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Self-Assembling Hydrogels

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Summary

The overview focuses on the design, synthesis, and characterization of peptide/protein-containing polymers, which self-assemble into stimuli-sensitive hydrogels. Hybrid hydrogels are composed of two classes of molecules, synthetic and biological. They self-assemble from graft copolymers, block copolymers and by association of soluble polymers and protein domains. Genetic engineering provides a powerful tool to produce biomaterials (macromolecules) with precise control of composition, length, and three-dimensional structure. Minimal changes in the composition of the coiled-coil forming protein domains in genetically produced triblock copolypeptides result in dramatic changes in the properties of the copolymers and, ultimately, the self-assembled hydrogels. The application of self-assembling hydrogels as biomaterials has been investigated and has shown a promising future.

Introduction

Hydrogels are hydrophilic polymers that can retain a significant amount of water while maintaining a distinct three-dimensional structure. As early as 1960, it has been proposed that hydrogels can be used as biomaterials [1]. Based on the nature of the crosslinking force, hydrogels can be categorized as chemical (covalent) or physical gels [2]. 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. Since the assembly of physical hydrogels depends on the spontaneous organization and specific association of molecules through a number of non-covalent interactions [3,4], physical hydrogels can also be called "self-assembling hydrogels". It is well known that hydrogel networks can have volume phase transitions. This phenomenon has been theoretically predicted by Dušek and Patterson [5] in 1968 and was experimentally verified by Tanaka and coworkers ten years later [6].

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Traditional methods of hydrogel synthesis include crosslinking copolymerization, crosslinking of polymeric precursors, and polymer-polymer reactions. These synthetic methods lack precise control of chain length, sequence, and three-dimensional arrangement. In addition, side reactions may occur, including formation of internal loops, unreacted pendant groups, and entanglements [7]. The structural variability of the crosslinked polymers can influence their physico-chemical properties and performance in the biological environment. For example, it has been shown that the detailed structure of hydrogels based on N,N-dimethylacrylamide copolymers varied when using different synthetic methods. Further the degradation rate of hydrogels containing azoaromatic linkages was dependent on the polymer structure [7].
An alternative method for hydrogel synthesis appears to be necessary in order to produce hydrogels with defined structures. The development of genetic engineering technology provides a powerful tool to produce biomaterials with precisely controlled sequences and even 3-dimensional structures [8,9]. Using this approach, hybrid hydrogels containing protein moieties [10] and protein-based hydrogels [11,12] have been synthesized.

The coiled-coil motif

A typical example of a protein motif used in the synthesis of hybrid hydrogels or protein-based hydrogels is the coiled-coil. Coiled-coils have been found in over two hundred native proteins. Structurally, it is a right- or left-handed supercoil formed by two or more strands of α-helices [13-20]. 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 conventionally 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 (Figure 1).

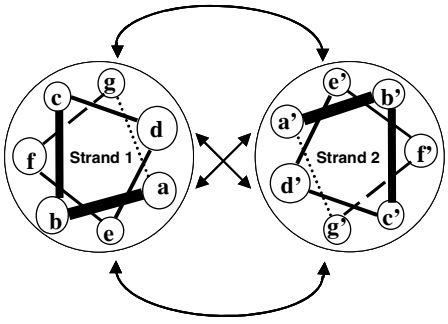

Figure 1. Helical wheel diagram of a two-stranded coiled-coil.

Hybrid hydrogels

Hybrid hydrogels are usually defined as hydrogel systems whose components are composed of at least two distinct classes of molecules (for example, synthetic polymers and biological macromolecules) interconnected either covalently or

non-covalently [21,22]. Conjugation of peptide domains and synthetic polymers may lead to novel materials with properties superior to those of the individual components. Compared to synthetic polymers, proteins and protein modules have well-defined and homogeneous structures, consistent mechanical properties, and cooperative folding/unfolding transitions. The peptide domain (graft, block, or crosslink) may allow a level of control over the structure formation at the nanometer level; the synthetic part can contribute to the biocompatibility of the hybrid material [21]. The synergistic combination of two types of structures may lead to new materials that possess unprecedented levels of structural organization and have novel properties [21]. In principle, the responsiveness of hydrogels may be directly related to the defined structure of the protein crosslinks. By optimizing the amino acid sequence, a tailored responsiveness of the hybrid hydrogels for a specific application may be achieved.

