Video Article

Site-Directed Immobilization of Bone Morphogenetic Protein 2 to Solid Surfaces by Click Chemistry

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Abstract

Different therapeutic strategies for the treatment of non-healing long bone defects have been intensively investigated. Currently used treatments present several limitations that have led to the use of biomaterials in combination with osteogenic growth factors, such as bone morphogenetic proteins (BMPs). Commonly used absorption or encapsulation methods require supra-physiological amounts of BMP2, typically resulting in a so-called initial burst release effect that provokes several severe adverse side effects. A possible strategy to overcome these problems would be to covalently couple the protein to the scaffold. Moreover, coupling should be performed in a site-specific manner in order to guarantee a reproducible product outcome. Therefore, we created a BMP2 variant, in which an artificial amino acid (propargyl-L-lysine) was introduced into the mature part of the BMP2 protein by codon usage expansion (BMP2-K3Plk). BMP2-K3Plk was coupled to functionalized beads through copper catalyzed azide-alkyne cycloaddition (CuAAC). The biological activity of the coupled BMP2-K3Plk was proven *in vitro* and the osteogenic activity of the BMP2-K3Plk-functionalized beads was proven in cell based assays. The functionalized beads in contact with C2C12 cells were able to induce alkaline phosphatase (ALP) expression in locally restricted proximity of the bead. Thus, by this technique, functionalized scaffolds can be produced that can trigger cell differentiation towards an osteogenic lineage. Additionally, lower BMP2 doses are sufficient due to the controlled orientation of site-directed coupled BMP2. With this method, BMPs are always exposed to their receptors on the cell surface in the appropriate orientation, which is not the case if the factors are coupled via non-site-directed coupling techniques. The product outcome is highly controllable and, thus, results in materials with homogeneous properties, improving their applicability for the repair of critical size bone defects.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56616/>

Introduction

The ultimate goal of bone tissue engineering and bone regeneration is to overcome the disadvantages and limitations occurring during common treatments of non-union fractures. Auto- or allo-transplantations are predominantly used as current therapy strategies, even though they both have several drawbacks. The ideal bone graft should induce osteogenesis by osteoinduction as well as osteoconduction, leading to the osteointegration of the graft into the bone. Nowadays, only auto-transplantation is considered as the "gold standard" since it provides all characteristics of an ideal bone graft. Unfortunately, it also presents important negative aspects, such as long surgery times, and a second trauma site that usually entails more complications (e.g., chronic pain, hematoma formations, infections, cosmetic defects, etc.). Allogenic grafts, on the other hand have suboptimal characteristics for all general aspects¹. Alternative bone graft technologies have been improved in the last few years, with the aim to produce scaffolds that are osteoinductive, osteoconductive, biocompatible, and bioresorbable. Since many biomaterials do not show all of these osteogenic characteristics, different growth factors, mainly BMP2 and BMP7, have been incorporated in order to improve the osteogenic potential of the particular scaffold².

As an essential criterion, such growth factor delivery systems should provide a controlled dose release over time in order to facilitate the essential events like cell recruitment and attachment, cell ingrowth, and angiogenesis. However, BMPs as well as other osteogenic growth factors have been commonly immobilized non-covalently³. Entrapment and adsorption techniques require the use of supra-physiological amounts of protein due to an initial burst release, which leads to severe disadvantages *in vivo*, typically affecting the surrounding tissues by inducing bone overgrowth, osteolysis, swelling, and inflammation⁴. Thus, the retention of growth factors at the delivery site for longer periods of time can be achieved by covalent immobilization methods. Chemically modified BMP2 (succinylated⁵, acetylated⁶ or biotinylated⁷), engineered heterodimers⁸, or BMP2 derived oligopeptides⁹ have been designed and used to overcome the limitations related to absorption. However, the bio-activity of these constructs is not predictable since the arrangement potentially inhibits the binding of the immobilized ligand to the cellular

receptors. As previously shown, it is essential that all four receptor chains involved in the formation of activated ligand-receptor complexes interact with the immobilized BMP2 in order to fully activate all downstream signaling cascades¹⁰.

