Published in final edited form as:

Acta Biomater. 2009 March ; 5(3): 805–816. doi:10.1016/j.actbio.2008.10.001.

Peptide-directed self-assembly of hydrogels

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Abstract

This review focuses on the self-assembly of macromolecules mediated by the biorecognition of peptide/protein domains. Structures forming α -helices and β -sheets have been used to mediate self-assembly into hydrogels of peptides, reactive copolymers and peptide motifs, block copolymers, and graft copolymers. Structural factors governing the self-assembly of these molecules into precisely defined three-dimensional structures (hydrogels) are reviewed. The incorporation of peptide motifs into hybrid systems, composed of synthetic and natural macromolecules, enhances design opportunities for new biomaterials when compared to individual components.

Keywords

Hydrogels; Self-assembly; Hybrid biomaterials; Peptides; Proteins; Coiled-coils; Graft copolymers; Block copolymers

1. Introduction

Hydrogels are hydrophilic polymers that can retain a significant amount of water while maintaining a distinct three-dimensional structure [1–3]. Based on the nature of the crosslinking, hydrogels can be categorized as chemical (covalent) or physical gels. The structure and properties of the primary chains, as well as the crosslinking density, contribute to the overall properties of chemical hydrogels. Physical gels are networks held together by molecular entanglement and/or secondary molecular interactions. These interactions can be disrupted by changes in the environment, such as temperature, pH, ionic strength, presence of specific solutes and stress; consequently, the formation of physical hydrogels may be reversible. Traditional synthetic methods, such as crosslinking copolymerization, crosslinking of polymeric precursors and polymer–polymer reactions, have produced numerous hydrogel materials with excellent properties [4,5]. However, these approaches do not permit a precise control of chain length, sequence and three-dimensional (3-D) structure. In contrast, self-assembly of macromolecules into physical hydrogels has the potential to produce precisely defined, hierarchical 3-D structures [2,3,6,7]. Self-assembly is highly specific, and is frequently found in biology in both the peptide/protein and DNA worlds. Self-assembly of proteins and peptides (within the scope of this review) has generated most of the structural and functional biological systems, such as actin filaments in the cytoskeleton, microtubules and the fibrous protein components of the extracellular matrix. The distinctive features of peptide/protein self-

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Part of the Self-Assembling Biomaterials Special Issue, edited by William L. Murphy and Joel H. Collier.

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assembly are: (i) high specificity, mediated by molecular recognition and binding among polypeptide building blocks; (ii) all the information needed for the folding and function of a polypeptide is usually encoded in the primary structure [8]; and (iii) precise dynamic control of the material's structure and properties due to the reversibility of the assembly and disassembly processes.

The incorporation of peptide motifs into hybrid systems composed of synthetic and natural macromolecules enhances design opportunities when compared to individual components. The design of hybrid materials augments the control over nanostructure formation and permits the formation of hierarchically organized materials [9]. The fact that properties of a peptide domain can be imposed on the whole hybrid hydrogel [10] suggests that the supramolecular organization of hybrid materials is driven mainly by the biorecognition of peptide segments. This suggests that the understanding of self-assembly processes will undoubtedly contribute to a better understanding of the processes occurring in nature [11].

Structures forming α -helices and β -sheets have been used to mediate self-assembly into hydrogels of peptides [12–14], reactive copolymers and peptide motifs [10,15,16], block copolymers [17–20] and graft copolymers [21–25]. In the present article we review these processes.

2. Self-assembling peptides

Peptides can spontaneously self-assemble into nanospheres, nanotubes, nanotapes, nanofibrils and hydrogels [26,27]. The structural factors decisive for the final shape of the self-assembling peptides have been analyzed by Zhang [28]. A typical design incorporates complementary ionic sites with regular repeats on the hydrophilic surface. The positively and negatively charged amino acid residues are alternating as unimers (+-+-), dimers (++--++--), trimers or tetramers. The presence of complementary groups within each peptide molecule governs the self-assembly [12,29]. For example, an ionic self-complementary peptide RADARADARADADA (RADA16-I) forms a stable β -sheet structure. It self-assembles into nanofibers and ultimately into a hydrogel that consists of >95% water. The reversibility of self-assembly may be employed to produce well-defined 3-D structures in systems composed from more than one component. Yokoi et al. [29] demonstrated that a homogeneous distribution of biological molecules within the peptide scaffold can be achieved by reassembling the system following disassembly by sonication.

