Dynamic Hydrogels

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Dynamic Hydrogels: Translating a Protein Conformational Change into Macroscopic Motion**

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The development of materials that undergo shape changes occupies a central theme in materials science and has proven important in several applications. Several classes of hydrogels, which are cross-linked water-soluble polymers, can change their properties (such as, volume, cross-link density) in response to temperature, pH value, or ionic strength.[1–5] These dynamic hydrogels can also be modified with biochemical moieties to give materials that change their properties in response to proteins and ligands.[3,6] For example, hydrogels that undergo volume changes because of antigen–antibody[2] and lectin–carbohydrate[7,8] interactions have been used as biosensors. The underlying principle of operation of dynamic hydrogel materials relates to a change in their physical or chemical cross-linking density in response to environmental cues. An unexplored alternative to these approaches relies on the use of a natural protein that undergoes a conformational change as a mechanism to alter the characteristics of a material. The functional importance of protein motions in biological systems, together with the wide range of protein motions that can be harnessed, offers a flexible approach to the preparation of dynamic materials.

We describe herein an example of a protein-based dynamic material, the functional nature of which is derived from the conformational properties of the protein calmodulin (CaM). Calmodulin is a 16.5-kDa protein with two distinct conformational states (Figure 1).[9–13] In the presence of calcium ions, CaM has an extended, dumbbell-shaped conformation (herein termed "extended CaM").[14] This calcium-bound CaM undergoes a transition from an extended dumbbell to a collapsed conformation (herein termed "collapsed CaM")[15] upon the binding of ligands, which include certain antipsychotic drugs (such as, trifluoperazine (TFP)),[10,13,16] peptides,[9] and a variety of proteins[12]. Recently, Daunert and co-workers described a class of hydrogels that incorporate CaM and a small-molecule ligand as pendant moieties within the network.[17] The binding of the CaM units and ligands resulted in an increased cross-linking density and a decreased swelling of the network. This approach is analogous to the development of dynamic hydrogels based on antigen–antibody[2] and lectin–carbohydrate[7,8] interactions, but differs from our approach in that the dynamic response of the gel is not due primarily to the conformational properties of CaM.

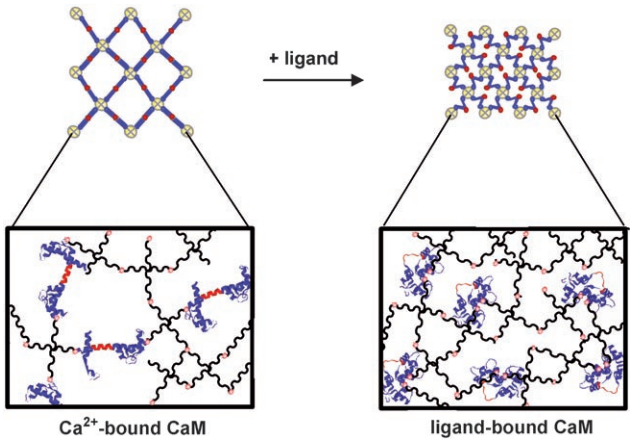

Figure 1. The two conformational states of calmodulin: an extended, dumbbell-shaped conformation in the presence of calcium ions (left), and a collapsed conformation upon binding to a ligand (right). Dark red: hinge region; blue: ends of dumbbell-shaped protein; light-red spheres: acrylate–thiol linkage; black ribbons, ⊗: poly(ethylene glycol) moiety.

We prepared an engineered version of CaM in which cysteine residues are included in place of tyrosine residues at the ends of the dumbbell-shaped protein (CaM Y34C, Y110C). The distance that separates the two cysteine residues is approximately 50 Å in the extended conformation,[18] but is decreased to approximately 15 Å in the collapsed conformation.[16] We incorporated the CaM building blocks into a poly(ethylene glycol)-based hydrogel by using a four-armed poly(ethylene glycol) (PEG) molecule terminated at each end with an acrylate group. The acrylate groups react selectively with the sulfhydryl groups on the engineered CaM and therefore serve to cross-link the CaM proteins into water-soluble conjugates. The Ellman test, which measures the amount of free sulfhydryl groups, was performed after a period of 5 minutes and showed that the reaction of the PEG tetraacrylate with the engineered CaM was complete (Figure 2a). Furthermore, MALDI-TOF mass spectrometry confirmed the conversion of the free CaM protein into cross-linked products (Figure 2b). The MALDI spectra, for example, showed a diffuse peak at ≈27 kDa, which represents a conjugate of a single PEG tetraacrylate chain (≈10.2 kDa) and a single CaM molecule (16.5 kDa). This peak is not observed when the PEG acrylate is present in a high concentration, and these data, along with the loss of the sulfhydryl groups, demonstrate the formation of higher-order conjugates by partial cross-linking. To complete the formation of the hydrogel, solutions containing conjugates of the CaM

