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Osteoinductive Surface by Adhesive Bone Morphogenetic Protein-2 Prepared using the Bioorthogonal Approach for Tight Binding of Titanium with Bone

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Abstract: Inorganic biomaterials are used in various orthopedic and dental implants. Nevertheless, they cause clinical issues such as loosening of implants and patient morbidity. Therefore, inspired by mussel adhesive proteins, we aimed to design an adhesive and dimer-forming highly active bone morphogenetic protein-2 (BMP-2) using bioorthogonal chemistry, in which recombinant DNA technology was combined with enzymatic modifications, to achieve long-term osseointegration with titanium. The prepared BMP-2 exhibited substantially higher binding activity than that with the wild-type BMP-2, and the adhered BMP-2 was more active than soluble BMP-2. The adhesive BMP-2 promoted the mechanical binding of titanium to bones, enabling efficient bone regeneration. Insert abstract text here.

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Figure S8 Implantation of coated Ti screws in rat femurs.

Table S1 Primers used for constructing BMP-2 variants.

Experimental Procedures

1. Materials

Unless specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Wild-type BMP-2 (commercial BMP-2) was purchased from R&D Systems (Minneapolis, MN, USA). The *Escherichia coli* BL21 (DE3) and DH5α cells were purchased from TAKARA, Inc. (Shiga, Japan). The pET15b expression vector was purchased from Novagen (Darmstadt, Germany). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All restriction enzymes and T4-DNA ligase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Gene synthesis and DNA sequencing were performed at Sangon Biotech (Shanghai, China) and RIKEN Research Resources Division (Saitama, Japan), respectively. The cOmplete™ protease inhibitor was purchased from Roche (Basel, Switzerland). Standard-size markers of proteins and DNA were purchased from Thermo Fisher Scientific. Ti wires (φ1.0 × 10 mm) and screws (φ1.6 × 5.0 mm) were purchased from Nilaco Corporation (Tokyo, Japan) and HOYA Technosurgical Corporation (Tokyo, Japan), respectively.

2. Preparation of recombinant BMP-2 variants

2.1. Construction of pET15b-BMP-2

A DNA sequence encoding human BMP-2 was constructed based on the National Center for Biotechnology Information-approved polypeptide sequence (MQAKHKQRKR LKSSCKRHPL YVDFSDVGWN DWIVAPPGYH AFYCHGECPF PLADHLNSTN HAIVQTLVNS VNSKIPKACC VPTELSAISM LYLDENEKVV LKNYQDMVVE GCGCR; GenBank accession no. AOQ30482.1). Codon optimization was performed according to the codon preferences of *E. coli*. The resulting DNA sequence was commercially synthesized in a pUC19 cloning vector between the *Nde*I and *Xho*I restriction sites at Sangon Biotech. The recombinant pUC19 vector was digested using the *Nde*I and *Xho*I restriction enzymes (Thermo Fisher Scientific) according to the manufacturer's instructions. The released fragment was excised from the agarose gel and purified using a DNA extraction kit (Thermo Fisher Scientific). The purified insert fragment was ligated to a pET15b expression vector linearized with the *Nde*I and *Xho*I restriction enzymes to construct pET15b-BMP-2.

2.2. Construction of Y-pep-BMP-2, BMP-2-Ypep, and BMP-2-linker-Ypep expression vectors

To construct the pET15b-Ypep-BMP-2 expression vector, pET15b was linearized using polymerase chain reaction (PCR) with the following primer pair: GGACATGGTTGTGGAaGGTTGTGGcTGTCGCTAAC (forward) and GTACTTGTATTTATAGCTGCCGCGCGGCACCAGGCCGCTG (reverse). The insert fragment of Ypep-BMP-2 was amplified using PCR. The following primer sets were used for the Ypep-BMP-2 gene: AGCTATAAATACAAGTACCATCAGGCGAAACACAAACAGCGTAAA (forward) and TCCACAACCATGTCCTGATAGTTTTTCAGCACAAC (reverse). PCR was performed using KOD-ONE DNA polymerase (TAKARA). The insert fragment was cloned into the linearized pET15b vector using an In-Fusion cloning kit (TAKARA). The constructed plasmid was transformed into *E. coli* DH5α cells, which were spread onto lysogeny broth (LB) agar (100 μg/mL ampicillin) plates. The colonies that appeared were sequenced using colony PCR. The confirmed plasmid was extracted from DH5α using a QIAprep® miniprep kit (Qiagen, Venlo, The Netherlands) and transformed into *E. coli* BL21 (DE3) expression host, which was spread onto an LB agar (100 μg/mL ampicillin) plate. Plasmid construction was confirmed using colony PCR and DNA sequencing. The confirmed plasmids were extracted from DH5α using the QIAprep® miniprep kit and transformed into the *E. coli* BL21 (DE3) expression host. BMP-Ypep and BMP-2-linker-Ypep expression vectors were prepared using the same methods. The information on primers used is provided in Table S1.

2.3. Batch culture of *E. coli*

Based on a previous study,[10] the temperature and concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) used for expressing the recombinant proteins in *E. coli* BL21 cells were optimized and found to be 37 °C and 400 μM IPTG, respectively.

To obtain large amounts of the BMP-2 variants, the BL21 (DE3) transformants were grown overnight in 20 mL LB medium containing ampicillin (100 μg/mL) in a 100-mL flask at 37 °C until an OD_{600} of approximately 1 was reached after 10 h. The cells were subsequently transferred to 5 L of sterilized terrific broth (TB)-modified medium (Sigma-Aldrich) containing ampicillin (100 μg/mL) and antifoam (0.01% [v/v]) (Wako Pure Chemical Industries Ltd.) in an inoculated flask at 37 °C and shaken at 130 rpm. The culture was induced at OD_{600} of 0.8 by adding IPTG (400 μM). After 12 h, the cells were harvested from the culture medium via centrifugation at 8,000 rpm for 15 min at 4 °C.

2.4. Protein extraction and purification of unfolded proteins

The obtained pellet (5 g) was resuspended in lysis buffer A (25 mL) (Tris-HCl [50 mM] containing ethylenediamine tetraacetic acid [EDTA] [1 mM], NaCl [10 mM], Triton-X 114 [2% (v/v)], and 1 tablet of cOmplete™ protease inhibitor, pH 8.0), 1 mL DNase I (0.8

mg/mL), and 1 mL lysozyme (0.1 mg/mL). The solution containing cell bodies was slowly rotated at 4 °C for 1 h. Next, the cells were lysed using a sonicator (Branson Sonifier 250, Danbury, CT, USA) on ice (30–40% duty cycles, 3–4 power output). The disrupted cells were centrifuged at 8,000 rpm for 30 min at 4 °C, and the resulting pellet was resuspended in lysis buffer A. The suspension was sonicated for 1 min and recentrifuged. The washing process was repeated thrice with buffer A. After the final centrifugation, the pellets were resuspended in lysis buffer B (25 mL) (Tris-HCl [50 mM] containing EDTA [1 mM], NaCl [10 mM], and 1 tablet of cOmplete™ protease inhibitor, pH 8.0) and washed thrice via centrifugation with buffer B.
To extract the inclusion bodies, the pellets were resuspended in a mixture of solubilization solution/buffer A (urea [8 M], dithiothreitol [5 mM], NaH_2PO_4 [100 mM], and Tris-HCl [50 mM] at pH 8.0) and rotated overnight at 4 °C. The supernatant containing solubilized proteins was obtained via centrifugation at 8,000 rpm for 30 min at 4 °C. The extracted protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (5 mL HisTrap Fast Flow, Cytiva Japan, Tokyo, Japan). The column was initially equilibrated with buffer A (150 mL; urea [8 M], dithiothreitol [1 mM], NaH_2PO_4 [100 mM] and Tris-HCl [10 mM] at pH 8.0) at the flow rate of 1 mL/min at 4 °C. Next, the proteins were eluted from the column using buffer B (urea [8 M], dithiothreitol [5 mM], NaH_2PO_4 [100 mM], Tris-HCl [50 mM], and imidazole [0.5 M], pH 8.0).