Yu et al. [14,30] used a system composed from a pair of oppositely charged peptides. The modular design strategy based on mutual attraction but self-repulsion prevents spontaneous gelation or aggregation. Gelation may be triggered by mixing two peptides (e.g. acetyl-WKVKVKVKVK-amide and acetyl-EWEVEVEVEV-amide) or by suppressing ionization by changes in pH and/or ionic strength. Similarly to other physically crosslinked hydrogels, these have limited inherent mechanical strength when exposed to shear stress. However, dynamic rheological characterization disclosed that these hydrogels were capable of rapid recoveries from repeated shear-induced breakdowns [14].

Messersmith and co-workers [31] evaluated a 16-amino-acid peptide, FEK-16 ($\text{H}_2\text{N}-(\text{FEFEFKFK})_2-\text{COOH}$), containing alternating hydrophobic and hydrophilic residues. Such peptides self-assemble into β -sheet structures, positioning all hydrophobic side chains on one side of the β -sheet and all hydrophilic side chains on the other [32]. This peptide self-assembled into highly crosslinked, fibrillar, β -sheet hydrogels at concentrations $>10 \text{ mg ml}^{-1}$ in the presence of mM concentrations of inorganic salts. Triggered self-assembly was achieved by control of salt release from liposomes [31].

Schneider et al. [33] developed a peptide system whose self-assembly can be triggered by conditions controlling the intramolecularly folded conformational state of the peptide. The intramolecular folding propensity of β -hairpins, a class of peptides, was used to be the driving force for self-assembly. The pH and temperature dependence of β -hairpin conformation imposed stimuli sensitivity and reversibility. Disassembly may be easily achieved by eliminating the folding stimulus (pH or temperature change). This results in unfolding and hydrogel dissolution. MAX1, a 20-residue peptide (VKVKVKVKV^DPPTKVVKVKV-NH₂) is composed of V and K residues, possessing high β -sheet propensity, flanking a tetrapeptide (V^DPPT) designed to adopt a turn structure [13]. The temperature at which the folding/self-assembly is triggered can be controlled by the hydrophobicity of the peptide. Whereas, at pH 9, MAX1 forms hydrogels at 25 °C, mutation V16T resulted in gelation of the peptide at 40 °C. Increasing the hydrophilicity of the peptide further by double mutation (V7T and V16T) resulted in a peptide whose gelation was triggered at 60 °C [34]. The evaluation of the gelation kinetics of MAX1 [35] and of the single mutant [36] determined the relationship between peptide concentration, pH, temperature and ionic strength on the one hand and the kinetics of self-assembly and structure of the hydrogel on the other.

Hydrogels from self-assembled peptides have a potential to serve as synthetic extracellular matrices. Frequently, however, their stiffness does not match that of the tissues to mimic. To enhance the hydrogel stiffness, Collier and co-workers [37] used native chemical ligation to produce peptide bonds between the termini of fibrillar β -sheet peptides, CQQKFQFQFEQQG-COSCH₂CH₂COOC₂H₅.

3. Poly(amino acid)-based hydrogels

Peptide and/or protein biorecognition blocks can be incorporated into synthetic poly(amino acid) with the aim of mediating the self-assembly of the macromolecules into defined 3-D structures [2,3,8]. Recognition motifs found in the nature, such as coiled-coils and β -sheets, have been employed as models for self-assembly.

The coiled-coil, a supercoil formed by two or more strands of α -helices [38–42], will be used as an example to demonstrate the potential for the design of well-organized hydrogel structures. The primary sequence of a typical coiled-coil is composed of 7-residue repeats, designated as heptads. The amino acid residues in a heptad are denoted as “a, b, c, d, e, f, g”. Hydrophobic residues at positions “a” and “d” form an inter-helical hydrophobic core, providing a stabilizing interface between the helices. Charged residues at positions “e” and “g” form electrostatic interactions, which contribute to coiled-coil stability and mediate specific association among helices. The versatility of this motif, especially the possibility of manipulating its stability and specificity by modifying the primary structure, bodes well for the successful design of a new class of hydrogel biomaterials [3].