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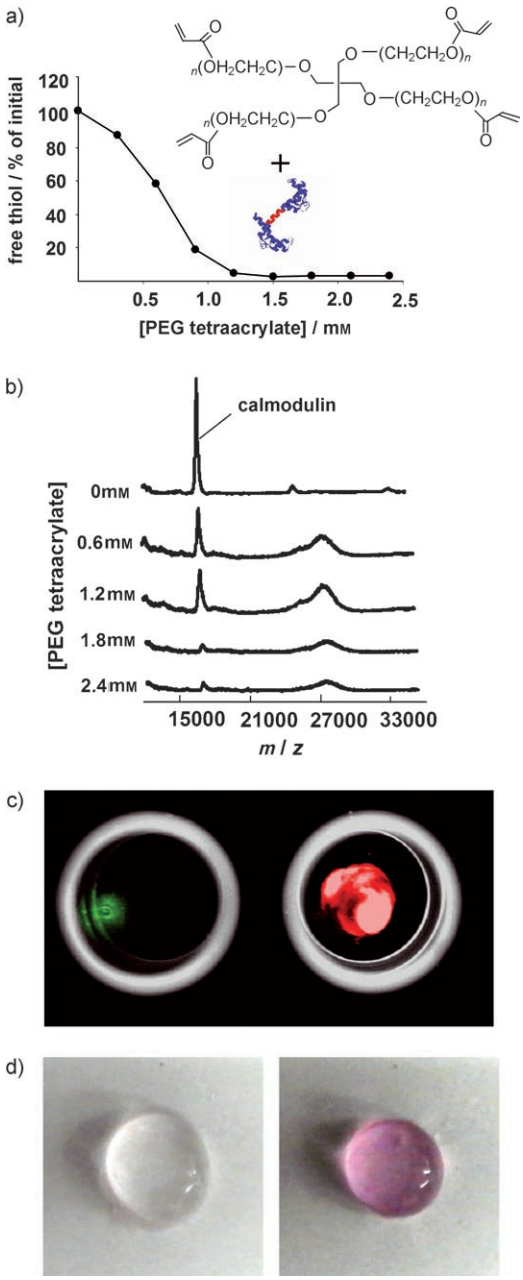

Figure 2. PEG tetraacrylate reacts efficiently with CaM (Y34C, Y110C) to allow the inclusion of functional CaM within a PEG hydrogel. a) A decrease in the amount of free sulfhydryl groups is observed with increasing concentration of the PEG tetraacrylate and indicates a Michael-type addition of the cysteine sulfhydryl group to the acrylate group. b) The reaction is confirmed by MALDI-TOF mass spectrometry, which shows a disappearance of the calmodulin peak upon the addition of the PEG tetraacrylate in increasing concentrations. c) Fluorescently labeled CaM is included within a PEG gel as a partial cross-linker (right), whereas CaM-free gels display only weak autofluorescence (left). d) The CaM included into PEG hydrogels is functional and capable of binding a labeled peptide ligand for calmodulin (right), whereas CaM-free PEG gels demonstrate no ligand binding (left).

protein and the PEG tetraacrylate were mixed with a solution of dithiothreitol (DTT) at room temperature. This procedure caused cross-linking of the remaining acrylate groups and gave a solid hydrogel. We confirmed the incorporation of CaM by repeating the assembly with a CaM protein that was labeled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Fluorescence microscopy showed that the protein was incorporated into the gel and did not diffuse out of the gel on standing (Figure 2c).

The formation of the CaM–PEG network did not compromise the function of the protein units. One measure of this function is the ability of CaM to associate with peptide ligands. We treated the CaM–PEG network with a Cy5-labeled (Amersham Biosciences) peptide ligand for CaM, ARRKWQKTGHAVRAIGRLSSC. This ligand binds to CaM with a dissociation constant of 1 nM.[9,19] After preparing the gels in the presence of the labeled ligand and washing extensively with Tris(tris(hydroxymethyl)aminomethane buffer, we found that gels containing CaM were stained red, whereas control PEG hydrogels contained no bound ligand (Figure 2d).

