Mechanobiochemistry

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Mechanically Modulating the Photophysical Properties of Fluorescent Protein Biocomposites for Ratio- and Intensiometric Sensors**

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Abstract: *Mechanically sensitive biocomposites comprised of fluorescent proteins report stress through distinct pathways. Whereas a composite containing an enhanced yellow fluorescent protein (eYFP) exhibited hypsochromic shifts in its fluorescence emission maxima following compression, a composite containing a modified green fluorescent protein (GFPuv) exhibited fluorescence quenching under the action of mechanical force. These ratio- and intensiometric sensors demonstrate that insights garnered from disparate fields (that is, polymer mechanochemistry and biophysics) can be harnessed to guide the rational design of new classes of biomechanophore-containing materials.*

Polymer mechanochemistry[1–4] is a rapidly growing field of study wherein mechanical energy is harnessed to drive useful chemical transformations,[5–11] many of which are otherwise inaccessible. Apart from their fundamental interest, mechanochemical phenomena can be applied toward the development of novel stress-sensing materials with the capacity to report damage in a qualitative as well as quantitative manner. Salient examples of mechanically facilitated transformations that have been exploited within such materials include the electrocyclic ring opening of spiropyran derivatives,[12–14] formal [4+2] cycloreversions of anthracene derived Diels–Alder adducts,[15,16] and formal [2+2] cycloreversions of 1,2-dioxetanes.[17] Collectively, these systems report stress either through mechanochromism[12–14] or mechanoluminscence,[16,17] which enables quantification of mechanical damage using standard optical spectroscopies. Unfortunately, such stimulus responsive materials typically require tedious chemical syntheses; consequently, tuning their mechanochemical reactivity (for example, through chemical diversification of mechanically labile scaffolds) can present a significant impediment to the development of new force-responsive sensors.

Recently, attention has been directed toward harnessing the biosynthetic machinery of living organisms to access mechanically sensitive biomolecules (that is, biomechanophores).[18] While nature is replete with examples of force-responsive systems,[19] there is a relative dearth of reports wherein biomolecules are used for mechanochemical applications. Indeed, although the modulation of enzymatic activity through mechanical stress has been reported,[20–23] few efforts have been directed toward developing biomechanophores that report mechanical stress through optical output. We envisioned that polymeric materials containing fluorescent proteins could serve as new classes of stress-sensing biocomposites. Fluorescent proteins,[24] which are ubiquitous within the purview of the biochemical sciences, can be modified by site-selective mutagenesis[25] to precisely alter their structural and photophysical properties. Additionally, fluorescent proteins have been extensively optimized to achieve high stability and high levels of recombinant overexpression. The photophysical properties of the canonical fluorescent protein, green fluorescent protein (GFP), stem from a 4-(*p*-hydroxybenzylidene)imidazolidin-5-one chromophore located in the center of the β-barrel structure of the protein.[24] Genetic mutations that alter the structure of the chromophore (as in the case of cyan fluorescent protein or blue fluorescent protein) or local residues that impact the stereoelectronic environment surrounding the chromophore (as in the case of yellow fluorescent protein) give rise to a vibrant array of proteins with unique emissive properties.[24]

The fluorescence of all photoemissive protein variants is highly dependent on proper folding of the protein;[24,26,27] as such, mechanical perturbation of the β-barrel structure results in modulation of any associated photophysical properties.[28–30] Yellow fluorescent protein (YFP) is particularly attractive for use as a biomechanophore, as the yellow fluorescence results from a weak arene interaction between the chromophore and tyrosine 203 (mutated from threonine in the parent GFP).[26] Gruner and co-workers reported that pressurized crystals of the YFP variant, citrine, exhibited a gradual hypsochromic shift in fluorescence as the pressure

