Functionalization of multilayered DNA-coatings with bone morphogenetic protein 2

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Abstract

The focus of the present study was to functionalize multilayered DNA-coatings with the osteoinductive factor bone morphogenetic protein 2 (BMP-2) using different loading modalities. The multilayered DNA-coatings were built up from either poly-D-lysine (PDL) or poly(allylamine hydrochloride) (PAH) and DNA using electrostatic self-assembly (ESA). The amounts of BMP-2 loaded into the multilayered DNA-coatings and its subsequent release characteristics were determined using radiolabeled BMP-2. Additionally, the effect of BMP-2 functionalized multilayered DNA-coatings on the in vitro behavior of bone marrow-derived osteoblast-like cells was evaluated in terms of proliferation, differentiation, mineralization, and cell morphology. The results demonstrate the feasibility of multilayered DNA-coatings to be functionalized by embedding BMP-2 according to three different loading modalities: superficial (s), deep (d), and double-layer (dl). All differently loaded multilayered DNA-coatings showed an initial burst release followed by an incremental sustained release of the remaining BMP-2. In vitro experiments demonstrated that the loaded factor remained biologically active, as an accelerated calcium deposition was observed on s- and dl-loaded multilayered DNA-coatings, without affecting cell proliferation. In contrast, d-loaded multilayered DNA-coatings influenced osteoblast-like cell behavior by decreasing the deposition of calcium.

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1. Introduction

In the field of implantology, control over tissue responses after the insertion of a biomaterial remains a challenge. Several approaches to modulate tissue responses have been investigated in the past decades, including topographical and/or chemical mod-

ulations at the biomaterial surface. Additionally, biomaterials have been combined with biologically active factors to induce desired tissue responses in the direct vicinity of the implant.

In the early 1990s, a versatile coating technique has been developed [1], known as layer by layer (LbL) assembly or electrostatic self-assembly (ESA). This technique is based on the sequential adsorption of oppositely charged polyelectrolytes into multilayered structures (polyelectrolyte multilayers; PEMs) [2]. Due to the wide choice of polyelectrolytes, this technique allows the generation of coatings with numerous different properties.

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Previously, the application of ESA to fabricate multilayered coatings consisting of DNA and a polycationic counterpart has been described [3–8]. In our previous work, we fabricated multilayered DNA-coatings onto glass and titanium substrates consisting of deoxyribonucleic acid (DNA) as the anionic polyelectrolyte and either poly-d-lysine (PDL) or poly(allylamine hydrochloride) (PAH) as its cationic counterpart [9]. In these studies, in particular DNA was chosen not for its genetic information, but for its beneficial biomaterial properties, which include non- or low-immunogenicity [10–12], capability to incorporate other compounds [13,14], and high phosphate content. In view of these properties, multilayered DNA-coatings are known to be cyto- and histocompatible [15], and proposed to be eligible for drug-delivery and favorable for the deposition of calcium phosphates in a bony environment. Additionally, the complexation of DNA with polycationic polyelectrolytes has demonstrated to prevent DNA from nucleolytic degradation [16], which is indicative for the stability of multilayered DNA-coatings.

For drug-delivery purposes, the use of multilayered coatings can be based on functional components of the (biodegradable) multilayered structure itself [17–20]. On the other hand, functionalization of multilayered coatings can be based on the incorporation of biologically active factors within the basic multilayered structure. Several reports have already demonstrated this approach for the regulation of biological responses, using both in vitro [21–27] and in vivo [28] experiments. In these latter studies, a variety of biologically active factors were used, which were embedded through covalent bonding [21,25,27,28], adsorption [22], or complexation (with or without chaperone molecules) [23,24,26,29]. However, the focus of these studies was to evaluate the potential of functionalized multilayered coatings, in which the biologically active factor was incorporated at a single location in a multilayer architecture. On the contrary, the present study is aimed at the functionalization of multilayered DNA-coatings with the osteoinductive factor bone morphogenetic protein 2 (BMP-2) using different loading modalities. Clinically, BMP-2 could be used to accelerate the process of osseo-integration of orthopedic devices and dental implants. It is hypothesized that the location of the factor in a multilayered coating influences its release profile and affects the behavior of osteoblast-like cells in vitro.

