Layer-By-Layer Films as a Biomimetic Reservoir for rhBMP-2 Delivery: Controlled Differentiation of Myoblasts to Osteoblasts**

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Efficient delivery of growth or survival factors to cells is one of the most important long-term challenges of current cell-based tissue engineering strategies. The extracellular matrix acts as a reservoir for a number of growth factors through interactions with its components. In the matrix, growth factors are protected against circulating proteases and locally concentrated. Thus, the localized and long-lasting delivery of a matrix-bound recombinant human bone morphogenetic protein 2 (rhBMP-2) from a biomaterial surface would mimic in vivo conditions and increase BMP-2 efficiency by limiting its degradation. Herein, it is shown that crosslinked poly(L-lysine)/ hyaluronan (HA) layer-by-layer films can serve as a reservoir for rhBMP-2 delivery to myoblasts and induce their differentiation into osteoblasts in a dose-dependent manner. The amount of rhBMP-2 loaded in the films is controlled by varying the deposition conditions and the film thickness. Its local concentration in the film is increased up to ≈500-fold when compared to its initial solution concentration. Its adsorption on the films, as well as its diffusion within the films, is evidenced by microfluorimetry and confocal microscopy observations. A direct interaction of rhBMP-2 with HA is demonstrated by size-exclusion chromatography, which could be at the origin of the rhBMP-2 "trapping" in the film and of its low release from the films. The bioactivity of rhBMP-2-loaded films is due neither to film degradation nor to rhBMP-2 release. The rhBMP-2-containing films are extremely resistant and could sustain three successive culture sequences while remaining bioactive, thus confirming the important and protective effect of rhBMP-2 immobilization. These films may find applications in the local delivery of immobilized growth factors for tissue-engineered constructs and for metallic biomaterial surfaces, as they can be deposited on a wide range of substrates with different shapes, sizes, and composition.

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1. Introduction

The ability to design surfaces that can direct cell fate is an important challenge in the field of tissue engineering and biomaterials.[1] Besides the bulk properties of a biomaterial or engineered tissue, which will give its mechanical properties and strength, the surface properties often dictate the direct interactions of the material with its environment, in particular cellular interactions.[2,3] Thus, functionalization of surfaces of different types of materials of various shapes, such as

prosthetic bones, vascular implants, or polymeric particles, has attracted considerable interest in the development of biomaterials.[4] In particular, the controlled delivery of growth factors from a biomaterial surface would offer the potential to concentrate the growth factor and deliver it locally, in contrast to a topical administration, thereby also protecting it from degradation by enzymes in tissue fluids. The transforming growth factor (TGF) family of proteins plays an essential role in bone formation through the regulation of osteoprogenitor and osteoblast proliferation and differentiation,[5] in the differentiation of cardiac progenitor cells,[6] and in the epithelial–mesenchymal transition.[7] Bone morphogenetic protein 2 (BMP-2), a member of the TGF family, stimulates differentiation of mesenchymal stem cells[8] and C2C12 myoblast cells toward an osteoblastic lineage,[9,10] when added to the culture medium.

BMP-2 belongs to the family of basic growth factors: it is characterized by a high isoelectric point of 8.5,[11] a poor solubility in physiological medium,[12] and is only active in its dimeric form. Recombinant human BMP-2 (rhBMP-2) is already used as a therapeutic protein for inducing bone growth in the reconstruction of osseous defects, but is more effective when delivered associated with a matrix.[13] Several publications have described the use of rhBMP-2 in the "bulk" of three main types of matrices:[14] biological materials, such as collagen gels (already used clinically);[15,16] inorganic materials, such as hydroxyapatite;[17] and synthetic polymers, such as polylactic acid, polylactic–polyglycolic acid copolymers,[18] or polyethylene glycol hydrogels.[19] Usually, the entire matrix is soaked in a rhBMP-2 solution prior to being introduced in vivo[11,15] or rhBMP-2 is noncovalently incorporated prior to gel polymerization.[20,21] More specifically for rhBMP-2, it is known that a localized and sustained delivery is the most appropriate method for obtaining an optimal efficacy in vivo.[13]

Presenting osteogenic growth factors in a surface-associated fashion may better mimic the native physiology and improve their effective delivery.[2] Indeed, association of many growth factors, including BMP-2, with extracellular matrix components is commonly present in vivo and greatly affects delivery.[22,23] Matrix immobilization allows a spatiotemporal regulation of BMP-2 concentration and contributes to its protection against proteases.[24] In addition, not only porous matrices but also nonporous metal implants, such as titanium or stainless steel[25] used for dental and orthopedic applications,[26] could benefit from a method allowing local delivery from the surface. The difficulty resides in designing a coating that can retain and locally concentrate growth factors, possibly in a tunable amount, and limit the initial burst release that often occurs with gels.[21,27] An attempt consisted in incorporating rhBMP-2 in a thick calcium phosphate-coated titanium surface (thickness of the coating 50 μm), but results were better when rhBMP-2 was simply adsorbed on it.[25]

The layer-by-layer (LbL) technique appears to be an alternative strategy for such an application because it allows for the precise control of various parameters, such as film architecture,[28,29] thickness, chemistry, and mechanical properties.[30,31] As the deposition is achieved in aqueous solution, incorporation of sensitive biomolecules and biopolymers, such as proteins and DNA, is possible.[29] Indeed, protein adsorbed

or embedded in the films, such as protein A[32] and brain-derived neurotrophic factor (BDNF),[33] have been shown to retain their activity, as well as peptides covalently coupled to one of the polyelectrolytes.[34,35] Cellular processes, such as adhesion, proliferation,[31] expression of transfected constructs,[36] or more recently differentiation,[37] can be controlled depending on the film composition and properties. Using the LbL technique, Jansen et al. embedded and/or adsorbed BMP-2 in poly(L-lysine)/DNA and poly(allylamine hydrochloride)/DNA films,[38] but the effects on bone-marrow-derived osteoblast-like cells were very limited and only comparable to those of control bare titanium surfaces. Jessel et al. showed that rhBMP-2 and TGFβ_1 embedded in a multilayered architecture containing β-cyclodextrins can synergistically induce embryonic stem cells within embryoid bodies to differentiate in cartilage and bone.[39] However, the concentration of rhBMP-2 in the film was not controlled or quantified and the mechanism of action was not elucidated.

In this study, we investigated the in vitro potential of LbL films as a delivery reservoir for rhBMP-2. Poly(L-lysine)/hyaluronan (PLL/HA) films, which are characterized by their exponential growth[40] and are known to be cell adhesive when crosslinked,[41] were chosen for this purpose due to their reservoir potential (thickness 1 μm or more). Our strategy was to build a reservoir and to load it with the bioactive molecule (rhBMP-2) instead of using rhBMP-2 as a film component.[38] Herein, we demonstrate that the amount of rhBMP-2 loaded in LbL films can be modulated over a large range by varying the film thickness and/or the initial BMP-2 concentration in the suspending medium. We further show that rhBMP-2 retains its bioactivity and induces the tunable differentiation of myoblasts in osteoblasts through contact with the surface-adsorbed rhBMP-2, without being significantly released from the film over several days in culture.