To overcome the problems of an inhomogeneous product outcome with limitations in terms of bioactivity, stability, and bioavailability of the immobilized factor, we designed a BMP variant capable of covalently binding scaffolds in a site-directed manner. This variant, termed BMP2-K3PIk, comprises an artificial amino acid which was introduced by genetic codon expansion¹¹. This variant has been successfully linked to scaffolds using a covalent coupling strategy while maintaining its biological activity.

Protocol

1. Production of the BMP2 Variant BMP2-K3PIk

1. Cloning of BMP2-K3PIk by site-directed mutagenesis using PCR¹²

1. Amplify human mature BMP2 (hmBMP2) from a p25N-hmBMP2 vector (see **Table of Materials**) with a forward primer (5' GACCAGGACATATGGCTCAAGCCTAGCACAAACAGC 3') and a reverse primer (5' CCAGGAGGATCCTTAGCGACACCCACAACCCT 3') introducing an amber stop codon (TAG) at the position of the first lysine of BMP2's mature part. Perform the PCR reaction using 33.5 μ L of H₂O, 10 μ L of PCR reaction mix, 1.5 μ L of 10 μ M dNTP stock solution, 1.5 μ L of forward primer (10 pmol/ μ L), 1.5 μ L of reverse primer (10 pmol/ μ L), 1 μ L of p25N-hmBMP2 (10 ng/ μ L), and 1 μ L of DNA polymerase (see **Table of Materials**) in a thermal cycler (denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min).
2. Purify the PCR product using a commercially available kit following manufacturer recommendations (see **Table of Materials**).
3. Digest the PCR product with the restriction enzyme NdeI at 37 °C for 45 min using 16 μ L of H₂O, 3 μ L of digestion buffer, 10 μ L of DNA (20 ng/ μ L), and 1 μ L of restriction enzyme. Heat-inactivate the enzyme at 65 °C for 5 min. Digest the PCR product with the restriction enzyme BamHI at 37 °C for 1 h using 16 μ L of H₂O, 3 μ L of digestion buffer, 10 μ L of DNA (20 ng/ μ L), and 1 μ L of restriction enzyme. Heat-inactivate enzyme at 80 °C for 5 min.
4. Digest the pET11a-pyrRNA vector with NdeI at 37 °C for 2 h and 45 min using 16 μ L of H₂O, 3 μ L of digestion buffer, 10 μ L of DNA (20 ng/ μ L), and 1 μ L of restriction enzyme. Heat-inactivate at 65 °C for 5 min. Digest the pET11a-pyrRNA vector with BamHI at 37 °C for 1 h using 16 μ L of H₂O, 3 μ L of digestion buffer, 10 μ L of DNA (20 ng/ μ L), and 1 μ L of restriction enzyme. Heat-inactivate at 80 °C for 5 min.
5. Separate the digested vector from undigested vector and digestion remnants by agarose gel electrophoresis (0.8% agarose, 60 min at 100 V). Ligate the digested BMP2-K3TAG insert with digested pET11a-pyrRNA backbone at room temperature (RT) for 2 h using 100 ng of pET11a-pyrRNA backbone and 34.5 ng of BMP2-K3TAG insert (1:3 molar ratio). The reaction mixture contains the DNA backbone and insert, 2 μ L of ligase buffer, 1 μ L of DNA ligase, and H₂O to a total volume of 20 μ L.
6. Perform a co-transformation of pET11a-pyrRNA-BMP2-K3PIk and pSRF-duet-pyrRNA_{synth} in BL21(DE3) bacteria:
 1. Add 50 ng of each plasmid to the bacteria, place the mixture on ice for 30 min, heat shock at 42 °C for 50 s, place on ice for 5 min, add 500 μ L of Lysogeny Broth (LB) medium in the mixture, and shake at 300 rpm for 60 min.
 2. Spread 100 μ L of the bacterial mixture onto pre-warmed kanamycin (50 μ g/mL)/ampicillin (100 μ g/mL) plates, and incubate overnight at 37 °C.