The current study focused on three types of functionalization of multilayered DNA-coatings built up from either poly-d-lysine (PDL) or poly(allylamine hydrochloride) (PAH) as the cationic component and DNA as the anionic component. The amounts of BMP-2 loaded into the multilayered DNA-coatings and its subsequent release characteristics were determined using radiolabeled BMP-2. Subsequently, the effect of BMP-2 functionalized multilayered DNA-coatings on the in vitro behavior of bone marrow-derived osteoblast-like cells was evaluated in terms of proliferation, differentiation, mineralization, and cell morphology.

2. Materials and methods

2.1. Materials

Polyanionic DNA (300 bp/molecule; sodium salt) was kindly provided by the Central Research Laboratory of Nichiro Corporation (Kawasaki-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, IL, US) and measured to be below 0.20% w/w (data not shown). Polycationic polyelectrolytes poly(allylamine hydrochloride) (PAH; MW ∼ 70000) and poly-d-lysine (PDL; MW 30,000–70000) were purchased from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). Recombinant human bone morphogenetic protein 2 (rhBMP-2; MW 32,000; in MFR buffer [containing 0.5% sucrose, 2.5% glycine, 30 mM L-glutamic acid, 0.01% polysorbate 80, pH 4.5]) was generously supplied by Yamanouchi Europe B.V. (Leiderdorp, the Netherlands). All materials were used without further purification.

2.2. Substrate preparation and cleaning

Disc-shaped titanium substrates (diameter 12 mm; as machined) were used. Prior to the fabrication of multilayered DNA-coatings, substrates were cleaned ultrasonically in nitric acid (10% v/v), acetone, and isopropanol, respectively. Subsequently, the substrates were air-dried.

2.3. Generation of multilayered DNA-coatings

Multilayered DNA-coatings were generated using the ESA-technique, as described previously [9]. Briefly, the cleaned substrates were immersed in an aqueous solution of either PDL (0.1 mg/ml) or PAH (1 mg/ml) for 30 min, allowing sufficient time for the adsorption of the first cationic polyelectrolyte layer onto the substrates. Subsequently, the substrates were washed in ultra-pure water (5 min, continuous water flow) and dried using a pressurized air stream. Thereafter, the substrates were alternately immersed in an anionic aqueous DNA solution (1 mg/ml) and the respective cationic polyelectrolyte solution for 7 min each, with intermediate washing in ultra-pure water (5 min, continuous water flow) and drying using a pressurized air stream. The build-up of the multilayered DNA-coatings was continued until a total of 5 double-layers were reached. These coatings were designated either [PDL/DNA]₅ or [PAH/DNA]₅.

2.4. Functionalization of multilayered DNA-coatings

Multilayered DNA-coatings were functionalized with rhBMP-2 according to three different loading modalities (Fig. 1). The loading modalities are designated superficial (s), deep (d), and double-layer (dl), depending on the location of the BMP-2. During the build-up of the multilayered DNA-coatings, rhBMP-2 was loaded at the appropriate location from a rhBMP-2 solution without precipitates (10 μl of a 10 μg/ml rhBMP-2 solution in 0.5% (w/v) BSA/PBS) and allowed to adsorb for 7 min. Unless the rhBMP-2 was applied on top of the multilayered DNA-coatings, substrates were washed in ultra-pure water, after which the build up of the coatings was continued as described above. rhBMP-2 applied on top of the coatings (in case of s-loading and the final loading step in dl-loading) was allowed to dry at room temperature.
2.5. Radioiodination of rhBMP-2

rhBMP-2 was labeled with $^{125}$I according to the iodogen method, as described previously [30]. Briefly, to a 500 µl eppendorf vial containing 100 µg iodogen, 10 µl 0.5 M phosphate buffer (pH 7.4), 80 µl 50 mM phosphate buffer (pH 7.4), 10 µg rhBMP-2 (in 2.6 µl PBS), and 3 µl $^{125}$I (0.3 mCi) were added. The vial was incubated at room temperature for 10 minutes. Subsequently, the quench reaction was initiated by adding 100 µl of a saturated Tyrosine solution in PBS. Finally, the reaction mixture was eluted with 0.5% BSA/PBS on a pre-rinsed disposable Sephadex G25M column (PD-10; Pharmacia, Uppsala, Sweden) to separate labeled rhBMP-2 from free $^{125}$I. To prevent sticking of the rhBMP-2, pipette tips and vials used during the radioiodination procedure were sanitized with SigmaCort® (Sigma).