2.5. Refolding of purified proteins
To efficiently prepare the active 3D forms of homodimeric BMP-2 variants, the unfolded proteins were refolded using a previously reported dropping method.[40] Briefly, to promote the refolding of denatured proteins by rapidly decreasing the urea concentration, the unfolded protein solution was slowly dropped into 1 L of refolding buffer (Tris-HCl [50 mM], NaCl [40 mM], EDTA [1 mM], L-arginine [1 M], glycerol [10% (v/v)], and glutathione disulfide [GSSG]/GSH [2 mM]) using a peristaltic pump (MINIPULS3, Gilson, Inc., WI, USA), and stirred using a magnetic stirrer. The refolding buffer was further concentrated to 30 mL using a concentrator (Amicon Stirred Cell 400 mL, EMD [Millipore, Billerica, MA, USA]) with a semipermeable membrane (molecular weight cut-off, 10 kDa; Amicon [Millipore]). At this concentration, GSSG (2 mM) was added to the concentrated solution, and the solution (30 mL) was dialyzed using a dialysis buffer (urea [6 M], Tris-HCl [100 mM], and EDTA [5 mM] at pH 6.0) for further purification.

2.6. Homodimer purification
The dialysis solution was loaded into a heparin column for further purification at 4 °C. The binding buffer of the heparin column (5 mL HiTrap Heparin HP column, Cytiva Japan) comprised urea (6 M), Tris-HCl (100 mM), and EDTA (5 mM) at pH 6.0. The elution buffer comprised urea (6 M), Tris-HCl (100 mM), EDTA (5 mM), and NaCl (1 M) at pH 6.0. The eluted fractions were analyzed using 15% non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples for analysis were resuspended in a 1× protein loading buffer (Tris-HCl [60 mM], 2% SDS, 10% glycerol, and 0.01% bromophenol blue), and the gels were stained with Coomassie Brilliant Blue R-250. The Bradford assay was performed for protein quantification at 595 nm using the Pierce™ Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific). The fractions containing only the dimers were obtained and dialyzed against ammonium acetate buffer (20 mM) (pH, 4.8). The dimer solution was lyophilized and stored at −20 °C until use.

2.7. Tyrosine hydroxylation
Hydroxylation of the Tyr residues was performed using tyrosine hydroxylase (50 µg/mL) (Sigma-Aldrich), L-ascorbic acid (20 mM), and dimers (5 mg/mL) in 20 mM ammonium acetate (1 mL) at pH 4.8 overnight at 4 °C.

3. MALDI-TOF-MS and LC-MS
MALDI-TOF-MS analyses were performed on the Applied Biosystems QSTAR Elite system (Life Technologies, Carlsbad, CA, USA) in the Support Unit for Bio-Materials Analysis Research Resources Center at the RIKEN Center for Brain Science. DOPApep-BMP-2 was digested with endoproteinase Arg-C (Sigma) for 2 h at 37 °C. The digested fragment was detected using Q-Exactive HF-X (Thermo Fisher Scientific) and EASY-nLC 1200 (Thermo Fisher Scientific) at the Support Unit for Bio-Materials Analysis Research Resources Center at the RIKEN Center for Brain Science.

4. Coating of Ti materials
The Ti materials were sonicated thrice in a hexane solution for 15 min. After drying, the samples were sonicated thrice in a detergent reagent (BD detergent solution, 660585, BD, NJ, USA) for 30 min and rinsed thrice with MilliQ water. The materials were exposed to UV light for 15 min before coating. The Ti materials were coated with BMP-2 derivative solutions and washed with phosphate-buffered saline (PBS at pH 7.4).

5. Binding assay
The adsorption characteristics of various concentrations on the Ti substrate were measured at 25 °C using QCM-D (Meiwafosis Co., Ltd, Tokyo, Japan). In particular, PBS was allowed to run until the baseline became stable. Next, each sample was run for 20 min at approximately 1.67 µL/s before elution with a PBS buffer. All experiments were conducted in triplicate for each concentration to calculate the average values. The thickness of the DOPApep-BMP-2 layer was determined using a spectroscopic ellipsometer (M-2000UI, J. A. Woollam, Co., NE, USA).
The proteins on the materials were observed using fluorescein isothiocyanate (FITC; Cytiva Japan). The Ti screws were incubated with a solution of Ypep-BMP-2 or DOPApep-BMP-2 for 2 h at 4 °C and washed thrice with PBS for 30 min. Subsequently, the screws were rinsed with PBS-Tween (0.2% v/v), blocked with ECL blocking agent (4% w/v) (Cytiva RPN2125, Merck, Kenilworth, NJ, USA) in Tris-buffered saline with Tween (TBS-T) for 30 min, washed thrice with TBS-T (0.2% v/v), and incubated with FITC for 1 h at 25 °C.

Surface images of the resulting screws were recorded using an AxioObserver fluorescence microscope equipped with an AxioCam MRc5 camera (Carl Zeiss Co., Ltd., Göttingen, Germany).

6. In vitro evaluation of biological activity

BRE-Luc C2C12 cells, harboring a luciferase reporter gene with a BMP-2-specific enhancer derived from the inhibitor of differentiation (Id)1 promoter, were kindly provided by Dr. K. Shiba at the Cancer Institute Hospital of JFCR and were used for in vitro evaluation. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Tokyo, Japan) supplemented with 5% bovine serum (Gibco, Thermo Fisher Scientific) and 1% penicillin–streptomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 37 °C in the presence of 5% CO_2. The starved cells were seeded with different concentrations of soluble proteins or those bound on a Ti disk, added to 1% DMEM culture medium, and incubated at a density of 2 × 10^4 cells at 37 °C in the presence of 5% CO_2. After 48 h, the cells were washed with PBS and disrupted using a lysis reagent (Promega, Madison, WI, USA). Luciferase activity in the lysate was measured using a luciferase assay reagent kit (Promega) and a multimode plate reader (PerkinElmer, Waltham, MA, USA). The observed activity was normalized to that of the protein content of the cell lysate, which was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). All experimental concentrations were assessed in triplicate.

7. In vivo assays

7.1. Animal experiments

Female Sprague–Dawley rats (8–10 weeks old, SLC, Japan) were anesthetized in an anesthesia chamber filled with 4% isoflurane (Forane; Abbott Japan Co., Ltd., Tokyo, Japan), and the modified BMP-2-coated and non-coated control implants were implanted in the left and right tibia, respectively. After the prescribed periods, the animals were euthanized, and the implanted tibias were resected and used for further studies. All animal protocols were approved by the Animal Care and Use Committee of Okayama University (approval numbers OKU-2021429 and OKU-2021493), and all experiments were performed following the relevant guidelines and regulations.

7.2. Sample preparation and implantation

Ti wires and screws were coated with modified BMP-2 (2 mg/mL) in PBS at different pH. The coated Ti was washed thrice for 30 min with PBS (pH 7.4) until release of the BMP-2 derivatives was not observed. The wire and screw were inserted in the femoral condyle after drilling a 1.1-mm diameter hole by cutting 10 mm of the skin in front of the knee. The medial tibia was exposed, and the screw was implanted slightly distal to the epiphyseal line of the tibia.

7.3. Pull-out test

Pull-out tests using a tensile tester (MX2–500 N, Imada Co., Ltd., Aichi, Japan) were used to evaluate the strength of binding between the implant and bone. Measurements were performed using a digital force gauge at a constant deformation rate of 100 mm/min until the sample was completely removed from the bone.

7.4. Micro-CT analysis

Micro-CT scanning was performed using a high-resolution micro-CT LaTheta LCT 200 (Aloka, Tokyo, Japan), and the specimens were placed vertically in a sample holder and scanned. The scanning voxel size was 24 mm, and the X-ray energy was 80 keV. In total, 1,592 projections per 360° were performed. Micro-CT was used to identify the exact position of the growth plate and analyze bone density.

