

Articles

Biosynthesis of an Amphiphilic Silk-Like Polymer

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An amphiphilic silk-like protein polymer was efficiently produced in the yeast *Pichia pastoris*. The secreted product was fully intact and was purified by solubilization in formic acid and subsequent precipitation of denatured host proteins upon dilution with water. In aqueous alkaline solution, the negatively charged acidic polymer assumed extended helical (silk III-like) and unordered conformations. Upon subsequent drying, it assumed a conformation rich in β -turns. In water at low pH, the uncharged polymer aggregated and the solution became turbid. Concentrated solutions in 70% (v/v) formic acid slowly formed gels. Replacement of the formic acid–water mixture with methanol and subsequent drying resulted in β -sheets, which stacked into fibril-like structures. The novel polymer instantaneously lowered the air–water interfacial tension under neutral to alkaline conditions and reversed the polarity of hydrophobic and hydrophilic solid surfaces upon adsorption.

Introduction

Material scientists have long been investigating ways of producing stimulus-responsive, nanostructured, and self-assembling polymers for high-end applications such as self-healing coatings, nanofibers, nanoelectronic assemblies, surgery, tissue engineering, and drug delivery. Because of the limited level of control over conventional polymerization processes, interest has increased for biological polymeric materials and proteins in particular. The monomers that constitute a protein polymer are monoenantiomeric (L - α) amino acids, and their sequence is exactly defined by an invariant genetic (DNA) template.

Proteins vary greatly in characteristics, such as their specific folding and 3D structure, their hydrophobicity and net charge, and their interaction with other molecules. Protein engineering allows to adapt the natural DNA templates or design novel templates and, thus, to produce monodisperse polymers, consisting of one or multiple blocks, each of which can be designed

to be inert, stimulus-responsive, or promoting self-assembly into specific supramolecular structures. Protein polymers that have thus far been produced by genetic engineering include elastin-like, collagen-like, silk-like, leucine zipper-like, and newly designed sequences. These biomimetic materials have been excellently reviewed elsewhere.^{1–5}

Real-life application of biosynthetic polymers requires that a sufficiently high productivity is achieved. We previously reported the secreted production of collagenous polymers in the yeast *Pichia pastoris* at very high levels.^{6–8} In the present work we studied the possibility of secreted production in this host of pH-responsive surface active polymers for coating applications. As a structural framework for the design of such polymers, we chose a silk-like polymer pioneered by the group of Tirrell,⁹ consisting of repeating $(GA)_3GE$ octapeptides. The acidic glutamate residues, in combination with the self-assembling properties of silk Gly-Ala repeats,¹⁰ impart pH-responsive properties. When dried from certain solvents^{9,11,12} it forms insoluble, silk crystal-like stacks of antiparallel β -sheets, bordered by Gly-Glu-Gly (3-residue) γ -turns, with successive glutamic acid residues on opposite sides of the sheet. To elicit amphiphilic characteristics, we replaced alternate glutamates with leucine, as a highly hydrophobic and otherwise inert,

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Table 1. Oligonucleotide and Protein Sequences

name	DNA or amino acid sequence
EE-FW ^a	AATTCGGTCTCGGTGCTGGTGCTGGTGC GTGAGGGAGCCGGTGCAGGAGCCGGCA AGGTGCCTAACGCCGC
EE-RV ^a	TCGAGCGGCCGCTTAGGCACCTTCGCCGGC TCCAGCACCGGCTCCCTCACCAAGCACCA GCACCAGCACCGAGACCG
EL-FW ^a	AATTCGGTCTCGGTGCTGGTGCTGGTGC GTGAGGGAGCCGGTGCAGGAGCCGGCT GGGTGCCTAACGCCGC
EL-RV ^a	TCGAGCGGCCGCTTAGGCACCCAAGCCGGC TCCAGCACCGGCTCCCTCACCAAGCACCA GCACCAGCACCGAGACCG
EE24 ^b	YVEFGL[(GA) ₃ GE(GA) ₃ GE] ₂₄ GA
EL28 ^b	YVEFGL[(GA) ₃ GE(GA) ₃ GL] ₂₈ GA

^a DNA sequence (5'-3') of oligonucleotides used for construction of gene monomers. ^b Theoretical amino acid sequence of mature protein.

aliphatic amino acid, resulting in poly-[(GA)₃GE(GA)₃GL)]. We describe here the efficient biosynthesis of this amphiphilic polymer in the yeast *P. pastoris*, its purification, and the characterization of its physicochemical and structural properties.

Materials and Methods

Generation of Recombinant Strains. A double-stranded DNA monomer EE, encoding the amino acid sequence (GA)₃GE(GA)₃GE, was produced by annealing of two complementary oligonucleotides EE-FW and EE-RV (Table 1). Likewise, a monomer EL encoding (GA)₃GE(GA)₃GL was produced by annealing of the complementary oligonucleotides EL-FW and EL-RV (Table 1). Both double-stranded adapters have EcoRI/XbaI overhanging ends. The adapters were ligated into an EcoRI/XbaI digested pMTL23 vector,¹³ which had been modified to remove the BsaI site normally present. The inserts were multimerized by digestion with BsaI/BanI and recursive directional ligation. Clones of similar size were selected for both protein types: EE24, bearing 24 repeats of the EE monomer, and EL28, bearing 28 repeats of the EL monomer. The inserts were cloned into the EcoRI/NorI sites of expression vector pPIC9 (Invitrogen, Breda, The Netherlands). The resulting vectors were linearized with SalI to promote homologous integration at the *his4* locus upon transformation of *P. pastoris* GS115 by electroporation, as described previously.⁶ See Table 1 for the EE24 and EL28 amino acid sequences expected upon expression of the cloned genes.

