Supplementary information for:

Modular Design of Optically Controlled Protein Affinity Reagents

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Protein production:

pET24b+ plasmids containing the sequence that codes for the 4C-E4 Fynomer, [1] as well as its L3CL29C double mutant were produced by Biobasic Inc. In each plasmid the coding sequence was followed by a 6-His tag for purification purposes. The modified loops are underlined.

Fynomer sequence:

MRGSGVTLFVALYDYNATRWTDLSFHKGEKFQILEFGPGDWWEARSLTTGETGYIPSNYVAPV DSIQGEQKLISEEDLHHHHHH*

L3CL29C-Fynomer sequence:

MRGSGVTCFVALYDYNATRWTDLSFHKGEKFQICEFGPGDWWEARSLTTGETGYIPSNYVAPV DSIQGEQKLISEEDLHHHHHH*

For producing each Fynomer, the plasmid was transformed into chemically competent *E. coli* (BL21*DE3) cells using the heat shock method and plated on agar/lysogeny broth (LB) plates containing 50 μg/mL kanamycin. A colony was used for inoculating 50 mL of LB culture media on the following day. The culture was grown overnight at 37°C in the presence of 50 μg/mL kanamycin. The overnight culture was transferred to 1 L LB containing the same concentration of kanamycin and incubated at 37°C on a shaker (250 rpm) until an optical density (OD) of 0.6 was reached. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 750 μM. The induced culture was then incubated/shaken for 4 hours at the same temperature. The culture was centrifuged at 4000 rpm for 1 hour and the harvested cell pellets were resuspended in 30 mL of 10 mM phosphate buffered 6 M GuCl solution (pH=8.0, containing 10 mM imidazole) and was stored at -20°C. On a different day, the cell lysis mixture was thawed on an orbital shaker for two hours at room temperature. Cell debris was removed by centrifugation at 12000 rpm for 2 hours. The crude supernatant was filtered through a 0.45 μm syringe filter and then applied to a Ni-NTA column which was pre-equilibrated with the same buffer. Once loaded with the His-tagged protein, the Ni-NTA resin was washed with 5 mL of the basic GuCl buffer three times. The protein was eluted by lowering the pH to 4.5 using a solution of 6 M GuCl, 200 mM sodium acetate. The expression yield was found to be 5 mg per litre of culture on average. The eluate was then used for either the crosslinking reaction or further purification for the enzymatic assays by reversed phase HPLC (*vide infra*).

Production of ^{15}N-labelled proteins:

A colony of transformed BL21*DE3 cells was used for inoculating 25 mL of LB media. The culture was grown overnight at 37 °C in the presence of 50 μg/mL kanamycin, then 6 mL of the overnight culture was centrifuged. The pellet was resuspended in 50 mL of M9 minimal media containing 50 μg/mL kanamycin and was incubated/shaken until the optical density reached OD=0.6. The culture was centrifuged and the pellet was resuspended in 1L of M9 media, supplemented with 1 g of $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Inc.), 1 mL of ^{15}N Bioexpress growth mixture (Cambridge Isotope Laboratories, Inc.), 10 mg of thiamine, 10 mg of biotin, 0.3% d-glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 50 μg of kanamycin.

The culture was induced after an OD of 0.6 was reached. Subsequent steps were identical to those described above for unlabelled proteins.

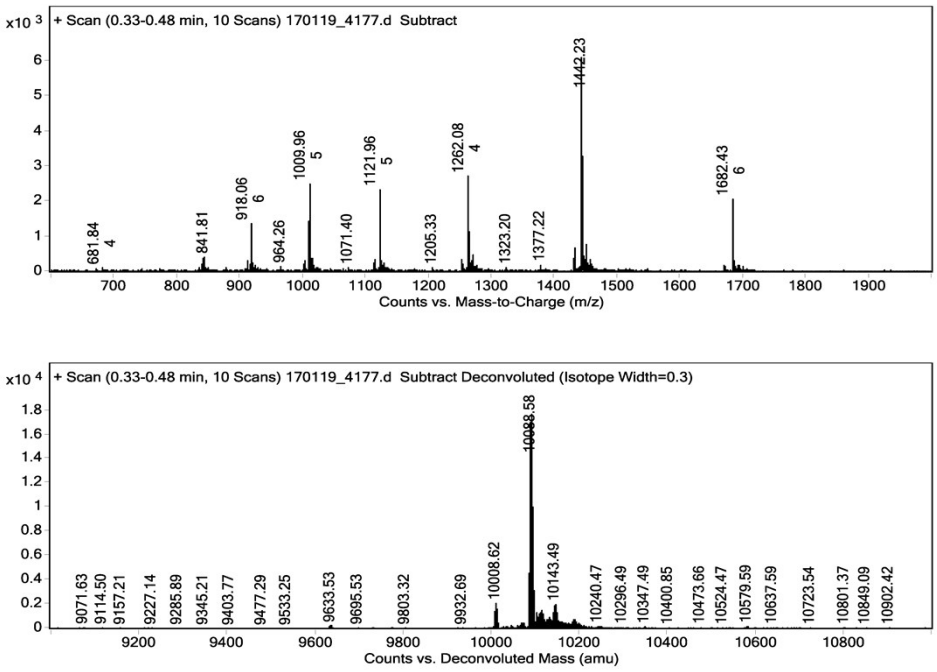
Crosslinking of Fynomer with azobenzene cross-linkers:

