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RESEARCH ARTICLE

Development of a system of heparin multilayers on titanium surfaces for dual growth factor release

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Abstract

The aim of the present study was to establish a modular platform of poly-L-lysine-heparin (PLL-Hep) polyelectrolyte multilayer (PEM) coatings on titanium surfaces for dual growth factor delivery of recombinant human bone morphogenic protein 2 (rhBMP2) and recombinant human vascular endothelial growth factor 165 (rhVEGF165) in clinically relevant quantities. Release characteristics for both growth factors differed significantly depending on film architecture. rhBMP2 induced activation of alkaline phosphatase in C2C12 cells and proliferation of human mesenchymal stem cells (hMSCs). rhVEGF mediated induction of von Willebrand factor (vWF) in hMSCs and proliferation of human umbilical vein endothelial cells. Osteogenic and angiogenic effects were modified by variation in cross-linking and architecture of the PEMs. By creating multilayer films with distinct zones, release characteristics and proportion of both growth factor delivery could be tuned and surface-activity modified to enhance angiogenic or osteogenic function in various ways. In summary, the system provides a modular platform for growth factor delivery that allows for individual composition and accentuation of angiogenic and osteogenic surface properties.

KEYWORDS
biofunctionalization, bone morphogenic proteins, controlled release, heparin polyelectrolyte multilayer, vascular endothelial growth factor

1 | INTRODUCTION

Biofunctionalization of titanium implant surfaces for the enhancement of bone integration has been subject to research for more than two decades. In particular, growth factors and adhesion molecules that enhance proliferation, angiogenesis and tissue differentiation have been employed to improve the performance of inserted implants (for review see References 1–4) Anchoring of bioactive molecules to the metal surface with subsequent controlled delivery of functionally active signals remains a challenge.

Attachment of growth factors to material surfaces has been approached using single layers of anchoring strands as well as multiple layers of organic molecules of various nature and origin.[5–13] It has been emphasized that the application of growth factors for the enhancement of bone regeneration should include the delivery of more than just one factor in order to arrive at a more physiological

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signaling pattern by a combination of angiogenic and osteogenic growth factors.[14] Unfortunately, delivery of dual growth factors from modified titanium implant surfaces has not yet been successful in showing beneficial effects of the addition of angiogenic signals to the use of osteogenic growth factors.[15–17] The accommodation of multiple growth factors on the surface of titanium implants requires surface coatings that provide a sufficient number of binding sites for growth factors to allow for binding and release of the individual signals. Glycosaminoglycans (GAGs) that are widely present in the extracellular matrix can offer favorable properties for binding and release of growth factors. Their chemical nature as poly-anionic molecules allows for the combination with poly-cationic electrolytes in a layer-by-layer (LbL) approach of surface engineering through polyelectrolyte multilayer (PEM) films. Other than single layers of anchoring strands, the potential of multilayer coatings to accommodate amounts of growth factors in the μg range[18,19] may allow for the incorporation of more than one growth factor in sufficient quantities to achieve a biological response. Among the group of GAGs, hyaluronic acid (HA), chondroitinsulfate (CS), and heparin (Hep) have been explored in conjunction with single growth factor applications such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP2).[8,10,19–22] A recent analysis of GAG-protein interactions has shown that Hep has by far the highest amount of protein interactions.[23] Additionally, Hep contains binding sites for different groups of polypeptide growth factors[24] and many growth factors involved in bone regeneration provide Hep binding domains.[25–27]

It was thus the aim of the present study to establish a modular platform of poly-L-lysine-heparin (PLL-Hep) multilayer coatings by systematic variation of film architecture to accomplish the release of angiogenic and osteogenic growth factors with different delivery strategies on titanium surfaces in order to modify periimplant cellular behavior. Sandblasted acid etched titanium discs were coated with PLL as polycation and Hep as polyanion in different thicknesses and layer structures combined with different degrees of cross-linking before and after loading with recombinant human (rh) VEGF165 and recombinant human bone morphogenic protein 2 (rhBMP2). The resulting modifications were assessed for release profiles of the incorporated growth factors and their biological effect on cellular behavior. To the best knowledge of the authors no reports have been published yet that have used surface engineering of Hep multilayer films on titanium surfaces for the delivery of dual growth factors with different biological characteristics from biomaterial surfaces.

2 | MATERIALS AND METHODS

2.1 | Specimen fabrication

Commercially pure titanium (Ti) discs of 14.7 mm diameter was prepared by sandblasting and subsequent acid-etching (KLS Martin, Tuttlingen, Germany). The samples were etched in 5.1 M hydrochloric acid and 4.6 M sulfuric acid solution for 300 s by 108°C as previously described in Reference 7.

2.2 | Multilayer coating of Ti discs

The experiments for multilayer coating of the Ti discs were designed in three different approaches: (i) Multilayer PLL-Hep coating loaded with rhBMP2 and recombinant human vascular endothelial growth factor 165 (rhVEGF165) (Figure 1A); (ii) Multilayer PLL-Hep coating with different degrees of cross-linking and variation in the sequence of growth factor loading (Figure 1B); (iii) Multilayer PLL-Hep coating with two distinct zones and variation in the sequence of growth factor loading (Figure 1C) through Figure 1E.

2.2.1 | Multilayer PLL-Hep coating

Chemicals and reagents were purchased from Sigma Aldrich (Taufkirchen, Germany) and used without further purification, unless stated otherwise. PEM films were assembled using PLL (30–70 kDa) and Hep (50 mg/ml, from porcine intestinal mucosa, (sulfate content 2.7/monomer, isoelectric point 1.87, specific activity ≥180 USP units/mg, mixture of polyanion chains having molecular weights ranging from 6 to 30 kDa, with most chains in the range of 17–19 kDa). The polyelectrolytes were dissolved in Na-acetate buffer (20 mM, pH 4.5) at a concentration of 1 mg/ml. Film construction was performed semi-automatically employing a dipping robot (DR3, Riegler&Kirstein, Germany). Briefly, the cleaned substrates were first soaked into the polycation solution (PLL) and left there for 5 min until an adsorption equilibrium was established. Subsequently the samples were soaked into three deionized water wash solutions to rinse the surface to remove unbound polyelectrolytes. The polyanion Hep was adsorbed likewise by an incubation for 5 min followed by three rinsing steps. The film construction was performed by repeating these cycles until reaching the desired number of double layers and film architecture, respectively. All samples were rinsed in deionized water and air dried in a gentle stream of pressurized air. The resulting films of 10 double layers $(PLL\text{-}Hep)_{10}$ were loaded with growth factors (**10-GF** and **10-GF$_1$ + GF$_2$**; Figure 1A) as described below. Uncoated Titanium surfaces with and without growth factor loading were used as controls (**Ti-Contr**, **Ti-Contr-GF**, and **Ti-Contr- GF$_1$ + GF$_2$**).

Surface characterization was done using Quartz Crystal Microbalance with dissipation monitoring (QCM-D, QSense, Biolin Scientific, Sweden), field emission scanning electron microscopy (FESEM; ULTRA plus, Carl Zeiss, Jena, Germany) with carbon sputtered specimens (EM ACE200, Leica, Wetzlar, Germany) and profilometry (Smartproof 5, Carl Zeiss, Jena, Germany) in conjunction with automated software analysis (ConfoMap ST, vers. 7.4.8076, Carl Zeiss). The $(PLL\text{-}Hep)_{10}$ multilayers on the titanium surfaces had a total mass of 6.33 ± 0.32 μg/cm^2 and a thickness of 63.4 ± 3.21 nm. Surface roughness (R_a,S_a) of the different specimens varied between R_a: 3.41 ± 0.43 μm, S_a: 3.62 ± 0.66 μm (Ti-Contr), R_a: 3.08 ± 0.49 μm,

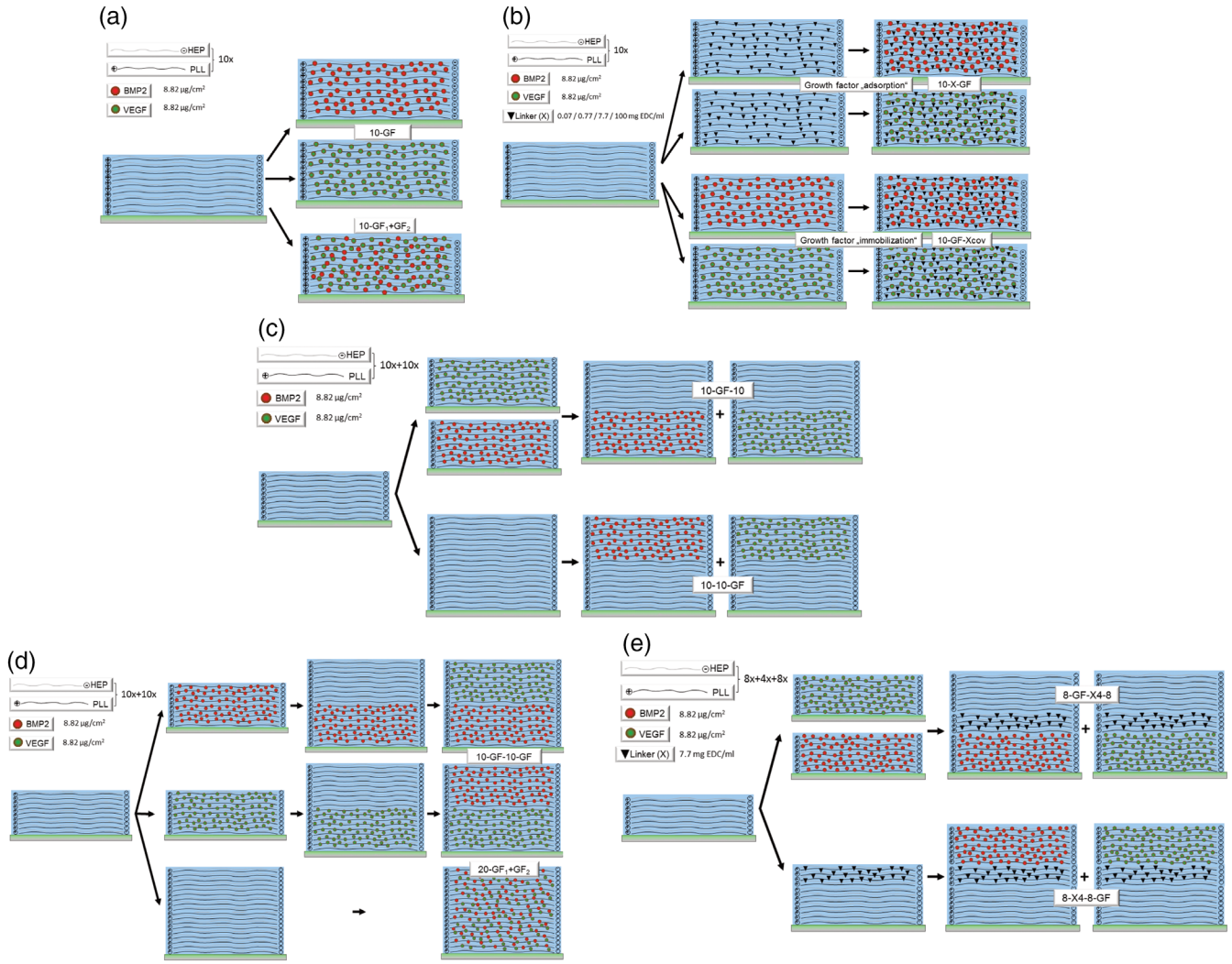

FIGURE 1 (a) Basic film construction loaded with rhBMP2 and rhVEGF165. (b) Cross-linking approach for films with adsorption and covalent immobilization of growth factors (GF). (c) Construction of films with two distinct zones of 10 double layers with alternating loading of growth factors in one zone only. (d) Construction of films with two distinct zones of 10 double layers with alternating loading of growth factors in both zones. (e) Construction of films with two distinct zones of 8 double layers with an intervening zone of four 100% cross-linked double layers. Growth factor loading in one zone only (see text). rhBMP2, recombinant human bone morphogenic protein 2; rhVEGF165, recombinant human vascular endothelial growth factor 165

