Supporting Information for

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

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Experimental Details

Materials and General Considerations.

Synthetic oligonucleotides were purchased from Integrated DNA Technologies. Lysozyme, PMSF, and DTT were obtained from Sigma. Antibiotics, $CaCl_2$, $MgCl_2$, and EDTA were purchased from Fisher. Tris base, Tris-HCl, NaCl, and culture media were purchased from EMD Bioscience. IPTG was obtained from US Biological. Imidazole was obtained from Fluka. DNaseI was purchased from Roche. Nickel-agarose resin was obtained from GBioscience. DNA polymerase and restriction enzymes were purchased from New England Biolabs. Purifications of plasmid DNA, PCR products, and enzymatic digestions were performed using kits purchased from Qiagen. Centrifugal concentrating devices were obtained from Millipore. Yellow-green fluorescent (505/515) carboxylate-modified polystyrene spheres (0.5 μm diameter) for particle-tracking microrheology were purchased from Molecular Probes. The gene encoding chicken calmodulin was kindly provided by Prof. A. R. Means (Duke University).

Plasmid manipulations were performed using *E. coli* TOP10F' cells (Invitrogen) that were transformed by electroporation. All new constructs were verified by DNA sequencing at the NSF-supported Center for Fundamental and Applied Molecular Evolution at Emory University.

Gene Construction.

Genes encoding the $((AG)_3PEG)_2$ linker, the leucine zipper, PGD CBD, and eNOS CBD were constructed by the extension of overlapping synthetic oligonucleotides with DNA polymerase to create modules that contained restriction sites compatible with "seamless" ligation.[S1,S2] Digestion with appropriate enzymes (typically *Bbs*I or *Bsm*BI) produced fragments that lacked the recognition site and could be subcloned into plasmids with compatible sticky ends to produce the desired in-frame protein sequences. The linker sequences $((AG)_3PEG)_8$ and $((AG)_3PEG)_{40}$ were created in pUC18 by a seamless recursive directional ligation approach beginning with a synthetic sequence encoding the homodimeric sequence $((AG)_3PEG)_2$.

To create vectors for protein expression, we created three triblock cassettes (CaM-(n)-Zip, PGD-(n)-PGD, and PGD-(n)-eNOS), in which a sacrificial DNA sequence was incorporated as the central block. These cassettes were subcloned into a derivative of the pET14b expression vector (Novagen) that introduced an N-terminal hexahistidine tag. Digestion of these vectors with *Bsm*BI excised the sacrificial sequence and revealed sticky ends that were compatible with linker sequences digested with *Bbs*I and *Bsm*BI. This modular strategy allowed various linkers to be cloned directionally and seamlessly to give genes encoding the desired triblock proteins in a single step.

Protein Expression and Purification.

For protein expression, *E. coli* BL21(DE3)/pLysS cells (Novagen) were transformed by electroporation with the desired vector, plated onto selective LB/agar containing ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL), and grown overnight at 37 °C. Single colonies were used to inoculate 5 mL starter cultures of selective LB broth containing ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL), which were grown overnight with shaking (300 rpm, 37 °C) and then used to inoculate 250 mL or 600 mL of selective TB containing ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL) which were grown with shaking (300 rpm, 37 °C). Overexpression of each protein from the T7 promoter was induced by the addition of IPTG (1 mM final concentration) during exponential growth phase (OD_{600} 0.65-0.85). Cultures were chilled on ice after approximately 3 hours, and cell pellets were harvested

[S1] Padgett, K. A.; Sorge, J. A. *Gene* **1996**, *168*, 31-5.
[S2] Goeden-Wood, N. L.; Conticello, V. P.; Muller, S. J.; Keasling, J. D. **2002**, *3*, 874-879.

by centrifugation at 2 °C. Pellets were washed with lysis buffer (50 mM Tris-Cl, 300 mM NaCl, 5 mM imidazole, 0.03 mM EDTA, 1 mM PMSF, pH 7.4), repelleted by centrifugation at 2 °C, and stored at -80 °C.

