Protein Engineering

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A Genetically Engineered Protein Responsive to Multiple Stimuli**

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Dedicated to Professor John Ralston on the occasion of his 65th birthday

Stimuli-responsive materials, including polymers, biopolymers, conjugates, and hydrogels, have emerged as an important class of materials for both fundamental study and applied research in many fields of science, including biology and medicine.[1–5] At present these materials are prepared from a limited number of synthetic polymers, biopolymers, and biomimetic polymers. Protein engineering approaches to biomaterials design offer powerful strategies to overcome challenges and provide new opportunities in the design and rapid development of responsive biomaterials.[2,6–8] Consequently, there is a significant paradigm shift in the design concept for new elastic biomaterials, from a synthesis-based approach to the utilization of dynamic structural motifs derived from extracellular matrix proteins. Responsive properties of the highly elastic protein elastin and elastin-mimetic proteins (EMPs) that exhibit tunable lower critical solution temperatures (LCST) have stimulated development of novel protein-based biomaterials.[2,3,7–11] The sol–gel transition of gelatin that exhibits responsive upper critical solution temperature (UCST) behavior has also been of significant importance in the food and pharmaceutical industries.[12] Green fluorescent protein (GFP) and genetically engineered GFP mutants that demonstrate tunable photophysical properties have revolutionized cell culture, biological assay, and targeted sensing.[13] Recently, dual-phase behavior (DPB, the occurrence of both UCST and LCST) in proteins was predicted by Li et al.[14] using molecular modeling. Herein we report for the first time the unique dual phase transitions and pH-responsive photophysical properties of the resilin mimetic elastic protein rec1-resilin.

Resilin is a member of the family of natural elastic proteins that include elastin, gluten, gliadin, abductin, and spider silks, and it is found in specialized regions of many arthropods (e.g., fleas, spittle bugs, cicadas, and dragonflies) where there are highly repetitive movements. It is purported to be one of the most resilient elastic materials known,[11,15] but the molecular origin of this unique characteristic is not yet understood nor has it been exploited. Recently, we reported the synthesis of the resilin-mimetic polymer rec1-resilin from the repeat sequences of the first exon of the *Drosophila melanogaster* CG15920 gene[15] using recombinant DNA technology and its film-forming characteristics.[16b] The composition of this polypeptide (Figure 1) is dominated by 18 copies of a 15-residue repeat sequence: GGRPSDSYGAPGGGN (Scheme S1 in the Supporting Information). It contains a very high content of Gly (ca. 34.5 mol%) and Pro (ca. 14 mol%) and lacks hydrophobic residues with long aliphatic or aromatic side chains. The predicted structure of the protein using the DSC (discrimination of secondary structure class) routine[17] suggests that the secondary structure of rec1-resilin is devoid of α-helices and is dominated by random-coil configurations (Figure 1). This result has also been supported from experimental investigations using FTIR spectroscopy.[16] Nonetheless, zeta potential measurement of aqueous solutions of rec1-resilin confirms that the experimental isoelectric point (PI, zeta potential, $\varsigma = 0$) is observed at pH ≈ 4.8 (Figure 2A) compared to the theoretical value of pH 9.2 (assuming that all residues are uniformly exposed to water). This discrepancy indicates complex organization of rec1-resilin in solution, with negatively charged protein surfaces exposed to water and positively charged residues forming the core. The small-angle neutron scattering (SANS) data of rec1-resilin in solution can be fitted to a core–shell model, which also supports this hypothesis of complex organization. (Figure S2 and Table S2 in the Supporting Information).