S_a: 3.04 ± 0.54 µm ($(PLL\text{-}Hep)_{10}$), and R_a: 2.81 ± 0.52 µm, S_a: 3.38 ± 0.62 µm ($(PLL\text{-}Hep)_{10}$) with growth factor loading) without statistical significance ($p = .382$ and 0.534, respectively). Hence, the multilayer films and growth factor loading did not alter the morphologic characteristics of the sandblasted and etched surfaces (Figure 2A through Figure 2D).

2.2.2 | Cross-linking

Cross-linking was done using EDC/NHS (1-ethyl-3-[3-dimethylamino propyl] carbodiimide/N-hydroxysuccinimide; abcr Gmbh, Karlsruhe, Germany) at four concentration levels. The number of possible binding sites for cross-linking were calculated and 0.077, 0.77, and 7.7 mg/ml EDC (corresponding to coverage of 1%, 10%, and 100% of theoretically available binding sites) were used to achieve cross-linking of the $(PLL\text{-}Hep)_{10}$ multilayer system. For the cross-linking reaction with 1%–100% EDC 10-fold concentrated stock solutions of EDC and NHS were prepared in ice cold 0.15 M NaCl solution, pH 5. First, the NHS-stock solution was diluted in a way that the final concentration of 0.16 M was reached after adding of the EDC stock solution. Hence, the prepared stock solutions from EDC were further diluted in ice cold buffer with NHS until the desired EDC concentration was reached. Cross-linking was performed before (**10-X-GF**) and after (**10-GF-Xcov**) loading with growth factors. In the former approach, growth factor molecules would diffuse into the multilayer films cross-linked to different degrees and bound by adsorption, in the latter approach growth factors molecules would be covalently immobilized in the multilayer films to different degrees by cross-linking after diffusing into the layer systems (Figure 1B). A clearly above-threshold concentration of 100 mg/ml EDC

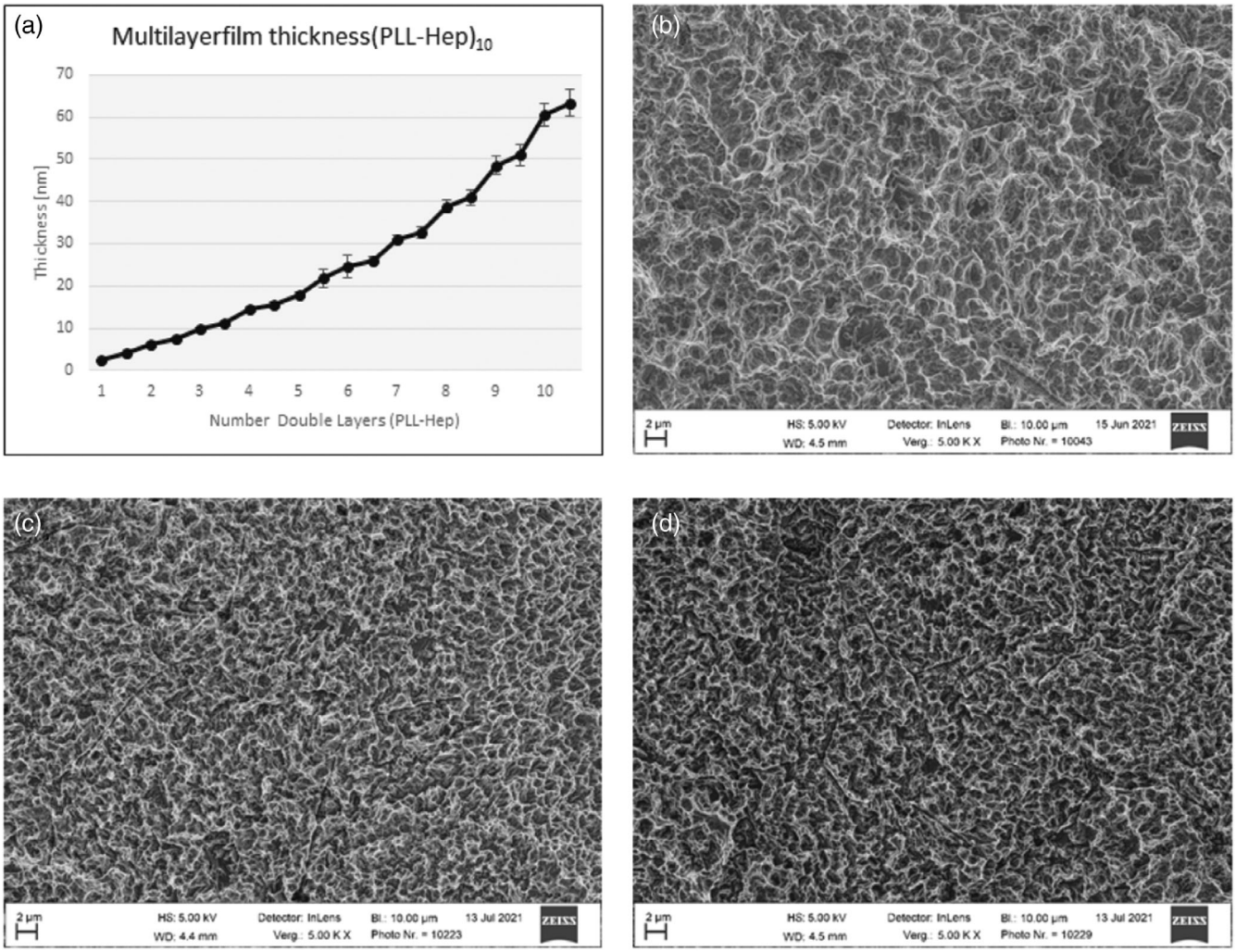

FIGURE 2 Surface characterization. (A) QCMD-analysis of film thickness on specimen surfaces. (B) FESEM image of uncoated Ti surface. (C) FESEM image of Ti surface coated with $(PLL\text{-}Hep)_{10}$ PEM. (D) FESEM image of Ti surface coated with $(PLL\text{-}Hep)_{10}$ PEM, loaded with rhBMP2. Profilometric analysis of the three surface conditions revealed no significant differences. PEM, polyelectrolyte multilayer; PLL-Hep, poly-L-lysine-heparin; rhBMP2, recombinant human bone morphogenic protein 2

was employed as positive control in both approaches. For the cross linking with 100 mg/ml EDC a 200 mg/ml EDC stock solution in ice cold 0.15 M NaCl, pH 5 was mixed in an equal volume with a 22 mg/ml NHS solution. Each cross-linking reaction was done in 1 ml EDC/NHS-solution in 24-well plates. All working steps were performed on ice with pre-cooled buffer and all samples were incubated over night at 4°C. After the cross-linking, all samples were washed at room temperature three times with 0.15 M NaCl, pH 8. Between each washing step, the samples were incubated for 1 h. Finally, the samples were rinsed with water and allowed to air dry at room temperature (RT).

2.2.3 | Variation in architecture

The PEM film architecture with two distinct zones used all in all 20 PLL-Hep multilayers and was designed in two different ways, using a two-step and a three-step procedure:

a. In the two-step procedure, a $(PLL\text{-}Hep)_{10}$ multilayer system was applied to the Ti surface and loaded with growth factor, after which a second $(PLL\text{-}Hep)_{10}$ multilayer system was added (**10-GF-10**). This procedure was modified by adding the second (upper) layer and then load this with the growth factor (**10-10-GF**) to determine the effect of the position of the growth factor within the 20-layer system (Figure 1C). The two zones were then subsequently loaded with two growth factors in alternating order (**10-GF_1-10-GF_2** and **10-GF_2-10-GF_1**) thereby changing the position of the individual growth factor within the multilayer systems. Finally, the two growth factors were loaded together onto a 20 layer PLL-Hep system (**20-GF_1 + GF_2**) (Figure 1D).
b. In the three step procedure, the architecture was built by using two $(PLL\text{-}Hep)_8$ multilayer systems that were separated by four layers of PLL-Hep that was 100% cross-linked to create an insolating layer between the two $(PLL\text{-}Hep)_8$ multilayer films. Corresponding to the procedure described under a), growth factors

were loaded onto the bottom (**8-GF-4-8**) and upper layer (**8–4-8-GF**) (Figure 1E).

2.3 | Growth factor loading

Unmodified or cross-linked PEM coated titanium samples were deposited into a single well each of a 24-well plate and incubated overnight with 200 µl rhBMP2 (75 µg/ml; Chinese Hamster Ovary cell-derived, PeproTech, Hamburg, Germany) or rhVEGF165 (75 µg/ml; Human Embryonic Kidney 293 cell-derived, ThermoFischer, GIBCO, Darmstadt, Germany) solutions. For definition of the final concentration of the loading solution see Supplementary Table 1.The samples where two growth factors were loaded together onto the surfaces (10-GF_1 + GF_2, 20-GF_1 + GF_2 and Ti-Contr-GF_1 + GF_2) were first incubated with rhVEGF165 (overnight) followed by rhBMP2.

After loading the supernatant was transferred to a reaction tube for further use and the discs were washed twice with deionized water and shortly air-dried at RT. The fully prepared discs were immediately used for further experiments or for a maximum of 48 h stored at 4°C.

For the preparation of two-zone modified PEMs, the cytokines were loaded in a sequential manner. Hence, the fabrication of the PEMs with 8–10 double layers PLL-Hep and the loading of the first cytokine was performed as described above. After this, the loaded discs were instantly used for the next dipping process until a PEM coating with 20 double layers was achieved. Next, the second cytokine was loaded on the coated disc, which was again incubated overnight with the cytokine solution of the second cytokine.

After the incubation the supernatant was taken from the discs and the loaded discs were washed twice with deionized water and shortly air-dried at RT.

