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REVIEW

Multi-responsive biomaterials and nanobioconjugates from resilin-like protein polymers

Cite this: DOI: 10.1039/x0xx00000x

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Received xxth xxxx xxxx,
Accepted xxth xxxxx xxxx

DOI: 10.1039/x0xx00000x

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Nature, through evolution over millions of years has perfected materials with amazing characteristics and awe-inspiring functionalities that exceed the performance of man-made synthetic materials. One such remarkable material is native resilin ─ an extracellular skeletal protein that plays a major role in the jumping, flying, and sound production mechanisms in many insects. It is one of the most resilient (energy efficient) elastomeric biomaterial known with resilience of ~97% and fatigue life in excess of 300 million cycles. Recently, resilin-like polypeptides (RLPs) with exquisite control over the amino acid sequence (comprising repeat resilin motifs) and tuneable biological properties and/or functions have been generated by genetic engineering and cloning techniques. RLPs have been the subject of intensive investigation over a decade and are now recognized to be multi-functional and multi-stimuli responsive; including temperature (exhibiting both an upper and a lower critical solution temperature), pH, moisture, ion and photo-responsive with tuneable photo-physical properties. Such unusual multi-stimuli responsiveness has scarcely been offered and reported for either synthetic or natural biopolymers. Furthermore, directed molecular self-assembly of RLPs also exhibit promise as efficient template for synthesis and stabilization of metal nanoparticles. These developments and observations reveal the opportunities and new challenges for RLPs as novel materials for nanotechnology, nanobiotechnology and therapeutic applications. In this review, we discuss and highlight the design and synthesis of different RLPs, their unique molecular architecture, advanced responsive behaviour; and functionality of hydrogels, solid-liquid interfaces, nanoparticles and nanobioconjugates derived from RLPs.

1. Introduction

Resilin, an extraordinary material with almost perfect elasticity, is a member of the family of elastic proteins that includes abductin, collagen, elastin, fibrin, gluten, spider silks and titin.[1] Resilin is normally found in specialised regions (where there are highly repetitive movements) of many insects, such as in the wing tendon of the adult dragonfly *Zyxomma* sp. (Fig. 1A).[2,3] Weis-Fogh first identified resilin in the 1960's and described it as a colourless, swollen, isotropic rubbery protein with remarkable elastic recovery. He elucidated its roles in the thorax of flying insects, elastic wing hinges in locusts and elastic tendons in dragonflies.[4-7] Since then, resilin has been well recognized for its outstanding mechanical properties; and has been explored in a wide variety of insects including vinegar flies, kissing bugs, click beetles, honey bees and cicadas.[8-12] Recently, resilin has also been identified in other members of the arthropod phylum including the crustacean crabs and crayfish.[13] The structure of resilin from the wing-hinge ligament of locusts and the elastic tendon of dragonflies was identified to consist entirely of a cross-linked protein with a unique composition of amino acid residues (66% of non-polar groups, 31% glycyl and 14% from hydroxyl, but lacking sulphur-containing residues and hydroxyproxyl with only traces of tryptophan). This composition of resilin, when compared to other elastic proteins such as collagen, silk fibroin and elastin, differs distinctly with a low isoelectric point and high hydrophilicity.[7] The natural resilin proteins occur as a di- and trityrosine cross-linked network; therefore, fluorescent under ultraviolet rays (Fig. 1A). It exhibits a Young's modulus of 50-300 kPa, an ultimate tensile strength of 60-300 kPa, outstanding resilience (ability to return to the original form after being stretched) of >92%; and can be stretched to three times its original length before breaking.[3,4] Over the years, RLPs have advanced as potential biomaterial substitutes for many biomedical applications including drug delivery, tissue engineering, and regenerative medicine.[14]

2. Engineering resilin-like polypeptides (RLPs)

The gene sequence (CG15920) identification of resilin from the fruit fly *Drosophila melanogaster* (Fig. 1B) by Ardell and Andersen in 2001 opened new routes to engineer RLPs with properties comparable to those of native resilin.[15] The full length resilin gene contains an N-terminal elastic repeat domain (Exon 1), the chitin-binding domain (Exon 2) and the C-terminal elastic domain (Exon 3), where Exons 1 and Exon 3 have repetitive sequences (common

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feature found in other elastomeric proteins).[3,15,16] Following this discovery, many RLPs and RLP-based multifunctional proteins (Table 1) have been developed through molecular cloning approach in recent years; and have generated significant interest in the scientific community for their unique characteristics, including multi-responsiveness and their potential applications.[17,18]

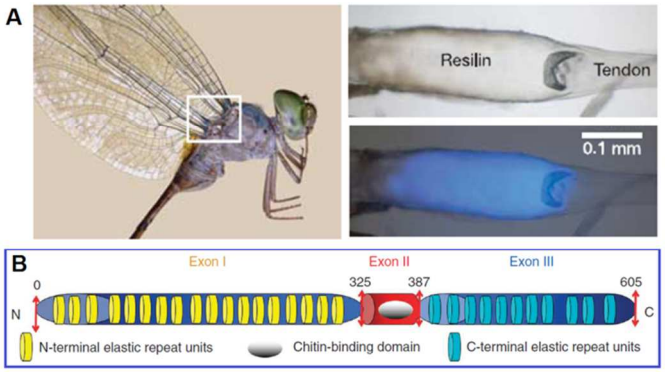

Fig. 1 (A) Fluorescence of resilin in the wing tendon of the adult dragonfly (*Zyxomma* sp.). The right panels show photomicrographs of the tendon in phosphate-buffered saline under white light and ultraviolet light (Reproduced with permission from ref. 3, Nature Publishing Group, Copyright 2005). (B) Amino-acid sequence scheme of the three exons in the full-length resilin protein (Reproduced with permission from ref. 16, Nature Publishing Group, Copyright 2012).

The recombinant synthesis approach involves insertion of the desirable gene sequence into a plasmid vector and cloning into a host cell. The recombinant protein synthesis approach has taken advantage of a diverse library of templates and peptide domains available in nature as frameworks; to not only synthesise pro-resilins with exquisite control over their amino acid sequence (comprising repeat resilin motifs) but also RLP with a range of customizable properties and activities.[19-21] The production of RLPs in bacteria or other expression organisms yields reasonable quantities of proteins with high purity, precise amino acid sequence and a virtually monodisperse molecular weight. Pro-resilins are un-crosslinked water soluble forms of resilin and are the precursors for cross-linked water insoluble rubbery resilin proteins. The first recombinant pro-resilin, rec1-resilin was reported by Elvin *et al.* in 2005.[3] Rec1-resilin was derived from the N-terminal elastic repeat domain (Exon 1) of the *D. melanogaster* CG15920 gene (Fig. 1C) comprises 18 repeats of a 15 amino acid sequence: GGRPSDSYGAPGGGN; expressed as a water soluble protein in *Escherichia coli* with yield of about 15 mgL^{-1} of culture.[3] Later, development of high-cell density fermentation by lactose induction resulted in an 8-fold increase (volumetric productivity) of purified rec1-resilin compared to that of auto-induction; leading to an overall 20-fold increased yield (300 mgL^{-1} of culture), compared to conventional Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction in Luria-Bertani (LB) medium.[22] Structurally, rec1-resilin consists of 310 amino acid residues with a molecular weight of 28.5 kD, containing repeat sequences with the YGAP motifs (a unique characteristics of resilin genes).[3] Rec1-resilin was later conjectured to be insufficient to store the energy required for the jumping or flying of insects; as it was

Table 1. Summary of resilin and RLPs reported.