Pellets were thawed on ice for 10 min, lysis buffer (15-20 mL) containing lysozyme (1 mg/mL) was added, and the suspensions were homogenized by sonication. Following sonication, DNaseI was added to a concentration of 5 μg/mL, the mixture was incubated on ice for 10 min, and the lysate was centrifuged at 2 °C.

The cleared lysate was applied to a column containing nickel resin that was pre-equilibrated with lysis buffer. After all lysate had entered the resin, the column was washed with additional lysis buffer (5 column volumes), followed by wash buffer (5 column vol., 50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 0.03 mM EDTA, pH 7.4). Finally, the column was washed with elution buffer (10–12 mL, 50 mM Tris-Cl, 300 mM NaCl, 200 mM imidazole, 0.03 mM EDTA, pH 7.4), and proteins were collected in 1.5 mL fractions that were analyzed by SDS-PAGE with Coomassie Blue staining. Fractions containing pure proteins were combined and concentrated using 4 mL or 15 mL centrifugal concentrating devices with appropriate molecular weight cutoffs. Several cycles of dilution with sample buffer (25 mM Tris-Cl, 0.05 mM EDTA, 1 mM DTT, pH 7.0) and concentration exchanged the buffers. The concentration of each protein solution was determined by absorbance at 280 nm.

Microrheology Studies.

Proteins (final concentration ~2 mM) were mixed for each experiment, and HCl (1 N) was added to each protein mixture to lower the final pH to 6.0 for each sample. Fluorescent polystyrene spheres (0.5 μm) were added to obtain a final concentration of ~10^7 particles/μL of sample, which resulted in 50-100 particles in the field of view at 100x magnification. Samples were mixed by stirring and were transferred into a chamber (15 mm x 4 mm x 340 μm) constructed with a glass slide and cover slips. Samples were imaged in two dimensions (93 nm/pixel resolution, 30 Hz frame rate) using an inverted microscope (Leica) equipped with a mercury arc lamp and a 100X objective (N.A. = 1.4). In all experiments, data were collected far from the glass surfaces to eliminate wall effects.

To measure cation-dependent viscosity changes, stock solutions of either $CaCl_2$ or $MgCl_2$ (1 M) were added to the samples to give a final concentration of 30 mM (~4 equivalents per Ca^{2+} binding site). For samples in which the addition of the ion did not visibly alter particle diffusion, data were collected after 15 min (6 Hz frame rate). For samples in which the diffusion of the particles slowed dramatically, we could typically observe the Ca^{2+} front in real-time (see Movie Links and Figure S1), and data were collected after 60 min (2 Hz frame rate). The reversible behavior of the Ca^{2+}-dependent systems was probed by the addition of excess EDTA, which rapidly diffused through the samples (see Movie Links); data were collected after the sample appeared homogeneous.

