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Order/Disorder in Protein and Peptide-Based Biomaterials

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Abstract: Nature utilizes both order and disorder (or *controlled disorder*) to achieve exceptional materials properties and functions, while synthetic supramolecular materials mostly exploit just supramolecular order, thus limiting the structural diversity, responsiveness and consequent adaptive functions that can be accessed. Herein, we review the emerging field of supramolecular biomaterials where disorder and order deliberately co-exist, and can be dynamically regulated by considering both entropic and enthalpic factors in design. We focus on sequence-structure relationships that govern the (cooperative) assembly pathways of protein and peptide building blocks in these materials. Increasingly, there is an interest in introducing dynamic features in protein and peptide-based structures, such as the remarkable thermoresponsiveness and exceptional mechanical properties of elastin materials. Simultaneously, advances in the field of intrinsically disordered proteins (IDPs) give new insights about their involvement in intracellular liquid-liquid phase separation and formation of disordered, dynamic coacervate structures. These have inspired efforts to design biomaterials with similar dynamic properties. These hybrid ordered/disordered materials employ a combination of intramolecular and supramolecular order/disorder features for construction of assemblies that are dynamically reconfigurable. The assembly of these dynamic structures is mainly entropy-driven, relying on electrostatic and hydrophobic interactions and is mediated in part through the adopted (unstructured) protein conformation or by introducing an oppositely charged guest for peptide building blocks. Examples include design of protein building blocks composed of disordered repeat sequences of elastin-like polypeptides in combination with ordered regions that adopt a secondary structure, the co-assembly of proteins with peptide amphiphiles to achieve reconfigurable, yet highly stable membranes or tyrosine-containing tripeptides with sequence-controlled order/disorder that upon enzymatic oxidation give rise to melanin-like polymeric pigments with customizable properties. The resulting hybrid materials with controlled disorder can be metastable, and sensitive to various external stimuli giving rise to insights that are especially attractive for the design of responsive and adaptive materials.

Keywords: Self-assembly · supramolecular order-disorder · biomaterials · peptide · protein

1. Introduction

Owing to advances in the field of supramolecular chemistry and especially that of peptide self-assembly,[1] programming building blocks that self-assemble at will to form biomaterials with desired structures and properties is now achievable.[2] High-resolution microscopy, scattering and spectroscopy methodologies have helped to unravel structural insights of the molecular basis underlying these biomaterials, increasingly enabling the design of next-generation of building blocks and materials. Despite these developments, the dynamic functionality of biological materials is not yet accessible in man-made biomaterials. This dynamic functionality is underpinned by the chemical and structural diversity of biological building blocks and by the rich set of properties that emerges from the polymeric and supramolecular interactions between components. For example, the extracellular matrix is composed of networks of polysaccharides, glycoproteins, and protein fibres. These complex proteins contain structured and unstructured domains that contribute to supramolecular order-disorder, resulting in properties that are simultaneously highly dynamic and mechanically robust.

Alongside complex cooperatively assembled biopolymers containing ordered and disordered domains, the emerging field of liquid-liquid phase separation, as the basis of membraneless organelles formation,[3] has triggered a renewed interest in intrinsically disordered proteins (IDPs) and design of materials based on their remarkable dynamic properties. Membraneless organelles, also called biomolecular condensates,[4] are supramolecular disordered compartments which include stress granules, nucleoli, and Cajal bodies. The commonly suggested mechanism for the formation of these disordered organelles is based on liquid-liquid phase separation[5] of intrinsically disordered proteins and other biomolecules (mainly nucleic

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acids), a process termed complex coacervation, that is mainly entropy-driven[6] and mediated by electrostatic interactions.[4] It has been proposed that repetitive, low-complexity domains within IDPs promote phase separation and formation of disordered organelles.[7]

These revelations have led several groups to focus on IDPs and their synthetic mimics as building blocks for the design of biomaterials, either on their own, or in combination with self-assembling structures. Notably, most of the efforts in the field are focused on unravelling the biological role of IDPs in formation of membraneless organelles,[3] while the chemical principles governing these disordered assemblies are just emerging and they have not been systematically reviewed yet.

Here, we will present a number of recent studies that exploit proteins and peptides as building blocks for construction of biomaterials by leveraging their intramolecular or supramolecular order/disorder. We will focus on the molecular and supramolecular design of these biomaterials, rather than their applications. We will first discuss studies of proteins or polypeptide-based materials, including those designed based on elastin sequences, containing disordered regions and sequences inspired by dynamic structures found in marine organisms. Thereafter, we will focus on combinations of designed peptides and biomacromolecules, including dynamic metastable assemblies and kinetically trapped or solid-like structures. Finally, we will summarize the different assembly pathways and provide an outlook of the field.

2. Protein-Based Biomaterials

2.1 Elastin-Like Protein Assemblies

Chilkoti and co-workers have utilized order and disorder as a design principle to develop stimuli-responsive materials[8] with designable hysteresis. They designed recombinant building blocks containing disordered regions, based on the repeating pentapeptide sequence VPGXG of elastin-like polypeptides (ELPs) and ordered domains composed of polyalanine sequences (Figure 1a). While the disordered regions are thermally responsive and promote phase separation, the polyalanine sequences are α-helix domains, which are thermally and structurally stable and thus facilitate intramolecular order. All designed polypeptides exhibited thermal hysteresis, a difference between the transition temperature (*T*t) of the material from an optically clear solution phase to a turbid phase during heating, compared to the *T*t during cooling (Figure 1b). Interestingly, this hysteresis was found to be sequence-encoded, with both the length and composition of the disordered ELP domains controlling the *T*t-heating, and the ordered helical domains' composition controlling the *T*t-cooling. Coarse grained simulations revealed that below *T*t-heating, the polypeptides are clustered as oligomers by their helical domains where the disordered segments are found on the exterior of these clusters. Above *T*t-heating, the oligomers start to cluster through hydrophobic interactions of the disordered ELP domains. The helical domains swap with neighbouring clusters and eventually entangle into a network (Figure 1c). The repulsion between disordered domains and their solubility decreases with temperature increase, resulting in transition from spherical clusters into less dynamic aggregates. Unlike the phase separation behaviour of disordered ELPs, which form liquid-like droplets, the ordered/disordered polypeptides exhibited arrested phase separation. Super resolution microscopy revealed a two-stage aggregation process where the polypeptides first nucleate and then connect into kinetically stable fractal networks, above *T*t-heating.[8]