The self-assembly of genetically engineered *ABA triblock copolymers* composed from a random coil block (B) flanked by two coiled-coil-forming blocks (A) has been intensively evaluated [17,19,43–45]. The self-assembly occurs as a balance between the oligomerization of the helical ends and swelling of the central water-soluble random coil segment. Consequently, temperature and/or pH-responsiveness may be achieved by manipulating the amino acid sequence of the coiled-coil domains. Minor modifications in their structure have a strong impact on the stimuli-sensitivity of self-assembled hydrogels. For example, the thermal stability of the coiled-coil containing proteins can be manipulated in a predictable way by substituting amino acids in the coiled-coil domain [19]. Intermolecular association of A blocks produces physical crosslinks in the hydrogel network. It has been shown, however, that the ABA chains have a strong tendency to form intramolecular loops, producing elastically ineffective chains [46]. The continuous rearrangement of A block associates results in the

erosion of the network from the surface. However, the loop formation can be easily avoided (and the erosion rate controlled) by designing ABC block copolymers, where A and C are coiled-coil-forming blocks which do not associate with each other [43].

Nowak et al. [18] designed and synthesized *amphiphilic diblock copolypeptides* with poly(L-lysine) or poly(L-glutamic acid) as the hydrophilic block and poly(L-leucine), poly(L-valine) or poly(D/L-leucine) as the hydrophobic block. They demonstrated that these low-molecular-weight diblock copolymers associate into hydrogels at very low polymer concentrations. In particular, the hydrogels maintain their mechanical strength up to high temperatures and rearrange rapidly after stress. Apparently, these copolymers employ a peptide gel assembly process not found in nature [47]. Amphiphilic block copolypeptides (such as K_xL_y) self-assemble into hydrogels via entirely hydrophobic α -helical domains. The α -helices associate perpendicular to fibril long dimensions and bear a resemblance to β -sheet fibrils (without the interstrand H-bonds). This arrangement results in a much higher stability at elevated temperatures and very fast recovery from shear thinning. However, due to their hydrophobic force-driven assembly, these hydrogel structures are probably less ordered than those held together by the formation of coiled-coils [47].

The gel-forming properties of *triblock amphiphilic copolymers*, $K_xL_yK_z$, were found to be similar to those of diblock copolymers. Evidently, both systems assemble in a similar manner. The triblock copolymers produced hydrogels of greater strength and enhanced salt tolerance when compared to diblock copolymers at comparable concentrations [48].

The design of protein-based hydrogels frequently reflects the structure of biological macromolecules. For example, *elastin- and silk-like polymers* are a class of widely studied self-assembling materials [49–51]. Elastin-based hydrogels [52–54] were designed and synthesized with a repeating pentapeptide sequence $(VPGVG)_m(VPGXG)_n$, where X may be any of the 20 naturally occurring amino acids. These hydrogels contract when temperature is raised above 25 °C. This phenomenon was termed an “inverse temperature transition”. At low temperature, the protein chains remain extended because of the surrounding pentagonal water cages. As the temperature increases, the water pentagons lose their structure and become bulk water, allowing the protein chains to fold properly into compact structures. The inverse transition temperature can be modulated by changing the protein composition, degree of ionization, pH, salt or phosphorylation [52]. Crosslinking of these polypeptide hydrogels by γ -irradiation enhanced their mechanical properties [55].

Tirrell and co-workers evaluated the mechanism of phase transition in an elastin-mimetic peptide, $MGLDGSMG(VPGIG)_{40}VPLE$. The temperature-responsive properties were a result of a mechanism involving conformational change, association and aggregation [56].

Manipulation of mechanical properties and phase transition can be also achieved by combination of several elastin-like structural motifs. Wright et al. [57] combined elastin-mimetic sequences, possessing different mechanical properties, into triblock copolymers. Following phase separation from aqueous solution, the copolymers form an elastic hydrogel. Redesigning these block copolymers to include larger endblock segments resulted in materials able to withstand significantly greater loads [58]. Incorporation of functional motifs into self-assembled elastin-like polypeptides does not impair the self-assembling properties. Elastin-like polypeptides containing the metal-binding polyhistidine sequence self-assembled into hydrogels and demonstrated a potential for heavy metal removal [59].

To achieve desired properties, protein-like polymers may be designed incorporating two structural motifs. For example, silk–elastin-like protein polymers have been designed [60] and self-assembled into hydrogels [61–67]. These protein polymers are composed of tandemly repeated silk-like blocks (GAGAGS) and elastin-like blocks (GVGV). The silk-like blocks

form hydrogen-bonded β -sheet crystals spontaneously, which impart thermal and chemical stability. The inclusion of elastin-like blocks decreases the crystallinity and increases the flexibility and water solubility of the copolymers. The introduction of an ionizable residue (glutamic acid) into the silk–elastin-like protein polymers increased the pH- and temperature sensitivity [65]. Evaluations on the swelling and transport properties of the silk–elastin-like hydrogels suggested that they have the potential to become controlled release bioactive materials [61,62].