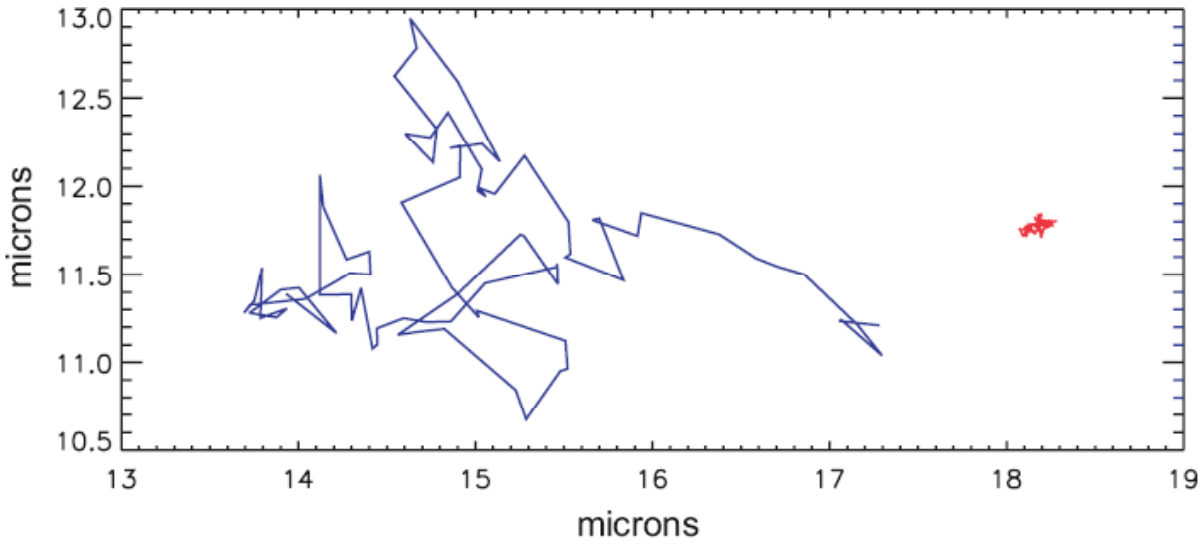

Figure S1. The trajectories of two particles on opposite sides of the diffusing Ca^{2+} front produced by the addition of Ca^{2+} to an equimolar mixture of CaM-(8)-Zip and PGD-(40)-PGD. The duration of the trajectories is 12 s. The particle at left (blue) is in a region where the sample is still fluid, while the particle at right (red) is in a region where Ca^{2+} ions have induced polymerization and increased the local viscosity by approximately 500 times. Both trajectories were observed simultaneously approximately 30 μm apart; they are artificially displaced to be close to one another in the figure.

Images were analyzed using macros within the Interactive Data Language (IDL), whereby particles were identified and their positions tracked as a function of time.[S3] The mean-square displacements (MSDs) of the particles were extracted from their positions (as shown in Figures

[S3] Crocker, J. C.; Grier, D. G. *J. Coll. Interface Sci.* **1996**, *179*, 298.

S2 and S3). The viscosities of the samples were determined by extrapolating the long lag time linear behavior of the MSDs using the Stokes-Einstein relation.