2. Results and Discussion

2.1. Adsorption of rhBMP-2 and Initial Release in a Physiological Buffer

For this purpose, rhBMP-2^{CF} (labeled with carboxyfluorescein) was employed and measurements were performed by
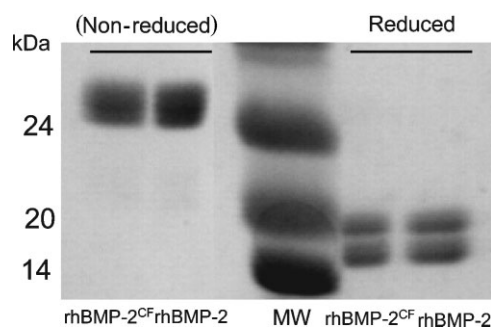

Figure 1. Gel electrophoresis of fluorescently labeled rhBMP-2 dimer (first line, rhBMP-2^{CF}) and rhBMP-2 dimer (second line) prior to reduction as well as after reduction by a DTT solution (fourth and fifth lines). The MW markers are also indicated (central column).

microfluorimetry in 96-well plates, which allowed working with only small amounts of rhBMP-2 in each well and screening of several conditions in a single experiment. To verify the labeling quality of rhBMP-2, we submitted rhBMP-2 and rhBMP-2^{CF} to gel electrophoresis (Figure 1). In nonreducing conditions, both rhBMP-2 and rhBMP-2^{CF} were found in the dimeric form (molecular weight, MW, of ≈27 kDa), which is known to be the active form.[42] In reducing conditions (in the presence of dithiothreitol (DTT)), both rhBMP-2 and rhBMP-2^{CF} were dissociated into monomers after gel electrophoresis. Also, exposure of the gel to UV light revealed fluorescence of the rhBMP-2^{CF} bands (not shown). This result indicated that grafting of the fluorophore onto rhBMP-2 was successful and did not affect its dimeric structure.

In a previous work, we examined the influence of the extent of crosslinking (i.e., 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) concentration) on the differentiation of myoblasts into myotubes, which is the regular differentiation pathway for the C2C12 myoblast cell,[43] and found that films crosslinked at EDC levels higher than 50 mg mL^{-1} allowed a long-term differentiation in myotubes.[44] Thus, PLL/HA films crosslinked at 50 mg mL^{-1} were chosen as a reference in the present study as these films allow myoblast adhesion, proliferation, and differentiation into myotubes.[44] We first established the experimental conditions for rhBMP-2 loading in crosslinked PLL/HA films by quantifying, for a fixed concentration of rhBMP-2 deposited onto the films (20 µg mL^{-1}), how the adsorbed amount varied with pH (for pH 3 and 7.4) and ionic strength (from 0 to 250 mM NaCl), parameters that are known to greatly affect rhBMP-2 solubility[15] (Figure 2A,B).

Maximum loading was reached in about 1 h (Figure S1 in the Supporting Information), exhibited only minor dependence on pH, and decreased with increasing ionic strength. The maximum loading was reached at low ionic strength (below 50 mM NaCl). Release in a physiological buffer was first investigated, as many matrices often exhibit a "burst release" after loading.[13] The lowest proportion of material released was obtained when rhBMP-2 was adsorbed at pH 3 without salt (less than 20% release when the initial BMP-2 concentrations were below 10 µg mL^{-1}; Table 1). We also noticed that, by deposition at pH 3 without salt, a more homogeneous layer could be visualized by confocal laser scanning microscopy (CLSM) compared to deposition at pH 7.4 with 0.15 M NaCl (Figure 2C,D). The
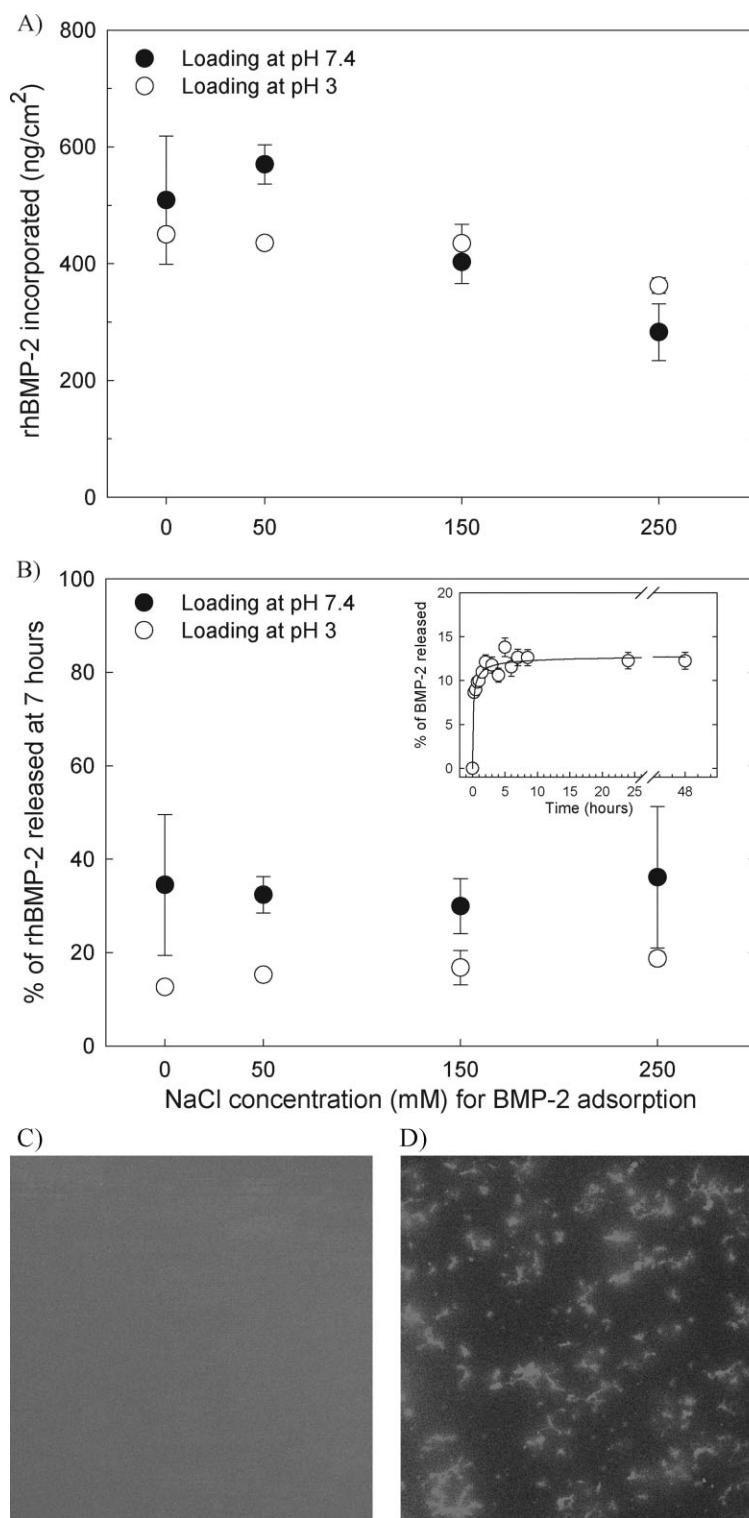
Figure 2. A) Incorporated amounts of rhBMP-2 expressed in ng cm^{-2} and B) percentage of release of the incorporated amount as a function of pH and ionic strength of the initial BMP-2 solution at 7 h of release, after which a plateau was reached (see inset in (B) for release after adsorption in 1 mM HCl without salt). Release was performed in HEPES/NaCl buffer at pH 7.4. In all cases, the initial solution concentration of BMP-2^{CF} was 20 µg mL^{-1}. C,D) CLSM images showing the difference of rhBMP-2^{Rhod} layer homogeneity when deposited at C) pH 3 or D) pH 7.4 (HEPES/50 mM NaCl, corresponding to the maximum incorporated amount in (A); image size: 143 × 143 µm^2). Images were acquired in a HEPES/NaCl buffer (pH 7.4, 0.15 M NaCl) after the rinsing steps. HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Rhod = rhodamine.

