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SHORT COMMUNICATION

Enzymatic synthesis of polypeptides in natural deep eutectic solvents

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Abstract

We are first to report the protease-catalyzed polypeptide synthesis in the natural deep eutectic solvent (DES) choline chloride/glycerol (ChCl:Gly) at a 1:2 molar ratio with 30 vol% of water using subtilisin Carlsberg (alkaline protease from *Bacillus licheniformis*). Poly-*L*-phenylalanine, poly-*a*-*L*-lysine, and the co-polypeptides poly(*L*-phenylalanine-*a*-*L*-lysine) as model polypeptides are produced from amino acid ester substrates in 40–70% yield and molecular weights ranging from 5 to 14 KDa according to dynamic light scattering (DLS). The natural DES is composed of 30 vol% water, whereas no polypeptides are formed at lower water contents. Poly-*L*-phenylalanine displays supramolecular self-assembly into homogenous nanotubes in water/isopropyl alcohol mixtures. This enzymatic route to polypeptides has advantages over previous reports in water and polar compressed fluids because of the higher molecular weights and greener process, respectively.

Introduction

The enzyme-mediated polymer synthesis contributes to mild and green bioprocesses. These routes, which include polyesters, polycarbonates, polyphenols, polyanilines, polysaccharides, and peptides are reported in conventional solvents, ionic liquids, and compressed fluids (CF) [1]. Comprehensive reviews and books in this subject are available in the literature [2–6].

De novo peptide linkages are achieved using proteases from amino acid ester substrates and several of these enzymes from bacterial, plant and fungal origin are successfully reported in water [7, 8]. Nonetheless, the drawback of these chemoenzymatic approaches is the proteolytic activities which results in low molecular weight oligopeptides in low yields [9–11]. This hampers further applications for this bio-catalyzed peptides in favor of conventional synthetic processes, which are often costly and time consuming with additional toxicity concerns [12, 13]. It is in this regard that we investigate enzymatic routes towards polypeptides for biomedical applications [14–16]. We recently reported the protease-catalyzed synthesis of high-molecular weight polypeptides in liquid 1,1,1,2-tetrafluoroethane solvent [17, 18], where a poly-*L*-phenylalanine of ca. 30 kDa displays supramolecular self-assembly into homogeneous nanotubes for controlled delivery of antibiotics under physiological conditions [18]. The highly ordered structure for long chain ePPhe is an outstanding result for further biological studies comparing to that same arrangement commonly reported for diphenylalanine or Fmoc-diphenylalanine dimers [18–20]. However, that work received some concerns on the deterioration of the greenish of the process by using this organic CF, despite its low toxicity [8].

Alternatively, natural deep eutectic solvents (DES) have recently emerged as low toxic solvent media [21], and among them the choline chloride (ChCl) mixtures with H-donors stand out due to low costs and high polarity, which can be tuned by adjusting the added water content in the DES formulation itself [22]. This paper discloses the first enzymatic long-chain polypeptide syntheses in a DES, for that we studied ChCl/glycerol (Gly) mixture at a 1:2 molar ratio with added water volumes (ChCl:Gly:water) and the protease from subtilisin Carlsberg (SC). The *L*-phenylalanine and *L*-lysine (*e*-CBz protected) esters are used as model substrates and the successful results are discussed herein.

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Experiments

Enzymatic synthesis of polypeptides in DES

In a typical experiment, 30 mg of SC, the endoproteinase EC.3.4.21.62 of 27 kDa from *Bacillus licheniformis* (initial enzymatic activity $U_0 = 212$), and 6 mmol of desalted amino acid esters, or 3:3 (mmol) for co-polypeptide synthesis, 10 g of ChCl and 13.4 g of Gly were placed in a round bottom flask (50 mL) equipped with a magnetic stirrer. Distilled water (6 mL; 30 vol%) was added to the flask and the contents were stirred at 40 ºC for 2–3 h until product precipitation was observed, which was recovered by filtration (0.45 µm) and washed with cold ethanol (5 °C). Then, ultrafiltered in Amicon Ultra-4 centrifugal filter 3KDa (MWCO Millipore). Products were neutralized to pH 7 with NaOH (0.1 N) and lyophilized to attain enzymatic poly-L-phenylalanine ethyl ester (ePPhe), *Nε*-protected poly-*a*-*L*-lysine methyl ester (ePLysCbz), and co-polypeptide ePPheLysCbz as slight yellow powder. For Cbz deprotection, 0.5 g of ePPheLysCbz or ePLysCbz was placed in a 50 mL flat-bottom flask with distilled water containing the minimum possible volume of CF_3COOH to dissolve the sample (final volume 100 mL), and 30 wt% of Pd-C catalyst (palladium on active carbon 10wt% Pd Evonik NOBLYST P1173). The flask was placed in a high pressure 300 mL Parr reactor and pressurized with hydrogen (6 atm) for 6 h. Products were recovered with distilled water and concentrated by ultrafiltration (3KDa), neutralized to pH 7 with NaOH (0.1 N) and lyophilized to attain enzymatic poly-*a*-*L*-lysine methyl ester (ePLys) and poly-*L*-phenylanine-co-*a*-*L*-lysine ester (ePPheLys) as slight yellow powder. Results of all experiments are obtained from triplicates. Control experiments without enzyme under all the experimental conditions yielded no peptide products.