Fermentation of *P. pastoris*. Fed-batch fermentations were performed in 2.5 L Bioflo 3000 fermenters (New Brunswick Scientific, Nijmegen, The Netherlands), essentially as described by Zhang et al.¹⁴ Minimal basal salts medium¹⁴ was used and no protease-inhibiting supplements were added. The pH throughout the entire fermentation was maintained at 5.0 (EE24) or 3.0 (EL28) by addition of ammonium hydroxide as base. The methanol fed-batch phase for protein production lasted three to four days. A homemade semiconductor gas sensor-controller, similar to that described by Kataoka et al.,¹⁵ was used to monitor the methanol level in the off-gas and to maintain a constant level of ~0.2% (w/v) methanol in the broth. At the end of the fermentation, the cells were separated from the broth by centrifugation for 10 min at 10000 × g and 4 °C in a Sorvall SLA-3000 rotor (Thermo Scientific, Breda, The Netherlands), followed by microfiltration of the supernatant. For EE24, the pH of the culture was raised to 10.0 by addition of sodium hydroxide prior to centrifugation to prevent the formation of a gel layer on top of the cell pellet.

Purification of EE24 and EL28. All centrifugation was done for 30 min at 20000 × g and 4 °C, interchangeably in a Sorvall SLA-1500 or SLA-3000 rotor (Thermo Scientific).

The EE24 protein was precipitated from the cell-free broth by adjusting the pH to 3.0 with sulfuric acid followed by centrifugation. The precipitate was resuspended in an equal volume (relative to the

cell-free broth) of Milli-Q water adjusted to pH 9.0 with sodium hydroxide. The pH was then adjusted to 3.0 with sulfuric acid, ammonium sulfate was added to 30% of saturation, and the sample was centrifuged. The ammonium sulfate precipitation was repeated once, and the final pellet was resuspended in 0.2 volumes (relative to the cell-free broth) of Milli-Q water, and the pH was adjusted to 9.0 with ammonium hydroxide. Ethanol was added to 80% (v/v), and the sample was centrifuged. The supernatant containing a portion of the pure EE24 protein was collected. The pellet was subjected to several more rounds of resuspension and ethanol precipitation to increase the recovery of the EE24 protein extracted in ethanol. The protein was then precipitated from the combined supernatants by addition of sodium chloride to 50 mM and centrifugation. The air-dried pellet was resuspended in Milli-Q water and extensively desalted by repeated isoelectric precipitation using a volatile acid (formic acid) and dialysis against Milli-Q water. The final EE24 product was lyophilized.

The EL28 protein was precipitated from the cell-free broth by addition of acetone to 40% (v/v) and centrifugation. The pellet was resuspended in an equal volume (relative to the cell-free broth) of 100% (v/v) formic acid and incubated overnight at room temperature. The liquid was diluted 15 times with Milli-Q water and centrifuged to pellet contaminating proteins. The EL28 protein was precipitated from the supernatant by addition of ammonium sulfate to 40% of saturation and centrifugation. This step was repeated once, and the final pellet was resuspended in an equal volume (relative to the cell-free broth) of Milli-Q water. The protein was then precipitated by addition of 40% (v/v) acetone and centrifugation. The air-dried pellet was resuspended, desalted and lyophilized as described above for EE24.

SDS-PAGE, Densitometry, and N-Terminal Protein Sequencing. The NuPAGE Novex system (Invitrogen) was used for SDS-PAGE, with 10% Bis-Tris gels, MES SDS running buffer, and SeeBlue Plus2 prestained molecular mass markers. All protein samples were preincubated for 30 min with 6 M urea, pH 8.5, prior to mixing with NuPAGE LDS loading dye (Invitrogen), and were not heated before electrophoresis. Gels were stained with Coomassie SimplyBlue SafeStain (Invitrogen). Quantification of protein bands by densitometry was performed with a GS-800 calibrated densitometer (Bio-Rad, Veenendaal, The Netherlands), relative to a calibration curve of the corresponding purified protein (see Results and Discussion for purity). The standard solution used to make the calibration curve was prepared by weighing a known amount of the pure lyophilized protein. Blotting of proteins for N-terminal sequencing by Edman degradation was as described previously.⁶ Protein sequencing was performed by Midwest Analytical (St. Louis, MO).

Mass Spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using an Ultraflex mass spectrometer (Bruker, Wormer, The Netherlands), following a procedure modified after Wenzel et al.¹⁶ Samples were prepared by the dried droplet method on a 600 μm AnchorChip target (Bruker), using 5 mg/mL 2,5-dihydroxyacetophenone, 1.5 mg/mL diammonium hydrogen citrate, 25% (v/v) ethanol, and 7% (v/v) trifluoroacetic acid as matrix. Measurements (50 shots at 20 Hz) were made in the positive, linear mode, with the following parameters: ion source 1, 20000 V; ion source 2, 18450 V; lens, 5000 V, pulsed ion extraction, 550 ns. Protein Calibration Standard II (Bruker) was used for external calibration.

Protein Crystallization. Crystallization of proteins was performed in 70% (v/v) formic acid (Merck, reagent grade, ≤10 ppm nonvolatile matter) under vapor diffusion of methanol, similar to Cantor et al.¹¹ Samples were incubated overnight at room temperature at 30 mg/mL in 70% (v/v) formic acid under gentle agitation. The cup containing the sample was then opened and placed in a bigger closed vessel containing a bottom layer of 100% (v/v) methanol. After incubation for 48 h at room temperature the protein gel was washed three times with 100% (v/v) methanol.

Infrared Spectroscopy. Protein gels in 100% (v/v) methanol were smeared onto a zinc selenide attenuated total reflection (ATR) crystal

and allowed to dry. Infrared spectra were recorded using a Vector 22 Fourier transform infrared spectroscopy (FTIR) spectrometer (Bruker) set as follows: resolution 4 cm^{-1} , scanner speed 10 kHz, number of scans 1024.