Of primary interest, we observed that gels prepared in the presence of the aforementioned peptide ligand had a lower volume than hydrogels without CaM included. We attribute this volume change to the distinct conformational state of the ligated CaM units. To better understand how the conformational changes of CaM affect the density of the gel, we performed the following experiments with cylindrical hydrogels (thickness: 1 mm, initial volume: 10 μL) cast between parallel glass plates. CaM-containing hydrogels were prepared in the presence of calcium ions, and therefore with CaM in an extended conformation. They underwent significant volume changes when exposed to a solution of TFP ligand (5 mM), which binds to CaM and induces the adoption of the collapsed conformation (Figure 3a,b). When the gels were washed with ethylene glycol tetraacetic acid (EGTA), which chelates calcium ions and therefore removes the calcium-dependent ligand from the gels, UV absorption showed that the total amount of TFP ligand that was released from the gels was directly correlated to the amount of CaM included during gel preparation (Figure 3c). The TFP/CaM ratio (5.4:1) indicates that included CaM binds the TFP ligand in a manner similar to that observed in previous studies of CaM–TFP binding in solution (TFP/CaM 4–7:1).[10,13,20]

To confirm that the volume changes in the hydrogel were indeed caused by the CaM and not by conformational properties of the PEG network, we prepared hydrogels in the absence of CaM. These gels exhibited no significant volume change in the presence of TFP ligand. In further control experiments, we incorporated a distinct engineered version of CaM (CaM Q41C, K75C), in which there is little difference in the distance between the two cysteine residues in extended (20 Å) and collapsed (18 Å) CaM. As expected, hydrogels containing this mutant did not undergo a volume change in response to TFP binding (Figure 3d).

The conformational changes in response to ligand binding were gradual and reversible. We created gels with extended CaM and then cycled the material between collapsed and extended conformations.[21] The volume of each gel was then analyzed at various intervals for 2 hours. These experiments showed that the gels are capable of responding to multiple cycles of CaM collapse and extension by changing their volume (Figure 4a). Importantly, these volume changes were

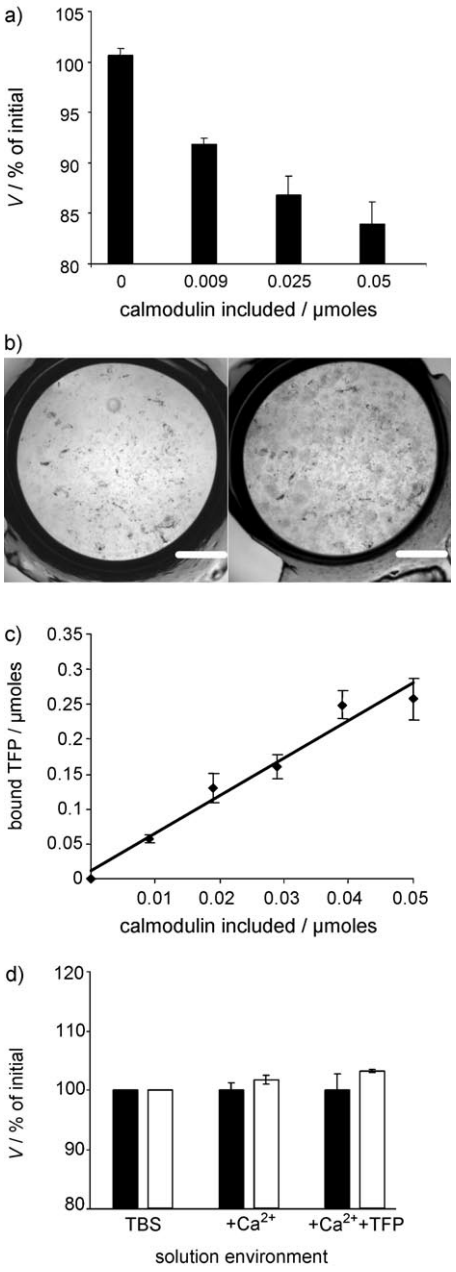

Figure 3. The volume of the hydrogel can be changed significantly by varying the conformational state of the calmodulin cross-linker, and the volume change is dependent on the total amount of CaM included in the gel. a) Hydrogels prepared with CaM in the extended state demonstrated significant changes in swelling upon the addition of TFP ligand. The magnitude of the volume change increases as the total amount of CaM included is increased. b) Shown is a hydrogel with CaM in a ligand-free state (left) and the same gel with CaM in a ligand-bound state (right); a decrease in gel volume is observed after ligand binding (scale bars: 1 mm). c) The total amount of bound TFP ligand is also dependent on the total amount of CaM included in the gel; the slope of the TFP-binding curve indicates that there are 5.4 bound TFP molecules per CaM molecule included in the gel, as expected on the basis of previous solution studies of CaM–TFP binding.[10,13,20] d) PEG hydrogels with (black bars) or without (white bars) the CaM Q41C, K75C mutant included were prepared in Tris-buffered saline (TBS) then exposed to calcium ions (extended CaM) or to both calcium ions and TFP ligand (collapsed CaM). There is no significant change in the volume of gels with the CaM Q41C, K75C mutant included, as expected on the basis of the lack of change in the Cys–Cys distance in this mutant upon ligand binding.

