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A Genetic Toolbox for Creating Reversible Ca^{2+}-Sensitive Materials

Shana Topp,[†,§] V. Prasad,[‡] Gianguido C. Cianci,[‡] Eric R. Weeks,[‡] and Justin P. Gallivan*[,†,§]

Departments of Chemistry and Physics and the Center for Fundamental and Applied Molecular Evolution, Emory University, 1515 Dickey Drive, Atlanta, Georgia 30322

Received June 27, 2006; E-mail: justin.gallivan@emory.edu

A major goal of polymer science is to develop "smart" materials that sense specific chemical signals in complex environments and respond with predictable changes in their mechanical properties.[1] The ability to sense and respond to environmental changes is a hallmark of living systems, which often employ proteins to recognize and relay chemical signals. While many reversible self-assembling polymers mimic or directly incorporate elements of natural biomaterials to provide mechanical strength, few of these materials capture the exquisite sensing properties of proteins.[2] Here, we describe a genetic toolbox of natural and engineered protein modules that can be rationally combined in manifold ways to create reversible self-assembling materials that vary in their composition, architecture, and mechanical properties. Using this toolbox, we created several materials that reversibly self-assemble in the presence of Ca^{2+} and characterized these materials using microrheology.

Changes in Ca^{2+} concentration mediate several biological functions, including neuronal communication and muscle contraction. Many Ca^{2+}-sensitive pathways are regulated by the protein calmodulin (CaM), which upon binding four Ca^{2+} ions, undergoes a conformational change that allows it to bind to one of over 100 different calmodulin binding domains (CBDs) within other proteins.[3] This process reverses upon removal of calcium, and it is well characterized both structurally and biochemically.[3]

Ehrick et al.[4] recently created a Ca^{2+}-sensitive hybrid hydrogel by a free-radical copolymerization of a chemically modified CaM with acrylamide, bisacrylamide, and an acryloyl-substituted phenothiazine. Removal of Ca^{2+} disrupted physical cross-links between the pendent CaM and phenothiazine groups and swelled the gel. While some bulk properties of these materials could be tuned by adjusting the monomer stoichiometries, the placement of functional groups in a given polymer chain is essentially random. Moreover, because the binding of phenothiazine to CaM mediates the Ca^{2+} response, opportunities for systematic tuning may be limited to subtle modifications of the phenothiazine scaffold.

We anticipated that a fully genetic approach would provide several important advantages to creating and systematically studying Ca^{2+}-sensitive self-assembling materials. First, genetic engineering provides precise control over the composition of a material. Second, gene sequences are highly modular and amenable to rapid combinatorial materials synthesis. Finally, genetic engineering provides access to the great diversity of natural and engineered CBDs, which can bind to CaM as monomers or dimers, with affinities spanning 5-orders of magnitude ($K_d = 10^{-7}-10^{-12}$ M), and with varying Ca^{2+}-dependencies.[3] This diversity enables systematic variations in network topology, junction strength, and Ca^{2+} sensitivity to create materials with new properties.

To build reversible Ca^{2+}-sensitive networks, we created a genetic toolbox (Figure 1) composed of several modules that were chosen

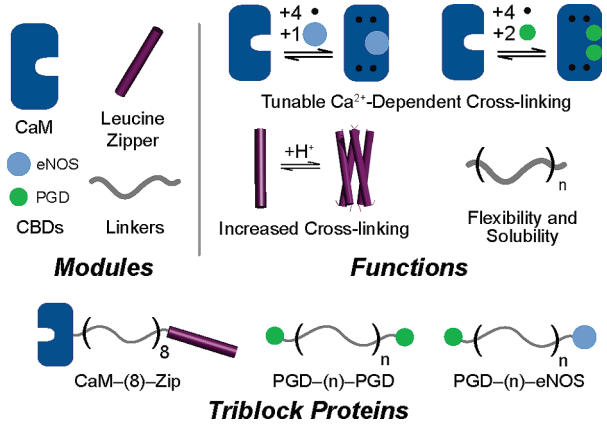

Figure 1. Each module in the genetic toolbox encodes a specific function. Modules can be combined in many ways to produce functional materials. The triblock protein architectures shown are studied here.

to perform specific functions in a material. To form Ca^{2+}-sensitive junctions, CaM was used in tandem with either the CBD from human endothelial NO synthase,[5] which binds as a monomer ($K_d \approx 3 \times 10^{-9}$ M, eNOS, light blue), or with the CBD from petunia glutamate decarboxylase,[6] which binds as a dimer ($K_d \approx 2 \times 10^{-8}$ M, PGD, light green). A previously described tetrameric leucine zipper domain[7] (Zip) and hydrophilic protein sequence[7] ($(AG)_3$-$PEG)_n$ were used to provide additional branching and to link components together, respectively. Genes encoding the modules were constructed with compatible restriction sites to allow rapid assembly.[8] Full-length genes were expressed in *E. coli*, and the resulting proteins were purified in high yields (typically >100 mg/L; see Supporting Information).

While this toolbox enables the construction of many modular proteins, we studied the three classes of triblock proteins shown in Figure 1. The first (CaM-(8)-Zip) is comprised of CaM linked to a leucine zipper domain via the sequence $((AG)_3PEG)_8$. The second (PGD-(*n*)-PGD) contains two copies of the PGD sequence (which binds to CaM as a dimer) that flank a variable number of repeats of the hydrophilic linker ($n = 8$, 40). The third (PGD-(*n*)-eNOS) is composed of the PGD sequence, a hydrophilic linker ($n = 8$, 40), and the eNOS sequence that binds to CaM as a monomer.