Circular Dichroic Spectroscopy. For measurement in solution the proteins were dissolved in 50 mM sodium borate, pH 9.0. Alternatively, proteins were dissolved in dilute ammonium hydroxide, pH 9.0, and dried as a film onto the outside of a cuvette before measuring. Circular dichroic (CD) spectra were recorded at 20 °C using a J-715 spectropolarimeter (Jasco, IJsselstijl, The Netherlands). Spectra were obtained as the average of 10 consecutive scans from 260 to 190 nm, using a scanning speed of 100 nm/min at a resolution of 0.2 nm. Data were smoothed by applying a moving average filter with a window size of 2 nm, and the spectra were normalized to similar peak intensities in view of their nonquantitative character.

Drop Tensiometry. The liquid–air surface tension as a function of time was measured at room temperature using an automated drop tensiometer¹⁷ (IT Concept, Longessaigne, France). Each measurement started with a clean interface of a newly formed drop of protein solution (10–15 μL) in air. Proteins were freshly prepared in 50 mM sodium phosphate buffer at 2 g/L (pH 8.0) or 0.2 g/L (pH 3.0). Surface tension was determined by drop shape analysis.

Contact Angle Measurements. Polystyrene discs cut from petri-dishes (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) or 0.03 mm aluminum discs (Wanit Universal GmbH & Co., Dietzenbach, Germany) were thoroughly washed with Milli-Q water and 100% (v/v) ethanol before use. After air drying, the discs were submerged for 48 h at room temperature in 70% (v/v) formic acid (blank) or in a 1 mg/mL protein solution in 70% (v/v) formic acid. The discs were then incubated for three hours at –20 °C and subsequently rinsed with 10 mM hydrochloric acid, followed by two agitated washes for 45 min in Milli-Q water and air drying. Sessile drop contact angles were determined using a VCA Optima XE surface analysis system (AST Products, Billerica, MA). Milli-Q water droplets of 2 μL (polystyrene) or 1.5 μL (aluminum) were applied, and contact angles (expressed as the average of the left and right values) were determined after a delay of five seconds. A total of 15 replicate droplets divided over three plates were measured for each sample.

Atomic Force Microscopy. Proteins were crystallized as described above, but at 1 mg/L protein. Si(100) wafers (n-type, 1–5 Ω/cm) modified with a carboxylic acid-terminated monolayer of undecylenic acid^{18–20} were submerged in the protein solution during crystallization. The discs were then washed with Milli-Q water to remove nonadsorbed material and with 100% (v/v) methanol to facilitate air drying. Atomic force microscopy (AFM) was in tapping mode in air using a Dimension 3100 or MultiMode system, a NanoScope IV controller and NanoScope 6.12r2 software (Digital Instruments, Santa Barbara, CA), and NSG10 cantilevers (NT-MDT, Zelenograd, Russia). Image processing (flattening and plane fitting) and analysis (line profiles) were done using WSXM 4.0 software.²¹

Results and Discussion

Biosynthesis and Purification. Fermentations were performed with *P. pastoris* strains expressing the EE24 nonamphiphilic prototype and the EL28 amphiphilic variant as extracellular proteins (see Table 1 for protein sequences). Initial cultures were done at pH 3.0, but for EE24 it was found necessary to culture at pH 5.0 because the secreted protein would otherwise form a gelly precipitate. Culture supernatants were analyzed by SDS-PAGE (Figure 1A, lanes 1,2). The samples subjected to SDS-PAGE contained urea (see Materials and Methods), because otherwise, the EL28 protein was often not detectable on gel except as halo-like bands just below the wells (as confirmed by N-terminal sequencing). The urea probably solubilized nonelectrophoresable aggregates. EE24 and EL28 migrate slower in SDS-PAGE than would be expected on the

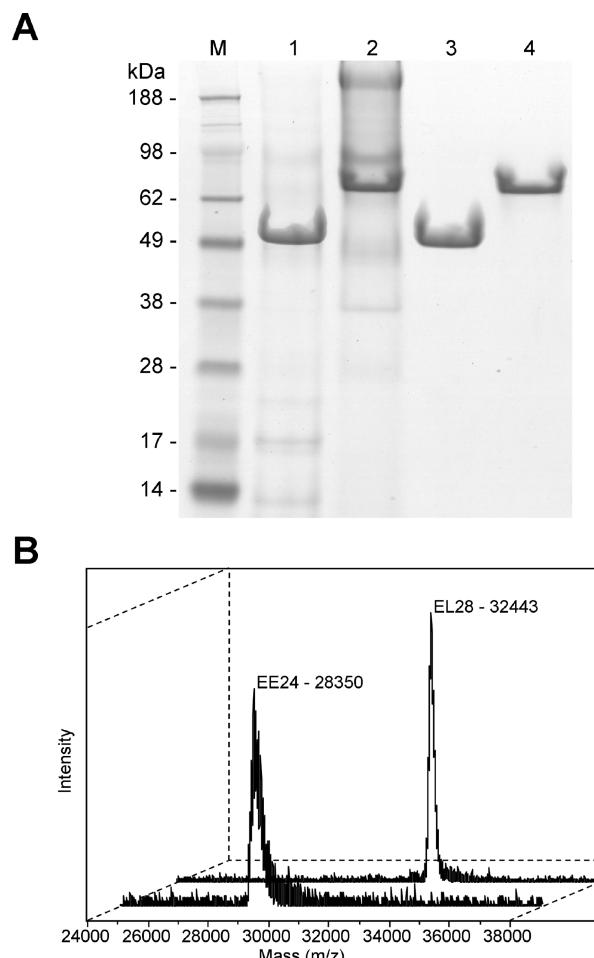


Figure 1. Production and purification of silk-like polymers. (A) SDS-PAGE of total extracellular protein and purified EE24 and EL28. Lane M, molecular weight marker; 1, EE24 culture supernatant (2 μL); 2, EL28 culture supernatant (6 μL); 3, purified EE24 (6 μg); 4, purified EL28 (6 μg). (B) MALDI-TOF mass spectra of purified EE24 and EL28.

basis of their molecular weight (28248 and 32366 Da, respectively), which is a known anomaly for this type of protein.²²