Figure 2B demonstrates the self-assembly pathways and occurrence of phase transitions in rec1-resilin (pH 7.4) as a function of temperature. From the heating cycle, an abrupt decrease in hydrodynamic diameter D_h is observed at approximately 6 °C, which is indicative of UCST behavior. The association and dissociation at the UCST is observed to

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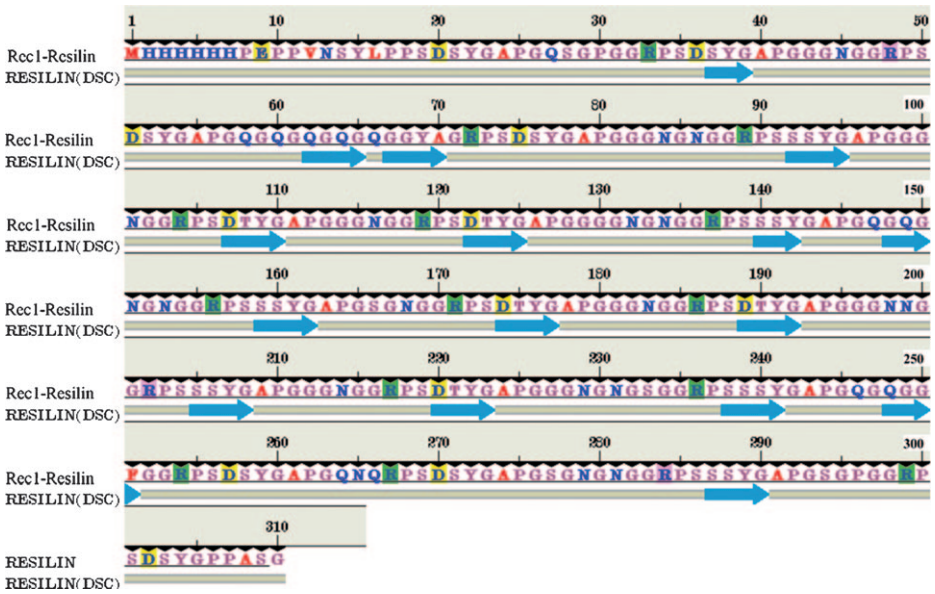

Figure 1. The primary structure and the DSC secondary structure cartoon of rec1-resilin. Single-letter amino acid codes are used for the primary structure. Residue color code: hydrophobic red, hydrophilic blue, neutral maroon. Background color code: negatively charged yellow, positively charged green, neutral white. Secondary structure color code: α helices red, β strands blue, random coils beige.

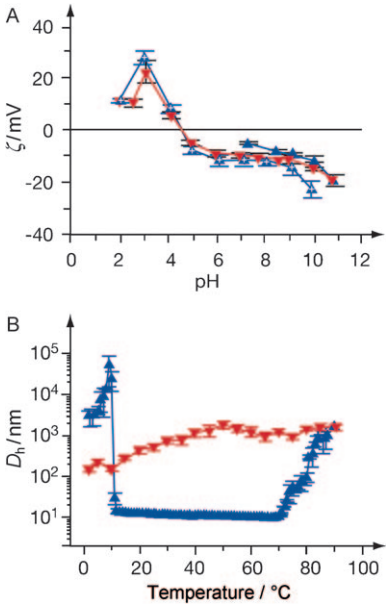

Figure 2. Responsive behavior of rec1-resilin. A) Plot of zeta potential as a function of pH value: blue triangles Fwd 1, red triangles Rev 1, blue triangles with crosses Fwd 2. B) Plot of hydrodynamic diameter D_h (pH 7.4) as a function of temperature: heating blue, cooling red.