The radiochemical purity of the $^{125}$I-labeled rhBMP-2 was determined by instant thin-layer chromatography (ITLC) on Gelman ITLC-SG strips (Gelman Laboratories, Ann Arbor, MI, USA) with 0.1 M citrate, pH 5.0 as the mobile phase. The radiochemical purity of the $^{125}$I-labeled rhBMP-2 preparation was 97.3%, which indicates that 97.3% of the $^{125}$I-label was covalently linked to rhBMP-2. The specific activity of the labeled rhBMP-2 was 14.1 µCi/µg.

2.6. Determination of rhBMP-2-loading and in vitro rhBMP-2 release

The amount of rhBMP-2 loaded in the functionalized multilayered DNA-coatings was determined using radiolabeled rhBMP-2. Functionalization was performed as described in Functionalization of multilayered DNA-coatings with the exception that radiolabeled rhBMP-2 was used. For each type of coating, three substrates ($n=3$) were coated with a functionalized multilayered DNA-coating.

The loaded amount of rhBMP-2 was determined by measuring activity of the experimental substrates in a shielded well-type gamma counter (Wizard, Pharmacia-LKB, Sweden). The amount of gamma radiation from the deep (d-functionalization) and double-layer (dl-functionalization) loaded multilayered DNA-coatings was calculated using that of superficially loaded (s-functionalization) multilayered DNA-coatings, which was artificially set at 100 ng.

To study the in vitro release characteristics of rhBMP-2, the substrates modified with a type of functionalized multilayered DNA-coating ($n=3$) were placed separately in 10 ml glass vials containing 4 ml PBS, and incubated statically at 37 °C for up to 8 weeks. At selected time points (4 h, 1, 7, 14, 22, 28, 42, and 56 days) the samples were carefully transferred into new vials containing fresh PBS. Subsequently, the activity on the substrates was measured in a gamma counter. Standards were counted simultaneously to correct for radioactive decay.

2.7. In vitro experiments

2.7.1. Experimental groups

For cell culture experiments, 7 experimental groups were used, based on coating composition and loading modality:

1. [PDL/DNA]$_s$-s (superficial)
2. [PDL/DNA]$_d$-d (deep)
3. [PDL/DNA]$_s$-dl (double-layer)
4. [PAH/DNA]$_s$-s (superficial)
5. [PAH/DNA]$_d$-d (deep)
6. [PAH/DNA]$_s$-dl (double-layer)
7. control (non-coated titanium)

The use of a control consisting of non-coated titanium is justified as previous in vitro experiments have demonstrated that osteoblast-like cells behave similar on non-coated titanium compared to both [PDL/DNA]$_s$ and [PAH/DNA]$_s$ multilayered coatings with respect to cell proliferation, differentiation, mineralization, and morphology (unpublished data).

All substrates, coated with either type of multilayered DNA-coating or non-coated control substrates, were sterilized using a UV-irradiation treatment (254 nm; 4 h). The entire cell culture experiment was performed in two independent runs, using rat bone marrow cells from one rat per experimental run.

2.7.2. Isolation and pre-culture of rat bone marrow cells

Rat bone marrow (RBM) cells were isolated and cultured according to the method adapted from Maniotopoulos et al. [31]. Briefly, the femora of male Wistar WU rats were retrieved, cleaned, and epithyses were cut off. The marrow was flushed out of the remaining diaphyses using cell culture medium (α-MEM (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 50 µg/ml ascorbic acid (Sigma), 10 mM Na-β-glycerophosphate (Sigma), 10⁻⁸ M dexamethasone (Sigma), and 50 µg/ml gentamycin (Gibco)). RBM cells of two femora were cultured under static conditions in cell culture medium in three 75 cm² culture flasks (Greiner Bio-One) for 1 day, after which the medium was refreshed to remove non-adherent cells. Subsequently, the attached cells were pre-cultured for another 6 days.

