Supplementary Information

Molecular crowding elicits the acceleration of enzymatic crosslinking of macromolecular substrates

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The amino acid sequences of all the proteins used in this study

Table S1. The amino acid sequences of all the proteins used in this study

EGFP-LLQG (28.6 kDa) MHHHHHHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT TLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKED GNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQ SALSKDPNEKRDHMVLLEFVTAAGITLGMDELYRGGGSLLQG
mTagBFP-LLQG (28.0 kDa) MHHHHHHMSELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILATSFL YGSKTFINHTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNGPVMQK KTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIANIKTTYRSKKPAKNLKMPGVYYVDYRLERIKEA NNETYVEQHEVAVARYCDLPSRLGHRLNGGGSLLQG
EGFP-MRHKGS (28.6 kDa) MHHHHHHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT TLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKIDFKEDG NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQS ALSKDPNEKRDHMVLLEFVTAAGITLGMDELYRMRHKGS
PolyTag-EGFP (29.5 kDa) MHKRWRHYQRGGGGSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPV PWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELK GIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD NHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYRHHHHHH
MTG inactive form (44.7 kDa) MHNHNHNHNHNHNDNGAGEETRSAAETYRLTADDVANINALNESAPAASSGGGSENLYFQ↓SGGGGSDS DDRVTPPAEPLDRMPDPYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWV NSGQYPTNRLAFASFDEDRFKNELKNGRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNRALENA HDESAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKERNGGNHDPSRMKAVIYSKHFWSGQDRSS SADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFVNFDYGWFGAQTEADADKTVWTHGNHYH APNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGWP
MTG active form (38.3 kDa) SGGGGSDSDDRVTPPAEPLDRMPDPYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYG CVGVTWVNSGQYPTNRLAFASFDEDRFKNELKNGRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVM NRALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKERNGGNHDPSRMKAVIYSKHFW SGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFVNFDYGWFGAQTEADADKTVWT HGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGWP

Black: Protein of interest

Orange: Q-tag

Green: 6 × His tag or 6 × HN tag

Blue: K-Tag

Cyan: GS linker sequence

Magenta: PolyTag

Red: Propeptide (K9R/Y11A)

Purple: TEV protease recognition site

Characterization of proteinaceous substrates

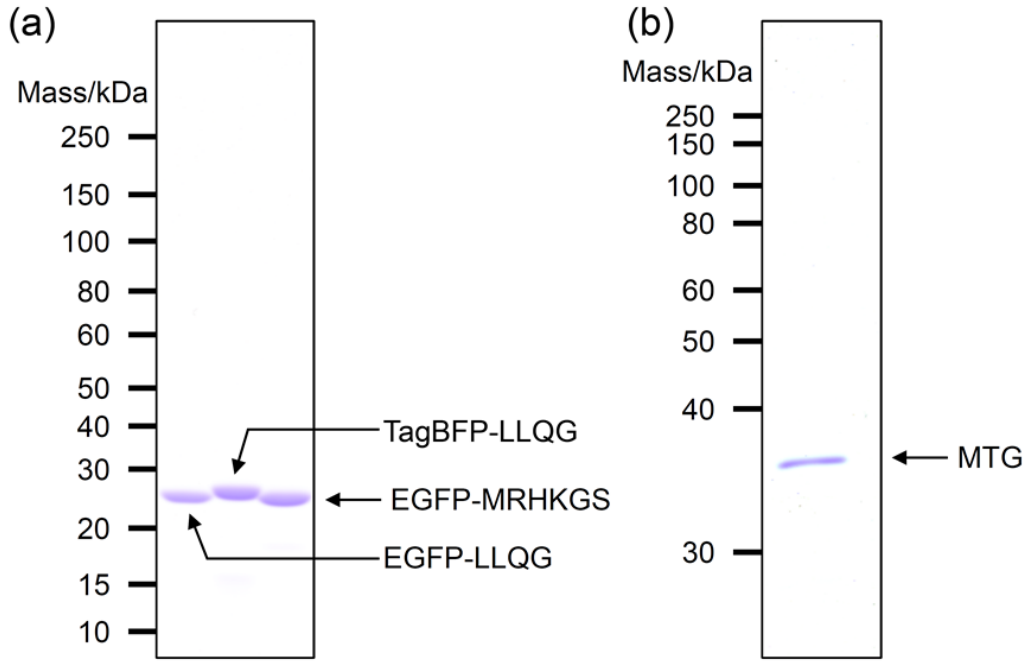

Fig. S1 SDS-PAGE of (a) recombinant fluorescent proteins and (b) MTG. The concentrations of fluorescent proteins and MTG were 10 μM and 1 Unit/mL, respectively.

