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The stability of BMP loaded polyelectrolyte multilayer coatings on titanium

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ABSTRACT

Immobilization of bone morphogenetic proteins (BMP) onto material surfaces is a promising, but still challenging, strategy for achieving dependable and consistent osseointegration of long-term metal implants. In the present study, we have developed an osteoinductive coating of a porous titanium implant using biomimetic polyelectrolyte multilayer (PEM) films loaded with BMP-2. The amount of BMP-2 loaded in these films was tuned – over a large range – depending on the cross-linking extent of the film and of the BMP-2 initial concentration. The air-dried PEM films were stable for at least one year of storage at 4 °C. In addition, they resisted exposure to γ-irradiation at clinically approved doses. The preservation of the growth factor bioactivity upon long-term storage and sterilization were evaluated both *in vitro* (using C2C12 cells) and *in vivo* (in a rat ectopic model) for the perspective of industrial and clinical development. BMP-2 loaded in dried PEM films exhibited shelf-life stability over one year. However, their bioactivity *in vitro* decreased from 50 to 80% after irradiation depending on the γ-irradiation dose. Remarkably, the *in vivo* studies showed that the osteoinductive potential of BMP-2 contained in PEM-coated Ti implants was fully preserved after air-drying of the implants and sterilization at 25 kGy. Film drying or irradiation did not affect the amount of new bone tissue formation. This "off-the-shelf" novel technology of functionalized implants opens promising applications in prosthetic and tissue engineering fields.

1. Introduction

Growth factor delivery is currently a great challenge in clinics [1] for both soft and hard tissue repair [2,3]. Researchers aim at better controlling the spatial and temporal delivery of growth factors as the natural extracellular matrix (ECM) does, in order to improve clinical efficacy while limiting side effects of these potent molecules. In view of their osteoinductive potential [4], bone morphogenetic proteins (BMPs) have been introduced into orthopedic clinical practice for the treatment of spinal fusion of either delayed or non-unions [5]. Although a wide range of carrier materials have been experimentally tested in combination with BMPs, the current clinical formulation of the rhBMP-2 (recombinant human) or rhBMP-7 (noted, hereafter, as BMP-2 and BMP-7, respectively) carrier used in practice is limited to collagen in the form of an adsorbable type I collagen sponge or paste. Currently, the growth factor is either soaked onto the collagen sponge or mixed into it; the wet matrix is then transferred to the implantation site. However, BMPs have only weak affinity for collagen, and the growth factor is completely cleared from the collagen carriers in less than 14 days *in vivo* [6,7]. Consequently, loss of BMP-2 solution due to mechanical manipulation during implantation as well as high burst release of BMP-2 [6] *in vivo* post-implantation must be considered. For these reasons, and also because BMP-2 signaling is highly regulated *in vivo* [8], very high doses of BMPs (specifically, several mg) are needed for the device to be osteoinductive [9]. At these supraphysiological doses, pathological events (such as osteolysis, heterotopic ossifications, immunological reaction and tumorigenesis) have been reported primarily in spinal application in humans [10–12]. A recent review on the safety of BMP-2 in spinal surgery concluded an incidence of 10–50% adverse events depending on

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the type of clinical application [13]. Thus, there is a strong motivation to develop delivery systems that both reduce the clinically efficient implanted BMP doses and improve safety and cost-effectiveness.