In an effort to establish design rules for phase separation of elastin-like polypeptides (ELP) based on variations in sequence composition and molecular weight, Lopez and co-workers designed four model ELPs that were composed of the repeating sequence VPGXG.[9] Above their *T*t, these polypeptides typically phase separate[10] into protein coacervates. The authors managed to control the *T*t by changing the ELPs sequence composition and length (Figure 2a). Substitution of valine to alanine or decrease in protein length from 160 to 80 block repeats decreased the hydrophobicity of the building block, leading to increase in the *T*t (Figure 2b). The phase separation behaviour of ELP was studied a water/oil microemulsion. Above their *T*t, the proteins phase separated inside the aqueous droplets and over time coalesce and coarsen to eventually form a core-shell arrangement of dense coacervates

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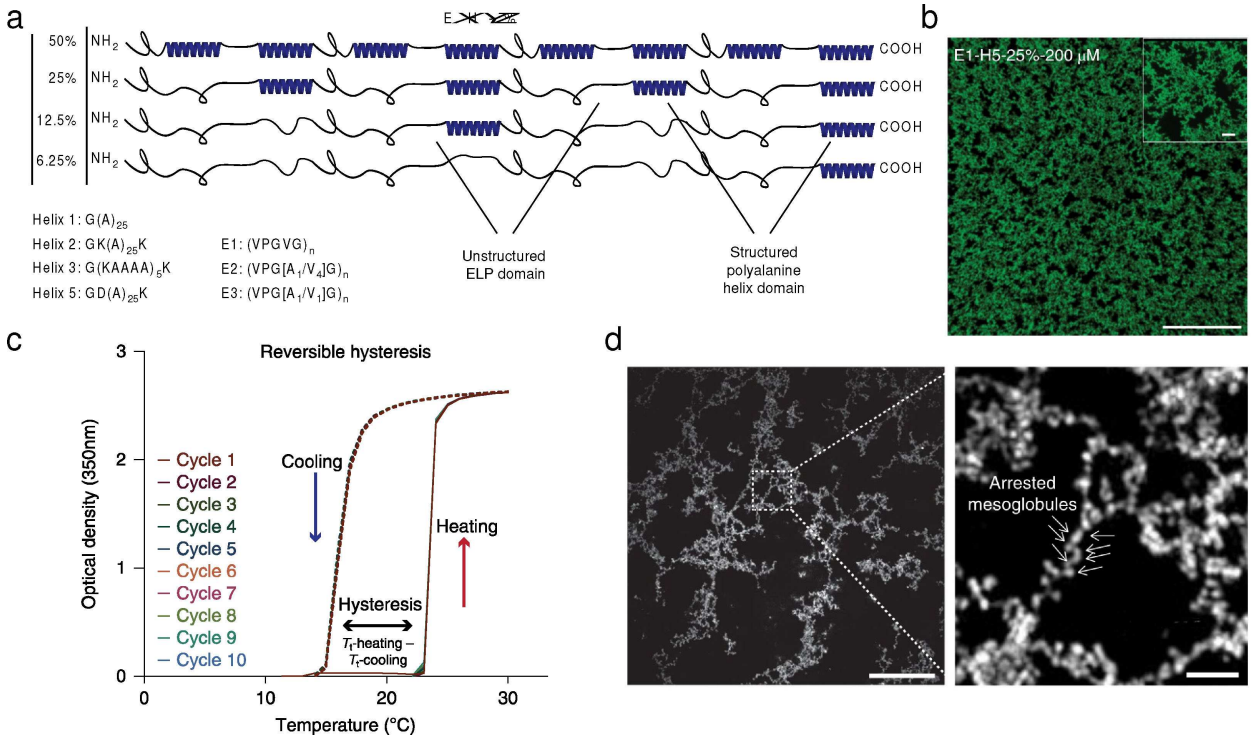

Figure 1. Ordered-disordered recombinant polypeptides. **a**. Design of recombinant polypeptides was based on three disordered ELP components and four polyalanine ordered domains at amino acid percentages up to 50%. **b**. The material formed by E1-H5-25% shows a reversible transition from an optically translucent liquid to an opaque solid-like structure during a heating and cooling cycle. **c**. Optical density measurements showing hysteresis and no change in thermal behavior of the materials upon repeating cycles of heating and cooling. **d**. 20-μm-thick three-dimensional reconstruction of E1-H5-25% using super resolution microscopy. Scale bars are 50 μm and 10 μm for the inset. **e**. Mesoscale network architecture showing interconnected globular structures. Scale bars are 10 μm (left) and 1 μm (right). Reproduced from ref. 8 with permission from Springer Nature.

within droplets. This process was completely reversible upon application of cooling-heating cycles. Next, a two-component core-shell coacervate arrangement inside the droplets was obtained by combining ELPs with different *T*t (Figure 2c). Following heating above the *T*t of both E1 and E2, the structure with two immiscible coacervates layers is formed. Reducing E1 length from 160 pentamer repeats to 40 repeats enhanced the mixing entropy, resulted in formation of miscible coacervates. These findings show that the layered organization of coacervates can be controlled by the ELP sequence, where high sequence similarity increases ELP-ELP hydrophobic interactions, resulting in a mixed miscible coacervate while dissimilar sequences forming immiscible layered coacervates.

Mata and co-workers have recently utilized a biomimetic approach that harnesses supramolecular order-disorder to guide mineralization.[11] This work was inspired by the biological role of IDPs in biomineralization, including the IDP amelogenin, which upon interaction with enamel crystals changes its conformation from random coil to β-sheet.[12] As the components of the organic matrix with controlled disorder, they designed an elastin-like recombinamer (ELR) containing a hydrophobic region (VPGIG), a positively charged region (VPGKG) that can be cross-linked through the lysine, and a highly acidic region known to promote mineralization (Figure 3a). The protein self-assembles into a membrane containing β-amyloid-like fibrils and 3D spherulites (Figure 3b) when dissolved in dimethylformamide (DMF) in the presence of a cross-linking agent and upon dehydration. At physiological conditions, ordered mineralized structures were observed to grow on both sides of the protein membrane when a supersaturated solution of fluorapatite was added to the pre-formed membrane. The mineralized structures nucleated from the protein spherulites, and mineralization nucleation was enhanced in the presence of acidic amino acids, compared to when the acidic region was omitted from the protein. The mineralized structures, which show similar crystalline XRD pattern to that of fluorapatite, grow radially and reach 1 mm in diameter and tens of microns in height. Scanning electron microscopy (SEM) analysis revealed that the ELR spherulites templated the formation of fluorapatite nanocrystals as well as vertically aligned nanocrystals on the membrane surface. Density-dependent colour SEM, which enable topographical and density measurements together with energy dispersive X-ray (EDX) spectroscopy, showed a thin, low-density material associated with the organic phase surrounded by high-density inorganic phase, indicating organic-inorganic interaction. High

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ELP	Repeating sequence	X	N
E1	VPGXG	V	160
E2	VPGXG	$V_{50\%}$, $A_{50\%}$	80
E3	$(VPGVG)_{10}GKG_8$	$V_{80\%}$, $A_{20\%}$	80
E4	VPGXG	A	80