HPLC analysis of MTG-mediated FITC-β-Ala-QG and TAMRA-cadaverine conjugation

The FRET donor molecule (FITC-β-Ala-QG; 10 μM) was mixed with the acceptor (TAMRA-cadaverine; 200 μM) in 1x PBS (pH 7.4) and solutions comprising 15twt% of Dex60k and Dex500k. The crosslinking reaction was started by adding 0.5 U/mL MTG (total volume: 80 μL). The reaction was performed at 37 °C. At a given time, 15 μL of the sample solution was added to 50 μL of 6 M guanidine hydrochloride/50 mM ammonium bicarbonate to stop the MTG-caralyzed crosslinking reaction. Then 65 μL of 99% of ethanol was added to the sample solutions and mixed them by vortex. The supernatant was separated from the dextran precipitate by centrifugation and subjected to HPLC analysis. HPLC analysis was conducted using Agilent 1260 Infinity II LC (Agilent Technologies, Santa Clara, CA, USA). In the RP-HPLC, 100 μL of sample solutions were injected into a COSMOSIL 5C4-AR-300 (4.6 × 150 mm) column (NACALAI TESQUE, Kyoto, Japan) at a flow rate of 1.2 mL/min and a detection wavelength of 565 nm (for TAMRA). The mobile phase gradients conditions were ACN/water (0.1% TFA) = 90:10 → 60:40 (over 15 min). From the peak area of TAMRA-cadaverine and FITC-TAMRA conjugate, the conversion rate of FITC-β-Ala-QG was calculated by following equations (1) and (2).

$$Consumption\ amount\ of\ TAMRA\ (\mu M) = \frac{peak\ area_{FITC-TAMRA\ conjugate}}{peak\ area_{FITC-TAMRA\ conjugate} + peak\ area_{TAMRA-cadaverine}} \times 200 \ldots (1)$$

where 200 represents the original concentration of TAMRA-cadaverine.

$$Conversion\ rate\ of\ FITC\ (\%) = \frac{Consumption\ amount\ of\ TAMRA}{10} \times 100 \ldots (2)$$

where 10 represents the original concentration of FITC-β-Ala-QG.