The amounts of rhBMP2 and rhVEGF165 bound to the multilayer systems were determined indirectly from the supernatant of the coating procedure. Growth factor concentrations in the supernatant were assessed using the bicinchoninic acid (BCA) Protein Assay Kit (ThermoScientific, Darmstadt, Germany) with bovine serum albumin (BSA) as standard according to manufacturer instruction. Absorbance was measured with an ELISA plate reader (SpectraMax M2, Molecular Devices, San Jose, CA, USA) at 562 nm, RT. All samples were measured in duplicate.

In case of dual loading with growth factors, the supernatant from the second loading procedure was tested for a possible elution of the first loaded growth factor during the second loading using the BMP2 or VEGF enzyme-linked immunosorbent assay (ELISA), respectively, as described below. The specificity of the ELISAs were tested. Only background absorption or between 0.01% and 0.16% of the determined amount of the cytokine first loaded on the discs were detected (see Supplementary Table 2).

2.4 | Release experiments

All coated titanium discs were placed into 24-well plates and incubated in 1 ml DMEM supplemented with 2% FCS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere, shaking at 70 rpm (Celltron, InforsHT, Einsbach, Germany). The medium was collected and replaced after 24, 48, 72 h and every 3 days thereafter until day 21. The supernatants were stabilized with protease inhibitor (ROCHE Diagnostics, Mannheim, Germany). The release profiles of rhBMP2 and rhVEGF165 were assessed using Human/Murine/Rat BMP2 and Human VEGF Standard TMB ELISA Development Kit (PeproTech, Hamburg, Germany), respectively, according to the instructions of the supplier. RhBMP2 (PeproTech) or rhVEGF-165 (ThermoFisher, Gibco) were used as standard. Absorbance was measured with an ELISA plate reader (SpectraMax M2) at 450 nm with wavelength correction set at 620 nm. All measurements were done in dupplicate on three specimens. Two series of experiments were performed for each growth factor and surface condition.

2.5 | Osteogenic and angiogenic activity of surface conditions

Osteogenic activity associated with the different surface conditions was measured in C2C12 cells (DSZM, Braunschweig, Germany) using the alkaline phosphatase (ALP) assay. Moreover, proliferation of human mesenchymal stem cells (hMSCs; Lonza, Verviers, Belgium) was analyzed.

C2C12 cells and hMSCs were cultivated in Dulbecco's Modified Eagle's Medium GlutaMAX (DMEM; ThermoFisher,GIBCO, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin) and 1% penicillin/streptomycin (PAN Biotech, Aidenbach, Germany) at 37°C in a 5% CO_2 atmosphere. The medium was exchanged every 3–4 days after washing the cells with PBS. Until reaching 70%–80% confluency, the cells were detached from the culture flask surface using a trypsin/EDTA solution (0.25% trypsin/0.02% EDTA in 1x PBS; PAN Biotech) and subcultivated.

For the ALP assay, the C2C12 cells were seeded at a density of 40.000 cells/well in 24-well plates and cultivated in DMEM with 2% FCS and 1% penicillin/streptomycin. After attachment of the cells, supernatants from the release experiments were diluted in culture medium and added at different concentrations. To analyze the BMP2 activity on the titanium discs, 40,000 cells were seeded onto each disc in 24-well plates and cultivated in DMEM with 2% FCS and 1% penicillin/streptomycin. The measurement of ALP was carried out after 72 h, whereby the titanium discs were transferred into new 24-well plates. The cells were washed with PBS and lysed with 300 µl lysis buffer/well for 1 h at RT on a plate shaker (THERMOstar) at 400 rpm. The lysis buffer was composed of 0.1 M glycin, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ and 1% NonidetP-40 (Octylphenoxypolyethoxyethanol). The lysates were centrifuged at 1500 rpm (Eppendorf benchtop centrifuge 5415) for 5 min at RT and 75 µl aliquots from each supernatant were transferred into a 96-well plate in duplicate. After addition of 75 µl substrate buffer (0.1 M glycin, 1 mM $MgCl_2$, 1 mM $ZnCl_2$) containing 2 mg/ml p-Nitrophenylphosphate (pNPP; SigmaAldrich Chemie, Taufkirchen, Germany) the samples were incubated at 400 rpm on a

plate shaker (THERMOstar) at 37°C. Absorbance was measured at 405 nm and 37°C at 60 and 90 min. (SpectraMax M2).

The influence of rhBMP2 on the proliferation of hMSCs was determined using the colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The assay solution contains the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) combined with the electron coupling reagent phenazine ethosulfate (PES).

Thirty-thousand hMSCs were seeded onto each titanium disc (without prior release) in 24-well plates and cultivated in DMEM with 10% FCS and 1% penicillin/streptomycin for 72 h. To measure the amount of soluble formazan produced by cellular reduction of MTS, the discs were transferred into new 24-well plates and, after washing the cells with PBS, 500 µl fresh culture medium and 100 µl assay solution per well were added. After incubation for 2 h at 37°C in a 5% CO_2 atmosphere, 100 µl aliquots from each well were transferred into a 96-well plate in duplicate and the absorbance was measured at 490 nm with an ELISA plate reader (SpectraMax M2).

Angiogenic properties were evaluated both for the surfaces and the released growth factor, separately. Angiogenic activity of the released growth factor was evaluated by assessing the induction of von Willebrand factor (vWF) in hMSCs grown separately on tissue plastic using real-time quantitative polymerase chain reaction (q-PCR). Forty-thousand cells/well were incubated with rhVEGF supernatants from the in vitro release experiments in DMEM supplemented with 2% FCS and 1% penicillin/streptomycin for 72 h. After washing the cells with PBS, RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and stored at −80°C. RNA quality was assessed using the NanoDrop 1000 spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The RNA samples were converted into cDNA using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Feldkirchen, Germany), quantified using the iQ™ SYBR® Green Supermix (Bio-Rad) and MyiQ™ real-time PCR Detection System (Bio-Rad) according to the instructions of the supplier. The gene-specific primer sets are listed in Table 1. The delta–delta Ct method was used to calculate the relative fold gene expression of the samples.

Moreover, to test the activity of the released rhVEGF165, proliferation of human umbilical venous endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) was analyzed.

TABLE 1 Primer sequences

Von Willebrand factor, 237Bp	
fw	GCTGCTGGACACAAGTTTGA
rev	ACTCATGGGGCTCTGCATAC
Von Willebrand factor, 237Bp	
fw	GCTGCTGGACACAAGTTTGA
rev	ACTCATGGGGCTCTGCATAC

HUVECs were grown in serum-reduced, VEGF-free Endothelia Cell Growth Medium (PromoCell) and cultivated at 37°C in a 5% CO_2 atmosphere. Every 3–4 days, the cells were washed with PBS and the medium was exchanged. Until reaching 70%–80% confluency, the cells were detached and subcultivated as described above. For the proliferation assay, 40.000 cells/well were cultivated in 24-well plates in basal medium (Endothelia Cell Basal Medium, PromoCell) with 10% FCS, 1% penicillin/streptomycin and incubated with the supernatants for 72 h. After removal of the medium, the cells were washed with PBS and incubated with 500 µl fresh basal medium supplemented with 10% FCS, 1% penicillin/streptomycin and 100 µl assay solution (MTS assay, Promega, see above) for 3 h at 37°C in a 5% CO_2 atmosphere. After incubation, 100 µl aliquots from each well were measured in 96-well plates in duplicate (490 nm, ELISA plate reader, SpectraMax M2).

Angiogenic activity of the multilayer films were assessed by evaluating the enhancement of proliferation of HUVECs on the multilayer film surfaces both on surfaces without prior release experiments to test the initial activity (day 1) and on surfaces after release experiments to test the remaining activity at the end of the observation period (day 21). Forty thousand HUVEC cells were seeded onto each disc in 24-well-plates and cultivated in basal medium with 10% FCS and 1% penicillin/streptomycin. After 72 h the discs were transferred into new 24-well-plates. To evaluate the proliferation of HUVECs on the discs, the MTS assay was undertaken as described above. Three discs from each group were evaluated at each interval with all measurements performed in duplicate.

2.6 | Statistics

Data are presented as means ± SD. Univariate ANOVA (SPSS Statistics 26.0, http://support.spss.com) were used to compare the release kinetics between the three different surfaces. Univariate ANOVA with Bonferroni correction was employed to compare growth factor release, induction of ALP and vWF through released growth factor and direct contact with the PEM films as well as proliferation of hMSCs and HUVECs. Univariate ANOVA with Bonferroni correction was also used to compare the R_a values of the uncoated/coated Ti specimens.

3 | RESULTS

3.1 | Release of growth factors

3.1.1 | Unmodified multilayer systems

The release of rhBMP2 from the unmodified layer system (10-BMP2) was characterized by a rapid delivery of 993.63 ± 208.15 ng/cm^2 during the first 24 h with a steep decline at day 2 and the following intervals. After 21 days, 55.85 ± 13.00% of the incorporated growth factor