Source	RLPs	Description	Mol. Wt. (kDa)	Crosslinking	Resilience (%)	Ref.
Fruit fly (*D. melanogaster*)	Full length resilin	Exons 1, 2 and 3	60	Peroxidase mediated crosslinking	~96	23
	Exon 1-RLP	Exon 1	30	Horseradish peroxidase-mediated crosslinking, citrate-modified photo-Fenton reaction crosslinking	~90	24
	Exon 3-RLP	Exon 3	23	Horseradish peroxidase-mediated crosslinking	~63	24
	Rec1-resilin	18 pentadecapeptide repeats (GGRPSDSYGAPGGGN) found entirely on exon 1	28.5	$Ru(II)(bpy)_3^{2+}$ mediated photochemical crosslinking	~97	3
	Dros16	16 Repeats of a consensus sequence (GGRPSDSYGAPGGGN) from exon 1	23	$Ru(II)(bpy)_3^{2+}$ mediated photochemical crosslinking	~91	28
	RLP12	12 repeats of a consensus sequence (GGRPSDSYGAPGGGN) where Y is replaced with F	28	Mannich-type reaction	90-98	29
	RLPX (X=24, 36 or 48)	12, 24, 36, or 48 repeats of a consensus sequence (GGRPSDSYGAPGGGN) where Y is replaced with F	28-93	Vinyl sulfone based Michael type addition reaction	-	30
	RLP-X (X=RGD, RDG or MMP)	12 repeats of a consensus sequence (GGRPSDSYGAPGGGN) where Y is replaced with F and M. Sequence flanked with K	23-24	Tris(hydroxymethyl phosphine)-mediated crosslinking	-	31
African malaia mosquito (*A. gambiae*)	An16	16 Repeats of a consensus sequence (AQTPSSQYGAP)	18.9	$Ru(II)(bpy)_3^{2+}$ mediated photochemical crosslinking	~94	28
	RZ10-X (X=RGD or RDG)	10 repeats of a consensus sequence (AQTPSSQYGAP), where Y is replaced with F and K is added	18	Tris(hydroxymethyl phosphine)-mediated crosslinking	-	32
Flea (*Ctenocephalides felis*)	Cf-resB	Isoform B transcript of full length resilin devoid of chitin-binding domain	47	$Ru(II)(bpy)_3^{2+}$ mediated photochemical crosslinking	~88	25
Buffalo fly (*Haematobia irritans exigua*)	Hi-resB	Isoform B transcript of full length resilin devoid of chitin-binding domain	52	$Ru(II)(bpy)_3^{2+}$ mediated photochemical crosslinking	~87	25

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thought that the Exon 2 (chitin-binding domain) and Exon 3 domains present in full-length resilin were required- in addition to Exon 1- in order for the protein to display both elastic and energy storagefunction, efficiently.[23]

In order to assess the importance of the two critical regions (Exons 1 and 3) of the native resilin-encoding genes, Qin *et al*.[24] cloned and expressed Exon 1 and Exon 3 of the *D. melanogaster* CG15920 gene. RLPs synthesised from Exon 1 and Exon 3 (yield of ~25 mgL^{-1} of culture) exhibited ~90% and ~63% resilience, respectively.[24] This observation confirmed that Exon 1 is the most critical domain to impart elasticity in functional materials that mimic native resilin.[23] The isoform B transcript of full length resilin, which is devoid of the chitin-binding domain has also been generated from the flea *Ctenocephalides felis* and the buffalo fly *Haematobia irritans exigua* resilin genes.[25] Following the synthesis of rec1-resilin, Elvin *et al.*[26] reported two other RLPs, namely An16- and Dros16-resilin generated from the African malaria mosquito *Anopheles gambiae* (BX619161 resilin gene) and the *D. melanogaster* (CG15920 resilin gene), respectively through the modular protein engineering approach. Interestingly, the volumetric productivity of protein expressed by auto induction varied significantly for rec1-, An16- and Dros16-resilin at 60, 220 and 20 mgL^{-1} of culture, respectively.[26,27] These RLPs were generated in order to determine whether different repetitive consensus motifs derived from different insect sources could generate similar highly resilient elastic proteins. The synthetic An16-resilin consisted of 16 copies of an 11-residue consensus repeat sequence: GAPAQTPSSQY with a molecular weight of 18.5 kD; whereas Dros16-resilin with a molecular weight of 20 kD consisting of 15 copies of a consensus repeat sequence: GGRPSDSYGAPGGGN.[26] The resilin gene from *A. gambiae* is homologous to the *D. melanogaster* resilin gene based upon the presence of N-terminal YGAP repeats.[26] Also, polypeptides containing 4, 8, 16 and 32 repeats of a resilin-inspired sequence AQTPSSYGAP were derived from *A. gambiae* to be used as tags on recombinant fusion proteins.[28]

Kiick and co-workers reported the synthesis of RLP12 (with a yield of ca. 70-80 mgL^{-1} of culture) as 12 repeats of a resilin putative consensus sequence: GGRPSDSFGAPGGGN with molecular weight of 28 kDa, derived from the first exon of *D. melanogaster*.[29] RLPs integrated with cell adhesion, cell-directed degradation, and heparin binding domains were also generated.[28] The group also demonstrated cysteine-containing (with the Ser19 residue of the RLPs mutated to a cysteine using site-directed mutagens) RLPs (RLP24, RLP36 and RLP48) having molecular weights of ~49, ~71 and ~93 kDa.[30] To expand future options for cross-linking of these materials through the incorporation of non-natural amino acids, tyrosine (Y) groups were substituted with phenylalanine (F) and methionine (M) in RLP12.[31] On the other hand, Liu *et al*.[32,33] demonstrated (with a yield of ca. 22-58 mgL^{-1} of culture) 10 and 30 repeats of the *A. gambiae* consensus sequence: AQTPSSQYGAP with Y replaced by F and lysine (K). In order to mimic the mechanical properties of muscles, Lv *et al*.[34] created a biomaterial with GB1 (the B1 domain of *Streptococcal* protein G)-resilin networks. The GB1 domain was used to mimic titin immunoglobulin domain. In another study, Bracalello *et al.*[35] reported chimeric resilin-elastin-collagen-like polypeptides (REC) comprising a resilin-mimetic sequence: $(SDTYGAPGGGNGGRP)_4$, an elastin-mimetic section and a collagen-like domain to combine the unique mechanical properties of multiple structural proteins into a single multifunctional material. Elastomeric hydrogels may be formed from the RLPs using physical or chemical cross-linking methods, as discussed in section 4.