Characterizations

1H NMR spectra were recorded on a Varian Unity Innova spectrometer (USA) at 400 MHz in D_2O using TMS as standard calibration. Dynamic light scattering analyses (DLS) were conducted in a Zetasizer µV instrument (Malvern) equipped with an 830 nm photodiode laser to determine molecular weights. Samples were prepared in a LiCl solution (0.2 M). After filtration, 500 µL of diluted sample (5 mg/mL) was allowed to equilibrate at 25 ºC for 10 min. Ten measurements were conducted in 30 s time intervals for each sample. Data were analyzed to determine the hydrodynamic radius value, which correlates with the molecular weight as the mean size of the dominant peak. For SEM analysis, glutaraldehyde (Sigma-Aldrich) was added to the sample (5 v/v%) and stored at 5 °C for 48 h. The preparations were dehydrated in a graduated series of alcohol and covered with gold. Samples were observed in a JEOL JSM-5900-LV scanning electron microscope (Japan).

Supramolecular self-assembly of ePPhe into nanotubes and scanning electron microscopy (SEM) analysis

To a stock solution (7.6 mg/mL) of ePPhe in distilled water was added isopropyl alcohol as co-solvent to a 1 vol% concentration of the alcohol in the final mixture ([ePPhe] = 7.52 mg/mL). Then, 20 µL from each solution were deposited over a microscope coverslip in a 24-well plate and dried at 4 °C for slow solvent evaporation.

Results and discussion

The protease SC catalyzes the successive polyaminolysis reactions in the proposed DES with yields ranging from 41 to 70% (Table 1). Initial attempts to synthesize polypeptides using neat ChCl:Gly mixtures at a 1:1, 2:1 and 3:1 molar ratios were unsuccessful. The addition of increasing volumetric amounts of water to ChCl:Gly at a 1:2 molar ratio allows the propagation of the peptide chains under study with molecular wights up to of 14 KDa (Table 1).

The spectra for the final achieved polypeptides with signal assignments are shown in Figs. 1 and 2 for the homopolypeptides and co-polypeptides, respectively. Best results are obtained using up to 30 vol% of water content in the final solvent mixture. Of note, experiments conducted with water contents from 5 to 20 vol% display only oligopeptides in low yields (data not shown). In this regard, the addition of water is reported to allow designing DESs for wider applications by decreasing viscosities, and generally small amounts of water are desired not to affect the network structure and preserve the bridging between ChCl and Gly [23]. However, recent studies using ChCl:Gly (1:2) indicate

Table 1 Molecular weight and yields for the protease-catalyzed polypeptides in DES

Entry	Molecular weight (kDa) by DLS	Molar fraction of *a*-Lys units by ^{1}H NMR[a]	Yield (%)
ePLysCbz	5.15 ± 1.91	100	76.21
ePLys	3.2 ± 0.50	100	34.6
ePPheLysCbz	14.35 ± 3.10	0.58	54.34
ePPheLys	1.95 ± 0.50	0.92	18.6
ePPhe	14.1 ± 0.85	0	41

