Supporting Information

Linear Polymerization of Protein by Sterically Controlled Enzymatic Cross-Linking with a Tyrosine-Containing Peptide Loop

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1. Supplementary information

1-1. Amino acid sequences of BAP-WT

MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPGGARR
LTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDALPLTGQYTH
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VMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVVGLTDQTDLFYTMKAALGLK

1-2. Amino acid sequences of BAP-Y

MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPGGARR
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1-3. Amino acid sequences of BAP-pG_2pA-Y

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PRGSGGGSGGGGY

1-4. Amino acid sequences of BAP-Loop-Y

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SIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHAHASQIVAPDTKAPGL
TQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVVGLTDQTDLFYTMKAALGL
K

1-5. Amino acid sequences of pG_2pA-Y

MHHHHHHTYKLVINGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE
KPEVIDASELTPAVTTYKLVINGKTLKGETTTKAVDAETAEKAFKQYANDNGVDGVWTYDDA
TKTFTVTEGGGGSDVDNKFNKEQQNAFWEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEA
KKLNDAQAPKGGGGY

Protein/peptide fragments are labeled with different colors:
Red: Signal peptide;
Orange: Hexahistidine tag;
Purple: Alkaline phosphatase;
Green: Flexible linker;
Cyan: Protein G;
Dark red: Protein A;
Grey: thrombin recognition sequence;
Blue: Y-tag.
Yellow Shaded: Y-Loop

2. Supplementary material and methods

2-1. Purification of BAPs and pG_2pA-Y

Competent cells of *E. coli* BL21Star (DE3) were transformed with expression vectors using a heat-shock method. A single colony of transformants was selected from the agar plates (containing 100 μg/mL of ampicillin sodium) and inoculated into 3.5 mL of LB media containing 100 μg/mL of ampicillin sodium. The media was cultured for 6-7 hours at 37 °C. The pre-cultured media were then poured into 500 mL of TB media containing 100 μg/mL of ampicillin sodium and incubated until the OD_{600} reached ~0.5. The expression of BAPs and pG_2pAs was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (final concentration of 0.1 mM) and followed by shaking for 18 h at 18 °C. The cells were then collected by centrifugation at 5,000 g for 20 min at 4 °C. The cell pellets were washed with TBS (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl, pH 7.4) 3 times. The suspensions containing the cells were frozen and stored at -80 °C.

The frozen cell suspensions were thawed and then sonicated on ice for 15 min. The supernatant was separated from insoluble cell components by centrifugation at 5,000 g for 20

min at 4 °C. The supernatants were initially applied to an equilibrated HisTrap FF Crude column (equilibrated with binding buffer: 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 35 mM imidazole). The column was washed with five column bed volumes of the binding buffer, and BAPs and pG_2pAs were eluted with a gradient of the elution buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 500 mM imidazole) from 0% to 100%. The fractions containing the proteins were collected and pooled. The pooled fractions were then desalted into 10 mM Tris-HCl (pH 8.0) using a HiPrep Desalting column and applied to an anion exchange column, HiTrap Q HP (equilibrated with 10 mM Tris-HCl, pH 8.0). The BAPs and pG_2pAs were then eluted with a gradient of the elution buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl) from 0% to 100%. The fractions containing the proteins were pooled and then concentrated to a volume of ~5 mL using an Amicon Ultrafiltration device (MWCO 50 kDa) (Merck Millipore, Tullagreen, Ireland). The concentrated solutions of BAPs and pG_2pAs were applied to a size exclusion chromatography (SEC) column (HiLoad Superdex 200 pg). The SEC mobile phase was 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. The fractions were analyzed by SDS-PAGE, and the fractions containing the target proteins in high purity were collected. The fractions were then concentrated using Amicon Ultrafiltration devices (MWCO 50 kDa), and finally, the concentration of the proteins was measured by the BCA assay using BSA as the standard.

2-2. Optimization of TL-catalyzed BAPs polymerization

We optimized the concentration of *Trametes sp.* laccase (TL) and reaction time. The volume of reaction was 50 μL. The final concentration of the BAP was 10 μM, and the final concentration of TL for optimization reaction was varied in the range of 0.5, 1.0, 1.5, 2.0, 3.0, and 5.0 μM. The mixture was then incubated for 1 h at 37 °C. After we obtained the optimum

concentration of TL, then we optimized the reaction time. Final concentrations of BAP and TL were 10 and 2 μM, respectively. The polymerization reaction was performed by incubating the reaction mixture at 37 °C for 5, 15, 30, 60, and 120 minutes. The samples were then analyzed by SDS-PAGE.

2-3. Horseradish peroxidase (HRP)-catalyzed polymerization of BAPs

The polymerization of BAP was conducted in 10 mM Tris-HCl (pH 8.0) solution with a total volume of 50 μL. The concentrations of BAP and HRP were 10 and 2 μM, respectively. The final concentration of hydrogen peroxide (H_2O_2) was 10 μM. One mole of H_2O_2 can oxidize two moles of Y-tags (thus one mole of dimeric BAPs). The reaction mixture was incubated at 37 °C for 2 h. The samples were analyzed by SDS-PAGE. The activity of the BAP monomers and polymers were also measured by using *p*-NPP as a substrate.