Affinity-purified Fynomer was buffer exchanged into pH 8.0 6M GuCl (10 mM phosphate, 10 mM imidazole) and concentrated using centrifuge concentrator tubes (Millipore Sigma, 3000 MWCO). The absorbance of the resulting protein stock solution was measured at 280 nm to determine protein concentration. (Molar extinction coefficients for wildtype and mutant Fynomers were obtained from the ProtParam online tool: 22,460 $M^{-1}cm^{-1}$)

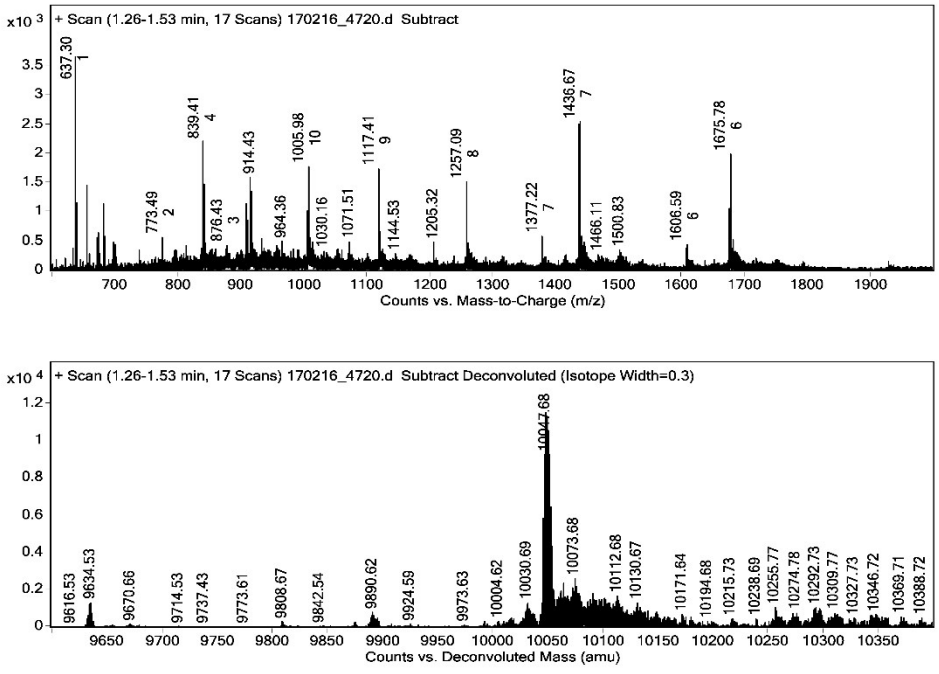
The protein was then treated with 10 equivalents of tris(2-carboxyethyl)phosphine (TCEP) and incubated at room temperature for at least 30 minutes to ensure the cysteine thiols were fully reduced. Stock solutions of BSBCA and TOM (synthesized as described previously[2,3]) were prepared in anhydrous DMSO (Sigma). To crosslink the Fynomer with BSBCA, 3 equivalents of BSBCA were added to the reduced protein and the reaction mixture was shaken at 50°C for 12 hours. To produce TOM-Fynomer, reduced protein was first diluted with DMSO such that the final concentration of DMSO would be at least 30% (v/v). Then 2 equivalents of TOM were added and the mixture was shaken at 50°C for 6 hours. Another 5 equivalents of TCEP followed by 1 equivalent of TOM were added to the mixture and shaking was continued for 12 more hours. The step-wise addition of cross-linker in this case prevented the formation of species with two azobenzenes attached.

