Thermally Reversible Hydrogels via Intramolecular Folding and Consequent Self-Assembly of a *de Novo* Designed Peptide

Darrin J. Pochan,*,[†] Joel P. Schneider,*,[‡] Juliana Kretsinger,[‡] Bulent Ozbas,[†] Karthikan Rajagopal,[‡] and Lisa Haines[‡]

Department of Materials Science and Engineering and Delaware Biotechnology Institute, and Department of Chemistry and Biochemistry, University of Delaware, 201 DuPont Hall, Newark, Delaware 19716

Received March 25, 2003; E-mail: pochan@udel.edu; schneijp@udel.edu

Advances at the interface of materials science and biomolecular chemistry are resulting in materials responsive to biologically relevant stimuli.[1] A primary example of biologically relevant stimuli responsive materials are hydrogels responsive to temperature designed for potential use as drug delivery matrices or tissue engineering scaffolds.[2] Traditionally, hydrogel materials that swell or contract with changes in temperature are termed thermally responsive.[3] For example, cross-linked synthetic polymer networks such as covalently cross-linked poly(*N*-isopropylacrylamide) (PNIPAAm) have been engineered to undergo volume transitions by taking advantage of thermally sensitive hydrophobic interactions within the polymer network.[4] Large biopolymers such as cross-linked elastin-based networks undergo inverse temperature volume transitions due to the ordering of hydrophobic regions within the cross-linked network on heating.[5]

Distinct from these temperature responsive examples are hydrogels that undergo a thermally reversible transition between a low viscosity aqueous solution and rigid hydrogel. Two distinct classes of thermally triggered hydrogelation systems exist: that in which gelation occurs on cooling or that in which it occurs with heating. Both synthetic[6] and biopolymeric[7,8] examples exist in which a physically cross-linked network forms upon cooling. Generally, the mechanism responsible for gelation on cooling is the formation of physical cross-links that are denatured at higher temperatures. Conversely, examples of synthetic polymers that undergo hydrogelation on heating include the Polaxamers and uncross-linked PNIPAAm derivatives.[9,10] Examples of bioderived systems include chitosan/glycerol formulations[11] and elastin-based polymers.[12,13] Most systems that undergo heat-induced hydrogelation take advantage of the ability of water to solubilize the hydrophobic moieties of amphiphiles at reduced temperature.[14,15] Heating the solution decreases the solubility of the hydrophobes, and consequent collapse/aggregation/phase separation of these groups results in physically cross-linked network formation.

Here, we describe a small, de novo designed peptide (MAX3) (Figure 1) that exhibits complete thermoreversible self-assembly into a hydrogel network. Importantly, a prerequisite to hydrogelation is that the peptide must first fold into a conformation conducive to self-assembly.[16] At ambient temperature and pH 9, MAX3 is unfolded, resulting in a low viscosity aqueous solution. Upon increasing the temperature, the peptide undergoes a unimolecular folding event, affording an amphiphilic β-hairpin that consequently self-assembles into a hydrogel network. Increasing the temperature serves to dehydrate the nonpolar residues of the unfolded peptide and trigger folding via hydrophobic collapse.[14,15] Cooling the
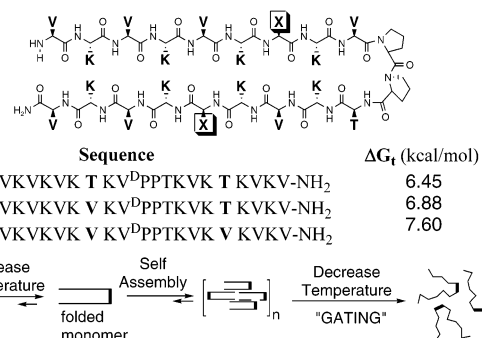
† Department of Materials Science and Engineering and Delaware Biotechnology Institute.
‡ Department of Chemistry and Biochemistry.