3. Supplementary results

3-1. Purification of BAP-WT

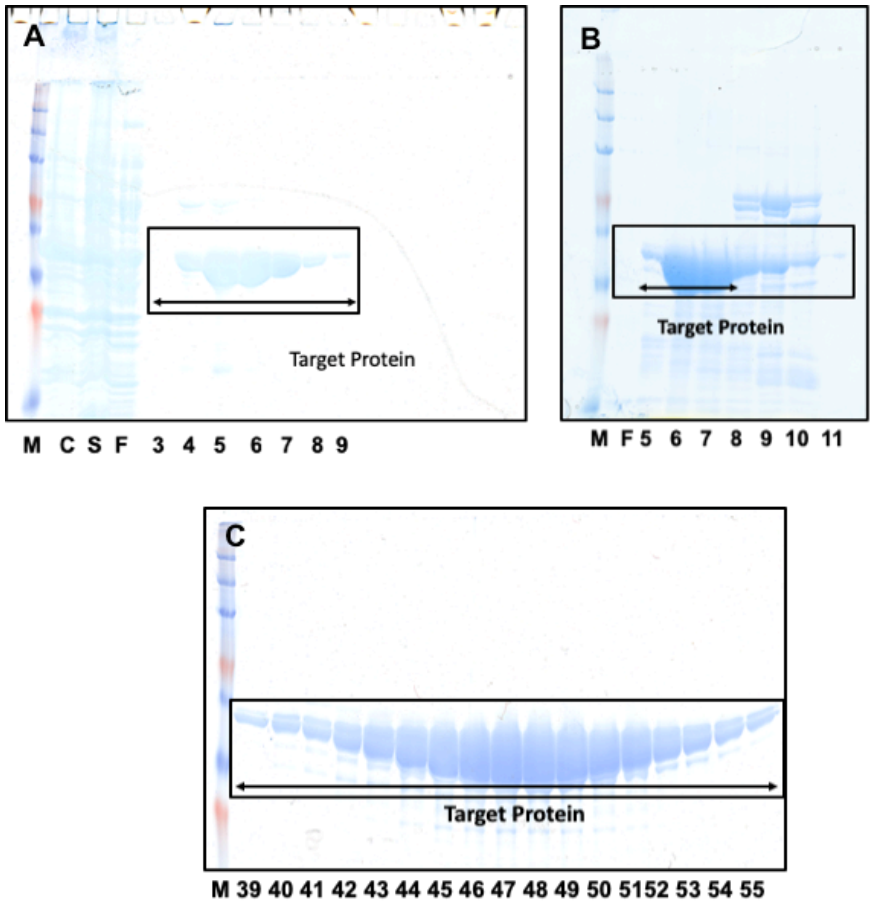

Figure S1 The purification results of BAP-WT. SDS-PAGE results of purified fractions; (A) fractions in the HisTag purification step, (B) fractions in the anion exchange chromatography (AEC) step, and (C) fractions in the SEC chromatography of BAP-WT. M: protein molecular weight marker; C: whole-cell extract; S: supernatant of the cell extract after centrifugation; F: flow-through fraction. The number indicated is the No. of the fractions, which showed a UV absorbance peak in the chromatograms. The arrow indicated the pooled fractions.

3-2. Purification of BAP-Y

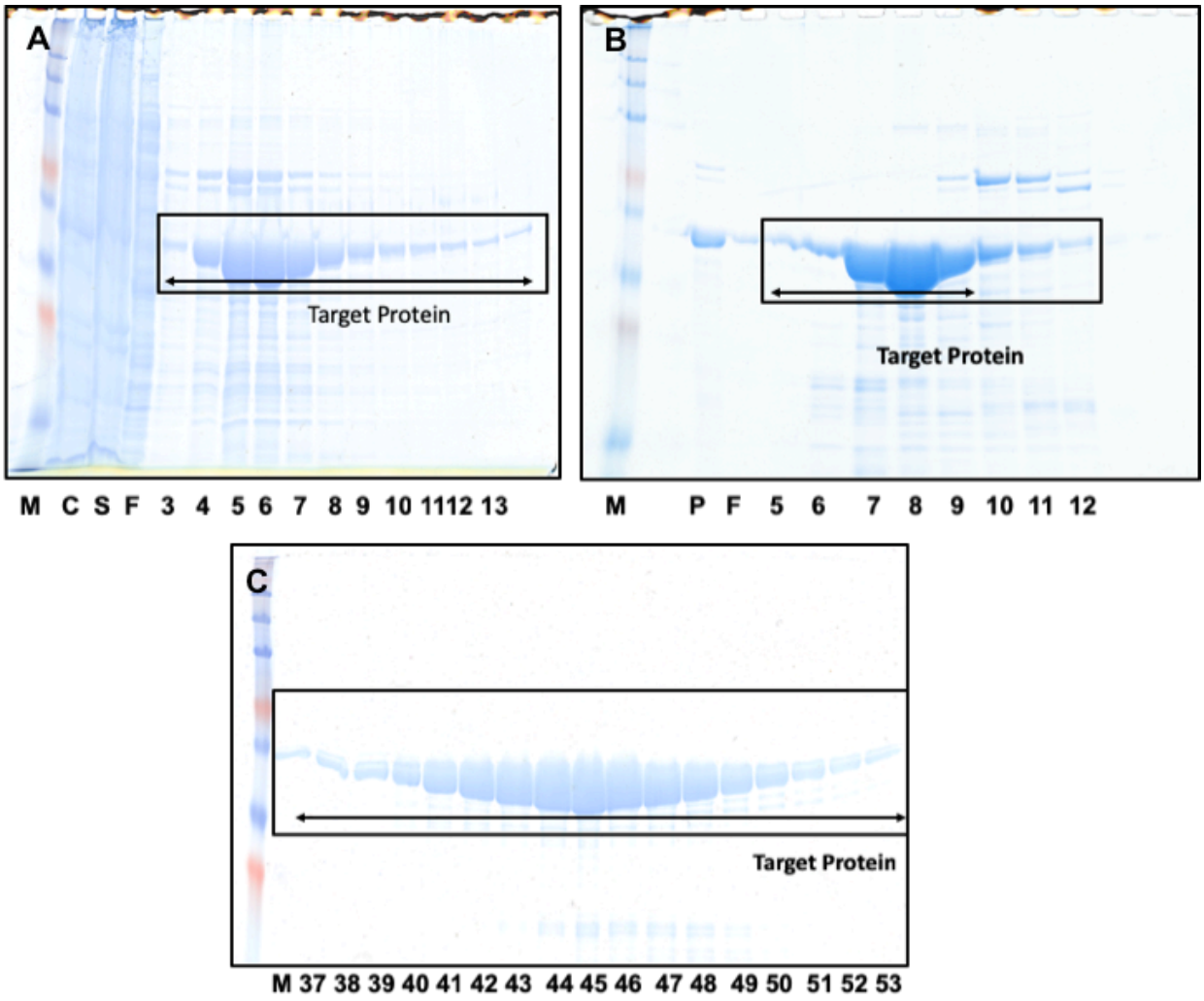

Figure S2 The purification results of BAP-Y. SDS-PAGE results of purified fractions; (A) fractions in the HisTag purification step, (B) fractions in the anion exchange chromatography (AEC) step, and (C) fractions in the SEC chromatography of BAP-Y. M: protein molecular weight marker; C: whole-cell extract; S: supernatant of the cell extract after centrifugation; F: flow-through fraction. The number indicated is the No. of the fractions, which showed a UV absorbance peak in the chromatograms. The arrow indicated the pooled fractions.