After the primary culture of 7 days to obtain osteoblast-like cells, cells were detached using trypsin/EDTA (0.25% (w/v) trypsin, 0.02% (w/v) EDTA) and the total cell number was determined using a Coulter® counter (Beckman Coulter Inc., Fullerton, CA, USA).
USA). Finally, cells were seeded at a density of $1 \times 10^4$ cells/cm$^2$ onto the experimental substrates, which were placed in a 24-wells plate (Greiner Bio-One). Cell culture medium was refreshed 1 day after cell seeding, and thereafter 3 times per week.

2.7.3. Cell proliferation

Cell proliferation curves were made based on a total cellular protein measurement. At 4, 8, 12, and 16 days post-seeding, the medium was removed and the cells were washed with PBS three times. Subsequently, the experimental substrates with attached cells were transferred into fresh 24-wells plates, and each experimental substrate was immersed in 1 ml ultra-pure water. These samples were frozen and thawed for 3 repetitive cycles, after which the cellular protein content in the aqueous samples was analyzed using a micro BCA protein assay (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer. In each experimental run, three samples per time point for each experimental condition ($n=3$) were used to ensure reproducibility.

2.7.4. Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity of the osteoblast-like cells was measured as a marker for early differentiation of osteoblast-like cells using the aqueous samples of the proliferation assay according to a previously described method [32]. A volume of 80 μl of sample or standard and 20 μl of buffer solution (5 mM MgCl$_2$, 0.5 M 2-amino-2-methyl-1-propanol) was pipetted into a 96-wells plate (Greiner Bio-One) in duplo, and 100 μl of substrate solution (5 mM p-nitro-phenyl-phosphate) was added per well. Subsequently, the plate was incubated for 1 h at 37 °C, after which the reaction was stopped by adding 100 μl 0.3 M NaOH. Serial dilution of 4-nitrophenol (final concentrations 0–25 nM) were used for the standard curve. The plate was read in an ELISA reader at 405 nm. In each experimental run, three samples per time point for each experimental condition ($n=3$) were used.

2.7.5. Calcium deposition

The deposition of calcium was used as a marker of late differentiation of osteoblast-like cells. The amount of calcium deposited after 4, 8, 12, 16, and 24 days of cell culture was measured by the orthocresolphtalein complexone (OCPC) method (Sigma), as described previously [33]. Briefly, the experimental substrates were washed twice using PBS, after which 1 ml 0.5 N acetic acid was added. After overnight incubation on a shaking apparatus, 300 μl working solution was added to 10 μl sample in a 96-wells plate (Greiner Bio-One). Working solution consisted of (a) OCPC solution (80 mg OCPC in 75 ml milliQ+0.5 ml 1 M KOH+0.5 ml 0.5 N acetic acid), (b) 14.8 M ethanolamine/boric acid buffer (pH=11), (c) 8-hydroxyquinoline (1 g in 20 ml 95% ethanol), and (d) milliQ, in a ratio of 5:5:2:88 (a/b/c/d). A standard curve was generated by preparing serial dilutions of CaCl$_2$ (0–100 μg/ml). For each experimental run, the calcium assay was performed using 3 substrates per experimental condition at each time point ($n=3$).

2.7.6. Cell morphology

Scanning electron microscopy (SEM) was performed to evaluate the morphological appearance of the cells. At 4 and 16 days post-seeding, substrates with attached cells were

![Fig. 2. Incorporation of rhBMP-2 into multilayered DNA-coatings. The amount of gamma radiation of s-loaded coatings was artificially set at 100 ng, after which d- and dl-loading were calculated accordingly. Results are presented as mean±S.D. ($n=3$). (Loading efficiencies are 100%, 15%, and 32% for s-, d-, and dl-loading, respectively.)](image)

![Fig. 3. In vitro release of rhBMP-2 from differently loaded multilayered DNA-coatings after immersion in PBS. (A) Release characteristics of [PDL/DNA]-based multilayered DNA-coatings. (B) Release characteristics of [PAH/DNA]-based multilayered DNA-coatings (s=superficial, d=deep, dl=double-layer). Results are presented as mean±S.D. ($n=3$).](image)