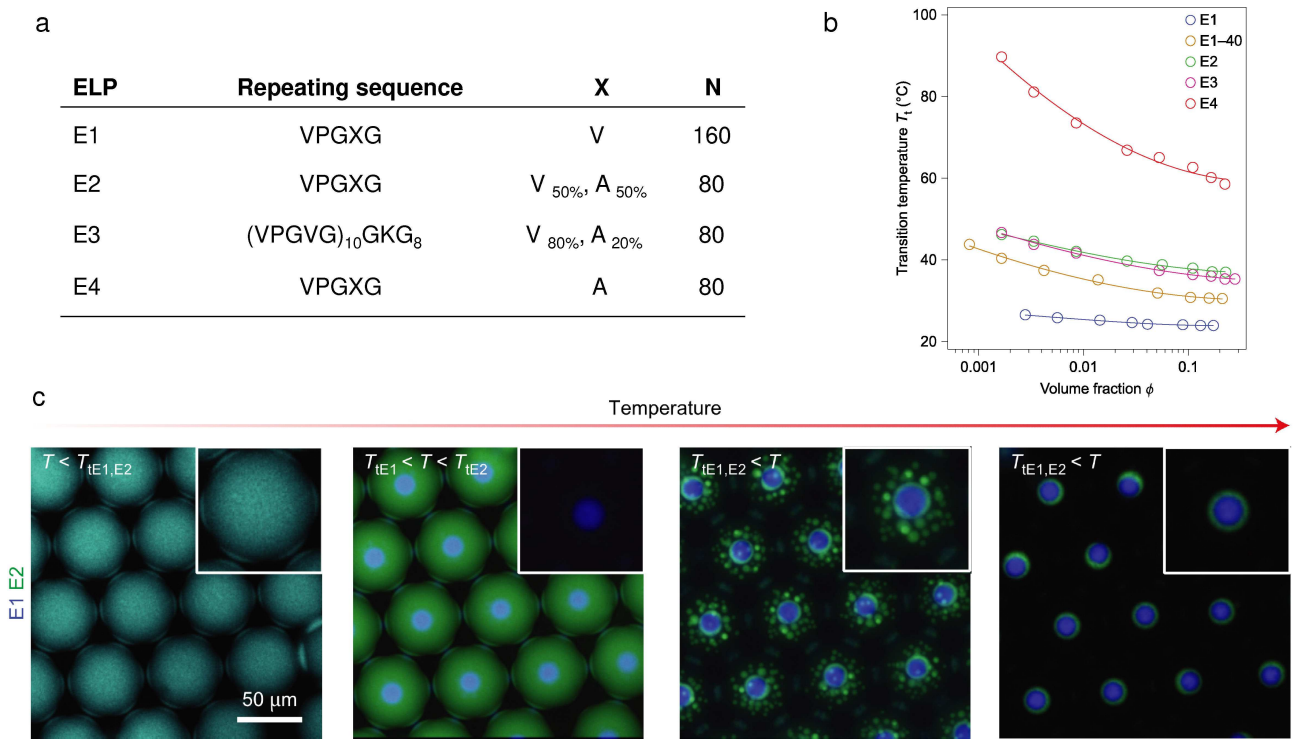

Figure 2. Programmable intrinsically disordered protein coacervates. a. Sequences of the designed elastin-like proteins, where N represents the number of pentamer repeats. **b**. Transition temperature T_t as a function of volume fraction (ϕ), solution volume fraction occupied by each of the ELP chains. E1-40 refers to an ELP containing 40 repeats of E1. **c**. Fluorescence microscopy images of double layered coacervates formed upon temperature increase by E1 (blue labeled) and E2 (green labeled). Reproduced from ref. 9 with permission from Springer Nature.

resolution transmission electron microscopy (HR-TEM) further confirmed that the ELR spherulites within the membrane template apatite crystal growth into hierarchical mineralized structures.

Next, the authors tuned the level of ELR intramolecular order from a β-sheet to a random coil conformation by modifying the cross-linker concentration and utilized the varying level of order to direct mineralization. Interestingly, increasing the amount of disordered (random coil) conformation compared to ordered β-sheet resulted in increase in spherulite structure formation and in turn in a change to the microscale geometry of mineralized structures, from concentric rings to radial prisms (Figure 3b). Moreover, tuning the ELR level of intramolecular order, by applying different cross-linker concentrations, enabled control over the mechanical properties of the mineralized material. Thus, higher Young's modulus (E) and hardness (H) were observed by structures grown within softer membranes (Figure 3b), that might arise from the ability of the nanocrystals to grow closer or denser in a softer membrane. The proposed mechanism of biomineralization involves a preliminary step of protein assembly where drying and evaporation of the organic solvent promoting ordered β-sheet conformation that in turn facilitate fibrils formation, while crosslinking promoting disordered random coil conformation which facilitates spherulites formation. The spherulites serve as a nucleation site for calcium phosphate ions to grow nanocrystals and eventually mineralized spherulites. Thus, this work shows how the interplay between intra- and supramolecular order and disorder can be leveraged to control templated mineralization processes.

An example of a hybrid protein-peptide system was reported by the Mata group, composed of an ELP and a peptide amphiphile (PA).[13] The ELP contains hydrophobic blocks of the pentamer consensus sequence (as described above), which facilitates coacervation and supramolecular disorder, whereas the PA, a nonapeptide attached to a C_{16} alkyl tail (Figure 4a), forms ordered β-sheet nanofibers upon charge screening.[14] Adding the positively charged peptide PAK3 to the negatively charged ELP5 and above ELP5 *T*t results in interfacial co-assembly into a nanofibrous multi-layered membrane that entraps the PAK3 solution inside and is surrounded by ELP5 solution (Figure 4b). The membrane can be easily disassembled and it can grow in an anisotropic manner by simply pinching and extending it (Figure 4c). Time-of-flight secondary-ion mass spectrometry (ToF-SIMS) showed that the outer surface of the membrane is composed of ELP5 and the inner surface mostly contains PAK3. Substituting the three lysines of PAK3 with glutamic acid resulted in no interaction with ELP5, demonstrating that electrostatic interaction between the protein and peptide are critical for membrane assembly. Notably, PAK3 was previously reported to form membranes and sacs upon complexation with a

a ELR Sequence

$[[(VPGIG)_2(VPGKG)(VPGIG)_2]_2$ DDDEEKFLRRIGRFG $[(VPGIG)_2(VPGKG)(VPGIG)_2]_2]_3$

b

ELR cross-links	0.25	0.5–1	3–6	12
Random : β ratio (Disorder : order)	0.26 ± 0.06	0.44 ± 0.02	0.87 ± 0.03	1.05 ± 0.17
ELR spherulite morphology				
No. of ELR spherulites	x	0.17 sp/mm^2	3.24 sp/mm^2	5.38 sp/mm^2
ELR stiffness	4.5 ± 0.8 kPa	48.3 ± 8.3 kPa	13.7 ± 5.7 MPa	11.4 ± 3.6 MPa
Apatite hierarchical structures	X	Rings	Mixture	Prisms