3. Structural organization in RLPs

The primary structures of resilins and RLPs consist of a large proportion of proline and glycine amino acid residues. Proline and glycine are thought to interrupt the regularity of the α-helical backbone conformation ('helix breakers'); commonly found in turns conformations.[36] Moreover, they exhibit a combination of low overall hydrophobicity (lower than elastin and silk) and large net charge that is a unique property of natively unfolded proteins.[14,37] These observations are reported to be related to the mechanical and biological properties of the elastic proteins.[38] Early examinations of morphological structures from insects composed of resilin using high/low-angle X-ray diffraction and electron microscopy reported that resilin is highly amorphous in nature.[39] The secondary-structure distribution of a synthetic resilin construct of An16-resilin was first investigated by Nairn *et al*.[40] Circular dichroism (CD), small angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) spectroscopy were used to determine the secondary-structure distributions of the An16-resilin construct in water. The results suggested that the structure of An16-resilin is intrinsically unstructured or disordered protein (IDP) (Fig. 2A) with no apparent α-helical or β-sheet features. Moreover, Raman spectroscopy revealed that the conformations of cross-linked An16-resilin are similar to that of the uncross-linked form of resilin.[40] Bochicchio *et al*.[41] reported the existence of a dynamic equilibrium between the folded (mainly β-turns) and extended (polyproline II (PPII) and β-strands) conformations of resilin from *D. melanogaster* that could be at the origin of the high entropy of the relaxed state. The observed PPII structure in resilin is a left-handed helix and is widely present in elastomeric proteins such as elastin and titin.[42,43]

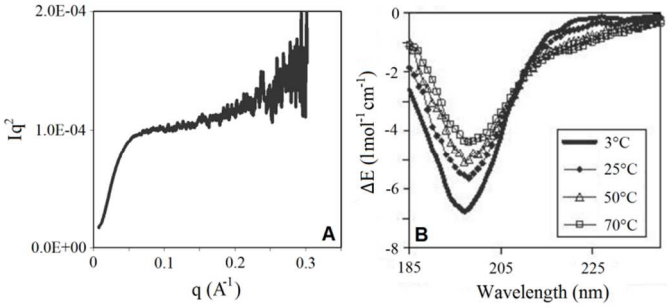

Fig. 2 (A) Kratky plot of An16 from SAXS data showing an overall "random coil" conformation through increase in intensity at higher q (Reproduced with permission from ref. 40, Elsevier, Copyright 2008). (B) CD spectra of An16 displaying minima around 195 nm; characteristics of random coil secondary structure (Reproduced with permission from ref. 27, American Chemical Society, Copyright 2009).

Lyons *et al*.[27] investigated the secondary structure of rec1-, An16- and Dros16-resilin using CD spectroscopy and reported rec1- and An16-resilin to be largely disordered with very little helical content and some contributions from sheets, turns, and PPII conformations; whereas Dros16-resilin was reported to be a more ordered structure than rec1-resilin with a high level of β-structure. Also, rec1- and An16-resilin were found to exhibit stronger temperature dependence than Dros16-resilin, with the PPII structure becoming less stable with increasing temperature (Fig. 2B).[27] Recombinant Exon-1 and Exon-3 encoded resilin proteins demonstrate the same random coil conformation suggesting that the chains of recombinant resilins are mobile and can adopt a wide range of conformations; which is also the case for the cross-linked proteins.[24] Moreover, chimeric recombinant proteins such as REC and bioactive domains (cell-binding or degradation domain)

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incorporated recombinant resilin-based proteins resulted in a similar secondary structure to that of other recombinant resilin-based proteins.[32,35,44] Tamburro *et al*.[45] studied the molecular and supramolecular structures of some polypeptides belonging to the Gly-rich repeated domain of *D. melanogaster* resilin. The sequence $(PGGGN)_{10}$ (this pentapeptide motif occurs 9 times in the *D. melanogaster* resilin protein) was reported to display a tendency to aggregate into several μm long and 80 nm diameter fibrous structures.[45] In support of this peptide-based evidence, Qin *et al*.[16] showed the fibrillar structure of the resilin exon-I protein via an AFM imaging study (explained further in section 5). It is tempting to speculate that this fibrillar promoting pentapeptide motif may serve to promote self-assembly of the protein in a manner that favours hydrophobic interaction between the phenolic tyrosine side chains of adjacent protein chains, where some of which (ca. 25%) are involved in dityrosine formation.

4. Crosslinking of RLPs

Native resilin occurs as a cross-linked (di- and tri-tyrosine crosslinks between their tyrosine residues) biopolymer exhibiting very high resilience.[4] These cross-links are essential for the rubber-like elasticity exhibited by resilin.[12] In order to mimic the properties of native resilin, RLPs can be cross-linked via physical or chemical methods to form cross-linked elastomeric hydrogels. The di-tyrosine cross-linking between tyrosine residues of the first recombinant resilin-like polypeptide, rec1-resilin, was achieved through a Ru(II)-mediated photo-cross-linking method using a 600 W tungsten-halide source to generate rubber-like biomaterial with ~97% resilience.[3,46] It is believed that photolysis of the ruthenium (II) from tris-bipyridyl dication ($Ru(II)(bpy)_3^{2+}$) produces Ru(III) based activated metal complex that extracts an electron from amino acids such as tyrosine or tryptophan, leading to a radical species that can then attack a wide variety of other groups including other tyrosine residues. The persulphate used in the crosslinking reaction, functions purely as an electron acceptor, serving to generate the Ru(III) intermediate from photo-excited $Ru(II)(bpy)_3^{2+}$. The two closely spaced tyrosyl radicals in resilin lead to spontaneous dityrosine formation via covalent crosslinking.[47] Lyons et al. reported Ru(II)-mediated photo-crosslinking of An16 and Dros16 pro-resilins to form cross-linked hydrogels that exhibited resilience values of ~94% and ~91%, respectively.[27] Despite the fact that An16 has a low glycine content (8.9 Mol%) compared to rec1-resilin (34.2 Mol%), it displays high resilience (close to rec1-resilin) upon crosslinking; which has be proposed to be due to the dynamic PPII and β-turn structures.[40] Lv *et al*.[34] also reported Ru (II)-mediated photo-crosslinking of the two chimeric-resilin constructs $(G\text{-}R)_4$ and GRG_5RG_4R (where G represents an individual GB1 domain of muscles and R represents a single resilin repeat) to develop a crosslinked biomaterial that mimics the mechanical properties of muscles.

Resilin-like polypeptides RLP12 and RZ10 synthesized from *D. melanogaster* and *A. gambiae* resilin genes were chemically cross-linked through a biocompatible Mannich-type condensation reaction.[29,32] The reaction involves cross-linking between the primary amine group (lysine) in polypeptides and the hydroxymethyl group in the cross-linkers ([tris(hydroxymethyl)phosphine]propionic acid or tris(hydroxymethyl)phosphine).[29,31,32] McGann *et al*.[30] reported RLP-poly(ethylene glycol)(PEG) composite hydrogels by crosslinking RLP12 (containing cysteine residues as crosslinking sites) with vinyl sulfone groups of a 4-arm PEG. Lately, Qin *et al*.[24] cross-linked proteins expressed from Exons 1 and 3 of *D. melanogaster* resilins into rubber-like biomaterials via horseradish peroxidase-mediated cross-linking using the citrate-modified photo-Fenton system. The reaction involved generation of reactive oxygen species (ROS) from the peroxidase-hydrogen peroxide (H_2O_2) system, which can further interact with the hydroxyphenyl groups to generate the desired *p*-hydroxyphenyl radicals for crosslinking (Fig. 3).[24] In addition, pH- and temperature-responsive behaviour of RLPs can also lead to physically assembled (hydrophobic interaction) hydrogels (discussed later in section 6.1).[48]

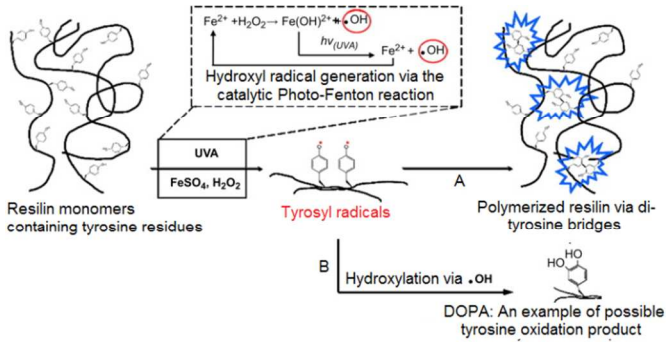

Fig. 3 Mechanism of resilin cross-linking via the photo-Fenton reaction. Hydrogen abstraction from tyrosine via strong oxidizing agent (.-OH) can generate tyrosyl radical, resulting in di-tyrosine bridges (A) and other tyrosine oxidation products (B). (Reproduced with permission from ref. 24, Elsevier, Copyright 2011).