2. Expression and Purification of BMP2-K3PIk^{13,14}

1. Propagate a single colony overnight in 50 mL of LB medium with 50 μ g/mL of kanamycin and 100 μ g/mL of ampicillin at 37 °C.
2. Dilute overnight cultures 1:20 into 800 mL of Terrific Broth (TB) medium supplemented with 50 μ g/mL of kanamycin and 100 μ g/mL of ampicillin, and grow at 37 °C until an OD₆₀₀ of 0.7 is reached. Add propargyl-L-lysine to a final concentration of 10 mM. Collect a 100 μ L sample from the culture before isopropyl β -D-1-thiogalactopyranoside (IPTG) induction.
3. Induce gene expression by the addition of IPTG to a final concentration of 1 mM. Grow the culture at 37 °C for 16 h in an orbital shaker at 180 rpm. Collect a 100 μ L sample from the culture.
4. Centrifuge the whole culture at 9000 x g for 30 min. Discard the supernatant, and resuspend the bacterial pellet in 30 mL of TBSE buffer (10 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)) with 1:1000 (v/v) 2-mercaptoethanol (freshly added). Caution: Handle 2-mercaptoethanol under the fume hood. Avoid contact with skin and eyes. Avoid inhalation of vapor or mist. Perform all the resuspension steps with buffers containing 2-mercaptoethanol under the fume hood.
5. Weigh an empty centrifuge beaker and transfer the resuspended pellet into the empty beaker. Centrifuge at 6,360 x g for 20 min. Discard the supernatant and weigh the beaker with the pellet. Subtract the weight of the empty beaker to calculate the weight of the pellet.
NOTE: The protocol can be paused here. Freeze the pellet at -20 °C in the short term or at -80 °C in the long term. Upon resuming the protocol, thaw the pellet at RT.
6. Resuspend the pellet in STE buffer (10 mM Tris pH 8.0; 150 mM NaCl; 1 mM EDTA, 375 mM sucrose; 1:1000 (v/v) 2-mercaptoethanol (freshly added)). Use 200 mL of STE buffer for every 10 g of pellet.
7. Sonicate the suspension on ice (10 min with 40 s pulse, 20 s brake, and 30% amplitude). Centrifuge at 6,360 x g for 20 min. Discard the supernatant and weigh the pellet. Repeat the sonication and centrifugation steps 4 times.
8. Resuspend the pellet in 100 mL of TBS buffer (10 mM Tris, 150 mM NaCl). Centrifuge at 6,360 x g for 20 min. Discard the supernatant and weigh pellet.
9. Resuspend the pellet in nuclease buffer (100 mM Tris, 1 mM EDTA, 3 mM MgCl₂) with freshly added 80 U/mL nuclease (e.g., Benzonase) using 10 mL/g of pellet. Incubate the suspension overnight at RT on a stirring plate (30 rpm).
10. Add Triton buffer (60 mM EDTA, 1.5 M NaCl, 6% (v/v) 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol) to the suspension. The volume of Triton buffer to add corresponds to a 0.5 volume part of the suspension. Incubate for 10 min at RT (no stirring). Centrifuge at 6,360 x g for 20 min. Discard the supernatant and weigh the pellet.
11. Resuspend the pellet in TE buffer (100 mM Tris, 20 mM EDTA) using 8 mL/g of pellet. Centrifuge at 6,360 x g for 20 min. Discard the supernatant and weigh the pellet.