Synthetic polymers have been crosslinked by oligopeptide sequences [23-26], full-length proteins such as bovine serum albumin [27], oligodeoxyribonucleotides [28], and polysaccharides [29]. Several examples of these hydrogel systems follow.

Concanavalin A (Con A) is a lectin, which exists as a homotetramer at physiological pH and has a glucose-binding site on each monomer. *Con A has been used as a physical crosslinker in hydrogel systems* based on poly(2-glucosyloxyethyl methacrylate) [30] and poly(acrylamide-co-allyl glucose) [31,32]. These hydrogels respond to free glucose, which competitively binds to Con A and disrupts the physical crosslinks. Antigen-antibody interactions have also been used to crosslinking primary chains in polyacrylamide hydrogels [33].

In another study, *polyacrylamide hydrogels were crosslinked by a genetically engineered immunoglobulin* (Ig) module from the muscle protein titin through metal complexation [34]. When the temperature increased to the point where the Ig module started a thermal unfolding from globular state to random coil, the hydrogels swelled to three times their initial volume. This is a unique and adjustable temperature response mechanism based on the folding transition of the protein module crosslink. The change in the swelling state of the hydrogels was studied using a thermodynamic model [35], which suggested that the structural change of the physical crosslinks may cause the phase-volume transition of the hybrid hydrogels.

Recently, calmodulin (CaM) was selected as the biological element in the stimuli-responsive CaM-phenothiazine hydrogels [36]. Both the modified CaM and the polymerizable phenothiazine were immobilized within the polymer network. In the presence of Ca^{2+}, the immobilized phenothiazine derivative was bound to CaM, forming physical crosslinks within the hydrogel. Upon the removal of Ca^{2+}, the immobilized phenothiazine derivative was released from the CaM binding site, and the CaM protein underwent a conformational change from a more constrictive conformation to its native conformation, which caused the hydrogel to swell.

Self-assembly of synthetic polymer chains mediated by genetically engineered protein domains is a new method of hydrogel synthesis developed in our laboratory. Novel hybrid hydrogels were assembled using water-soluble synthetic polymers and coiled-coil protein domains [10]. Two coiled-coils, CC1 and CC2, were studied. CC1 was a segment of the stalk region of the *Drosophila* motor protein, kinesin. CC2 contained a *de novo* designed coiled-coil sequence ($[VSSLESK]_6$), in which valine and leucine occupy the first and the fourth positions of the heptad repeating unit. Specific charge patterns were also engineered in order to favor coiled-coil homodimerization. A 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. The two proteins were separately attached to a hydrophilic synthetic copolymer of N-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-(*N'*,*N'*-dicarboxymethylaminopropyl)ethacrylamide through a metal complex formed by the protein's terminal histidine residues, Ni^{2+} ion, and iminodiacetate ligands from the HPMA copolymer backbone. A highly swollen hydrogel was formed upon coiled-coil multimerization. Hydrogels formed by crosslinking HPMA copolymers with CC1 coiled-coil modules underwent dramatic volume transitions (de-swelling up to 10-fold) at the melting temperature of the coiled-coil modules [10]. 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 [37]. These results seem to indicate that the properties of a well-defined coiled-coil protein motif can be imposed onto a hybrid hydrogel containing synthetic polymer based primary chains. This adds a new dimension to the field of "smart" hydrogel-based biomaterials given the immense potential of tailoring material properties with genetically engineered proteins [38].
Graft copolymers composed of HPMA copolymer backbone and coiled-coil sequences as grafts self-assemble into reversible hydrogels. The grafts were composed either of coiled-coils of different lengths (three to seven heptads) or the design involved two oppositely charged peptide grafts. The impact of parallel homodimer or antiparallel heterodimer formation on the self-assembly process was assessed [39].

Biopolymer-based hydrogels

There is an upsurge of interest in hydrogels derived from biological macromolecules, since their components are analogous to those in living cells. Polysaccharides have been widely used in hydrogel formation, including agar [40], dextran, chitosan, and alginate [41-47]. This overview, however, is focused on peptide/protein-based materials. Major advances related to protein-based hydrogels are discussed below.