Figure 3. Templating and tuning mineralization by ELR spherulites. a. ELR sequences. Lysine is colored blue and acidic amino acids red. **b.** Summarizing table showing the tuneability of ELR levels of order (ratio between disordered random coil to ordered β-sheet), polarized light microscope (PLM) images of the resulting spherulite structures, number of spherulites and their measured stiffness, as well as SEM images of mineralized structures grown on spherulites. Scale bars: 3 μm (ELR spherulite morphology) and 20 μm (apatite hierarchical structures). Reproduced from ref. 11 with permission from Springer Nature.

negatively charged polysaccharide, formed upon liquid-liquid contact and physical separation of both components where the PA self-assembles at the interface of the sacs by charge screening.[15]

Further experiments showed that a specific combination of oppositely charge density of ELP/PA is required to form robust membranes. Thus, reducing the charge density of PAK3 by omitting one lysine resulted in formation of a thin and transparent ELP5/PA membrane that shrank over time and could not be dynamically manipulated. Increasing PAK3 charge density by adding one lysine resulted in formation of a weak and loose membrane upon mixing in ELP5 solution.

In addition to electrostatic interactions, the ELP and PA also interact through hydrophobic interactions. ELPs undergo conformational changes above their *T*t, due to dehydration of their hydrophobic domains, leading to exposure of these domains enabling their interaction with the hydrophobic domain of PA. Indeed, only disordered aggregates could be formed below the *T*t of ELP5, and cooling the temperature of pre-formed membrane resulted in its shrinking, indicating that ELP5 conformational change and dehydration of its hydrophobic domains are critical for the interaction with the PA. Furthermore, small angle x-ray scattering (SAXS) measurements showed that the interaction with PAK3, and specifically its self-assembly, is required for ELP5 conformational change and hydrophobic domains opening above its *T*t.

Thus, cooperative assembly of charge (mis-) matched peptides and biopolymers can give rise to materials with mechanical properties that can be dynamically regulated, while maintaining exceptional mechanical strength.

2.2 Biomimetic Protein Assemblies

Various marine organisms including tubeworms, squids and mussels produce biomaterials that have outstanding adhesive and mechanical properties. The formation of these materials is mediated by complex coacervation of adhesive protein building blocks,[16] in particular to promote surface adhesion.

Waite and co-workers[17] reported that the squid beak is made of two families of proteins: chitin-binding proteins and histidine-rich proteins. It was previously suggested that squid beak proteins can form complex coacervates. To study the mechanism of assembly, the authors cloned, recombinantly expressed and purified one of these His-rich proteins. The protein contains repeats of the pentapeptide GHGXY, where X

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Sequence		pI	ζ (mv)
ELP5	MESLLP-[(VPGVG VPGVG VPG**E**G VPGVG VPGVG)$_{10}$(VGIPG)$_{60}$]$_2$-[(VPGIG)$_{10}$-AVTGRGDSPASS(VPGIG)$_{10}$]$_2$-V	3.4	-30.5±4.0
PAK3	$C_{15}H_{31}$CONH-VVVAAA**KKK**-CONH$_2$	10.3	67.5±3.3

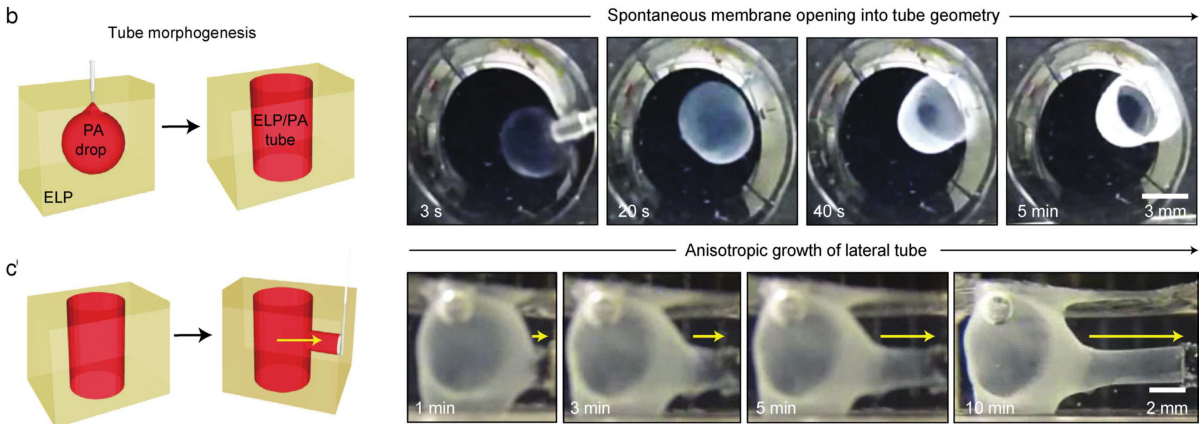

Figure 4. Interfacial membrane formed by protein-peptide co-assembly. a. Sequences of elastic-like polypeptide (ELP5) and peptide amphiphile (PAK3). **b**. Schematic illustration and time-lapsed images of ELP/PA immersion and rapid membrane (tube) assembly. **c**. ELP5/PAK3 assemble into robust membranes that can be grown on demand by simply touching and displacing the membrane. Reproduced from ref. 13 with permission from Springer Nature.

is often valine or leucine, which shares sequence similarity with elastin repeats discussed in above. These proteins are known to drive coacervation through hydrophobic interactions. The recombinant beak protein spontaneously formed coacervates upon addition of phosphate buffer (100 mM). CD analysis showed that the protein adopts a helical conformation in the condensed coacervate phase. However, dispersion of the coacervates resulted in a transition to a β-sheet conformation. Next, the authors designed a 25-mer peptide based on the beak proteins consensus domain. The peptide ((GHGVY)$_2$-(GHGPY)$_2$-GHGLY)) was found to self-coacervate in the presence of 0.5 M ionic strength. Interestingly, upon adjustment of the ionic strength of 1 M, the peptide transitioned from a solution to solid-like structures to coacervates (from pathways 2 to 1, Figure 7).[17] This work therefore demonstrates that biomaterials can easily reconfigure from ordered to disordered assemblies by responding to reaction conditions.