7.5. SEM imaging

After fixation, the samples were embedded in resin and cross-sectioned using a cross-sectional polisher (SM-09020CP Cross-section Polisher, JEOL, Tokyo, Japan). The specimens were observed using field emission SEM (FE-SEM) (JSM-6701F, JEOL), with a backscattered electron operated at 5 kV, using an annular semiconductor detector. The cancellous bone-to-wire (bone/wire) ratio was calculated in the range of 1.3 mm^2 using a numerical analysis software (MATLAB, MathWorks, Natick, MA, USA). The specimens were also analyzed using energy-dispersive X-ray spectroscopy (SEM-EDX) to evaluate the Ca and P concentrations.

7.6. Histology

Differences between the implants and bone tissues were histologically analyzed. The bone samples were resected at the indicated intervals after implantation, fixed in 10% formalin, decalcified in 10% EDTA, embedded in paraffin, and stained with hematoxylin/eosin (H&E).

8. Statistical analysis

Independent experiments were performed at least thrice. Triplicate samples were analyzed in each experiment, and their representative data are shown. Data from the control and treated groups were statistically examined using a paired Student's *t*-test, and statistical significance was set at $p < 0.05$.

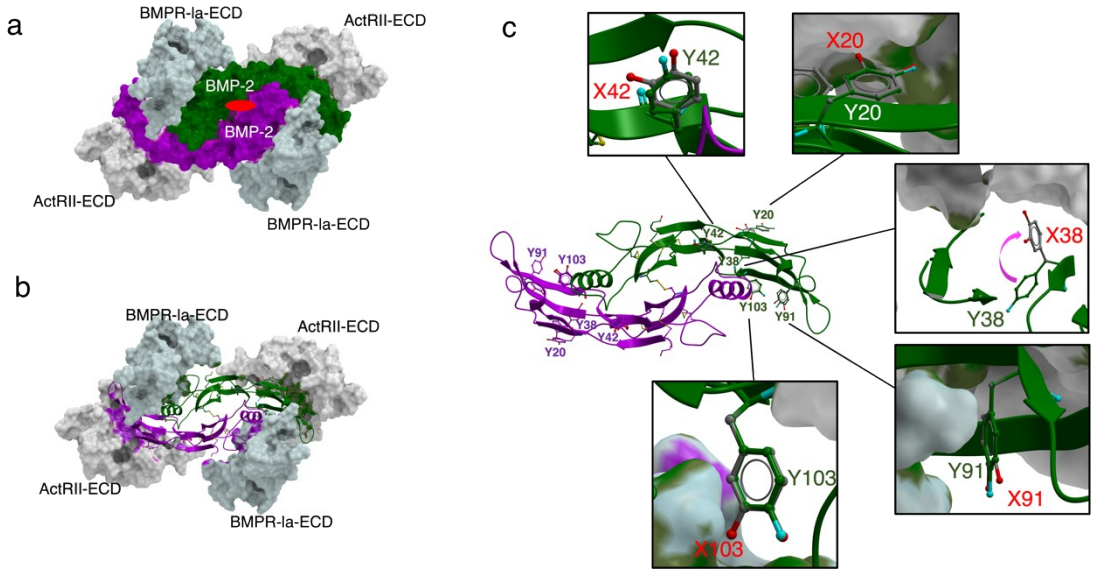
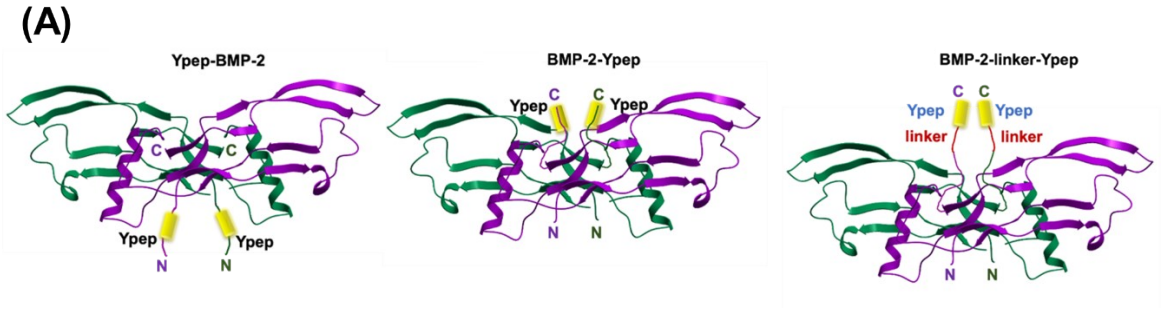


Figure S1. Schematic drawing of the bone morphogenetic protein-2 (BMP-2) homodimer complexed with the BMPR-Ia-ECD and AcRII-ECD domains of the BMP receptor (BMPR) (PDB ID: 2GOO). (a) Surface representation of BMP-2 homodimer (green and purple, respectively), complexed with BMPR-Ia-ECD (light cyan) and ActRII-ECD (white). The red ellipse indicates the position of the 2-fold axis. (b) BMP-2 homodimer (ribbon representation) complexed with BMPR. The interacting sections between BMP-2 and BMPR are schematically represented with graduated green and purple colors, respectively. (c) Tyrosine residues (Y20, Y38, Y42, Y91, and Y103) in BMP-2 are zoomed in, showing the interaction with the BMPR surfaces. The L-DOPA residues (X20, X38, X42, X91, and X103) are superposed onto the corresponding Y residues with local energy optimization. The side-chain conformation of X38 is largely flipped toward the surface of ActRII-ECD from Y38, whereas other residues show subtle displacements upon L-DOPA formation. Residue numbers correspond to those in the crystal structure. The picture drawing and energy calculation were conducted using ICM-Pro 3.9-3a.



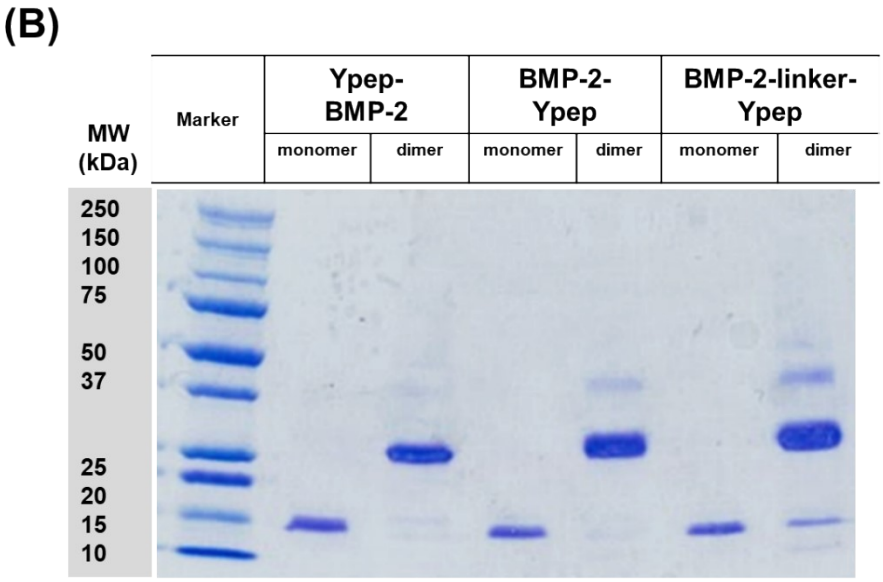


Figure S2. **Molecular structures and characterization of the prepared bone morphogenetic protein-2 (BMP-2) derivatives. (A)** Structures of the dimerized Ypep-BMP-2, BMP-2-Ypep, and BMP-2-linker-Ypep proteins, generated using AlphaFold. The 3,4-dihydroxyphenethylamine residues are depicted based on the length of the N-terminal loops. The monomer chains of BMP-2 are colored green and purple. Yellow cylinders indicate Ypep, and the linkers are in red. **(B)** Characterizing the monomer (mono) and dimer forms of recombinant Ypep-BMP-2, BMP-2-Ypep, and BMP-2-linker-Ypep using non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One microgram of protein was loaded in each lane.