be reversible without any hysteresis upon temperature cycling (Figure S3.1 in the Supporting Information). Note that dynamic light scattering (DLS) data are accurate between 0.6 nm and about 5 μm, and the data above 5 μm shown in Figure 2B are only a qualitative indication of the magnitude of change in size. This transition is also apparent visually (Figure S3.2 in the Supporting Information) as the solution changes from turbid to transparent. Between approximately 15 and 70 °C, the D_h value of rec1-resilin in aqueous solution is about 11 nm (polydispersity index width PDI < 0.1; see the Supporting Information, S1.2.3) and remains approximately constant, indicating the near-monodispersity and stability of the molecular organization. A second transition during the first heating cycle (Figure 2B) at approximately 70 °C is characterized by a sharp increase in D_h and polydispersity (PDI ≈ 0.21), which is indicative of LCST behavior. This transition is reversible, but in contrast to the UCST, the kinetics of reversal is slower (Figure S3.3 in the Supporting Information). Whilst LCST behavior has been observed in many thermoresponsive synthetic polymers[1,5] and proteins including EMPs,[2,6] UCST behavior is not common. UCST behavior has been reported in complex multisystem interpenetrating networks[18] of poly(acrylamide) and poly(acrylic acid), polyzwitterions,[19] and in gelatin.[12] The occurrence of DPB in a single macromolecule within the accessible experimental temperature range has been predicted[14,20] but is rare. Whilst there are similarities in the protein compositions of rec1-resilin and other elastic proteins such as EMPs and gelatin, there are also significant fundamental structural differences. All three proteins are rich in amino acid residues Gly and Pro and hence exhibit significant chain flexibility. However, EMPs have very few polar side chains and are hydrophobic in nature (Figure S4.1 in the Supporting Information). They are considered to be elastic owing to the presence of β-turn structures, and their LCST behavior is related to hydrophobic interactions.[6] Gelatin (Gly-Pro-Pro or Gly-Pro-Hyp) demonstrates gelation (UCST) owing to intermolecular triple-helix formation related to strong hydrogen bonding.[11] In rec1-resilin, high concentrations of both polar and charged side chains exist; it is hydrophilic (Figure S4.2 in the Supporting Information) and predominantly random-coil in structure (Figure 1). DPB of rec1-resilin results from its unique molecular composition and architecture as well as its interaction with water (Section S5 in the Supporting Information).

Cryo-TEM has been used to investigate the morphology of rec1-resilin below the UCST and above the LCST, in order to further elucidate the thermoresponsive behavior. The Cryo-TEM image of vitrified rec1-resilin solution (0.3 mg mL^{-1}) at 20 °C shows only dispersed spherical particles of approximately 9.5 nm diameter with poor contrast (Figure 3A), indicating a soluble state with random conformation. The diameter of the spherical cores derived from Cryo-TEM correlates well with the size of the particles

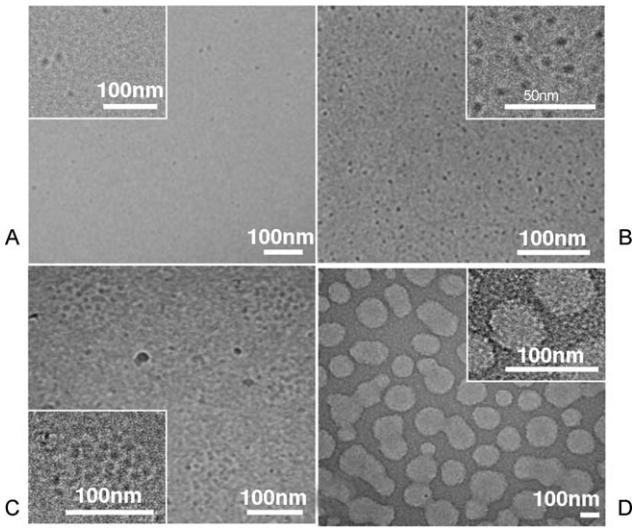

Figure 3. Cryo-TEM micrographs of rec1-resilin for solutions of A) 0.3 mg mL^{-1} at 20 °C, B) 0.3 mg mL^{-1} just below UCST (4 °C), C) 0.3 mg mL^{-1} above LCST, and D) 10 mg mL^{-1} at 4 °C.

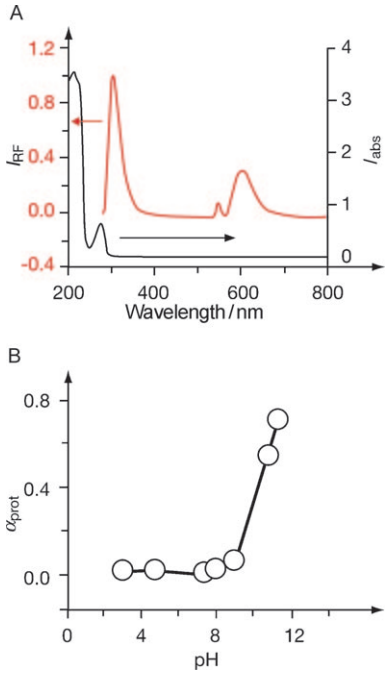

Figure 4. A) UV/Vis absorption (black) and emission (red) response of rec1-resilin in solution. B) The degree of tyrosyl ionization (α_{prot}) as a function of pH value.