Local administration of BMPs onto implantable osteosynthetic materials, such as metal fixative devices, will broaden the clinical applications of BMPs. Most endoprostheses—used in orthopedic and maxillo-facial surgery—are manufactured from titanium (Ti) and titanium alloys. Ti alloys are widely used as implantable materials because of their excellent mechanical properties compared to other metals, their chemical inertness, corrosion resistance, bone compatibility, and ease of use during surgery [14,15]. Dependable and consistent osteo-integration of long-term metal implants, however, is still a clinical problem, especially in patients with poor osseous healing capabilities and impaired bone regeneration potential [16]. To address this issue, several studies have focused on the modification of titanium surface in order to increase new bone formation around such implants, *i.e.*, its osteo-integration [17]. The strategies to modify the Ti surface have essentially focused on improving Ti osteo-conductivity, *i.e.*, increasing bone apposition to the implant surface. The most popular approaches have used either physical or chemical modifications such as plasma treatment, sand-blasting, and exposure to chromosulfuric acid to increase the metal surface roughness [18], as well as the coating of those surfaces with biomimetic layers such as hydroxyapatite (HAP) [17]. In addition, research endeavors aim at rendering the metal surface osteoinductive to promote new bone formation. One method to render a metal implant osteoinductive is by incorporating or grafting BMPs at the material surface. Unfortunately, direct covalent grafting of BMP-2 onto a metal surface allows only very small amounts of BMP-2 to be delivered; whereas direct adsorption of BMP-2 leads to a rapid burst of the protein because of its low affinity with metal surfaces [19,20]. In order to increase the affinity between BMP-2 and Ti surfaces, biomimetic coatings (for example HAP [21–23] or natural biopolymers [24–26]) have been used. These formulations are promising because of the similarity of HAP and biopolymers with bone tissue constituents. The underlying strategy is that a biomimetic matrix can trap, retain, and deliver BMPs locally in a more efficient manner.

Recently, we have developed an osteoinductive coating in the form of a polyelectrolyte multilayer (PEM) film loaded with BMP-2 [27]. The biomimetic PEM films, which are prepared by assembly of poly(L-lysine) (PLL) and hyaluronan (HA), can be used as carriers of BMP-2. Our initial studies showed that such PEM films, deposited on tricalcium phosphate/hydroxyapatite granules, retained sufficient amounts of BMP-2 to induce bone formation *in vivo* in a rat ectopic model [28]. A major advantage of such films is that they can coat various material surfaces of either two- or three-dimensional structure [29].

From the industrial and clinical perspectives, shelf-life stability, and sterilization of BMP-2 coated on implants are important issues. To date, these issues have not yet been addressed and/or resolved [30]. Medical devices have to be sterilized after packaging and must preserve their functional properties upon storage for several years [31]. The presence of biological active molecules makes these devices more difficult to handle, store, and sterilize than traditional biomaterials. Such difficulties can hamper the clinical diffusion of these innovative solutions, limiting them to the research level [32]. Developing a device coated with BMP-2 that has shelf-life stability and sterilization resistance is of high clinical interest.

The objective of the present research project was twofold. First, we aimed to assess the osteoinductive properties of the BMP-2 loaded PEM coatings onto porous titanium scaffolds. Second, our aim was to assess its performance *in vitro* and *in vivo* pertinent to long-term storage in a dry state and to sterilization by γ irradiation.

2. Materials and methods

2.1. Materials

Planar titanium samples (pure grade 2 titanium; 0.4 mm in thickness and 1 cm^2) used for *in vitro* studies were kindly donated by TIMET Savoie S.A (Ugine, France). Titanium implants used for *in vivo* studies were Ti 6 Al 4V ELI (TA6V) custom-made implants (cylinders of 3 mm height and 5 mm diameter), one of the most commonly used titanium alloy for medical applications. The implants had open, communicating pores with a diameter of 500 μm, and a porosity of 22%; the surface roughness was characterized by a R_a of 1.6 μm as measured by contact profilometry.

2.2. Polyelectrolyte multilayer film deposition on titanium and BMP-2 loading

PEM deposition was performed using polyethyleneimine (PEI, Sigma) at 2 mg/mL, poly(L-lysine) hydrobromide (PLL, Sigma) at 0.5 mg/mL, and hyaluronic acid (HA, 360 kDa, Lifecore, USA) at 1 mg/mL dissolved in a buffered saline solution (0.15 M NaCl, 20 mM Hepes pH 7.4, called hereafter Hepes–NaCl buffer). The $(PLL/HA)_{24}$ film (PLL being the polycation, HA the polyanion and 24 the number of layer pairs of the film) buildup using an automatic dipping machine (Dipping Robot DR3, Kierstein GmbH, Germany) and the subsequent cross-linking were done as previously described [33].