12. Resuspend the pellet by adding 4 mL/g of 25 mM NaAc pH 5.0 and 5 mL/g of 6 M GuCl with 1 mM dithiothreitol (DTT) (freshly added). Incubate the suspension overnight at 4 °C on a stirring plate (30 rpm).
13. Centrifuge at 75,500 x g for 20 min. The supernatant now contains the unfolded monomeric BMP2 protein. Collect the supernatant and concentrate this extract to 20 OD/mL using a 3 kDa molecular weight cut-off (MWCO) membrane in a concentrating cell (see **Table of Materials**).
14. Add the concentrated monomers in single drops to the Renaturation buffer (2 M LiCl, 50 mM Tris, 25 mM CHAPS, 5 mM EDTA, 1 mM glutathione disulfide (GSSG), 2 mM glutathione (GSH)) while stirring (30 rpm). Incubate at RT for 120 h in the dark.
NOTE: During this incubation period, refolding occurs. The solution from this step onwards contains the folded dimeric BMP2-K3PIk.
15. Adjust the pH of the solution to 3.0 using concentrated HCl. Dialyze the solution against 1 mM HCl. Concentrate the dialyzed solution using a 10 kDa molecular weight cut-off (MWCO) membrane in a concentrating cell.
16. Equilibrate the solution by adding buffer A (20 mM NaAc, 30% isopropanol). Add a volume of buffer A corresponding to a 30% volume of the solution. Centrifuge the solution at 4700 x g for 15 min.
17. Equilibrate the column for ion exchange on a fast protein liquid chromatography (FPLC) system¹⁵ with 150 mL of buffer A. Load the protein solution onto the equilibrated column. Elute fractions using a linear gradient of buffer B (20 mM NaAc, 30% isopropanol, 2 M NaCl) using 100 mL of buffer B. Collect 2 mL fractions.
NOTE: Concentrate the protein before loading to the column according to the total column volume. The final protein volume should be <5% of the total column volume.
18. Analyze 20 µL of each fraction by SDS Polyacrylamide gel (12%) electrophoresis (SDS-PAGE)¹⁶ and Coomassie Brilliant Blue staining¹⁷ (see **Table of Materials**).
NOTE: The Coomassie Brilliant Blue stained SDS-PAGE gel shows fractions containing dimers (26 kDa) and fractions containing monomers (13 kDa).
19. Pool dimer-containing fractions. Dialyze the pool of dimer containing fractions overnight at 4 °C against 1 mM HCl (5 liter volume). Concentrate the final product by using a 10 kDa concentrating centrifugal filter unit (see **Table of Materials**). Analyze the final product by SDS Polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining.
NOTE: The band on the Coomassie Brilliant Blue stained SDS-PAGE gel should show only one band at 26 kDa. This is the final BMP2-K3PIk product.

2. Optimization of Copper (I)-Catalyzed Alkyne-Azide Cycloaddition (CuAAC) Conditions

1. Effect of sodium ascorbate (NaAsc) and copper (II) sulfate (CuSO_4) on wild type BMP2 (BMP2-WT)
 1. Incubate 20 µM BMP2-WT with different ratios of NaAsc to CuSO_4 . Use 0.5 mM NaAsc and 0.5 mM CuSO_4 (starting concentration) in a 50 µL volume. Proceed with the following ratios of NaAsc to CuSO_4 : (1:1), (1.7:1), (10:1), (20:1), keeping the CuSO_4 concentration constant at 0.5 mM and adjusting the concentration of NaAsc accordingly. Perform the reaction in H_2O on a rotating mixer (20 rpm) overnight at RT.
 2. Prepare reduced samples by adding loading buffer containing 5% 2-mercaptoethanol and incubate sample at 95 °C for 6 min. Analyze reduced and non-reduced samples by SDS Polyacrylamide gel (12%) electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining. The Coomassie stained SDS-PAGE gels show bands in the range of 13 kDa (reduced conditions) to 26 kDa and higher molecular weight (non-reduced conditions).
2. Effect of Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) on the CuAAC Reaction
 1. Incubate 20 µM BMP2-K3PIk with different ratios of THPTA to CuSO_4 . Use 5 mM NaAsc, and 200 µM 3-Azido-7-hydroxycoumarin (keep NaAsc and 3-Azido-7-hydroxycoumarin constant). Use 50 µM THPTA and 0.5 mM of CuSO_4 as starting concentrations. Use different ratios of THPTA to CuSO_4 : (7:1); (10:1); (12:1); (15:1); (20:1). Perform reaction in H_2O (50 µL total volume) for 24 h at RT on a rotating mixer (20 rpm).
 2. Upon coupling, 3-Azido-7-hydroxycoumarin becomes a fluorescent dye. Prepare samples in reduced and non-reduced conditions and analyze them by SDS-PAGE. Visualize the gels under the excitation channel with the specific wavelength for the 3-Azido-7-hydroxycoumarin ($\lambda_{\text{abs}} = 404 \text{ nm}$; $\lambda_{\text{em}} = 477 \text{ nm}$).