derived from DLS experiments. However, when the same solution was vitrified at 4 °C (Figure 3B), it demonstrated a high-density network of well-dispersed interconnected spherical particles of (5.4 ± 0.4) nm diameter with excellent contrast. Above the LCST in the rec1-resilin solution, the formation of large discrete spherical aggregates with a size range of approximately 100 to 130 nm is observed (Figure 3C). On increasing the solution concentration (ca. 10 mg mL^{-1}) the formation of an interconnected gel particle network at the UCST is clearly demonstrated (Figure 3D). The tunability of the UCST of rec1-resilin by adjustment of the pH value is also revealed from consecutive heating and cooling profiles (Figure S5 in the Supporting Information). The UCST behavior is dependent on the pH value because the hydrogen-bonding capabilities of the different constituent amino acid residues depend strongly on the pH value. However, the LCST is observed not to be significantly dependent on pH value, and the association at LCST can be attributed to the kinetically controlled nonspecific aggregation (S5.2 in the Supporting Information).

In addition to the unique DPB, rec1-resilin also exhibits distinctive photophysical properties arising from the presence of twenty Tyr fluorophore residues in different local environments. Figure 4A shows both the optical density and fluorescence emission spectra of an aqueous solution of rec1-resilin at pH 7.4. It absorbs with maximum intensity at 275 nm; however, on excitation at 275 nm it emits at 304 and 602 nm, with a minor peak at 548 nm. These observations may be attributed to the presence of the Tyr fluorophore in two distinctly different environments, Ser(n−1)-Tyr(n)-Gly(n+1) and Thr(n−1)-Tyr(n)-Gly(n+1). The small Stokes shift for the major emission peak (304 nm) suggests total and presumably rigid encapsulation of the Tyr fluorophore of Ser(n−1)-Tyr(n)-Gly(n+1) in its microenvironment (Section S6 in the Supporting Information). The optical density spectrum changes dramatically with the change in pH value from 8 to 12, with a distinct increase in the molar absorption coefficient in the 250 nm region and red-shifting of the peak absorbance from 275 to 293 nm (Figure S6A in the Supporting Information). This change is attributed to the ionization of the amino acid residue Tyr. The degree of tyrosyl ionization α_{prot} as a function of pH value was calculated from UV/Vis absorption spectra (Figure 4B).[21] Thus at pH ≈ 9.5 (pH > pK_a of Tyr), Tyr is deprotonated and most of the Tyr residues remain in the "tyrosinate" form, which transforms the optical density spectrum. The quenching of the fluorescence emission spectra with increasing pH value also follows the same trend. (Figure S6B in the Supporting Information). These pH-dependent oxidation and reduction processes of the Tyr amino acid residue are reversible.

In summary, to our knowledge this discovery reveals GGRPSDSYGAPGGGN as the first UCST motif in biology that also exhibits LCST and pH-responsive photophysical properties. The unique chemical composition, molecular flexibility, self-organization, and physical attributes enable rec1-resilin to function as an intelligent biomaterial, such as a separating agent or reporter molecule. The data presented herein also reveal the opportunities and challenges for development of other rec1-resilin mutants for novel biomaterials design—an exciting finding for protein engineering and materials science.

Experimental Section

Preparation of rec1-resilin was performed using our published protocols.[15] Self-assembly and phase transition were observed on a Malvern Zetasizer NanoZS. Cryo TEM was performed on a cryoelectron microscope (FEI, Tecnai G2 F30). SANS experiment was performed using the SANS facility at ANSTO. Photophysical

properties were measured using a Cary UV/Vis Spectrophotometer and a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc.; see S1 in the Supporting Information for details).

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