Inspired by the adhesive properties of mussel proteins, Tirrell and Waite designed adhesive biomaterials that are formed by protein-polysaccharide complex coacervation. They used a recombinant mussel adhesive protein fused to RGD as an anionic building block and hyaluronic acid (HA), one of the main components of the ECM, as the cationic building block for electrostatically driven complexation.[18] By using a low pH which is found in between the protein (2.5) and HA (9.3) isoelectric points, the two components interact to form coacervates.

The adhesion properties of marine mussel proteins are thought to be mediated by coacervation that is not driven by electrostatic interactions, as these proteins are cationic (and no anionic proteins are present) and contain high prevalence of aromatic amino acids. Thus, cation-π interactions were suggested as the driving force for this like-charge coacervation. Inspired by this unique mode of coacervation, Hwang and co-workers have designed materials formed by assembly and coacervation of two positively charged building blocks.[19] They used the recombinant mussel foot protein-1 (Rmfp-1), composed of 12-repetition of the decapeptide (AKPSYPP-TYK) and the polyelectrolyte poly(2-(trimethylamino)ethyl methacrylate). Rmfp-1 is rich in cationic and aromatic amino acids (20 mol% tyrosine and 20 mol% lysine) and the trimethylammonium group of the polyelectrolyte is predicted to form strong cation–π interactions with the protein lysines. By keeping the reaction conditions at pH 3, the cationic building blocks assemble into coacervates that resemble those formed by oppositely charged polyelectrolytes, yet they have lower density. Raman spectroscopy analysis shed light on the coacervation mechanism, showing enhancement of the tyrosine ring stretching vibrations and ring-O stretching vibration, indicating that coacervation is mediated by cation-π interactions between the phenol tyrosine of the protein and the cationic groups of the polyelectrolyte. The cationic protein alone self-assembles into bundles only upon charge screening by salt addition. The authors suggested that the short range cation-π attraction, which is stronger between the protein and polyelectrolyte compared to the protein alone, is strong enough to exceed the electrostatic repulsion between the two, resulting in coacervate formation. [19]

A clear trend is emerging from the protein-focused studies discussed thus far (Table 1), showing a critical role for the

Table 1. Summary of ordered-disordered protein- and peptide-based building blocks.

Reference	Building blocks	Conformation	Assembly type	Assembly trigger	Interactions
Chilkoti and co-workers[8]	Polypeptides	Disordered α-helix	Coacervates Solid-like structures	Thermal Thermal	Hydrophobic H-bonding
Lopez and co-workers[9]	Polypeptides	Disordered	Coacervates	Thermal	Hydrophobic
Mata and co-workers[13]	Protein-peptide	Disordered* (protein) β-sheet (peptide)	Membrane	Thermal	Electrostatic; hydrophobic
Mata and co-workers[11]	Polypeptide	Random coil β-sheet	3D spherulites Fibrillar	Organic solvent; crosslinker	Electrostatic; hydrophobic
Tirrell & Waite[18]	Protein-polysaccharide	Not reported	Coacervates	Low pH	Electrostatic
Hwang and co-workers[19]	Protein-polyelectrolyte	Not reported	Coacervates	Low pH	Cation–π
Waite and co-workers[17]	Protein Peptide	α-helix Not reported	Coacervates Coacervates Solid-like structures	Ionic strength 0.5 M ionic strength 1 M ionic strength	Hydrophobic Hydrophobic
Tirrell and co-workers[22]	Peptides	Random coil β-sheet	Coacervates Solid precipitates	Ionic strength	Electrostatic H-bonding
Mann and co-workers[23]	Peptide-nucleotides	α-helix	Coacervates	Spontaneous, pH 8	Electrostatic
Mann and co-workers[24]	Peptide-polyelectrolyte	Non	Coacervates Nanofibers, hydrogel	pH 8 pH 4.5	Electrostatic Hydrophobic; H-bonding
Keating and co-workers[25]	Peptide-RNA	Non	Coacervates	Phosphorylation	Electrostatic
Ulijn and co-workers[21]	Peptides	Syn Anti	Aggregates; fibers Nanofibrils; crystals	Thermal annealing	H-bonding; electrostatic; π-stacking
Stupp and co-workers[26]	Peptides	Random coil β-sheet**	Short fibers Long fibers	Ionic strength; Thermal annealing	H-bonding; electrostatic

* A conformational change of the protein is triggered upon complexation with the peptide, although there is no indication for a defined conformation (helical or β-sheet). **Refers to intermolecular structure.

proteins' intramolecular order and nature of the adopted conformation to the level of entropy in the system and to the fate of resulting assemblies. The possible assembly pathways for a given protein conformation are summarized in Figure 7. The emerging relationship between protein conformation and assembly pathway is as follows: (i) a random coil conformation promotes formation of dynamic assemblies (pathway 1, Figure 7) that might occur through coacervation,[8,9,17,19] which is driven by either (or combination of) electrostatic,[8] hydrophobic,[9,13] or cation-π[19] interactions; (ii) an adopted helical or β-sheet secondary structure formed by intramolecular H-bonding facilitates formation of intermolecular interactions, resulting in either kinetically trapped (pathway 2, Figure 7) or thermodynamically favourable crystalline assemblies[8] (pathway 3, Figure 7). The dynamic assemblies in pathway 1 are thermodynamically metastable and can spontaneously transition into kinetically trapped structures[8,11,17] with a deeper thermodynamic well, while encounter a higher energetic barrier in transitioning to pathway 3. Notably, in this review we have not included a discussion on protein liquid crystals, another related type of dynamic assemblies. For an extensive discussion on protein liquid crystals we refer the reader to a review article by Knight and Vollrath.[20]

Thus, protein building blocks that are designed for assembly pathway 1 should contain disordered low complexity domains that promote phase separation, ionic amino acid blocks to promote electrostatic interactions, or aliphatic amino acids to promote hydrophobic interactions. Accordingly, proteins designed for pathways 2 or 3 should include amino acids that promote a specific secondary structure including alanine and asparagine (α-helix) or valine, threonine and isoleucine (β-sheet), as well as aromatic amino acids to promote stacking and cation-π interactions, when found in proximity to basic amino acids. Distinguishing between pathway 2 and 3 by design is not trivial and depends on reaction conditions (temperature, pH, ionic strength) in addition to the molecular composition of the building block, yet, this transition can be obtained by overcoming the energy barrier using thermal annealing (as will be discussed in section 2.2).

3. Peptide-Based Biomaterials

The biological systems discussed so far typically include designed protein (repeat) sequences of considerable length and structural complexity. In contrast, oligopeptides and especially short peptides, which are popular building blocks for self-assembly,[1,2] do not easily adopt intramolecular secondary structures (instead relying on intermolecular interactions). Moreover, in certain supramolecular short peptide systems, the chemical nature and position of each amino acid plays a critical role in formation of intermolecular interactions and the consequent overall supramolecular structure.[1,21] Therefore, peptides offer significant opportunities for systematic study of ordered-disordered supramolecular materials and for sequence-structure relations to be established. While the studies discussed in the previous section were focused predominantly on intramolecular order/disorder and its impact on assembly pathways, herein, intermolecular order/disorder and its effect on the resulting structure and materials properties will be mainly discussed.