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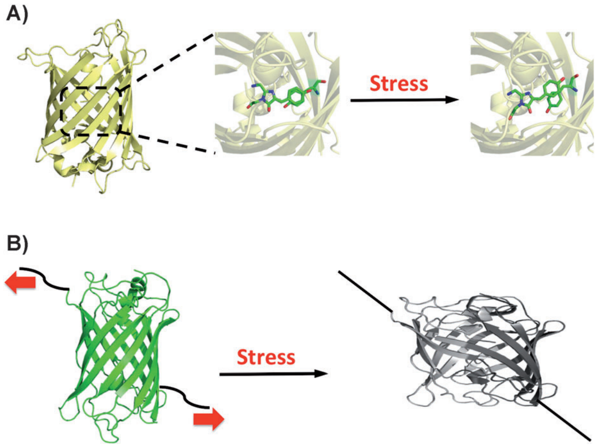
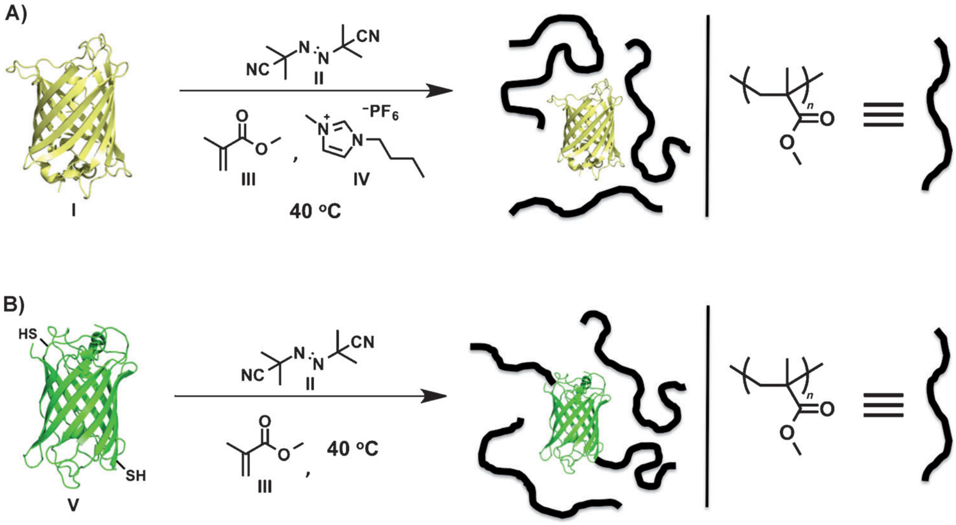
was increased from 0 to 360 MPa at low temperatures (77 K).[31,32] The fluorescence was subsequently found to bathochromically shift upon reducing the pressure and warming the crystals to 180 K. While this work clearly revealed the potential to develop YFP as a mechanosensor, there have been no reports to date wherein an analogous modulation of YFP fluorescence was harnessed for applications in mechanically responsive materials. Furthermore, surprisingly few efforts have been directed toward developing stress-sensing materials that employ fluorescent proteins. For example, Clark and co-workers utilized Förster resonance energy transfer (FRET) between YFP and cyan fluorescent protein (CFP) to develop stress-reporting poly(acrylamide) composites.[33,34] Stretching these materials under uniaxial strain resulted in increased FRET interactions between YFP and CFP near microscopic cracks that formed within the material, as determined by fluorescence confocal microscopy and fluorescence lifetime imaging (FLIM).[34] Bruns and co-workers more recently reported that eYFP could serve as a mechanically sensitive link between glass substrates and epoxy resins, where delamination of the resin resulted in denaturation of the protein and subsequent fluorescence quenching.[35] While these examples elegantly demonstrated that fluorescent proteins could be adapted for applications in stress reporting, there have been no reports showcasing diverse and tunable mechanochemical responses from biocomposite materials containing fluorescent proteins, features expected to be valuable for the design of precisely tailored force-sensing materials. Herein we present the facile preparation of biocomposite materials containing either: 1) an enhanced YFP (eYFP[36]) that exhibits shifts in λ_{em} under mechanical stress; or 2) a genetically modified GFP (GFPuv[37,38]) that exhibits fluorescence quenching under the action of mechanical force.

As eYFP was predicted to exhibit greater mechanical sensitivity than GFPuv,[29] our initial efforts were directed toward the development of eYFP-containing biocomposites. We reasoned that embedding eYFP within a polymeric matrix and subjecting the resulting material to bulk compression would elicit the desired photophysical modulation, as local areas of high pressure generated during material compression could disrupt the arene interaction responsible for yellow fluorescence by subtle distortions of the protein structure (Scheme 1).



Scheme 1. Representations of proposed mechanical activations of fluorescent proteins. A) Compression of composite materials containing eYFP distorts the arene interaction between the chromophore and tyrosine 203. B) The incorporation of cysteine residues at strategic sites in GFPuv facilitates the covalent attachment of polymer chains to the protein; subsequent compression of the composite mechanically denatures GFPuv and quenches the protein fluorescence.

