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Design and Production of a Chimeric Resilin-, Elastin-, and Collagen-Like Engineered Polypeptide

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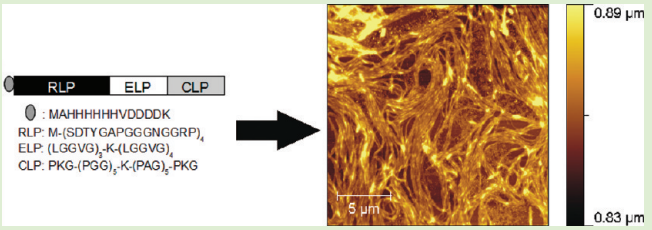
Supporting Information

ABSTRACT: Protein-inspired biomaterials have gained great interest as an alternative to synthetic polymers, in particular, for their potential use as biomedical devices. The potential inspiring models are mainly proteins able to confer mechanical properties to tissues and organs, such as elasticity (elastin, resilin, spider silk) and strength (collagen, silk). The proper combination of repetitive sequences, each of them derived from different proteins, represents a useful tool for obtaining biomaterials with tailored mechanical properties and biological functions. In this report we describe the design, the production, and the preliminary characterization of a chimeric polypeptide, based on sequences derived from the highly resilient proteins resilin and elastin and from collagen-like sequences. The results show that the obtained chimeric recombinant material exhibits promising self-assembling properties. Young's modulus of the fibers was determined by AFM image analysis and lies in the range of 0.1−3 MPa in agreement with the expectations for elastin-like and resilin-like materials.



INTRODUCTION

Protein-based biomaterials are of great interest for the biomedical field because of their potential use as prosthesis as well as scaffolds in tissue engineering applications.[1] In recent years many efforts have been made in order to obtain protein-based biomaterials with the aim to mimic the biological and mechanical properties of the natural tissues, with particular attention to the problems of best characteristics of biocompatibility. Furthermore, when the biomaterials are designed as scaffolds for tissue engineering the requisites are more stringent because they must be suitable also for cellular invasion, proliferation and differentiation.[2] The proteins used as models for the production of protein-based biomaterials are mainly proteins, such as elastin, collagen and silk, well-known for their mechanical properties.

Elastin-inspired biomaterials are of paramount interest for their intrinsic elastic properties as well as for their straightforward design.[3,4] The simplicity of polymer design for elastin-inspired biomaterials is due to the presence of small-sized repetitive sequences in elastin primary structure, such as, for example, VPGXG (X = V, I, A) or JGGZG (J, Z = V, L, A). Sometimes, a little variation was introduced by the sporadic insertion of charged residues such as K, E, and Orn in order to cross-link the polypeptides. Up to now, several elastin-like biopolymers were produced and many research groups focused their attention on the development of polypeptides containing mainly the sequences (VPGXG)*n*, highlighting their physicochemical properties and their high self-assembling propensities.[5−7] Alternatively, Tamburro and co-workers designed and chemically synthesized elastin-like polymers, containing the glycine-rich JGGZG repeats.[8−10] Among the glycine-rich polypentapeptides different molecular and supramolecular results were observed. Poly(VGGVG) and poly(VGGLG) characterized by circular dichroism (CD), Fourier Transform Infrared (FT-IR) spectroscopies and Atomic Force Microscopy (AFM) show typical features of amyloid-like aggregates.[11−13] Differently, transmission electron microscopy (TEM) shows for poly(LGGVG) an elastin-like supramolecular aggregation pattern.[8]

Recently, a different elastomeric protein, resilin, with remarkable mechanical properties gained great attention in the biomaterial field.[14−17] Resilin is an elastomeric protein present in specialized regions of insect cuticle, which plays a key role in insect flight, jumping mechanism of fleas and vocalization of cicades.[18] Resilin confers to the insect tissues high resilience and very high fatigue lifetime. The putative *Drosophila melanogaster* resilin sequence has been identified and highlighted the presence of two different types of repeats: the N-terminal region, dominated by a 18 times repeated peptide sequence,

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SDTYGAPGGGNGGRP, and the C-terminal domain where a 13 residue sequence, GYSGGRPGGQDLG, is 11 times repeated.[19]

Thanks to the versatility of DNA recombinant methodologies, in recent years a great number of different protein-inspired polypeptides were produced. Silk-like[20,21] and elastin-like,[22−24] as well as resilin-like, polypeptides were obtained and their mechanical and biological properties were investigated. Furthermore, the introduction of different biological active sequences, such as cell adhesion sequences,[25−28] as well as the combination of sequences[29−31] derived from different proteins has expanded the possibility to fine-tune the characteristics of the produced biomaterial, adapting it to its potential use. The noteworthy progress in the field is essentially due to the possibility to chemically synthesize genes encoding for any repeat unit thus offering almost limitless possibilities for creating novel protein polymers with unique and functional properties. As a result, it is possible to produce "chimeric" polypeptides containing repeat units of different proteins, and combine them in any order thus obtaining finely tunable biomaterials.[32,33]

In this paper, we report on the biosynthesis of resilin-elastin-collagen-like chimeric polypeptide (REC) by taking advantage of the recursive directional ligation (RDL) approach, a recombinant DNA method proposed by Chilkoti and co-workers.[34] The aim of our work is to explore the design and production of new type of biomaterials by combining the rubber-like proteins, resilin and elastin, that exhibit reversible deformation, very high resilience and elasticity, with a collagen-like sequence, that limits tissue deformation.

MATERIALS AND METHODS

Design and Synthesis of RLP, ELP, and CLP DNA Sequences. The DNA sequences are shown in Figure S1. The DNA sequences for RLP (flanked at 3′ by *Sfi* I) and CLP monomer (flanked at 5′ by *Bgl* I and at 3′ by *Sfi* I) were synthesized and introduced in pCR 2.1 vector (Invitrogen) by MWG Biotech (Germany). The ELP monomer gene was obtained from a recombinant pDrive+ELP plasmid by double digestion with *Bgl* I and *Sfi* I restriction endonucleases.

