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Production of lysozyme nanofibers using deep eutectic solvent aqueous solutions

Nuno H.C.S. Silva[a,b], Ricardo J.B. Pinto[a], Carmen S.R. Freire[a], Isabel M. Marrucho[b,c,*]

[a] CICECO – Aveiro Institute of Materials and Chemistry Department, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal
[b] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. República, Ap. 127, 2780-901 Oeiras, Portugal
[c] Centro de Química Estrutural, Instituto Superior Tecnico, Universidade de Lisboa, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal

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ABSTRACT

Amyloid fibrils have recently gained a lot of attention due to their morphology, functionality and mechanical strength, allowing for their application in nanofiber-based materials, biosensors, bioactive membranes and tissue engineering scaffolds. The *in vitro* production of amyloid fibrils is still a slow process, thus hampering the massive production of nanofibers and its consequent use. This work presents a new and faster (2–3 h) fibrillation method for hen egg white lysozyme (HEWL) using a deep eutectic solvent based on cholinium chloride and acetic acid. Nanofibers with dimensions of 0.5–1 μm in length and 0.02–0.1 μm in thickness were obtained. Experimental variables such as temperature and pH were also studied, unveiling their influence in fibrillation time and nanofibers morphology. These results open a new scope for protein fibrillation into nanofibers with applications ranging from medicine to soft matter and nanotechnology.

1. Introduction

Protein misfolding and aggregation into amyloid fibrils was first studied due to its association with several pathological disorders, such as Alzheimer's and Parkinson's diseases, which affect a large number of people worldwide [1]. Such structures are typically defined as unbranched protein fibers of aligned cross-β structures, which are easily detected *in vitro* using fluorescence dyes as thioflavin T, and morphologically observed by electron microscopy [2,3].

Several proteins have been used to unveil the mechanism of amyloid fibrils formation [4]. Hen egg white lysozyme (HEWL) has been widely used as a model protein in aqueous solution, and the influence of a variety of conditions [5], including pH of the media (using acidic [6,7] or alkaline [8,9] solutions), temperature [10–12], high pressure [13], and the addition of sodium azide [14,15], urea [16,17] or ethanol [18], in fibrillation induction have been researched. Although the exact mechanism of protein structure induction by different solvents is still debatable, it is generally accepted that the nonpolar to polar balance of the solvents used in fibrillation is an important parameter in the disruption of the protein intramolecular hydrogen bonds allowing the formation of β dimers, which presence has been confirmed by circular dichroism. The protofilaments are formed by stacking of the dimers with their long axis (nearly) perpendicular leading to the formation of the nanofibers [19–21]. These studies contributed to the understanding of protein fibrillation process and consequently to the development of new drugs targeted against amyloidosis.

Due to their singular mechanical properties, comparable to dragline silk and much greater than most biological filaments [22], a range of potential technological applications of protein fibrils rely on their efficient preparation and use in the fabrication of nanofiber-based materials. For instance, amyloid-hydroxyapatite composites have been developed as reinforced materials to mimic bone tissues [23–25] and other bioactive nanomaterials based on protein nanofibers were synthesized for applications in sensing, neuronal tissue engineering, and electrostimulated stem cell differentiation [26,27]. Furthermore, such fibrils have been used as templates for the synthesis or assembly of several metallic nanoparticles and nanowires [28–30], as well as other inorganic (micro)nanophases, like fluorapatite [31], calcium carbonate [32,33], carbonate apatite [34], silica [35] and CdSe nanofibers [36]. These functional hybrid nanomaterials find application in biosensors, electronic and energy devices, bioactive membranes and tissue engineering scaffolds, among many others [37,38]. Due to their exceptional features, such as high strength and thermochemical stability, protein nanofibers are indeed emerging as a

* Corresponding author at: Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. República, Ap. 127, 2780-901 Oeiras, Portugal.
E-mail address: imarrucho@itqb.unl.pt (I.M. Marrucho).

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unique and novel class of building nanoblocks for the construction of innovative functional nanocomposites. Despite the fact that most of the amyloid fibrils used for these applications are being produced by denaturing methods, there are also functional amyloid fibrils being produced by bacteria which are used as building blocks for bionanomaterials and nanotechnologies [39,40]. For example, Zhong et al. [39] reported the development of a strong and multifunctional underwater adhesives obtained from fusing mussel foot proteins (Mfps) of *Mytilus galloprovincialis* with CsgA proteins, the major subunit of *Escherichia coli* amyloid curli fibres. Although the kinetics to produce the amyloid fibrils by bacteria is relatively fast, the process is complex and expensive.

One of the major limitations in the production of fibrils for the fabrication of intelligent materials is the time required to obtain these fibrils, since most of the fibrillation procedures developed so far can take days, weeks and even months. Only one work [41], reported the production of lysozyme fibrils in less than 3 h, through the use of guanidine hydrochloride as an amyloid inducer. However, the fibril dimensions in terms of length are shorter (~300 nm) comparing to other studies approaching material-based fibrils, which usually display lengths around or even higher than 1 μm [25,27,42]. During the last years, different fibrillation procedures have been developed for the production of material-based fibrils, usually taking 8–15 h [9,16].