Hydrogels based on natural proteins

Hydrogels have been formed based on natural proteins, such as collagen and gelatin. Collagen is a structural protein with a triple helix in its secondary structure. At high temperature or high salt content, collagen macromolecules change their conformation from semi-crystalline and elongated triple helices to amorphous and compact coils, resulting in contraction of collagen hydrogels [48,49]. These hydrogels have been studied as injectable or protein-releasing biomaterials [50-52].

Hydrogels based on protein-mimetic polypeptides

The design of protein-based hydrogels frequently reflects the structure of biological macromolecules. Elastin-based hydrogels [53-55] 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. Unlike most of the other hydrogels, these hydrogels contract when temperature is raised above 25 ^oC. 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 protein composition, degree of ionization, pH, salt, or phosphorylation [53]. It could also be modulated by electrochemical reduction/oxidation and light, when the protein polymer was incorporated with prosthetic groups, such as N-methylnicotinamide [56,57] and azobenzene [58]. Genetically produced polypeptides, $(GVGVP)_{251}$ and $(GVGIP)_{260}$, have been crosslinked into hydrogels by γ-irradiation [59]. Detailed investigation showed that with increasing temperature, hydrophobic folding and assembly phase transition occurred, which in turn had a dramatic influence on elastic moduli and fracture properties. The self-assembly of elastin-mimetic block copolymers have been designed and biologically synthesized [60-63]. The morphology and the structure of the hydrogels were studied by electron microscopy and solid-state NMR.

To achieve desired properties, protein polymers may be designed incorporating two structural motifs. For example, silk-elastinlike protein polymers have been designed [8,9,64] and self-assembled into hydrogels [65-71]. These protein polymers are composed of tandemly repeated silk-like blocks (GAGAGS) and elastin-like blocks (GVGVP). 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-elastinlike protein polymers increased the pH- and temperature-sensitivity [69]. Evaluations on the swelling and transport properties of the silk-elastinlike hydrogels suggested that they have the potential to become controlled release bioactive materials [65,66].

Hydrogels based on protein folding modules

Hydrogels have been formed from oligopeptides [72-74], diblock [75] and triblock [11,76] copolypeptides. Diblock copolypeptides were designed and synthesized to form hydrogels at low concentrations [75]. Block copolypeptides containing 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 were synthesized and evaluated. These low-molecular-weight diblock copolymers associate into hydrogels at very low polymer concentrations, maintain their mechanical strength at high temperatures and recover (rearrange) rapidly after stress [75]. The shape of the polymer chains was an important factor in the hydrogel self-assembly (gelation) process. It was demonstrated that α-helical segments were better gelators than β-strands, which in turn were better than random coils. The formation of hydrogels from diblock copolymers at low concentrations is a curious phenomenon, and could one day supplement tools for the design, synthesis and self-assembly of novel biomaterials and drug-delivery systems [77].

The self-assembly of triblock copolymers into hydrogels is being intensively studied. ABA block copolymers, where the block A is a coiled-coil forming peptide and block B a random coil, self-assemble into hydrogels [11]. These hydrogels reversibly respond to changes in temperature and/or pH. The stimuli-sensitivity and the dynamic structure of the hydrogels are closely related to the three-dimensional structure. Solid-state NMR spectroscopy data are consistent with the hypothesis that the block B acts as the flexible swelling agent of the hydrogel network while the terminal domains form intermolecular aggregates [76].

Minor structural changes may result in dramatic changes in properties

It has been shown that the microscopic changes in the coiled-coil structures can result in macroscopic changes in the physico-chemical properties of the hydrogels. Therefore, design of coiled-coils with specific associations may lead to the desired hydrogel formation. For example, a change of one amino acid residue in the hydrophobic region of the coiled-coil may change the interaction of two helices. A buried polar Asn residue in the hydrophobic core of a coiled-coil can change the orientation specificity of the coiled-coils [78]. This demonstrates the enormous design potential of coiled-coil containing materials.