Production of the RLP Monomer Gene. The plasmidic DNA of pCR 2.1 vector containing the RLP monomer gene was used as template to amplify the RLP sequence. The forward and reverse primers sequences were, respectively, 5′ TCG GAT ACC TAT GGC GCT CCT G 3′ and 5′ CAG GCC GCC CAG GCC CGG A 3′ (*Sfi* I restriction site is underlined). The PCR cycle included 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and finally, 72 °C for 5 min. PCR reaction utilized Taq polymerase (EuroClone) in a Thermal Cycler PTC-100 (MJ Research).

The PCR product was separated on a 1% agarose (Sigma) gel. The band corresponding to the desired product (189 bp) was purified from the PCR reaction mixture using the MinElute Reaction Cleanup kit (Qiagen). The purified RLP monomer gene was inserted into pDrive vector (Qiagen) and the recombinant plasmid was used to transform TG1 competent *E. coli* cells. Colonies were screened on LB plates supplemented with 100 μg/mL ampicillin and the correct construct was verified by DNA sequence analysis (MWG Biotech, Germany) of recombinant plasmid isolated from a single transformant using standard procedures.

Production of the ELP Monomer Gene. A single colony of TG1 competent *E. coli* cells containing pDrive+ELP recombinant vector (unpublished data) was grown in LB medium supplemented with 100 μg/mL ampicillin. The DNA plasmid was isolated using Qiaprep Spin Mini Prep kit (Qiagen) and digested with *Sfi* I and *Bgl* I (New England Biolabs). Digestion products were separated on a 1% agarose gel. The band corresponding to the desired product (99 bp) was purified from the gel using a MinElute gel Extraction kit (Qiagen).

Production of CLP Monomer Gene. The CLP monomer gene were amplified from recombinant pCR2.1 vector containing the CLP monomer gene. Forward and reverse primers sequences for CLP monomer gene amplification were, respectively, 5′ GGG GTG GGC CTG GGC GG 3′ and 5′ CAG GCC GCC CAG GCC GCC 3′.

The PCR cycle included 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and finally, 72 °C for 5 min. PCR reaction utilized Taq polymerase (EuroClone) in a Thermal Cycler PTC-100 (MJ Research). The PCR product, purified from the PCR reaction mixture (MinElute Reaction Cleanup kit, Qiagen), was digested with *Sfi* I and *Bgl* I (New England Biolabs). Digestion products were separated on a 1% agarose gel. The band corresponding to the desired product (156 bp) was purified from the gel using a MinElute gel extraction kit (Qiagen).

Construction of the REC Chimeric Gene. The recombinant plasmid pDrive+RLP was digested with *Sfi* I, dephosphorilated with alkaline phosphatase (NEB), and the resulting linearized vector was retained and purified. The *Bgl* I/*Sfi* I ELP fragment and the linearized vector were religated, using T_4 DNA Ligase (New England Biolabs) by overnight incubation at 16 °C, resulting in a larger construct containing RLP and ELP consensus motifs. A colony with the desired polymer gene size was selected. The recombinant DNA plasmid was isolated using Qiaprep Spin Mini Prep kit and afterward *Sfi* I digested and dephosphorylated. The *Sfi* I pDrive+RLP+ELP linearized vector and the *Bgl* I/*Sfi* I CLP fragment were religated, resulting in a construct containing RLP, ELP and CLP monomer genes. The methodology is schematically shown in Figure 2. The polymeric gene was selectively cloned into pET46EK/lic expression plasmid.

Expression of Recombinant REC Chimeric Polypeptide. For the expression of the REC gene fragment, recombinant pDrive+RLP+ELP+CLP vector was used as template for PCR with primers designed to amplify a 408 bp fragment, inserted into a pET 46EK/lic T7-promoter expression vector (Novagen) containing a N-His_6 tag. Primers sequences are shown below:

Forward: 5′ GAC GAC GAC AAG ATG TCG GAT ACC TAT GGC GCT CCT 3′.

Reverse: 5′ GAG GAG AAG CCC GGT TAG CCT TTG GGC CCT GCT GGG 3′.

The PCR cycle included 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and finally, 72 °C for 5 min. PCR reaction utilized Taq polymerase (EuroClone) in a Thermal Cycler PTC-100 (MJ Research). The PCR product, purified from the 1% agarose gel, was cloned into pET46EK/Lic vector. The recombinant plasmid was used to transform BL21DE3 competent *E. coli* cells (Novagen) and selected on LB plates containing 100 μg/mL ampicillin. After cloning into the expression plasmid the sequence of the chimeric gene was confirmed by DNA sequencing.

Overnight culture of bacterial cells containing the construct was grown in LB medium containing antibiotic as previously described, and subsequently used to inoculate 1 L of LB medium, supplemented with 100 μg/mL ampicillin and incubated at 37 °C with shaking until the OD_{600} was 0.6−0.8. The polypeptide expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Cells were then collected by centrifugation (8000 g for 10 min at 4 °C) and the cell pellet was frozen at −80 °C. This protocol has yielded about 40−50 mg of REC per liter of culture.

Immunoblotting Analysis. Samples containing cell pellets from 250 μL of induction culture, was resuspended in lysis buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2.5% SDS, 1% β-mercaptoethanol, bromophenol blue 0.01%) and were fractioned by 12−4% SDS-PAGE gel and then transferred by electroblotting onto PVDF Immobilon-P Transfer Membrane (Pierce). After blotting, the membrane was blocked for 1 h in

PBS containing 0.05% Tween 20 and 1% milk powder, followed by incubation for 2 h with the monoclonal antipolyhistidine peroxidase conjugate antibody to test the fusion protein expression (Figure S2).

