Supporting Information

Hydrolytically labile linkers regulate release and activity of human Bone Morphogenetic Protein-6

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**Materials and methods**

Commercial grade reagents were purchased from Sigma-Aldrich unless stated otherwise and were used without further purification. Solvents were dried over molecular sieves. Recombinant human BMP-6 (hBMP-6) was purchased from R&D Systems as a carrier-free, 0.2 µm filtered solution (0.04 mg/mL) in acetonitrile and trifluoroacetic acid (TFA) and further diluted to the desired working concentration in PBS buffer. Water used was of high-purity (Millipore, R = 18.2 MΩcm). Detailed descriptions of the sample preparation, functionalization and characterization as well as of the cell activity assessment experiments are included in the Electronic Supplementary Information (ESI). All experiments were repeated at least three times in triplicate. Error bars represent standard errors, and statistical analysis was performed using the one-way analysis of variance test (ANOVA) to evaluate the statistical differences and a Tukey’s Post-hoc test (*p < 0.05 or **p < 0.005) among all samples or between samples and controls. In the case where only differences between sample and control were to be assessed a t-test was used to determine statistical differences (*p < 0.05 or **p < 0.005)

**Preparation of (3-glycidoxypropyl)trimethoxysilane (GPTMS).** Either single side polished p-type Si(100) wafers (Okmetic) or microscope glass slides (76 x 26 mm, Menzel-Gläzer) were cut into pieces of approximately 1 cm². The substrates were immersed in piranha solution (3:1 concentrated H₂SO₄/ 30% H₂O₂) for 30 min to install silanol groups. After rinsing several times with water, the substrates were dried under a flow of dry N₂ and immediately taken into the N₂ filled glove box and immersed in a 1 v/v% GPTMS toluene solution for 16 h.
Subsequently, the substrates were removed from solution and rinsed several times with toluene and ethanol. To remove unbound deposited materials, the substrates were placed in a beaker with ethanol in an ultrasonic bath for 5 min, rinsed with ethanol and dried under a flow of dry N$_2$. The samples were immediately used for further experiments.

**Preparation of poly(glycidyl methacrylate) (PGMA) layers.** These substrates were prepared following a modified literature procedures.$^{1, 2}$ Either single side polished p-type Si(100) wafers (Okmetic) or microscope glass slides (76 x 26 mm, Menzel-Gläser) were cut into pieces of approximately 1 cm$^2$. The substrates were activated in piranha solution (3:1 concentrated H$_2$SO$_4$/ 30% H$_2$O$_2$) for 30 min and then rinsed several times with water. The substrates were dried under a flow of dry N$_2$ and immediately placed at the bottom of a dessicator with a vial containing 100 µL of (3-aminopropyl)-triethoxysilane (APTES). After evacuating the desiccator with a diaphragm vacuum pump for 10 minutes, the substrates were left in an atmosphere of APTES vapor overnight. Subsequently, the substrates were thoroughly rinsed with ethanol and dried under a flow of dry N$_2$. The cleaned substrates were then immersed into a solution of triethylamine in 40 mL of dry toluene. At 0 °C, 300 µL of ATRP initiator 2-bromo-2-methylpropionyl bromide were added dropwise and left to stir for 1.5 h under a N$_2$ atmosphere. After rinsing the substrates with toluene, ethanol and water, the substrates were dried under a flow of dry N$_2$. Then a solution of glycidyl methacrylate (5 mL, 36.7 mmol), 2,2'-dipyridyl (bpy) (141 mg, 0.904 mmol) in methanol (4 mL) and water (1 mL) was prepared and purged with argon for 30 min in a round-bottom flask sealed with a septum. In another flask, CuCl (36.4 mg, 0.368 mmol) and CuBr$_2$ (3.9 mg, 0.017 mmol) were added together and also purged with argon for 30 min. Monomer, ligand, and catalyst were then combined, stirred and degassed for another 30 min to facilitate the formation of the organometallic complex. Subsequently, this solution was transferred with a degassed syringe into an argon-degassed flask containing the functionalized substrates with the initiator. Upon addition, the ATRP
reaction was allowed to proceed for 2 h at room temperature after which the substrates were removed from the flask. After thorough rinsing with water, ethanol, a gentle sonication in dichloromethane and rinsing with dichloromethane, the substrates were dried under a flow of dry N₂. The samples were immediately used of further experiments.