Let's discuss, as an example, the possibility to manipulate the properties of the triblock copolymers and self-assembled hydrogels by minor, but tailored, modifications of the structure. In particular, the relationship between the structure of the coiled-coil block on the self-assembly of triblock copolymers was studied [12]. Coiled-coil containing triblock polypeptides with structures of ABA and CBC were designed and genetically synthesized [12]. Block B was a central water-soluble random coil segment, which had an Ala-Gly-rich sequence $[(AG)_3PEG]_{10}$ [11]. Blocks A and C were terminal coiled-coil domains, whose amino acid sequences were $(VSSLESK)_6$ [79] and $(VSSLESK)_2$-VSKLESK-KSKLESK-VSKLESK-VSSLESK, respectively. Four amino acid residues (of block A) were modified to produce the C block (Fig. 1). Lys replaced Val in the **a** position of the fourth heptad to destabilize the hydrophobic interactions in the core, and three additional Lys residues replaced Ser in the **c** positions of the third, fourth, and fifth heptads to introduce an electrostatic repulsive force between **c** and **g** residues. As a result, the structural modification of block A should decrease the thermal stability of the coiled-coil association. Furthermore, the insertion of Lys residues should increase the pH-sensitivity of the coiled-coil domains. Circular dichroism (CD) spectrophotometry indicated that the thermal stability of CBC was indeed substantially lower than that of ABA (Figure 2).

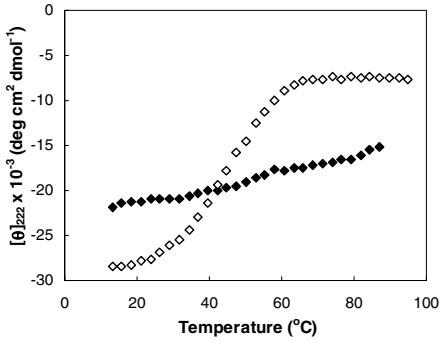

Figure 2. Temperature dependence of the secondary structures of the protein polymers. CD signal (ellipticity) at 222 nm as a function of temperature. Filled diamonds, ABA copolymer; open diamonds, CBC copolymer (modified from ref. [12].)

What was the impact of structural changes on the self-assembly of block copolymers into hydrogels? Hydrogels self-assembled from these copolypeptides (ABA and CBC) at different polymer concentrations. From ABA to CBC, the concentration required for hydrogel formation decreased from 35 wt. % to 5 wt. %. The gelation concentration was directly correlated to the oligomerization state of the coiled-coil domains, as determined by analytical ultracentrifugation. Whereas the ABA copolymer exhibited a monomer-dimer equilibrium, the oligomerization state of the CBC triblock copolymer displayed a dimer-tetramer equilibrium [12]. The self-

assembly of hydrogels was also followed using microrheology. In microrheology, a small sample volume is employed and micrometer sized particles are used as embedded probes. With the use of high-resolution microscopy, the rheological properties of the materials can be measured [80-84]. Depending on how the probe particles are manipulated, microrheology can be categorized as active or passive. In passive microrheology, no external force is applied on the particles, and the particles are driven by thermal energy, k_BT. During a measurement, the thermal motion of the embedded particles is observed under a microscope and recorded as movies using a CCD camera. The positions of each tracer particle are analyzed in each frame of the movie, which are used to obtain the MSD (mean-squared displacement). At this point, the storage G' and loss G" moduli can be extracted from MSD using a generalized Stokes-Einstein relation. However, it is usually sufficient to deduce the viscoelasticity of a material by plotting the graph of MSD versus lag time τ on a double logarithmic plot [84], since the slope of the graph must fall between the two extremes — viscous (slope = 1) and elastic (slope = 0) [11]. Moreover, the power of microrheology lies not only in the ability to measure low viscosity samples with exquisite sensitivity, but also in the ability to provide heterogeneity information of complex fluids [83, 85-88]. To demonstrate the technique, the viscoelastic properties of solutions of CBC copolymers at different concentrations are shown on Figure 3.

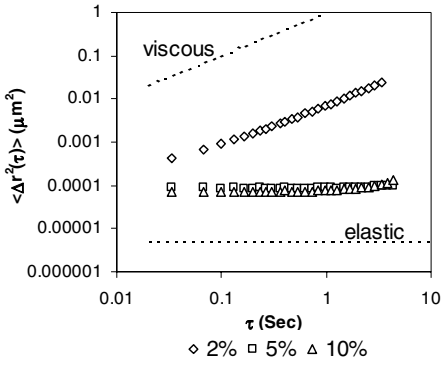

Figure 3. Mean-square-displacement as a function of lag time for 0.5 μm amidine-modified particles in aqueous solutions of CBC copolymer (modified from ref. [12]).