<table>
<thead>
<tr>
<th>Coating architecture</th>
<th>Burst release (ng) (0–24 h)</th>
<th>Sustained release (ng/week) (1–56 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PDL/DNA]-s</td>
<td>75.8 (75.8%)</td>
<td>1.6 (6.7%)</td>
</tr>
<tr>
<td>[PDL/DNA]-d</td>
<td>7.4 (47.6%)</td>
<td>0.5 (5.8%)</td>
</tr>
<tr>
<td>[PDL/DNA]-dl</td>
<td>105.1 (65.0%)</td>
<td>3.8 (6.6%)</td>
</tr>
<tr>
<td>[PAH/DNA]-s</td>
<td>61.3 (61.3%)</td>
<td>3.1 (8.1%)</td>
</tr>
<tr>
<td>[PAH/DNA]-d</td>
<td>4.7 (34.8%)</td>
<td>0.5 (6.3%)</td>
</tr>
<tr>
<td>[PAH/DNA]-dl</td>
<td>121.1 (73.7%)</td>
<td>3.0 (7.0%)</td>
</tr>
</tbody>
</table>
washed twice with PBS and fixed using gluteraldehyde (4% in 0.1 M cacodylate buffer) for 20 min. Subsequently, the substrates were washed twice with 0.1 M cacodylate buffer and dehydrated in a graded series of ethanol. Finally, the substrates were dried with tetramethylsilane, sputter coated with gold and examined using a JEOL 6310 SEM at an acceleration voltage of 10 kV.

2.8. Statistical analysis

Measurements were statistically evaluated with Graphpad® Instat 3.05 software (GraphPad Software Inc., San Diego, CA, USA). Data of the in vitro release experiment and the cell culture experiments were analyzed using a one-way ANOVA, combined with a post-hoc Tukey–Kramer Multiple Comparisons test. The significance level was set at $p<0.05$.

3. Results

3.1. Loading of multilayered DNA-coatings with rhBMP-2

The amounts of rhBMP-2 loaded into the multilayered DNA-coatings are presented in Fig. 2. The results demonstrate that the amount of rhBMP-2 incorporated into the differently loaded multilayered DNA-coatings was highest with dl-loading ($\pm 160$ ng $\approx 140$ ng/cm$^2$), intermediate with s-loading ($100$ ng $\approx 88$ ng/cm$^2$), and lowest with d-loading ($\pm 15$ ng $\approx 13$ ng/cm$^2$). No statistically significant differences were observed between [PDL/DNA]-based coatings and [PAH/DNA]-based coatings. The loading efficiency was 100% for s-loading, 15% for d-loading, and 32% for dl-loading.

Fig. 5. Alkaline phosphatase (ALP) activity of bone marrow-derived osteoblast-like cells on differently loaded multilayered (A) [PDL/DNA]- and (B) [PAH/DNA]-coatings (*$p<0.05$ compared to controls).

Fig. 6. Mineralization (calcium deposition) by bone marrow-derived osteoblast-like cells on differently loaded multilayered (A) [PDL/DNA]- and (B) [PAH/DNA]-coatings (*$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to controls).
3.2. In vitro release of rhBMP-2 from multilayered DNA-coatings

The in vitro release characteristics of rhBMP-2 from multilayered DNA-coatings were determined using radiolabeled rhBMP-2. In Fig. 3A and B, the cumulative release of rhBMP-2 out of the differently loaded multilayered DNA-coatings is depicted. All differently loaded multilayered DNA-coatings revealed an initial burst release within the first 24 h of incubation in PBS, ranging from 35% to 75% of the initially loaded amount rhBMP-2. Proportionally, the burst release was low for d-loaded (47.6% for [PDL/DNA]-based and 34.8% for [PAH/DNA]-based multilayered DNA-coatings) and high for both s- and dl-loaded multilayered DNA-coatings (>60%). After the burst release, all differently loaded multilayered DNA-coatings showed an incremental sustained release, in which a continuous fraction of approximately 6–8% of the remaining rhBMP-2 was released in each week (Table 1). After an incubation period of 8 weeks, the cumulative rhBMP-2 release of the d-loaded multilayered DNA-coatings approximated 70%, whereas both the s-loaded and the dl-loaded DNA-coatings released approximately 85%, cumulatively. Statistical analysis revealed that actual cumulative amounts of released rhBMP-2 were highest for dl-loading, intermediate for s-loading, and lowest for d-loading. No statistically significant

![Fig. 7. Scanning electron microscopy images of bone marrow-derived osteoblast-like cells after 16 days of culture on differently loaded multilayered DNA-coatings. (A) Non-coated control, (B) [PAH/DNA]5-s, (C) [PAH/DNA]5-d, and (D) [PAH/DNA]5-dl. Note the difference in globular accretions at the high magnification images (E–H).](image-url)
differences were observed between equivalently functionalized coatings based on either [PDL/DNA] or [PAH/DNA] regarding release characteristics in percentage terms \((p > 0.05)\).