[a]Obtained by integration of phenyl H of Phe and methylene H of *a*-Lys units

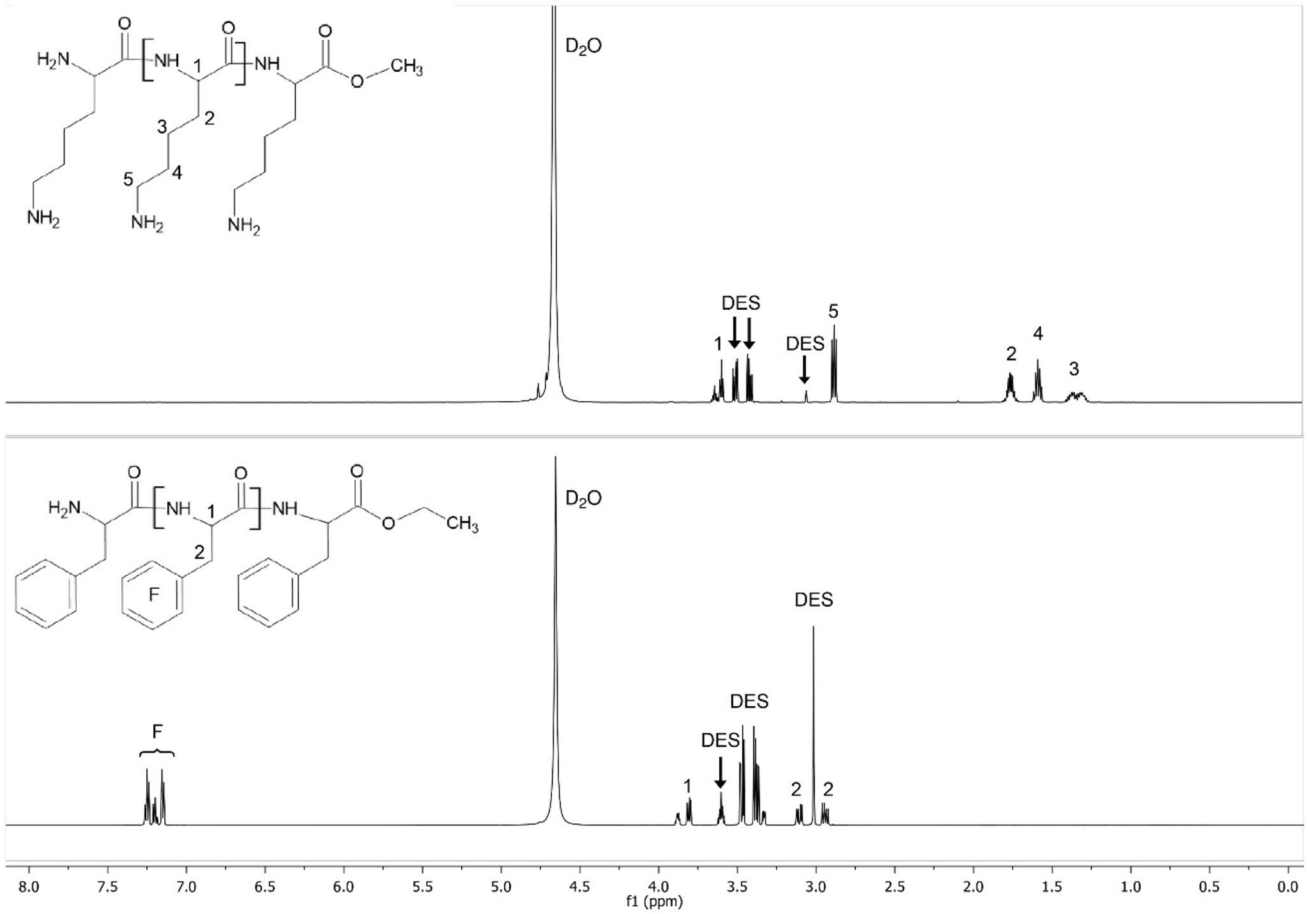

Fig. 1 ^{1}H NMR spectra for enzymatically synthesized Plys (above) and PPhe (below) in ChCl:Gly:water

a Janus-face role of water in the supramolecular ChCl/Gly/water nanostructured network. The water molecules hydrogen-bond with chloride, Gly and Ch, with a decrease in the interactions of the halide anion with the latter two [24]. According to molecular dynamics simulation, water is able to bridge Ch and Gly as chloride does, reaching its maximum with 35.8 wt% of water content in the mixture [24]. More recently, NMR studies also using ChCl:Cl at a 1:2 molar ratio demonstrate that between 11 wt% and 35 wt% water, the solvation of the Ch, chloride and Gly occurs, but the DES nanostructure is still present. However, the authors state that beyond 35 wt% this supramolecular structure begins to disrupt, thus transiting into a DES-in-water solution [25]. Clearly, more efforts are needed to understand the characteristics of ChCl/Gly/water mixtures as intrinsically "designer solvents", since besides of being a suitable solvent for enzymatic reactions, other authors have pointed out the great potential of incorporating bioactive components in this fluid for biological applications.