To test the aforementioned hypothesis, we overexpressed hexahistidine-tagged eYFP in *E. coli* BL21 (DE3) and subsequently purified the isolated protein by nickel affinity chromatography. As summarized in Scheme 2, poly(methyl methacrylate) (PMMA) composites were prepared by adding eYFP (**I**) directly to a mixture of methyl methacrylate (**III**), azobisisobutyronitrile (AIBN, **II**), and the plasticizer, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM-PF_6, **IV**), at 40 °C. The stability of eYFP under these relatively harsh conditions was remarkable, and the composite materials isolated following consumption of the free monomer exhibited strong fluorescence (λ_{ex} = 485 nm; λ_{em} = 540 nm; Φ = 0.64) and uniform protein distribution. Although significant denaturation of eYFP was



Scheme 2. Synthesis of fluorescent protein biocomposites. A) General conditions: eYFP (**I**; 1.0 equiv), AIBN (**II**; 1.3×10^3 equiv), MMA (**III**; 2.6×10^5 equiv), and BMIM-PF_6 (**IV**; 3.1×10^4 equiv) were combined in a single vessel under N_2 and heated to 40 °C. B) General conditions: GFPuv(Y39C/D103C) (**V**; 1.0 equiv), AIBN (**II**; 1.3×10^3 equiv), and MMA (**III**; 2.6×10^5 equiv) were combined in a single vessel under an N_2 atmosphere and heated to 40 °C.

observed upon dissolution of the isolated biocomposites in tetrahydrofuran (as evidenced by fluorescence quenching), this obstacle was circumvented by cutting and polishing the materials to afford specimens with defined geometries. The BMIM-PF_6 additive, which is known to serve as a highly effective plasticizer of acrylate-derived polymers,[39] allowed precise modulation of the physical properties exhibited by the composites. Specifically, the addition of BMIM-PF_6 enabled the glass transition (T_g) of PMMA to be reduced to approximately 40 °C (as determined by differential scanning calorimetry), which was found to be beneficial for sample processing.

To explore the mechanical sensitivity of our composite materials, a 50 mg sample was mounted in a hydraulic press and subjected to compression at incrementally increasing pressures (0–360 MPa) for periods of 45 s, after which time the solid-state fluorescence was measured. As shown in Figure 1 A, the λ_{em} of the sample gradually shifted from 539 nm at 0 MPa to approximately 534 nm at 360 MPa (a hypsochromic shift commensurate with that previously reported by Gruner[32]). Frictional heating during compression appeared to contribute the overall response of the material through thermal denaturation of the protein (as evidenced by a reduction in fluorescence intensity; see the Supporting Information). Importantly, though, the observed change in λ_{em} correlated monotonically with the applied force and was, thus, consistent with a mechanical process.[1–4] Compressing the composites for 1 h did not cause their λ_{em} to shift beyond what was measured after compression for 45 s at the same pressure. Collectively, these data suggested to us that mechanical forces generated upon compressing the composites were inducing subtle distortions of the protein chromophore.

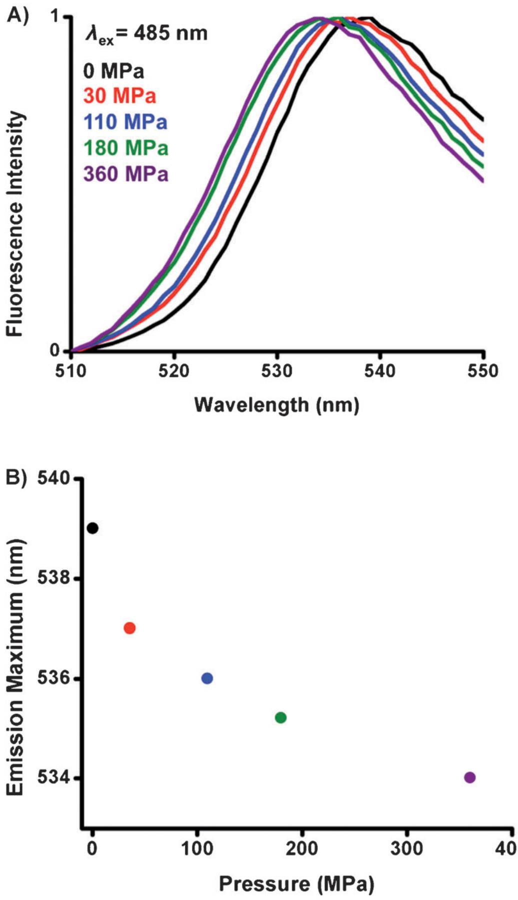


Figure 1. A) Compression of PMMA composites containing eYFP caused the λ_{em} to gradually undergo a hypsochromic shift. Normalized fluorescence intensities are shown. B) The fluorescence maxima of the compressed PMMA composite containing eYFP plotted as a function of applied pressure.