EE24 was purified essentially by a combination of isoelectric precipitation and subsequent removal of remaining host proteins by ethanol precipitation. We were unable to purify EL28 using the same or other common approaches (differential salt precipitation, filtration, ion exchange, and hydrophobic interaction chromatography), in that the product consistently copurified with host proteins. In view of the above-mentioned aggregation problems during SDS-PAGE, it seems plausible that the amphiphilic EL28 formed insoluble complexes with these contaminating proteins. Indeed, a successful purification method was found that relies on the use of pure formic acid as an effective organic solvent to disrupt intra- and intermolecular protein interactions. Upon dilution of the solubilized proteins with water, the denatured host proteins precipitated nearly quantitatively, while the silk-like protein remained soluble, at least for some time. The purification methods developed for EE24 and for EL28 both resulted in highly pure and intact protein (Figure 1A, lanes 3,4). The purity of both polymers on the protein level was estimated to be at least 98%, based on amino acid analysis and subsequent linear least-squares fitting of the observed data to a combination of (1) the theoretical composition of the respective pure protein and (2) the composition determined for host-derived proteins present in the medium

(not shown). Polysaccharides are the major nonprotein contaminant in these preparations and were present at less than 1% (m/m), as determined by a carbohydrate assay.²³ MALDI-TOF mass spectrometry (Figure 1B) showed that the proteins have the expected molecular weights and confirmed the monodispersity apparent from SDS-PAGE. N-terminal sequencing revealed the expected sequence (see Table 1) for both proteins (YVEFGLG), with no heterogeneity resulting from their proteolytic maturation in the host organism.

To estimate the yield of EE24 and EL28 at the end of fermentation, an SDS-PAGE gel with final culture supernatants from duplicate fermentations and known quantities of the corresponding purified protein was analyzed by densitometric scanning. The yield calculated for EE24 was 2.9 ± 0.3 g/L of clarified broth and for EL28 0.9 ± 0.2 g/L (\pm SD). With purified EL28 (as with both purified and unpurified EE24), a linear relationship was found between the densitometric response and the amount of protein over a wide range, while for unpurified EL28, this range was very narrow. This indicates that the above-mentioned aggregation problems of EL28 culture supernatant during SDS-PAGE are not completely resolved by the presence of urea in the samples. It cannot be ruled out that even within the linear range used, part of the unpurified EL28 might be obscured, and the yield estimated for EL28 could be an underestimation. In any case, the range of roughly 1–3 g/L of secreted protein described here rates as high-yield expression for *P. pastoris*²⁴ or microbial hosts in general.²⁵

The differences encountered between EL28 and EE24 with respect to their aggregation behavior are noteworthy. While EE24 precipitated from the fermentation broth at pH 3.0, EL28 did not. In purified form, however, both proteins could be precipitated at low pH, suggesting that in the broth EL28 may have bound other components that prevent its self-aggregation. Moreover, the formation of such complexes may explain why only unpurified EL28 shows migration and detection problems in SDS-PAGE.

Secondary Structure. Poly-[((GA)₃GE)] self-organizes into crystalline stacks of antiparallel β -sheets when dried after gel formation in 70% (v/v) formic acid and methanol vapor diffusion.¹¹ We verified if EL28, which has alternate glutamic acids replaced by leucine, could also form such a structure under these conditions. Unfortunately, CD spectroscopy of the turbid dry films of EE24 and EL28 prepared under these conditions could not be used due to scattering artifacts. Therefore, the protein gels were dried onto an ATR crystal and ATR-FTIR was performed. Indeed, the spectra of both EE24 and EL28 (Figure 2) show amide I, II, and III vibrations around 1622, 1522, and 1230 cm⁻¹, respectively, characteristic of the β -sheet conformation, while the weak amide I band at \sim 1697 cm⁻¹ is indicative of the antiparallel β -sheet conformation.⁹ The amide I component at \sim 1652 cm⁻¹ may arise from a disordered conformation, chain folds, or a trace of silk I-like structures^{9,12} and has been shown to correlate with the level of hydration of the molecule.²⁶ This suggests that, given its lower absorbance at this frequency, a lower fraction of EL28 than of EE24 is present in disordered form. Accordingly, tentative fitting of the spectra to reference spectra and Lorentzian/Gaussian peak shapes proposed by Venyaminov et al.²⁷ indicated that the contribution of β -sheet in EL28 was around 82–92% and approximately 1.3 times higher than that in EE24. This may result from packing constraints or because in 70% (v/v) formic acid–30% (v/v) water some hydrophobic effects play a role and a juxtaposition of dehydrated nonpolar leucine residues, as compared to

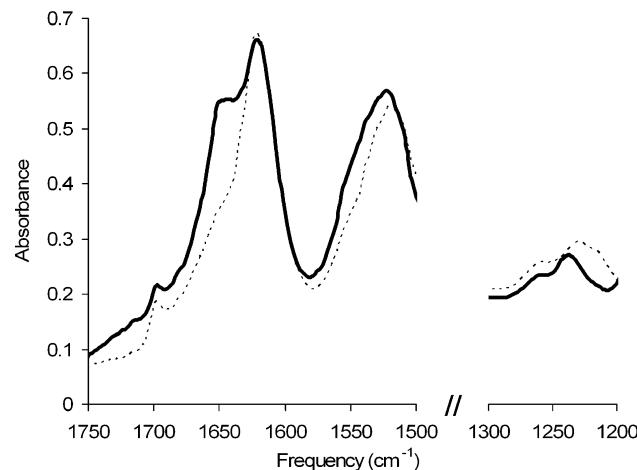


Figure 2. ATR-FTIR spectra of crystallized EE24 (solid line) and EL28 (dotted line).