Purification. The cell pellet was resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0, 2 M urea). Complete cell lysis was obtained by sonicating for two 10 min periods, using Sonoplus Ultrasonic Homogenizers (Bandelin). The soluble protein fraction was recovered in supernatant following centrifugation at 14000 g for 20 min at 4 °C. The His_6 tag fusion polypeptide was purified by an affinity method using Ni-NTA Agarose beads (Qiagen). The resin was pre-equilibrated with lysis buffer and loading of the supernatant, the mixture was shaked for 1 h. The resin was washed with washing buffer (50 mM Tris, pH 8.0 containing 1 M NaCl, 20 mM imidazole) and the His_6 tag polypeptide was eluted from the resin using elution buffer (50 mM Tris, pH 8.0 containing 300 mM NaCl, 250 mM imidazole). To remove the salts and imidazole, the elute was dialyzed using a dialysis tube with a cut off of 1000 Da (Spectrapore Float-A-lyzer, Spectrum Laboratories Inc., Rancho Dominguez, U.S.A.) against pure deionized water. For cleavage with Recombinant Enterokinase (Novagen), the His_6 tag fusion polypeptide was digested using 1 U of enzyme for 50 μg fusion protein at 22 °C for 24 h in buffer containing 500 mM NaCl, 200 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.4. Following proteolytic cleavage, fragments were separated by RP-HPLC, using a Phenomenex Jupiter C18 300 Å (250 × 10 mm, 5 μm) column with a flow rate of 3 mL/min and UV detection at 220 and 280 nm. Products were eluted with a gradient of acetonitrile from 5 to 80% over 60 min. Pure fraction was pooled, lyophilized to a white powder. Identity and purity of the final product were confirmed by MALDI-MS spectrometry (CEINGE, Naples, Figure S4) and amino acid analysis (CCiT, University of Barcelona, Spain, Table S1).

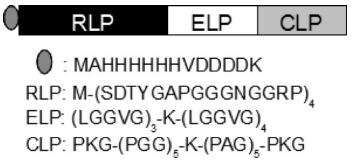

Figure 1. Scheme of the modular structure of REC polypeptide (top) and amino acid sequences of His_6-tag (ellipse), resilin-like (RLP), elastin-like (ELP), and collagen-like (CLP) sequences (bottom).

Circular Dichroism Spectroscopy. Circular dichroism spectra were recorded at 0, 25, and 70 °C using a Jasco (Tokyo, Japan) model J-815 spectropolarimeter, equipped with a Haake thermostat (Thermo Fisher Scientific, Waltham, MA, U.S.A.), averaging 16 scans. Baselines were corrected by subtracting the solvent contribution. Cylindrical, fused quartz cells of 0.1 cm path length (Hellma, Mullheim, Germany) were employed. The data are expressed in terms of $[\theta]$, the total molar ellipticity (deg cm^2 $dmol^{-1}$). 2,2,2-Trifluoroethanol (TFE) were purchased from Romil. Deionized water was further purified using a Milli-Q reagent grade water system from Millipore.

Atomic Force Microscopy (AFM). The samples were solubilized in ultrapure water (concentration of 0.5 mg/mL) and incubated at 50 °C for 7 days.[17] Each 24 h, an aliquot (10 μL) of the incubated sample was withdrawn and deposited as drops on Si 100 substrates. After air drying at room temperature, the AFM images were carried out by using the XE-120 microscope (Park Systems) in air and at room temperature.[35,36] Data acquisitions were carried out in intermittent contact mode at scan rates between 0.4 and 0.7 Hz, using rectangular Si cantilevers (NCHR, Park Systems) having the radius of curvature less than 10 nm and with the nominal resonance frequency and force constant of 330 kHz and 42 N/m, respectively. AFM images were elaborated by using Gwyddion 2.22 software (http://gwyddion.net). The resolution enhancement was obtained by applying the *Local Contrast* procedure. The method is useful for visualizing features in areas with low and high variation of the *z*-axis at the same time.

Elasticity Measurement. Fibers formed from the REC polymer, when dissolved in solution, will assume a conformation that strongly