**Preparation and functionalization of polymer films.** 1000PEGT70PBT30 (PA, Mₘ of PEG is 1000 g/mol and a 70/30 ratio of PEGT/PBS) was a gift from PolyVation (The Netherlands). Thick PA-films were made by casting a clear 3.4 wt% PA solution in chloroform (40 mL) onto a glass petri dish (Ø = 14 cm). After standing overnight at room temperature, the as-dried cast film further dried in a vacuum oven at 60 °C for several hours. An average thickness of 100 µm was found. The film was then cut into pieces of approximately 1 cm² and used for immunostaining, cell adhesion and activity experiments. Thin PA-films were made by spin coating a clear 1 wt% PA solution in chloroform (500 rpm during 3 s and 3000 rpm during 30 s) on either gold substrates (Ø = 2.5 cm, 2-5 nm Ti on glass and 200 nm gold, SSENS, The Netherlands) or 1 cm² cut single side polished p-type Si(100) wafers (Okmetic). An average thickness of 100 nm was found using an ellipsometer. A Plasma-Prep II plasma etcher (SPI supplies, USA) was used at an oxygen pressure of 1.0 bar, a vacuum pressure of 160 mbar and a current of 40 mA for 15 sec to activate the surface of the PA-films. The films were subsequently modified with GPTMS using the same procedure as described above. In the case of PA-films, dry hexane was used in all steps instead of dry toluene to minimize swelling of the polymer.

**Reactive µCP.** Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master’s surface was fluorinated using 1H,1H,2H,2H-perfluorododecyltrichlorosilane (PFDTS). Poly(dimethylsiloxane) (PDMS) (Dow Corning) stamps were fabricated by curing the degased mixture (10:1 elastomer, curing
agent) of the components of SYLGARD® 184 silicone elastomer kit on the surface of the master at 60 °C overnight. PDMS stamps were first oxidized in an O₂-plasma reactor (Plasma-Prep II plasma etcher, SPI supplies, USA) at an oxygen pressure of 1.0 bar, a vacuum pressure of 160 mbar and a current of 40 mA for 20 sec. The stamps were then stored in water. Prior to use, the stamps were dried with a flow of dry N₂ and then incubated with 50 µL of a hBMP-6 solution (20 µg/mL) in PBS for 5 min at room temperature. The excess of inking solution was then removed and the stamps were dried with a flow of dry N₂ and brought into conformal contact with the epoxide-terminated layers for 2 h with 15 g/cm² of pressure. After printing, the substrates were washed three times 10 min with PBS buffer including 0.005% Tween-20, then washed with PBS and dried with a flow of dry N₂. For fluorescence microscopy imaging, a final washing with water was performed to reduce the amount of salts present on the surface. Prior to the washing procedure upon printing on the activated PA films, a solution of 100 mg of NaBH₃CN in 40 mL of PBS with 10 mL of EtOH for 5 minutes was applied in the case when reduction of the imine bond was required.

**hBMP-6 immunostaining.** Following protein immobilization via reactive µCP, the background was blocked by incubating the samples for 2 h in a solution of 0.1 wt% O-(2-aminoethyl) polyethyleneglycol 3,000 (NH₂-PEG) or BSA in PBS buffer. Subsequently, the substrates were washed with PBS buffer, rinsed with water and dried with a flow of dry N₂. Then, the substrates were incubated with 100 µL of a solution of 5 µg/mL of monoclonal anti-BMP-6 antibody produced in mouse (mouse IgG2B isotype, clone 74219.11, Invitrogen) in PBS buffer including 1 mM Tween-20. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N₂. Subsequently, the substrates were incubated with 100 µL of a solution of 40 µg/mL of Alexa Fluor® 546 labeled goat anti-mouse IgG2b (γ2b) (Invitrogen) produced in goat in PBS buffer
including 1% BSA. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20 during, rinsed with water and dried with a flow of dry N₂.