3-3. Purification of BAP-pG$_2$pA-Y

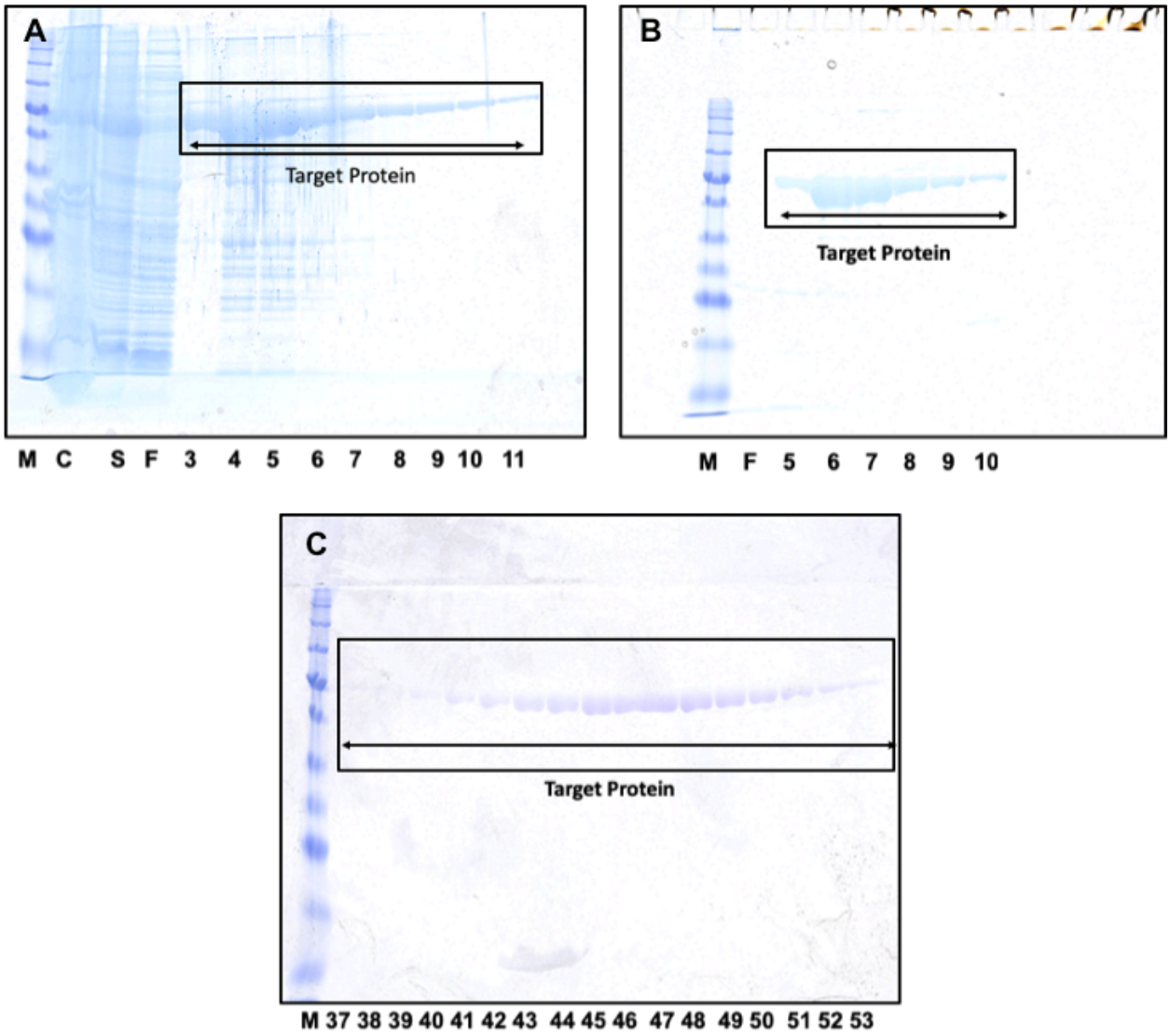

Figure S3 The purification results of BAP-pG$_2$pA-Y. SDS-PAGE results of purified fractions; (A) fractions in the HisTag purification step, (B) fractions in the anion exchange chromatography (AEC) step, and (C) fractions in the SEC chromatography of BAP-pG$_2$pA-Y. M: protein molecular weight marker; C: whole-cell extract; S: supernatant of the cell extract after centrifugation; F: flow-through fraction. The number indicated is the No. of the fractions, which showed a UV absorbance peak in the chromatograms. The arrow indicated the pooled fractions.