3.3. Osteoblast-like cell behavior on rhBMP-2-loaded multilayered DNA-coatings

The behavior of osteoblast-like cells on the differently loaded multilayered DNA-coatings was evaluated to detect biological activity of the incorporated rhBMP-2.

3.3.1. Cell proliferation

The proliferation of osteoblast-like cells, based on total cellular protein content measurements, is depicted in Fig. 4. Osteoblast-like cells showed a similar proliferation pattern on all types of differently loaded multilayered DNA-coatings and non-coated control substrates. After cell seeding, osteoblast-like cells started proliferating, reaching a maximum around day 12, after which a decrease was observed. Somewhat lower, but significantly different levels of cellular protein content were observed in both types of d-loaded multilayered DNA-coatings on day 12 ([PDL/DNA]5-d vs. control, \(p < 0.01\); [PAH/DNA]5-d vs. control, \(p < 0.05\)) after 16 days of osteoblast-like cell culture, no significant different levels were observed between both types of d-loaded multilayered DNA-coatings and controls \((p > 0.05)\).

3.3.2. Alkaline phosphatase activity

Osteoblast-like cells showed a normal ALP-activity expression on all experimental substrates. The ALP-activity increased on all experimental substrates during the first 12 days of culture, after which a rapid decrease in ALP-activity was observed (Fig. 5). Significant differences compared to controls \((p < 0.05)\) were observed on day 12 for both types of d-loaded multilayered DNA-coatings.

3.3.3. Calcium deposition

The deposition of a mineralized extracellular matrix by osteoblast-like cells was determined by measuring the amounts of calcium deposited on the experimental substrates during cell culture (Fig. 6). An accelerated calcium deposition by osteoblast-like cells was observed on s- and dl-loaded multilayered DNA-coatings compared to non-coated controls. On day 12, osteoblast-like cells on s- and dl-loaded multilayered DNA-coatings had deposited significantly increased amounts of calcium compared to non-coated controls \((p < 0.001)\). On the other hand, d-loaded multilayered DNA-coatings demonstrated to decrease calcium deposition by osteoblast-like cells. Significantly decreased amounts of deposited calcium were observed on these types of functionalized multilayered DNA-coatings on days 16 and 24 \((p < 0.001)\).

3.3.4. Cell morphology

The morphological appearance of the osteoblast-like cells cultured on the differently loaded multilayered DNA-coatings was evaluated using scanning electron microscopy. At day 4, all differently loaded multilayered DNA-coatings and non-coated controls were covered with a layer of osteoblast-like cells. No apparent differences in cell morphology were observed. In contrast, at day 16 d-loaded multilayered DNA-coatings showed an aberrant morphological appearance of osteoblast-like cells compared to all other experimental groups (Fig. 7). Many calcified globular accretions associated with collagen bundles were present on s-, and dl-loaded multilayered DNA-coatings, and non-coated controls. Less characteristics of mineralization were observed on d-loaded multilayered DNA-coatings.

4. Discussion

The functionalization of biomaterial coatings with biologically active factors is of interest, as localized drug delivery reduces systemic side effects and increases drug efficiency. In view of this, the functionalization of polyelectrolyte multilayers offers increased application due to the possibility of generating ESA-based coatings onto many different materials used for both soft and hard tissue implants without restrictions regarding geometry. The aim of the current study was to explore the potential of multilayered DNA-coatings, based on [PDL/DNA] and [PAH/DNA] double-layers, for functionalization with the osteoinductive factor BMP-2. To that end, three different modalities of BMP-loading were chosen, based on the location of BMP-2 in the multilayered DNA-coating (i.e. superficial, deep, and double-layer). The incorporation and release of BMP-2 were quantitatively assessed using radiolabeled BMP-2. Additionally, functionalized multilayered DNA-coatings were used in cell culture experiments to assess their biological effects on rat bone marrow-derived osteoblast-like cells. The results demonstrated the feasibility of multilayered DNA-coatings to be functionalized with BMP-2 according to all three loading modalities, and that the loading and release characteristics are independent of the cationic counterpart of DNA in the multilayered coating (i.e. PDL or PAH). All types of functionalized multilayered DNA-coatings showed an initial bulk release within 24 h followed by an incremental sustained release. The in vitro experiments with osteoblast-like cells revealed that the loaded BMP-2 remained biologically active. Superficial and double-layer loading resulted in accelerated calcium deposition, whereas deep loading decreased the deposition of calcium by osteoblast-like cells.