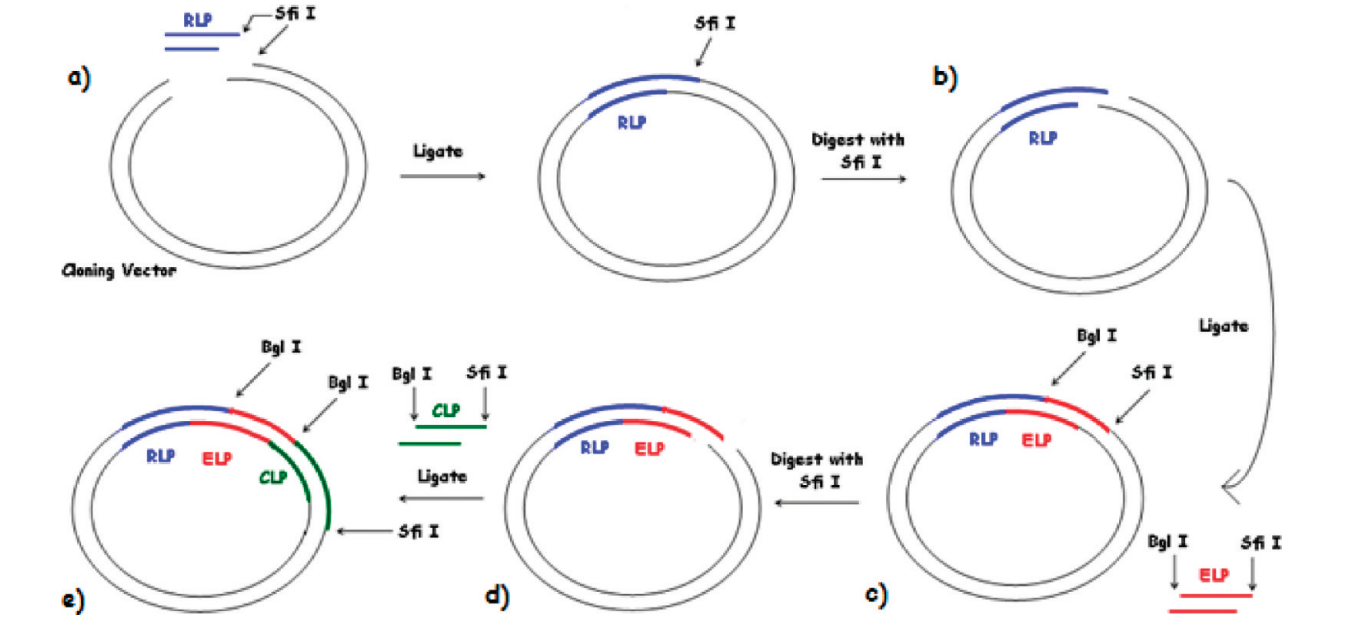

Figure 2. Cloning scheme of REC gene. (a) The RLP monomer gene is inserted into pDrive cloning vector, the RLP gene is designed to contain recognition site for *Sfi* I restriction endonuclease at the 3′ end; (b) the recombinant vector is linearized by *Sfi* I digestion; (c) the ELP monomer gene is obtained by digestion of the pDrive recombinant vector with both *Bgl* I and *Sfi* I and, subsequently, ligated into the vector that has been linearized by digestion with only *Sfi* I; (d) the recombinant vector contains RLP and ELP monomer genes, with the *Sfi* I site maintained only at the 3′-end of the recombinant gene; then the recombinant vector is linearized by *Sfi* I digestion; and (e) the CLP monomer gene is obtained by digestion of the pDrive recombinant vector with both *Bgl* I and *Sfi* I and subsequently ligated into the RLP-ELP-pDrive vector that has been linearized by digestion with only *Sfi* I.

depends on their torsional elasticity: softer polymers will have higher tendency to fold into a compact shape, whereas more rigid molecules will appear as straight lines. From a geometrical point of view, this effect can be described in terms of the persistence length P, the autocorrelation length of the tangent vector. If we call θ the angle between a vector that is tangent to the polymer at a give position L and the tangent vector at a position $L + x$ away from it, the mean value for the cosine of the angle is expected to decay exponentially

$$\langle \cos \theta \rangle \propto e^{-x/P}$$

where P is exactly the persistence length. A measure of P was carried out on several AFM images, by means of a semiautomated procedure[53] that identifies isolated fibers by the application of a segmentation filter and then calculates the value of P from its geometrical definition. For each fiber, the value of the radius was also measured from the height of the fiber, to avoid any tip convolution problem. To verify the stability of the fibers and the effect of hydration on the apparent dimensions measured with AFM, the samples where rehydrated in pure water and the measurements acquired in the same working mode.

RESULTS AND DISCUSSION

The design and production of biomaterials with tailored mechanical and biological functions is a complex process and great interest is devoted to understand the relationship among amino acid composition, primary sequence, and mechanical and biological properties.

Polypeptide Design. The choice of the sequences composing REC polypeptide was made taking into account the mechanical properties of the inspiring proteins and their self-assembling propensities. A scheme of the modular protein and its amino acid sequence is shown in Figure 1. The 15 amino acid-long repeating unit SDTYGAPGGGNGGRP derived from the N-terminal exon of the *Drosophila melanogaster* resilin gene was repeated four times and used as a hydrophilic elastomeric domain to impart elasticity to the scaffold. The elastin-like domain (ELP) contained the seven time repeated hydrophobic sequence LGGVG with one lysine residue in the middle, for cross-linking purpose. Also the collagen-like sequence (CLP) contained three lysine residues interspersed inside the GPY (Y = G, A) triplets. Resilin and elastin are classified as rubber-like proteins, characterized by a suite of properties such as high resilience, large strains and low stiffness, that function in the storage of elastic-strain energy. Furthermore, resilin and elastin are stretchy, reaching maximal extensions in excess of 100%, with a very low modulus of elasticity.[37] Nevertheless, they have very diverse physicochemical properties, being the resilin-like sequences highly hydrophilic, while the elastin-like sequences are hydrophobic. The dissimilar polarity could contribute to the different self-assembling pattern that elastin- and resilin-derived sequences show. As a consequence, the presence of defined blocks of compositionally dissimilar sequences, having significantly different polarities and self-assembling patterns, could drive selective aggregation of the different sequences. Furthermore, the introduction of resilin-like sequences could increase the hydrophilicity and consequently the solubility of the polypeptide sequence.[15] The presence of lysine residues is fundamental in order to ensure the possibility to perform cross-links among the polypeptide chains. In literature numerous chemical cross-linking agents have been investigated, including tannic acid,[38] hexamethylene diisocyanate,[39,40] glutaraldehyde,[25,41,42] and (hydroxymethyl)phosphine.[43] The main aim of cross-links is to confer a stable and resistant 3D network to the biomaterial in order to support mechanical stress without ruptures. This characteristic is particularly important if the biomaterial has to be employed for the production of a 3D scaffold for cell proliferation and differentiation.