Hydrogels self-assembled from CBC triblock copolymers showed pH- and temperature-sensitivity, which corresponded well with the pH- and temperature-responsiveness of non-assembled CBC. It can be interred that the primary sequence of CBC determined the structural properties of CBC, as well as the rheological properties of the hydrogels self-assembled from CBC. The four lysine residues present in the coiled-coil (C) block sequence of CBC, not only successfully decreased the thermal stability, but also enhanced the pH responsiveness of CBC, compared to similar polypeptides [12].

An important finding is that these structures were reversible after denaturation. The coiled-coil sequences in CBC copolymer, after denaturation by guanidium hydrochloride, fully refolded after the denaturant was removed by hydrolysis, as indicated by CD spectra. The reversibility of the self-assembly of CAC copolymer hydrogels when temperature was temporarily increased above the melting point of the C block was monitored by microrheology. After the temperature was increased from room temperature to 55 ^{o}C, a change from elastic to mostly viscous properties

occurred, suggesting a loss of organization and disassembly of the three-dimensional structure of the hydrogel. As the sample was cooled back to room temperature, the MSD vs. lag time curve almost overlapped with the original one, suggesting a recovery of the self-assembled hydrogel structure [12]. The scanning electron microscopy (SEM) of the hydrogels from the polypeptides strongly suggested that the self-association among the coiled-coil blocks mediated the self-assembly of the hydrogels, since porous inter-connected networks were found in each of the hydrogel samples (see example in Figure 4).

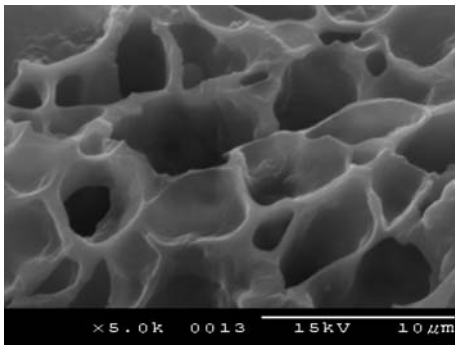

Figure 4. SEM image of hydrogels self-assembled from 10 wt. % aqueous solution of CBC copolymer.

Applications

Numerous applications have been proposed and investigated for the self-assembled hydrogels. Extensive studies have been focused on the development of controlled release drug delivery systems using these materials [38,89-92]. These hydrogels can respond to environmental stimuli, such as temperature, pH, electric field, light, or chemical signals. The glucose-sensitive insulin-releasing system is a typical example [31,32]. Physically crosslinked hydrogels were formed from copolymers of allyl glucose and N-vinyl-2-pyrrolidone when Con A was added. When exposed to free glucose, the pre-loaded insulin inside the hydrogels could be released. Because of their high water content and biocompatibility, hydrogels have been widely used as scaffolds in tissue engineering [46,93-95]. The polymer network not only can provide growth scaffolds for tissue formation, but can also act as a depot for various growth factors to facilitate tissue regeneration. Self-assembling hydrogels may also be used as medical devices. For example, elastin-based hydrogels have been evaluated in ophthalmic surgery and cell adhesion [96,97].

Besides biomedical applications, hydrogels have showed their practical use in other fields, including bioseperation [98], immobilized enzymes and cells [45,99], biomimetic actuators [100], and microfluidic valves [36,101]. In a recent study, a microfluidic valve was fabricated using CaM-phenothiazine hydrogels [36]. The hydrogel responded to stimuli by shrinking in the presence of Ca^{2+}. In an ideal system, the hydrogel would reversibly respond to the stimuli, opening and closing the microfluidic valve (Figure 5).

As complexity and variety is revealed by Mother Nature, mimicry of biological systems suggests a promising future for the application of both hybrid and biopolymer based hydrogels.

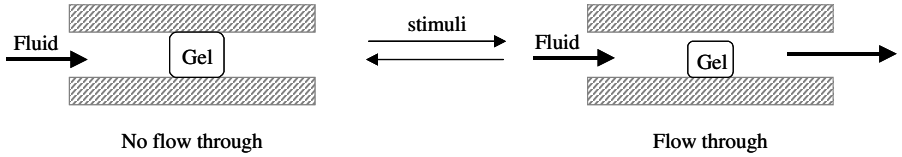

Figure 5. Schematic diagram of a putative microfluidic device (adapted from [36]).

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