To create a highly branched Ca^{2+}-sensitive network (Figure 2A), we mixed equimolar solutions of CaM-(8)-Zip and PGD-(8)-PGD (2 mM, pH 6.0) and used particle-tracking microrheology[9] to measure the viscosity of the solution. In the absence of Ca^{2+}, this sample behaves as a relatively nonviscous liquid (Figure 2B). Addition of Ca^{2+} (30 mM, ~4 equiv/binding site) rapidly caused a 5000-fold increase in viscosity, which was fully reversible upon addition of the Ca^{2+} chelator EDTA. In addition to quantifying the extreme viscosity of these materials ($>10^5 \times \eta_{water}$, Figure 2B), the microrheology data show that the tracer particles embedded in the samples were motionless within the resolution limit on short time scales (<10 s); such behavior has previously been attributed

[†] Department of Chemistry.
[‡] Department of Physics.
[§] Center for Fundamental and Applied Molecular Evolution.

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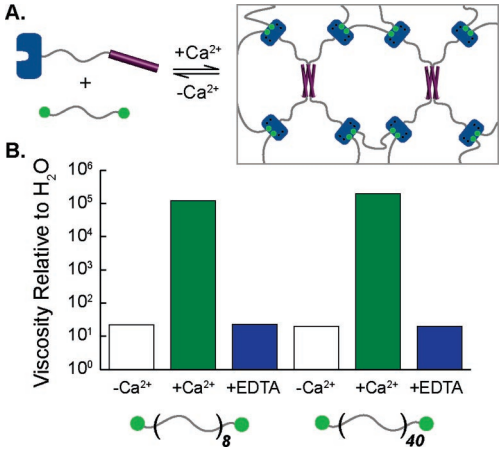

Figure 2. (A) Idealized representation of network formation between an equimolar mixture of PGD-(*n*)-PGD and CaM-(*n*)-Zip; (B) viscosities of equimolar mixtures of PGD-(*n*)-PGD and CaM-(8)-Zip in the absence of Ca^{2+}, the presence of Ca^{2+} (30 mM), and after Ca^{2+} chelation by EDTA.

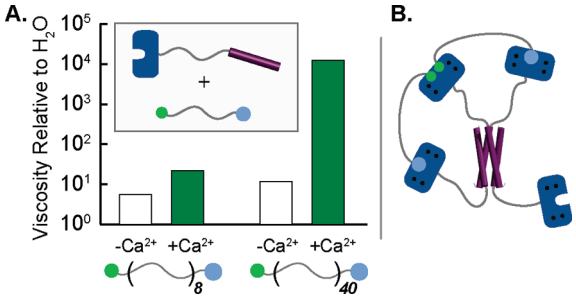

Figure 3. (A) Viscosities of stoichiometric mixtures of PGD-(*n*)-eNOS and CaM-(8)-Zip in the absence and presence of Ca^{2+}; (B) model for capping of CaM-(8)-Zip with the short PGD-(8)-eNOS cross-linker.

to hydrogel formation.[7b,9b] In parallel experiments with the longer PGD-(40)-PGD cross-linker, similar behaviors were observed (Figure 2B).

Substituting Mg^{2+} for Ca^{2+} produced only a 3-fold increase in viscosity, reflecting both the selectivity of CaM and the absence of nonspecific ionic interactions. Solutions lacking cross-linker or in which CaM-(8)-Zip was replaced with a CaM-(8) diblock did not form networks in the presence or absence of Ca^{2+}, indicating the importance of these domains (Supporting Information).

Compared to samples containing PGD-(*n*)-PGD, samples containing PGD-(*n*)-eNOS were less viscous and showed more complicated behavior in the presence of Ca^{2+} (Figure 3A). The PGD-(8)-eNOS cross-linker effected only trivial Ca^{2+}-dependent viscosity increases, consistent with a model (Figure 3B) in which the eNOS modules of two different cross-linkers bind quickly and tightly to two of the CaM binding sites of a CaM-(8)-Zip tetramer. This binding increases the effective concentration of the two pendent PGD modules near the remaining CaM binding sites, and the highly cooperative nature of PGD binding favors binding to one of these sites, which precludes extended network formation. Providing further support for this model, the longer PGD-(40)-eNOS cross-linker effected a significant (1000-fold) Ca^{2+}-dependent viscosity increase (Figure 3A), which was fully reversible by EDTA (not shown). The viscosity increase likely results from a reduced propensity for intramolecular cyclization and an increased probability of intermolecular cross-linking.

In conclusion, we used a genetic toolbox comprising natural and engineered protein modules to create several materials that form reversible networks in the presence of Ca^{2+}. By choosing appropriate modules on the basis of their dilute solution behavior, we rationally controlled the architectures, sensing behavior, and rheological properties of these materials. The modular nature of this system is highly amenable to creating precisely tuned materials through variations in composition, network architecture, and junction strength, which provides an excellent model system for our ongoing investigations into the fundamental nature of the gel transition. These efforts are greatly facilitated by the diversity of natural and engineered CBD sequences and CaM variants with altered binding affinity or ion sensitivity. We anticipate that this modular framework, in which a natural protein sensor directly mediates self-assembly, can be extended to create a variety of new sensing materials using modules from the diverse array of signaling pathways that are mediated by reversible protein−protein interactions. Because these materials incorporate elements that evolved to sense specific biological signals in complex, physiologically relevant environments, they may be useful in the fields of tissue engineering and controlled drug delivery.

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Supporting Information Available: Experimental procedures, additional supporting figures, and video of particle tracking experiments (qt). This material is available free of charge via the Internet at http://pubs.acs.org.

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