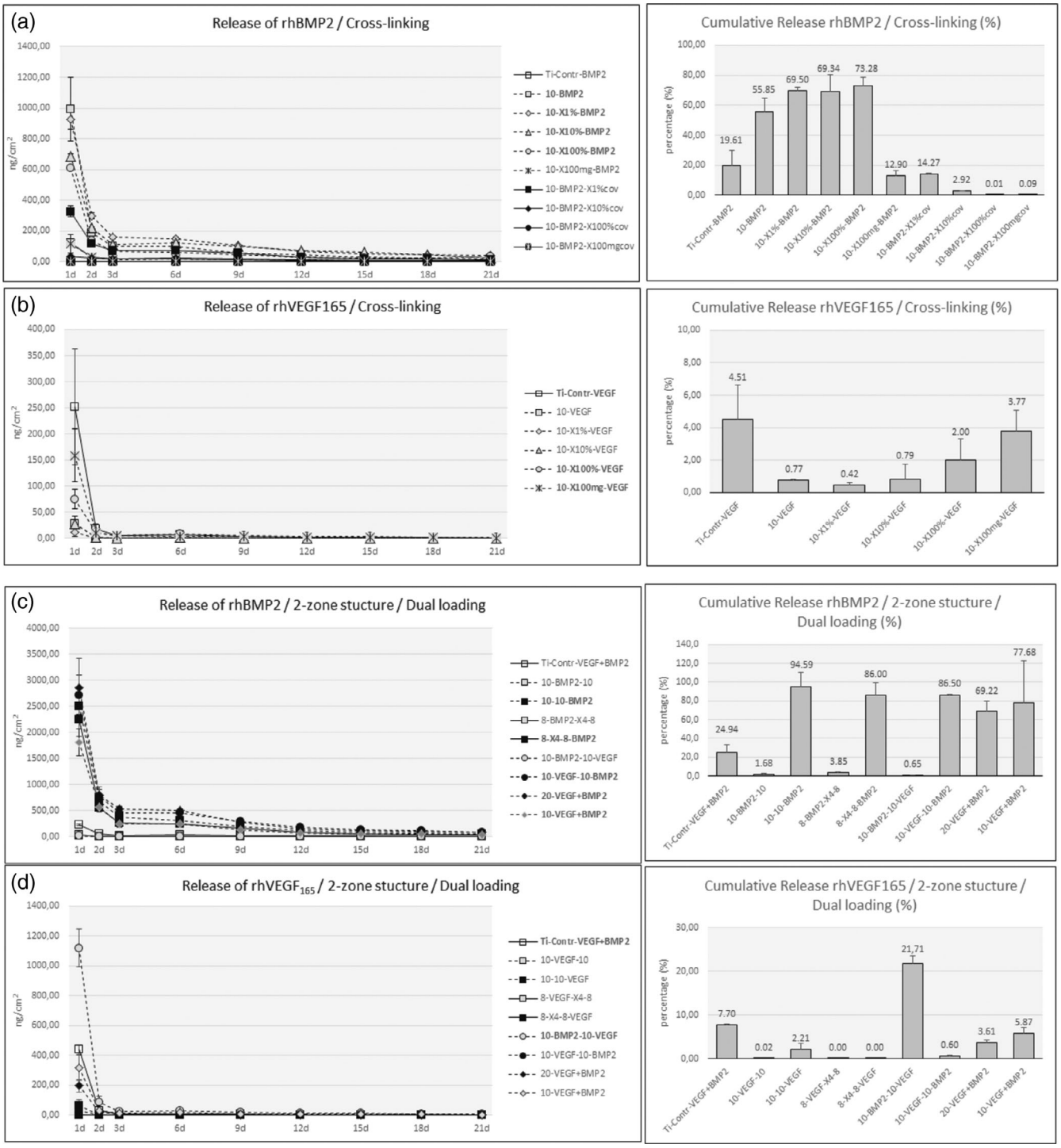

FIGURE 3 (a) Left side: Release profile of rhBMP2 from films with different degrees and modes of cross-linking; right side: Cumulative percentage of total release of rhBMP2 from these films. (b) Left side: Release profile of rhVEGF165 from films with different degrees and modes of cross-linking; right side: Cumulative percentage of total release of rhVEGF165 from these films. (c) Left side: Release profile of rhBMP2 from films with two-zone architecture and dual growth factor loading; right side: Cumulative percentage of total release of rhBMP2 from these films. (d) Left side: Release profile of rhVEGF165 from films with two-zone architecture and dual growth factor loading; right side: Cumulative percentage of total release of rhVEGF165 these films. Data are presented as means ± SD with $n = 3$. The abbreviations for samples with high release highlighted in bold in the release curves. rhBMP2, recombinant human bone morphogenic protein 2; rhVEGF165, recombinant human vascular endothelial growth factor 165

had been delivered. The release of rhVEGF165 (10-VEGF) showed a comparable characteristic, however, at a level that was up to two orders of magnitude lower (27.52 ± 1.39 ng/cm^2) during the first day and a rapid decline at the following intervals. After 21 days, only 0.77 ± 0.07% of the incorporated rhVEGF165 on average had been released (Figure 3A,B).

TABLE 2 Protein loading cross-linking

Specimens	VEGF µg/cm²	BMP2 µg/cm²
Ti-Contr-BMP2	–	1.22 ± 0.15
10-BMP2	–	2.78 ± 0.03
10-X1%-BMP2	–	2.67 ± 0.19
10-X10%-BMP2	–	2.08 ± 0.09
10-X100%-BMP2	–	1.43 ± 0.18
10-X100mg-BMP2	–	1.99 ± 0.50
10-BMP2-X1%cov	–	5.22 ± 0.09
10-8MP2-X10%cov	–	5.03 ± 0.25
10-BMP2-X100%cov	–	5.28 ± 0.43
10-BMP2-X100mgcov	–	2.92 ± 0.63
Ti-Contr-VEGF	6.67 ± 0.34	–
10-VEGF	4.54 ± 0.42	–
10-X1%-VEGF	3.65 ± 0.42	–
10-X10%-VEGF	4.63 ± 0.54	–
10-X100%-VEGF	6.08 ± 0.03	–
10-X100mg-VEGF	5.18 ± 0.54	–

Note: Data are presented as means ± SD with $n = 3$.

3.1.2 | Cross-linked multilayer systems

The loading behavior of the layer system that had been cross-linked *before* loading with rhBMP2 ("growth factor adsorption") differed with regard to incorporation in that higher cross-linking rates resulted in a significant decrease in uptake of rhBMP2 (1%, 10%, and 100% cross-linked layers, Table 2). The release profile was very much comparable to that of the unmodified layer system. The 21 day release rate in percentage of total protein loading was at a slightly higher level for all three cross-linking levels (between 69.34 ± 2.70% and 73.28 ± 10.70%) without reaching significance. Only the layers that had been cross-linked with 100 mg/ml EDC showed a significantly reduced percentage of release which is comparable with the release from the uncoated Ti discs (Figure 3A).

The rhBMP2 release from layer systems that had been cross-linked *after* growth factor loading ("covalent growth factor immobilization") was significantly lower than the release from films cross-linked (1%, 10%, and 100%) before loading with growth factor ($p = .000$). After 21 days, the percentage of released growth factor was significantly decreased (between 0.01 ± 0.00% and 14.24 ± 3.50%) (Figure 3A).

For rhVEGF165, cross-linking *before* growth factor loading had resulted in significantly increased uptake of growth factor from 1% to 100% cross-linking rate ($p = .000$). In contrast to rhBMP2, the uncoated Ti discs showed a high uptake of rhVEGF165 (Table 2). The released amount of growth factor between the cross-linking groups differed as well in that the percentage of release was significantly increased with an increased mean cross-linking level ($p = .000$) (Figure 3B).

Cross-linking *after* loading with rhVEGF165 had been abandoned, as it could be expected from the effect shown for rhBMP2 with significantly reduced release levels already at 1% cross-linking would have led to results for rhVEGF165 below the threshold level of the ELISA used (approx.10 pg/ml of growth factor).

TABLE 3 Protein loading two-zone structure

Specimens	VEGF µg/cm²	BMP2 µg/cm²
Ti-Contr-VEGF + BMP2	6.51 ± 0.21	1.98 ± 1.01
10-BMP2-10-VEGF	6.08 ± 0.34	7.45 ± 0.34
10-VEGF-10-BMP2	7.25 ± 0.34	6.04 ± 1.29
20-VEGF + BMP2	6.67 ± 0.34	7.65 ± 1.02
10-VEGF + BMP2	7.06 ± 0.59	4.90 ± 2.07
10-BMP2-10	–	2.75 ± 0.90
10-10-BMP2	–	4.71 ± 0.59
8-BMP2-X4-8	–	2.55 ± 0.90
8-X4-8-BMP2	–	4.37 ± 0.34
10-VEGF-10	5.20 ± 0.15	–
10-10-VEGF	4.98 ± 0.09	–
8-VEGF-X4-8	5.49 ± 0.53	–
8-X4-8-VEGF	5.76 ± 0.33	–

Note: Data are presented as mean ± SD with $n = 3$.

3.1.3 | Two-zone multilayer systems/loading with one growth factor

Experiments with loading of only one growth factor to the two-zone architecture were performed to test the retarding effect of the upper zone on the release of the growth factor from the lower zone.

a. The 10-10-GF variation with rhBMP2 loaded in the upper zone accommodated significantly more protein than the 10-GF-10 variation that had been loaded with rhBMP2 in the lower zone (Table 3) and showed significantly higher percentages of delivered growth factor whereby the release profiles were comparable to all other layer systems (Figure 3C).

For rhVEGF165, location in the lower zone (10-GF-10) almost completely ablated the release whereas 2.2 ± 1.35% were released when rhVEGF165 was incorporated in the upper zone (10-10-GF) (Figure 3D).

a. In the 8-X4-8-GF variation with rhBMP2 incorporated in the upper multilayer zone that was isolated against the lower layer (inbuilt 100% cross-linked zone), more rhBMP2 were accommodated than in the 8-GF-X4-8 architecture, where rhBMP2 was incorporated in the lower multilayer zone (Table 3). The profiles of delivery did not differ from the ones already known from the other groups whereas the amount of released growth factor was significantly higher in the 8-X4-8-GF variation (Figure 3C).

For rhVEGF165, the total release was negligible (<0.01%) independent from the location within the film architecture (Figure 3D).

3.1.4 | Two-zone multilayer systems/loading with two growth factors

a. Sequential loading of the two-zone multilayer systems with rhBMP2 and rhVEGF165 without separation by a 100% cross-linked zone of 4 PLL-Hep layers (10-BMP2-10-VEGF and 10-VEGF-10-BMP2) resulted in high amounts of incorporated growth factors in both zones (Table 3). The incorporation of rhBMP2 in the lower zone led to only 0.65% ± 0.20% release of the incorporated growth factor after 21 days, whereas 21.71% ± 2.61% of the rhVEGF165 in the upper zone was delivered during this period. When locations of rhBMP2 and rhVEGF165 in the two zones were swapped, 86.50% ± 5.31% of the incorporated rhBMP2 in the upper zone was delivered on average within 21 days, while only 0.60% ± 0.20% of the rhVEGF165 from the lower zone was released during the observation period (Figure 3C, D). Dual loading of the PLL-Hep layers with rhBMP2 and rhVEGF165 (20- VEGF+BMP2 and 10-VEGF+BMP2) exhibited both a significantly higher release for rhBMP2 (Figure 3C,D).
b. Release experiments with sequential dual growth factor loading of the two-zone multilayer systems with a separation by a 100% cross-linked zone of 4 PLL-Hep layers (8–GF-X4-8-GF) were abandoned, after the experiments with a single factor incorporated into the lower zones and the dual growth factor loading of both zones without intervening 100% cross-linked zone (10-GF-10 and 10-GF-10-GF architecture) had shown that retardation of the growth factor release from the lower zones by a single upper zone had shown to be equally effective as the 8-GF-X4-8 architecture with the intervening 100% cross-linked zone. Therefore, no additional retarding effect for the delivery from the lower zone had been expected through this intervening 100% cross-linked zone.

3.2 | Osteogenic and angiogenic properties

3.2.1 | Induction of ALP using unmodified and cross-linked multilayer systems with single growth factor loading

Induction of ALP using only the released rhBMP2 from the Ti discs in C2C12 cells grown separately on tissue plastic is shown in Figure 4A, B. The threshold concentration of rhBMP2 was identified at 50 ng/ml. The osteogenic activity of the release from the unmodified multilayers correlated well with the concentrations of the stock solution of rhBMP2 (Figure 4A). The amount of 50 ng/ml was available from the release until day 9. At 50 ng/ml rhBMP2, the release from the unmodified multilayer systems had a clear osteogenic activity normalized by the controls and averaged across the release intervals (Figure 4B).

The release from the multilayer surfaces that had been cross-linked *before* growth factor loading exhibited a significantly decreasing activity with increasing degree of cross-linking at 10 and 100% ($p = .002$) (Figure 4B).