3.1 Dynamic Peptide Assemblies

Tirrell and co-workers[22] have demonstrated the contribution of hydrogen bonding in driving the formation of ordered peptide assemblies (pathway 3, Figure 6) versus disordered peptide coacervates. They studied the effect of ionic polypeptides' chirality on assembly into ordered/disordered complexes. The complexation of polylysine (pK) and polyglutamic acid (pE) with varying MW (9 Da to 24 KDa for pK and 7 Da to 14 KDa for pE) with different stereochemistry was analysed. Upon complexation, the two peptides form either liquid coacervates or solid amorphous precipitates, in the presence of various salt concentrations. When a racemic polypeptide was used in combination with oppositely charged polypeptides, coacervates exclusively formed, indicating a clear role of backbone stereochemistry. FTIR analysis suggested a random coil conformation for polypeptide complexes forming coacervates while β-strands where observed for solid precipitates. In addition, the solid precipitates dissociated in the presence of urea, known to disrupt hydrogen bonding, whereas coacervates' stability was entirely dependent on salt concentration. These findings demonstrate that the formation of solid precipitates involve hydrogen bonding and (pathway 3, Figure 7) that of liquid coacervates dominate by electrostatic interactions (pathway 1, Figure 7). The authors suggested that the polyelectrolyte chirality dictates neutralization of charge, which in turn enables both secondary structure hydrogen bonding (α-helix or β-sheet) and intermolecular hydrogen bonding. Molecular dynamics simulations showed that the homochiral combination pLK + pLE formed an electrostatic complex in the presence of NaCl, where the polypeptides adopt a disordered structure. The complex subsequently transitioned into a parallel β-sheet compact structure, which stayed stable throughout the simulation and showed high levels of peptide-peptide hydrogen bonding. In contrast, the complex pLK + p(L,D)E, experimentally forming liquid coacervates, showed a less compact structure where the polypeptides remained mainly unstructured, had preferential interaction with water, and very few intermolecular hydrogen bonding.[22] This work opens opportunities to manipulate materials properties from dynamic liquid droplets to solid-like structures by controlling building blocks chirality rather than their chemical composition.

Mann and co-workers reported on a number of peptide-based systems that employ intermolecular disorder to form dynamic functional compartments. Their early work from 2011[23] provided the first demonstration of oligopeptides and nucleotides co-assembly into dynamic droplets. They obtained a set of spherical compartments formed by liquid-liquid phase separation triggered by complexation of cationic oligolysines (2–10 or 5—24 mer) or polylysine (~115 mer) and anionic nucleotides. Assembly of oligolysine and ATP resulted in small microdroplets that dynamically assemble and disassemble upon thermal cycles. In contrast, droplets formed by polylysines/ATP were less stable as they coalesce or sediment. Interestingly, formation of polylysine-ATP coacervate microdroplets was found to be mediated by conformational transition of the polylysine from random coil to α-helix, due to charge screening of the lysine side chains by ATP. The microdroplets were used to sequester organic dyes, porphyrins, nanoparticles, a protein and enzymes.

The Mann group has also reported on the simplest aromatic peptide amphiphile thus far able to form microdroplet coacervates by complexation with poly(diallyldimethylammonium chloride) (PDDA).[24] At pH 8, the protected dipeptide *N*-(fluorenyl-9-methoxy-carbonyl)-D-Ala-D-Ala (Fmoc-AA) formed coacervates through electrostatic interactions between its carboxylic acid group and the cationic PDDA. Microdroplet coarsening was observed upon protonation of Fmoc-AA by slow pH decrease to 4.5 with glucono-d-lactone (GDL), as the peptide self-assemble into nanofibers (Figure 5a). By using confocal fluorescence microscopy and labelling the PDDA with rhodamine B isothiocyanate (RITC), the authors observed that PDDA is mainly located in the centre of the droplets during fibrils growth. The fibrils grow from the microdroplets, which gradually transition into a peptide-PDDA self-supporting hydrogel (transition from pathway 1 to 2, Figure 7). Droplet re-assembly from the hydrogel could be achieved by Fmoc-AA deprotonation with pH increase (Figure 5b), which promotes electrostatic repulsion between the peptide molecules, resulting in fibres disassembly, and triggering electrostatic interactions with the cationic PDDA. Thus, this work demonstrates a pH dependence transition between two types of materials: dynamic liquid droplets and fibrils containing hydrogel, formed by simple polymer-dipeptide building blocks.[24]

A different approach to controlling peptide charge and consequently electrostatic interactions with RNA, resulting in coacervates formation, was reported by Keating and co-workers.[25] The cationic peptide RRASLRRASL was selected

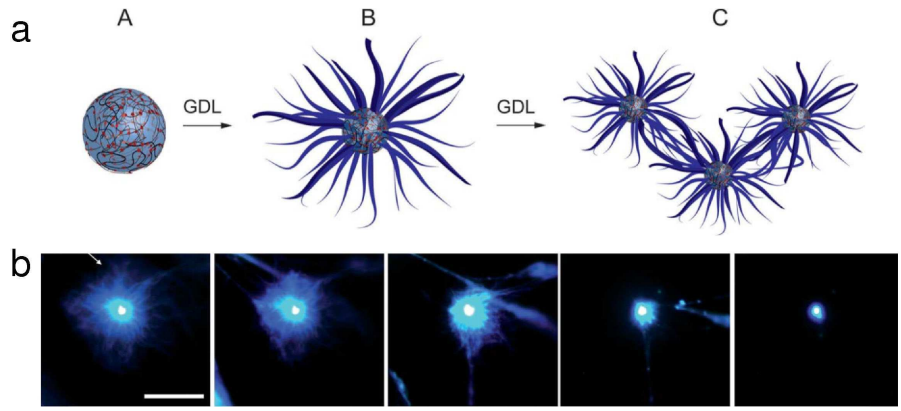

Figure 5. Short peptide-polymers pH responsive system transitioning from coacervates to nanofibrillar hydrogel. a. Schematic representation of coacervates (A) forming by complexation of Fmoc-AA and PDDA and emergence of nanofibrils (B) forming a hydrogel (C) upon a gradual pH decrease. **b**. Time dependent epifluorescence images recorded at 10 sec intervals showing nanofibrils disassembly and re-formation of a coacervate upon pH increase by sodium hydroxide. Arrow indicates the direction of sodium hydroxide gradient. Scale bar = 50 μm. Reproduced from ref. 24 with permission from The Royal Society of Chemistry.