More recently, the use of ionic liquids (ILs) has been shown to alter the fibrillation process and to stabilize different amyloid species, opening new perspectives of probing protein conformational states and exploring new protein-solvent interactions [43–46]. Bae et al. [46] demonstrated that the presence of specific ILs creates a hydrophobic ionic media that induces the formation of amyloid fibrils of α-lactalbumin. Diluted (5 wt%) solutions of 1-butyl-3 methyl imidazolium ([C4mim])-based ionic liquids, combined with different anions, in 20 mM glycine buffer (pH = 2) at room temperature were used and amyloid fibrils were observed after few days when $[C_4mim][BF_4]$ and $[C_4mim][PF_6]$ are used [46]. These authors show that the morphology and properties of the aggregates could be modulated depending on the chemical structure of the IL and pH. Although the exact mechanism of protein structure induction is still debatable, it is generally accepted that the nonpolar to polar balance of these solvents is an important parameter in the disruption of the intramolecular hydrogen bonds, which are essential for beta sheets formation.

Very recently, deep eutectic solvents (DES) have been gaining much attention as versatile alternatives to ILs [47]. DES can be regarded as a new generation of ionic solvents composed of a mixture of two or more compounds, where one of them is a salt. The formation a new liquid compound at room temperature is due to the formation of hydrogen bonds between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), usually a halide anion present in the salt [48–50]. The most popular DESs synthesized so far are those based on cholinium chloride (used as HBA) because of its low cost, low toxicity, biodegradability and biocompatibility, since it is considered an essential nutrient, which can be extracted from biomass, and often regarded as a part of the B-complex vitamins [51]. Cholinium chloride has been combined with several classes of HBD such as renewable polyols, carbohydrates, amides, amines, alcohols and carboxylic acids [52]. Cholinium and other ammonium cations, for example betaine, combined with convenient anions, such as saccharinate, lactate, and hexanoate, have been largely explored in the search for natural, biocompatible, renewable and "drinkable" solvents [53]. DES share many of the ILs appealing features, such as low volatility, high thermal stability and conductivity, wide liquid range and high solvation capacity [53–55] and possess other interesting advantages over ILs: they are easier to synthesize, since the components are easily mixed without any further purification; they have low production cost due to the low price of starting materials; and most of the synthesized DES are biodegradable, biocompatible and non-toxic [55,56]. DES are also considered to be cheaper, efficient and greener solutions, and, in this way, are finding many applications from metal finishing processes [57] up to, more recently, compound extraction and separation media for azeotropic mixtures [58–61], with reported performances even superior to conventional organic solvents and ILs. Regarding protein fibrillation, partial denaturation caused by solvent environment or changes in temperature or pressure is prerequisite for fibril formation. To the best of our knowledge, protein fibrillation using DES has only been researched in terms of protein stability studies. The stability of HEWL in different choline chloride-based deep eutectic solvents has been studied and showed the accumulation of discrete, partially folded intermediates that displayed a high content of secondary structure and disrupted tertiary structure when using non-diluted and 10%-diluted urea:choline chloride (2:1) at 70 °C [62].

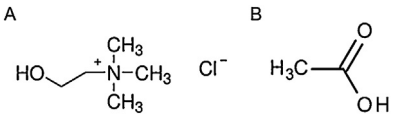

Fig. 1. Chemical structure of (A) cholinium chloride ([Ch]Cl) and (B) acetic acid.

In this work, one widely used DES based cholinium chloride, and a simple acid, acetic acid (Fig. 1), in a proportion of 1:1, ([Ch]Cl:Ac) was studied as a possible promoter of HEWL fibrillation. As mentioned by several authors [51,63], DES having organic acids as HBD do not present melting points on DES, but instead less transition temperatures are observed. The influence of experimental variables such as temperature (room temperature (RT), 50, 70 °C) and pH (2, 5) in the process of fibrillation induction was also researched, as well as the role of DES in the production of protein nanofibers.

2. Experimental details

2.1. Preparation of the DES

As proposed by Florindo et al. [63], the preparation of [Ch]Cl:Ac was performed by first mixing the two components, cholinium chloride (Sigma-Aldrich, ≥98%) and acetic acid (Sigma-Aldrich, ≥99,7%) in a 1:1 mol proportion, and then grinding them in a mortar with a pestle at room temperature until a homogeneous liquid is formed.

2.2. Preparation of protein nanofibers

Lysozyme from hen egg white (Fluka, ~70000 U/mg), was dissolved (2 mg/mL) in an aqueous buffer solution of 10 mM HCl at pH = 2 with 20 mM glycine (Sigma-Aldrich, ≥98,5%) with 1, 5 and 10% (v/v) of the DES. The samples were incubated at different temperatures (RT, 50 °C and 70 °C), under magnetic agitation. The assays at 50 and 70 °C were conducted using an oil bath. For the test at pH = 5, 0.1 M phosphate buffer solution was used. Triplicates of each sample were carried out so that standard deviations can be calculated. The protein nanofibers were separated from the solution with the DES after centrifugation at 15,000 rpm, during 45 min, using a Megafuge 16 R centrifuge (Thermo Scientific). The supernatants were exchanged with Milli-Q ultrapure water. This separation step was repeated 2 times.

Controls using [Ch]Cl and acetic acid were prepared according to the amounts of individual components in the DES, 3.5% (m/v) of [Ch]Cl and 1.5% (v/v) of acetic acid.