3-4. Purification of BAP-Loop-Y

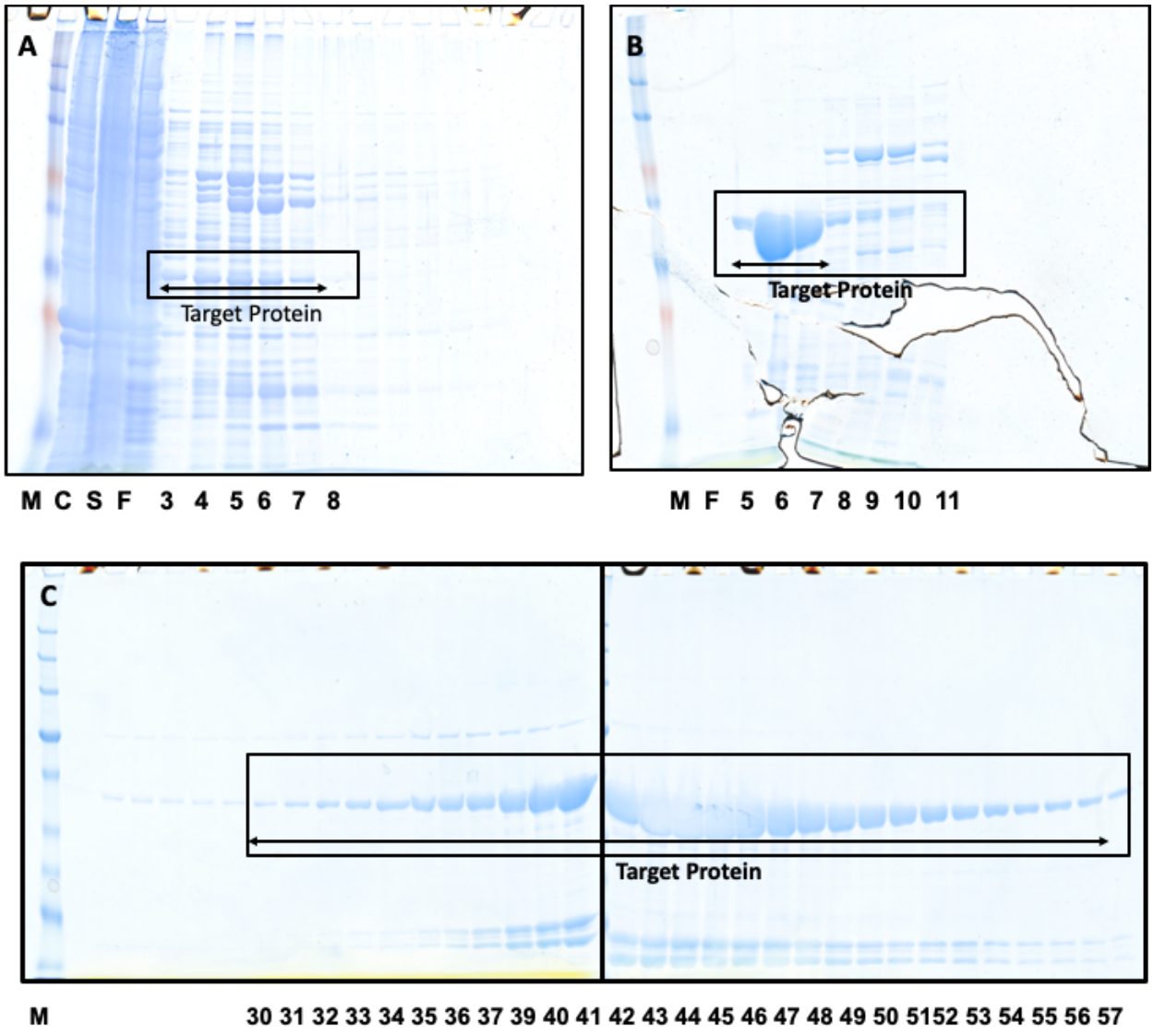

Figure S4 The purification results of BAP-Loop-Y. SDS-PAGE results of purified fractions; (A) fractions in the HisTag purification step, (B) fractions in the anion exchange chromatography (AEC) step, and (C) fractions in the SEC chromatography of BAP-Loop-Y. M: protein molecular weight marker; C: whole-cell extract; S: supernatant of the cell extract after centrifugation; F: flow-through fraction. The number indicated is the No. of the fractions, which showed a UV absorbance peak in the chromatograms. The arrow indicated the pooled fractions.

3-5. Purification of pG_2pA-Y

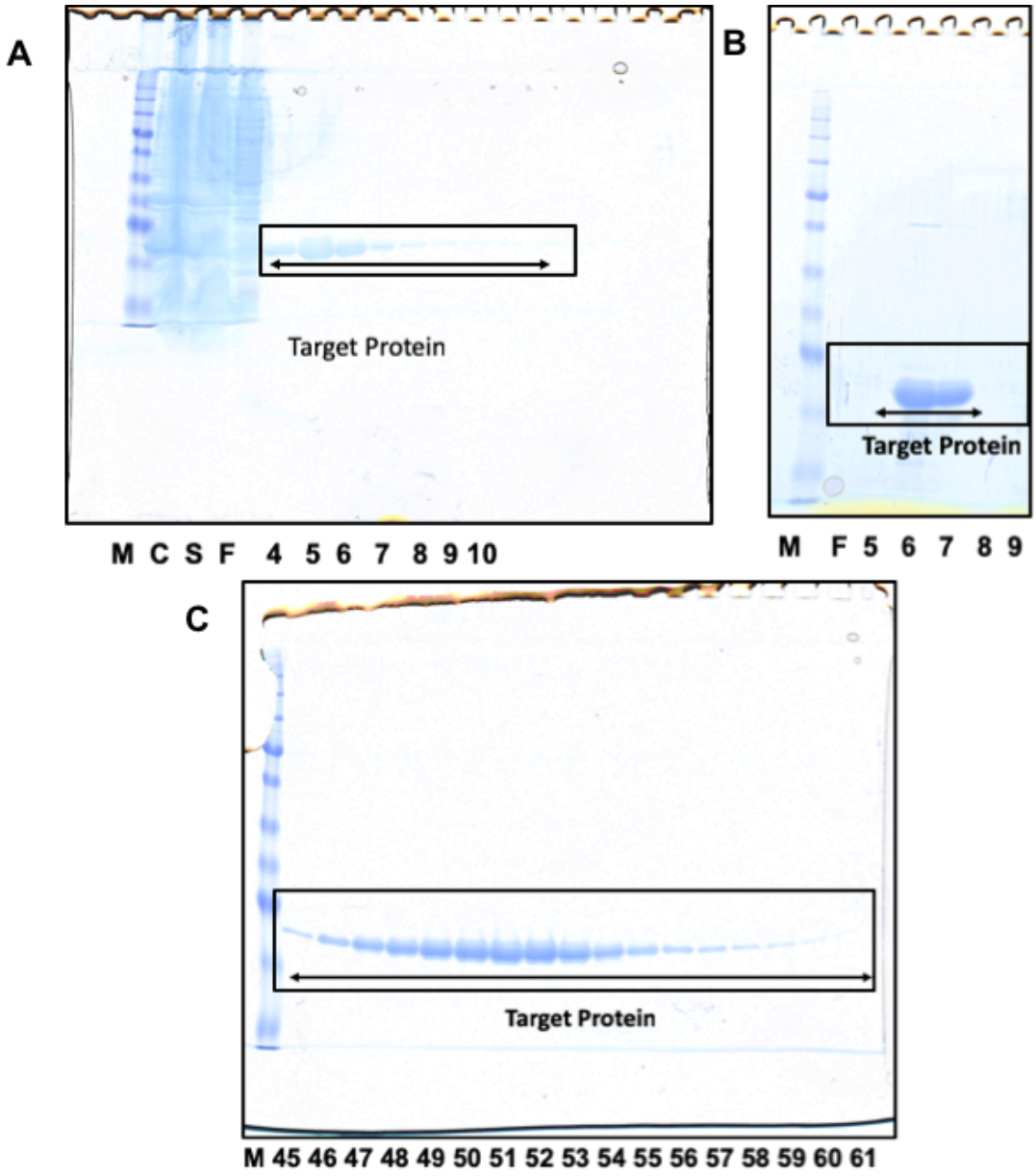

Figure S5 The purification results of pG_2pA-Y. SDS-PAGE results of purified fractions; (A) fractions in the HisTag purification step, (B) fractions in the anion exchange chromatography (AEC) step, and (C) fractions in the SEC chromatography of pG_2pA-Y. M: protein molecular weight marker; C: whole-cell extract; S: supernatant of the cell extract after centrifugation; F: flow-through fraction. The number indicated is the No. of the fractions, which showed a UV absorbance peak in the chromatograms. The arrow indicated the pooled fractions.