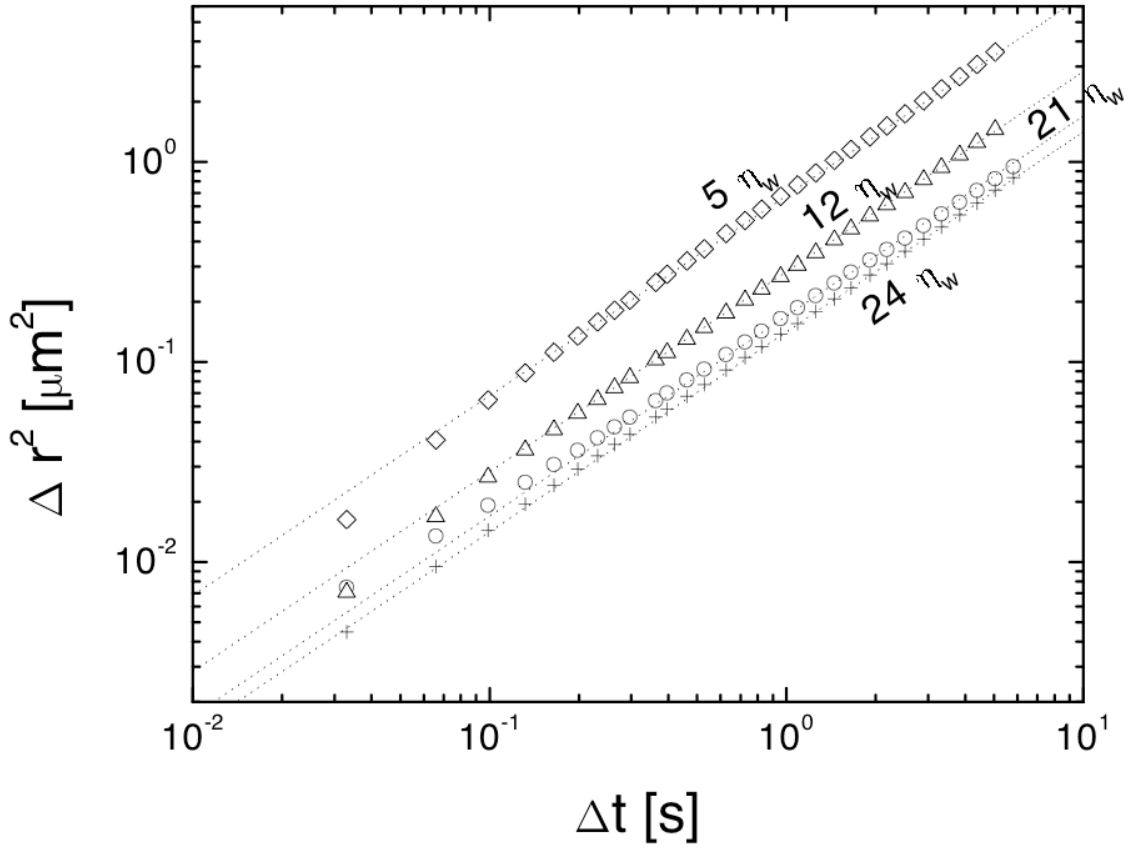

Figure S2. Mean-square displacements versus lag time for samples before addition of Ca^{2+}. Lines with a slope equal to unity on the log-log plot were fitted to the data to calculate the viscosities relative to water.
◇ = CaM-(8)-Zip + PGD-(8)-eNOS; △ = CaM-(8)-Zip + PGD-(40)-eNOS;
+ = CaM-(8)-Zip + PGD-(8)-PGD; ○ = CaM-(8)-Zip + PGD-(40)-PGD.