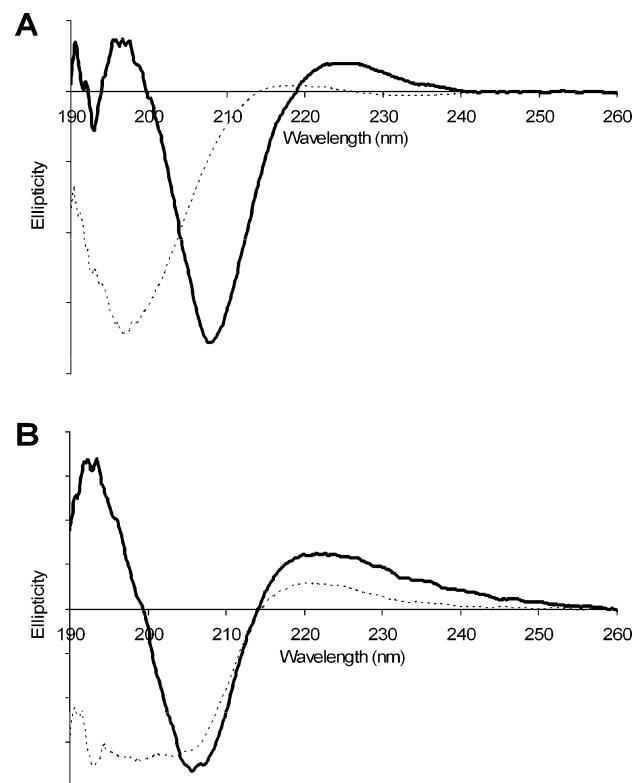


Figure 3. CD spectra of purified EE24 (A) and EL28 (B). Broken lines, aqueous solution at pH 9.0; solid lines, films dried from aqueous solution at pH 9.0. Spectra are normalized to similar peak intensities.

H-bonding glutamic acid residues, promotes the transition to the crystalline state.

In view of the potential formulation of the protein in aqueous solutions or dispersions for coating applications, the behavior of EL28 under these conditions and after drying was analyzed next by CD spectroscopy and compared to EE24 (Figure 3). At low pH, both EL28 and EE24 aggregated, and the resulting turbidity of the samples did not permit the registration of reliable UV-CD spectra. The spectra obtained in alkaline buffer suggest a mixture of random and extended (polyglycine II-like²⁸ or silk fibroin type III-like²⁹) conformations, where the more positive relative ellipticity around 220 nm for EL28 indicates a higher contribution of extended conformation elements as compared to EE24. Drying the polymers dissolved in (volatile) alkaline

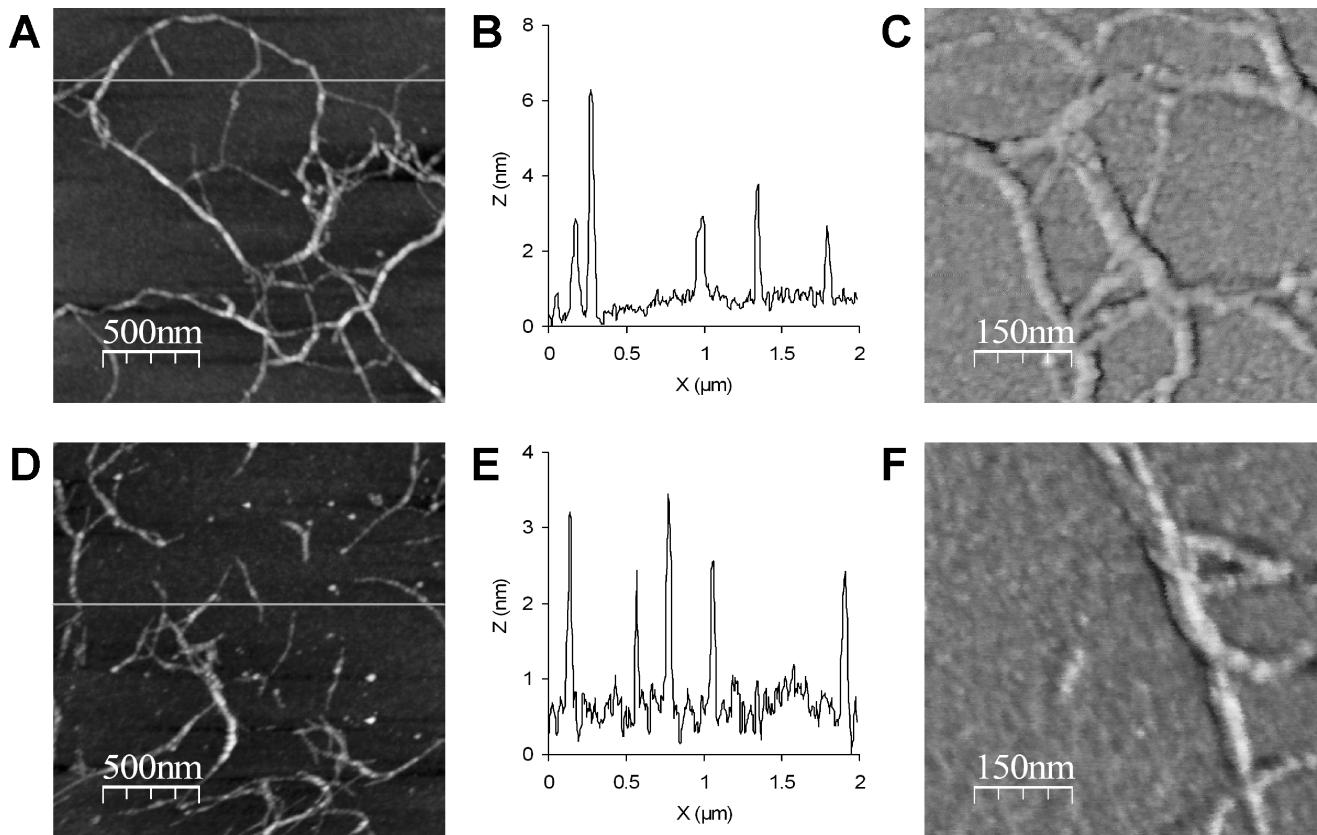


Figure 4. AFM of EE24 (A–C) and EL28 (D–F) fibrils deposited on modified Si(100) wafers under crystallizing conditions. (A, D) Topography image. (B, E) Topography profiles for the cross-sections indicated in A and D. (C, F) Detail of fibrils: shown are phase images to allow optimal resolution of fibril structure.