The reaction mixture was purified by reversed-phase HPLC using a C18 semi-preparatory column (Zobrax, RxC18) using 0.1% (v/v) TFA water and 0.1% TFA (v/v) acetonitrile as solvents with a linear gradient of 5% to 70% acetonitrile in the course of 25 minutes. The eluate was monitored using a dual wavelength Waters detector at 280 nm (chromophore: protein and azobenzene) and 360 nm (only azobenzene). The crosslinked Fynomers eluted at ~52% acetonitrile. The eluate was then lyophilized and reconstituted using 50 mM sodium phosphate pH 8.0. The purified product was analyzed with ESI or MALDI mass spectrometry. The expected and observed mass values are as follows:

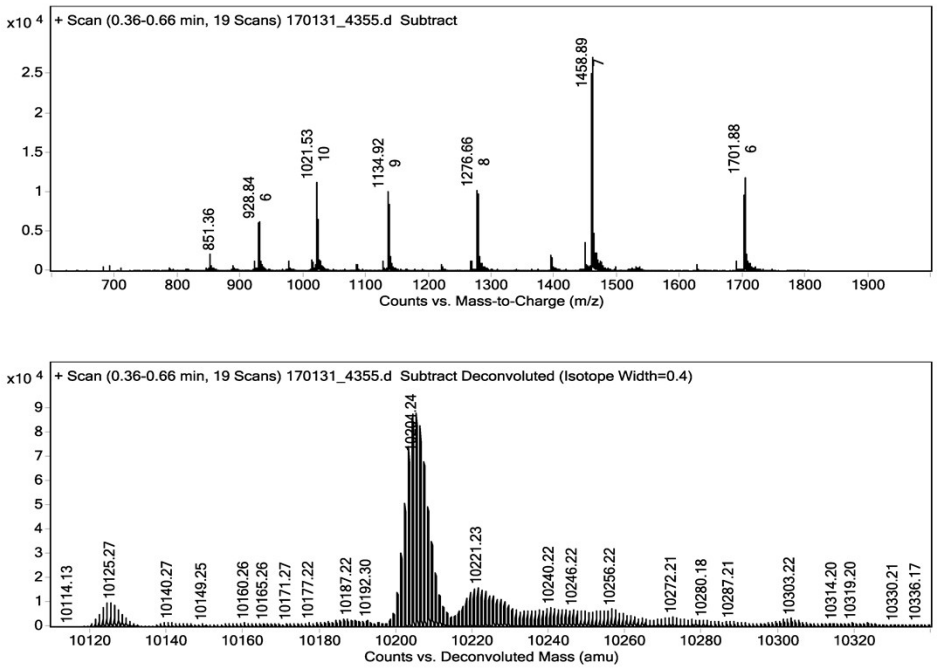
BSBCA-Fynomer: Expected 10089.0, Observed (QTOF ESI-MS) 10088.89
BSBCA-^{15}N-Fynomer: Expected 10206.7, Observed (QTOF ESI-MS) 10205.44
TOM-Fynomer: Expected 10049.0, Observed (QTOF ESI-MS) 10049.05
TOM-^{15}N-Fynomer: Expected 10166.7, Observed (MALDI-TOF-MS) 10167.7



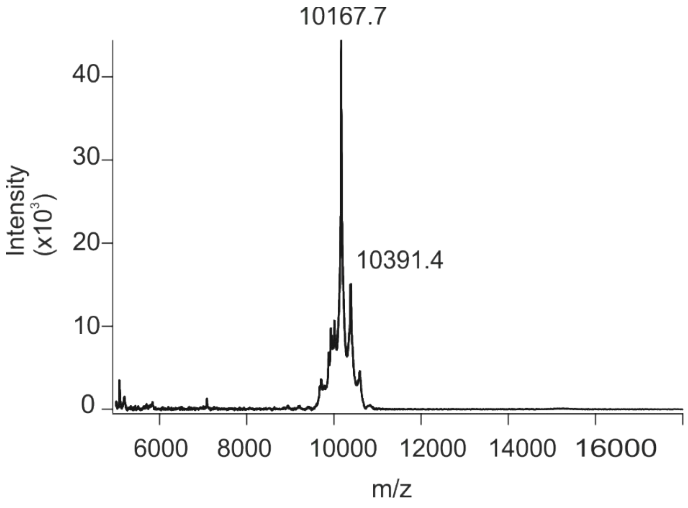
ESI-MS spectrum of HPLC purified BSBCA-Fynomer



ESI-MS spectrum of HPLC purified TOM-Fynomer



ESI-MS spectrum of HPLC purified BSBCA-^{15}N-Fynomer



MALDI-MS spectrum of HPLC purified TOM-^{15}N-Fynomer

Electronic absorption spectroscopy:

UV-Visible spectra were recorded on Perkin Elmer Lambda 35, Shimadzu UV-2401PC, or a diode array spectrophotometer (Ocean Optics Inc., USB4000). Temperature was maintained at 22°C for all measurements (Quantum Northwest), using 10 mm or 1.5 mm quartz cuvettes (Hellma Analytics).