4. Hybrid hydrogels

Hybrid hydrogels are usually referred to as hydrogel systems that possess components from at least two distinct classes of molecules, e.g. synthetic polymers and biological macromolecules, interconnected either covalently or non-covalently [68]. The combination of two types of structures may produce new materials that possess unprecedented levels of structural organization and novel properties. The peptide domain may impose a level of control over the structure formation at the nanometer level; the synthetic part may contribute to the biocompatibility of the hybrid material [9].

4.1 From hybrid block copolymers

Hybrid block copolymers can self-assemble into numerous nanostructured materials, such as vesicles, micelles, rod-like aggregates and hydrogels [69]. There is a vast literature on the self-assembly into micelles of hybrid block copolymers composed of PEG and poly(amino acid)s for anticancer drug and/or gene delivery [70]. These polymers and applications are beyond the scope of this review.

Diblock copolymers of PEG and β -sheet forming peptides have been studied extensively (reviewed in Refs. [9,11]). Different peptides, such as β -amyloid mimics [71–73], elastin mimics [74,75] and silk mimics [76–78], have been used as the protein/peptide block.

The first example of *block copolymers of PEG and a β -sheet forming peptide* was published by Lynn and co-workers [71–73]. The attachment of PEG (mol. wt. 3000) to the C-terminus of residues 10–35 of A β (A β _(10–35)) of the amyloid peptide rendered the formation of fibrils, in contrast to native peptide, completely reversible. The hybrid material took on a structure composed of up to six (extended) laminated parallel β -sheets surrounded by the PEG block. Apparently, PEG prevented lateral association of the fibrils, and, therefore, inhibited the irreversible step of fibrillogenesis [71–73].

Diblock copolymers of PEG (mol. wt. ~600) and elastin-like polypeptides ((VPGVG)₄ and (VPAVG)₄) self-assembled to reversible supramolecular thermoresponsive assemblies [74]. Triblock ABA copolymers containing a central PEG (B) block and A blocks from *N*-methacryloylated elastinlike single repeat, VPGVG, were synthesized by atom transfer radical polymerization. CD measurements indicated that the VPGVG side chains maintained their characteristic lower critical solution temperature. Heating of the block copolymers resulted in aggregation as pointed out by turbidity measurements [75].

Hybrid block copolymers containing *silk mimic peptide blocks* were studied intensively apparently due to the excellent mechanical properties of silk. Van Hest and co-workers [77, 79] used β -sheet polypeptide as the central (B) block and PEG as the end (A) blocks in ABA triblock copolymers. The polypeptide block, consisting of tandem repeats of (AG)₃EG, was prepared by genetic engineering and conjugated via two terminal cysteine residues to maleimide-functionalized PEGs of various molecular weights. These polymers assembled into fibrillar microstructures with a fibrillar height of ~2 nm. PEG demonstrated an effect on assembly only with the highest molecular weight (5000) used. In this case, shorter fibers were

produced when compared to block copolymers containing PEGs of lower molecular weight. Apparently, fibril formation in the β -sheet stacking direction was not affected, whereas the PEG chains prevented further lateral aggregation.

Hybrid multiblock copolymers of PEG and GAGA peptides were prepared by step-growth polymerization. Their design was based on the structure of *Bombyx mori* silk; when the amorphous regions of the protein structure were replaced by PEG, the GAGA peptides assembled intra- or intermolecularly into parallel or antiparallel β -sheets. In fact, these multiblock copolymers assembled into nanostructures with a β -sheet content as high as 90%. A microphase-separated morphology was detected where 20–50 nm peptide domains dispersed in a continuous PEG phase [80]. Similarly, hybrid multiblock copolymers mimicking *Nephila clavipes* silk were synthesized by end-capping PEG with polyalanine sequences [76].

Hybrid diblock and triblock copolymers containing a central amphiphilic peptide block capped with one or two PEG blocks self-assembled into fibrils. It was hypothesized that these fibrils result from the hierarchical self-assembly of peptide β -strands into helical tapes, which then stack into fibrils [81].