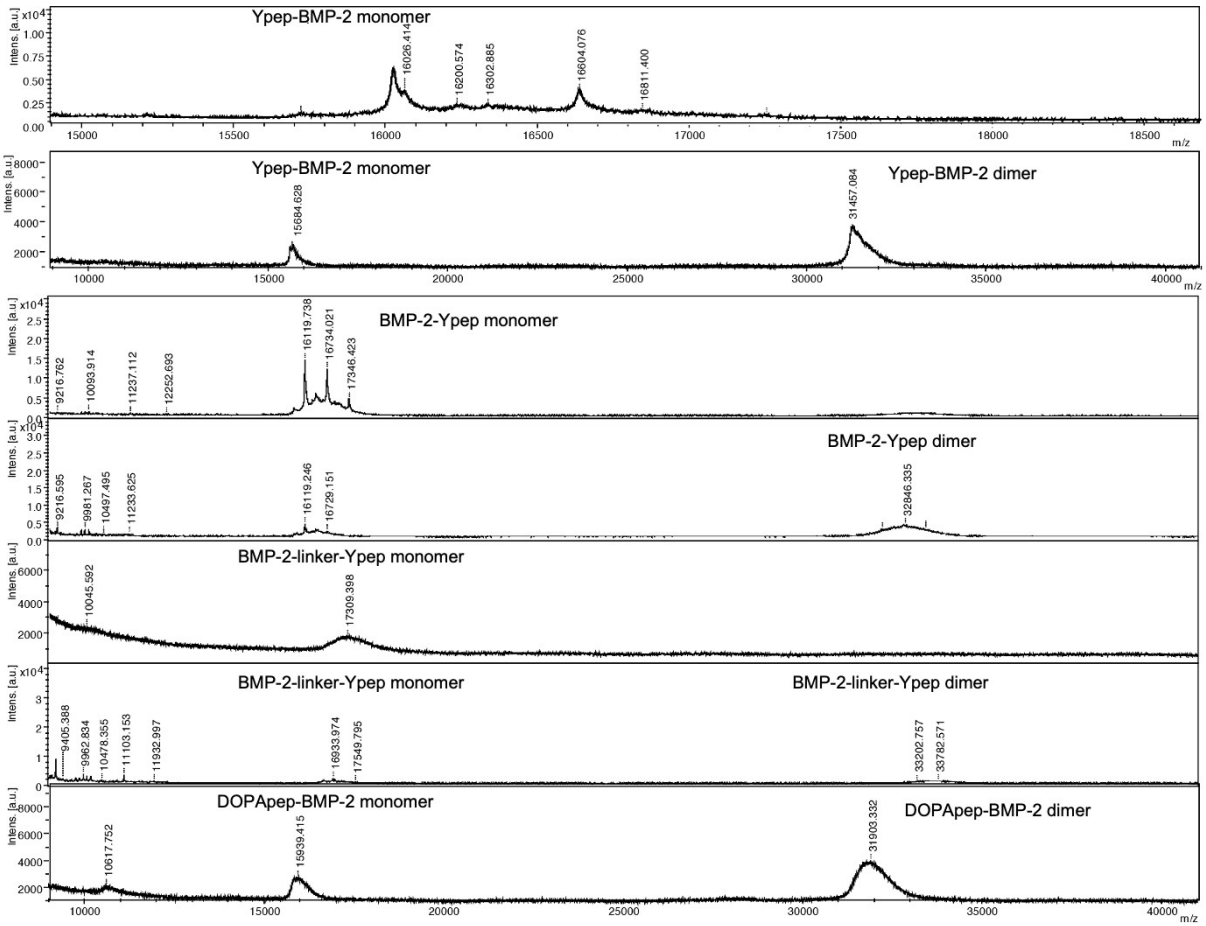
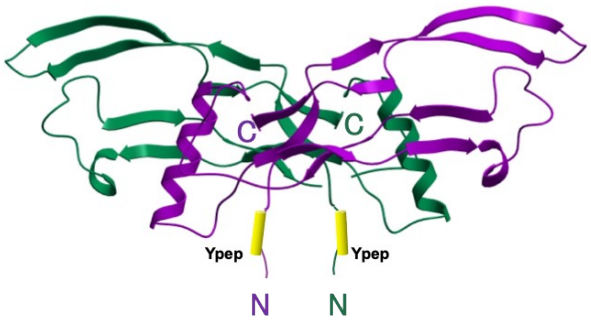
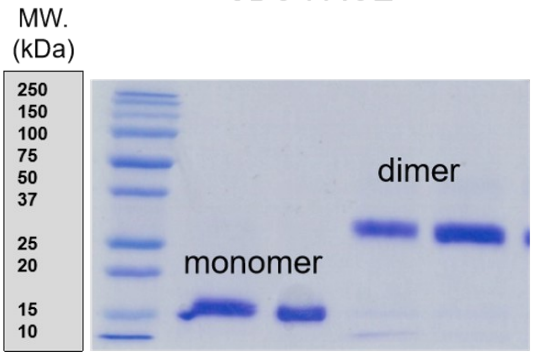


Figure S3. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy results of prepared proteins.