Photoisomerization: For BSBCA-Fynomer, protein samples in 50 mM sodium phosphate pH 8 were dark adapted by incubation at 37°C overnight in light protected containers. UV irradiation was performed by placing a 365 nm LED above the sample tube for 1 minute. (897-LZ440U610 LedEngin LED, San Jose, CA, USA, operating at 68 mW/cm^2) For blue light irradiation, a 445 nm LED was used for 1 minute. (Luxeon III Star LED Royal Blue Lambertian operating at 40 mW/cm^2 at 700 mA). For TOM-Fynomer, a fan-cooled bright red LED was used for 30 minutes (LedEngin LZ4-40R200-0000, 700 mA, 635 nm, 90 mW/cm^2)

Estimates of the percentage of cis/trans in photostationary states (PSS):

Spectra for purely cis BSBCA and cis TOM (both conjugated to peptide FK11) from the literature[2,4] were used to approximate the cis isomers' contribution to absorbance at λ_{max} in the dark adapted spectra (367 nm and 400 nm for BSBCA-Fynomer and TOM-Fynomer, respectively). It is also assumed that the cis isomer in the fully dark-adapted samples is negligible. This allows for the calculation for %cis using the following equation and the absorbance values at these wavelengths:

$$PSS\ cis\% = \frac{A_{PSS} - A_{pure\ cis}}{A_{dark-adapted} - A_{pure\ cis}}$$

Thermal relaxation rates:

For studying the effect of chymase binding on the relaxation rate of the azobenzene, a sample of BSBCA-Fynomer was mixed with chymase to reach final concentrations of 4 μM and 4.5~9.0 μM, with respect to BSBCA-Fynomer and chymase, respectively. The final solution contained 12.5% glycerol, 20 mM sodium phosphate, 20 mM Tris, and 0.4 M NaCl, and its pH was measured to be 7.8. The sample was placed in a 1 cm cuvette. The cuvette was irradiated with UV (365 nm) for 15 seconds, gently mixed by pipetting, then immediately capped with Parafilm to prevent evaporation, and placed inside the spectrophotometer. The sample was periodically scanned (250 to 600 nm for each scan, integration time: 2s; scan speed: 480 nm/min) with 20 minute intervals for 6 hours. Similar kinetic measurements were performed on a sample of 4 μM BSBCA-Fynomer in the same buffer with the same pH. (Figure S4)

NMR spectroscopy

Lyophilized isotopically labelled compounds were dissolved in 10 mM HEPES (pH 7.2), 10% D_2O. They were then centrifuged at 15000 rcf for 10 minutes to ensure that no insoluble precipitate was present in the final samples. All HSQC experiments were performed at CSICOMP (Department of Chemistry, University of Toronto) using an Agilent DD2 700 MHz spectrometer equipped with an HFCN cold probe. An NH HSQC Watergate pulse sequence from the Varian Biopack library was used. All spectra were acquired at 25°C with 128 transients and 64 increments in the ^{15}N dimension. Sample concentrations of ~200 μM were used since higher concentrations promoted self-association of the analyte and decreased the efficiency of switching due to higher optical density. Spectra were processed using the NMRPipe

processing suite.[7] FID signals were zero filled to double the original data size and apodized using a sine window function prior to Fourier transformation. In the indirect dimension, linear prediction was applied to double the original data size. To achieve the difference spectra, subtraction of FIDs was done by the addNMR program of NMRPipe. Analysis was aided by NMRViewJ (One Moon Scientific).

Dark-adapted BSBCA-Fynomer was prepared by incubating a foil-wrapped NMR tube containing the sample at 37°C overnight. The foil-wrap was removed immediately prior to insertion in the magnet to minimize its exposure to ambient light. To irradiate a sample inside the magnet, a 1 mm fiber optic (Thorlabs) was inserted through the top of the NMR tube. The aforementioned LEDs were placed directly in front of the other end of the tube (with a 2 mm gap to avoid touching). The following irradiation methods were employed to ensure that the photostationary was reached for each sample.