**Characterization of substrates.** Modified surfaces were examined by dynamic contact angle (sessile droplet) measurements using a Krüss G10 contact angle measuring instrument, equipped with a CCD camera. Droplets (1.5-2 µL) of water were placed randomly over the surface and advancing and receding (Θ\(a\) and Θ\(r\)) contact angles were automatically determined during growth and shrinkage of the droplet by a drop shape analysis routine. All reported values were an average of at least three measurements.

Ellipsometric layer thickness measurements were performed on a Plasmos ellipsometer (Plasmos SD 2002 ellipsometer, \(\lambda = 632.8 \text{ nm}\)) with a fixed angle of incidence of 70°. The indexes of refraction were considered to be constant and equal to the “bulk” values of 1.500 and 1.457 for organic layers and the underlying native oxide, respectively. The average thickness of the SiO\(_2\) layer was measured separately on an unmodified Si substrate and subtracted from the total layer thickness. All reported thickness values were averaged from at least five different measurements on the same substrate.

XPS spectra were measured using a Physical Electronics Quantera Scanning X ray Multiprobe instrument, equipped with a monochromatic Al K\(\alpha\) X-ray source operated at 1486.6 eV and 25 W. Spectra were referenced to the main aliphatic C 1s peak set at 284.8 eV. The X-ray beam size was 100 µm and the data were collected from surface areas of 100 µm x 300 µm with a pass energy of 224 eV and a step energy of 0.8 eV for survey scans at a detector input angle of 45°. The measurement was collected after 3 cycles scanning. Charge neutralization was achieved by low-energy electrons and low-energy Argon ions. Atomic force microscopy (AFM) experiments were carried out using a digital NanoScope III multimode AFM (Digital Instruments, Veeco Metrology Group, USA) in tapping mode, equipped with a Si3N4 tip and with a J-Scanner at a scan rate of 0.6 Hz. AFM imaging was performed at ambient conditions.
Polarization modulation (PM) FT-IRRAS spectra were recorded using a Tabletop Optical Module (TOM) connected to a Nicolette 6700 spectrometer (Thermo Scientific) and equipped with a PEM-100 controller (HINDS instruments), a synchronous sampling Demodulator (GWC Technologies) and with a liquid nitrogen-cooled MCT-A detector at a reflection angle of 81°. Data of a dual-channel polarization modulation experiment were collected for 250 scans at a resolution of 8 cm\(^{-1}\).

**Fluorescence microscopy.** Fluorescence microscopy images were recorded using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition using the following Olympus filter cubes: 300 nm ≤ \(\lambda_{\text{ex}}\) ≤ 400 nm for blue emission (410 nm ≤ \(\lambda_{\text{em}}\) ≤ 510 nm), 510 nm ≤ \(\lambda_{\text{ex}}\) ≤ 550 nm for red emission (\(\lambda_{\text{ex}}\) ≥ 580 nm) and 460 nm ≤ \(\lambda_{\text{ex}}\) ≤ 490 nm for green emission (\(\lambda_{\text{em}}\) ≥ 520). All fluorescence microscopy images were acquired in air.