5. Origin of elasticity in resilins

The mechanism of the outstanding rubber-like elasticity in resilin and RLPs is attributed primarily to entropic origin.[49] A possible mechanism for resilin elasticity was initially proposed by Bochicchio *et al*.[41], who advocated that resilin behaves like an entropic spring. It is proposed that in a relaxed state the structure of resilin is in equilibrium between an open and folded structure; whereas when stressed, there is a significant decrease in entropy. When the stress is released, resilin is likely to return to its original relaxed state of high entropy.[41,45] Recently, Qin *et al*.[17] investigated the thermal transition behaviour of full length resilin; and reported that Exon 1 contributes to the superior elasticity of resilin, whereas Exon 3 contributes to a reversible transition in structure related to energy storage. The authors also proposed that when stress is applied to a relaxed state of resilin (unordered conformation), Exon 1 responds immediately and transfers energy to Exon 3 leading to more ordered structure formation in Exon 3 (Fig. 4A-E). Subsequently, when the stress is removed, Exon 3 is likely to return to an unordered structure releasing the stored potential energy to the Exon 1 region.[16] Therefore, on stretching resilin, the chain entropy is reduced and upon strain release the high entropy condition is restored.[24,49]

However, in order for a system to possess rubber-like elasticity there are two required structural criteria. Firstly, conformational flexibility of the components is important in order to allow for rapid response to external forces; and secondly, a network structure is required that is made up of cross-linking interactions between components.[49-51] The purpose of the cross-links is to uniformly distribute the imposed stress throughout the matrix, and to ensure that the structural integrity of the material is maintained when force is applied.[49] In addition, the rubber-like properties of resilin only exist when the protein is in a hydrated or polar solvent environment, where, water and/or polar solvents act as plasticisers (explained later in section 6.4) causing decrease in energy barrier between different conformational states.[46] The experimental temperatures were further observed to only marginally affect the resilience of cross-linked rec1-resilin, as long as water was in the

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liquid state. Furthermore, at equilibrium hydration, near-perfect elastic behaviour of the gel was demonstrated confirming the theory of elasticity of RLPs to be entropic in origin.[46]

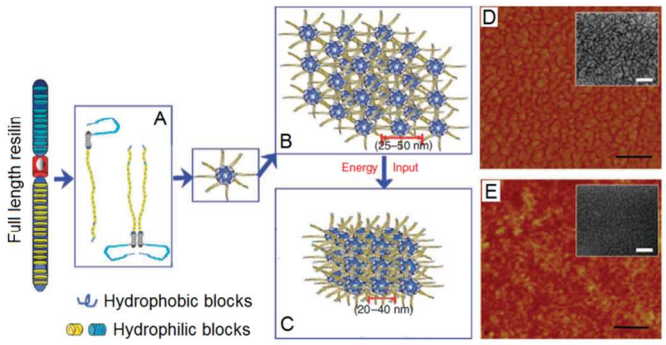

Fig. 4 Proposed model of resilin elasticity by Qin *et al*.[16] in the process of energy input and release. (A) Possible chain folding intra- and inter-molecular schemes. (B) Structure assembly and relaxed network driven by hydrophilic–hydrophobic–hydrophilic polymers to form water-swollen structures with irregular sized micelles cross-linked by fibrils. (C) The assembly process upon heating and tighter elastic network after energy input. The surface morphology of full-length resilin observed in AFM (phase images); before (D) and after (E) thermal treatments. (Reproduced with permission from ref. 16, Nature Publishing Group, Copyright 2013).

6. Multi-stimuli responsiveness of RLPs

6.1. Temperature responsiveness

Thermo-responsive polymers exhibit critical solution temperature(s) such as lower (LCST) and/or upper (UCST); above or below which the components of a mixture are completely or partially immiscible.[52] The application of the majority of smart materials relies on abrupt changes in solubility/phase transition behaviour at either at LCST or at UCST. Since the earliest report of the LCST behaviour of poly(N-isopropylacrylamide) (PNIPAM) in 1967, a large number of synthetic and biopolymers have been identified that exhibit LCST behaviour. Elastin and elastin-mimetic protein (EMPs) polymers also exhibit a reversible LCST (~37 ^{o}C); and have been the subject of numerous investigations that prompted useful applications including drug delivery, bio-analytical devices and injectable gels for tissue engineering.[53]

Elvin *et al*.[3] observed the thermo-responsive nature of rec1-resilin, which forms protein-rich and protein-poor phases at low temperatures. Lyons *et al*.[26] reported formation of coacervates in An16- and Dros16-resilin at low temperatures. This UCST behaviour of resilin-based proteins is reversible, and the coacervates disappeared upon heating.[26,47] Dutta *et al*.[48] first demonstrated precisely and quantitatively the dual phase behaviour (DPB, the appearance of both UCST and LCST) of rec1-resilin through its hydrodynamic size change with temperature measured using the dynamic light scattering (Fig. 5A) technique. The appearance of DPB in a single macromolecule has been predicted; but is indeed rare.[54,55] Rec1-resilin exhibits a UCST and a LCST at ~6 °C and ~70 °C, respectively.[48] In order to further elucidate the thermo-responsive behaviour, the morphology of rec1-resilin below the UCST (spherical particles as shown in Fig. 5B) has been studied using cryo-transmission electron microscopy (TEM). The authors also demonstrated that the UCST behaviour of rec1-resilin is reversible without hysteresis; however, the LCST behaviour displayed slower reversal kinetics.[48] The UCST behaviour of rec1-resilin is also apparent visually from optical properties of protein solutions changing from transparent (above UCST) to turbid (below UCST) and vice versa.[48] The observed UCST coacervation of RLPs is likely to be a consequence of intermolecular self-association of protein chains in solution. Recent studies of self-assembly and the observed coacervation of elastin in aqueous solution suggest that self-assembly is promoted by association of hydrophobic domains contained within the tropoelastin sequence.[56]

In elastin, the crosslinking domains are rich in Lysine residues, while in resilin, the cross-linking domains are tyrosine-rich (YGAP-repeats motifs). In the case of elastin, the crosslinking domains were shown to be structurally labile during assembly, adapting to changes in their environment and aggregated state. The sequence of crosslinking domains has a dramatic effect on self-assembly properties of elastin-like polypeptides, and the presence of lysine residues in these domains may serve to prevent inappropriate ordered aggregation.[56] By analogy, it is proposed that the YGAP-motif found as a multiple repeat sequence in all insect resilin protein sequences (with some variation on the motif; eg. YGPP) represents the crosslinking domain that is involved in dityrosine crosslink formation in native resilins.