3-6. Protein concentration determination of BAPs and pG_2pA-Y

Table S1 protein concentration of purified BAPs

Samples	MW (g/mole)	Protein (mg/mL)	Volume (mL)	Total protein (mg)
BAP-WT	97618.96	8.23	0.95	7.82
BAP-Y	99621.18	7.92	1.10	8.71
BAP-pG_2pA-Y	142009.50	6.94	1.15	7.99
BAP-Loop-Y	100510.34	7.37	0.90	6.63
pG_2pA-Y	22041.13	6.88	2.40	16.52

3-7. Optimization of polymerization reaction

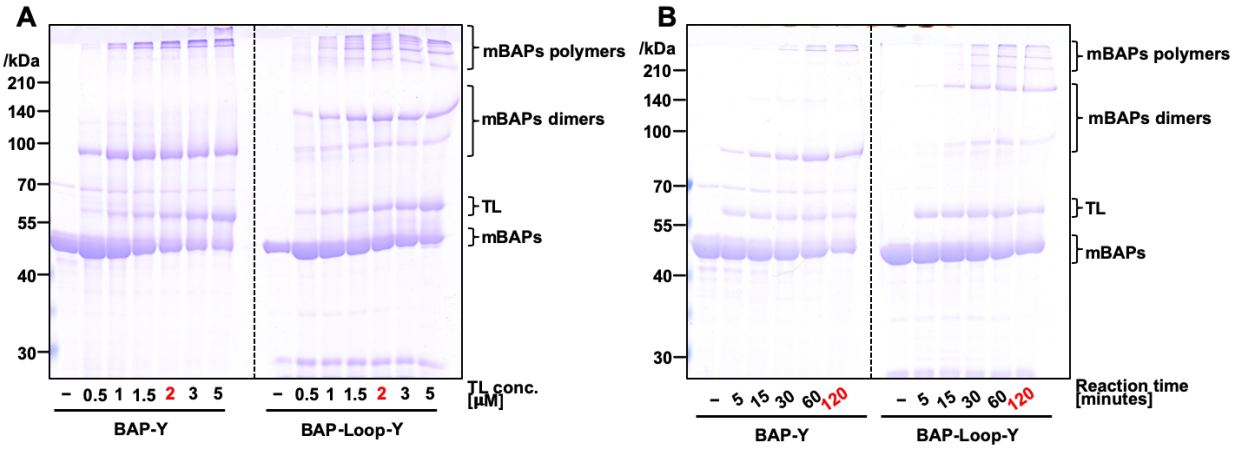

Figure S6 Results of optimization of polymerization reaction of the BAPs against time and TL concentration. (A) Optimization of TL concentration. (B) Optimization of reaction time. The final concentration of the BAPs was 10 μM. The reaction was conducted in 10 mM Tris-HCl pH 8.0 and incubated at 37 °C for an appropriate time.

3-8. Horseradish peroxidase (HRP)-catalyzed BAPs polymerization

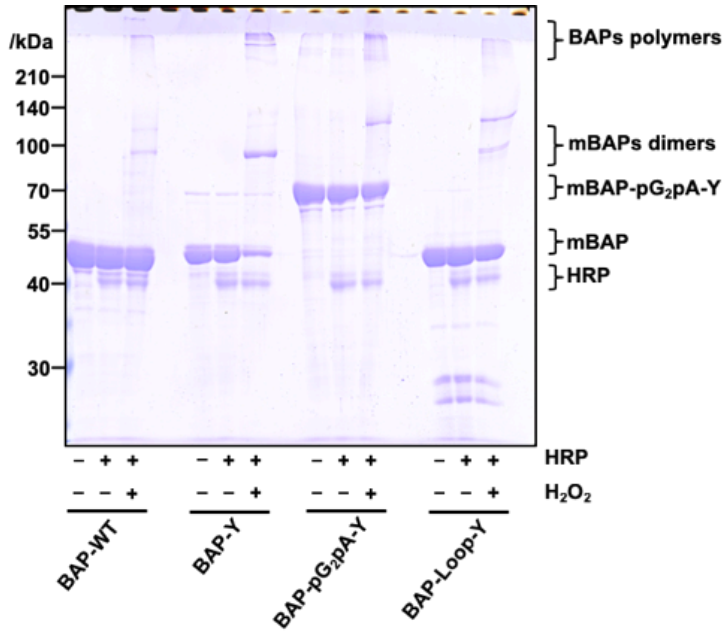

Figure S7 Results of the SDS-PAGE analysis of the HRP-catalyzed polymerization reaction of the BAPs. The final concentrations of the BAPs, HRP, and hydrogen peroxide (H_2O_2) were 10, 2, and 10 μM, respectively. The reaction was conducted in 10 mM Tris-HCl pH 8.0 and incubated at 37 °C for 2 h.

3-9. TL-catalyzed polymerization of BAPs

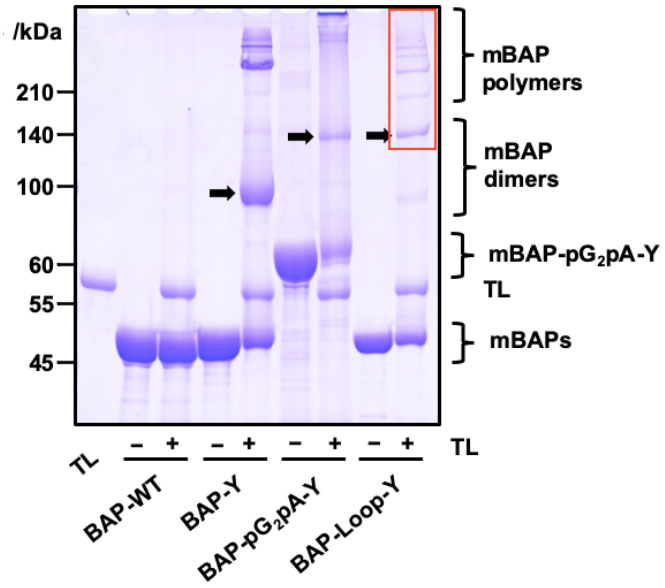

Figure S8 Results of the SDS-PAGE analysis of BAPs before and after TL treatment. The concentration of the BAPs and TL were 10 and 2 μM, respectively. The cross-linking reaction of the BAPs was conducted in 10 mM Tris-HCl pH 8.0 at 37 °C for 2 h. The percentage of the separating gel used in the native-PAGE analysis was 10 % (wt%).