There is an upsurge of interest in block copolymers containing α -helix forming blocks [2,3]. Hybrid block copolymers composed from synthetic blocks and coiled-coil-forming peptide blocks were first studied by Pechar et al. [82]. The secondary structure and self-assembly of block copolymers composed of peptides with the general structure (VSSLESK)_n ($n = 3, 4, 5$ or 6) and an α -methoxy-PEG (mPEG) block (mol. wt. 2000). Conjugation of mPEG did not have a disturbing effect on coiled-coil formation, but improved the thermal stability of the coiled-coil superstructure [82]. In a similar study, a coiled-coil-forming peptide, GEAK (LAEIEAK)₂LAEIY, was modified with PEG (mol. wt. 750 and 2000). While the PEG attachments did not interfere with the coiled-coil formation, analytical ultracentrifugation experiments showed a decrease in the relative concentration of coiled-coil aggregates with increasing PEG chain length [83]. The enhanced thermal stability of these block copolymers was explained by the formation of a hydrophilic shell around the hydrophobic interior stabilizing the peptide secondary structure [83]. Electron paramagnetic resonance experiments support this hypothesis on the superstructure of PEG-coiled-coil-forming peptide copolymers [84].

Mardsen et al. [85] demonstrated the formation of noncovalent triblock copolymers from two diblock copolymers containing oppositely charged peptide blocks (E and K). Mixing of two diblock copolymers, PEG-K and polystyrene-E, resulted in the formation of coiled-coil heterodimers (E/K) and formation of a triblock copolymer. The triblock copolymer self-assembled into rod-like micelles.

4.2 From synthetic polymers and peptide domains

A new method of hydrogel synthesis – self-assembly of synthetic polymer chains mediated by genetically engineered protein domains demonstrated that it is possible to impose properties of a well-defined coiled-coil protein motif onto a hybrid hydrogel containing synthetic polymer primary chains [10]. Hybrid hydrogels were assembled from an *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer (containing side chains terminated in iminodiacetic acid moieties charged with Ni⁺⁺) and His-tagged coiled-coil-forming peptides (CC1 and CC2) through metal complexation, and temperature-induced changes in hydrogel swelling were monitored. CC1 was a segment of the stalk region of *Drosophila* motor protein, kinesin. CC2 was a de novo designed coiled-coil sequence ([VSSLESK]₆). Thermal melting study showed that CC₁ unfolds cooperatively with a major mid-point temperature (T_m) of 35°C, whereas CC2 does not unfold below 90°C. Hydrogels assembled using CC1 underwent a sharp volume transition (deswelling up to 10-fold) upon heating with a mid-point around 39°C, which is in

good agreement with the T_m of CC₁ in solution. In contrast, gels containing CC₂ did not show any appreciable change in volume. Thus, the behavior of self-assembled hybrid hydrogels closely resembled the behavior of the coiled-coil domains. This is a new temperature response mechanism for hydrogels that can be tuned over a wide temperature range by assembling gels with coiled-coils that have different melting temperatures. These results add a new dimension to the field of “smart” hydrogel-based biomaterials providing the immense potential of tailoring material properties using genetically engineered proteins [7,10].

To investigate the influence of coiled-coil protein structure and stability on hydrogel volume transition, a series of block proteins containing interspersed naturally derived recombinant coiled-coils was synthesized. Three recombinant proteins, designated KS₁, KS₂ and KS₃, were modular in structure and contained alternating blocks of coiled-coil and non-coiled chains. KS₁ was an amino acid sequence from the N-terminal segment of the *Drosophila* kinesin stalk (from N340 to A410); KS₂ and KS₃ were the dimer and trimer of KS₁, respectively. Similar to in Ref. [10], hybrid hydrogels were assembled from an HPMa copolymer and His-tagged block proteins through metal complexation, and a temperature-induced decrease in hydrogel swelling was observed. The onset temperature of the hydrogel volume transition correlated well with the onset temperature of protein unfolding [15]. Instead of adopting extended linear structures, the KS block proteins formed self-associating oligomers and displayed thermal unfolding profiles indicative of a hierarchic higher-order structure (Fig. 1). Based on sedimentation experiments, CD measurements and temperature stability, a model of peptide higher order structure was proposed [15].