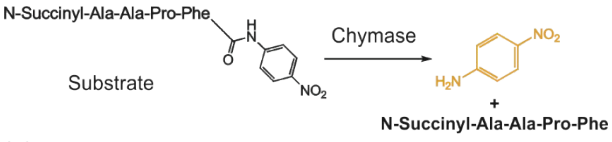
BSBCA-Fynomer, UV: 30 minutes, inside the magnet
TOM-Fynomer, Red: 1 hour inside the magnet following offline irradiation of the tube for four hours
TOM-Fynomer, Blue: 1 hour inside the magnet

In all three cases, light was kept on during acquisition.

The spectra for both the dark-adapted and UV-irradiated states exhibited some unresolved signal in the centre of the spectrum consistent with the formation of soluble aggregates at higher concentrations needed for 2D-NMR. Subtraction of the processed spectrum files resulted in a spectrum with two sets of crosspeaks with opposite phases which correspond to the changes of peak intensities after irradiation. (Figure 2 and Figure 4a in the main text). The disappearance of the unresolved peaks after subtraction suggested that the soluble aggregates did not undergo significant changes upon irradiation.

Activity assays:

The activity assay was based on measurement of the production of *p*-nitroaniline:



Chymase and the substrate peptide were purchased from Sigma (CS1140). A stock solution of the substrate peptide was prepared in DMSO. Substrate working solutions with various concentrations were prepared by diluting the stock solutions with a phosphate buffer (50 mM sodium phosphate, pH 8.0). DMSO was added to maintain the same final DMSO percentage in all dilutions for consistency.

All kinetic measurements were performed in triplicate. For each kinetic measurement, 100 µL of the enzyme, or enzyme/Fynomer solution was added to 50 µL of substrate solution in a 1 cm UV-rated plastic micro-cuvettes (Sigma, BR759235) and immediately placed in the Lambda 35 instrument equipped with a moving cuvette rack. Absorbance at 400 nm (ε=12300 $M^{-1}cm^{-1}$) [5] was measured every 30 seconds for 10 to 15 minutes following the initiation of the reaction. The spectrophotometer slit width and integration time were adjusted to 0.5 nm and 0.5 s to minimize the effect of the measuring light on the azobenzene.

Uninhibited reaction:

A 333-fold dilution of chymase was prepared with the addition of phosphate buffer and the assay buffer provided in the chymase assay kit (Sigma, CS1140). The final percentage of the assay buffer was 20%.

Inhibition assays:

Enzyme/Fynomer solutions were first made by diluting the chymase stock (~9 μM) 333-fold with the phosphate and assay buffers, and adding the inhibitor solution (unmodified, dark-adapted BSBCA-Fynomer, or TOM-Fynomer). Once mixed with the substrate solution, the final percentage of the assay kit buffer was 17% v/v, and the nominal enzyme concentration was ~18 nM. For the estimation of apparent IC_{50} values for the BSBCA-Fynomer, Triton-X100 (Sigma) was added to achieve a final concentration of 0.02% v/v to help prevent the formation of aggregates. The unmodified Fynomer was also treated with Triton to the same final concentration for consistency.

All samples were made in a dark room under a dim red light.

For the lit-state of BSBCA-Fynomer, the enzyme/inhibitor working solution was divided into two aliquots. The first aliquot was irradiated with UV light for 30 seconds while inside a temperature controller jacket. The LED was fan-cooled during the experiment. The light was then switched off and the solution was mixed by inversion to ensure that the percentage of cis-azo is not higher near the top of the tube (which is closer to the light source). The solution was then quickly mixed with the substrate and the measurement initiated.

IC_{50} data (Fig. 3a, Fig. S2) were fitted to a standard 4-parameter equation[1]:

$$V_o = D + ((A-D)/(1+10^{((\log([\text{BSBCA-Fynomer}])-\log(IC_{50}))*B)}))$$

Rate data as a function of substrate concentration (Fig. 3b) were fitted to the Michaelis-Menten (MM) equation and the MM equation for competitive inhibition using the global fitting method in IgorPro (Wavemetrics). The total concentration of inhibitor was adjusted to give an observed rate similar to that seen in IC_{50} data, (~80 nM). Irradiated state data were included in the global fitting by fitting to the following modified MM (competitive inhibition) equation:

$$V_o = V_{max}*[S]/((K_m*(1 + (0.2*[I]/K_{i\_trans}) + (0.8*[I]/K_{i\_cis}))) + [S]);$$

Which assumes the BSBCA-Fynomer is 80% cis when irradiated based on UV-Vis data (see above). Mean initial rates obtained from triplicate measurements were subjected to t-test statistical analysis. The analysis confirmed that the difference between the rates corresponding to dark-adapted and UV-irradiated inhibitors was significant with 99.9% confidence.