The release from the multilayer films that were cross-linked *after* loading was sufficient to test the separate effect of the release only for the 1% cross-linked multilayers. The osteogenic activity of the rhBMP2 released from these surfaces was equal to the release from the unmodified layers (Figure 4B).

Induction of ALP in C2C12 cells grown *directly* on the surfaces of the Ti discs showed significantly increased levels of ALP activity (at day 1) on unmodified multilayer surfaces and all cross-linked multilayer systems loaded with rhBMP2 that had been modified before growth factor loading ("Growth factor adsorption") ($p = .000$). After release for 21 days, the surfaces still induced ALP at a significantly higher level than the controls in cells grown directly on the surface with the exception of the 100 mg cross-linked multilayer systems. Cells grown on multilayer films cross-linked after growth factor loading ("Growth factor immobilization") exhibited the same pattern of activity both at day 1 (without prior release) and after 21 days release with the exception of the 100% and 100 mg cross-linked multilayer systems ($p = .000$) (Figure 4C).

3.2.2 | Induction of ALP using two-zone multilayer systems/loading with single and dual growth factor loading

Induction of ALP in cells on the multilayer surfaces was assessed for the 10 + 10-architecture with single loading of rhBMP2 (10-10-BMP2) and with sequential loading of rhVEGF165 and rhBMP2 (10-VEGF-10-BMP2 and 10-BMP2-10-VEGF) as well as dual loading (20-VEGF+BMP2). The 8 + 4 + 8-architecture was abandoned as reported above.

In C2C12 cells grown on these surfaces, only the multilayer systems with rhBMP2 in the upper zone of the layer architecture (10-10-BMP2 and 10-VEGF-10-BMP2) and the multilayer films with dual loading of rhVEGF165 and rhBMP2 (20-VEGF+BMP2) have induced significant enhancement of ALP activity ($p = .000$). After 21 days, the activity was still clearly increased in cells growing on those films that presented rhBMP2 in their upper layer (Figure 4D).

3.2.3 | Angiogenic gene transcription

In hMSCs cells grown on tissue plastic, the growth factor released from the multilayer systems loaded with rhVEGF165 had induced a level of transcription of vWF in hMSCs, that was higher than the growth factor released from Ti control surfaces. This difference was significantly increased for the release from films that had been cross-linked to higher degree ($p = .032–.016$). Interestingly, the level of transcription of vWF was significantly decreased with the release from the two-zone architecture that had a substantial co-release of

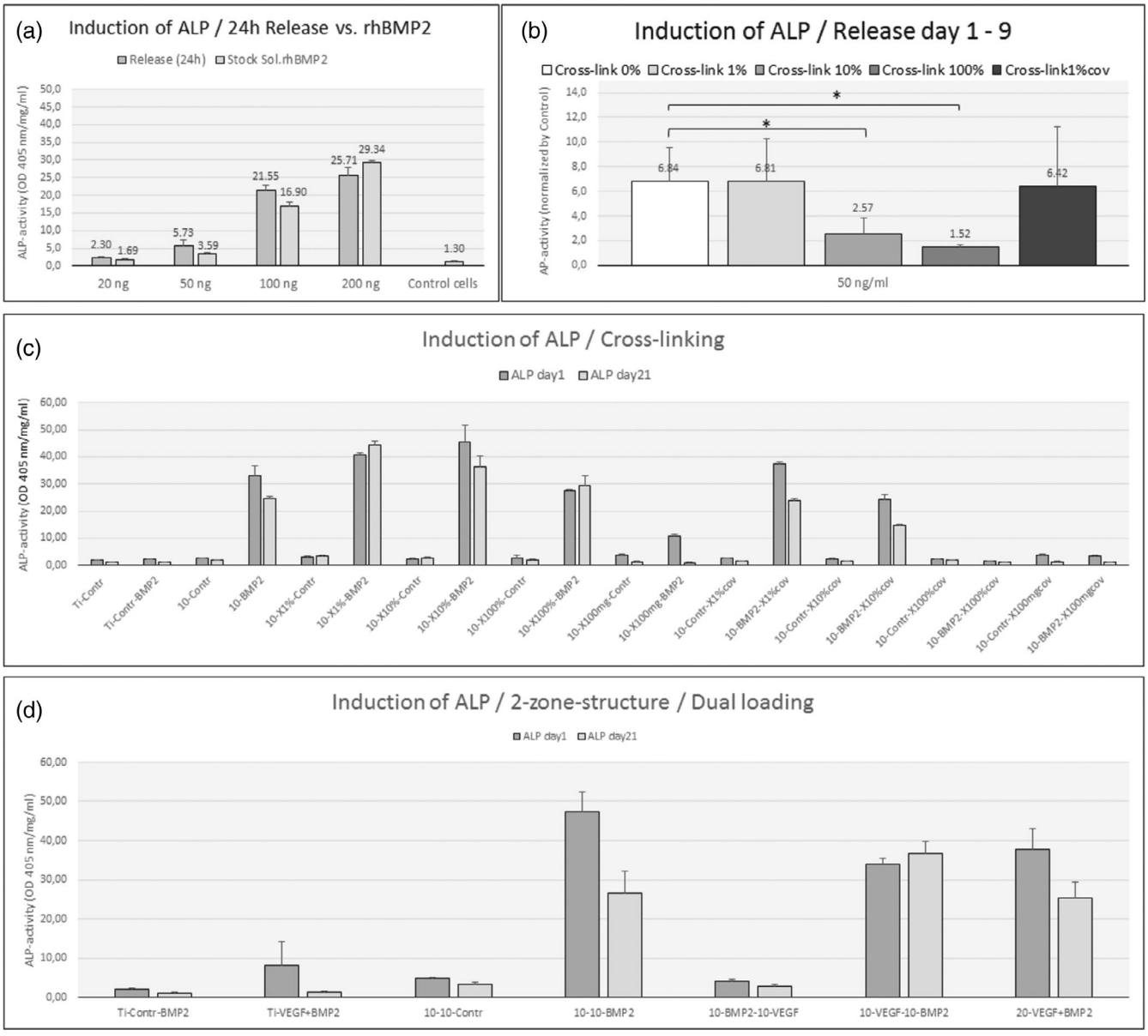

FIGURE 4 (a) Induction of ALP in C2C12 cells grown on tissue plastic by the released rhBMP2 from $(PLL\text{-}Hep)_{10}$ films compared to stock solution. (b) Induction of ALP in C2C12 cells grown on tissue plastic by the released rhBMP2 from $(PLL\text{-}Hep)_{10}$ films with different degrees of cross-linking. (c) Induction of ALP in C2C12 cells grown on PEM covered Ti surfaces with different degrees of PEM cross-linking. (d) Induction of ALP in C2C12 cells grown on PEM covered Ti surfaces with PEM films in two-zone architecture and dual growth factor loading. Data are presented as means ± SD with $n = 2$ (a, b) and $n = 3$ (c, d). ALP, alkaline phosphatase; PEM, polyelectrolyte multilayer; PLL-Hep, poly-L-lysine-heparin

rhBMP2 (i.e., when rhVEGF165 was located in the bottom layer or had been loaded simultaneous with rhBMP2) ($p = .003$) (Figure 5B).

3.3 | Cell proliferation

3.3.1 | Human MSCs

Human MSCs exhibited a significantly increased rate of proliferation after 3 days on unmodified multilayer surfaces loaded with rhBMP2 and on the cross-linked surfaces regardless of the rate of cross-linking if rhBMP2 was loaded after cross-linking. Interestingly, the 1%–100% cross-linked multilayer systems without incorporation of rhBMP2 exhibited a significantly increased proliferation even compared to non-cross-linked multilayer PLL-Hep surfaces (Figure 6A). The level was comparable to Ti discs loaded with rhBMP2 without multilayer coating. In the multilayer systems where the growth factor had been covalently immobilized by cross-linking after loading with rhBMP2, there was a decrease in proliferation of hMSCs with increasing degree of cross-linking. Surfaces

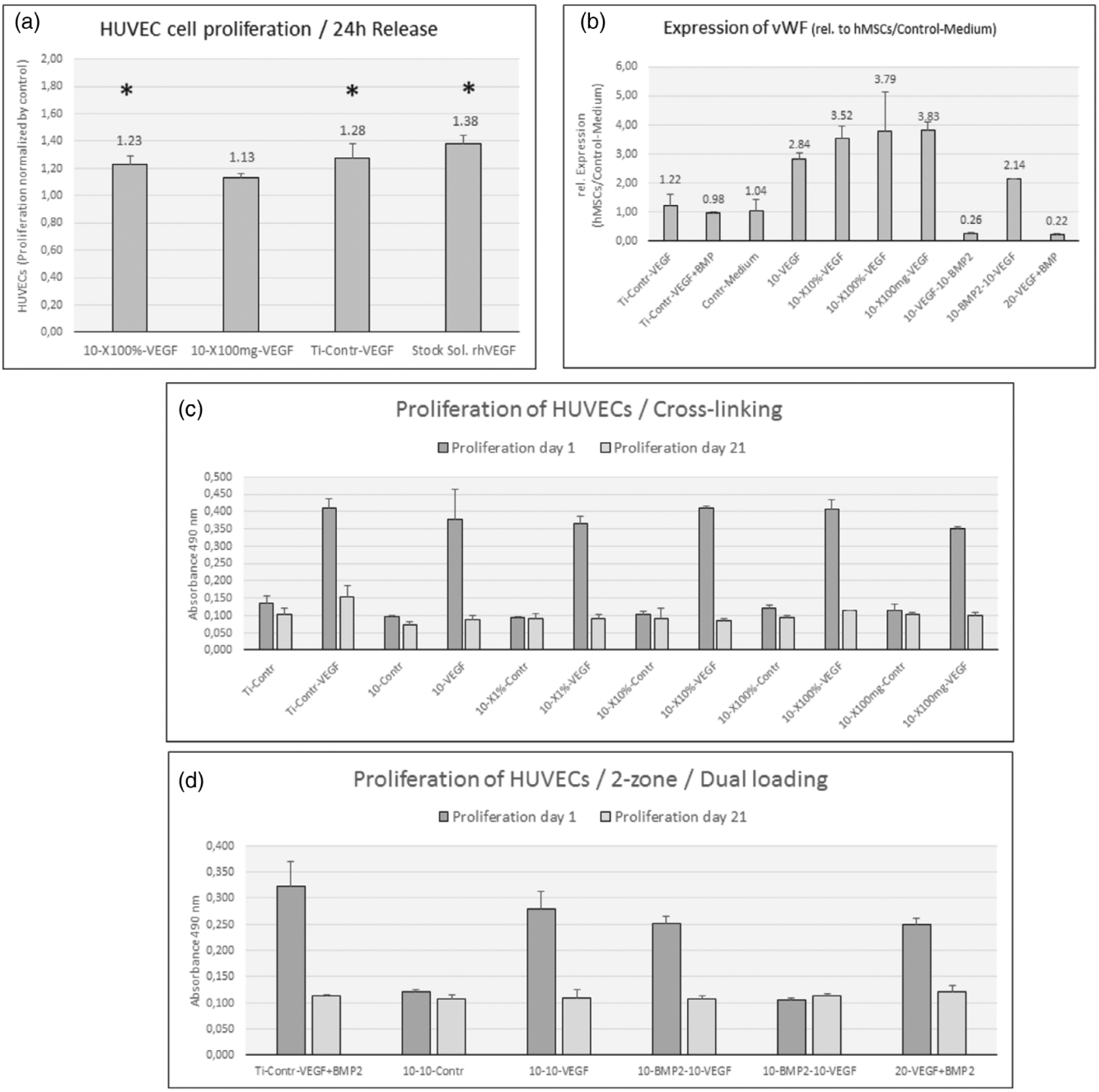

FIGURE 5 (a) Proliferation of HUVECs grown on tissue plastic by the released rhVEGF165 from $(PLL\text{-}Hep)_{10}$ films with different degrees of PEM cross-linking. (b) Induction of vWF in hMSCs grown on tissue plastic, exposed to eluates from different degrees of PEM cross-linking and from PEM films with a two-zone architecture and dual growth factor loading. (c) Proliferation of HUVECs grown on PEM covered Ti surfaces with different degrees of PEM cross-linking. (d) Proliferation of HUVECs grown on PEM covered Ti surfaces with PEM films in two-zone architecture and dual growth factor loading. Data are presented as means ± SD with $n = 2$ (a, b) and $n = 3$ (c, d). HUVECs, human umbilical venous endothelial cells; PEM, polyelectrolyte multilayer; PLL-Hep, poly-L-lysine-heparin