However, it is worth to remark that the residual enzymatic activity of SC in this medium after 3 h exposure at 40 °C displays a 7-fold decrease, from the initial 7.9 ± 0.29 U/mg in water to 1.12 ± 0.31 U/mg. The enzymatic activity considerably decreases for the other mixtures with lower water content, and it depletes when incubation in neat DES. This evidence an inhibition of this enzyme by one or both organic components of the DES, and further work is needed to assess the activities for proteases in these reaction media.

The precipitation of white solids starts approximately after 1.5-2 h of reaction time, thus indicating the termination of the growing peptide chains by earlier precipitation from the reaction media. In this regard, ePPhe displays the highest molecular weight (ca. 14 KDa) according to DLS analyses (Table 1), but in lower yield compared to ePLysCbz, which is attained in the lowest molecular weight. Therefore, the experimental evidence indicates that growing poly-*a*-lysine chains with *e*-amine protected Cbz group are less soluble in this media. A molar ratio of 0.58 for LysCbz units in the co-polypeptide, which is obtained by integration of assigned signals in 1H NMR, agrees with the previous report by Espinoza-González et al. using liquid 1,1,1,2-tetrafluoroethane solvent medium [26] with random incorporation of statistically equal molar ratio of both amino acids in the polypeptide. Of note is that increasing enzyme