V_{max} , k_{cat}, and K_m values for chymase in the absence of inhibitor were: V_{max} = 110 ± 15 nM/s; k_{cat} = 7 ± 1 s^{-1} (if [chymase] is 15 nM), K_m = 225 ± 20 μM. These values are similar to those reported previously for the same substrate.[6] We note these fits for Ki values are approximate since the concentration of chymase in these assays is ~ 15-20 nM (similar to the fitted K_is).

For the irradiated states of TOM-Fynomer, the enzyme/inhibitor working solution was first irradiated with red light for 30 minutes. After the measurement of the red-irradiated samples, the same working solution was irradiated with blue light for 5 minutes. Both LEDs were fan-cooled during irradiation.

Control experiment with unmodified Fynomer:

To ensure that UV irradiation has no effect on the enzyme or any other component of this system, the inhibition assay was performed using the unmodified Fynomer with and without UV irradiation. Kinetic traces from both sets of experiments did not differ significantly (Figure S3).

Testing the reversibility of activity change:

A solution of enzyme and BSBCA-Fynomer was prepared as described above and was divided into two aliquots. The first aliquot was irradiated with blue light (445 nm) for 1 minute. A 100 μL aliquot of the enzyme/inhibitor solution was mixed with 50 μL of substrate solution for each of the triplicate cuvettes and absorbance was monitored as a function of time. The second aliquot was irradiated with UV light (365 nm) for 30 seconds and immediately irradiated with blue light (445 nm) for 1 minute and then mixed with substrate in the same fashion. Kinetic traces for both sets of samples are given in Figure S5.

Supplementary Figures:

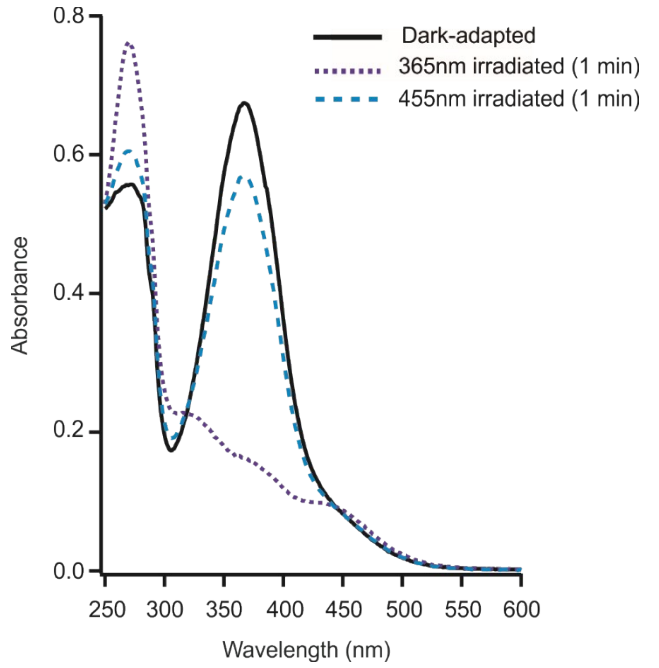


Figure S1. UV-Vis spectra of BSBCA-Fynomer when dark adapted (solid black), 365 nm-irradiated (dotted purple), and 445 nm irradiated (dashed blue)(sodium phosphate buffer, pH 8.0)

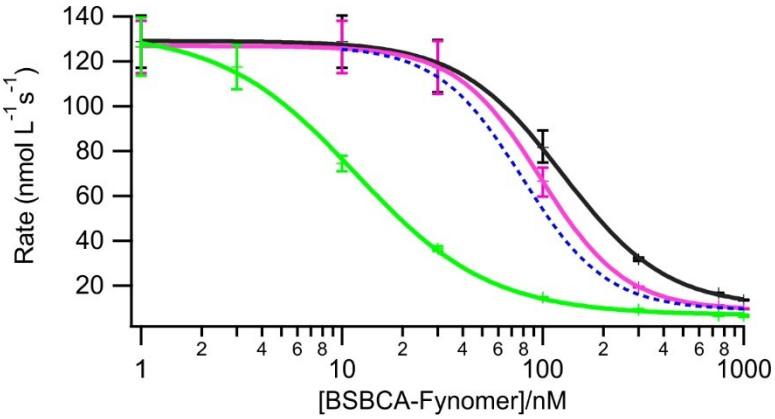


Figure S2. IC_{50} curves for dark-adapted (black line)(126 ± 4 nM), UV-irradiated (pink line)(97 ± 2 nM), (calculated) pure cis state (dotted blue line)(79 ± 2 nM) and unmodified Fynomer (green line)(12 ± 1 nM). See ESI text above for assay details.