3-10. Height analysis of BAPs monomers and polymers in SPM analysis

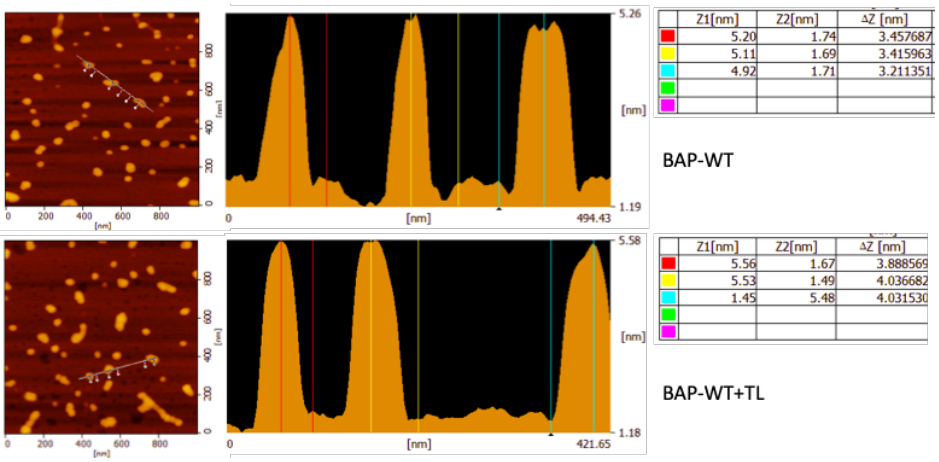

Figure S9 Height analysis of BAP-WT monomers and polymers

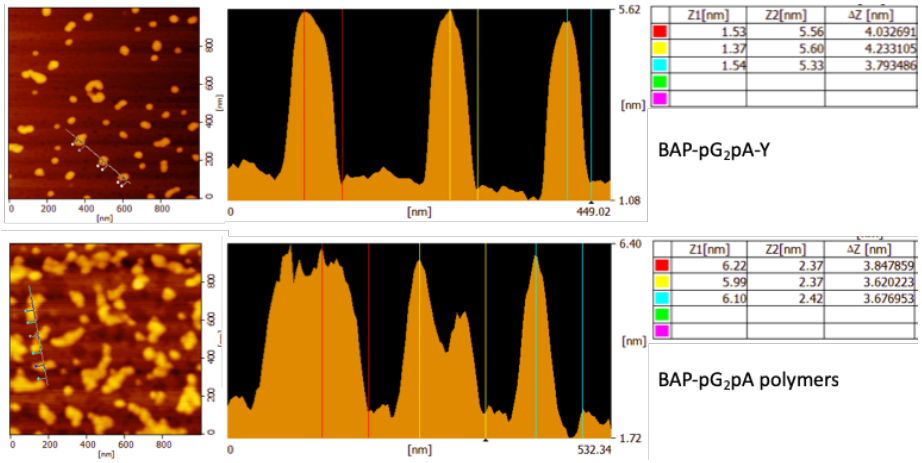

Figure S10 Height analysis of BAP-pG$_2$pA-Y monomers and polymers

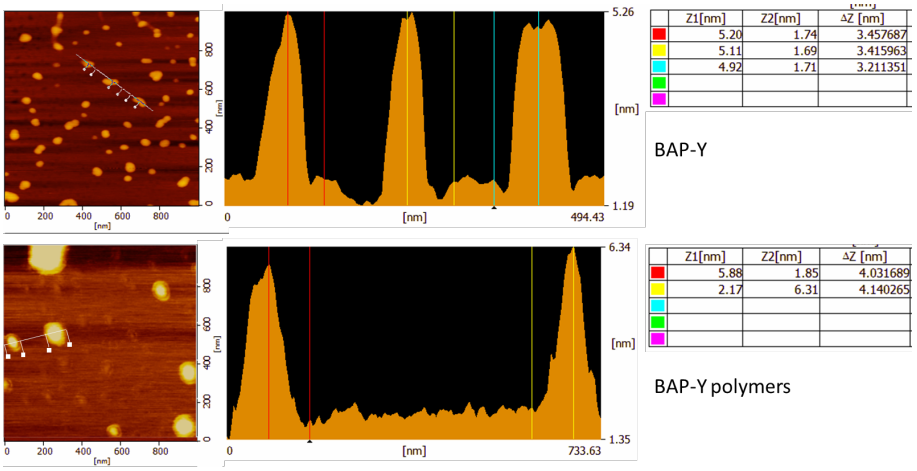

Figure S11 Height analysis of BAP-Y monomers and polymers

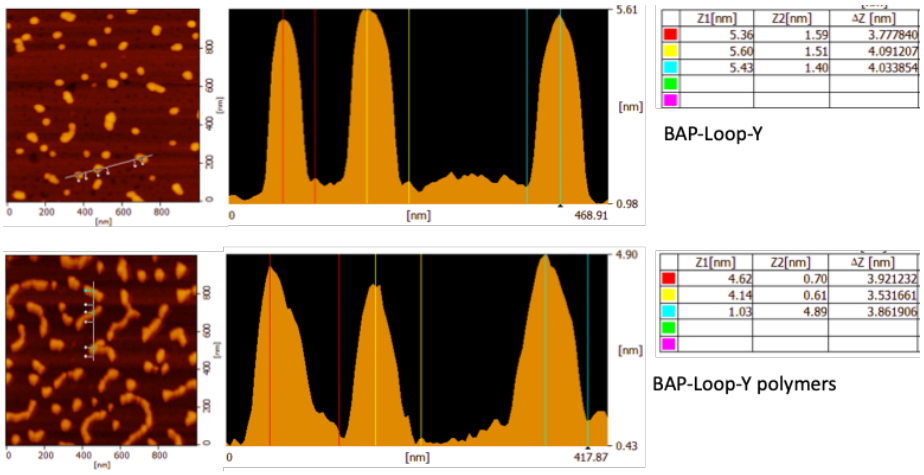

Figure S12 Height analysis of BAP-Loop-Y monomers and polymers

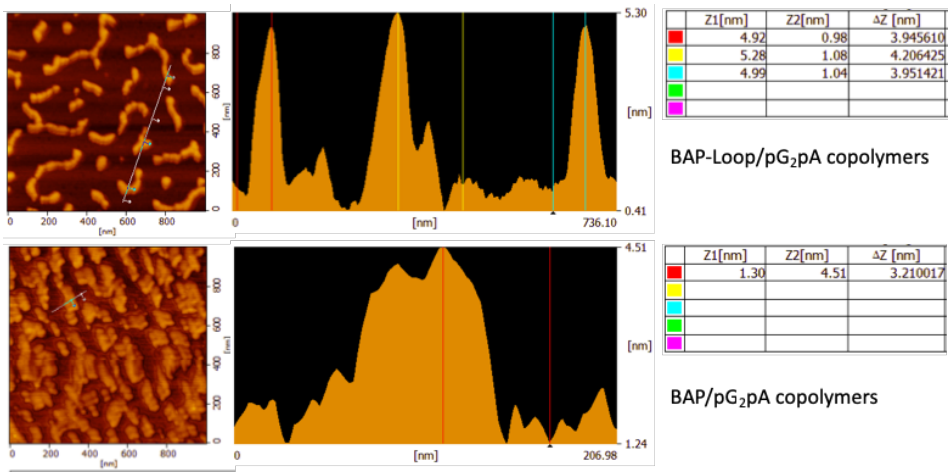

Figure S13 Height analysis of BAP-Loop-Y/pG$_2$pA-Y and BAP-Y/pG$_2$pA-Y copolymers