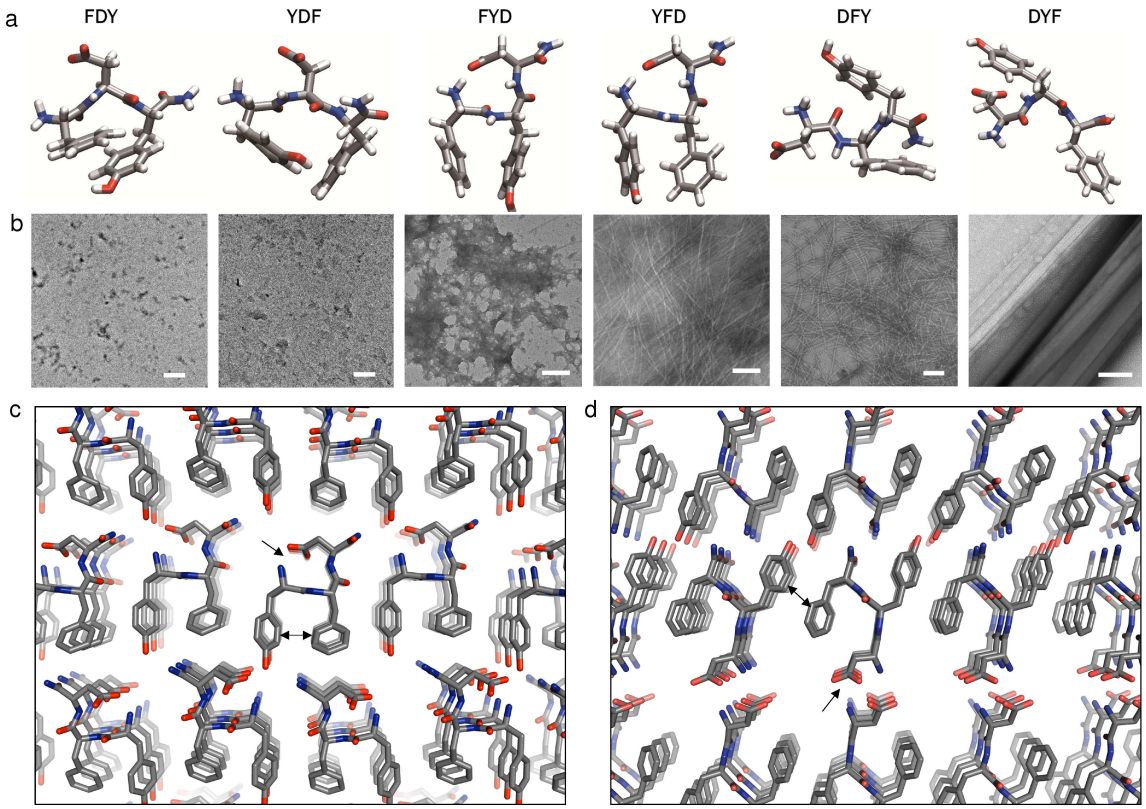

Figure 6. Tripeptide sequence and conformation control of supramolecular order. a. Preferred conformation of tripeptides in solution based on atomistic MD simulations. A syn conformation is presented by FYD and YFD and an anti conformation by DFY and DYF. **b**. TEM images of structures forming by the tripeptides self-assembly showing different levels of supramolecular order. Scale bars = 100 nm. **c–d**. Single crystal x-ray diffraction structures of YFD (c) and DYF (d). Arrows in indicate intramolecular (for YFD, c) vs. intermolecular (for DYF, d) salt bridge and π-stacking interactions. Reproduced from ref. 21 with permission from the American Association for the Advancement of Science.

as a building block, inspired by LRRASLG, the model synthetic substrate for protein kinase A (PKA), and poly uracil (polyU; MW = 600–1,000 kDa) or transfer RNA (tRNA) were used as anionic building blocks. As expected, interaction between the cationic peptide and polyU was found to be mediated by electrostatic interactions, as charge screening by

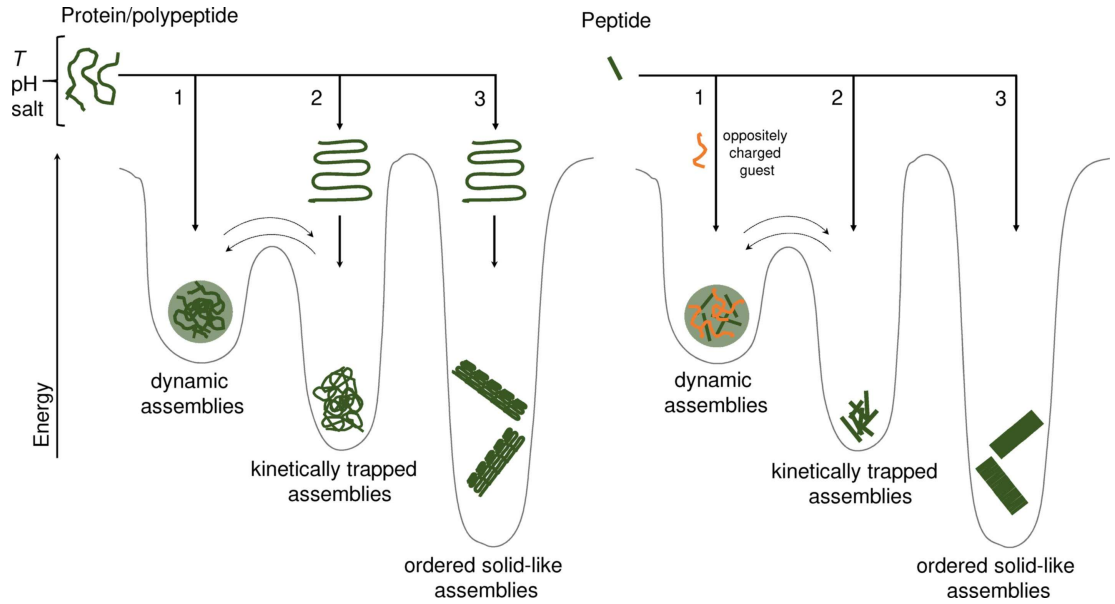

Figure 7. Schematic summary of the different assembly pathways of proteins/polypeptides (left) or peptides (right) and their energy landscape.

addition of NaCl resulted in droplets disassembly. Increasing peptide chain to triple RRASL pentamer repeats (15-mer) decreased the critical coacervate concentration (CCC) and increased the stability of formed droplets against high NaCl concentrations, due to increase in the number of ionic groups.