buffer to a film resulted in uncommon spectra, having a minimum at about 208 nm and maxima around 195 and 220 nm. Lednev et al.³⁰ have recorded very similar spectra of the related protein poly-[(GA)₃GY(GA)₃GE(GA)₃GH(GA)₃GK]. Such spectra were also seen by Safar et al.,³¹ who showed that, depending on the conditions, two clearly different CD spectra could be recorded in dry poly-L-lysine films. One was a prototypical β -sheet spectrum, observed under conditions where FTIR also indicated mainly β -sheet components. β -Sheet CD spectra of for example poly-L-lysine³² and silk fibroin type II¹⁰ have a single negative peak at \sim 217 nm and lack a positive band around 220–225 nm. The other spectrum observed by Safar et al. in poly-L-lysine films³¹ was found under conditions where FTIR indicated non- β -sheet structures, and it closely resembled the unusual film spectra shown in Figure 3. As Crisma et al.³³ determined highly comparable spectra for the model peptides Z-Aib-L-Pro-Aib-L-Pro-Ome and Piv-L-Pro-Aib-NHMe when present in a mixed β -turn conformation, we tentatively ascribe the observed spectra of dried EE24 and EL28 to a conformation rich in β -turns. Although the exact types of β -turns in our material are not necessarily the same, it is interesting to note that also poly(Ala-Gly) in type II conformation³⁴ and *Bombyx mori* silk fibroin in type I conformation³⁵ are composed of repeated β -turns.

Supramolecular Fibrils. Proteins, dissolved under crystallizing conditions, were adsorbed to surface-modified Si(100) wafers. The wafers were thoroughly rinsed and analyzed by AFM (Figure 4). Fibrils were observed with a length in the order of hundreds of nanometers to several micrometers. To analyze the height and width distribution of the fibrils, 50 random small line profiles (each perpendicular to a fibril) were made, and the height relative to the baseline and the width of each peak was

determined. The average fibril height was 2.4 ± 1.3 nm (\pm SD; range 0.7 to 5.9 nm) for EE24 and 2.7 ± 1.3 nm (\pm SD; range 1.0 to 7.6 nm) for EL28. Although these average heights are similar to the fold-to-fold distance of 2.8 nm calculated for poly-[(GA)₃GE] in antiparallel β -sheet conformation (with Glu present in the turns) or to the value of 3.6 nm obtained for this structure with X-ray diffraction,⁹ the thinnest EE24 and EL28 fibrils are considerably smaller. Topilina et al.³⁶ also observed lower fibril heights for the related poly-[(GA)₃GY(GA)₃GE(GA)₃GH(GA)₃GK], but they propose a different structure for that particular polymer. The apparent fibril width found for EE24 was 50 ± 11 nm (\pm SD; range 25–74 nm) and 49 ± 14 (\pm SD; range 23–87 nm) for EL28, although the true width of the fibrils will be smaller due to tip convolution (the tip used had a typical radius of 10 nm). For comparison, the expected length of a fully stretched monomolecular β -sheet structure (with 0.474 nm interstrand spacings, as determined for poly-[(GA)₃GE])⁹ is \sim 23 nm for EE24 and \sim 27 nm for EL28.

Krejchi et al.⁹ observed the formation of needle-shaped lamellae upon crystallization of their poly-[(GA)₃GE]-type protein. Smeenk et al.³⁷ chemically cross-linked poly(ethylene glycol) (PEG) at its N- and C-termini, which resulted in the formation of fibrils upon crystallization. They found that the PEG groups prevent macroscopic crystallization, as a nonconjugated control showed only few fibrils due to the formation of larger aggregates. In this light it was surprising to find that EE24 and EL28 also formed fibrils. A possible explanation could be the fact that we used a 10000-fold lower protein concentration during the crystallization (i.e., 1 mg/L), which may promote fibril formation over isotropic aggregation. Also the fact that we crystallized the proteins in the presence of the modified silicon wafers might be of influence. On

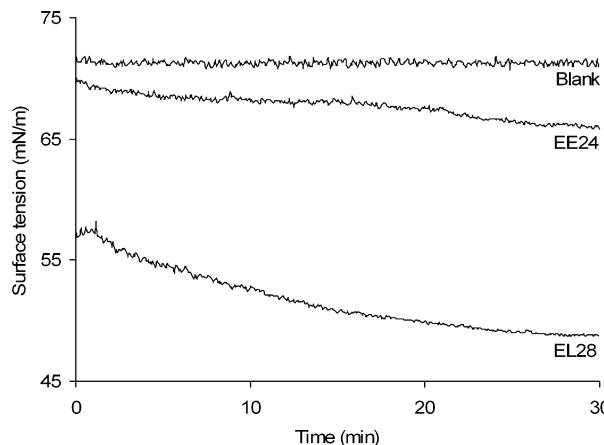


Figure 5. Drop tensiometry of EE24 and EL28 at 2 g/L and pH 8.0. For clarity, the results at pH 3.0 are not shown as they completely coincided with the blank.

Table 2. Effect of Protein on Contact Angle

substrate	mean contact angle (degrees, $N = 15$) ^a		
	blank	EE24	EL28
polystyrene	87 ± 4	83 ± 3 (6×10^{-3})	75 ± 7 (1×10^{-5})
aluminum	58 ± 12	33 ± 8 (3×10^{-7})	78 ± 8 (9×10^{-6})

^a Standard deviations are indicated. Probability values of two-sided *t*-tests for lack of effect are shown in parentheses.

the other hand, a remarkable morphological difference with the PEG-conjugated fibrils is evident. The fibers described here have a tortuous, crooked appearance, whereas the PEG-lined fibers³⁷ had a much stiffer appearance with a persistence length in the μm range.