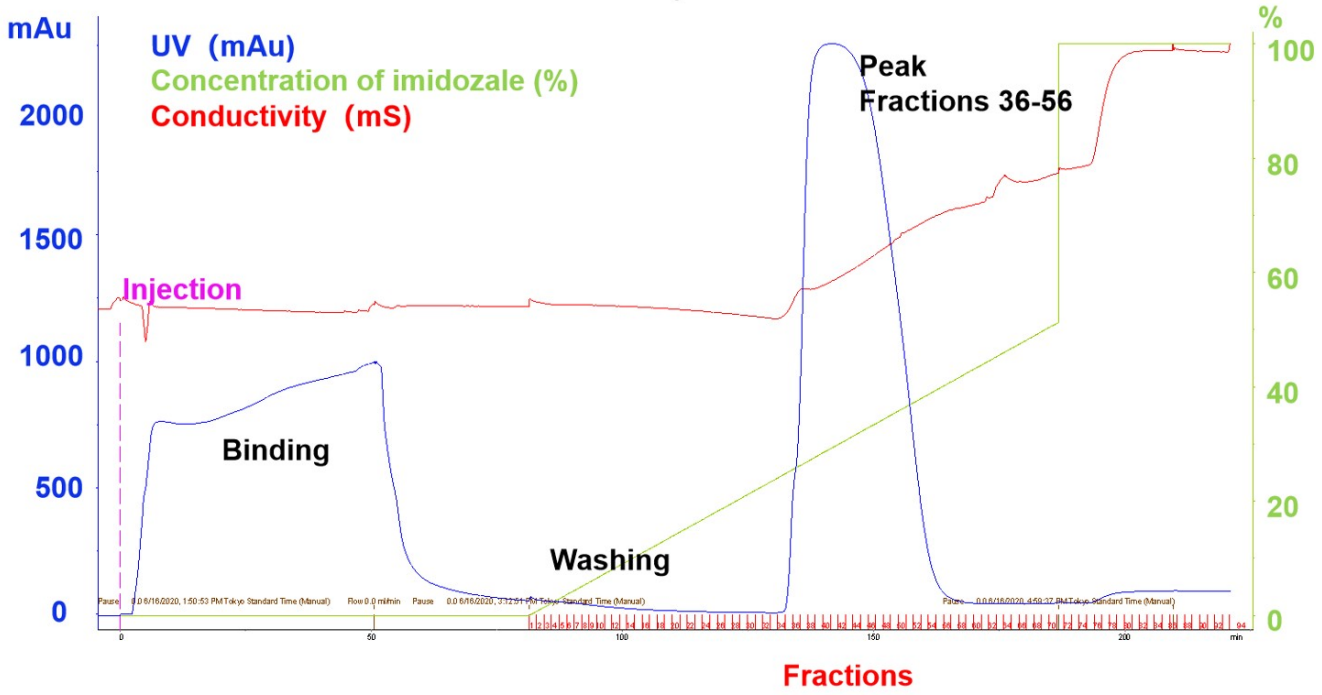
Ypep-BMP-2



SDS-PAGE



FPLC of 1st purification



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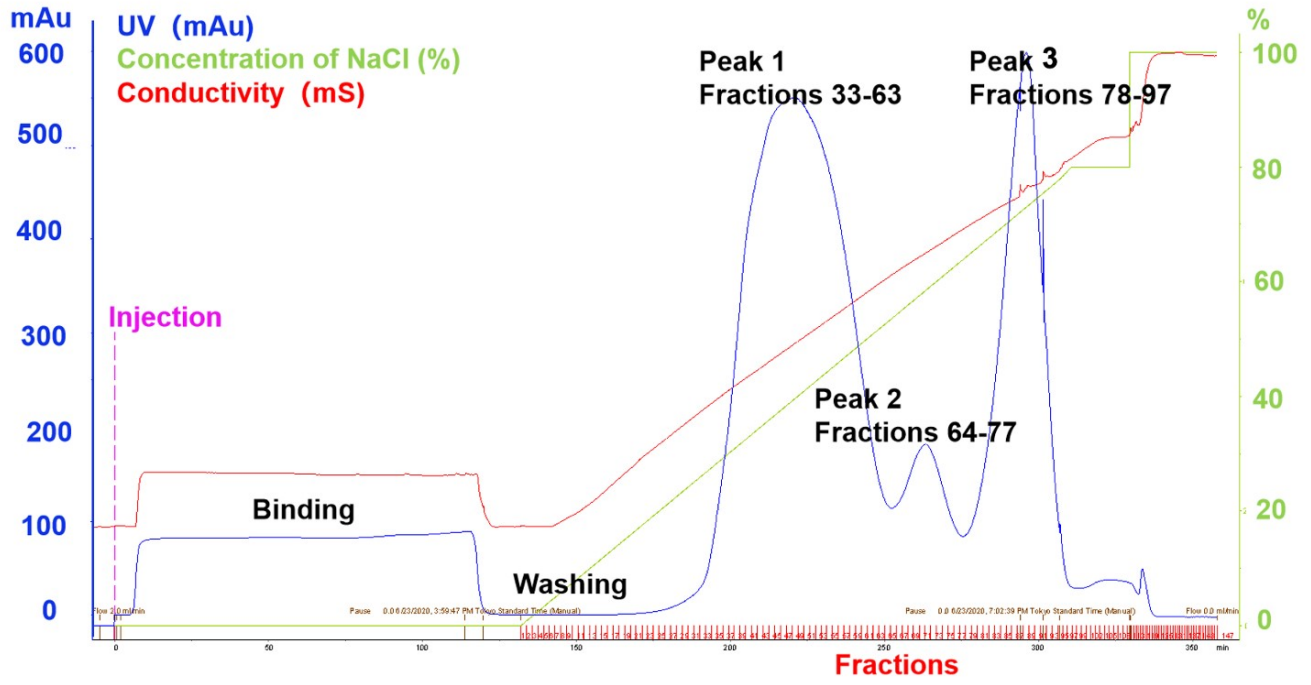
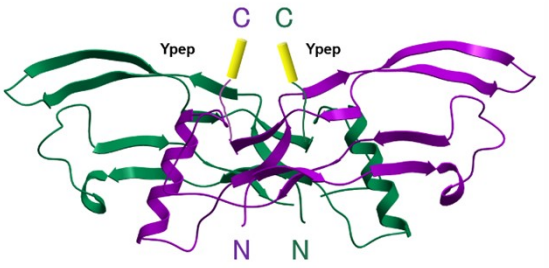


(A)

(B)

BMP-2-Ypep



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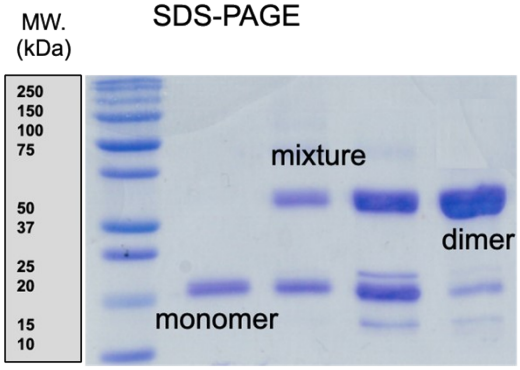

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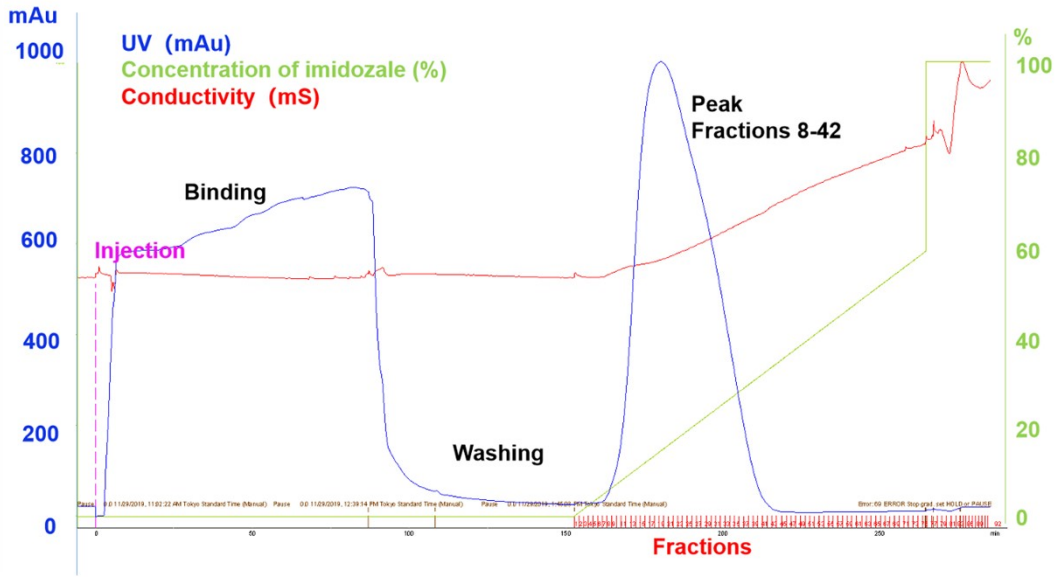
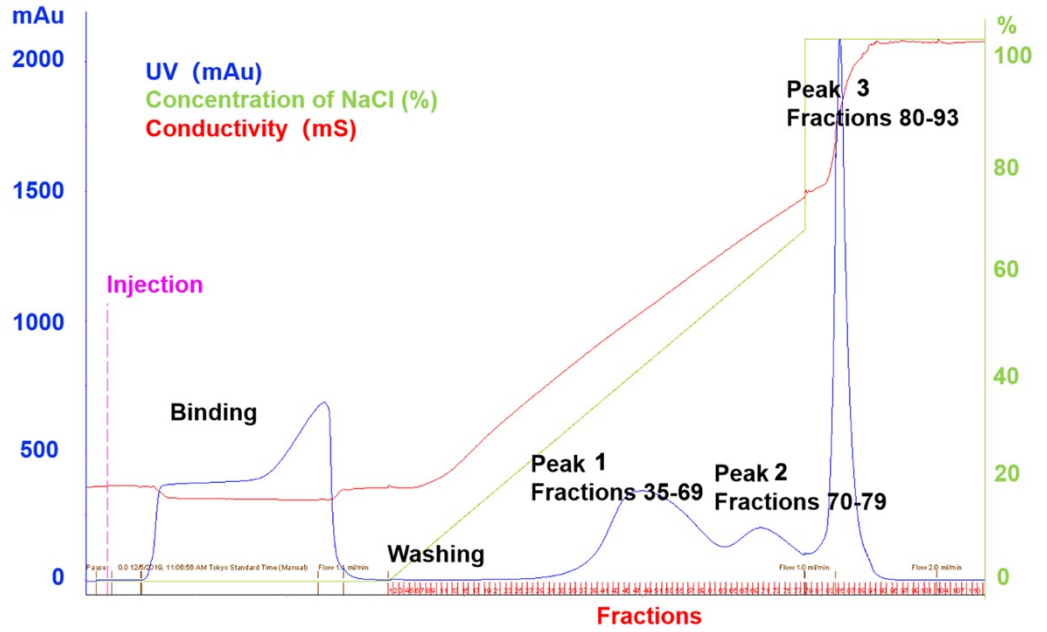