**Cell culture and staining.** All substrates were sterilized prior to cell seeding with 70% ethanol and subsequently washed twice with PBS to remove any excess of ethanol. Mouse pre-osteoblast cell line KS483-4C3 was used as a model for osteogenic differentiation. Cells were plated at a density of 5000 cells/cm\(^2\) and cultured in Minimal Essential Medium (MEM) alpha supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin (Gibco) and 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL). After 24 h, adhered cells were carefully washed twice with sterile PBS to remove loosely attached cells. Cells were then fixed in 10% buffered formalin for 1 h at room temperature and stained for F-actin with Alexa Fluor® 568 phalloidin or Alexa Fluor® 488 phalloidin (Invitrogen) or cell nuclei with DAPI dilactate (Invitrogen) following the protocol from the provider. After washing the substrates two times with PBS and water, the substrates were carefully blown-dry with N\(_2\). Cell numbers were assessed after 24 h of adhesion using a
particle counting routine of the free software ImageJ, using the fluorescence pictures corresponding to the DAPI staining. Cell spreading was assessed in a similar way by quantifying the total cell area of at least 5 different fluorescence pictures corresponding to the F-actin staining samples and normalized by the corresponding cell number.

**ALP staining and quantification.** For osteogenic differentiation, 0.2 mM ascorbic acid was added to the medium either together with 100 ng/mL hBMP-6 delivered in soluble form or hBMP-6 was bound to the substrate. Cells were harvested at different time points for detection and quantification of alkaline phosphatase (ALP). For ALP staining, cells were fixed using 10% buffered formalin for 1 h. ALP staining was performed using the Leukocyte Alkaline Phosphatase kit according to manufacturer’s protocol. For ALP quantification, KS483-4C3 cells were lysed in 200 µL lysis buffer (0.1 M K₂HPO₄ + 0.1 M KH₂PO₄ + 0.1% Triton X100, pH 7.8), and ALP was quantified using the CDP-star® Chemiluminescent Substrate. ALP production was corrected for DNA content, measured using the CyQuant Cell Proliferation assay (Invitrogen). To avoid contact between cells and substrates, Transwell® inserts (Corning Life Sciences) were used according to the manufacturer’s protocol.

**Luciferase reporter activity.** C2C12 cells were stably transfected with an expression construct (BRE-Luc) containing a BMP responsive element (BRE) fused to the firefly luciferase reporter (Logeart-Avramoglou, 2006). Cells were cultured at a density of 5000 cells/cm² in Dulbecco’s Modified Eagles Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U penicillin/streptomycin (Gibco). At different time points, cells were lysed in Glo lysis buffer (Promega) and luciferase activity was measured using Steady-Glo luciferase reagent (Promega). Luciferase activity was normalized for DNA content, as measured by CyQuant Cell Proliferation assay (Invitrogen).
**Gene expression analysis.** Cells were lysed in Trizol for RNA isolation, RNA was extracted using the Nucleospin RNA II kit (Bioke) according to manufacturer’s protocol. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized using GAPDH and expressed as fold induction compared to controls. Primer sequences are listed in the following table.

| Gene | Primer sequence | Product size | Annealing temperature |
|------|-----------------|--------------|-----------------------|
| GAPDH | For 5' CGCTCTCTGCTCCTCCTGTT 3'<br>Rev 5' CCATGGGTGCTCTGAGCGATGT 3' | 82 bp | 60 °C |
| RUNX2 | For 5' GGAGTGGAGAGGGCAAGATTT 3'<br>Rev 5' AGCTTCTGTCTGTGGGCTTCTGG 3' | 133 bp | 60 °C |
| OCN | For 5' ACTGGCTCAAGAACAGTCCTG 3'<br>Rev 5' GAGAGAATCCCGGTACTGTGG 3' | 438 bp | 60 °C |

**Quantification of released hBMP-6.** Cell culture medium was collected at different time points and the amount of hBMP-6 was quantified using an ELISA assay according to the manufacturer’s instructions (Duo Set ELISA development kit, R&D systems) and expressed as a cumulative amount throughout the period of time under study. The quantification of hBMP-6 bound to the substrates at different time points was performed via the fluorescence intensity quantification of protein arrays by using the particle counting routine of the free software ImageJ. Prior to imaging, the substrates were washed twice with PBS and rinsed with water and after imaging they were placed back in cell culture media until further measurements.
References.