Having established a straightforward preparation of ratiometric stress sensors, we sought to explore the mechanochromism of another fluorescent protein to realize an intensiometric stress reporter. Such reporters are desirable because they exhibit changes in their optical properties that often facilitate rapid assessment of mechanical damage. As mechanical unfolding of GFP has previously been shown to quench the protein's fluorescence,[28] efforts were directed toward expanding the results of these atomic force microscope (AFM) pulling experiments to bulk materials. While the mechanical response of eYFP presumably resulted from the distortion of a weak, local interaction, we reasoned that modulation of the photophysical properties of GFP would involve more global phenomena (that is, complete denaturing of the protein); thus, we surmised that significantly higher forces would be required to mechanically denature the protein. As such, we hypothesized that GFP would need to be chemically cross-linked to the polymer matrix to sufficiently harness the mechanical forces generated during compression to achieve the desired fluorescence quenching. Guided by the work of Dietz, Rief, and Lorimer,[28] which revealed that the *N*-terminal β-sheet in GFP is mechanically labile, we concluded that polymeric appendages should be introduced on opposing sides of the aforementioned β-sheet to direct mechanical forces to this putative Achilles' heel (that is, the most mechanically labile structural element within the protein). The strategic incorporation of cysteine residues within the polypeptide backbone was predicted to facilitate the desired polymer ligation, as Bowman and Cramer have shown that thiyl radicals (which can be generated from thiols under free radical polymerization conditions) react efficiently with propagating acrylate radicals provided that the initial thiol concentration is relatively low.[40]

Site-directed mutagenesis was performed to incorporate cysteine residues on opposing sides of the β-barrel (replacing tyrosine 39 and aspartate 103) in GFPuv, as the attachment of polymer chains at these sites could direct mechanical forces to the aforementioned β-sheet and induce mechanical denaturation (and, consequently, fluorescence quenching). GFPuv was selected due to its high stability in bacterial expression systems and its stronger fluorescence signal than wild-type GFP.[37,38] The resultant hexahistidine-tagged GFPuv(Y39C/D103C) double mutant was overexpressed in *E.* coli BL21 (DE3) and purified by nickel affinity chromatography. As summarized in Scheme 2, PMMA composites were prepared by polymerizing methyl methacrylate (**III**) in the presence of GFPuv(Y39C/D103C) (**V**). Gel-permeation chromatography (GPC) visualized with ultraviolet-visible detection at 280 nm (a λ_{max} of tyrosine) revealed that the resulting polymeric material displayed an increased absorbance at this wavelength relative to a PMMA homopolymer that was prepared in the absence of GFPuv(Y39C/D103C) at the same concentration (see the Supporting Information). Moreover, mass spectrometry studies revealed that GFPuv(Y39C/D103C) was coupled to methyl methacrylate under the polymerization conditions, which confirmed that the cysteine residues were solvent exposed. Taken together, these results were consistent with the covalent attachment of GFPuv(Y39C/D103C) to the growing polymer chains during the preparation of the aforementioned composites. As shown in Figure 2, the solid-state fluorescence properties of the composites ($\lambda_{em} = 507$ nm; $\lambda_{ex} = 420$ nm) were in agreement with the successful incorporation of GFPuv(Y39C/D103C) into the polymeric matrix. Compression of these materials in a hydraulic press (41 MPa; 45 s) resulted in a significant reduction in fluorescence intensity (Figure 2A). Moreover, the fluorescence intensity was found to decrease monotonically with increasing pressure (0–41 MPa), which is a hallmark of mechanical phenomena (Figure 2B).[1–4] Compression of the materials for 1 h did not cause their fluorescence intensity to change beyond what was measured after 45 s at the same pressure. As such, these results suggested to us that mechanical forces were indeed denaturing the GFPuv(Y39C/D103C) upon compression of the composites (Scheme 1).