FPLC of 2nd purification



(C)

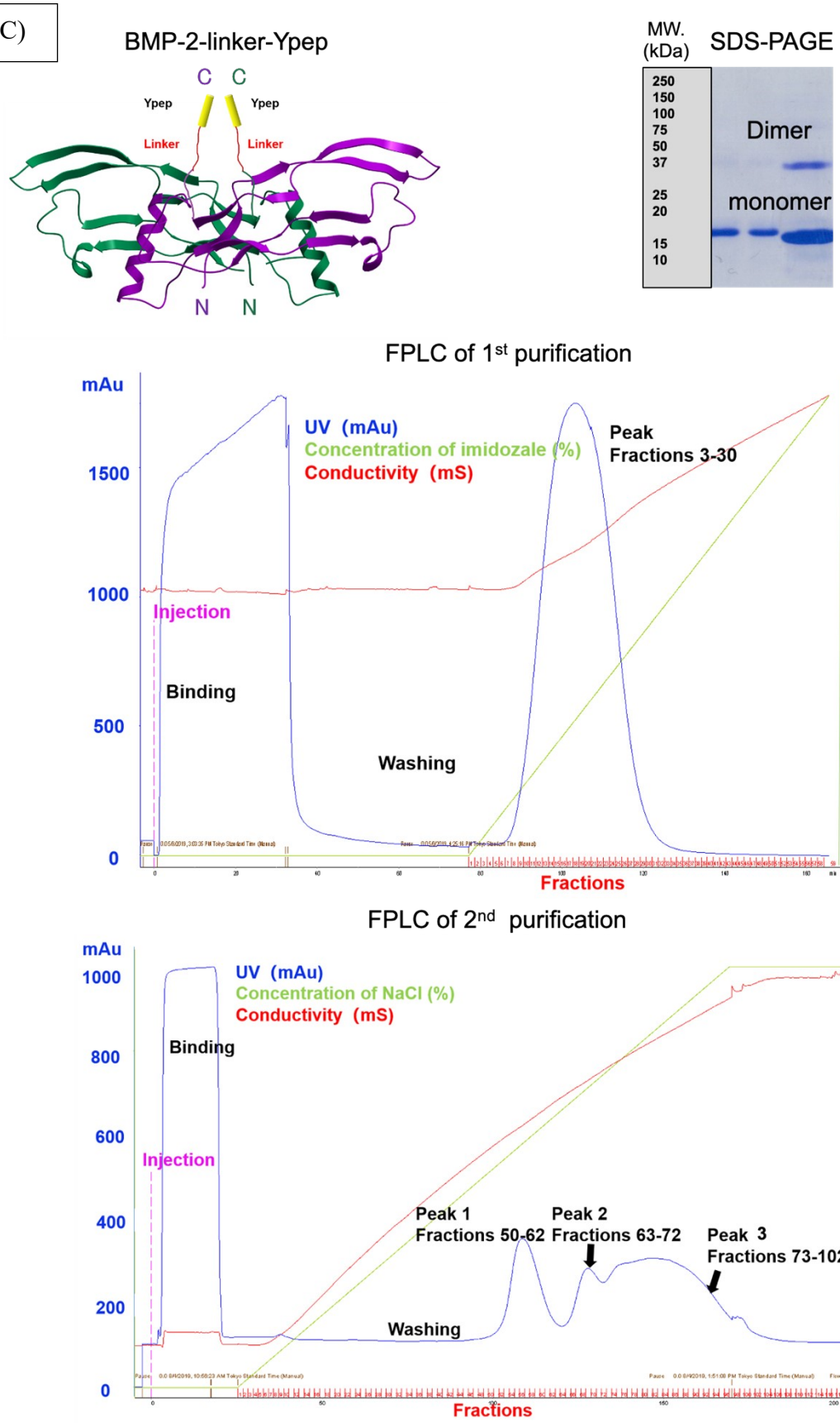

BMP-2-linker-Ypep
C C
Ypep
Ypep
Linker
Linker
N N
MW. (kDa)
SDS-PAGE
250
150
100
75
50
37
25
20
15
10
Dimer
monomer
FPLC of 1st purification
mAu
1500
1000
500
0
%
60
50
40
30
20
10
0
UV (mAu)
Concentration of imidozale (%)
Conductivity (mS)
Peak
Fractions 3-30
Injection
Binding
Washing
Fractions
FPLC of 2nd purification
mAu
1000
800
600
400
200
0
%
100
80
60
40
20
0
UV (mAu)
Concentration of NaCl (%)
Conductivity (mS)
Binding
Injection
Peak 1
Fractions 50-62
Peak 2
Fractions 63-72
Peak 3
Fractions 73-102
Washing
Fractions

Figure S4. Purification of bone morphogenetic protein-2 (BMP-2) variants. Each type of variant was prepared using (1) His-tag affinity purification (1st purification) to obtain proteins expressed from E. coli, (2) refolded to obtain the correctly folded protein, and (3) purified by heparin column chromatography (2nd purification) to obtain BMP-2 homodimers. Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis images are presented. (A) Ypep-BMP-2, (B) BMP-2-Ypep, and (C) BMP-2-linker-Ypep.

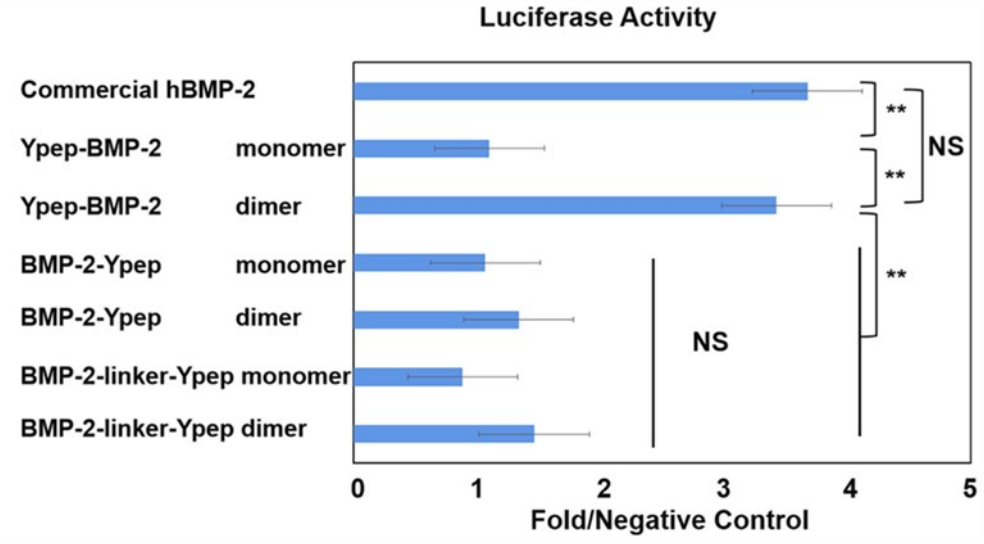
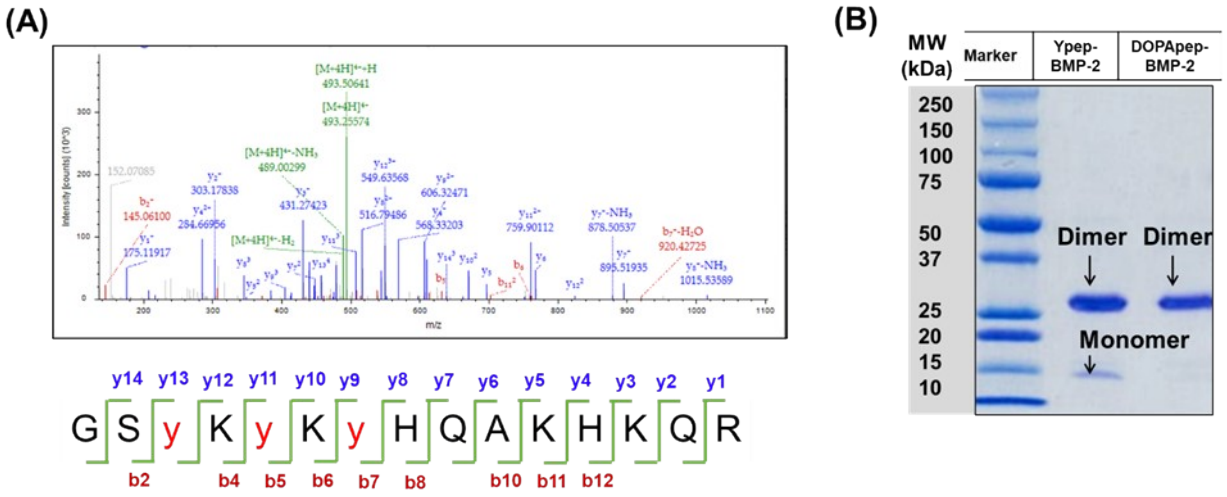