Table 1. Summary of BMP-2 loading into crosslinked (PLL/HA)$_{12}$ films (1 µm in thickness). The initial adsorbed amounts were measured by microfluorimetry directly after loading from BMP-2 solutions at different concentrations in HCl (1 mM, pH 3) and release in HEPES/NaCl (0.15 M, pH 7.4). As a plateau in cumulative release was observed after about 7 h (see Figure S2), the percentage of BMP-2 released is given at this time. The corresponding "effective" incorporated amount is thus taken as the amount retained in the film after 7 h in the rinsing buffer. The fold increase of the BMP-2 volume concentration of adsorbed BMP-2 (the volume concentration in the film being calculated by dividing the adsorbed amount by the film thickness, i.e., 1 µm), as compared to its initial bulk concentration, is also given.
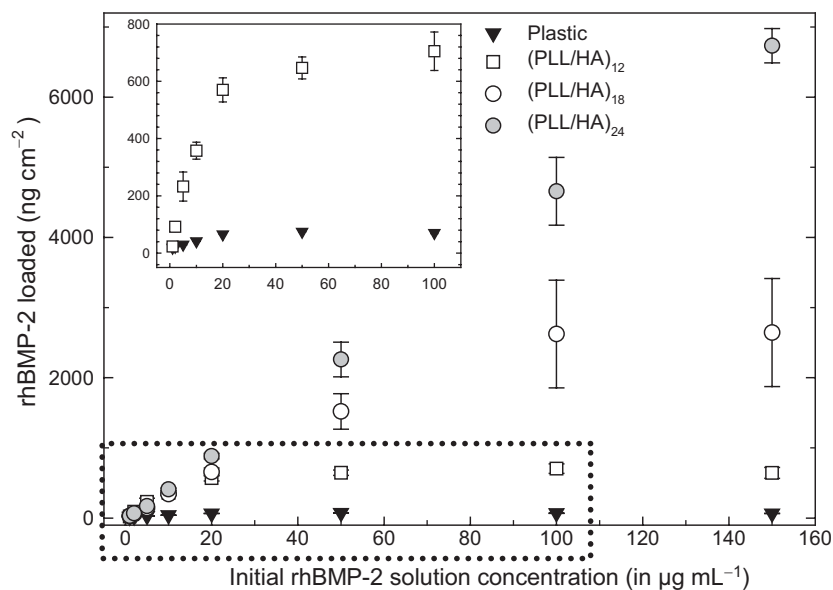
BMP-2 initial solution concentration [µg mL^{-1}]	1	2	5	10	20	50	100	150
Initial adsorbed amounts [ng cm^{-2}]	28 ± 8	121 ± 19	318 ± 58	480 ± 18	782 ± 35	975 ± 40	1319 ± 28	1258 ± 42
% released after 7 h	9 ± 5	17 ± 6	20 ± 8	19 ± 2	20 ± 2	28 ± 2	42 ± 4	44 ± 6
Effective incorporated amount [ng cm^{-2}]	25 ± 7	100 ± 20	254 ± 57	390 ± 21	622 ± 32	706 ± 31	769 ± 61	702 ± 65
Fold increase in volume concentration	256	501	507	390	310	141	77	47

higher solubility of rhBMP-2 at low pH and ionic strength[12] may explain such results. Indeed, soluble rhBMP-2 is thought to diffuse more easily within the film and in a more uniform fashion. When the film is rinsed with HEPES/NaCl solution at pH 7.4, rhBMP-2's solubility diminishes dramatically, which may lead to rhBMP-2 trapping in the film. Notably, release occurred mostly during the first 5 h, after which a steady state was reached without further detectable loss of rhBMP-2 for several days and several rinsing steps (Figure 2B, inset). For the PLL/HA films, the "burst" is thus rather limited from 0–20% for an initial rhBMP-2 concentration of 10 µg mL^{-1} to 44% for higher rhBMP-2 initial concentrations (Table 1 and Figure S2). As the films were always thoroughly washed in the physiological buffer prior to being introduced into the culture medium, the quantity of rhBMP-2 remaining in the film after 7 h of release will be taken as the "effective" incorporated amount. These effective incorporated amounts are gathered in Table 1 for (PLL/HA)$_{12}$ films (where 12 is the number of layer pairs).

Based on this preliminary study, rhBMP-2 suspended in 1 mM HCl (pH 3, no added salt) was subsequently used for film loading. We next varied the film thickness and investigated how we could modulate the incorporated amount of rhBMP-2 for films of various thicknesses (i.e., containing 12, 18, and 24 layer pairs) by raising the rhBMP-2 initial concentrations (Figure 3). For a given film thickness, the adsorbed amounts were higher when the initial rhBMP-2^{CF} concentration was raised. But over the range of concentrations investigated, the adsorbed amounts leveled off only for the films containing 12 and 18 layer pairs (at initial rhBMP-2 concentrations of 40 and 100 µg mL^{-1}, respectively). Importantly, rhBMP-2^{CF} adsorption on control plastic was always negligible compared to the amount adsorbed on PLL/HA films, which indicates a real reservoir effect of the film.

We can thus finely tune the amount of rhBMP-2 present in the films by varying the initial solution concentration of BMP-2 and maximize that amount by increasing the film thickness. An advantage of using PLL/HA films as a delivery reservoir is to obtain a significantly higher local concentration of rhBMP-2. This value can indeed be estimated, by knowing the incorporated amounts of rhBMP-2 in PLL/HA films, and was found to be up to ≈500-fold more concentrated than the corresponding bulk rhBMP-2 initial concentration (for a 5 µg mL^{-1} rhBMP-2 initial concentration and a film containing 12 layer pairs, 1 µm in thickness), which is the highest-fold increase obtained under our experimental conditions (Table 1). Thus, the local concentration of growth factor is dramatically raised when it is confined to the film.
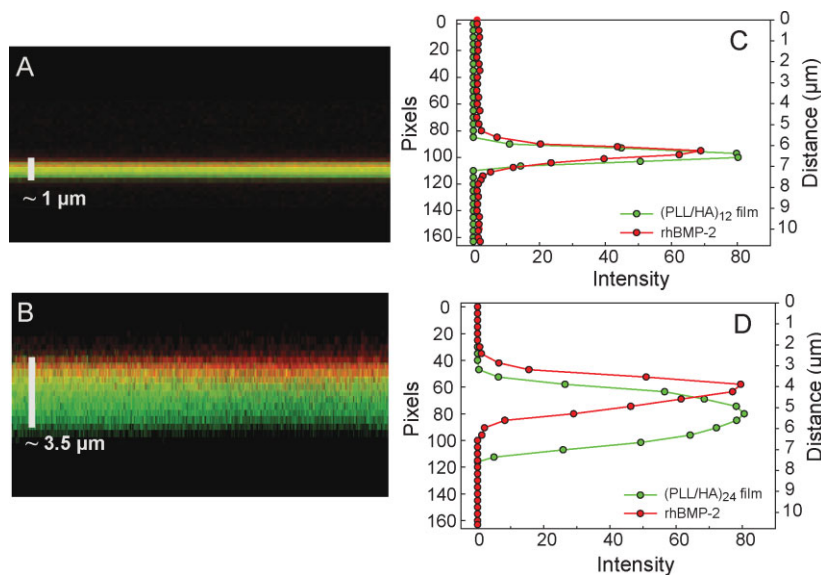
2.2. Visualization of Partial Diffusion of rhBMP-2 in PLL/HA Films