Figure 1. Generalized structure and sequences of β-hairpins. ΔG_t is the calculated free energy of transfer of unfolded peptide having an overall +8 charge state from octanol into water at 25 °C. Also shown is the proposed thermally triggered folding and self-assembly mechanism leading to hydrogelation.

Sequence	ΔG_t (kcal/mol)
MAX3: VKVKVK **T** KV^{D}PPTKVK **T** KVKV-NH$_2$	6.45
MAX2: VKVKVK **V** KV^{D}PPTKVK **T** KVKV-NH$_2$	6.88
MAX1: VKVKVK **V** KV^{D}PPTKVK **V** KVKV-NH$_2$	7.60

resultant hydrogel results in β-hairpin unfolding and consequent complete dissolution of the hydrogel.

MAX3 is composed of a central tetrapeptide having high type II′ β-turn propensity flanked by two extended strands.[17,18] These strands contain alternating hydrophilic and hydrophobic residues, an arrangement known to facilitate the formation of β-sheet structure.[19−21] Lysine occupies the hydrophilic positions, while valine, a residue of high β-sheet propensity, is used as the primary hydrophobic residue. Threonine, isostructural with valine but slightly less hydrophobic,[22] is incorporated at positions 7, 12, and 16. These amphiphilic strands are poised to intramolecularly fold, affording a β-hairpin at appropriate temperatures. The self-assembly of resulting hairpins is driven by both intermolecular hydrogen bonding and the association of hydrophobic faces, uniquely presented in the folded conformation, of individual hairpins.

Circular dichroism (CD) spectra of a 150 μM solution of MAX3 demonstrate that this peptide is unfolded at 5 °C, Figure 2a. Heating the solution to 80 °C results in a spectrum consistent with a β-sheet structure (θ_{min} at 218 nm). Subsequent temperature cycles show that folding and unfolding are reversible. Aqueous solutions of MAX3 at higher concentration (2 wt %) undergo thermally reversible self-assembly leading to gelation. Figure 2b shows the storage modulus, G' (a measurement of hydrogel rigidity), as a function of temperature for several heating/cooling cycles. At 75 °C, a 2 wt % aqueous preparation of MAX3 exists as a rigid (nonflowing, self-supporting) hydrogel ($G' = 1100$ Pa). Cooling to 5 °C results in hydrogel dissolution and G' values consistent with a low viscosity (freely flowing) solution. The temperatures used in both CD and rheology bracket the exact temperature ($T_{gel} = 60$ °C, Figure 3) at which folding and consequent self-assembly is triggered. This sol−gel transition is totally reversible with subsequent temperature cycles as shown in Figure 2b. The CD and rheology data taken together suggest a mechanism of hydrogelation
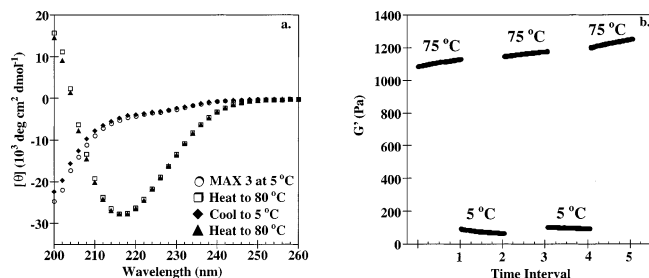
Figure 2. (a) Temperature-dependent CD of a 150 μM solution of MAX3 (125 mM Borate, 10 mM NaCl, pH 9). (b) Temperature dependency of the storage modulus (G') for a 2 wt % aqueous preparation of MAX3 under identical conditions; data were collected at the indicated temperatures for 20 min time intervals, allotting time for approximate instrumental/sample equilibrium between intervals.
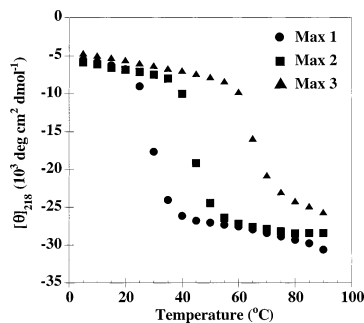

Figure 3. Temperature dependence of $[\Theta]_{218}$ for 150 μM solutions of MAX1, 2, and 3 (125 mM Borate, 10 mM NaCl, pH 9).

consistent with temperature-induced unimolecular folding followed by self-assembly, Figure 1. In fact, under identical conditions, a control sequence that disfavors intramolecular folding does not undergo hydrogelation (see below).