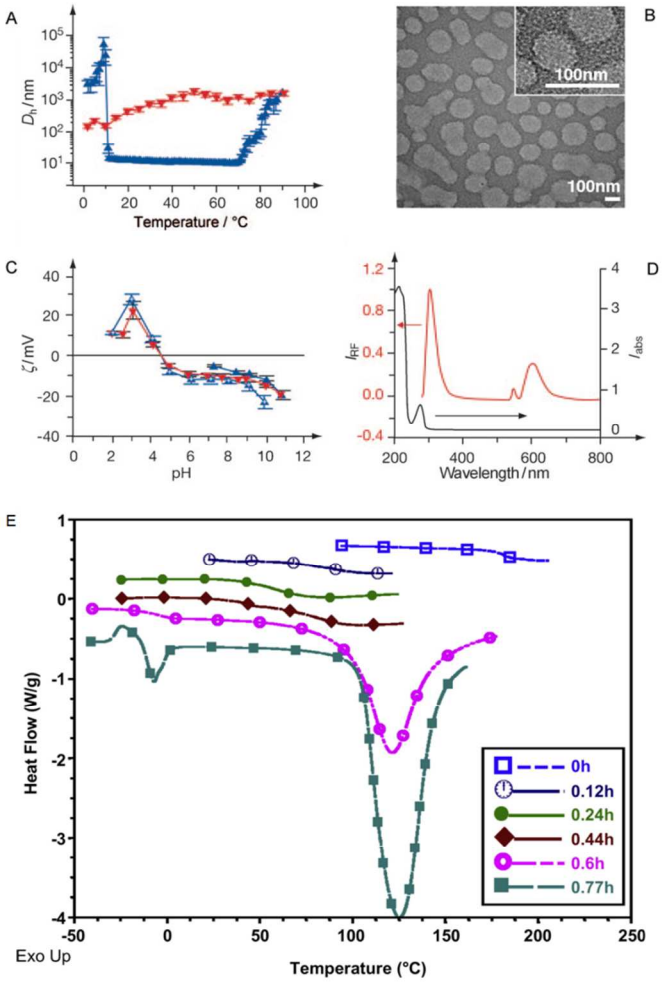

Fig. 5 Responsive behaviour of rec1-resilin. (A) Plot of hydrodynamic diameter, Dh at pH 7.4 as a function of temperature (heating is blue and cooling is red). (B) Cryo-TEM micrographs of rec1-resilin for solutions of 10 mg/mLat 4°C. (C) Plot of zeta potential Vs pH (blue triangles are forward and red triangles are reverse cycles). (D) UV/Vis absorption (black) and emission (red) response of rec1 in solution (Reproduced with permission from ref. 48, John Wiley & Sons, Copyright 2011). (E) Effect of hydration on the Tg of dry cross-linked rec1-resilin. The effect of non-crystallizable water on chain dynamics is suggested by

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significant decrease in T_g with the level of hydration, h. (Reproduced with permission from ref. 46, Elsevier, Copyright 2011).

6.2. pH and ion responsiveness

Zeta (ζ)-potential measurements of rec1-resilin in aqueous solution clearly demonstrate its pH-dependent surface charge characteristics of RLPs. At physiological and basic pH (negative ζ-potential) rec1-resilin forms protein particles (~9.5 nm) with positively charged residues forming the core and negatively charged residues exposed to water. The ability of rec1-resilin to rapidly change its structural conformation as a function of pH is evident from the charge reversal observed below the isoelectric point, IEP (pH 4.8).[48] Fig. 5C shows the effect of pH on surface charge of rec1-resilin. Quartz crystal microbalance with dissipation monitoring (QCMD) studies confirmed that rec1-resilin undergoes globule to coil to extended coil conformations as a function of pH with varied packing density at the solid–liquid interfaces.[57] Moreover, the tune-ability of the UCST of rec1-resilin solution by pH adjustment has also been revealed.[48] With difference in hydrogen-bonding capabilities of amino acids, UCST behaviour of rec1-resilin solution depends strongly on the pH (UCST increases with decreasing pH and vice versa).[48] However, the LCST is observed to be not significantly dependent on pH value, whereas the association at LCST is kinetically controlled with nonspecific aggregations (hydrophobic).[48]

6.3. Photo responsiveness

Native resilins are auto-fluorescent with a bright blue fluorescence under UV illumination.[3] The primary source of auto-fluorescence by cross-linked resilin was identified to be from the di- and trityrosine bridges in native resilin.[58] Like native resilins, recombinant cross-linked RLPs are also auto-fluorescent (Fig 5D).[3,48] This intrinsic blue fluorescence comes from photo absorption and emission of tyrosine (303 nm emission) and di-tyrosine (420 nm emission) from pro-resilin and cross-linked resilins.[48,59] However, this auto-fluorescence property of resilin in aqueous solution is sensitive to pH.[59] As the tyrosine phenolic group has a pKa of ~10.5, the emission maximum of resilin shifts from ~303 nm to ~350 nm above pH of 10.5.[48] This shift is attributed to the ionization state of the phenolic groups (tyrosine to tyrosinate) in the resilin.[60] Moreover, this pH dependent fluorescence is completely reversible with protonation of tyrosinate below pH of 10.5.[48,59] The fluorescence property of RLPs was also observed to be affected by the presence of surface plasmon active metal nanoparticles in solution (discussed later in section 7.3).[61]

6.4. Moisture sensitivity and responsiveness of RLPs

The characteristic mechanical properties of resilin and RLPs-based hydrogels are highly moisture sensitive. Dehydrated cross-linked rec1-resilin is brittle with a glass transition temperature (T_g) >180 °C (Fig. 5E).[46] Rec1-resilin constantly changes its T_g with change in the moisture level, and when hydrated above a critical level exhibits rubber-like elasticity. This moisture responsive viscoelastic behaviour of rec1-resilin is related to the modification of the molecular chain mobility- provided by alternative mobile hydrogen bond donors and acceptors for peptide groups when hydrated. The mechanical properties of rec1-resilin gels were demonstrated to change dramatically (examined through nano-indentation studies), even with marginal hydration (<10%).[46] Detailed investigation on the moisture sorption kinetics of dehydrated cross-linked rec1-resilin confirmed that the hydration process is complex and involves three different stages of hydration: first the water molecules interact strongly with charged hydrophilic groups, followed by condensation of water molecules around the already hydrated sites, and finally clustering of crystallisable water. At high levels of hydration (h>0.7, where, h = mass of water/mass of dry protein) cross-linked rec1-resilin showed presence of crystallisable water (bulk water) in the system, confirmed by a free water melting peak in differential scanning calorimetric (DSC) thermograph (Fig. 5E).[46]