The evolution of the adsorbed amounts with the film thickness given in Figure 3 indicates that rhBMP-2 most probably diffuses within the film. In fact, if rhBMP-2 was simply

Figure 3. Amount of rhBMP-2 loaded in crosslinked (PLL/HA)$_i$ multilayer films of various thicknesses ($i = 12$, ≈1 µm; $i = 18$, ≈1.6 µm; $i = 24$, ≈3.5 µm) as a function of the initial rhBMP-2 solution concentration. Inset: zoom on the data in the rectangular dotted box (i.e., for plastic and for the 12-layer-pair film). 50 µL of rhBMP-2 solution was deposited per well (surface of the well, 0.36 cm^2). Plastic is also given as a control. Data are means ± standard deviation (SD) of three samples.

adsorbed on the film surface, the adsorbed amounts should be independent of the film thickness. A visual and qualitative proof of rhBMP-2 diffusion in the film was obtained from confocal microscopy images. For these experiments, rhBMP-2 was labeled with rhodamine (rhBMP-2[Rhod]) and the entire film was visualized by confocal microscopy using PLL[FITC] (FITC = fluorescein isothiocyanate) thanks to its known diffusion inside the exponentially growing film before cross-linking[40] (Figure 4). The overlay of the green and red channels for films containing 12 or 24 layer pairs (Figure 4A,B) and the intensity profiles along the z-direction (Figure 4C,D) indicate that rhBMP-2 diffuses throughout the entire (PLL/HA)$_{12}$ film, thus leading to its homogeneous distribution in films containing 12 layer pairs. In thicker films of (PLL/HA)$_{24}$, rhBMP-2 seems to accumulate in the upper part of the film with a limited diffusion within the film. This concentration gradient may originate from the interplay between the diffusion of rhBMP-2 within the film and rhBMP-2 interaction with HA. Knowing the z resolution of these confocal images ($\approx$500 nm), it was difficult to assess precisely the thickness over which rhBMP-2 is diffusing. A further proof of rhBMP-2 diffusion down to the glass substrate was obtained from total internal reflection fluorescence (TIRF) microscopy[45] (Figure S3). The penetration depth of the evanescent wave being of the order of few hundreds of nanometers above the glass substrate, the visualization of the rhBMP-2[Rhod]-loaded films by TIRF proves the presence of rhBMP-2 in the evanescent field. As the rhBMP-2 dimer is about $7 \times 3 \times 3$ nm in size[46] (27 kD), its diffusion also indicates that the pore size of the film is at least 10 nm.

All the subsequent experiments with cells were carried out on crosslinked (PLL/HA)$_{12}$ films, as the amounts of rhBMP-2 inserted in these films (Figure 3B, inset) were largely sufficient to induce C2C12 myoblast differentiation in osteoblasts.
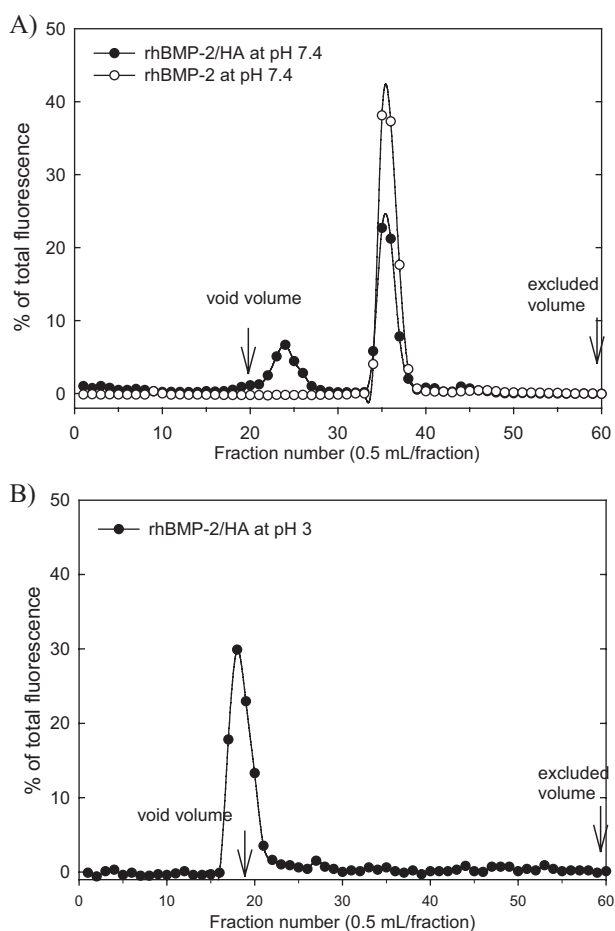
2.3. Interaction of BMP-2 with HA

A rhBMP-2[CF]/HA mixture ($\approx$1:10 mol/mol) was analyzed by size-exclusion chromatography. When elution was performed in 1 mM HCl, all rhBMP-2 was eluted in the void volume of the column, which indicates that it associates with HA. At pH 7.4 (without added salt), a fraction of the rhBMP-2[CF], presumably associated with HA, was excluded sooner than the other fraction being recovered in the retention volume expected from the size of rhBMP-2 (Figure 5). These data show that rhBMP-2 binds to HA (at pH 3 and 7.4), and provide an explanation for the trapping of rhBMP-2 within the film. This interaction has not been described previously, although structural interactions of rhBMP-2 with sulfated polysaccharides, specifically heparin, have been previously reported.[47] Consistent with our findings, Kim et al. showed that rhBMP-2 release was much lower from HA gels than from collagen gels.[48] Although the exact mechanisms of these interactions remain unclear, one may hypothesize that ionic bonds between negatively charged HA and positively charged rhBMP-2 and the numerous hydrogen bonds along HA chains[49] are likely to be important, as well as possible hydrophobic interactions between rhBMP-2 hydrophobic patches and surface-bonded HA. Such interactions have been observed for HA molecules adsorbed on a hydrophobic graphite surface.[50] This interaction most probably occurs in vivo, although it is not yet evidenced, thus participating in its delivery regulation and its protection against proteolysis.