3. Covalent Coupling Technique of BMP2-K3PIk to Azide Functionalized Agarose Beads

1. Incubate 20 µM of BMP2-K3PIk or BMP2-WT (used as negative control) with 20 µL of azide-activated agarose beads in a total volume of 500 µL in reaction buffer (0.1 M HEPES pH 7.0, 3.9 M urea, 50 µM CuSO_4 , 250 µM THPTA, 5 mM sodium ascorbate) for 2 h at RT on a rotating mixer (20 rpm). Stop the reaction by adding 5 mM EDTA (final concentration).
2. Incubate at RT for 15 min on a rotating mixer (20 rpm). Centrifuge samples at 20,000 x g for 1 min at RT. Collect the supernatants.
3. Wash the pellet containing the beads three times with 1,000 µL of HBS₅₀₀ buffer (50 mM HEPES, 500 mM NaCl), three times with 1,000 µL of 4 M MgCl_2 , and two times with 1,000 µL of phosphate buffered saline (PBS). At every washing step, centrifuge at 20,000 x g for 1 min at RT and collect supernatants. Store coupled beads in 1,000 µL of PBS at 4 °C.
Caution: Do not disturb the bead pellet while removing the supernatant.

4. Validating the Presence and Biological Activity of Immobilized BMP2-K3PIk Using Texas Red Labeled BMP Receptor I A Ectodomain (BMPIR-IA_{EC})

1. Incubate 50 µL of the BMP2-K3PIk functionalized beads with 1 µM of Texas Red labeled BMPIR-IA_{EC} with 150 µL of HBS₅₀₀ buffer (50 mM HEPES, 500 mM NaCl) at RT for 1 h on a rotating mixer (20 rpm).
2. Centrifuge the beads at 20,000 x g for 1 min at RT. Discard the supernatant. Wash the beads with 1000 µL of HBS₅₀₀ buffer. Repeat the washing step an additional 3 times. Centrifuge at 20,000 x g for 1 min at RT after each washing step.

- Resuspend the beads in 500 μ L of PBS and pipette 50 μ L on a glass cover slide. Set the microscope filter between 561 or 594 nm. Detect Texas Red–BMPR-IA_{EC}–BMP2-K3PIk functionalized beads using fluorescent microscopy and take pictures.

5. Measuring Alkaline phosphatase (ALP) Expression to Prove the *In Vitro* Bioactivity of the Produced BMP2-K3PIk Before and After the Coupling Reaction.