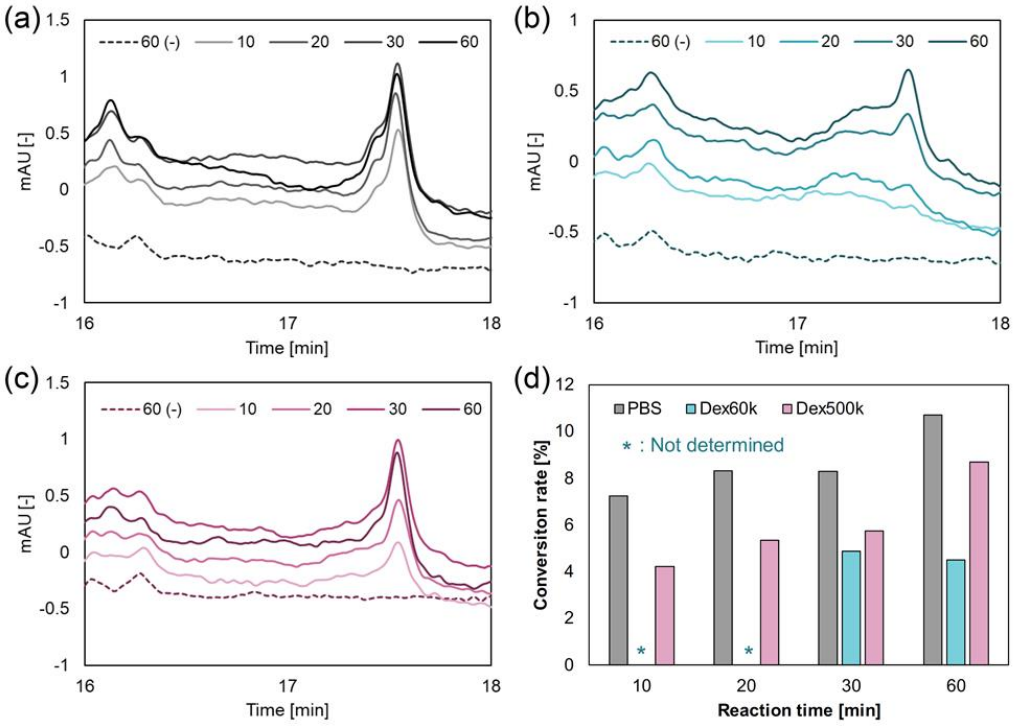

Fig. S2 HPLC chromatogram of MTG-mediated conjugation of FITC-β-Ala-QG and TAMRA-cadaverine performed in (a) PBS, (b) 15 wt% of Dex60k and (c) 15 wt% of Dex500k. (d) Conversion rate of FITC-β-Ala-QG calculated by peak areas in PBS and in solutions comprising 15 wt% of Dex60k or Dex500k.

MALDI-TOF-MS analysis of TAMRA-cadaverine modified EGFP-LLQG by MTG reaction
The FRET donor molecule (EGFP-LLQG; 10 μM) was mixed with the acceptor (TAMRA-cadaverine; 200 μM) in 1x PBS (pH 7.4). MTG (0.1 U/mL) was then added to start the crosslinking reaction (total volume: 40 μL). The reaction was performed at 37 °C. After 20 min, 15 μL of the sample solution was added to 50 μL of 6 M guanidine hydrochloride/50 mM ammonium bicarbonate to stop the MTG-catalyzed crosslinking reaction, and 65 μL of 99% of ethanol was added to the sample solutions. They were mixed well by vortex and supernatant and dextran precipitate were separated. After mixing 100 μL of sample solution with 20% of ACN (0.1% TFA), the solutions were exchanged to 20% of ACN (0.1% TFA) using Amicon Ultra-0.5 Centrifugal Filter Unit (3 kDa MWCO). EGFP-TAMRA conjugate solutions were spotted on a CHCA matrix, and the mass spectra were obtained with a Bruker Autoflex max MALDI-TOF mass spectrometer (Bruker, Billerica, MA, USA) in linear positive highmasses mode. Theoretical masses were calculated based on the program ChemDraw (Cambridge Soft Corporation, Waltham, MA, USA) and ExPAsy ProtParam.