3-11. Length analysis of BAP-Loop-Y polymers and BAP-Loop-Y/pG$_2$pA-Y copolymers

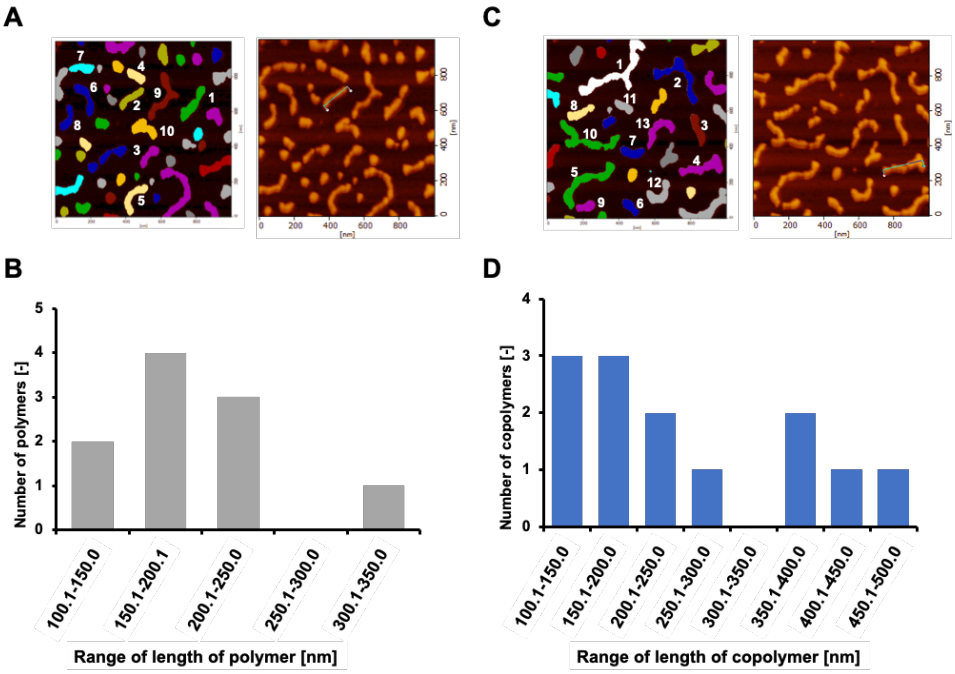

Figure S14 Results of the length analysis of BAP-Loop-Y polymers and BAP-Loop-Y/pG$_2$pA-Y copolymers. Grain analysis of the BAP-Loop-Y polymers (A) and the BAP-Loop-Y/pG$_2$pA-Y copolymers (C) to confirm whether each molecule observed in SPM analysis are connected each other or not. The numbers in grain analysis results indicates the molecules that have been analyzed. Some polymers and copolymers cannot be measured because the edge of the polymers was not clear. Histogram of range of length of the BAP-Loop-Y polymers (B) and the BAP-Loop-Y/pG$_2$pA-Y copolymers (D).

3-12. SDS-PAGE analysis and enzymatic activity of BAPs/pG$_2$pA-Y copolymers

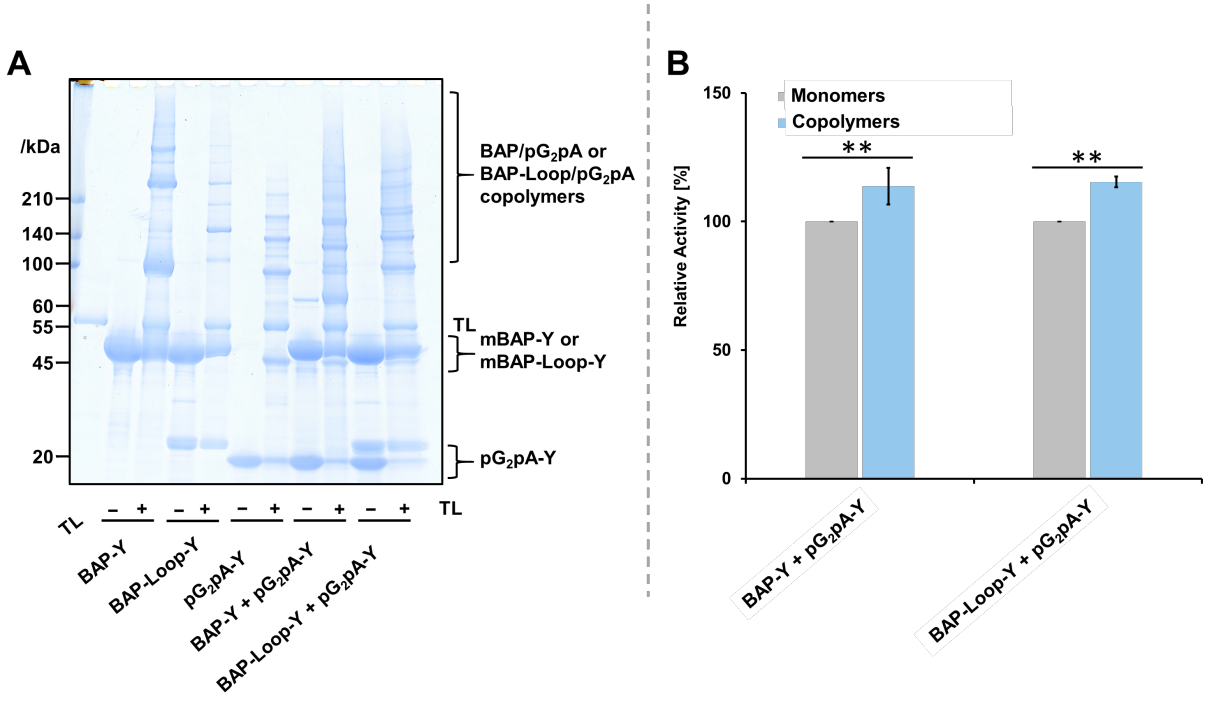

Figure S15 Results of the SDS-PAGE analysis (A) and enzymatic activity (B) of the BAPs/pG$_2$pA copolymers after TL treatment. The concentration of both proteins was 10 μM. The molar ratio of BAP and pG$_2$pA-Y were fixed to 1:1. The TL concentration was 2 μM. The reaction was conducted for 2 hours at 37 °C. A 10 % (wt.%) separating gel was used in the SDS-PAGE analysis.

3-13. SDS-PAGE analysis of TL-catalyzed copolymerization of two Y-tagged proteins with various molar ratios.