7. Directed self-assembly in RLPs and their applications

7.1. RLP-based biomedical hydrogels

Owing to their impressive elastomeric and multi-stimuli responsive properties, resilin and RLP-based materials have been fabricated and examined for both biomedical and industrial applications. RLP12-based hybrid constructs (Fig. 6A) developed by Kiick's group revealed interesting properties of cell and/or protein binding and biodegradation with suitable mechanical properties (storage modulus G′ within the range of 500 Pa to 10 kPa), which are highly comparable to the mechanical properties of targeted vocal fold tissues.[44] Moreover, their potential for vascular tissue engineering applications have been validated through cyto-compatibility proofs with NIH 3T3 fibroblasts (spread cells with well-formed and organized stress fibres), human aortic adventitial fibroblasts (transition from a round to extended morphology over the course of a week suggesting local degradation of protein matrix and focal adhesion of cells) and primary human mesenchymal stem cell (even distribution throughout the three dimensional gel characterized via a live/dead assay) culture assays.[29-31,44] Cross-linked RLPs namely $(G\text{-}R)_4$ and GRG_5RG_4R developed by Lv *et al*.[34] displayed Young's modulus values of ~70 kPa and ~50 kPa, respectively, which are comparable to that of myofibrils/myocytes (60-100 kPa). The developed biomaterials behave as rubber-like materials showing high resilience at low strain without hysteresis and as shock-absorber-like materials comparable to the behaviour of muscles.[34] RLP-based hydrogels developed by Qin et al.[24] from cross-linked Exon 1 proteins (expressed from *D. melanogaster* gene) using photo-Fenton reaction demonstrated excellent adhesive properties. The cross-linked protein was highly adhesive due to the formation of DOPA by the citrate-modified photo-Fenton reaction that resulted in the formation of di-tyrosine crosslinks.[24] This method of crosslinking thus may be useful for the development of in vivo polymerisation of RLPs. Dutta and co-workers demonstrated tuning of crosslink density of rec1-resilin based hydrogels by regulating the ratio of the rec1-resilin to cross-linking agent used for a photo-crosslinking reaction.[46] Such crosslink density modulation offers a unique opportunity to adjust the dynamic mechanical behaviour of the gels with significant potential in micro-electromechanical systems (MEMS) and biomedical micro/nano-manipulation systems.[62] The complex modulus and yield strain values of the cross-linked gels developed by Renner *et al*.[32,33] from the *A. gambiae* resilin gene were determined to be 22±1 kPa and 63%; significantly differing from that of the lysine cross-linked RLPs (10.6 kPa and 450%) developed by Charati *et al*.[29] from the *D. melanogaster* resilin sequence. This observation indicates the property difference between RLPs developed from different sources, which may be related to the change in the sequence and higher degree of cross-link density. Recently, Vashi *et al*.[63] demonstrated two important features of the cell compatibility of resilin-like sequences. Firstly, RLPs do not carry any native cell-recognition peptide sequences (such as the integrin-binding - motif RGD) and in consequence, cells do not bind to resilin surfaces. If the integrin-binding motif (-RGD) is supplied

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in the form of a peptide during photochemical crosslinking, the cells can bind to the previously passivated tissue culture polystyrene surfaces. Secondly, the photochemical reaction that drives dityrosine formation in RLPs is a cell-compatible process (cells in vitro survive the photochemical reaction). Renner et al.[32] demonstrated that RZ10-RGD based gels were suitable for tissue engineering applications, as they supported the adhesion and spreading of human mesenchymal stem cells (hMSCs) with 95% cell viability (demonstrated by LIVE/DEAD assay after 3 days) and cell morphology similar to that

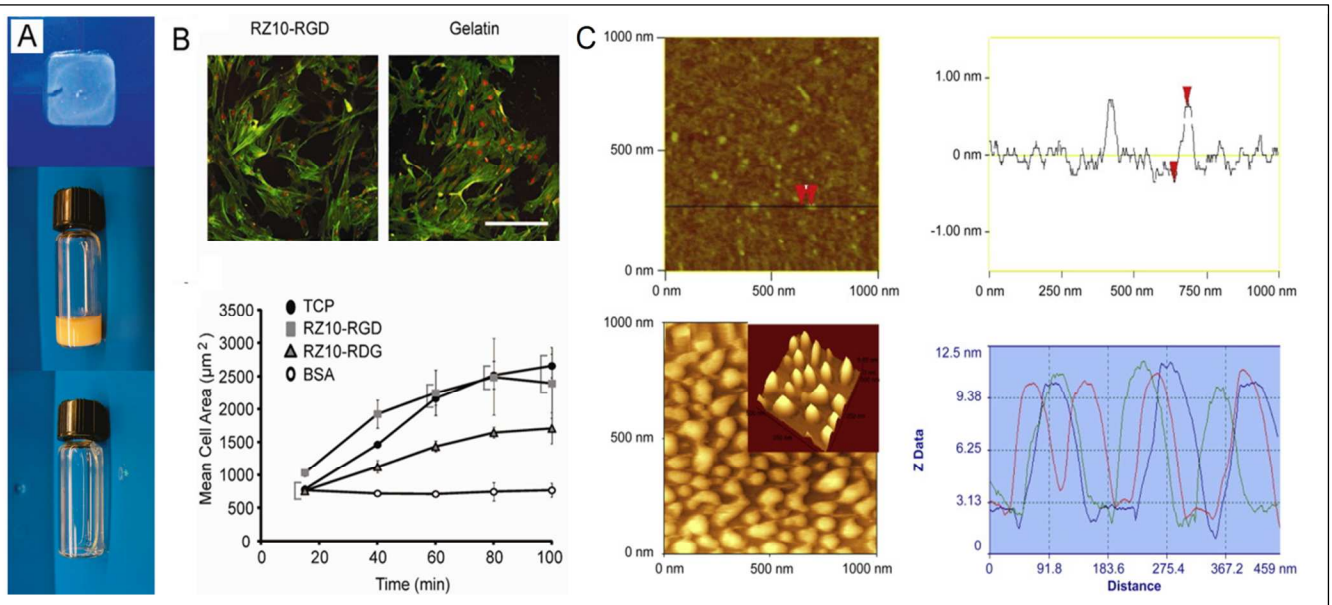

Fig. 6 (A) Top to bottom: a film of a 20 wt % RLP hydrogel with 1:1 cross-linking ratio (amine/hydroxymethylphosphine), a 20 wt % RLP hydrogel with a 1:1 cross-linking ratio and an un-cross-linked 20 wt % RLP solution (Reproduced with permission from ref. 44, with permission from American Chemical Society, Copyright 2011). (B) Human MSCs cultured for three days on RZ10-RGD and gelatin (positive control) with quantitatively measured comparison on RZ10-RGD, TCP, RZ10-RDG, and BSA indicating that cells interact with the cell binding domain on RGD (Reproduced with permission from ref. 32, American Chemical Society, Copyright 2012). (C) AFM images and cross-section analyses of rec1-resilin on the mica substrate (top) and graphite (bottom with 3D inset). The corresponding Z data is given in the right panel. (Reproduced with permission from ref. 64, Elsevier, Copyright 2010).

gelatine structures (Fig. 6B).[32] Recently, Li et al [31] have further confirmed that the RLP-RGD hydrogel are able to 3D encapsulation, support, attachment and spreading of hMSCs.