not dependent on the ionic strength of the media over a range of 15–150 mM NaCl (Figure 4b). These results are significant because they establish that it is possible to repeatedly cycle a bound protein between con-

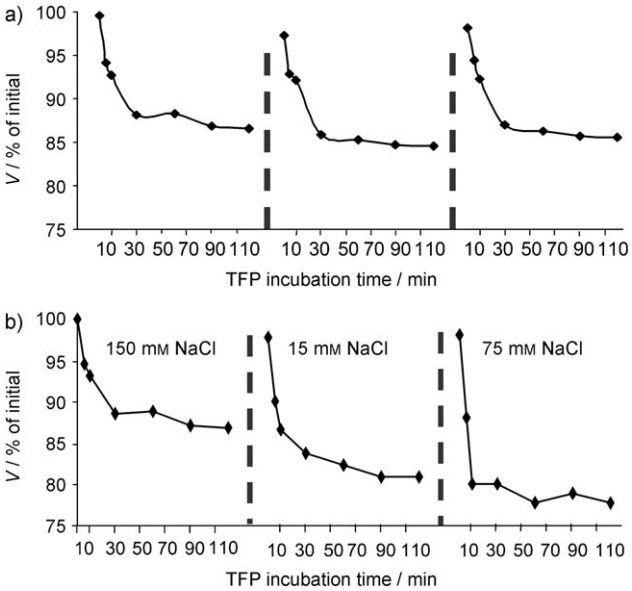

Figure 4. CaM can be repeatedly switched between extended and collapsed conformations with the corresponding changes in hydrogel volume. a) Hydrogels were exposed to TFP ligand, and the volume was measured at various intervals for 2 h. The gel was then washed repeatedly with EGTA and incubated in a calcium-containing buffer to restore the extended CaM conformation (the beginning of each new experiment with extended CaM is represented by dotted lines). Conformational changes occurred over minutes in a solution of TFP, and the initial volume of the gel was recovered completely upon removal of the ligand. b) The ionic strength of the medium surrounding the CaM-containing hydrogels did not influence substantially their change in volume in response to TFP binding.

formations to create smart materials that are "switchable". We note, however, that the magnitudes of the volume changes are probably far from optimized for this system and presumably reflect incomplete cross-linking of the proteins with the PEG molecules.

These studies have provided an early example of a bioinspired functional material that undergoes macroscopic shape changes derived from the conformational properties of their component proteins. Indeed, this concept can be extended to materials that display macroscopic changes in response to a host of stimuli. There are over 200 well-characterized protein motions,[22] and motor proteins can be engineered readily by using standard recombinant techniques. We therefore suggest that a broad range of functional materials can be engineered by creating modular proteins.

Experimental Section

Four-armed PEG molecules were derivatized with terminal acrylate groups as described previously.[23,24] Two calmodulin

mutants were constructed, expressed, and purified by routine molecular-biology techniques. To characterize the reaction of the protein and polymer, PEG tetraacrylate chains (0–2.4 mM) were treated with CaM (Y34C, Y110C; 1 mM) in Tris-HCl (20 mM) containing DTT (2 mM, pH 8.1) at 37 °C. For gel formation, solutions of CaM were treated with the PEG tetraacrylate (15 mM) for 4 h at 37 °C, followed by the addition of DTT (12.5 mM) in HEPES buffered saline (HBS; pH 7.4; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and incubation for 4–8 h at 37 °C (hydrogel equilibrium swelling ratio = 19 ± 1, which is similar to that found in previous studies[25]). For detailed analysis of CaM binding to TFP, hydrogels were prepared and swelled in HBS (pH 7.4) with $CaCl_2$ (10 mM), then incubated in a solution of $CaCl_2$ (10 mM) with TFP (5 mM). The mass was measured by removing the hydrogel from the solution, removing excess solution from the gel surface, and weighing. Mass measurements were converted into volume by considering the mass and density of the polymer, protein, and water included, and measurements were confirmed by light micrographs. After incubation in a TFP-containing solution, the gels were washed four times with EGTA. The amount of TFP removed was measured by UV absorption at $\lambda = 306$ nm, and [TFP] was determined from a standard curve that relates [TFP] to A_{306} ($R^2 > 0.99$).

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