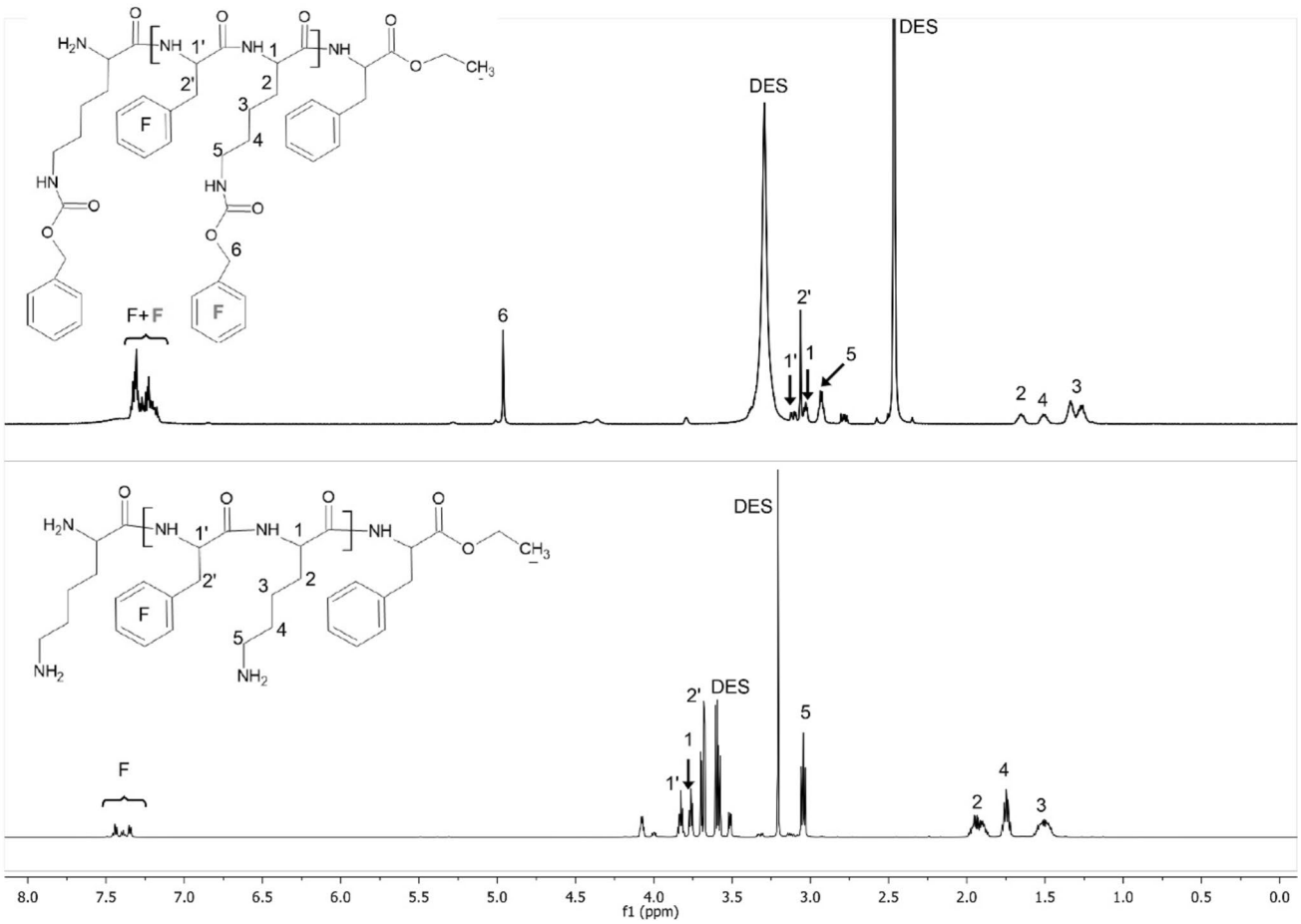

Fig. 2 ^{1}H NMR spectra for enzymatically synthesized co-polypeptide Cbz-protected (above) and deprotected (below) in ChCl:Gly:water

amounts, that is increasing enzymatic activity, might result in increasing yields, since the main limitation for the growing chains is the solubility in the media. Concomitantly, the amino acid feed could control the final unit ratios in these non-conventional solvent systems with SC biocatalyst, although other amino acid ester substrates must be also investigated in this system to corroborate this statement.

Noteworthy, the removal of Cbz group suffers from depolymerization which is attributed to the trifluoroacetic acid used to dissolve the polymer in the deprotection step. The co-polypeptide requires more CF_3COOH than that for ePLysCbz in our experimentation, which might explain the drop in molecular weights (Table 1). On the other hand, the highly significant reduction in Phe units in the Cbz-free co-polypeptide shown in Fig. 2 is a clear indication of the fractionation of the product in Lys rich chains according to the deprotection procedure. Therefore, catalytic hydrogenation by Pd over carbon for Cbz removal might not be best for our purposes and other less aggressive Cbz deprotection procedures can be conducted, as reported in the literature [27, 28].

Another important feature is that all 1H NMR spectra show the presence of residual Gly and Ch as indicated in assigned signals in Figs. 1 and 2, which remain in products as non-toxic residues from synthesis. This evidence suggests strong hydrogen bonding or dipole-dipole interactions between polypeptides and the eutectic mixture forming molecules as neither the purification processes using ethanol washings, nor the ultrafiltration (3KDa cut) could deplete these signals. These purification requirements are also found for ionic liquids in enzyme-mediated polymer syntheses [29–31] owing to the low boiling and high polarities in this type of solvents, and opposite to the use CFs solvents which evaporate after reaction [18, 19, 32, 33].

Finally, Fig. 3 shows the SEM micrograph for a sample of enzymatically synthesized ePPhe (14 KDa) arranged into homogeneous nanotubes. It is worth noting that other reported nanotubes from dimers and oligomers