1. Alkaline phosphatase (ALP) assay

- Culture promyoblastic C2C12 cells (ATCC CRL-172) in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Calf Serum (FCS), 100 U/mL penicillin G and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere at 5% CO₂.
- Seed C2C12 cells at a density of 3×10^4 cells/well into a 96-well microplate. Let cells attach overnight. Remove medium and incubate C2C12 cells in the presence of 0.5–200 nM of BMP2-WT or BMP2-K3PIk in 100 μ L/well of DMEM (2% FCS, 100 U/mL penicillin G and 100 μ g/mL streptomycin) at 37 °C in a humidified atmosphere at 5% CO₂ for 72 h.
- Remove medium and wash cells with 100 μ L of PBS per well. Lyse cells at RT for 1 h with 100 μ L/well of lysis buffer (1% NP-40, 0.1 M glycine, pH 9.6, 1 mM MgCl₂, 1 mM ZnCl₂) on a shaking plate (220 rpm).
- Add 100 μ L/well of ALP buffer (0.1 M glycine, pH 9.6, 1 mM MgCl₂, 1 mM ZnCl₂) with 1 mg/mL p-nitrophenylphosphate (freshly added) to the cell lysate.
- Measure absorption at 405 nm in a multiplate reader after adding the ALP buffer, and every 5 min until the development of the color is complete. Generate dose response curves and EC50 values using a logistic model, $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$.

2. Alkaline phosphatase (ALP) staining

- Culture promyoblastic C2C12 cells (ATCC CRL-172) in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Calf Serum (FCS), 100 U/mL penicillin G and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere at 5% CO₂.
- Seed C2C12 cells at a density of 3×10^4 cells/well in a 96-well microplate. Let cells attach overnight. Remove medium and add 20 μ L/well of BMP2-K3PIk coupled beads. BMP2-K3PIk coupled beads are in 1000 μ L of a PBS suspension.
- Prepare a solution of 0.4% low-melting-point agarose in DMEM. Melt in the microwave (600 W for 3 min) and let it cool down in a water bath at 37 °C until use.
- Add 20 μ L of 0.4% low-melting-point agarose in each well (covering beads and cells). Centrifuge at 2000 x g for 5 min at 20 °C. Add 80 μ L DMEM (2% FCS, 100 U/mL penicillin G and 100 μ g/mL streptomycin) and incubate at 37 °C in a humidified atmosphere at 5% CO₂ for 72 h.
- Remove medium, being careful not to detach the solidified 0.4% agarose. Add 100 μ L/well of 1-Step NBT/BCIP (Nitro-blue tetrazolium chloride, 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) substrate solution.
- Analyze alkaline phosphatase staining using light microscopy immediately after the purple staining becomes apparent. Use bright field microscopy and take pictures.

Representative Results

In this article, we describe a method to covalently couple a new BMP2 variant, BMP2-K3PIk, to commercially available azide functionalized agarose beads (**Figure 1**). The bioactivity of the produced BMP2-K3PIk variant was validated by the induction of alkaline phosphatase (ALP) gene expression in C2C12 cells. The *in vitro* test shows similar ALP expression levels induced by wild type BMP2 (BMP2-WT) and BMP2-K3PIk (**Figure 2**).

The redox reactions between copper (II) sulfate (CuSO₄) and sodium ascorbate (NaAsc) generate reactive oxygen species that might affect the structural integrity and thus affect the bioactivity of BMP2-K3PIk. To verify the structural integrity of the protein, we performed an overnight incubation with CuSO₄ and NaAsc, showing BMP2-WT degradation in a concentration-dependent manner (visualized by SDS-PAGE under reduced conditions (**Figure 3A1**)) and the formation of multimers or aggregates visible at approximately 40 kDa (under non-reduced conditions) (**Figure 3A2**). To avoid protein degradation, Tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA) was used as a protective agent (**Figure 3B**). The use of THPTA prevents BMP2 fragmentation, while the formation of higher molecular weight structures could not be prevented by the addition of THPTA. Further improvements of the reaction addressed the composition of the reaction buffer, the reaction temperature, and reaction time (data not shown). The protocol described here details the final achievements regarding reaction buffer composition, temperature, and reaction time.