with the two-zone architecture of PEM films exhibited a significantly increased proliferation of hMSCs when rhBMP2 had been incorporated in the upper zone or when it had been loaded simultaneously with rhVEGF165. When rhBMP2 was located in the lower zone, proliferation was equal to the controls without growth factor (Figure 6B).

3.3.2 | Human umbilical vein endothelial cells

Angiogenic activity of rhVEGF165 loaded surfaces as reflected by proliferation of human endothelial cells (HUVECs) was tested two-fold: HUVEC proliferation on tissue plastic induced by released rhVEGF165 and HUVEC proliferation on the multilayer coatings

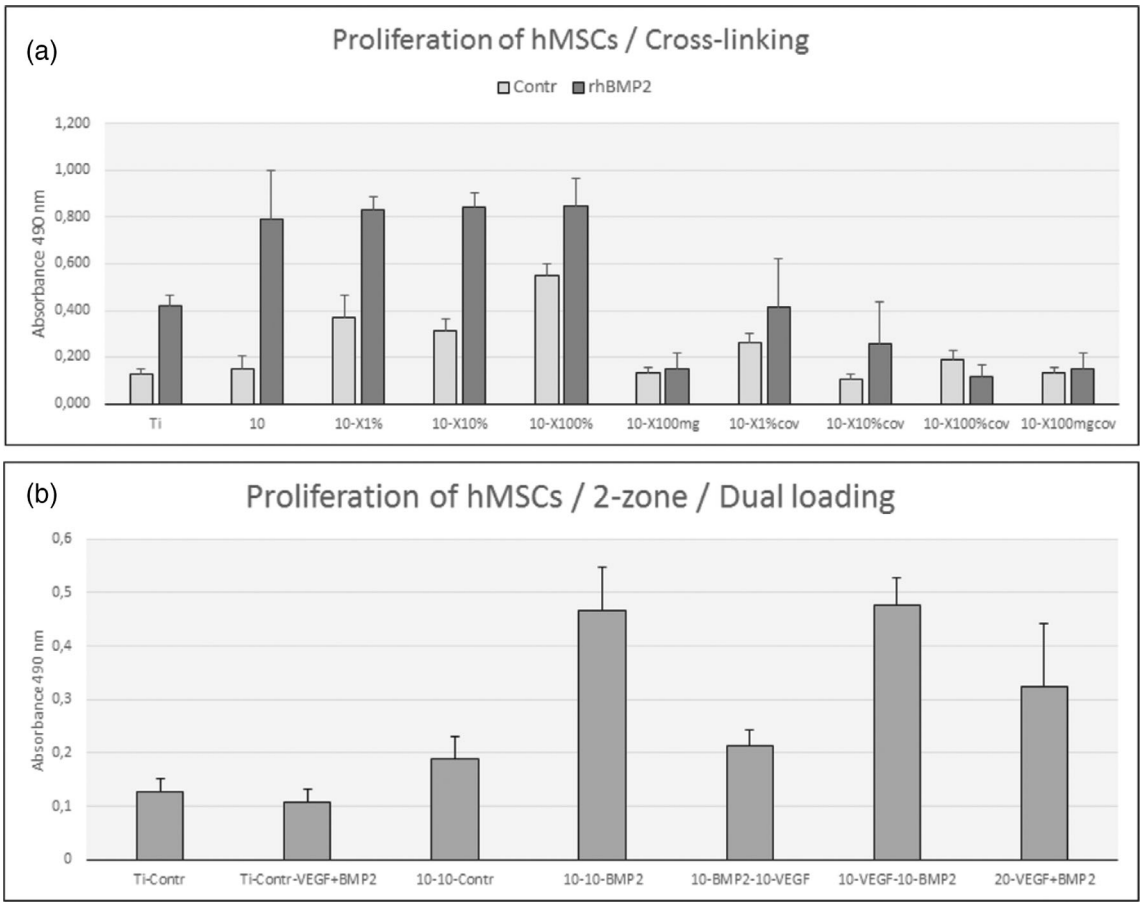

FIGURE 6 (a) Proliferation of hMSCs grown on PEM covered Ti surfaces with different degrees of PEM cross-linking. (b) Proliferation of hMSCs grown on PEM covered Ti surfaces with PEM films in two-zone architecture and dual growth factor loading. Data are presented as means ± SD with $n = 3$. hMSCs, human mesenchymal stem cells; PEM, polyelectrolyte multilayer

induced by the total biological activity of the surface. For HUVECs grown separately on tissue plastic, a threshold value of 50 ng/ml was identified. Due to the low-release rate of rhVEGF165 only the 24 h release from a limited number of surface modifications could be used. These releases significantly increased the proliferation of HUVECs as compared to the controls (Figure 5A). Other than with rhBMP2 the use of EDC did only affect the biological effect on the proliferation of HUVECs in very high amounts (100 mg EDC). Proliferation of HUVECs on the multilayer surfaces showed a significant increase on all multilayer surfaces that had been loaded with rhVEGF165 regardless of the degree and timing of cross-linking. When cells were cultured on the surfaces after 21 days at the end of the release period, this effect was not visible anymore (Figure 5C). HUVECs grown on surfaces with the two zone architecture exhibited a significantly increased proliferation only on those surfaces that presented rhVEGF165 in the upper zone (10-10-VEGF; 10-BMP2-10-VEGF; 20-VEGF+BMP2). If rhVEGF165 was incorporated in the lower zone (10-VEGF-10-BMP2) no enhancement of proliferation was observed. After 21 days of release, the enhancing effect on HUVEC proliferation had disappeared (Figure 5D).

4 | DISCUSSION

The present study systematically varied the architecture of Hep multilayer coatings on microrough titanium surfaces, a standard surface of today's dental implants, to explore their potential as a modular system for controlled binding and release of multiple growth factors involved in bone regeneration. The two growth factors tested behaved differently with respect to incorporation and release. The amount of rhVEGF165 incorporated in the Hep multilayer system was higher than rhBMP2 at the same concentration of the loading solution, but the release from the multilayer films was two orders of magnitude lower. The release of rhBMP2 from the PLL-Hep system was characterized by a rapid delivery of approx. 35% of the incorporated growth factor at day 1, followed by a much slower release during the following 3 weeks, indicating that roughly one third of the amount of rhBMP2 is only loosely attached to the substrate of the multilayer system. The rapid release during day 1 also occurred with rhVEGF165 but accounted for only 1% of the incorporated amount of growth factor indicating that the initial retaining capacity of the Hep multilayer system is more than 30 times higher for rhVEGF165 than for rhBMP2. It may be speculated that despite the structural similarity of the two

homodimeric cysteine knot proteins, the different quantities of bound and released rhVEGF165 and rhBMP2 are based on the differences in structure and position of the Hep binding domains of the two growth factors that affect interactions between individual growth factors and the biopolymers of the film and vary even among members of the same growth factor family.[25,28]

Binding and release of growth factors to PEM films has been extensively studied for rhBMP2 and PLL-HA systems indicating that the growth factor is adsorbed to the multilayer substrate and diffuses into the film to a certain extent while maintaining its ß-structure.[8,18] Confocal laser scanning microscopy (CLSM) imaging had shown penetration of rhBMP2 into PLL/HA multilayer films up to 3.5 μm.[18] Distribution of the incorporated growth factor molecules within the multilayer film has been shown to be concentrated in the superficial layers of these films, indicating a diffusion driven process of incorporation.[29] Release of the incorporated growth factor from these films is therefore most likely to occur through diffusion back to the surface. The released amount of rhBMP2 from these films has been shown to be up to 77% within the first 5 h.[18,19] The PLL/Hep PEMs evaluated in the present study are likely to behave differently as Hep is a stronger polyanionic partner than HA, which is expected to lead to higher water uptake between the polymer chains[30] which on the one hand may facilitate diffusion of proteins through the film. On the other hand, the use of a strong polyelectrolyte pairing partner has been shown to reduce the diffusivity of PEMs[31] and the replacement of HA by Hep has been shown to be associated with much lower film thickness and a lower amount of incorporated growth factor.[32] Moreover, Hep is highly sulphated with a lower isoelectric point than HA and has a higher specific affinity for growth factors. Nonspecific interactions of the incorporated growth factor with the higher number of anionic groups and specific interaction with the Hep binding site of the growth factor may result in slower diffusion and a lower release rate. Unfortunately, other than in studies analyzing PLL/HA multilayer films, visualization of fluorescent labeled proteins in the PEMs of the present study has not been possible as the film thickness of 65 nm was far below the z resolution of CLSM. It appears, nevertheless, from the present results that rhBMP2 incorporated into PLL/Hep multilayer coatings undergo a slower release than growth factors loaded on HA.[10,19] Interestingly, the resulting amount of rhBMP2 both incorporated and released in the present study is in contrast to a previous report showing a faster delivery of rhBMP2 from PLL/Hep than from PLL/HA films.[32] This shows, that not only the nature of the polyanionic and –cationic polymers is important for the amount of protein loaded and released but also the pH and ionic strength at film preparation as well as during growth factor loading has an impact on the polymer interactions inside the multilayer film and can affect the amount of protein bound to and released from the multilayer films.[18,33–35]

Differences in interactions between GAG substrates and the incorporated growth factor in conjunction with different surface engineering approaches may also explain the variety of reported release kinetics for other GAG multilayer systems: PEM films of Hep or CS in combination with chitosan as polycationic partner had shown a release of BMP2 between 6% and 35% after 4 days.[10] For VEGF, a similarly slow release has been reported by Ye et al. (2013) from Hep-based PEMs.[22] Loading of PEMs with FGF2 had resulted in incorporation of up to 45 ng/cm^2,[9] which is around two orders of magnitude lower compared to what has been fund for rhBMP2 and rhVEGF165 in the present study and to what has been reported for BMP2 in HA-PEMs.[12] The large variety of modifications and biopolymer combinations (in particular polycationic partners for Hep), the differences in loading strategies for the growth factor (in some studies, the growth factor had been directly used as polycationic partner for Hep[9,21] and the inhomogeneity of reporting (incorporated/released amount of growth factor, release kinetics) make it difficult to directly compare the present results with those derived from other Hep-based PEMs.