Polyacrylamide-based hydrogels crosslinked with genetically engineered immunoglobulin (Ig) modules from the muscle protein titin produced a positive swelling response following temperature-induced unfolding of the Ig module from a globular state to a random coil [16]. Titin is a gigantic, modular protein consisting of more than 200 Ig and fibrinectin type III domains. Structurally, the repeating titin Ig domain is a sandwich of two antiparallel β -sheets held together by hydrophobic interactions between the sheets in the core of the domain and by hydrogen bonds between the β -strands forming β -sheets [86]. The chemical potential of the hydrogel polymer matrix generated the driving force stretching titin Ig modules from their ends. The cooperative unfolding of the Ig crosslinks lead to the unique swelling profile in response to environmental (temperature) stimulus. This is another unique and adjustable temperature response mechanism based on a folding transition of a protein module crosslink. A quantitative thermodynamic model describing these phenomena, i.e. swelling changes of crosslinked gels caused by unfolding of folded chains built-in the gel matrix, was recently proposed [87,88].

4.3 From hybrid graft copolymers

Another polymer architecture evaluated for peptide-mediated self-assembly are graft copolymers composed of a synthetic polymer backbone and associative peptide grafts. An example of such graft copolymers are HPMa copolymer backbone and coiled-coil-forming peptide grafts. The first design involved peptide grafts forming homodimers and higher aggregates [23,24]. Microrheology and dynamic light-scattering measurements revealed that the length and the number of coiled-coil grafts per macromolecule had a decisive impact on the gelation process [23,24]. Grafts composed of at least four heptads were needed to achieve association of graft copolymers into hydrogels. In addition, a threshold amount of grafts per macromolecule was needed to form a 3-D structure. The kinetics of self-assembly could be manipulated by the concentration of the copolymer in aqueous solution and by temperature [24]. The limitation of this design is the possibility of dimer formation from grafts attached to the same macromolecule. The structure of physical crosslinks will then be diverse, involving many conformations, including two dimers associating in an antiparallel fashion.

To avoid association along the macromolecule and to guarantee the formation of a well-defined 3-D network, a new design of graft HPMA copolymers was proposed that consisted of the hydrophilic polymer backbone and a pair of oppositely charged peptide grafts [22,25]. Two distinct pentaheptad peptides (CCE and CCK) were anticipated to create a dimerization motif and serve as physical crosslinkers. Their structure was designed based on generally accepted principles governing coiled-coil conformation [38] with the goal of achieving an antiparallel heterodimeric conformation. Three major stabilizing interactions were taken into account: the hydrophobic interactions in the core, electrostatic attractions across the interface and helical propensity effects.¹ Consequently, the graft copolymers, CCE-P and CCK-P (P is the HPMA copolymer backbone), self-assembled into hybrid hydrogels in situ; the process was modulated by the formation of antiparallel heterodimeric coiled-coils. This approach minimizes the steric hindrance of the polymer backbone on the “in-register” alignment (total overlap of sequences) of peptide grafts (Fig. 2). Indeed, equimolar mixtures of the graft copolymers, CCE-P/CCK-P, have been observed to self-assemble into hydrogels in phosphate-buffered saline solution at neutral pH at concentrations as low as 0.1 wt.%. Circular dichroism spectroscopy, sedimentation equilibrium experiments and microrheology data revealed that the self-assembly process corresponded to the two-stranded α -helical coiled-coil formation between CCE and CCK. Moreover, the formation of hybrid hydrogels was reversible. Denaturation of the coiled-coil domains with guanidine hydrochloride (GdnHCl) solutions resulted in disassembly of the hydrogels. Removal of GdnHCl by dialysis caused coiled-coil refolding and hydrogel reassembly. Similarly, dynamic light scattering data indicated that exposure of the self-assembled hydrogel to a competing peptide (CCK) resulted in a decrease in crosslinking density and partial disassembly of the hydrogel [25]. As expected, concentration of graft copolymers had a significant impact on the kinetics of self-assembly [25] and on the structure and morphology of self-assembled hydrogels [22].

4.4 From hybrid star copolymers

Star-architecture-type macromolecules may self-assemble into 3-D structures. Hydrogels were synthesized by the self-assembly of two 4-arm PEGs [89,90]. The first star-type macromolecule was prepared by the attachment of heparin-binding domain of the heparin interacting protein (HIP) to 4-arm PEG. The HIP peptide carrying N-terminal cysteine (CRPKAKAKAKDQTK) was reacted via Michaelis addition with vinyl sulfone-modified 4-arm star PEG. The second star-type macromolecule was a 4-arm PEG–low-molecular-weight heparin (LMWH) conjugate. It was prepared by the reaction of thiol end-terminated 4-arm star PEG ($M_n=10,000$) with maleimide-functionalized LMWH. The mixing of two star copolymers resulted in hydrogel self-assembly (Fig. 3).