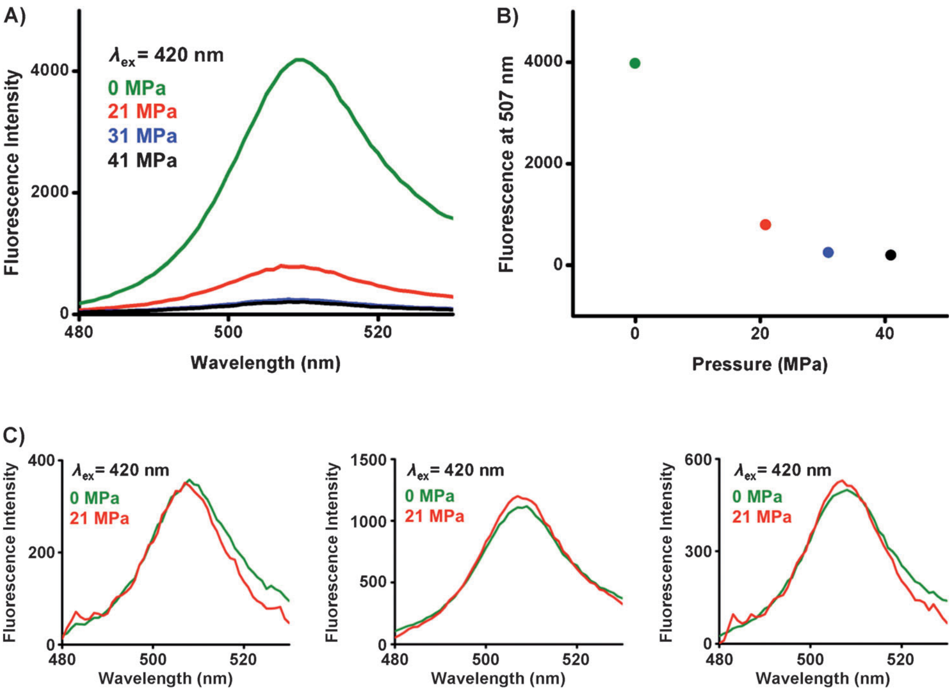


Figure 2. A) Compressing PMMA composites (0–41 MPa) containing double mutant GFPuv(Y39C/D103C) resulted in a monotonic decrease in the fluorescence intensity of the material. B) The fluorescence intensities at $\lambda_{em} = 507$ nm are plotted for clarity. C) Compression of PMMA composites containing GFPuv (left), GFPuv(Y39C) (center), or GFPuv(D103C) (right) did not significantly alter the fluorescence intensities of the materials.

To further demonstrate the mechanical origin of the observed activity, we also explored the mechanophoricity of the Y39C and D103C single mutants, wherein only one attachment site was present on the surface of the protein. Presumably, these mutants would function in a manner analogous to semi-telechelic derivatives of chemical mechanophores (that is, the mutants would not exhibit the same modulation in fluorescence as the Y39C/D103C double mutant).[1–4] Overexpression in *E. coli* and subsequent purification by nickel affinity chromatography afforded the desired semi-telechelic biomechanophores, which were subsequently polymerized with methyl methacrylate as described above. As shown in Figure 2, compression of the resulting composites did not significantly alter their photophysical properties. Similarly, compression of composites containing unmodified GFPuv did not result in modulation of their associated photophysical properties, and mass spectrometry confirmed that GFPuv was not coupled to methyl methacrylate under the polymerization conditions. Collectively, these data supported the conclusion that mechanical forces were responsible for the observed fluorescence modulation in the GFPuv(Y39C/D103C)-containing composites.

In summary, we have demonstrated that mechanical forces may be used to modulate the photophysical properties of fluorescent proteins embedded within polymeric matrices. Our work constitutes the first example of modulating the λ_{em} of eYFP-containing polymer composite materials through the action of mechanical forces, as well as the first example of mechanically modulating the photophysical properties of GFPuv in a bulk material. Moreover, we have shown that mechanical perturbation of various fluorescent proteins alters their photophysical properties in a distinct and tunable manner (that is, the reported systems exhibited either ratiometric or intensiometric responses to mechanical stress). The ability to precisely manipulate the optical output of composite materials containing biomechanophores through targeted mutations could afford new opportunities for the facile development of stress-responsive materials with tailored sensitivities. Moreover, we have demonstrated that single-molecule experiments can guide the rational design of biomechanophores, and insight garnered from mechanochemical studies involving chemical systems can be translated to those involving force-sensitive biomolecules. Finally, the technical simplicity associated with preparing the mechanically responsive biocomposites described herein holds promise for their development and utility as new classes of force-responsive materials.

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