Amphiphilicity. To explore the anticipated amphiphilic character of EL28, as compared to EE24, the effect of both polymers on the surface tension of an air–water interface was analyzed, initially, at pH 8.0 (Figure 5). Clearly, EL28 is more surface active than EE24. In view of the above CD results, both polymers are expected to be in random or extended conformation at high pH, unless other conformations are induced specifically at the air–water interface. The observed instantaneous effect of EL28 on the surface tension argues against such a conformational rearrangement. The amphiphilic character of EL28 under these conditions is therefore most likely inherent to its more hydrophobic composition and not related to any specific tertiary or supramolecular structure. At pH 3.0, no effect of EL28 and EE24 was observed. Both polymers aggregated and, probably because of this, their availability at the air–water interface was negligible.

To see if EL28 is capable of altering the surface hydrophobicity of solid substrates, a hydrophobic substrate (polystyrene) and a moderately hydrophilic substrate (aluminum) were submerged in solutions of each of the two polymers in 70% (v/v) formic acid, followed by thorough water washes to remove nonadhering material. As shown in Table 2, both substrates became more hydrophilic upon adsorption of EE24. The effect was small on polystyrene but very pronounced on aluminum, suggesting that the biopolymer may not have adhered well to the hydrophobic substrate. Interestingly, adsorbed EL28 rendered the hydrophobic substrate more hydrophilic and, conversely, the hydrophilic substrate markedly more hydrophobic. This finding confirms the amphiphilic nature of EL28, also in the solid state. In view of the ATR-FTIR results, β -sheet stacks of EL28 must have formed under the conditions applied. At present, it is unknown to what extent the leucine-containing

β -sheet edges were all oriented to the same side of the stack, thus, possibly contributing to the amphiphilicity of EL28.

Conclusions

Efficient biosynthesis of the amphiphilic silk-like polymer $[(\text{GA})_3\text{GE}(\text{GA})_3\text{GL}]_{28}$ was demonstrated in the yeast *P. pastoris*. The polymer was monodisperse and was produced at the g/L level. The secreted polymer could be purified to near homogeneity making use of the much higher tendency of the contaminating host proteins to precipitate from solution under acidic conditions, after complete denaturation with formic acid.

The CD spectrum of the negatively charged polymer in aqueous solution at high pH indicates a dominance of random and extended helical (silk III-like) structures, whereas the spectrum of a coating prepared from such a solution shows an unusual combination of a minimum at about 208 nm and maxima around 195 and 220 nm, indicating a conformation rich in β -turns.

The polymer is pH-responsive, in that it self-aggregates in water at low pH. It forms β -sheets and fibril-like structures upon crystallization in 70% (v/v) formic acid under vapor diffusion of methanol, as shown by ATR-FTIR and AFM.

The amphiphilic nature of the polymer is clear from tensiometry, and sessile drop contact angle measurements. Upon adsorption it renders a hydrophobic solid substrate hydrophilic, and a hydrophilic substrate hydrophobic. Given the biocompatibility of silk-like materials, the polymer may be of interest for biomedical applications, such as the coating of surgical implants or pH-responsive controlled drug release.

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References and Notes

- (1) Heslot, H. Artificial fibrous proteins: A review. *Biochimie* **1998**, *80*, 19–31.
- (2) Van Hest, J. C.; Tirrell, D. A. Protein-based materials, toward a new level of structural control. *Chem. Commun. (Cambridge)* **2001**, *19*, 1897–1904.
- (3) Sanford, K.; Kumar, M. New proteins in a materials world. *Curr. Opin. Biotechnol.* **2005**, *16*, 416–421.
- (4) Woolfson, D. N.; Ryadnov, M. G. Peptide-based fibrous biomaterials: Some things old, new and borrowed. *Curr. Opin. Chem. Biol.* **2006**, *10*, 559–567.
- (5) Zhao, X.; Zhang, S. Designer self-assembling peptide materials. *Macromol. Biosci.* **2007**, *7*, 13–22.