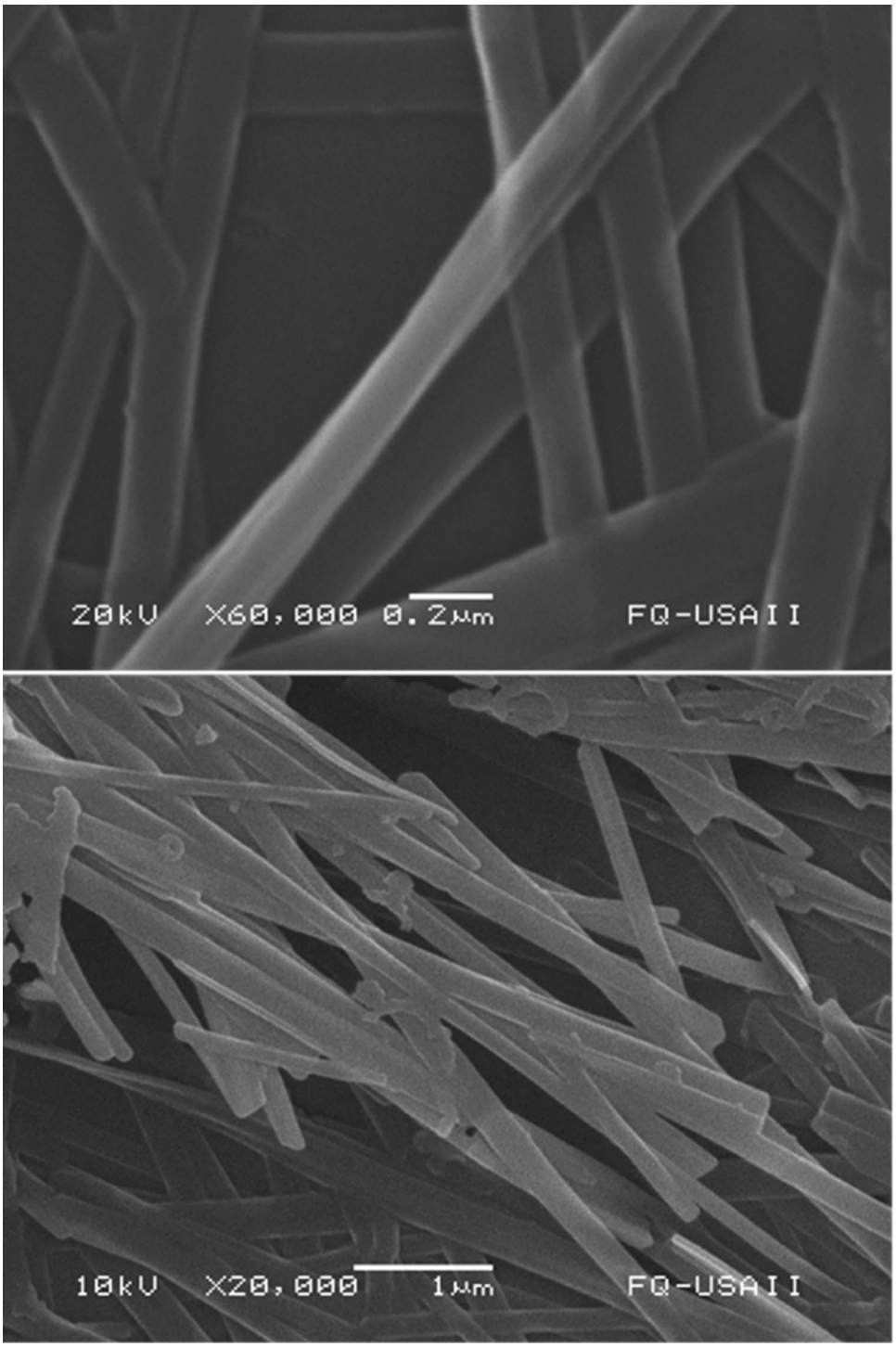

Fig. 3 SEM micrographs for supramolecular self-assembly of ePPhe into nanotubes at different enlargements

of phenylalanine require higher concentrations and co-solvent contents than the SC-mediated ePPhe to attain homogeneous nanotubes [19, 20, 34]. A supramolecular self-assembly for the above-mentioned SC-mediated long chain ePPhe (30 KDa) using the organic CF solvent medium, is described in Romero-Montero et al. from aqueous/hexafluoro isopropanol (1 vol%) solution [18]. In the present work, however, this highly ordered supramolecular structures are also achieved from a water solution of ePPhe and less toxic isopropyl alcohol (final alcohol concentration of 1 vol%).

Conclusively, we evidence a low-toxic enzymatic route to high molecular weight polypeptides with great potential for further biomedical applications, such as supramolecular hydrogels for tissue engineering and drug delivery studies, which are currently underway.

Conclusions

An enzymatic synthesis to polypeptides in high molecular weights (up to 14 KDa) is demonstrated using ChCl/Gly (at a 1:2 molar ratio) with 30 vol% of water. The addition of at least more than 20 vol% water content is crucial for enzymatic activities since the results display inhibition of the subtilisin Carlsberg by the DES adducts. The synthesized ePPhe has higher molecular weight than PLys under our experimental conditions, but in lower yields. Strong dipole-dipole interactions between polypeptides and glycerol/choline renders eutectic solvent residues in products not affecting further biological applications. This chemoenzymatic route in ChCl:Gly:water DES media can be extended to other protease-catalyzed polypeptide synthesis.

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Declarations

Conflict of interest This is to confirm that there is no conflict of interest to disclose.

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