The use of multilayered coatings to modulate cell/tissue responses has been described previously and was based on the functionality of basic multilayered structure components (i.e. polyelectrolytes) [17–20] or additional biologically active components [21–26,28]. In the present study, BMP-2 was incorporated as an additional component in multilayered coatings, which consisted of either PDL or PAH as the cationic counterpart of anionic DNA. These coatings demonstrated to contain approximately 3 μg DNA per double-layer per square centimeter, irrespective of the cationic polyelectrolyte used [9]. Recombinant human BMP-2 was incorporated after the adsorption of a negatively charged DNA-layer, as the isoelectric point of BMP-2 is 8.5, which indicates a positive charge of BMP-2 under physiological conditions [34]. However, due to the presence of (negatively charged) BSA in the BMP-2 solution, interference of electrostatic interactions between the
BMP-2 and DNA is likely. Additionally, the interaction of BSA with BMP-2 potentially results in the incorporation of BSA–BMP-2 complexes rather than pure BMP-2, which could also affect its release.

Functionalization of multilayered DNA-coatings according to the chosen loading modalities (Fig. 1) demonstrated that the differently loaded multilayered DNA-coatings bear the following proportion to one another: s +(4 × d)−d. From this proportion, it becomes evident that constant amounts of BMP-2 of approximately 15 ng can be incorporated into the coatings after a DNA-layer in case of subsequent wash steps. Additionally, this proportion indicates that the build-up of the multilayered DNA-coatings is not affected by the incorporation of factor. In view of this, the continuous build-up of multilayered coatings with adsorbed factors has already been demonstrated previously using Optical Waveguide Lightmode Spectrometry [22]. Calculations on the loading of multilayered DNA-coatings correspond to a BMP-2 incorporation of ~ 13 ng/cm² (1 μg DNA immobilizes ~ 4.5 ng BMP-2), which are well in line with incorporation efficiencies of other factors into other types of multilayers. For instance, brain-derived neurotrophic factor (BDNF) could be incorporated through adsorption to an amount of 96.4 ng/cm² into [PSS/PAH]-based multilayers [22]. Similarly, the chemorepulsive protein Semaphorin 3A could be incorporated to an amount of 25.4 ng/cm² into identical multilayer structures [22]. Both BDNF and Semaphorin 3A were adsorbed after polystyrene sulphonate in [PSS/PAH]-based multilayers. Although these studies as well as the present study show loading efficiencies of tens of nanograms per square centimeter, it has to be emphasized that due to differences in the properties of the multilayer components, the factors used for functionalization, and the conditions during multilayer build-up (e.g. salt concentration, pH, etc.), the actual loading efficiency through adsorption is likely to be influenced. Additionally, an increase in the amount of functionalization factor can be achieved using several approaches. The most feasible one is to increase the number of double-layers in a multilayer structure, which will increase the total amount of biologically active factor and has been demonstrated to be effective [35]. Another approach is the complexation of the biologically active factor with chaperone molecules. This method has demonstrated to be applicable for anti-inflammatory agents, including piroxicam [23] and a lipid A-antagonist [24], and increases the loading efficiency without affecting the biological activity of the factor. Other approaches include covalent bonding of factors to polyelectrolytes [21,25,28] prior to the generation of multilayered structures. Although this last method seems preferable in terms of controlling the loaded amount of factor, the biological activity of the factor might be affected by covalent bonding. For instance, covalent bonding might change the native structure of the factor, which impairs the availability of functional epitopes for cell surface receptors. Additionally, covalent bonding only allows release of the factor in case of degradation of the multilayered coating, which might limit its availability in the peri-implant surroundings. This has been demonstrated previously by Jessel et al. [21], who showed a reduced biological activity of a covalently bound factor underneath poly-D-lysine-containing compared to biodegradable poly-L-lysine-containing double-layers. Consequently, covalent bonding of factors in an effort to introduce biological activity into multilayered coatings is only functional if (i) the polyelectrolytes are biodegradable, (ii) the factor is located such, that it is available for cells via cellular membrane extension, or (iii) a combination of both.