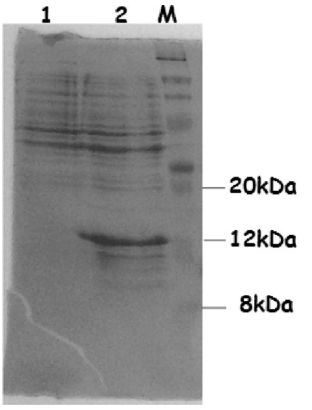

Figure 3. Expression of REC polypeptide. The figure shows SDS-PAGE of expressed polypeptide, with a prominent band at the appropriate molecular weight of 11.5 kDa. Results are shown for uninduced and induced culture. Lane M: electrophoresis marker (8−220 kDa; Sigma); lane 1: BL21DE3 cell culture before IPTG treatment; lane 2: BL21DE3 cell culture after 3 h treatment with IPTG (0.1 mM).

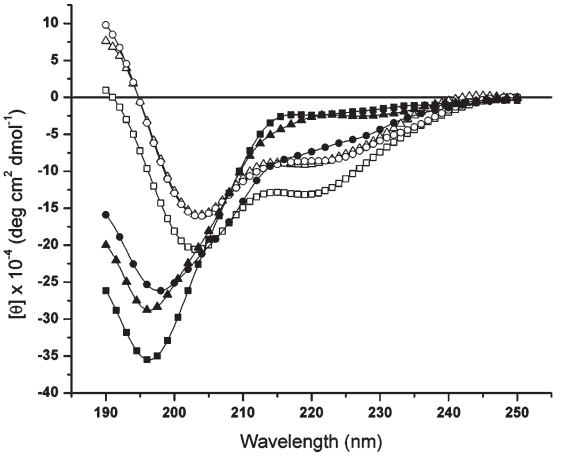

Figure 4. Temperature-dependent CD spectra of REC polypeptide recorded in water at 0° (■), 25° (▲), and 70 °C (●) and in TFE at 0° (□), 25° (Δ), and 70 °C (○).

The monomer genes of each block were designed to encode a defined number of repeats belonging to the RLP, ELP, and CLP sequences, and appropriate restriction endonuclease recognition sites were introduced at the ends of the coding sequences, compatible with the RDL method[34] (Figure S1). In order to overcome the instability of the synthetic gene containing tandem repeated sequences, careful use of the degeneracy of the genetic code was employed, limiting in this way the exact repetition in the polymer gene.[44] The REC gene was successively obtained by oligomerization of the three RLP, ELP, and CLP monomer genes in a recursive way (Figure 2).

Polypeptide Expression. The REC gene fragment was cloned into a pET 46EK/Lic T7-promoter expression vector containing the $[His]_6$ tag sequence at the N-terminus. The recombinant plasmid

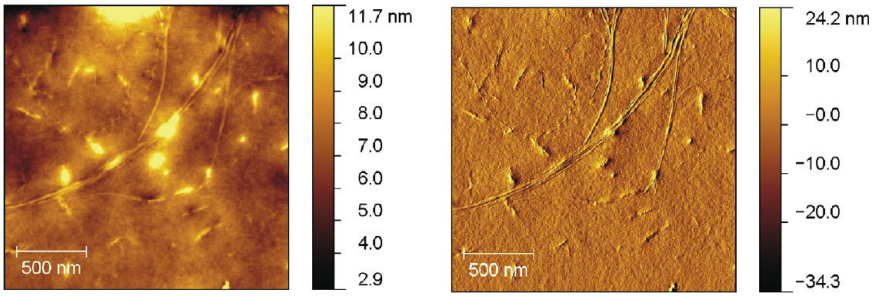

Figure 5. AFM images of REC polypeptide (0.5 mg/mL) incubated in water at 37 °C recorded after t_3 = 3 days of incubation. On the left, we observe the topography and on the right the error signal that more clearly evidenced the presence of fibrils.

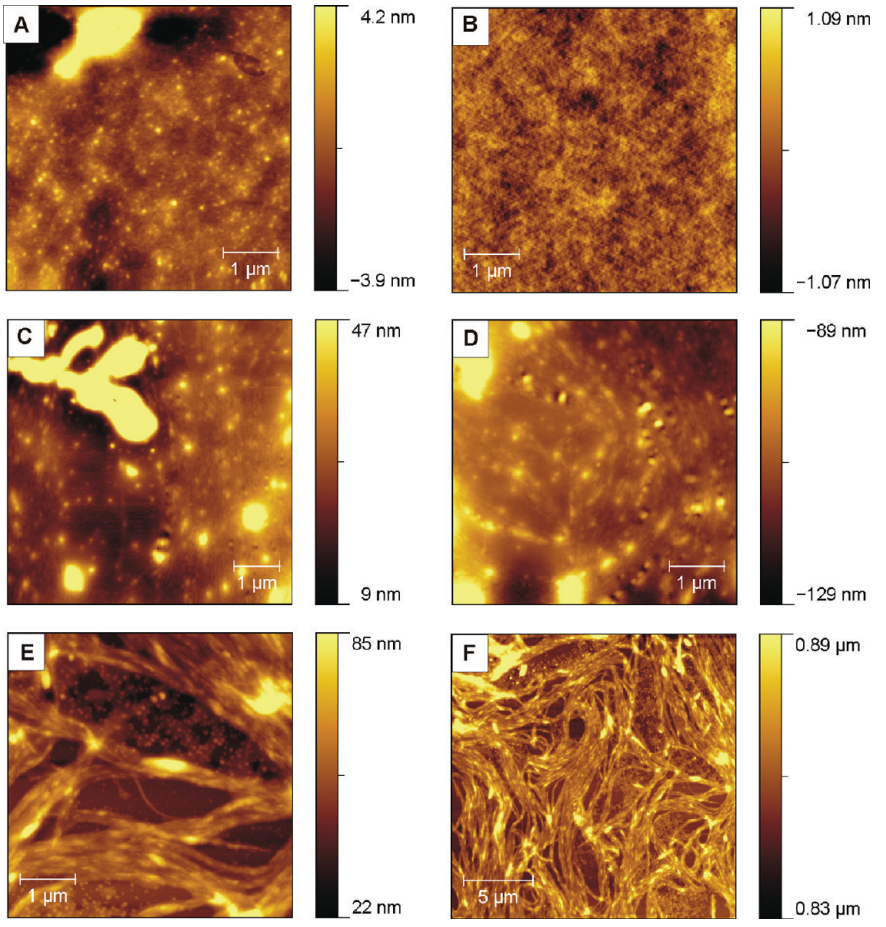

Figure 6. Time-course studies of REC polypeptide assembly (0.5 mg/mL) by AFM. The sample was incubated in water at 50 °C for t_0 = 0 (A); t_1 = 1 day (B), t_2 = 2 days (C), t_3 = 3 days (D), and t_5 = 5 days (E, F).