Ghosh and co-workers used *zero generation PAMAM dendrimers (4-arms)* decorated with the pH-sensitive leucine zippers [91] EZ and KZ [92].² Both D-KZ₄ and D-EZ₄ supported the assembly of cognate peptides into fibrillar structures with concomitant enhancement of helicity [91].

4.5 Other designs of hybrid hydrogels

Peptide/protein segments (within crosslinks) were used to induce degradability into hydrogels [93–95]. Depending on the degree of swelling and the size of the particular enzyme, the degradation proceeds either in bulk or as surface erosion [93].

¹The structures were as follows [22]: CCE: CYGG E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK; CCK: CYGG K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE.

²The structure of peptides was as follows [86]: EZ: H₂N-AQALEKELQALEKELQALEWELQALEKELSGSGC-COOH; KZ: H₂N-AQALKKKLQALLKKKLQALKWKLQALKKKLSGSGC-COOH.

Protein-graft-PEG hydrogels, mimicking the natural extracellular matrix, were prepared in a three-step process. First, an artificial protein was produced by genetic engineering, followed by grafting of PEG diacrylate. In the third step the acrylate groups were photopolymerized yielding hydrogels. The protein sequence contained the RGD biorecognition peptide and two plasmin degradation sites. These hydrogels are versatile biomaterials that permit cell attachment and proteolytic penetration [96]. A modified design of materials that permit 3-D cell migration within the hydrogel matrix was based on the reaction of cysteine thiols (inside the protein structure) with vinyl sulfone moieties of end-functionalized PEG [97,98].

Hybrid hydrogels composed of poly(acrylamide-co-allyl glucose) physically crosslinked by glucose-binding protein concanavalin A were synthesized to determine the concentration level of external glucose [99,100]. Free glucose competes for the concanavalin A binding sites, disrupting the physical crosslinks with a concomitant increase in swelling. Similarly, introducing concanavalin A into poly(2-glucosyloxyethyl methacrylate) crosslinked chemically with methylene-bis-acrylamide resulted in a glucose-sensitive hydrogel [101]. These hydrogels were designed to release entrapped insulin following changes in the hydrogel's equilibrium degree of swelling [99–101].

Antigen-responsive hydrogels were also studied [102,103]. Miyata et al [102]. prepared a semi-interpenetrating network (IPN) composed of a soluble poly[acrylamide-co-acryloyl rabbit IgG (antibody)] embedded in a hydrogel prepared by copolymerization of acrylamide, methylene-bis-acrylamide and acryloyl-goat-anti rabbit IgG (antigen). The resulting semi-IPN was sensitive to the presence of free antigen. Binding of free antigen resulted in a decrease in crosslinking density with a concomitant increase in the equilibrium degree of swelling. Lu et al. [103] synthesized antigen-sensitive hydrogels by copolymerization of *N*-isopropylacrylamide, a polymerizable antibody Fab' fragment from monoclonal anti-fluorescein BDC1 antibody and *N,N'*-methylene-bis-acrylamide. When the hydrogel was alternatively exposed to fluorescein and polyamidoamine dendrimer-fluorescein, significant reversible volume changes were observed.

Recently, the conformational changes accompanying protein–ligand recognition were used to trigger volume change in smart hybrid hydrogels [104–107]. Three designs were based on the changes in calmodulin conformation upon binding its ligand(s) [104–106]. Calmodulin changes its conformation from the native state to a “dumbbell” state when bound to calcium. Then, in the presence of Ca^{2+} , calmodulin changes from the “dumbbell” state to a collapsed state when bound to one of its ligands, phenothiazine.

Daunert and co-workers [104] mutated calmodulin to contain one cysteine residue to bind an allyl group. Copolymerization of acrylamide, *N,N'*-methylene-bis-acrylamide, allylcalmodulin and a polymerizable derivative of phenothiazine produced a ligand (Ca^{2+} , phenothiazine)-sensitive hydrogel. Its applicability in a microfluidic device was demonstrated [104].

Mrksich and co-workers genetically engineered calmodulin to contain two cysteine residues [105]. This mutant was used as a crosslinker (in addition to dithiothreitol) of 4-arm PEG containing acrylate groups. The resulting hydrogels underwent changes in the equilibrium degree of swelling upon binding of calmodulin ligand, trifluoperazine [105].

Murphy et al. modified calmodulin mutant (T34C, T110C) with poly(ethylene glycol) diacrylate to produce a protein with two polymerizable groups [106]. The latter was photocrosslinked into a stimuli-sensitive hydrogel, changing swelling upon exposure to trifluoperazine.