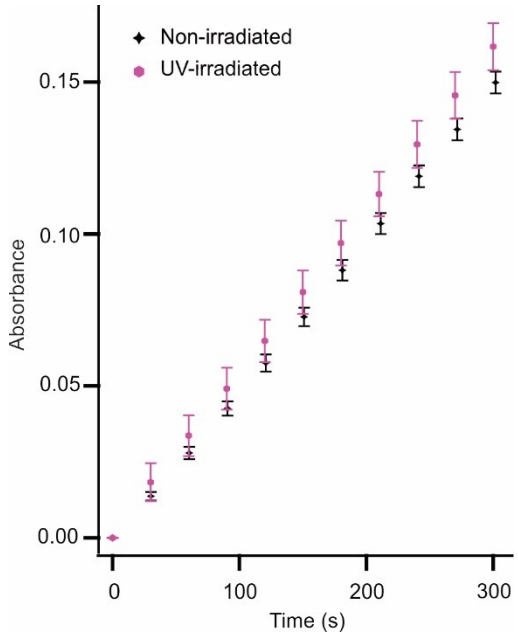


Figure S3. Chymase catalysed hydrolysis of peptide substrate in the presence of un-cross-linked Fynomer (~30 nM) ([substrate] = 350 μM). UV irradiation does not cause a significant change of activity when the Fynomer is not cross-linked with BSBCA. See ESI text above for assay details.

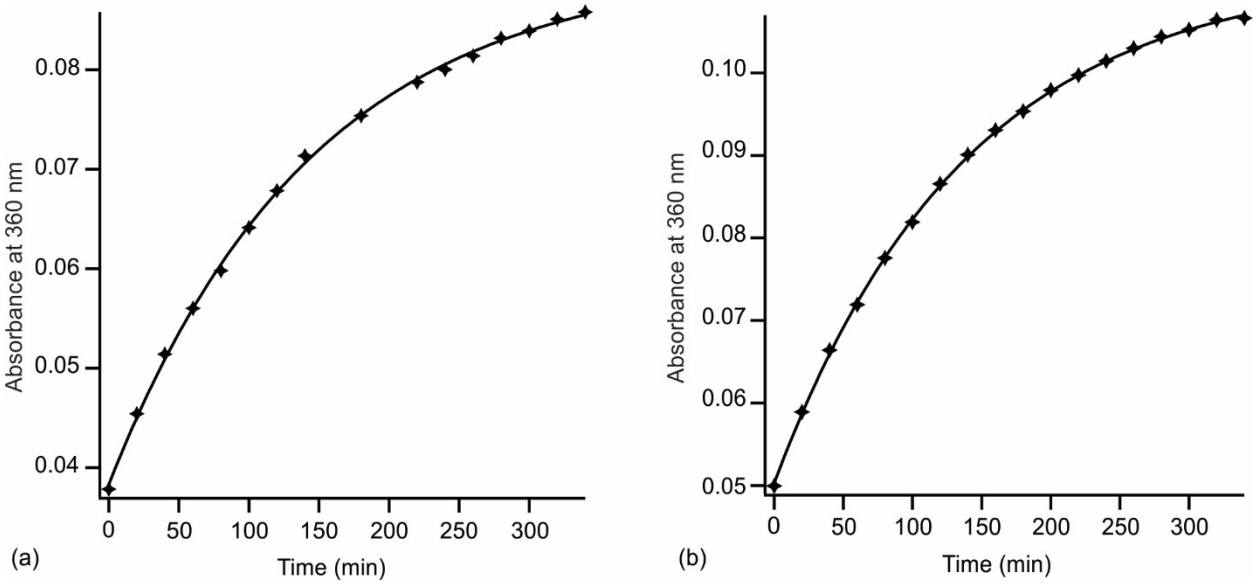


Figure S4. (a) Thermal relaxation of BSBCA-Fynomer (22ºC, phosphate/Tris buffer containing 12.5% glycerol and 0.4 M NaCl, pH 7.8). Absorbance at 360 nm was monitored. Solid curve shows fitted mono exponential curve ($\tau_{½}$=101 ± 15 min). Addition of chymase (b) does not significantly alter the relaxation rate ($\tau_{½}$=96 ± 15 min).