Controlling peptide charge by phosphorylation, either on a single serine (resulting in a +2 net charge) or on both serines (no charge), resulted in coacervation arrest, indicating that this RNA-peptide co-assembly system is mainly driven by charge. A control over coacervates formation could be achieved by using the enzyme lambda protein phosphatase, which cleaved the phosphate groups from the peptide, as the trigger. Similarly, coacervates dissolution was demonstrated by peptide phosphorylation using protein kinase A and ATP as a substrate. Reversible formation and disassembly of coacervates was demonstrated by utilizing both phosphatase and kinase in the double-phosphorylated peptide to trigger coacervates assembly and thereafter coacervates dissolution by kinase addition.

3.2 Kinetically Trapped and Solid-Like Peptide Assemblies

Stupp and co-workers have studied the link between function and energy landscape of PA.[26] They designed a building block containing the peptide sequence $V_3A_3K_3$ conjugated to 16-carbon alkyl chain at the N-terminus (also used in previously discussed studies[13,15]). The two main driving forces for the self-assembly of the PA are hydrogen bonding of the peptide backbone to form β-sheets and electrostatic repulsion by the lysine amines. The competition between these two driving forces was studied by applying different ionic strength. Below the critical ionic strength (I_c, 6 mM), the PAs self-assemble into short fibres with a polydisperse length distribution and a random coil secondary structure. Thermal annealing of these fibres resulted in a short monodisperse length and retention of a random coil structure, suggesting that they are the thermodynamically favoured product, representing the deepest energy well (pathway 3, Figure 7). A second type of fibres that are long and have a β-sheet structure are formed by tuning the ionic strength from $I_{>}I_c$ to $I_{<}I_c$. These fibres are kinetically trapped and found in a higher energy well (pathway 2, Figure 7). Reannealing of these fibres resulted in transition into the short thermodynamically stable random coil fibres, with a calculated energy barrier of 171 kJ mol^{-}.[1] Above the I_c, polydisperse β-sheet fibres are formed. These fibres lose their β-sheet structure upon heating and recover it upon cooling and increase in their length. At this ionic strength, metastable fibres that are short and have a β-sheet structure are formed by increasing the ionic strength of short monodisperse random coil fibres from $I<I_c$ to $I>I_c$ by salt addition. Thus, a control over the structure and length of the PA fibres could be achieved by regulating two conditions: (i) thermal energy of the reaction enables a control over PA fibres length; (ii) the ionic strength of the reaction and thus charge screening of the PA enables a control over hydrogen bonding and β-sheet vs. random coil structures formation.

While the role of proteins and polypeptides secondary structure in self-assembly is well studied, the relationships between peptide conformation, especially that of peptides that are too short to adopt a helical or β-sheet conformation, and the resulting assembled structures are not fully understood. We have recently studied the relationships between peptide sequence and adopted conformation and its contribution to

molecular, and supramolecular order.[21] We used sequence isomers of tripeptides containing the aromatic amino acids phenylalanine (F) and tyrosine (Y) and the acidic aspartic acid (D), where C-terminal amides were used to promote assembly at neutral pH. A clear sequence-dependency was found for the level of supramolecular order. First, the proximity of the aromatic amino acids position is critical for supramolecular disorder, when the aromatics Y and F are found in an unpaired position, the preferred dihedral angle is ~90 °C (Figure 6a), restricting the formation of aromatic stacking and resulting in a complete disorder (Figure 6b). When D is at the C-terminal position, the tripeptides are in a *'syn'* conformation (Figure 6a), resulting in an intramolecular salt bridge and π-stacking between adjacent aromatic residues as shown by single crystal x-ray diffraction (XRD) (Figure 6c). This compact conformation results in disordered aggregates by FYD and opaque hydrogel containing nanofibers and aggregates by YFD (Figure 6b). When the D is at the N-terminal position (DFY and DYF), the preferred conformation is '*anti*' (Figure 6a), promoting formation of well-ordered intermolecular interactions network (Figure 6d), resulting in extended nanofibrils assembly by DFY and crystals by DYF (Figure 6b). To the best of our knowledge, this study provides the first evidence of a control over supramolecular order-disorder by tripeptide sequence.[21]

We leveraged the tripeptides' different level of supramolecular order and their ability to pre-organize tyrosine in a differential manner to construct melanin-like materials, by using the assembled structures as templated substrates for enzymatic oxidation. Oxidation of the phenol side chain within the assemblies induced supramolecular disorder and emergence of new properties by tripeptide crosslinking, including broad UV-Vis absorption and micron-sized pigment particles, that are encoded by the peptide substrate sequence.[21] The highest levels of morphology control, storage capacity and conversion yields resulted from pigments formed by ordered assemblies (DFY and DYF. Wide angle XRD analysis showed that these peptide polymers retain the d-spacing typical for β-sheets, thus demonstrating a 'controlled disorder' and suggesting the role of supramolecular order in templating the catalytic process.

To summarize, the above studies present a variety of design principles to control the assembly pathway, thermodynamic well and consequently properties of peptide materials (Table 1). Since peptides are inherently simpler than proteins as building blocks, their chemical composition,[21] chirality[22] and charge[25] can be easily manipulated, which, in addition to reaction conditions,[26] dictate the level of their supramolecular order and disorder. However, unlike proteins that present high multivalency, formation of dynamic peptide assemblies, *i.e.* coacervates, requires complexation with an oppositely-charged guest molecule (pathway 1, right panel Figure 7) including an oppositely charged peptides[22] polyeletrolytes[24] or nucleotides.[23,25] Formation of thermodynamically favourable solid-like assemblies (pathway 3) can be achieved through a control of reaction conditions (thermal energy, ionic strength)[26] or by applying sequence design tools[21] to promote formation of multiple intermolecular interactions that increase the level of supramolecular order. It is clear that there is much scope in combining peptides and biomacromolecules to achieve dynamic functions by design, by taking advantage of aromatic, cation-π, electrostatic and hydrophobic interactions.

4. Outlook

While peptides and proteins have been employed as materials building blocks for decades,[27,28] these are typically designed as ordered, thermodynamic stable assemblies (deep well) with a desired functionality, whereas incorporating elements of intramolecular and supramolecular order/disorder has only recently been reported. The studies presented here show that intra- and supramolecular disorder can be controlled and designed for specific function, including formation of mineralized scaffolds,[11] encapsulation and delivery of biomolecules,[23] formation of pigments,[21] and cell matrices.[26]

This approach opens opportunities for designing adaptive materials with wider range of properties than that of conventional ones (based on ordered static structures), as the disordered dynamic assemblies they rely on respond to external triggers[24–26] and can easily transition into more ordered static structures. Yet, as this is an emerging and relatively young field, there is still a need in gaining more insights into the chemical principles underlying dynamic disordered structures, especially those formed by peptides, to enable the design of building blocks from first principles.

Acknowledgements

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