2.4. Release of rhBMP-2 in Culture Medium

Release of surface-adsorbed rhBMP-2 in the cell culture medium was also measured either directly by quantifying the amount in solution by enzyme-linked immunosorbent assay (ELISA;[51] Figure 6) or by measuring the rhBMP-2 remaining in the film by micro-fluorimetry (data not shown). This was achieved in the absence and in the presence of cells to determine whether media containing 10% serum and/or cells were able to induce rhBMP-2 release. Noticeably, the released amounts in the medium were low, from $\approx$2 to 25 ng mL^{-1} (Figure 6A) and even lower (below 8 ng mL^{-1}) when cells were cultured on the films (Figure 6B). For all conditions tested, these low release amounts corresponded to less than 3% of the effective adsorbed material. Consistent with these findings, no significant loss of rhBMP-2[CF] fluorescence was measured by microfluorimetry (data not shown).
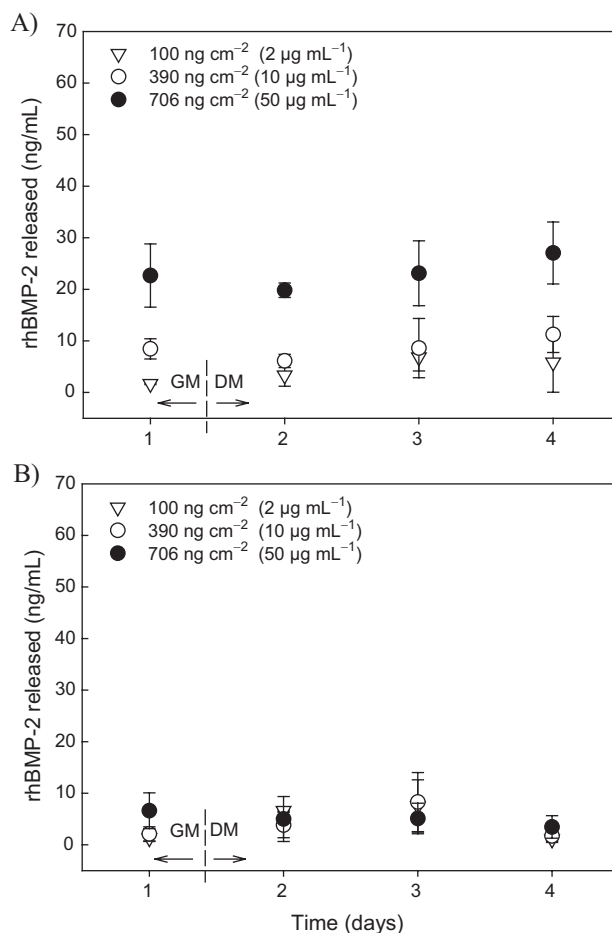
2.5. rhBMP-2-Induced Myoblast Differentiation in Osteoblasts

C2C12 myoblast cells normally differentiate into myotubes when cultured in a low-serum-containing medium (differentiation medium, DM) on tissue culture polystyrene,[52]

Figure 4. A,B) CLSM observations in HEPES/NaCl buffer (pH 7.4) of crosslinked (PLL/HA)$_i$–PLL[FITC]–HA multilayer films loaded with rhBMP-2; A) $i = 12$ and B) $i = 24$. PLL[FITC] was used to visualize the whole film and rhBMP-2[Rhod] was adsorbed at 20 µg mL^{-1} (in 1 mM HCl) and rinsed for 7 h in HEPES/NaCl buffer (pH 7.4) before observation. C,D) The corresponding z-intensity profiles are given along with the film thickness (left y axes) for rhBMP-2[Rhod] (red) and for rhPLL[FITC] (green).
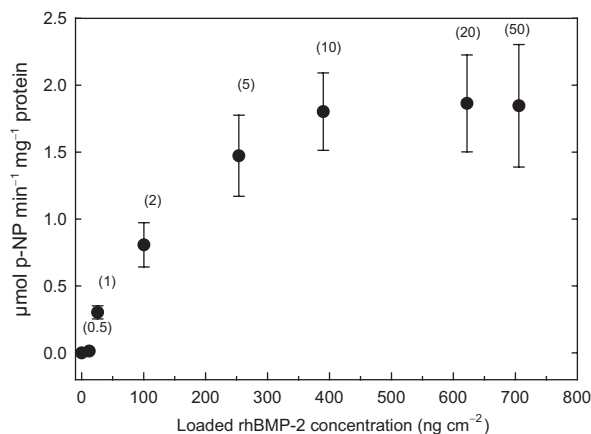
Figure 5. A) Size-exclusion chromatography elution profiles of a mixture of rhBMP-2^{CF} and HA ($\approx$1:10 mol/mol, first peak) and of rhBMP-2^{CF} alone (second peak) suspended in 40 mM Tris (pH 7.4). B) The same experiment was repeated with rhBMP-2 and HA suspended in 1 mM HCl (pH 3 without salt). The results suggest an association of rhBMP-2 with HA.
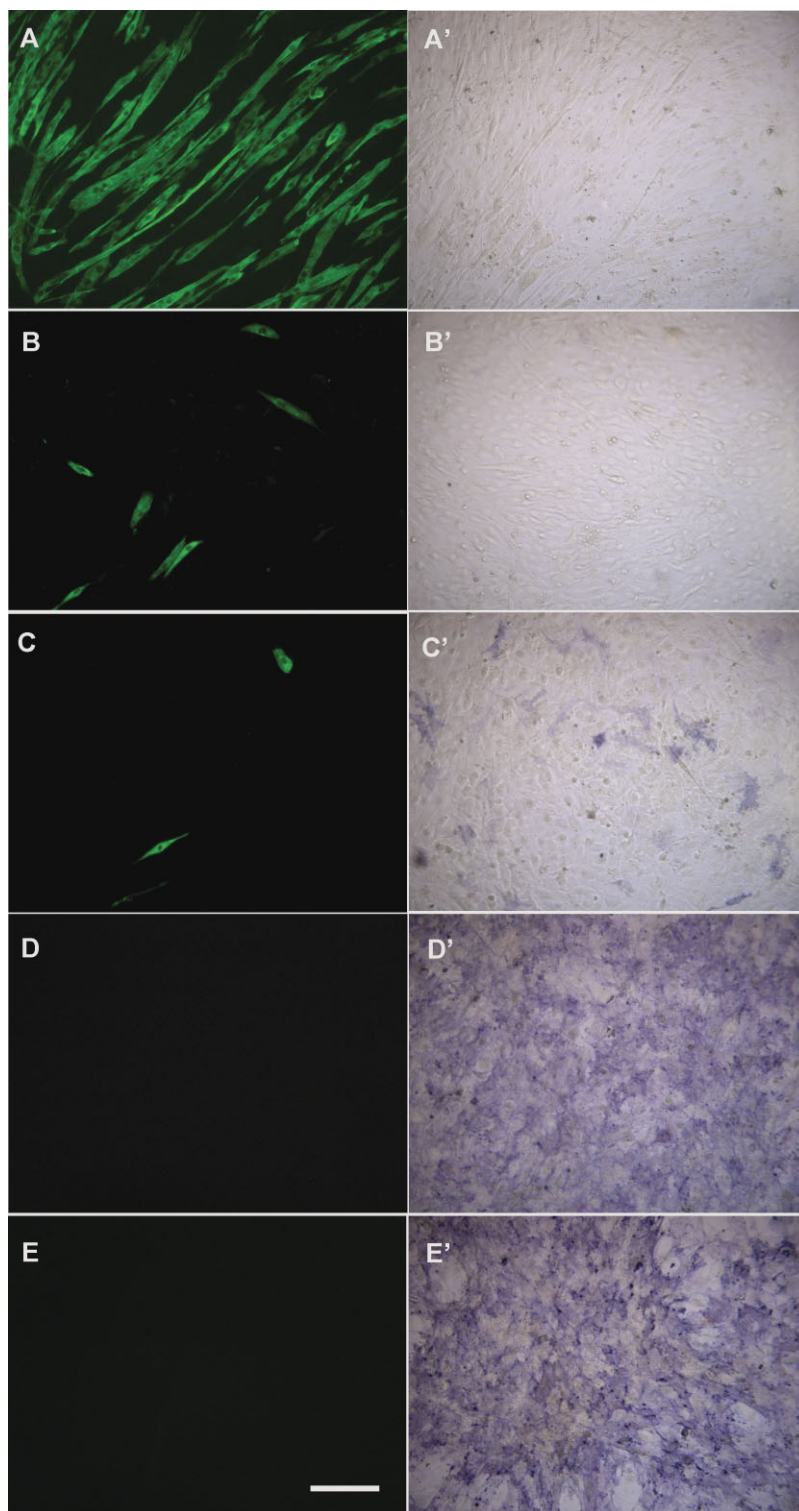