Finally, Yuan et al. [107] designed a dynamic hybrid hydrogels capable of translating enzyme–substrate recognition into mechanical motion. The design principle was based on incorporating

controlled motion of macromolecules into three-dimensional materials. To this end, HPMA copolymer-based hydrogels containing a covalently attached enzyme (adenylate kinase) were designed, synthesized and characterized. Upon binding of a substrate, the induced conformational transition of the enzyme translated into molecular motion with concomitant de-swelling of the hydrogel. To demonstrate the general applicability of the approach, similar hydrogels were synthesized from 4-arm PEG terminated in maleimide [107].

5. Concluding remarks

The knowledge of basic scientific principles governing the self-assembly of molecules of different structures into hierarchically organized materials made dramatic progress in the last decade. It is possible to control the structure of self-assembled materials on the nanoscale. The possibility of selectively placing functional groups into a protein structure permits the careful placing of functional groups within the 3-D assembly and allows the response to physiological stimuli to be manipulated. The option to incorporate non-canonical amino acid residues adds another dimension to the design of new biomaterials [108–110]. New polymerization techniques, atom transfer radical polymerization [111] and reversible addition-fragmentation chain transfer polymerization [112], as well as developments in peptide/protein–synthetic polymer conjugation techniques [113,114], enhance the synthetic potential and permit better control of the structure of the synthetic component in hybrid materials. Techniques to precisely analyze the 3-D structure of self-assembled materials should be routinely used. Fluorescence energy transfer experiments provide a tool to analyze the extent of “in-register” alignment of α -helices in the coiled-coil structure [115]. Nuclear magnetic resonance analysis provides another important tool to analyze complex structures [116].

This review has focused on the basic science problems of self-assembly. We are aware, however, of the enormous application potential of the systems discussed herein. Progress that may be expected in the near future includes: development of 3-D tissue engineering scaffolds; the in situ formation of hydrogels for drug delivery; sensors sensitive to environmental changes; and various microfluidic systems. The ability to control self-assembly on the nanoscale will permit the miniaturization of precisely designed hydrogel components of biomedical devices, sensors, microfluidic systems, nanoreactors and interactive surfaces with highly accurate biorecognition.

Acknowledgements

The hydrogel research in the authors' laboratory was supported by NIH grants EB00251 and EB005258, and by a seed grant from University of Utah Research Foundation Project No. 51003289.

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Fig. 1. Hybrid hydrogels self-assembled from HPMA copolymer and His-tagged block proteins by metal complexation. (A) Chemical structure of the copolymer of HPMA and *N*-[3-(*N*',*N*'-dicarboxymethyl)aminopropyl]methacrylamide (a); amino acid sequence of the KS segment from the *Drosophila kinesin* stalk region (b); schematic representation of the KS block proteins – KS₁, KS₂ and KS₃. Stripped rectangles present the sequence with high propensity of coiled-coil formation. (B) Thermal stability of the KS block proteins by CD spectroscopy. The arrows indicate the onset of temperatures of transition. (C) Hypothetical model for the higher order structure and thermal unfolding of the KS block proteins. Cylinders depict helical regions,

thick lines are non-coiled-coil sequences and thin lines are unfolded chains. The amino termini of the protein blocks are highlighted in gray. Adapted from Ref. [15].

Fig. 2.

(A) Self-assembly of the HPMA graft copolymers, CCK-P and CCE-P, containing oppositely charged peptide grafts (P is the HPMA copolymer backbone; see Ref. [53] for structure of CCE and CCK). Aqueous solutions of CCE-P or CCK-P did not form gels. In contrast, gel-like materials were formed from equimolar mixtures of CCE-P/CCK-P at low concentrations. (B) Microrheology of 1% w/v solutions of CCE-P, CCK-P and equimolar mixture of CCE-P/CCK-P. Mean square displacement of tracking particles as a function of time. (C) CD spectra of the equimolar mixture CCK-P/CCE-P before (full tilted squares) and after (empty triangles) denaturation by guanidine hydrochloride at 25 °C at a 20 μM peptide concentration and after removal of guanidine hydrochloride by dialysis (empty squares). Adapted from Ref. [22].

Fig. 3. Self-assembly of hydrogels from two complementary star copolymers. Biorecognition moieties, a sequence from HIP and LMWH, are indicated by half circles and rectangles, respectively. Adapted from Ref. [89].