7.2. RLP-based responsive interfaces

Dutta *et al.*[64] reported the molecular architecture control (using physical approaches) of rec1-resilin on various substrates such as graphite, silicon wafers and mica. The authors demonstrated tuning of the protein structure and conformation on substrates by tuning the physical conditions at the substrate surfaces (Fig. 6C).[64] The astonishing variations in adsorbed protein nanostructure arise due to difference in gain in entropy on different surface energy substrates. With surface charge distribution of the substrate and the protein, the morphology and surface coverage of the adsorbed rec1-resilin is reported to change significantly. An energetically favourable monomolecular layer structure or columnar structure was demonstrated on hydrophilic and hydrophobic surfaces respectively (Fig 6C).[64] Moreover, formation of a bio-mono/bi-layer rec1-resilin film (thickness range of 0.98-8.3 nm) triggered by substrate surface characteristic suggesting application of this approach to be useful in the design of nanoscale hydrogel structures with controlled morphology of rec1-resilin was reported.[64] The developed hydrogel structures could be used as a template/reservoir for drug delivery and sensor applications.

7.3. RLP-directed and stabilized noble metal nanoparticles

The environment induced self-assembly behaviour of rec1-resilin has been employed successfully to synthesise, stabilize and protection noble metal nanoparticles (NPs).[61,65] Mayavan *et al.*[61] have demonstrated a one-step non-covalent mode of binding protocol (unlike thiol, cysteine or citrate stabilized) to synthesize optically coupled hybrid architectures of 1-5 nm gold nanoparticles (AuNPs)-rec1-resilin nanobioconjugates. The synthesis (at room temperature) involved the formation of zero-valent AuNPs in the presence of rec1-resilin as soft template by reduction of Au (III) cation using an excess of sodium borohydride ($NaBH_4$). The size and quality of the NPs could be controlled through adjusting the ratio between Au(III) cation to pre-organized protein concentration (Table 2, Fig. 7); and tuning the conformations of rec1-resilin though tuning the pH.[61]

Table 2. Sample designation and physical characteristics of AuNPs synthesized using rec1-resilin as template (Reproduced with permission from ref. 61, Elsevier, Copyright 2011).

S	C_1 μM	C_2 μM	C_3 μM	C_r	N_{Au}
Au_2	2.015	6.59	4.28	2.12	62
Au_21	2.015	65.9	42.8	21.24	427
Au_106	2.015	329.6	214	106.2	376

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Au_212	2.015	659.2	428	212.4	-
Au_425	2.015	1318.6	856.1	424.86	-
S_Au_425	2.015	1318.6	856.1	424.86	835

S = Sample designation, C_1 = Concentration of rec1-resilin, C_2 = Concentration of $AuCl_3$, C_3 = Concentration of Au(III), C_r = molar ratio of Au(III) to rec1-resilin, N_{Au} = Average number of Au particles per nanocluster. S_Au is seeded growth; all others are normal growth.

The presence of uniformly distributed amino acid residue tyrosine (Tyr) along the molecular structure of rec1-resilin (twenty Tyr residues with 18 -Ser (or Thr)-Tyr-Gly- sequences) provides rec1-resilin unique optical density and fluorescence characteristics as shown in Fig 5D. The pronounced quenching of Tyr fluorescence of rec1-resilin due to the presence of AuNPs has been observed (Fig 7C) that confirms a highly efficient energy-transfer process between Tyr and AuNP with the Tyr residue of rec1-resilin as the energy donor and the AuNP as the acceptor. Moreover, from bimolecular quenching constant determined using the Stern–Volmer equation, the dominant mechanism of quenching of rec1-resilin fluorescence in the presence of AuNPs was confirmed to be static.[65] Such quenched fluorescence of rec1-resilin by AuNPs has considerable potential for fluorescence-based detection schemes and sensors. Mayavan *et al*.[66] demonstrated rec1-resilin-AuNPs decorated multi walled carbon nanotube (MWCNT) hybrid architectures; where, functionalized MWCNT was used as a substrate for depositing biosynthesized The high catalytic activity and durability of the synthesized hybrid architectures were also demonstrated for methanol oxidation.

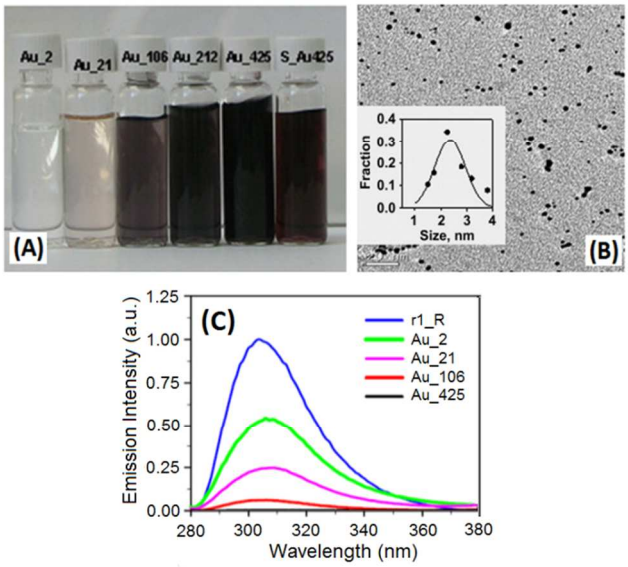

Fig. 7 (A) Optical micrograph of AuNPs-rec1-resilin nanobio-conjugates exhibiting surface plasmon resonance at different Au cation:rec1-resilin molar ratios. (B) TEM images of rec1-resilin capped gold nanoparticles synthesized at Au cation:rec1-resilin molar ratio of 2.12. (C) Fluorescence emission spectrum from the aqueous sols of rec1-resilin protected AuNPs (sample designations per Table 2). (Reproduced with permission from ref. 61, Elsevier, Copyright 2011).

Nanoclusters are a new class of materials that are made up of tens to hundreds of atoms and have intermediate composition between bulk and molecular regimes. In NCs the electronic band structure of the bulk gets modified to discrete electronic states as a result of quantum confinement. Such noble metal NCs are of considerable interest because of their distinct size dependency electronic properties; nanoclusters with sizes comparable to the Fermi wavelength of the electron (ca. 0.7 nm) show discrete electronic transitions among the quantized levels. Such NCs exhibit size-dependent fluorescence depending on the energy differences between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) - a scale function of the number of atoms within the cluster. The emission energies of the NCs fit with the sizes (number of atoms, N) via the simple relation, $E_{Fermi}/N^{1/3}$, predicted by the spherical Jellium model,[67] where E_{Fermi} is Fermi energy level.

Recently, RLP-templated green synthesis of highly fluorescent water soluble noble-metal nano-clusters (NCs)/quantum dots, have been reported by Dutta *et al*.[68] using single-step strategy using rec1-resilin as sole multifunctional agent at high pH in physiological temperature. The process is considered to be similar to a bio-mineralization. Rec1-resilin molecules have the ability to sequester noble metal ions (Pt, Au, Ag); and entrapped them upon addition of the precursor salts (e.g. chloroplatinic acid, chloroauric acid, silver chloride) to the aqueous protein solution. The entrapped ions undergo progressive reduction to form NCs (particle size < 2nm) in situ when activating the reduction ability of rec1-resilin molecules (transfer of tyrosine amino acid residue tyrosine to tyrosinate ion) by adjusting the reaction pH to ~12. This simple and convenient method has also been attempted using other proteins; particularly, bovine serum albumin (BSA), for the synthesis of NCs including gold, silver, their alloys,[69,70] and semiconductor nanoparticles.[71] The intrinsically disordered nature and non-covalent mode of stabilisation of rec1-resilin makes it unique and distinct from other globular proteins such as transferrin, bovine serum albumin (BSA) reported to stabilize AuNPs.[72,73] The fluorescent NCs are efficient biomarkers and cellular imaging agent as they can be internalized by cells through endocytosis- either receptor-mediated or nonspecific.[74] In all protein-NC bio-conjugates, the proteins provide biocompatible interface upon NC formation; moreover post synthesis surface modification with functional ligands through ligand exchange can offers effective ways to further modify the properties of the fluorescent NCs.