Figure 6. Release of rhBMP-2 in the culture medium quantified by ELISA. GM was removed at day 1 and replaced by DM. The media in contact with the rhBMP-2 loaded films (at 2, 10, and 50 μg mL^{-1} initial concentrations, 50 μL per well, surface of the well 0.36 cm^2) contained less than 25 ng mL^{-1} of rhBMP-2 for films without cells seeded on the films (A) and less than 10 ng mL^{-1} rhBMP-2 above cultured films (B). Data are means $\pm$ SD of three samples.

but can also differentiate into osteoblasts in the presence of rhBMP-2 in the culture medium.[9,10] On crosslinked films without rhBMP-2, we verified that cell differentiation into myotubes occurs effectively.[44] Then, we measured the bioactivity of film-adsorbed rhBMP-2 by quantifying the alkaline phosphatase (ALP) activity, an early marker of the osteogenic phenotype (Figure 7). We established a control dose–response curve for rhBMP-2 added to the culture medium (1 day in growth medium (GM) followed by 3 days in DM; Figure S4) and kept the same sequence of medium changes for the film experiments, except that rhBMP-2 was substrate-adsorbed and not in solution. We observed that ALP production increased as the amount of loaded rhBMP-2 (expressed here as a surface concentration) was increased, saturating at $\approx$400 ng cm^{-2} rhBMP-2 (Figure 7). Correlatively, the levels of troponin T expression (a marker of myogenic differentiation[9]) decreased (Figure 8). On the control film (no rhBMP-2), cells differentiated in myotubes and expressed troponin T (Figure 8A,A′). When the loading rhBMP-2 concentration was increased from 0.5 to 50 μg mL^{-1}, the cells

Figure 7. ALP activity of C2C12 cells seeded on rhBMP-2-loaded (PLL/HA)$_{12}$ films as a function of the loaded rhBMP-2 concentration (the initial rhBMP-2 concentration in solution is indicated in parentheses above each data point). ALP was measured for cells cultured 4 days (1 day in GM followed by 3 days in DM) in 24-well plates. Data are means $\pm$ SD of three samples.

Figure 8. Immunochemical and histochemical staining of troponin T (A–E) and ALP (A′–E′) of C2C12 on rhBMP-2-loaded films for increasing BMP-2 initial concentrations: A) 0, B) 0.5, C) 1, D) 10, and E) 50 μg mL^{-1} (scale bar: 150 μm).

Thus, low doses of surface-adsorbed rhBMP-2 can block C2C12 differentiation into myotubes, and significantly higher doses (10 μg mL^{-1} rhBMP-2 loading solution corresponding to ≈400 ng cm^{-2} rhBMP-2 loaded) allow the development of an osteogenic phenotype as measured by ALP production. We also checked that the ALP of cells cultured on the unfunctionalized films was null (Figure 7). Interestingly, after 9 days of culture on rhBMP-2-loaded films (650 ng cm^{-2}), C2C12 cells began to aggregate and formed "nodules". These nodules resemble those formed by bone-derived cell cultures that have the potential to mineralize in the presence of the appropriate minerals, thus forming a structure close to bone in vivo (data not shown).[53]

2.6. Long-Lasting Film Bioactivity over Several Cell-Culture Cycles

The persistence of surface-adsorbed rhBMP-2 bioactivity is a key issue, as rhBMP-2 in solution is known to be rapidly degraded in few hours.[51] rhBMP-2-functionalized films loaded at two rhBMP-2 concentrations were thus tested for their ability to retain rhBMP-2 activity over time. Three cell plating/replating culture sequences were performed one after the other on the same rhBMP-2-loaded films every 4 days after cells were gently detached without trypsin to preserve film and rhBMP-2 integrity. The ALP activity was measured 4 days after (re)plating at the end of each culture sequence. After a first sequence of culture, the film supported at least two additional cell-culture sequences and still triggered induction of ALP, albeit to a lower extent (Figure 9). Films loaded at high initial rhBMP-2 concentration (20 μg mL^{-1}) are more efficient in keeping their bioactivity than films loaded at low concentration (2 μg mL^{-1}). This finding demonstrates that films can remain bioactive for at least 12 days in cell culture medium in contact with cells, and that longer culture periods could be considered by simply increasing the amount of rhBMP-2 loaded in the film.

The results also demonstrate the resistance of the rhBMP-2-loaded films toward cell traction forces during migration and differentiation, as well as toward enzymatic secretions. In this respect, the PLL/H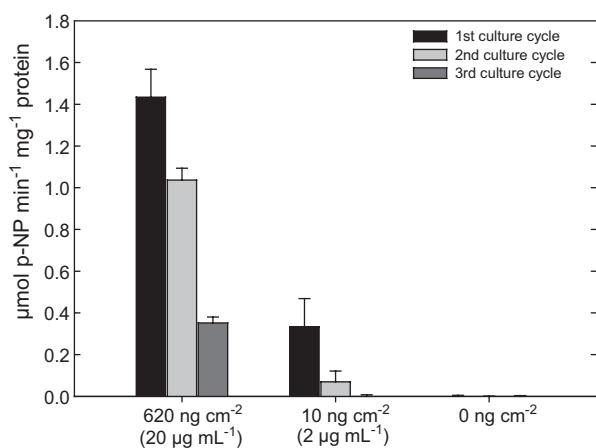A thin-film reservoir can be seen as a biomimetic system for rhBMP-2 delivery, which increases its local concentration and its lifetime through binding with the film. One of the film components, HA, is indeed present in many extracellular matrices.

progressively expressed more ALP (Figure 8B′–E′). It is worth noting that the cell differentiation program is significantly altered for BMP-2 initial concentrations as low as 0.5 μg mL^{-1} (corresponding to a loaded amount of 12 ng cm^{-2}; Figure 8B–E).

Figure 9. ALP activity of C2C12 cultured on rhBMP-2-loaded (PLL/HA)$_{12}$ films for three successive culture sequences (1 day in GM followed by 3 days in DM), the cells being detached between each culture sequence. Initial adsorbed rhBMP-2 amounts were 620 and 20 ng cm^{-2} (corresponding to initial rhBMP-2 concentrations in solution of 20 and 2 µg mL^{-1}, respectively, as indicated in parentheses). The control was a crosslinked (PLL/HA)$_{12}$ film without rhBMP-2 loaded. The raw activities represented the different culture sequences and initial rhBMP-2 concentrations.