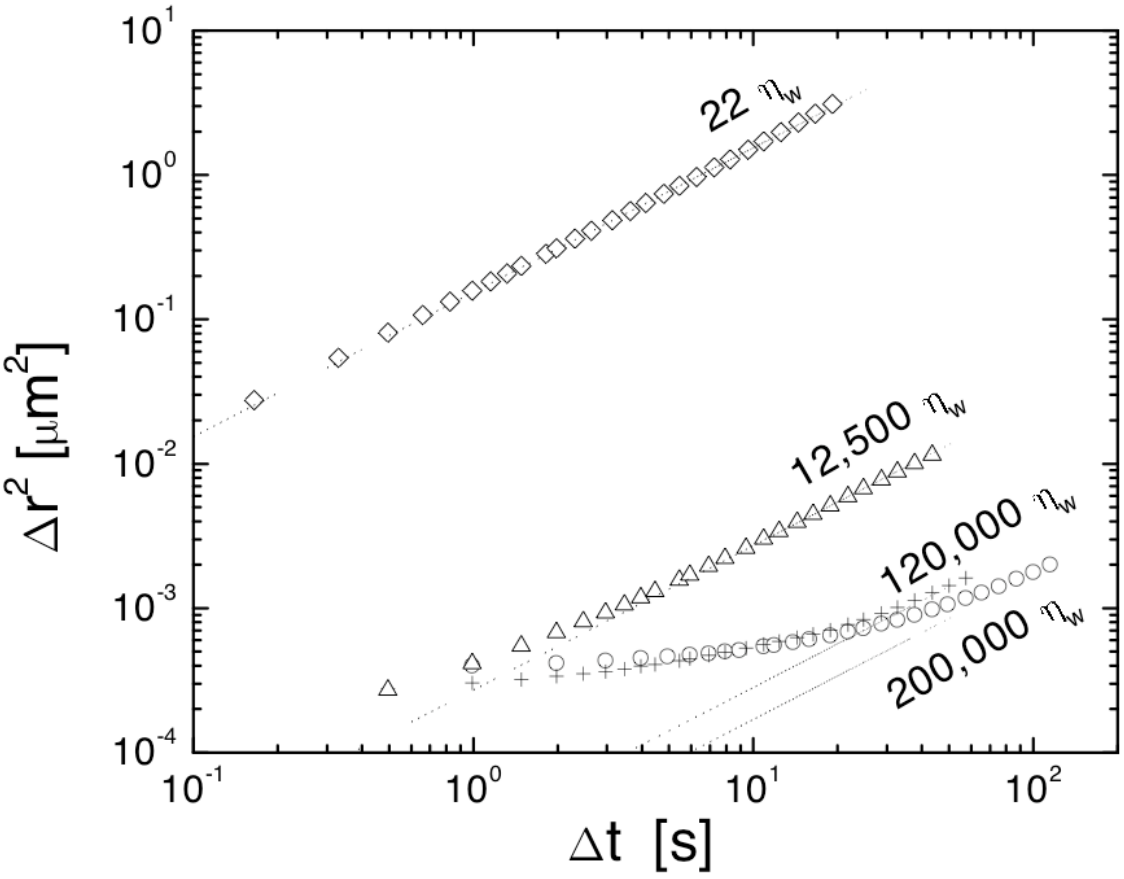

Figure S3. Mean-square displacements versus lag time for samples after addition of Ca^{2+}. Lines with a slope equal to unity on the log-log plot were extrapolated from the data at long lag times to calculate the viscosities relative to water.
◇ = CaM-(8)-Zip + PGD-(8)-eNOS; △ = CaM-(8)-Zip + PGD-(40)-eNOS;
+ = CaM-(8)-Zip + PGD-(8)-PGD; ○ = CaM-(8)-Zip + PGD-(40)-PGD.

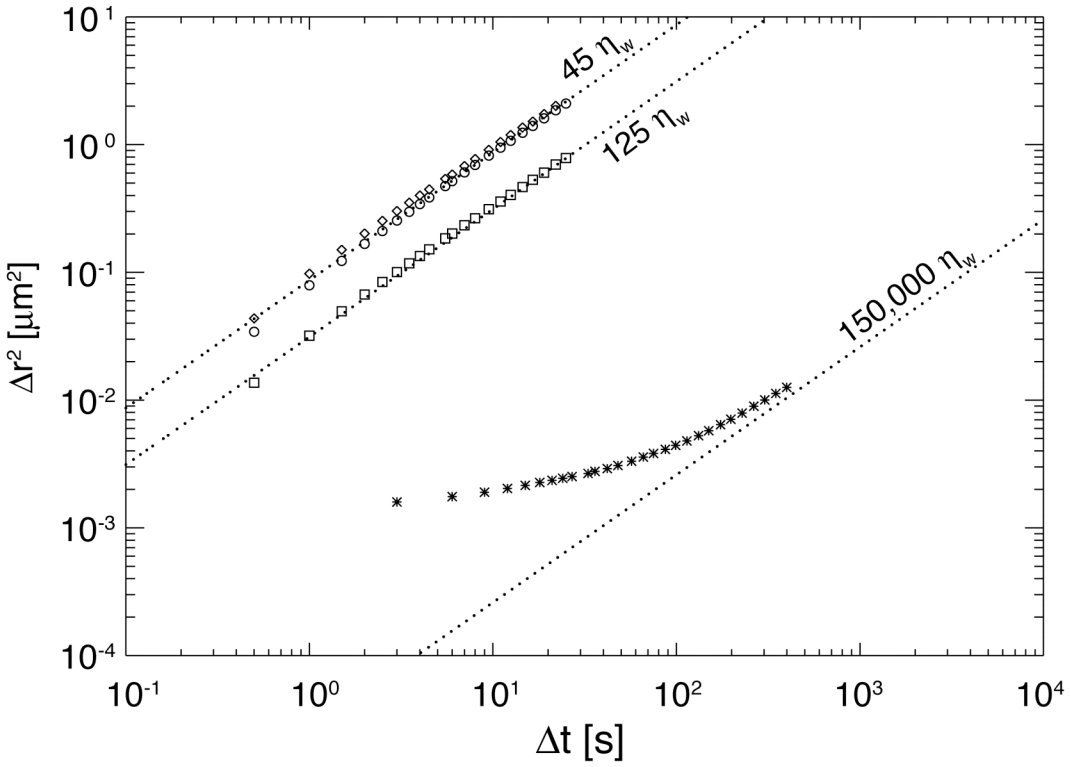

Figure S4. Mean-square displacements versus lag time for samples of CaM-(8)-Zip + PGD-(8)-PGD (each 2.8 mM) before addition of Ca^{2+} (○); after addition of Ca^{2+} (✳); after addition of Ca^{2+}, followed by EDTA (◇); and after addition of Mg^{2+} (□). Lines with a slope equal to unity on the log-log plot were extrapolated from the data at long lag times to calculate the viscosities relative to water.