Figure S5. Bioactivity of the monomer/dimer forms of bone morphogenetic protein-2 (BMP-2) variants. Bioactivity was measured using a luciferase reporter gene assay with a BMP-2-specific enhancer in modified C2C12 cells. Data are presented as the mean ± standard deviation, n = 3. *, $p < 0.05$; **, $p < 0.001$. NS, not significant.



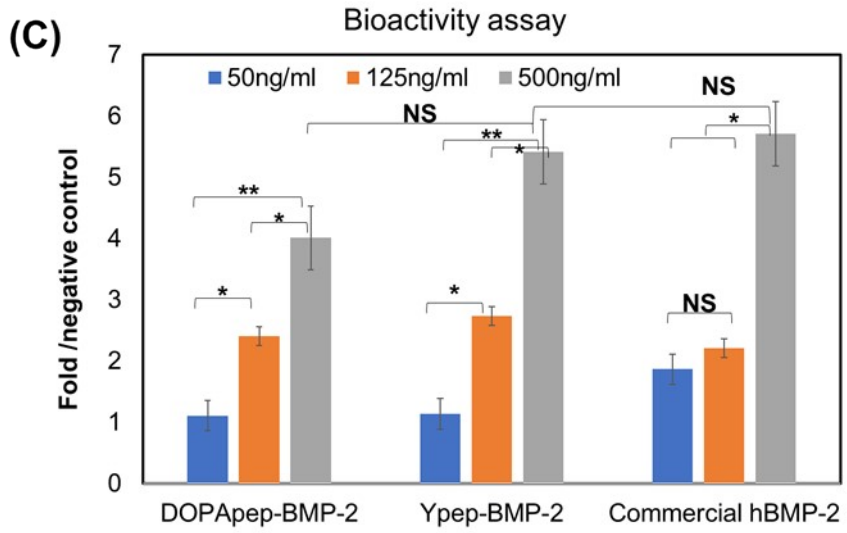


Figure S6. Characterization of recombinant Ypep-BMP-2 and DOPApep-BMP-2. **(A)** Liquid chromatography-tandem mass spectrometry analysis of the recombinant protein to validate the hydrolysis of tyrosine. The fragment GSyKyKyHQAKHKQR was detected, where *y* indicates hydrolyzed tyrosine, a 3,4-dihydroxyphenethylamine residue. **(B)** Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant proteins; 1 µg protein was loaded in each lane. **(C)** Luciferase activity of C2C12 in the presence of soluble DOPApep-BMP-2, Ypep-BMP-2, and commercial BMP-2 after 2 days. Data are presented as the mean ± standard deviation n = 3. *, $p < 0.05$; **, $p < 0.001$. NS, not significant.

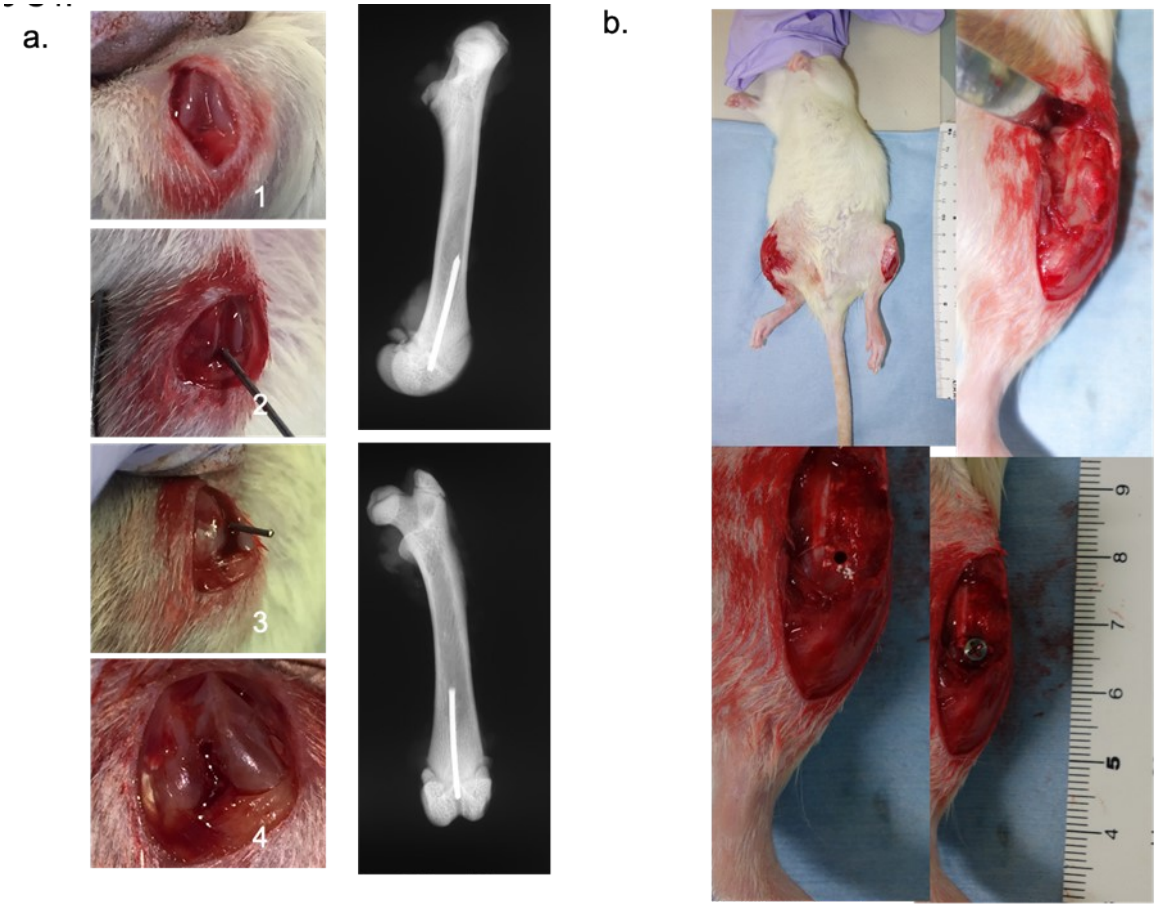


Figure S7. Implant surgery process: (a) Wire implantation and (b) screw implantation. The wire was inserted in the femoral condyle after drilling a 1.1 mm diameter hole by cutting 10 mm of skin in front of the knee. In the case of the screw, the medial tibia was exposed, and the screw was implanted slightly distal to the epiphyseal line of the tibia.