vector was transformed into competent *E. coli* BL21DE3 cells and expression of the REC polypeptide was induced by IPTG (Figure 3).

The recombinant REC polypeptide was purified by Ni-NTA affinity chromatography and the $[His]_6$ tag was cleaved with recombinant enterokinase. In the final purification step the fragments were separated by RP-HPLC (Figure S3). SDS-PAGE and MALDI-MS confirmed the size of the REC polypeptide (Figure S4).

Circular Dichroism Spectroscopy. Circular dichroism spectroscopy was used to determine the secondary structure of the REC polypeptide. The spectra were recorded in aqueous solution and trifluoroethanol (TFE) at different temperatures (Figure 4). In water at 0 °C, the spectrum shows a strong negative band at 196 nm. On increasing the temperature to 25 and 70 °C, the band is red-shifted and reduced in intensity. The CD data suggest that in water at low temperature the predominant conformation adopted by the polypeptide is the poly proline II left-handed helix (PPII), whose stability is reduced on heating. In the CD spectra recorded in TFE, a less polar solvent, we observe a negative band at 204 nm and a shoulder at 220 nm and at 190 nm a trend to assume positive ellipticities values. On increasing the temperature to 25 and 70 °C, we observe the reduction of the negative bands and the formation of a positive one at

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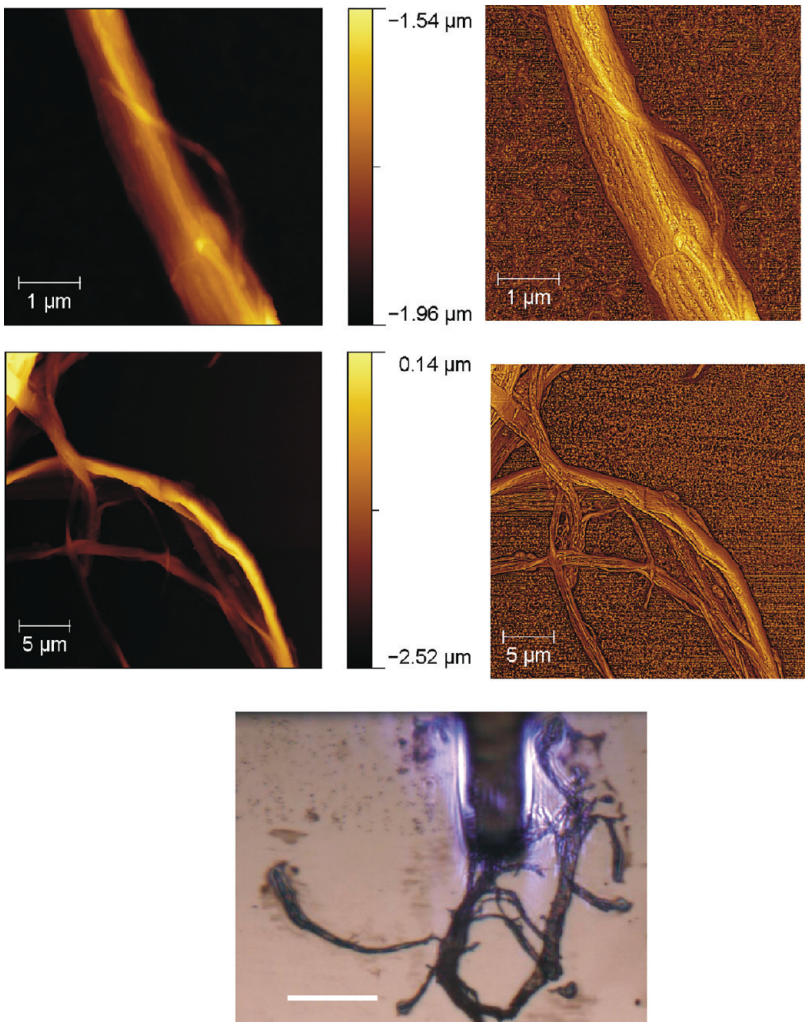

Figure 7. On the top, AFM images and the corresponding resolution-enhanced images of REC polypeptide (0.5 mg/mL) incubated in water at 50 °C for t_7 = 7 days. On the bottom, the optical microscopy image of the observed AFM sample (bar: 50 μm).

190 nm. These spectral findings could be assigned to the presence of a mixture of β-turns stable at high temperatures a mixture of β-turns and random coil.[45] This behavior is not dissimilar from those observed by CD for the isolated resilin (RLP) and elastin (ELP) sequences.[17,46] Nevertheless, even if no data are available for the isolated collagen-like sequence, we could suppose that, as observed for model collagen-peptides,[47] the organic solvent destabilizes the PPII structure typical of collagen and collagen-like peptides.

