector. The emission spectra were monitored at 482 nm under the excitation at 412 nm. Excitation slit width was 1 nm and emission slit width was 0.8.

Circular dichroism (CD). CD spectra were recorded using a Jasco J-815 CD spectrometer at 298.15 K in the far UV region ($\lambda = 180 - 260$ nm). Spectra were collected in a 0.1 cm path length quartz cuvette at a scan rate of 50 nm min^{-1} and sensitivity of 100 mdeg. The response time and the bandwidth were 2 s and 0.5 nm, respectively. The fibrils were dispersed in distilled water via sonication until a turbid solution was formed. The turbid solution was further diluted to 1:10 (v:v) to record the spectra with the HTV value below 600 which marks the accuracy of the CD spectrum. The supernatants spectra were recorded in a similar way.

Fourier transform infrared (FT-IR). FT-IR spectra were recorded using a Perkin Elmer Spectrum Bx spectrophotometer in the wavelength range from 1800 to 1300 cm^{-1} . For each spectrum 64 scans were carried out at a resolution of 2 cm^{-1} . The secondary structural analysis was done in the amide I region ($1600 - 1700 \text{ cm}^{-1}$) by a deconvolution method, using the Origin 8.5 software in the wavelength range of $1590 - 1710 \text{ cm}^{-1}$. Prior to deconvolution, the spectra were smoothed in five points of window using the SavitzkyGolay method in second polynomial order and normalized. The fitting of spectra at pH 2.0 corresponded to $R^2 = 0.997$ and $\chi^2 = 0.00038$ and that of fibrils corresponded to $R^2 = 0.999$ and $\chi^2 = 0.00011$, validating the curve fitting upon deconvolution.

UV-vis spectroscopy. UV-visible spectra of the protein fibrils solution were acquired using an UV-1800 SHIMADZU UV-spectrophotometer in a quartz cuvette of 1 cm path length. The spectra of Cytochrome c were measured in quartz cuvette of path length 0.1 cm.

Fluorescence spectroscopy. Fluorescence intensity of the alamarBlue® assays were acquired in a multimode microplate reader (Synergy HTX), equipped with a tungsten halogen lamp and a PMT detector. Intensities were acquired from the bottom of the well plate by filter-based fluorescence optics (excitation wavelength: 540/35 nm; Emission wavelength: 600/40) and processed in the Gen5™ software.

Zeta potential. The Zeta potential was determined using a Zetasizer Nano ZS light scattering apparatus (Malvern Instruments, UK) with a He-Ne laser (633 nm, 4 mW). The fibrils were dispersed in aqueous solutions by adding 4.4 mg of lyophilized fibrils at different pH values after sonication.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of the supernatants of the aqueous solutions containing fibrils were diluted at 1:1 (v:v) in a dissociation buffer consisting of 2.5 mL of 0.5 M Tris-HCl pH 6.8, 4.0 mL of 10 % (w/v) SDS solution, 2.0 mL of glycerol, 2.0 mg of bromophenol blue and 310 mg of dithiothreitol (DTT). This solution was heated at 95°C for 5 min to denature the proteins by reducing disulfide linkages, and thus overcoming some forms of the tertiary protein folding and breaking up the quaternary protein structure. Electrophoresis was run on polyacrylamide gels (stacking: 4% and resolving: 20%) with a running buffer constituted by 250 mM Tris-HCl, 1.92 M glycine, and 1% SDS. The proteins were stained with Coomassie Brilliant Blue G-250 0.1% (w/v), methanol 50% (v/v), acetic acid 7% (v/v), and water 42.9% (v/v). All gels were placed overnight in an orbital shaker at a moderate speed at room temperature. Gels were destained in a solution containing acetic acid at 7% (v/v), methanol at 20% (v/v) and water at 73% (v/v) in an orbital shaker at a moderate speed during 3–4 h at 40°C . SDS-PAGE Molecular Weight Standards, namely marker molecular weight full-range from VWR, were used as protein standards. Gels were analyzed by taking digital snapshots.