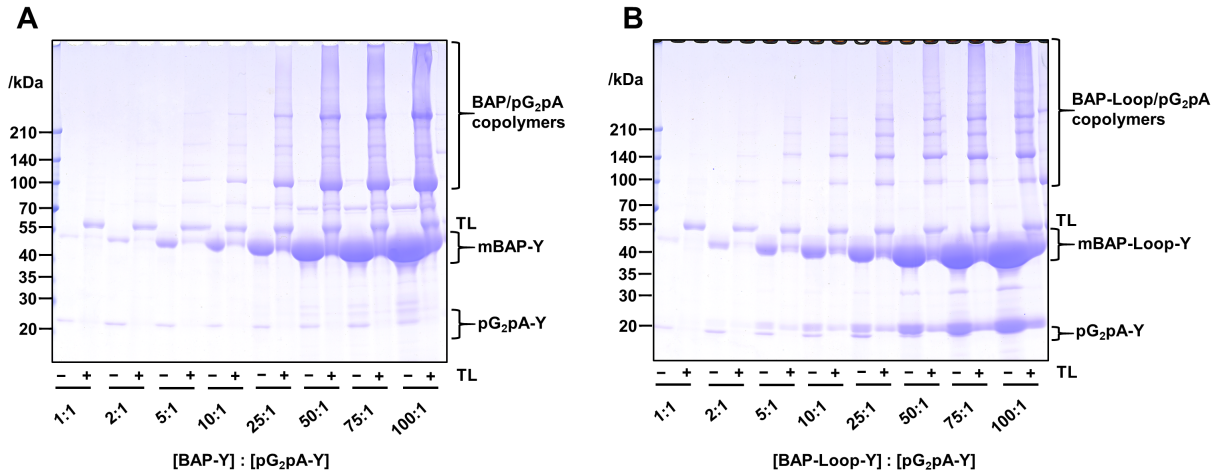

Figure S16 Results of the SDS-PAGE analysis of copolymers that were prepared from the copolymerization reaction of BAP-Y (A) or BAP-Loop-Y (B) with pG_2pA-Y with various molar ratios. The concentration of the antibody binding protein, pG_2pA-Y, was fixed to 0.5 μM. While the BAP-Y and BAP-Loop-Y concentrations were varied from 0.5–50 μM depending on the molar ratio. The concentration of TL was 2 μM. The reaction was conducted in 10 mM Tris-HCl pH 8.0, at 37 °C, for 2 h.

3-14. Size exclusion chromatography (SEC) of BAPs and pG_2pA-Y copolymers.

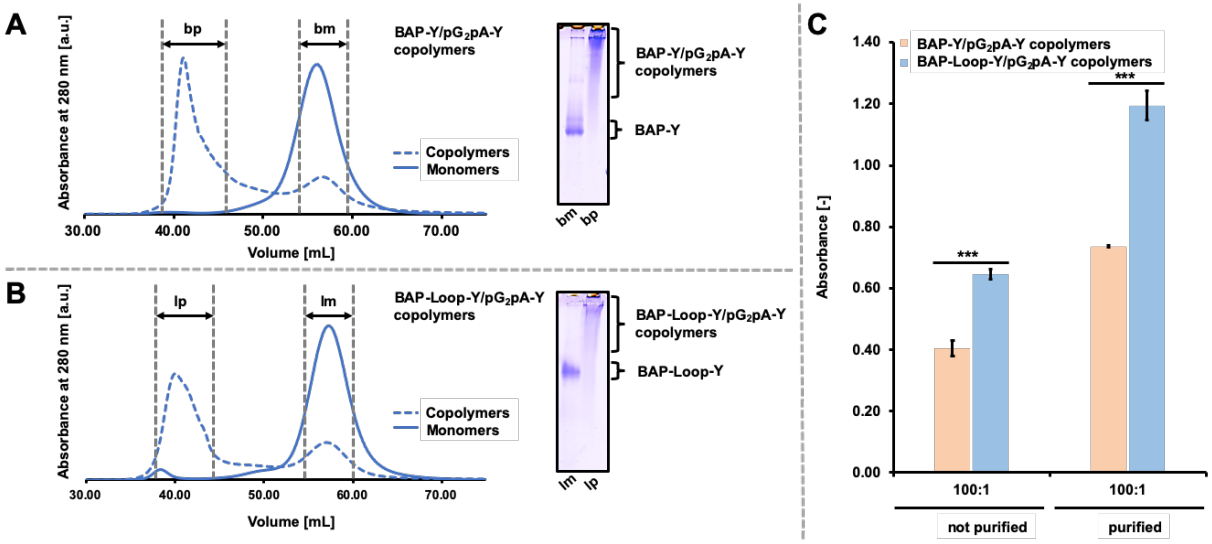

Figure S17 SEC, native-PAGE, and OVA-detecting ELISA results of the BAP/pG_2pA copolymers that were prepared at a 100:1 molar ratio of BAP-Y or BAP-Loop-Y to pG_2pA-Y. (A) BAP-Y/pG_2pA-Y copolymers. (B) BAP-Loop-Y/pG_2pA-Y copolymers. The SEC results of copolymers are shown as a dashed line. The mixed solution containing the BAPs and pG_2pA-Y without TL treatment was employed as the control (solid line). Native-PAGE analysis results of BAP-Y/pG_2pA-Y and BAP-Loop-Y/pG_2pA-Y copolymers stained using Coomassie blue. The separating gel was 10 % (%wt). (C) Results of the OVA-detecting ELISA of BAP-Y/pG_2pA-Y and BAP-Loop-Y/pG_2pA-Y copolymers from the pooled and concentrated fraction of polymers from SEC. The concentration of OVA that coated the well was 1 μg/mL in TBS (pH 7.4), while the concentration of the BAP/pG_2pA copolymers was fixed to 0.5 U/mL. Error bars denote the standard error of the measurements from three individual samples ($***p < 0.001$).

4. References

1. Minamihata, K.; Goto, M.; Kamiya, N. Control of a Tyrosyl Radical Mediated Protein Cross-Linking Reaction by Electrostatic Interaction. *Bioconjug. Chem.* **2012**, *23*, 1600–1609.
2. Selinheimo, E.; Lampila, P.; Mattinen, M.-L. L.; Buchert, J. Formation of Protein - Oligosaccharide Conjugates by Laccase and Tyrosinase. *J. Agric. Food Chem.* **2008**, *56* (9), 3118–3128.
3. Wakabayashi, R.; Yahiro, K.; Hayashi, K.; Goto, M.; Kamiya, N. Protein-Grafted Polymers Prepared Through a Site-Specific Conjugation by Microbial Transglutaminase for an Immunosorbent Assay. *Biomacromolecules* **2017**, *18* (2), 422–430.