For bone tissue engineering approaches, in which BMP-2 is the most powerful osteoinductive factor to be used [36], it is important to tailor the availability of the osteoinductive factor in such a way that the behavior of mesenchymal stem cells is controlled in terms of migration, differentiation, and secretion of extracellular matrix. The current study showed that the BMP-2 release pattern of the differently loaded multilayered DNA-coatings consisted of two phases: (i) a bulk release of 35–75% within 24 h, and (ii) an incremental sustained release of approximately 6–8% of the remaining BMP-2 weekly for (at least) 8 weeks. The bulk release might be due to differences in the composition of the solutions used during multilayer build-up and the release medium, which contained ionic species that are likely to affect electrostatic interactions. Bulk release was lowest for both types of d-loaded multilayered DNA-coatings. This is most likely due to the absence of dropcast BMP-2 on top of these multilayered DNA-coatings and increased interactions of BMP-2 with the polyelectrolytes in the coating. Different BMP-2 release profiles have been reported by others using different carriers. For instance, ceramics have demonstrated to bind BMP-2 with high affinity, resulting in a limited release [37,38]. On the other hand, biodegradable scaffolds consisting of either polycaprolactone (PCL) or a composite of PCL with calcium phosphate showed BMP-2 release profiles in which 100% release was established within 3 weeks [39]. The specific requirements for BMP-2 to be effective in vivo, however, remain unclear, since both release profiles induced osteogenic differentiation in vivo and in vitro, respectively.

The bioactivity of the BMP-2 loaded into the multilayered DNA-coatings was assessed using an in vitro cell culture model with rat bone marrow-derived osteoblasts. The results indicate the presence of a threshold for the osteoinductive capacity of BMP-2, as only functionalized multilayered DNA-coatings containing relatively high amounts of BMP-2 (s- and dl-loaded coatings) accelerated the deposition of calcium by osteoblast-like cells. Although the actual comparative fraction of biologically active BMP-2 under the different loading modalities is unknown, these results corroborate those obtained by van den Dolder et al. [40] and Vehof et al. [41]. In their studies, in which rat bone marrow-derived osteoblast-like cells were cultured in medium to which 0, 10, 100, or 1000 ng/ml BMP-2 was supplemented, effects of the supplemented BMP-2 became apparent from 100 ng/ml. A remarkable difference, however, is the altered behavior of osteoblast-like cells at low BMP-2 concentrations. Whereas the supplementation of 10 ng/ml BMP-2 to the culture medium did not affect osteoblast-like cell behavior in terms of proliferation and ALP expression [40,41], in our study osteoblast-like cells on d-loaded multilayered DNA-coatings proliferated and differentiated to a lesser extent compared to the control substrates. Additionally, the deposition of calcium was significantly decreased compared to controls.
Although no elusive explanation is available currently for this observation in osteoblast-like cell cultures with sub-threshold BMP-2 concentrations, it can be speculated that the amount and the temporal and spatial availability of factor are causal issues in the ultimate effect of factors.

5. Conclusion

This study demonstrates the feasibility of multilayered DNA-coatings to be functionalized by embedding BMP-2 according to three different loading modalities: superficial (s), deep (d), and double-layer (dl). BMP-2 was incorporated proportionally into the multilayered DNA-coatings as: s+(4*d)=dl. The release profiles of all differently loaded multilayered DNA-coatings showed an initial burst release, followed by a sustained release of the remaining BMP-2 for (at least) 8 weeks. In vitro culture experiments with rat bone marrow-derived osteoblast-like cells demonstrated that the loaded factor remained biologically active, as an accelerated calcium deposition was observed on s- and dl-loaded multilayered DNA-coatings, without affecting cell proliferation. In contrast, d-loaded multilayered DNA-coatings influenced the behavior of osteoblast-like cells by decreasing the deposition of calcium.

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