4.1 | Cross-linking and zonal architecture

When the PLL-Hep layers had been cross-linked *before* growth factor loading, rhBMP2 and rhVEGF165 again behaved differently in that the amount of incorporated rhBMP2 decreased and that of rhVEGF165 increased with increasing degree of cross-linking. Interestingly, the percentage of release increased slightly for rhBMP2 and significantly for rhVEGF165. As mentioned above, binding of rhBMP2 to multilayer films of PLL/HA has been shown to be based on diffusion.[8,18] Assuming that this diffusion process is also involved in the incorporation of growth factors in PLL-Hep systems,[32] it can be speculated that the covalent linking of carboxylic groups of the Hep molecule to the amine groups of the PLL through EDC/NHS has changed the molecular architecture of the multilayer system accounting for differences in initial diffusion and uptake of the loaded growth factors. This cross-linking may have reduced the accessibility of the Hep sulfate motifs that are important for binding of growth factors to the Hep binding domains.[24] This may affect rhVEGF165 more than rhBMP2, resulting in a larger portion of loosely attached growth factor and thus a higher rate of release for rhVEGF165. Cross-linking of PLL/HEP multilayer films has been less effective than in PLL/HA, where increasing degrees of cross-linking using 30–70 mg/ml EDC had resulted in a strong reduction of released amounts of incorporated BMP2.[12,19] A comparable reduction of release in the presently evaluated PLL-Hep films treated alike in the present study was only observed when much larger amounts of EDC/NHS (100 mg/ml) were employed. This may be explained by the fact that PLL-HA films contain no sulfate groups and thus will have shown a much higher degree of cross-linking compared to PLL-Hep films.[32]

Crosslinking of PLL-Hep multilayer systems *after* loading with growth factors has been much more efficient in retarding growth factor release showing that already 0.077 mg/ml EDC (corr. to 1% of possible binding sites) has significantly reduced the release of rhBMP2 and 0.77 mg/ml has reduced it to a level lower than what has been achieved by using 100 mg/ml EDC before loading with rhBMP2. This higher efficacy could be either due to the fact that larger portions of the growth factor are trapped within the cross-linked multilayer films or that a substantial amount of growth factor molecules are covalently

bound to the film substrate through primary amine groups as it has been described for activated phosphonic acid monolayers on titanium surfaces.[6] A matter of concern may be that the amounts of rhBMP2 released from the cross-linked films have shown reduced biological activity at increasing degrees of crosslinking, that is, when larger amounts of EDC are used. This may indicate an interaction between unbound EDC and growth factor molecules that occurs inside the multilayer films. Interestingly, the absence of function of released BMP2 from highly EDC-cross-linked PEMs has also been shown for PLL-HA films.[18] The present results, therefore, suggest that the amount of EDC used should be as low as possible by directly immobilizing the growth factor with EDC after loading rather than completely cross-linking the multilayer film before loading with rhBMP2.

The release characteristics of rhBMP2 from the two zone architecture was somewhat unexpected as the top zone of 10 PLL-Hep layers obviously reduced the release of rhBMP2 from the bottom zone to negligible amounts while the release after rhBMP2 loading into the top zone was substantially increased. Interestingly, the effect of an intermediate zone of four 100% cross-linked PLL-Hep layers had no additional effect. This may indicate that the portion of growth factor that is released during the observation period of 3 weeks originates mainly from the superficial layers of the multilayer films and will be sealed off by a second zone of PLL-Hep layers constructed on top of them. When considering these results, it should be kept in mind that bio-polymer based PEM films have an intrinsic dynamic with respect to diffusion of polyelectrolytes and film growth that is affected by environmental factors such as ionic strength and pH leading to the formation of fractions of biopolymers within PEMs with different diffusivity.[35] For PLL/HA multilayer systems, diffusion of PLL molecules across the film depending on their molecular weight has been reported,[36,37] leading to exponential growth of film thickness between 1 and 3.5 μm.[18,35] In contrast, the growth of the PLL/Hep films in the present study has shown a near linear growth characteristic with the resulting film thickness of appr. 65 nm being substantially thinner than reported for PLL/HA-based PEMs. Given the strength of the poly-anion Hep molecule and the strong polyion–polyion interactions with PLL molecules, it is unlikely that the polymer chains will migrate as readily as in PLL/HA PEMs and portion of growth factor bound to Hep through the Hep binding domains will remain buried in the lower zone or move much slower into the upper zone. Moreover, interactions between the negatively charged polymer groups and the positively charged groups of the growth factor molecule may limit diffusion of the unbound growth factor portions. In two-zone films with growth factor loading of the upper zone, a redistribution/diffusion of polymer molecules during the construction of the second layer could have been associated with for a larger portion of loosely attached growth factors when they are loaded to the upper zone leading to a higher early release. Interestingly, it has been reported for PLL/HA PEMs that part of the released rhBMP2 was associated with liberated HA through hypothesized ionic bonds between negatively charged HA and positively charged rhBMP2.[18] It may therefore be that part of the released rhBMP2 from the present PLL/Hep PEMs is bound to fractions of Hep liberated from the surface of the multilayer film. A possible "co-release" of fractions of Hep together with rhVEGF165 may also explain the significantly higher activity in the expression of vWF through the small amounts of rhVEGF165 released from Hep multilayer surfaces compared to the much higher release from Titanium control surfaces. Wijelath and co-workers had shown that the activity of rhVEGF is significantly enhanced in the presence of exogenous Hep.[38]

4.2 | Osteogenic activity

The biological activity of the multilayer systems as tested by cells grown on the film surfaces has shown for rhBMP2 containing films that ALP was clearly induced in C2C12 cells not only at the start of the observation period but also after 21 days of release when growth factor delivery has almost subsided. This indicates that for osteogenic differentiation of mesenchymal cells growing on the surface of the films, the portion of BMP molecules inside the films is of greater importance than the one released. A similar effect, observed with rhBMP2 loaded PLL-HA films with minimal release of the growth factor has been hypothetically explained by the cells either sensing the immobilized rhBMP2 or producing an extremely localized leak of rhBMP2 from the film following degradation of the film immediately underneath the cells by enzymes, such as metalloproteases and hyaluronidases.[18] It is interesting to note that C2C12 cells grown on BMP2 loaded PLL/HA films have been shown to internalize BMP2 molecules through the cell surface facing the material depending on the degree of cross-linking.[29] This is paralleled by the finding of the present study that the induction of ALP in C2C12 cells grown on films with cross-linking after growth factor loading has been significantly increased only up to a rate of 10% cross-linking, whereas rhBMP2 incorporated in films with 100% cross-linking and beyond (100 mg/ml EDC) after growth factor loading failed to induce ALP activity. This suggests that the cross-linking process at this level may have either completely bound the BMP2 molecules from the to the film substrate or changed the structure of the multilayer film thereby rendering the growth factor inaccessible for the cells growing on the surface. There is an ongoing discussion about the function of tethered vs. free growth factors in particular for BMPs, in bio-functionalized material surfaces. The induction of ALP by the immobilized portions of the rhBMP2 in the PLL/Hep films suggests that also growth factors tethered in the multilayer films activate osteogenic differentiation in C2C12 cells. As ALP is an early marker of osteogenic differentiation, it is unclear if terminal differentiation into osteoblasts would occur requiring internalization of the BMP/receptor complex. As mentioned above, cellular internalization of BMP2 occurs from PLL/HA films but is significantly reduced by one order of magnitude in strongly cross-linked films.[29] Hence, it is unclear, whether the osteogenic differentiation in C2C12 cells grown on heavily cross-linked films can be based on internalized rhBMP2. Moreover, previous studies using covalent grafting of rhBMP2 on PET surface as well as tethered BMP2 on polyrotaxane surfaces have shown to induce also late markers

of osteogenic differentiation such as osteopontin (OPN)[39] and osteocalcin[40] indicating that also tethered BMP2 signals can induce the complete cascade of osteogenic differentiation. Following this line of thoughts, it is likely that the failure of rhBMP2 to induce ALP in the multilayer films that had been highly cross-linked after growth factor loading is not due to tethering of the growth factor but rather due to modification of the film structure or the binding domain of the rhBMP2.

In cells grown on films with the two zone architecture, ALP was only induced in cells when the upper zones contained rhBMP2 supporting the hypothesis that cells get into contact with incorporated rhBMP2 immediately underneath them inside the film and fail to do so if an upper PLL-Hep zone devoid of rhBMP2 or loaded with a non-osteogenic growth factor is separating them from the incorporated rhBMP2 in the lower zone.

RhBMP2 loaded multilayer films have also enhanced the proliferation of hMSCs grown on the films after 3 days. Interestingly, only the multilayer films that were unmodified and cross-linked before incorporation of the growth factor have clearly shown this effect, whereas the difference was much lower on the films cross-linked after growth factor loading at low cross-linking rates and completely disappeared at higher levels (>10%) of cross-linking. The decreasing effect of rhBMP2 in the films with covalently immobilized growth factor suggests that other than C2C12 cells, hMSCs grown on the surface rely on the released portion of rhBMP2 and not on the fraction retained inside the film. This is supported by the finding that on the multilayer films with the two zone architecture, only films with rhBMP2 incorporated in the upper zones had shown enhanced hMSC proliferation, as films with rhBMP2 incorporated in the lower zone have only released negligible quantities of rhBMP2. In conjunction with the results of ALP induction, this indicates that rhBMP2 incorporated in the lower zone does not have an effect on the biology of cells grown on the surface as long as they are isolated by an unloaded zone of PLL-Hep multilayers. It may, however, be speculated that the release of growth factors from the lower zone will occur during degradation of the PEM films caused by pH changes[35] in the milieu of the tissue adjacent to the implant surface during periimplant healing.