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S3. Logeart-Avramoglou D, Bourguignon M, Oudina K, Ten Dijke P, Petite H. An assay for the determination of biologically active bone morphogenetic proteins using cells transfected with an inhibitor of differentiation promoter-luciferase construct. *Anal. Biochem.* **2006**, *349*, 78-86.
Figure S1. Atomic force microscopy (AFM) height image and line profile of hBMP-6 micro-patterned (10 µm diameter dots and 5 µm spacing) on an epoxide-terminated surface without using a passivated background. Scale bar 10 µm.
Figure S2. Effect of linker and immobilized hBMP-6 on cell adhesion and spreading observed 24h after seeding, studied via actin and nucleus staining of KS483 cells on a, d) a glass control, b, e) a GPTMS SAM and c, f) a PGMA layer both without hBMP-6 (a, b, c) and with hBMP-6 (d, e, f). The growth factor added for the glass control is presented in suspension contrary to the one bound to the GPTMS or PGMA layers. The scale bar is 200 µm. Insets show fluorescence microscopy image of cells after performing a Live-Dead assay. g) The cell surface area was quantified and normalized by the cell number after 24 h for the different cases. # p < 0.05, ## p < 0.01 when compared to all other platforms and * p < 0.05, ** p < 0.01 when comparing cases with or without hBMP-6 for a given platform.

Figure S3. Fluorescence microscopy images of immunostained micro-patterned hBMP-6 (100 x 100 µm dots) on a passive background on a GPTMS (left) or PGMA layer (right). Scale bars indicate 200 µm.
Figure S4. X-ray photoelectron (XPS) spectra of the C1s region for a PA film after the O$_2$-plasma treatment (left) and after introducing the epoxide-terminated silane (right). 1 was assigned to aromatic and aliphatic carbon bonds (C-C), 2 to ether bonds (C-O) and 3 due to its broadness could be assigned to ester (O-C=O), aldehyde (H-C=O) and ketones (C=O).

Figure S5. a) FT-IRRAS spectra of PA films at different stages of functionalization. The inset corresponds to the region associated to the O-H stretching. b) Ratio between the intensity of the asymmetric C-O-C and the C=O stretching bands.
Figure S6. a) Fluorescence images of immunostained microarrays of hBMP-6 (100 x 100 µm dots) with passive background. Left: PAOx-GPTMS-bhBMP-6. Middle: imine PAOx-bhBMP-6. Right: secondary amine after reduction of the imine PAOx-rbhBMP-6.

Figure S7. Effect of the linker and immobilized hBMP-6 on cell adhesion and spreading observed 24h after seeding, studied via actin and nucleus staining of KS483 cells on a, e) a PA control, b, f) oxidized PA, c, g) oxidized and reduced PA and d, h) PA functionalized with GPTMS both without hBMP-6 (a, b, c, d) and with hBMP-6 (e, f, g, h). The growth factor added for the PA control is presented in suspension while for the rest of cases it is bound to the platform. The scale bar is 200 µm. e) The cell surface area was quantified and normalized by the cell number after 24 hours for the different cases. # p < 0.05, ## p<0.01 when compared to all other platforms and *p < 0.05, ** p < 0.01 when comparing cases with or without hBMP-6 for a given platform.
**Table S1.** Advancing ($\theta_a$) and receding ($\theta_r$) water contact angle values and selected XPS data of PA films at different stages of functionalization.

| Sample      | $\theta_a$ (°) | $\theta_r$ (°) | C (XPS) | O (XPS) | Si (XPS) |
|-------------|----------------|----------------|---------|---------|----------|
| PA          | 52 ± 1         | 15 ± 1         | N/A     | N/A     | N/A      |
| PAOx        | 38 ± 1         | 15 ± 1         | 70.3 ± 1.4 | 29.2 ± 0.7 | 0.5 ± 1.0 |
| PAOx-GPTMS  | 53 ± 1         | 15 ± 1         | 60.3 ± 1.9 | 33.3 ± 0.2 | 6.4 ± 1.8 |