Cytochrome c assays. The activity of cytochrome c was measured using ABTS as substrate in presence of H_2O_2 . The conversion of ABTS to ABTS^{+} upon oxidation was monitored at 420 nm. For native Cyt c the typical reaction mixture contained 5 mM of ABTS + 1.7 μM of Cyt c + 1.66 mM of H_2O_2 . The reaction was initiated with the addition of H_2O_2 . The reaction was subjected to 1 min of incubation with the continuous acquisition of the absorbance data at 420 nm. For the activity assays of Cytochrome c supported in the fibrils, 0.014 g of fibrils were dipped in 0.5 mL of 12.71 μM Cytochrome c solution for 12 h. The sample was centrifuged at 5000 rpm for 5 min and the pink orange supernatant (indicating the Cyt c reduced form) was removed. 25.7 μg of Cyt c was attached to fibrils by centrifugation as detected from UV-vis spectra. The fibrils with attached Cyt c were then subjected to activity assays. The typical reaction mixture contained 5 mM of ABTS + 1.38 μM of Cyt c + 1.66 mM of H_2O_2 . The concentrations of both oxidized and reduced Cyt c were determined by UV-vis spectroscopy.

Cell culture and fibrils incubation. Mouse fibroblasts (L929) cells (European Collection of Authenticated Cell Cultures) were cultured in DMEM-low glucose

supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Cells were maintained in a CO_2 incubator at 37°C and the medium was replaced every 2 days. One day before each assay, cells were seeded at a density of 1×10^4 and 5×10^4 cells/well on a 96-well plates and 13 mm round-treated coverslips, respectively. Thereafter, the old culture medium was replaced with fresh medium along with the addition of various percentages (25–5%) of newly prepared EWP fibrils and cells were incubated for 24 h at 37°C .

Cell viability and morphology assays. Cell viability was determined by alamarBlue® (ThermoFisher Scientific, USA) and Live-Dead assays. The alamarBlue® assay was performed according to the manufacturer's guidelines. Briefly, 10% of alamarBlue® buffer was added to each well and the incubation was continued for 3 h. Afterwards, aliquots of 100 μL were taken from each well and transferred to a non-treated 96-well black plates with clear bottom, and the obtained fluorescence intensity was measured by a multimode microplate reader. For the Live-Dead assay, cells seeded on round coverslips were labeled with Calcein-AM (3 $\mu\text{g/mL}$) and Propidium Iodide (PI) (6 $\mu\text{g/mL}$) for 30 min at 37°C . Following incubation, cells were rinsed with DPBS and immediately observed by upright fluorescence microscopy. Cell morphology was evaluated by F-actin and nucleus staining with phalloidin and DAPI, respectively. To this end, after 24 h incubation with each concentration of EWP fibrils, cells seeded on round coverslips were fixed with 4% formaldehyde during 20 min, rinsed with DPBS and incubated with fluorescein phalloidin (25 $\mu\text{g/mL}$) for 30 min at 37°C followed by DAPI (1 $\mu\text{g/mL}$) for 5 min. Following incubation, cells were rinsed with DPBS and analyzed by upright fluorescence microscopy.

Data availability

The original datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

P.B. conceived, designed, and performed the experiments (fibrillation, optical microscopy, FTIR, CD, and zeta potential), interpreted the data and wrote the original version of the paper. D.M. conceived, designed, and performed the experiments (ILs synthesis, mechanical properties, and activity assays) of this work, interpreted the data, and contributed to the paper preparation. M.G.F. conceived and designed the work, interpreted the data, and contributed to the paper preparation. J.A.P.C. interpreted the data and contributed to the paper preparation. M.M.P. performed the computational analysis and wrote the respective part in the paper. M.R.A. performed SDS-PAGE and assisted in the Thioflavin experiments. M.C.N. performed the TEM images acquisition. M.C.G. performed the cell viability assays and wrote the respective part in the paper. J.F.M. interpreted the cell viability data and helped in the writing of the respective part in the paper. I.B. performed the AFM imaging. R.A.S.F. performed the thioflavin experiments and wrote the respective part in the paper.

Competing interests

The authors declare no competing interests.

Additional information

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