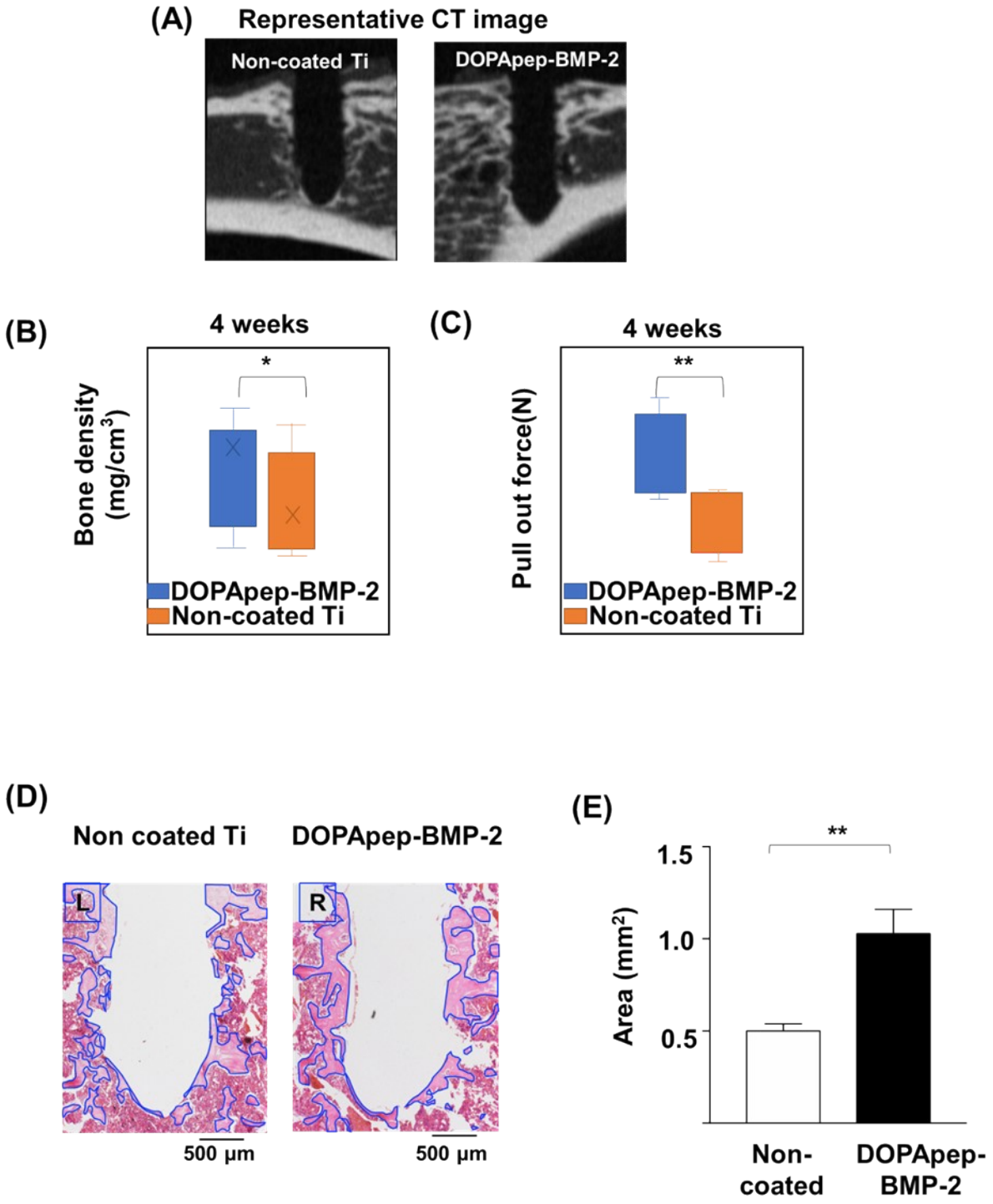


Figure S8. Implantation of coated Ti screws in rat femurs. **(A)** Micro-computed tomography images around non-coated and DOPApep-BMP-2-coated Ti screws. **(B)** Bone density around non-coated and DOPApep-BMP-2-coated Ti screws. **(C)** After 4 weeks of implantation, pull-out tests using a tensile tester were performed with non-coated and DOPApep-BMP-2-coated Ti screws to evaluate binding strength. **(D)** Hematoxylin and eosin (H&E) staining of non-coated and DOPApep-BMP-2-coated Ti screws. Blue lines indicate the bone-formation areas. **(E)** Quantified bone area within 500 μm of contact with the screws, as measured in the H&E-stained sections. Data are presented as the mean ± standard deviation, n = 5. *, $p < 0.05$; **, $p < 0.001$. NS, not significant.

Primer		Sequences (5' to 3')	Annealling temperature (°C)	Size (bp)
pET15b Ypep-BMP-2 vector	Forward	GGACATGGTTGTGGAAGGTTGTGGCTGTCGCTAAC	68	35
	Reverse	GTACTTGTATTTATAGCTGCCGCGCGGCACCAGGCCGCTG	72	40
Ypep-BMP-2 gene	Forward	AGCTATAAATACAAGTACCATCAGGCGAAACACAAACAGCGTAAA	64	45
	Reverse	TCCACAACCATGTCCTGATAGTTTTTCAGCACAAC	63	35
pET15b-BMP-2-Ypep Vector	Forward	GCTGCCGCGCGGCACCAGGCCGCTGCTGTGATGATG	76	36
	Reverse	TGTCGCTATAAATACAAGTATTAACCCAAGCTTATCGATGA	68	41
BMP-2-Ypep Gene	Forward	GTGCCGCGCGGCAGCCATCAGGCGAAACACAAACAGCG	74	38
	Reverse	TACTTGTATTTATACGCGACAGCCACAACCTTCCA	62	35
BMP-2-linker-Ypep Vector	Forward	GCTGCCGCGCGGCACCAGGCCGCTGCTGTGATGATG	76	36
	Reverse	GGTGGAGGGTCGGGCGGTGGCGGATCATATAAATACAAGTATTAACCCAAGCTTGG GAGCTTATCGATGA	74	70
BMP-2-linker-Ypep	Forward	GTGCCGCGCGGCAGCCATCAGGCGAAACACAAACAGCG	74	38
	Reverse	GCCCGACCCTCCACCCCCGCTCCCGCCACCTCCGCGACAGCCACAACCTTCCAC AACCATGTCCTGATAG	80	70

Table S1. Primers used for constructing BMP-2 variants.

BMP-2, bone morphogenetic protein-2

Table S2. LC/MS/MS Analysis to Confirm Hydroxylation of Tyrosine Residues in Ypep-BMP-2

Accession	Description	Coverage [%]	
x00001	user protein1	85	
Confidence	**Annotated Sequence**	**Modifications**	**Corresponding Y**
High	[G].LVPRGS**Y**.[K]	1xOxidation [Y7]	**Introduced Y**
High	[R].HPL**Y**VDFSDVGWNDWIVAPPGYH.[A]	1xOxidation [Y4]	**Y20**
High	[R].HPL**Y**VDFSDVGWNDWIVAPPGYHA.[F]	1xOxidation [Y4]	**Y20**
High	[R].HPL**Y**VDFSDVGWNDWIVAPPG**Y**HAF**Y**.[C]	1xOxidation [Y22]	**Y38**
High	[R].HPL**Y**VDFSDVGWNDWIVAPPG**Y**HAF**Y**CHGECPFPLADHLNSTNHAIVQTLVNSVNSK.[I]	2xOxidation [Y22; Y26]	**Y38;Y42**
High	[H].AF**Y**CHGECPFPLADH.[L]	1xOxidation [Y3]	**Y42**
High	[H].AF**Y**CHGECPFPLADHLNSTNHAIVQTLVNSVNSK.[I]	1xOxidation [Y3]	**Y42**
High	[A].F**Y**CHGECPFPLADHLNSTNHAIVQTLVNSVNSK.[I]	1xOxidation [Y2]	**Y42**
High	[K].ACCVPTELSAISML**Y**LDENEK.[V]	1xOxidation [Y15]	**Y91**
High	[T].ELSAISML**Y**LDENEK.[V]	2xOxidation [M7; Y9]	**Y91**
High	[K].ACCVPTELSAISML**Y**LDENEKVVLKN**Y**QDMVVEGCGCR.[-]	1xOxidation [Y27]	**Y103**