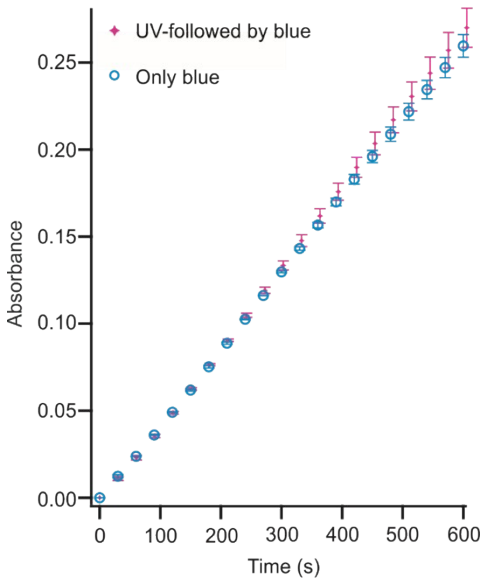


Figure S5. Chymase catalysed hydrolysis of peptide substrate in the presence of BSBCA-Fynomer (~100 nM) ([substrate] = 350 μM). No significant change in rate is observed following the two irradiation patterns (see legend in figure) indicating that the isomerization-driven change in the activity of Fynomer is reversible using blue light. See ESI text above for assay details.

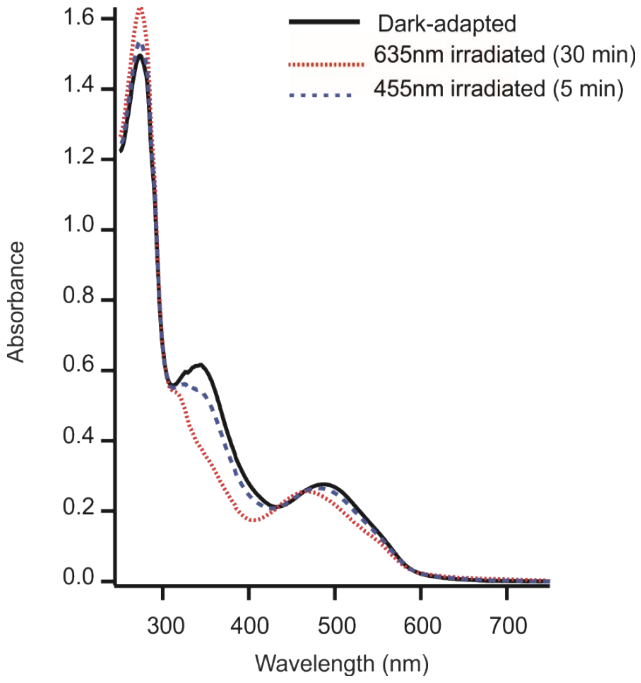


Figure S6. UV-Vis spectra of TOM-Fynomer when dark adapted (solid black), 635 nm-irradiated (dotted red), and 445 nm-irradiated (dashed blue)(sodium phosphate buffer, pH 8.0)

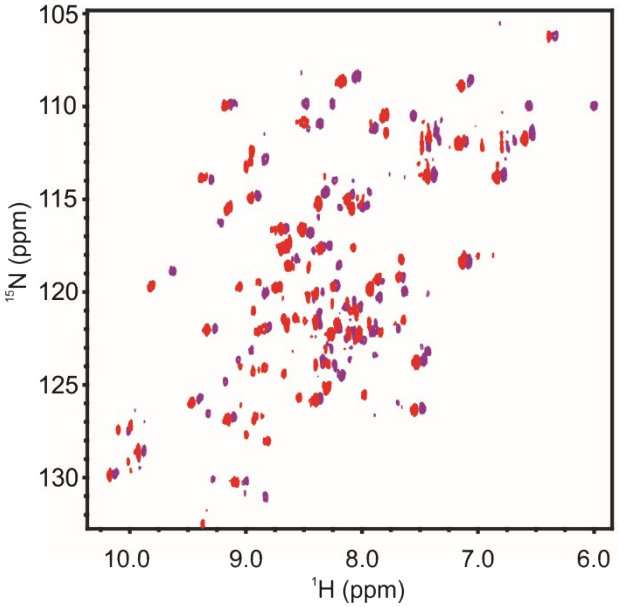


Figure S7. The folded state (UV-irradiated) of BSBCA-Fynomer (purple cross-peaks) corresponds closely to the folded state (red-irradiated) of TOM-Fynomer (red cross-peaks).

References:

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6. E. T. Smith, E. T. Perry, M. B. Sears and D. A. Johnson, *Protein Expr. Purif.*, 2014, **102**, 69-75.