- (6) Werten, M. W. T.; van den Bosch, T. J.; Wind, R. D.; Mooibroek, H.; de Wolf, F. A. High-yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast* **1999**, *15*, 1087–1096.
- (7) Werten, M. W. T.; Wisselink, W. H.; Jansen-van den Bosch, T. J.; de Bruin, E. C.; de Wolf, F. A. Secreted production of a custom-designed, highly hydrophilic gelatin in *Pichia pastoris*. *Protein Eng.* **2001**, *14*, 447–454.
- (8) Werten, M. W. T.; de Wolf, F. A. Reduced proteolysis of secreted gelatin and Yps1-mediated α -factor leader processing in a *Pichia pastoris* kex2 disruptant. *Appl. Environ. Microbiol.* **2005**, *71*, 2310–2317.
- (9) Krejchi, M. T.; Atkins, E. D.; Waddon, A. J.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. Chemical sequence control of β -sheet assembly in macromolecular crystals of periodic polypeptides. *Science* **1994**, *265*, 1427–1432.
- (10) Iizuka, E.; Yang, J. T. The disordered and beta conformations of silk fibroin in solution. *Biochemistry* **1968**, *7*, 2218–2228.
- (11) Cantor, E. J.; Atkins, E. D.; Cooper, S. J.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. Effects of amino acid side-chain volume on chain packing in genetically engineered periodic polypeptides. *J. Biochem. (Tokyo)* **1997**, *122*, 217–225.
- (12) Parkhe, A. D.; Cooper, S. J.; Atkins, E. D.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. Effect of local sequence inversions on the crystalline antiparallel β -sheet lamellar structures of periodic polypeptides: Implications for chain-folding. *Int. J. Biol. Macromol.* **1998**, *23*, 251–258.
- (13) Chambers, S. P.; Prior, S. E.; Barstow, D. A.; Minton, N. P. The pMTL nic^- cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **1988**, *68*, 139–149.
- (14) Zhang, W.; Bevins, M. A.; Plantz, B. A.; Smith, L. A.; Meagher, M. M. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. *Biotechnol. Bioeng.* **2000**, *70*, 1–8.
- (15) Katakura, Y.; Zhang, W.; Zhuang, G.; Omasa, T.; Kishimoto, M.; Goto, Y.; Suga, K. Effect of methanol concentration on the production of human β_2 -glycoprotein I domain V by a recombinant *Pichia pastoris*: A simple system for the control of methanol concentration using a semiconductor gas sensor. *J. Ferment. Bioeng.* **1998**, *86*, 482–487.
- (16) Wenzel, T.; Sparbier, K.; Mieruch, T.; Kostrzewa, M. 2,5-Dihydroxyacetophenone: a matrix for highly sensitive matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of proteins using manual and automated preparation techniques. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 785–789.
- (17) Benjamins, J.; Cagna, A.; Lucassen-Reynders, E. H. Viscoelastic properties of triacylglycerol/water interfaces covered by proteins. *Colloids Surf., A* **1996**, *114*, 245–254.
- (18) Sun, Q. Y.; de Smet, L. C.; van Lagen, B.; Wright, A.; Zuilhof, H.; Sudholter, E. J. Covalently attached monolayers on hydrogen-terminated Si(100): Extremely mild attachment by visible light. *Angew. Chem., Int. Ed.* **2004**, *43*, 1352–1355.
- (19) Sun, Q. Y.; de Smet, L. C.; van Lagen, B.; Giesbers, M.; Thune, P. C.; van Engelenburg, J.; de Wolf, F. A.; Zuilhof, H.; Sudholter, E. J. Covalently attached monolayers on crystalline hydrogen-terminated silicon: Extremely mild attachment by visible light. *J. Am. Chem. Soc.* **2005**, *127*, 2514–2523.
- (20) Faucheu, A.; Gouget-Laemmel, A. C.; Henry de Villeneuve, C.; Boukherroub, R.; Ozanam, F.; Allongue, P.; Chazalviel, J. N. Well-defined carboxyl-terminated alkyl monolayers grafted onto H-Si(111): Packing density from a combined AFM and quantitative IR study. *Langmuir* **2006**, *22*, 153–162.
- (21) Horcas, I.; Fernandez, R.; Gomez-Rodriguez, J. M.; Colchero, J.; Gomez-Herrero, J.; Baro, A. M. WSXM: A software for scanning probe microscopy and a tool for nanotechnology. *Rev. Sci. Instrum.* **2007**, *78*, 013705.
- (22) McGrath, K. P.; Tirrell, D. A.; Kawai, M.; Mason, T. L.; Fournier, M. J. Chemical and biosynthetic approaches to the production of novel polypeptide materials. *Biotechnol. Prog.* **1990**, *6*, 188–192.
- (23) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.
- (24) Cregg, J. M.; Cereghino, J. L.; Shi, J.; Higgins, D. R. Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* **2000**, *16*, 23–52.
- (25) Schmidt, F. R. Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 363–372.
- (26) Chen, C. C.; Krejchi, M. T.; Tirrell, D. A.; Hsu, S. L. Effect of water on the structure of a model polypeptide. *Macromolecules* **1995**, *28*, 1464–1469.
- (27) Venyaminov, S.; Kalnin, N. N. Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. II. Amide absorption bands of polypeptides and fibrous proteins in α -, β -, and random coil conformations. *Biopolymers* **1990**, *30*, 1259–1271.
- (28) Rippon, W. B.; Walton, A. G. Optical properties of the polyglycine II helix. *Biopolymers* **1971**, *10*, 1207–1212.
- (29) Valluzzi, R.; Gido, S. P.; Zhang, W.; Muller, W. S.; Kaplan, D. L. Trigonal crystal structure of *Bombyx mori* silk incorporating a 3-fold helical chain conformation found at the air–water interface. *Macromolecules* **1996**, *29*, 8606–8614.
- (30) Lednev, I. K.; Ermolenkov, V. V.; Higashiya, S.; Popova, L. A.; Topilina, N. I.; Welch, J. T. Reversible thermal denaturation of a 60-kDa genetically engineered β -sheet polypeptide. *Biophys. J.* **2006**, *91*, 3805–3818.
- (31) Safar, J.; Roller, P. P.; Ruben, G. C.; Gajdusek, D. C.; Gibbs, C. J., Jr. Secondary structure of proteins associated in thin films. *Biopolymers* **1993**, *33*, 1461–1476.
- (32) Townend, R.; Kumosinski, T. F.; Timasheff, S. N.; Fasman, G. D.; Davidson, B. The circular dichroism of the beta structure of poly-L-lysine. *Biochem. Biophys. Res. Commun.* **1966**, *23*, 163–169.
- (33) Crisma, M.; Fasman, G. D.; Balaram, H.; Balaram, P. Peptide models for β -turns. A circular dichroism study. *Int. J. Pept. Protein Res.* **1984**, *23*, 411–419.
- (34) Lotz, B.; Keith, H. D. Crystal structure of poly(L-Ala-Gly)II. A model for silk. I. *J. Mol. Biol.* **1971**, *61*, 201–215.
- (35) Asakura, T.; Ohgo, K.; Komatsu, K.; Kanenari, M.; Okuyama, K. Refinement of Repeated β -turn structure for silk I conformation of *Bombyx mori* silk fibroin using ^{13}C solid-state NMR and X-ray diffraction methods. *Macromolecules* **2005**, *38*, 7397–7403.
- (36) Topilina, N. I.; Higashiya, S.; Rana, N.; Ermolenkov, V. V.; Kossow, C.; Carlsen, A.; Ngo, S. C.; Wells, C. C.; Eisenbraun, E. T.; Dunn, K. A.; Lednev, I. K.; Geer, R. E.; Kaloyerous, A. E.; Welch, J. T. Bilayer fibril formation by genetically engineered polypeptides: Preparation and characterization. *Biomacromolecules* **2006**, *7*, 1104–1111.
- (37) Smeeenk, J. M.; Otten, M. B.; Thies, J.; Tirrell, D. A.; Stunnenberg, H. G.; van Hest, J. C. Controlled assembly of macromolecular β -sheet fibrils. *Angew. Chem., Int. Ed.* **2005**, *44*, 1968–1971.

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