2.7. Mechanism of Action of Surface-Adsorbed rhBMP-2

Our results show that rhBMP-2 adsorbed in LbL films retains its bioactivity. In fact, it seems more generally that LbL films are compatible with the biological activity of other embedded or adsorbed proteins (such as intercellular photo-receptor matrix,[54] BDNF,[33] protein A,[32] and fibroblast basic growth factor.[55]) However, no explanation for this activity of surface-adsorbed factors has been found and two main hypotheses have been evoked to explain the mechanism for this bioactivity:[36,56] 1) the growth factor could be released from the films or 2) cells could come into contact with the growth factors via the film and, possibly, locally degrade it by secreting enzymes. Whereas some cell types, such as macrophages, are characterized by a phagocytic activity toward LbL,[32] it is unlikely that myoblasts could degrade the crosslinked films that were found to be resistant to several types of enzymes.[57] In addition, our results demonstrate that active rhBMP-2 is extremely weakly released in the medium, and that the release was even lower in the presence of cells (Figure 5). One could argue that some inactive (or degraded) rhBMP-2 (and therefore not detected) might be released from the film, but this is in contradiction with the observation that ≈95% of the initial fluorescence of rhBMP^{CF} remains in the film. Importantly, we verified that doses of rhBMP-2 similar to those released from the films could not induce ALP production (Figure S4). A further check that the negligible amount of rhBMP-2 released in the medium was not responsible for the induced bioactivity was obtained from Transwell experiments. In these tests, cells were cultured in Transwell inserts without direct contact with the film surface, the rhBMP-2-loaded films being introduced at the bottom of the well. For a highly loaded film (initial BMP-2 concentration

50 µg mL^{-1}, ≈700 ng cm^{-2}), only a weak expression of ALP was measured (Figure S5). In addition, we observed the films by CLSM after the cells were cultured on top of them for 4 days and no film degradation was revealed at this resolution (Figure S6). Thus, our results strongly support the hypothesis that cells come into contact with the films and either "sense" the immobilized rhBMP-2 without significantly degrading the film or are sensitive to an extremely localized leak of rhBMP-2 from the film following degradation of the film beneath the cells by cell enzymes, such as metalloproteases and hyaluronidases.

For BMP-2, the cascade of events leading to signal transduction has been described.[58] Receptor signaling occurs through the hetero-oligomerization of two types of receptor chains (BMPRs), BMPR I and BMPR II upon BMP-2 binding. The ligand binds to the extracellular domain of BMPR I, which is then confined to the membrane surface. BMPR II chains are then recruited until the final hetero-oligomer signaling complex is formed.[58] In the present case, the surface-bonded (immobilized) BMP-2 may thus favor direct contact with the receptor chains by restricting their diffusion and/or internalization.

3. Conclusions

Our results provide evidence that very thin films (1 µm in thickness) can be used as a tunable reservoir for rhBMP-2 delivery to cells, BMP-2 being trapped in the film and remaining bioactive for more than 10 days. The optimal loading conditions were defined and the loaded amount was tuned by varying both the initial rhBMP-2 solution concentration and film thickness. Substrate-adsorbed rhBMP-2 was bioactive and induced myoblast differentiation into osteoblasts in a dose-dependent manner. In addition, film bioactivity was persistent over three successive culture cycles. This type of coating combines the ease of construction by dip coating and the localized delivery of bioactive growth factors. These results should have an important impact on the development of biofunctionalized surfaces for tissue-engineered constructs or for metallic implants. Indeed, as PLL/HA films were recently successfully deposited on polyethylene terephthalate prostheses[59] and onto nickel–titanium implant surfaces,[60] it may be envisioned that the present concept could be applied to biomaterial surfaces for investigating rhBMP-2 effects in vivo. Much larger doses of rhBMP-2 could indeed be loaded in PLL/HA films if necessary, by varying the number of layer pairs in the films and the deposition conditions (see Figure 3). Additionally, applications of such films in the differentiation or self-renewal of stem cells might be foreseen, as a recent study showed a very important role of HA gels in stem cell differentiation.[61]

4. Experimental Section

Film preparation and crosslinking procedure: HA (sodium hyaluronate, 2×10^5 g mol^{-1}) was purchased from Medipol

(Switzerland) and PLL (2×10^4 g mol^{-1}) was purchased from Sigma (France). PLL (0.5 mg mL^{-1}) and HA (1 mg mL^{-1}) were dissolved in a HEPES/NaCl buffer (20 mM HEPES, 0.15 M NaCl, pH 7.4). For all cellular experiments, the films were prepared as previously described[31] with a dipping machine (Dipping Robot DR3, Kierstein GmbH, Germany) on 14-mm-diameter glass slides (VWR Scientific, France). For BMP-2 integration and release experiments, films were manually constructed in 96-well plates (Nunc, Denmark) starting with a first layer of poly(ethyleneimine) (7×10^4 g mol^{-1}, Sigma, France) at 5 mg mL^{-1}. Briefly, polyelectrolyte solution (50 μL) was deposited in each well and allowed to adsorb for 8 min before being washed twice with rinsing solution (70 μL, 0.15 M NaCl, pH $\approx$ 6) for 1 min. The sequence was repeated until the buildup of a (PLL/HA)$_i$ film, i being the number of layer pairs, was achieved.

Films were crosslinked by following the protocol previously described[31] using EDC (50 mg mL^{-1}) and N-hydrosulfosuccinimide (11 mg mL^{-1}; both purchased from Sigma, France). After introduction of the coated glass slides in the culture plates, 0.5 mL of crosslinking solution was deposited in each well for 24-well plates (0.12 mL for 96-well plates) and left for 18 h at 4 °C. Final washing was performed with NaCl (0.15 M) at pH 8 for 1 h. These crosslinking conditions were chosen to ensure that the C2C12 cells adhered, proliferated, and differentiated optimally onto the films not containing rhBMP-2.[44] In addition, only HA-ending films were studied as previous results indicated no effect of the outermost layer on cell adhesion.[41]

rhBMP-2 labeling: rhBMP-2 (0.3 mg mL^{-1}; clinical grade, Wyeth BioPharma, USA) was dissolved in HCl (4 mM) and frozen at −20 °C until use. For fluorescent labeling, the pH of the protein was raised to 8 with a bicarbonate buffer (50 mM). BMP-2 was reacted for 4 h at room temperature with 1:20 (mol/mol) of 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (CF; Boehringer, Mannheim, Germany) or 1:100 (mol/mol) tetramethylrhodamine isothiocyanate (Rhod; Aldrish, Milwaukee, WI), before being reacidified with acetate buffer (0.2 M, pH $\approx$ 3). The labeled protein (rhBMP-2^{CF} and rhBMP-2^{Rhod}) was separated from the reagents by using a Sephadex G25 column (GE Healthcare, France) eluted with HCl (1 mM). The molar grafting ratios were estimated by determining the respective molar concentrations of dye and protein and calculating the grafting ratio (11 ± 2%).

Gel electrophoresis: BMP-2 (3 μg) and BMP-2^{CF} (4.5 μg) samples were mixed with Laemmli buffer (LB), loaded on a 20% polyacrylamide gel, and then stained with Coomassie blue. The two rhBMP-2 chains were dissociated under reducing conditions by adding DTT containing LB to the protein.