Atomic Force Microscopy (AFM). To study the potential self-assembling propensity, the REC polypeptide was incubated in water at 37 (Figure 5) and 50 °C (Figures 6 and 7) for 7 days and then observed by AFM. At both temperatures we observed the formation of fibrillar supramolecular aggregates during the time course. At 37 °C, the formation of isolated fibrils is discernible at 2 days. At 3 days, the fibrillar structures align and form bundles of fibrils (Figure 5). At higher incubation time no evidence of further aggregation was visible (Figure S5). At 50 °C, the tendency to form fibrillar supramolecular self-organized structures is higher. The rationale for that could be found into the stronger hydrophobic interactions driving the self-assembly on increasing of the temperature.[48,49] As a matter of fact, starting from globular oligomers (t_0 = 0), we observe the formation of highly aligned fibrils (t_2 = 2 and t_3 = 3) that evolve in a complex network of fibers and fibrillar bundles with time. The formation of fibrils from the merging of the globules is visible in Figure 6C, D. At t_5 = 5 days (Figure 6E,F), the fibrils are extensively collected in bundles of extremely flexible fibers with a highly peculiar structure. Further incubation of the sample to t_7 = 7 days, highlighted the formation of very huge fibers, about 1 μm in diameter, observable also by optical microscopy (Figure 7). Selected samples where rehydrated after imaging, showing a strong stability of the fibers and only a small difference in the geometrical properties (especially for height and width of the fibers).

The introduction of amino acids, such as lysine or glutamic acid, containing ionizable side chains in the polypeptide design, is usually performed to introduce potential cross-linking sites. However, it could also be performed to have a pH-responsiveness of the biomaterials. The phase separation phenomenon, for example, coacervation, could be influenced by the pH of the solution. This finding is crucial for elastin-derived polymers, based on the repetition of VPGXG (X = V, I, A, K, E) motif, able to undergo a phase transition in response to temperature changes. In our case, given the low number of lysine residues, 4/134, as well as the high distance in the primary sequence, we considered their contribution to the polypeptide self-assembly of minor impact. As a matter of fact,

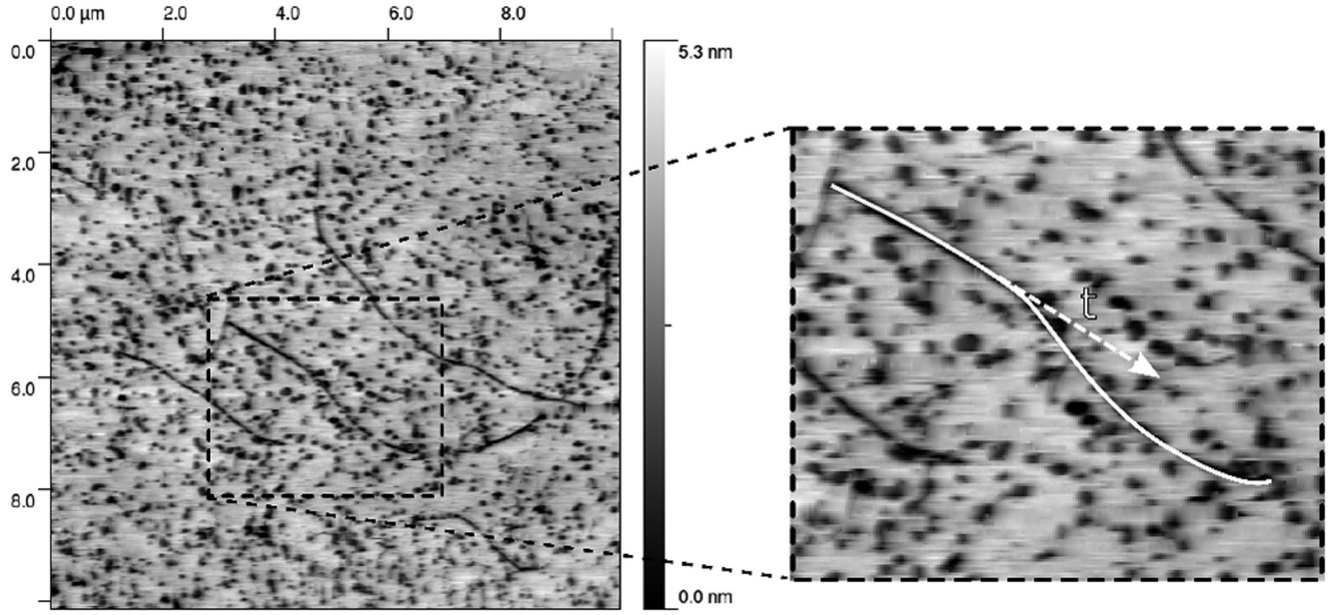

Figure 8. AFM analysis of fiber elasticity. Left panel: single AFM image taken in noncontact mode in water clearly showing the presence of polypeptide fibers. Right panel: zoom of the dashed region in the left panel. The white line indicates the fiber profile identified by the software procedure; as a reference, the tangent vector ***t*** in a specific point is also plotted (dashed arrow).

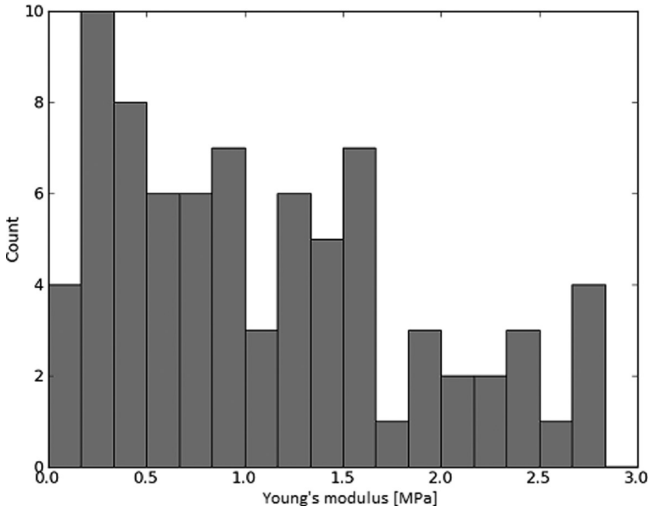

Figure 9. Distribution of the Young's modulus estimated for a set of 88 fibers measured by AFM microscopy.