Because the folding and consequent self-assembly of the hairpin are partially governed by hydrophobic interactions, the exact temperature at which this transition occurs can be modulated by varying the hydrophobic character of the peptide. More hydrophobic peptides should fold and assemble at lower temperatures. This behavior is reminiscent of the ability to tune the lower critical solution temperature phase transition as demonstrated in cross-linked poly(N-alkylacrylamide) polymers[4] and elastin-like polypeptides[22] to produce responsive hydrogels. The exact temperature at which folding/self-assembly is triggered (T_{gel}), and to what extent this transition can be tuned by modulating the hydrophobicity of this class of molecules, is shown in Figure 3. Max3, which contains three threonine residues at hydrophobic positions, folds and assembles at ~60 °C. Replacing the threonine at position 7 with valine results in a slightly more hydrophobic peptide, MAX2. Relative hydrophobicities were assessed by calculating the free energy of transfer from octanol to water, Figure 1.[23] MAX2 displays a $T_{gel} \approx$ 40 °C, 20 °C less than MAX3. Finally, replacing two threonines of MAX3 (at positions 7 and 16) with valine results in the most hydrophobic peptide, MAX1. This peptide displays the lowest T_{gel} value (~25 °C). A control peptide was synthesized to verify that this family of peptides first intramolecularly folds before self-assembling into hydrogels. Control 1 (VKVKVKVKV^LPPT-KVKVKVKV-NH$_2$) is identical to MAX1 with the exception that the ^DPro at position 10 has been replaced with ^LPro. Unlike the dipeptide ^DPro^LPro contained within MAX1 which favors type II′ turn formation[17] (Φ, Ψ; 59, −136; −59, −24), the ^LPro^LPro motif of Control 1 favors an open conformation[24] (Φ, Ψ; −60, 138; −95, −7). The two strands emanating from an open ^LPro^LPro conforma-

tion would be projected in opposite directions; intramolecular folding resulting in β-hairpin would be highly unfavored, and any observable self-assembly would likely result from the direct intermolecular association of extended peptide conformers. CD of a 150 μM solution of Control 1 under folding conditions (pH 9, 45 °C) showed only random coil (Supporting Information). Continued heating to higher temperatures (~65 °C) results in the irreversible formation of sheet-rich soluble aggregates. Also, 2 wt % solutions of Control 1 failed to undergo hydrogelation, instead producing an insoluble, fibril-like precipitate. This behavior is consistent with the irreversible self-assembly of extended conformers rather than reversible, intramolecular hairpin folding and consequent gelation. The inability of the control to undergo hydrogelation demonstrates the importance of the turn sequence of MAX1, 2, and 3 in facilitating intramolecular folding prior to self-assembly. Interestingly, in contrast to MAX3, the folding transitions of MAX1 and MAX2 are not reversible on cooling within reasonable, kinetically accessible time frames. Kinetic investigations into the hysteresis of the unfolding/disassembly transition for MAX1 and 2 as well as the dependence of T_{gel} on peptide concentration of all three peptides are currently being investigated.

The thermal behavior of this class of β-hairpins demonstrates that *de novo* design can be used to construct predictably responsive materials. Because this general design links intramolecular folding to intermolecular self-assembly, environmental factors that influence molecular folding also influence material properties.

Supporting Information Available: Experimental, HPLC, MS, and CD data for all peptides and frequency sweep data for MAX3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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