Quantification of rhBMP-2 and confocal observations: Incorporation and initial release studies were performed on (PLL/HA)$_i$ (i = 12, 18, 24) films constructed in 96-well plates. The films were always pre-equilibrated for 30 min in the medium in which rhBMP-2 was suspended (either 1 mM HCl or 20 mM HEPES, pH 7.4, with or without added NaCl). rhBMP-2^{CF} (50 μL) at increasing concentrations (from 0.5 to 150 μg mL^{-1}) was deposited on the films and allowed to adsorb overnight at 4 °C. HEPES/NaCl (150 μL) was added to each well and the plates were kept at room temperature for 15 min. The solution was then removed and replaced by fresh HEPES/NaCl buffer (pH 7.4) before measuring the fluorescence (excitation 485 nm/emission 535 nm) with a

Twinkle LB970 microfluorimeter (Berthold, Germany). For release studies, the wells were washed with the HEPES/NaCl solution (pH 7.4) and the fluorescence measured at various time intervals. The incorporated amount was calculated based on a calibration curve obtained with known amounts of rhBMP-2 in solution. rhBMP-2 loading on film-coated glass slides for cell-culture studies was achieved in a similar way. Coated slides were washed for 7 h in HEPES/NaCl before being sterilized for 15 min under UV light. For rhBMP-2 incorporation, the adsorbed amounts are expressed in ng cm^{-2} for ease of comparison, even though the films had a small roughness of 7 nm.[31] The amounts of rhBMP-2 released in the culture medium (GM, 10% serum) were determined with the Quantikine BMP-2 immunoassay (R&D Systems, France) according to the manufacturer's instructions. Experiments were performed at least in triplicate, with three independent samples per condition in each experiment.

Film topography and vertical structure were imaged using an LSM 510 META confocal microscope (Carl Zeiss, Germany) for films on 14-mm glass slides as previously described.[40] For rhBMP-2 deposition, the procedure described above was followed except that the deposited volume was larger (0.3 mL of rhBMP-2^{Rhod}).

Size-exclusion chromatography: rhBMP-2^{CF} (MW $\approx$ 27 kDa) or a mixture of rhBMP-2^{CF} and HA (MW $\approx$ 200 kDa) at a molar ratio of about 1:10 were suspended in HCl (1 mM) or Tris (40 mM, pH 7.4) at room temperature and left 1 h before being injected onto a Superdex 75 column (GE Healthcare, UK) with a size exclusion of 100 kDa. The protein was eluted with HCl (1 mM) or Tris (40 mM, pH 7.4) at a flow rate of 1 mL min^{-1} and fractions (0.5 mL) were collected and neutralized with HEPES (50 μL, 400 mM, pH 7.4) if needed. The fluorescence (excitation 485 nm/emission 535 nm) of each fraction was then measured by microfluorimetry to generate an elution profile.

C2C12 culture: C2C12 cells (<20 passages) were maintained in polystyrene flasks in an incubator at 37 °C and 5% CO$_2$, and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1; Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (PAA Laboratories, Les Mureaux, France) containing 10 U mL^{-1} penicillin G and 10 μg mL^{-1} streptomycin (GM; Gibco, Invitrogen, Cergy-Pontoise, France). Cells were subcultured prior to reaching 60–70% confluence (approximately every 2 days). For all experiments, C2C12 cells seeded on films at 4.5×10^4 cells cm^{-2} in GM were allowed to grow for 1 day and were then switched to the DM composed of DMEM/F12 (1:1) supplemented with 2% horse serum (PAA Laboratories, Les Mureaux, France), which contained 10 U mL^{-1} penicillin G and 10 μg mL^{-1} streptomycin, for 3 days.

To test C2C12 differentiation by medium-released BMP-2, 3000 cells were seeded in the upper compartment of Transwell inserts (6.5 mm in diameter, 0.4 μm pore membrane; Costar, Cambridge, MA) and rhBMP-2-loaded films were placed at the bottom of the wells (in 24-well plates), avoiding direct contact of the cells with the film. Control cultures were carried out with media supplemented with rhBMP-2 (300 ng mL^{-1}, without films) and with unfunctionalized films.

Film bioactivity: ALP assay: After 4 days in culture on BMP-2-loaded films, C2C12 cells were assayed for ALP activity, a marker for osteoblast differentiation.[9] After removal of culture medium,

cells were lysed by adding 0.1% Triton-X100 (0.5 mL) in phosphate-buffered saline (PBS) and sonicated. A buffer containing 2-amino-2-methyl-l-propanol (0.1 M; Sigma, St Quentin-Fallavier, France), $MgCl_2$ (1 mM), and p-nitrophenyl phosphate (9 mM; Euromedex, Mundolsheim, France), adjusted to pH 10 with HCl, was used to assay the cell lysate for ALP. The reaction was followed over 5 min in a 96-well plate by measuring the absorbance at 405 nm using a Multiskan EX plate reader (Labsystem, Helsinki, Finland). The activity was expressed as μmoles of p-nitrophenol produced per minute per milligram of protein. The total protein contents of the samples were determined by using a BCA protein assay kit (Interchim, Montluçon, France). Experiments were performed in triplicate, with three independent samples per condition in each experiment.

To assess the ability of rhBMP-2-loaded films to retain their bioactivity in long-term cell culture, the films were seeded with cells and followed a standard cell-culture sequence (1 day in GM, 3 days in DM). Cells were then rinsed with PBS without calcium or magnesium and were gently detached with ethylene glycol tetraacetic acid (2 mM) in PBS added at 4 °C (without trypsin). A gentle flux was applied by pipetting to proceed to cell detachment, while care was taken to avoid scratching the film. Films were then rinsed, sterilized by UV light, and reseeded. After three culture sequences, ALP activities were measured as described above.

Troponin-T and ALP histochemical analyses: Cells were fixed with 3.7% formaldehyde for 20 min, then permeabilized in 0.2% Triton X-100 for 4 min. As a marker for myogenic differentiation, cells were labeled with monoclonal mouse anti-troponin T (1:100; Sigma, St. Quentin-Fallavier, France). Primary antibodies were revealed with Alexa Fluor 488-conjugated goat antimouse (1:1000; Molecular Probes–Invitrogen, France). ALP, as a marker for osteodifferentiation, was stained with fast blue BB salt. Briefly, a mixture of naphthol (0.1 mg mL^{-1}; Fluka, Gillingham, UK) in DMF, $MgCl_2$ (2 mM), and fast blue BB salt (0.6 mg mL^{-1}; Fluka, Gillingham, UK) was deposited on the cells until coloration appeared. Observation of the stained cells was performed using a binocular microscope (Carl Zeiss SAS, Stemi SV11) or an Axiovert 200M microscope (Carl Zeiss SAS, Le Pecq, France) using a 10× objective.

Statistics: All experiments were repeated at least three times. Error bars represent standard deviations.

Supporting Information: The kinetics of rhBMP-2 adsorption (S1), the time course of rhBMP-2 release (S2), TIRF observations of the rhBMP-2-loaded films (S3), the dose–response curve for ALP to increasing amounts of rhBMP-2 added in solution (S4), the ALP activity of cells seeded in Transwell inserts (S5), and confocal images after 4 days in contact with cells in the culture medium (S6) are available as Supporting Information.

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