To confirm that BMP2-K3PIk retains bioactivity after coupling, we used fluorescently labeled receptor ectodomain protein of the type I receptor (BMPR-IA_{EC}) to demonstrate that the immobilized protein is still able to bind to this receptor. Beads coated with BMP2-K3PIk via CuAAC chemistry yielded fluorescence when incubated with the dye-labeled BMPR-IA_{EC} (**Figure 4**). In contrast, non-coated beads or beads that were incubated with BMP2-WT showed no fluorescence signal above background levels (data not shown)¹⁴.

After confirming receptor binding capabilities of the immobilized ligand *in vitro*, we also tested whether biological responses can be triggered by the functionalized beads in a cell-based assay. ALP mediated staining occurred only in those cells which were in direct contact with the BMP2-K3PIk-functionalized beads (**Figure 5**). This confirms that the protein is indeed covalently linked to the beads and not just absorbed, since a more spread-out staining at larger distances to the beads would otherwise have been observed.

Figure 1: Depiction of BMP2-K3Plk. (A) Depiction of the BMP2-K3Plk variant with the localization of the introduced amino acid substitution. (B) Coupling scheme: BMP2-K3Plk CuAAC reaction with azide functionalized beads. Reprinted (adapted) with permission from Tabisz *et al.* (2017): Site-Directed Immobilization of BMP-2: Two Approaches for the Production of Innovative Osteoinductive Scaffolds. *Biomacromolecules*. **18** (3), 695-708. (Copyright 2017 American Chemical Society) [Please click here to view a larger version of this figure.](#)

Figure 2: Bioactivity of BMP2-variants. Comparison of the bioactivities (ALP assay) of BMP2-K3Plk and wildtype BMP2 (BMP2-WT). The x-axis represents the concentration of BMP2-WT or BMP2-K3Plk used in the assay. The EC₅₀ data represent mean values and standard deviations of 4 individual experiments (N=4). Reprinted (adapted) with permission from Tabisz *et al.* (2017): Site-Directed Immobilization of BMP-2: Two Approaches for the Production of Innovative Osteoinductive Scaffolds. *Biomacromolecules*. **18** (3), 695-708. (Copyright 2017 American Chemical Society) [Please click here to view a larger version of this figure.](#)

Figure 3: Effect of the reducing agent on the integrity of the BMP2. (A) BMP2-WT was exposed to different molar ratios of sodium ascorbate (NaAsc) to copper (II) sulfate (CuSO_4). The samples were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining under reduced (Figure A1) and non-reduced conditions (Figure A2). In the reduced conditions (A1), the red arrows represent cleaved BMP2-WT. In the non-reduced conditions (A2), the red arrows indicate multimeric BMP2-WT. The bands representing cleaved BMP2 species are indicated by a blue arrow. Legend: NC_R - reduced BMP2-WT untreated; NC - BMP2-WT untreated; 1:1 to 20:1 - increasing molar ratios of sodium ascorbate to CuSO_4 . (B) BMP2-K3Plk was coupled to 3-Azido-7-hydroxycoumarin using CuAAC reaction conditions supplemented with THPTA. Upon coupling 3-Azido-7-hydroxycoumarin becomes a fluorescent dye. Legend: NC - BMP2-WT reacted with 3-Azido-7-hydroxycoumarin; 7:1 to 20:1 increasing molar ratios of THPTA to CuSO_4 . [Please click here to view a larger version of this figure.](#)

Figure 4: Bioactivity of immobilized BMP2-K3Plk in vitro. Representative picture of the interaction of immobilized BMP2-K3Plk with Texas-Red-labelled BMPR-IA_{EC}. Reprinted (adapted) with permission from Tabisz *et al.* (2017): Site-Directed Immobilization of BMP-2: Two Approaches for the Production of Innovative Osteoinductive Scaffolds. *Biomacromolecules*. **18** (3), 695-708. (Copyright 2017 American Chemical Society) [Please click here to view a larger version of this figure.](#)