Additional Control Experiments

To further establish that the observed viscosity changes were the result of the specific assemblies described in the main text and not other less-specific interactions, we performed several additional control experiments. To confirm the essential nature of the leucine zipper domains, we prepared mixtures analogous to those in Figure 2A, but with the Zip domain deleted (PGD–(8)–PGD + CaM–(8)). Addition of Ca^{2+} to this solution produced no detectable change in viscosity (Figure S5A).

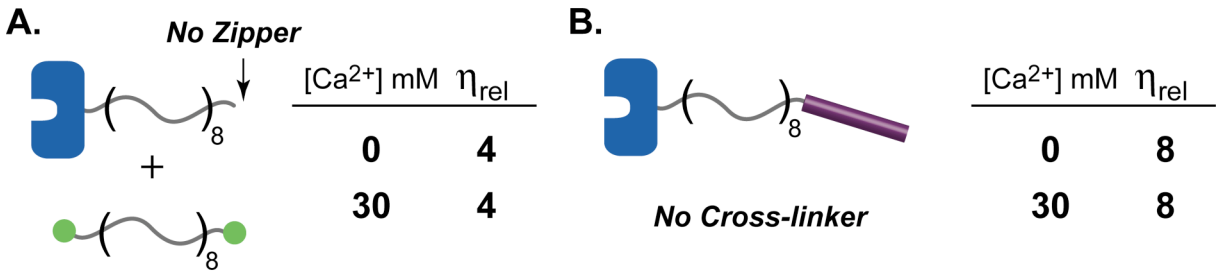

Figure S5. A. An equimolar mixture of CaM-(8) + PGD-(8)-PGD (each 1.7 mM) does not show significant changes in viscosity upon addition of Ca^{2+} (30 mM) indicating the importance of the zipper domain. **B.** A solution of CaM-(8)-Zip (2.6 mM) does not show significant changes in viscosity upon addition of Ca^{2+} (30 mM) indicating the necessity of the cross-linker for extended network formation. Viscosities are reported relative to H_2O.

To establish the role of the bifunctional cross-linker, we prepared a solution of CaM-(8)-Zip that lacked the cross-linker. Addition of Ca^{2+} had no significant effect on viscosity, which indicates the necessity of the cross-linker (Figure S5B).

Addition of Ca^{2+} to solutions of the cross-linkers alone induces aggregation of the anionic colloidal particles used for the particle tracking; this effect is not observed in solutions containing CaM, which presumably results from its high affinity for Ca^{2+}. Although this aggregation prevented us from obtaining microrheological data, visual analysis revealed that these samples acted like liquids (the solutions could be pipetted easily in the presence of Ca^{2+}, whereas the samples reported in Figures 2 cannot be pipetted in the presence of Ca^{2+}). We note that cationic microspheres are available, however, our protein linkers are polyanions and we wished to avoid non-specific charge-charge interactions with the microspheres.

Protein Sequences

MH_6-**CaM-**(8)-*Zip*

MHHHHHH**AADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFL**
TMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMT
AK(AGAGAGPEG)$_8$*AGSGDLENEVAQLEREVRSLEDEAAELEQKVSRLKNEIEDLKAE*

MH_6-**PGD-**(n)-**PGD**

MHHHHHH**AGHKKTDSEVQLEMITAWKKFVEEKKKK**(AGAGAGPEG)$_n$**AGHKKTDSEVQLEMITAWKKFVEEKKKK**

MH_6-**PGD-**(n)-*eNOS*

MHHHHHH**AGHKKTDSEVQLEMITAWKKFVEEKKKK**(AGAGAGPEG)$_n$*AGRKKTFKEVANAVKISASLMGAERLI*