in the condition we used for self-assembling studies, lysine side chains are positively charged and mutual repulsion should disfavor the polypeptide self-assembly. Nevertheless, our data show that the REC polypeptide has a high propensity to self-assemble in fibrous structures, regardless of the unfavorable conditions. Other authors have shown the influence of amino acids with ionizable side chains in the self-assembly of protein-derived polypeptides. Reguera et al.[6] and Hu et al.[50] showed that the presence of Glu determined the formation of nanopores because of segregation of the more hydrophilic carboxyl side group of the glutamic acids. No evidence of similar effect are reported for lysine containing polypeptides.

Elasticity Measurements. Any mechanical system in aqueous solution and at a given temperature T is subject to thermal motions that tends to randomize the position of each atom. If the system is a linear filament, this effect results in an effective force trying to bend the system with the final conformation depending on the elasticity of the filament itself. Therefore, it is possible to obtain information about the intrinsic mechanical properties of the sample by measuring the shape of a statistically relevant set of filaments and comparing it to a theoretical model. It has been shown that the elasticity of biological polymers can be well described by means of the so-called worm-like chain model[51,52] (WLC) in which the fiber is described as a thin homogeneous filament of a given length, L, elastically bending under an applied force. The quantity used in this model to describe the elasticity is the persistence length P, a measure of the distance over which the polymer is able to remain straight in liquid medium or, more precisely, the decay length of the autocorrelation function of the versor ***t*** tangent to the trajectory defined by the filament (see zoom image in Figure 8).

Several AFM images were analyzed and, using a semiautomated procedure,[53] the persistence length P was calculated for a set of isolated fibrils of different lengths. Given the starting hypothesis assuming that the WLC model is valid, it is also possible to translate the persistence length P into a measure of the Young's modulus Y of the fiber, using eq 1.[54]

$$YI = k_B TP \tag{1}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and I is the cross-sectional moment of inertia. Treating the fiber as an elastic cylinder of radius r, this equation can be recast in the final form of eq 2:[54]

$$Y = \frac{4k_B T}{\pi r^4} P \tag{2}$$

By measuring the radius and the persistence length for each curve, the Young's modulus was evaluated and the results are reported in Figure 9 for the whole data set, showing that the

elasticity of the polypeptide fibers lies in the range 0.1−3 MPa. The 78 fibers analyzed for the determination of the Young's modulus were obtained after 1 week of incubation at 37 degrees, and they were reasonably caught at different growth stages (longer and shorter, but also slightly thinner and thicker fibers), thus resulting in a broad distribution for Young's modulus values. Nevertheless, this value for the molecular elasticity is comparable with the expectations for elastin-like and resilin-like materials that are the softer components of the engineered molecule, whereas collagen-like bundles normally express Young's modulus up to 1 GPa (see ref 37 for a comparative table). This result is in agreement with a representation of the polymer as a series of three springs, in which the whole elasticity is closer to that of the softer components.

Self-Assembling Pattern of REC Polypeptide. Proteins such as elastin, resilin, and collagen are involved in different self-assembling processes, producing well-defined fibrils and fibers characterized by specific mechanical and supramolecular properties. While collagen-like peptides show a random network of fibrillar material when imaged by AFM,[55] collagen type I molecules self-assemble into fibrils and a highly ordered fiber network with the typical D-band periodicity of 67 nm.[56,57] AFM studies performed on elastin-derived peptides show string-of-beads-like fibrils,[58,59] while resilin-derived peptides evidenced more complex fiber morphology going from isolated insulae[16] to rod-like aggregates.[17] The comparison of the self-assembling pattern of REC polypeptide with the known inspiring proteins (resilin; elastin and collagen) highlights for REC polypeptide a peculiar morphology with great tendency to form higher order fibrillar structures. Furthermore, AFM images showed that the aggregation pattern of REC polypeptide is definitively different from twisted rope-helical structures found for some glycine-rich elastin-derived polypeptides. As a matter of fact, we observe a network of flexible, often aligned fibers (Figure 6F), rather than straight and isolated fibrils, typical of amyloid-like structures.

Accordingly, the high tendency in bending of the fibers and the estimated Young's modulus (0.1−3 MPa) of REC polypeptide is lower than those observed for amyloid-like fibers, usually straight and stiff from a mechanical point of view. As a matter of fact, Young's moduli of amyloid-like fibers are expected to be in the range of tens GPa, both for elastin-derived amyloidogenic polypeptides[60] and for different amyloidogenic proteins.[61]

CONCLUSIONS

This report describes the design, production and preliminary characterization of an artificial engineered polypeptide inspired by repeated sequences of resilin, elastin and collagen. The presented data show that the REC polypeptide is a promising candidate to be further developed for the production of protein-inspired biomaterials with potentially tunable mechanical and biological properties. The construction of the gene by the recursive ligation method renders the expression system highly flexible and easily modifiable, with the possibility to permute the different blocks in multiple arrangements, to repeat the blocks several times, and to introduce additional sequences as a function of the final use of the designed biomaterial. The designed REC polypeptide represents a model system from which we obtained the first indication on self-assembling properties and mechanical testing. A polypeptide with higher molecular weight coming out from the concatamerization of the chimeric gene is under development.

ASSOCIATED CONTENT

Supporting Information. Oligonucleotide sequences, Western blot analysis, HPLC chromatogram, MALDI-MS data, amino acid composition analysis, and AFM images of sample incubated at 37 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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