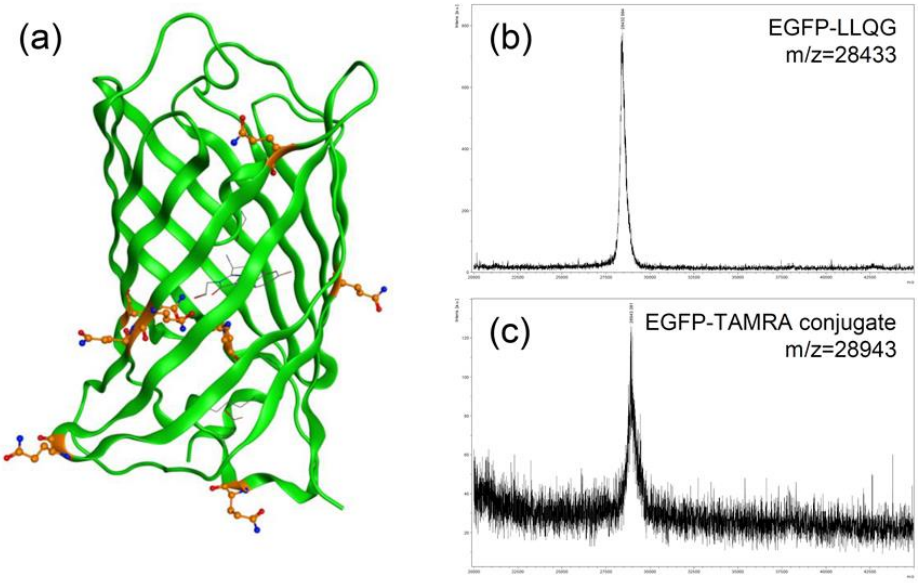

Fig. S3 (a) Crystal structure of EGFP from Protein Data Bank ID of 2Y0G. Orange sticks represent the glutamine residues. MALDI-TOF-MS results of (b) EGFP-LLQG and (c) EGFP-TAMRA conjugate, respectively.

SDS-PAGE analysis of TAMRA-cadaverine modified EGFP-LLQG by MTG reaction

The crosslinking reaction was carried out by mixing EGFP-LLQG (10 μM) and TAMRA-cadaverine (200 μM) with 0.1 Unit/mL of MTG in PBS (pH 7.4) and solutions comprising 15 wt% of Dex60k and Dex500k. The solution was incubated at 37 °C for 20 min. An aliquot of 4 × SDS-PAGE sample buffer was added to the reaction solution and then it was heated at 95 °C for 2 min. After denaturation treatment, SDS-PAGE was conducted using a 5–20% gradient gel. The fluorescent image was obtained by iBright CL1500 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) before CBB staining. The band intensities were measured using ImageJ software.

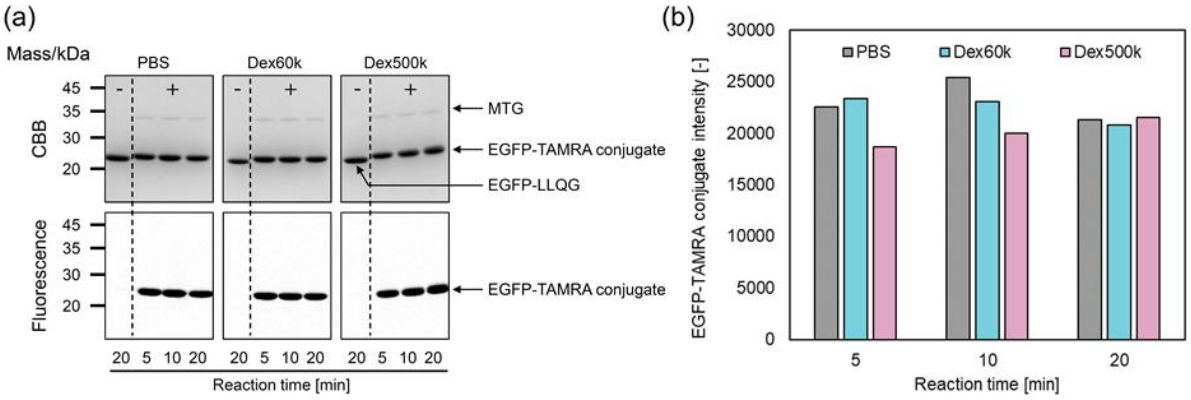

Fig. S4 (a) SDS-PAGE analysis of MTG-mediated EGFP-TAMRA conjugation. (b) Results of band intensity measurements of EGFP-TAMRA conjugates.

SDS-PAGE analysis of MTG-mediated TagBFP-LLQG and EGFP-MRHKGS conjugation

The crosslinking reaction was carried out by mixing TagBFP-LLQG (10 μM) and EGFP-MRHKGS (20 μM) with 0.05 Unit/mL of MTG in PBS (pH 7.4) and solutions comprising 15 wt% of Dex60k and Dex500k. The solution was incubated at 37 °C for 60 min. An aliquot of 4 × SDS-PAGE sample buffer was added to the reaction solution and then it was heated at 95 °C for 2 min. After denaturation treatment, SDS-PAGE was conducted using a 5–20% gradient gel and stained with CBB. The band intensities were measured using ImageJ software. The BFP-EGFP conjugate ratio was calculated by the following equation (3).