8. Conclusion and future perspective

Native resilins have attracted significant attention in 1960's due to their outstanding rubber-like elasticity, remarkable resilience, and presumably long fatigue life. The identification of the resilin gene sequence (CG15920) from *D. melanogaster* in 2002; followed by successful cloning and expression of Exon-1 of CG15920 gene in 2005 to synthesize a soluble resilin-like polypeptide, rec1-resilin has opened up possibilities for new RLP design and synthesis. Since then a variety of recombinant DNA molecules encoding different RLPs including the full length CG15920 gene, pro-resilin repeat from African malaria mosquito (*A. gambiae*), flea (*C. felis*) and buffalo fly (*H. exigua*) resilin genes have been reported. Several methods including the use of peroxidase enzymes (e.g. Horseradish peroxidase (HRP), photosensitizers and photo-Fenton reaction have been explored for the in vivo and in vitro cross-linking of RLPs. These crosslinking reactions result crosslinked RLPs and RLP hydrogels that are highly resilient, and exhibit ideal rubber-like elasticity. The nature of the crosslinker used and the density of crosslinking employed also offer the possibility to tune the mechanical properties (e.g. enhanced elastic modulus and reduced creep strain) and the adhesiveness of the gels, moderately. These studies have confirmed the possibility of generating highly elastic

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and resilient soft RLPs by genetic engineering; and established RLPs as a promising class of biomaterial. The facile and rapid photochemical crosslinking of RLPs into elastic hydrogel has been explored to form highly elastic tissue sealant, implantable/injectable gels and 3D encapsulation of hMSCs. RLP crosslinked hydrogels also offer physicochemical properties to mimic the extracellular matrix to support cell growth, and elasticity for vascular/cardiovascular prosthesis and vocal cord tissue engineering. It should also be feasible to design new generation of responsive hydrogels for recruitment, activation, control and homing for cell based therapies and preventative treatment.

The identification of unusual multi stimuli-responsive properties including dual phase behaviour of rec1-resilin in 2011 makes RLPs unique among the elastomeric proteins, and has generated significant excitement to the scientific community. The unique responsiveness, chemical composition, molecular flexibility, self-organization and physical attributes enable rec1-resilin to function as an intelligent biomaterial, and make them potentially useful for may applications including separating agent, reporter molecule, templating agent for nano/sub-nanoparticle synthesis and control drug delivery vehicle. Moreover, the demonstrated surface-guided self-assembly of RLPs could be exploited as a smart interface, reservoir for drugs, nanoparticles, enzyme delivery, and sensor applications. The multi-stimuli responsiveness has recognized RLPs as versatile biomaterials for both industrial and biomedical applications; however, the understanding of the origin of multi-responsiveness is still incomplete and need significant further in-depth investigation. Once the mechanism and structural origin are appropriately understood it will extend the opportunity for designing of a wide range of smart materials using recombinant, combinatorial biosynthesis and even synthetic methodology.

The emergence of new class of RLPs through recombinant DNA technology has the potential to enable the development of designer-polypeptides with specific sequences; allowing their mechanical, chemical, biological and responsiveness properties to be tailored according to requirements. The most recent revelation of the modification of RLPs with functionally-specific domains including cell adhesion, attachment and cell-directed degradation regions has prompted the potential of RLPs in many different smart biomedical applications in the future. The possibility of developing 'intelligent' biomaterials encoding biological signals that are designed to respond to the changes in the immediate environment; and consequently stimulating specific cellular responses, triggering biological events, or eliciting specific interactions with cells to direct cell proliferation/ differentiation, extracellular matrix production/organization are also emerging.

Currently, labelling the fixed cells using organic dyes or semiconductor quantum dots is prevalent in biomedical research. However, in many such applications the photo-bleaching of organic dyes and toxicity of the QDs are undesirable. The fluorescent noble metal nano-clusters particularly AuNCs have decent quantum yield, photo-stability. Therefore, RLP-capped AuNCs are highly promising as cell markers for long-term studies including cell-cell interactions, cell differentiation, and tracking because of their nontoxicity and biocompatibility. RLP-caped nano-clustres also hold significant promise to be useful as advanced theranostic material-targeted delivery of therapeutic molecules/drug/gene with the ability to identify its consequences using minimum invasive surgery- on appropriate post modification. Such composite theranostic materials also expected to have high therapeutic efficiency with minimum cytotoxicity, however significant long-term research on many aspects including post-modification of RLP-NCs are critical for the progress.

The combination of recombinant DNA technology and large-scale culture process, currently available, has the potential to synthesize commercial quantity of RLPs of predetermined structure and resulting functionality. We anticipate that the increased research focused on RLP based elastomers will result in new opportunities to mimic the natural intelligence of biological systems and development of powerful new technologies including RLP based micro/nanoscale structures for microelectromechanical systems (MEMS) that can serve as sensors, carriers. and diagnostic tools. However, before we may have such systems, processing of RLPs using modern techniques such as electrospinning, direct patterning and self-assembly has to be developed and optimized to manufacture a diverse range of structures, such as nanofibres of well-defined diameters and surface morphologies, porous scaffolds, nanowires, nanospheres. The successful co-assembly of such RLP based structures with a variety of other materials is going to be a prerequisite to make complex functional structure and significant further multidisciplinary research is critical.

Acknowledgements

The authors are grateful to the Australian Research Council (ARC) for financial support through the ARC Discovery grant DP1092678.

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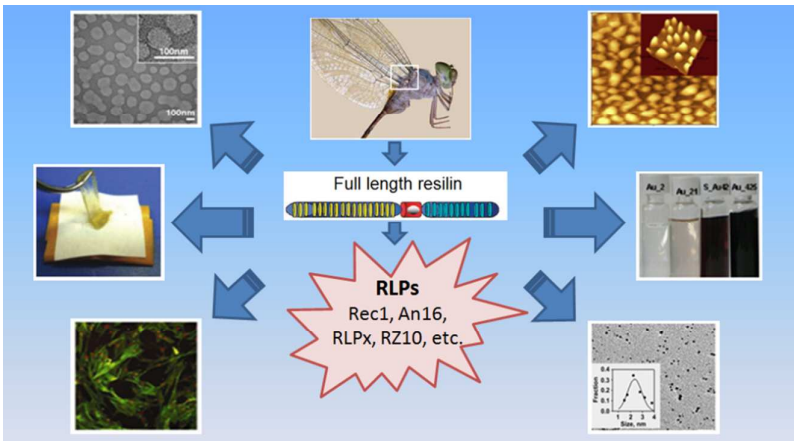

In this review, we highlight and discuss the design, synthesis, unique molecular architecture, advanced responsive behaviour and functionality of hydrogels, solid-liquid interfaces, nanoparticles and nano-biohybrids derived from resilin-mimetic protein polymers.

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