4.3 | Angiogenic activity

Angiogenic activity as shown by proliferation of HUVECs on the rhVEGF165 loaded surfaces mirrored the induction of osteogenic differentiation to a certain extent in that all surface films that contained rhVEGF165 induced significant enhancement of HUVEC proliferation at day one of release irrespective of cross-linking or dual zone architecture (apart from where rhVEGF165 is in the lower zone). However, in contrast to rhBMP2, the angiogenic effect on day 1 had disappeared after 21 days of release. It thus appears that the clear angiogenic effect at day 1 is accounted for by the portion of loosely attached rhVEGF165 that is released early from the multilayer films. The loss of angiogenic activity after 21 days of release indicates that similar to the proliferation of hMSCs on the rhBMP2 loaded films, rhVEGF165 incorporated deeper in the films may be not be available for the HUVECs grown on the surface of the undegraded films, which may be due to their inability to access them through localized degradation as it has been speculated for the C2C12 cells. The induction of vWF in hMSCs confirmed the angiogenic activity of the released growth factor. Interestingly, the effect has been significantly higher for rhVEGF165 release from PLL-Hep multilayer films than from Ti control surfaces and had been almost abolished with a substantial co-release of rhBMP2. The former effect may be accounted for by a simultaneous release of small amounts of Hep into the supernatant as Hep molecules that are supportive for the signal transduction of VEGF at its receptors.[38] The latter effect may be based on a BMP2 induced switch to the osteogenic lineage with suppression of the angiogenic effect of VEGF.

5 | CONCLUSION

The present results suggest that by modification of cross-linking strategies as well as layer architecture and sequence of growth factor loading of PLL-Hep PEMs, variable release profiles of individual growth factors can be achieved when multiple growth factors are to be employed. Cross-linking using EDC/NHS reduced the biological activity of the released rhBMP2 making it advisable to minimize the amounts used employing direct immobilization of the adsorbed growth factor in the multilayer film. The use of two zones allows for retardation of the growth factor activity in the deeper zone. For rhBMP2 incorporated in the superficial zone, the soluble portion of growth factor released plays a minor role in the osteogenic differentiation of cells growing on the surface of the films whereas the film-bound portion of rhBMP2 appears to provide the support for osteogenic differentiation. For rhVEGF165 functionalization, angiogenic activity is dependent on the released portion of the growth factor.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations

REFERENCES

1. Souza JCM, Sordi MB, Kanazawa M, et al. Nano-scale modification of titanium implant surfaces to enhance osseointegration. *Acta Biomater*. 2019;94:112-131.

2. Wang Q, Zhou P, Liu S, et al. Multi-scale surface treatments of titanium implants for rapid Osseointegration: a review. *Nanomaterials (Basel)*. 2020;10(6):1244. doi:10.3390/nano10061244
3. Meng HW, Chien EY, Chien HH. Dental implant bioactive surface modifications and their effects on osseointegration: a review. *Biomark Res*. 2016;4:24.
4. Schliephake H, Scharnweber D. Chemical and biological functionalization of titanium for dental implants. *J Mater Chem*. 2008;18:2404-2414.
5. Seol YJ, Park YJ, Lee SC, et al. Enhanced osteogenic promotion around dental implants with synthetic binding motif mimicking bone morphogenetic protein (BMP)-2. *J Biomed Mater Res A*. 2006;77(3):599-607.
6. Adden N, Gamble LJ, Castner DG, Hoffmann A, Gross G, Menzel H. Phosphonic acid monolayers for binding of bioactive molecules to titanium surfaces. *Langmuir*. 2006;22(19):8197-8204.
7. Schliephake H, Bötel C, Förster A, Schwenzer B, Reichert J, Scharnweber D. Effect of oligonucleotide mediated immobilization of bone morphogenic proteins on titanium surfaces. *Biomaterials*. 2012;33(5):1315-1322.
8. Gilde F, Maniti O, Guillot R, et al. Secondary structure of rhBMP-2 in a protective biopolymeric carrier material. *Biomacromolecules*. 2012;13(11):3620-3626.
9. Macdonald ML, Rodriguez NM, Shah NJ, Hammond PT. Characterization of tunable FGF-2 releasing polyelectrolyte multilayers. *Biomacromolecules*. 2010;11(8):2053-2059.
10. Anouz R, Repanas A, Schwarz E, Groth T. Novel surface coatings using oxidized Glycosaminoglycans as delivery systems of bone morphogenetic protein 2 (BMP-2) for bone regeneration. *Macromol Biosci*. 2018;18(11):e1800283.
11. Böhrnsen F, Krier M, Grohmann S, et al. MSC differentiation on two-photon polymerized, stiffness and BMP2 modified biological copolymers. *Biomed Mater*. 2019;14(3):035001.
12. Guillot R, Gilde F, Becquart P, et al. The stability of BMP loaded polyelectrolyte multilayer coatings on titanium. *Biomaterials*. 2013;34(23):5737-5746.
13. Guduru D, Niepel MS, Gonzalez-Garcia C, Salmeron-Sanchez M, Groth T. Comparative study of osteogenic activity of multilayers made of synthetic and biogenic polyelectrolytes. *Macromol Biosci*. 2017;17(8). doi:10.1002/mabi.201700078
14. Bayer EA, Gottardi R, Fedorchak MV, Little SR. The scope and sequence of growth factor delivery for vascularized bone tissue regeneration. *J Control Release*. 2015;219:129-140.
15. Ramazanoglu M, Lutz R, Rusche P, et al. Bone response to biomimetic implants delivering BMP-2 and VEGF: an immunohistochemical study. *J Craniomaxillofac Surg*. 2013;41(8):826-835.
16. Ramazanoglu M, Lutz R, Ergun C, von Wilmowsky C, Nkenke E, Schlegel KA. The effect of combined delivery of recombinant human bone morphogenetic protein-2 and recombinant human vascular endothelial growth factor 165 from biomimetic calcium-phosphate-coated implants on osseointegration. *Clin Oral Implants Res*. 2011;22(12):1433-1439.
17. Kim JE, Kang SS, Choi KH, et al. The effect of anodized implants coated with combined rhBMP-2 and recombinant human vascular endothelial growth factors on vertical bone regeneration in the marginal portion of the peri-implant. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2013;115(6):e24-e31.
18. Crouzier T, Ren K, Nicolas C, Roy C, Picart C. Layer-by-layer films as a biomimetic reservoir for rhBMP-2 delivery: controlled differentiation of myoblasts to osteoblasts. *Small*. 2009;5(5):598-608.
19. Bouyer M, Guillot R, Lavaud J, et al. Surface delivery of tunable doses of BMP-2 from an adaptable polymeric scaffold induces volumetric bone regeneration. *Biomaterials*. 2016;104:168-181.
20. Almodóvar J, Bacon S, Gogolski J, Kisiday JD, Kipper MJ. Polysaccharide-based polyelectrolyte multilayer surface coatings can enhance mesenchymal stem cell response to adsorbed growth factors. *Biomacromolecules*. 2010;11(10):2629-2639.
21. Wang HG, Yin TY, Ge SP, et al. Biofunctionalization of titanium surface with multilayer films modified by heparin-VEGF-fibronectin complex to improve endothelial cell proliferation and blood compatibility. *J Biomed Mater Res A*. 2013;101(2):413-420.
22. Ye X, Wang H, Zhou J, et al. The effect of heparin-VEGF multilayer on the biocompatibility of decellularized aortic valve with platelet and endothelial progenitor cells. *PLoS One*. 2013;8(1):e54622.
23. Vallet SD, Clerc O, Ricard-Blum S. Glycosaminoglycan-protein interactions: the first draft of the glycosaminoglycan Interactome. *J Histochem Cytochem*. 2021;69(2):93-104.
24. Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi AH, Kimata K. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. *J Biol Chem*. 2004;279(13):12346-12354.
25. Billings PC, Yang E, Mundy C, Pacifici M. Domains with highest heparan sulfate-binding affinity reside at opposite ends in BMP2/4 versus BMP5/6/7: implications for function. *J Biol Chem*. 2018;293(37):14371-14383.
26. Babik S, Samsonov SA, Pisabarro MT. Computational drill down on FGF1-heparin interactions through methodological evaluation. *Glycoconj J*. 2017;34(3):427-440.
27. Rider CC, Mulloy B. Heparin, Heparan Sulphate and the TGF-β cytokine superfamily. *Molecules*. 2017;22(5):713. doi:10.3390/moleclues220570713
28. Billings PC, Pacifici M. Interactions of signaling proteins, growth factors and other proteins with heparan sulfate: mechanisms and mysteries. *Connect Tissue Res*. 2015;56(4):272-280.
29. Gilde F, Fourel L, Guillot R, et al. Stiffness-dependent cellular internalization of matrix-bound BMP-2 and its relation to Smad and non-Smad signaling. *Acta Biomater*. 2016;46:55-67.
30. Almodóvar J, Place LW, Gogolski J, Erickson K, Kipper MJ. Layer-by-layer assembly of polysaccharide-based polyelectrolyte multilayers: a spectroscopic study of hydrophilicity, composition, and ion pairing. *Biomacromolecules*. 2011;12(7):2755-2765.
31. Park J, McShane MJ. Dual-function nanofilm coatings with diffusion control and protein resistance. *ACS Appl Mater Interfaces*. 2010;2(4):991-997.
32. Crouzier T, Szarpak A, Boudou T, Auzély-Velty R, Picart C. Polysaccharide-blend multilayers containing hyaluronan and heparin as a delivery system for rhBMP-2. *Small*. 2010;6(5):651-662.
33. Lee H. Effects of temperature, salt concentration, and the protonation state on the dynamics and hydrogen-bond interactions of polyelectrolyte multilayers on lipid membranes. *Phys Chem Chem Phys*. 2016;18(9):6691-6700.
34. Salvi C, Lyu X, Peterson AM. Effect of assembly pH on polyelectrolyte multilayer surface properties and BMP-2 release. *Biomacromolecules*. 2016;17(6):1949-1958.
35. Campbell J, Vikulina AS. Layer-by-layer assemblies of biopolymers: build-up, mechanical stability and molecular dynamics. *Polymers (Basel)*. 2020;12(9):1249. doi:10.3390/polym12091949
36. Picart C, Mutterer J, Richert L, et al. Molecular basis for the explanation of the exponential growth of polyelectrolyte multilayers. *Proc Natl Acad Sci U S A*. 2002;99(20):12531-12535.
37. Alkekhia D, Shukla A. Influence of poly-l-lysine molecular weight on antibacterial efficacy in polymer multilayer films. *J Biomed Mater Res A*. 2019;107(6):1324-1339.
38. Wijelath E, Namekata M, Murray J, et al. Multiple mechanisms for exogenous heparin modulation of vascular endothelial growth factor activity. *J Cell Biochem*. 2010;111(2):461-468.
39. Padiolleau L, Chanseau C, Durrieu S, Chevallier P, Laroche G, Durrieu MC. Single or mixed tethered peptides to promote hMSC differentiation toward osteoblastic lineage. *ACS Appl Bio Mater*. 2018;1(6):1800-1809.

40. Arisaka Y, Yui N. Tethered bone morphogenetic protein-2 onto sulfonated-polyrotaxane based surfaces promotes osteogenic differentiation of MC3T3-E1 cells. *J Biomater Sci Polym Ed*. 2017;28(10–12):974-985.

SUPPORTING INFORMATION

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