$$BFP-EGFP\ conjugate\ ratio\ (\%) = \frac{Band\ intensity_{BFP-EGFP\ connjugate}}{Band\ intensity_{BFP\ and\ EGFP\ monomer} + Band\ intensity_{BFP-EGFP\ conjugate}} \times 100 \quad \cdots (3)$$

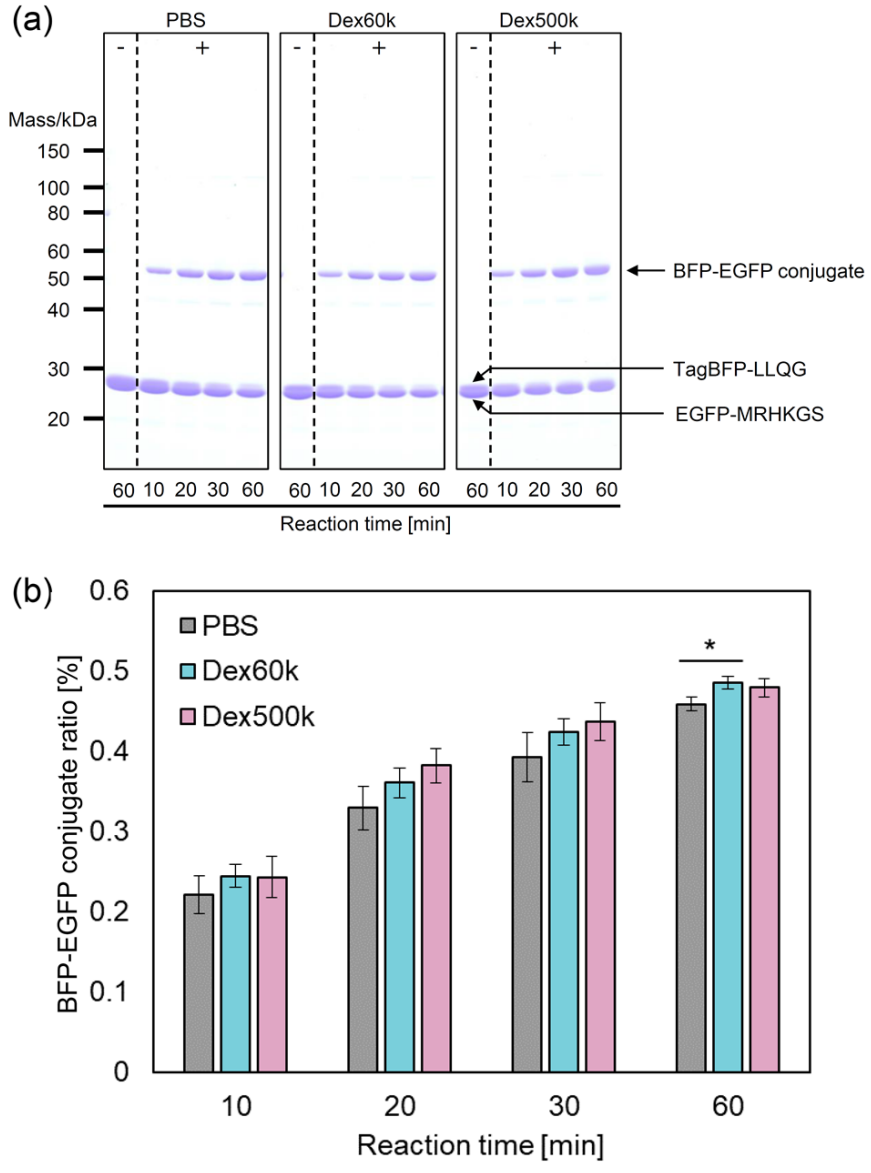

Fig. S5 MTG-mediated BFP-EGFP conjugate formation in PBS and solutions comprising Dex60k and Dex500k. Comparison of the crosslinking products produced from 10 to 60 min of MTG reaction (a) SDS-PAGE gel analysis and (b) band intensity measurements (n = 3). The error bars represent the standard deviations from three independent trials (*p < 0.05).