Figure 5: Bioactivity of immobilized BMP2-K3PIk in a cell-based assay. (A) Representative picture of alkaline phosphatase (ALP) staining upon treatment with beads coupled to BMP2-K3PIk functionalized beads. (B) Alkaline phosphatase (ALP) staining upon treatment with soluble 25 nM BMP2-K3PIk. Reprinted (adapted) with permission from Tabisz *et al.* (2017): Site-Directed Immobilization of BMP-2: Two Approaches for the Production of Innovative Osteoinductive Scaffolds. *Biomacromolecules*. **18** (3), 695-708. (Copyright 2017 American Chemical Society) [Please click here to view a larger version of this figure.](#)

Discussion

Generating tagged protein variants by genetic codon expansion allows the introduction of various non-natural amino acid analogs principally at any position of the primary protein sequence. In case of BMPs like BMP2, common tags such as a 6-Histidine (His) tag can only be introduced N-terminally, since the protein's C-terminal end is buried within the tertiary protein structure, and is thus not accessible from the outside. At other positions, the size of the introduced tag may very likely cause structural alterations that consequently obliterate the BMP's bioactivity. Further, introducing a mutation into the BMP2 sequence can affect the refolding efficacy, and can also change other protein parameters such as the isoelectric point of the modified protein. Thus, every single step in an established protein production protocol might need to be adapted according to the altered protein variant's characteristics. The production of the BMP2-K3PIk indeed required a modification of the established method for the expression of wild type BMP2. Different culture times or propargyl-L-lysine (PIk) concentrations were tested (data not shown) in order to reach the highest expression yield. Refolding, separation and purification steps fortunately did not require further adaptations. We have already reported that in case of other BMP2 variants produced in our lab, some protein characteristics were altered significantly, which required the modification of several steps of the BMP production method¹⁸. The produced BMP2-K3PIk showed biological activity comparable to that of the wild type protein. A difference occurs at high BMP2 concentrations, probably as a consequence of a lower solubility of the BMP2-K3PIk variant in medium compared to that of wild type BMP2.

Despite the known advantages of copper-catalyzed azide-alkyne cycloaddition (CuAAC)¹⁹, CuAAC reactions should be carefully adapted to particular applications. We showed how BMP2 can be fragmented or create aggregates or multimers due to the strong reducing reaction conditions. Thus, all reaction parameters had to be analyzed in detail in order to find conditions that leave the protein unaffected.

Our approach to covalently couple the BMP2 variant to azide-functionalized agarose beads by click chemistry could be realized with high efficiency resulting in functionalized beads triggering osteogenic differentiation *in vitro*. When wild type BMP2 protein was used in the reaction with the beads instead, we could observe only weak staining of ALP expression, indicating that coupling indeed occurred highly specifically via the propargyl-L-lysine residue of BMP2-K3PIk.

This positional coupling specificity reflects a great improvement compared to the immobilization procedures involving classical NHS/EDC chemistry. In such cases, random coupling occurs, mainly involving the primary amine groups present in lysine residues. The coupled protein has a variable osteogenic activity, due to the multiplicity of possible connections to the scaffold, leading to different orientations of the protein towards cell receptors.

The use of site-direct immobilized BMP2 might overcome the mentioned drawbacks related to the high doses of soluble BMP2, which are needed to induce bone formation. In addition, the results clearly show that certain cellular responses, such as the osteogenic differentiation of C2C12 cells, are entirely initiated by immobilized BMP2, despite recent studies claiming that signal transduction requires endocytosis of the ligand/ligand-receptor complex^{20,21}. Our findings are in agreement with other studies demonstrating that BMP2, which is coupled covalently (but not site-directed) to non-endocytosable surfaces, is still able to induce osteoblast differentiation²².

Considering that we have used supra-physiological concentrations of BMP2 for the coupling reaction, the amount of the immobilized BMP2 might be further reduced while still conserving the osteogenic properties of the beads. Prior to such optimization steps, however, the BMP2-K3PIk-functionalized scaffold needs to be tested in animal experiments to prove its osteogenic potential *in vivo*.

Disclosures

The authors declare no competing financial interests.

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