A previously established protocol for BMP-2 loading in cross-linked $(PLL/HA)_{24}$ films was followed [27,28]. Briefly, each PEM-coated titanium sample was deposited into multiple well plates and incubated with the BMP-2 solution (at 20 μg/mL for *in vitro* studies and 100 μg/mL for *in vivo* studies). The amounts BMP-2^{CF} carboxyfluorescein (CF) used as a tracer (2%) initially loaded into the PEM films [27] and subsequently released after several washes with Hepes–NaCl buffer were determined using a fluorescence spectrometer (TECAN Infinite 1000, Austria).

2.3. Film characterization by Fourier transform infrared spectroscopy (FTIR)

The $(PLL/HA)_{24}$ films were built on a ~1 cm^2 silicon substrate (Siltronix, France) coated with a 40 nm thick TiO_2 layer, rinsed with milli-Q water to remove the salt and dried for 1 h at 37 °C [34]. They were then analyzed by FTIR spectroscopy as previously described using a Vertex 70 spectrophotometer (Bruker Optics Gmbh, Ettlingen, Germany) [34]. The film spectra in transmission were acquired by summing 256 spectra acquired between 400 and 4000 cm^{-1} with a 2 cm^{-1} resolution using the liquid nitrogen-cooled MCT detector. For long-term stability measurements, the films were stored in dry state at 4 °C. Before each FTIR acquisition, they were incubated at 37 °C for 1 h. For γ-irradiation, dried BMP-2 loaded PEM films were sterilized at two different doses (25 and 50 kGy) and then characterized by FTIR.

2.4. Film imaging by scanning electron microscopy (SEM) and by fluorescence macroscopy

Films were imaged by scanning electron microscopy (SEM) using a FEI-Quanta 250 SEM-FEG and by a Leica Macrofluo (Z16 Apo) fluorescence system using an ×0.8 objective.

2.5. AFM imaging

AFM images of $(PLL/HA)_{24}$ films deposited on Ti substrates were obtained in tapping mode by means of a DI 3100 AFM (Veeco) with NanoScope IIIa controller using silicon cantilever (OMCL-AC240TS, Olympus). The PEM-coated titanium samples were washed in water and air-dried before observation. Substrate topographies were imaged with 512 × 512 pixels at a frequency of 1 Hz.

2.6. C2C12 cell culture and in vitro BMP-2 bioactivity assay (ALP assay)

Murine C2C12 skeletal myoblasts (<25 passages, obtained from the American Type Culture Collection, ATCC) were cultured in tissue culture Petri dishes, in a 1:1 Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 medium (Gibco, Invitrogen, France) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, France) and 100 U/mL penicillin G and 100 μg/mL streptomycin (Gibco, Invitrogen, France) in a 37 °C, 5% CO_2 incubator. This medium will be named hereafter C2C12 growth medium (C2C12 GM).

The bioactivity of BMP-2 on C2C12 cells was determined by assaying the BMP-2 induced alkaline phosphatase (ALP) activity, a marker of osteogenic differentiation following a previously established protocol [27]. First, the film was thoroughly washed with the Hepes–NaCl solution for at least 2 h to discard any weakly bound or unbound BMP-2. Then, 90,000 C2C12 cells were seeded on each sample in a 24-well plate. After 3 days of culture, the growth medium was removed and the cells were washed with PBS and lysed by sonication over 5 s in 500 μL of 0.1% Triton-X100 in PBS. The ALP activity of these samples was then quantified using standard protocol [27] and normalized to the corresponding total protein content, which was determined using a bicinchoninic acid protein assay kit (Interchim, France). For bioactivity assay after film drying, the loaded titanium samples were rehydrated for 30 min in Hepes–NaCl buffer and sterilized under UV irradiation before performing the ALP test.

2.7. Ectopic bone formation assay

The osteoinductive potential of BMP-2 containing TA6V scaffold was assessed *in vivo* in a rat ectopic model. Eight different groups were designed as described in Table 1. For each group, 8 implants were prepared. For experiments on dried films, PEM-coated titanium implants were washed with Hepes–NaCl buffer for 1 min and then quickly washed in ultrapure water before being dried 2 h under a laminar flow hood at room temperature. Seven week old Sprague–Dawley female rats were purchased from Janvier (Legenest-Saint-Isle, France) and handled according to the *European Guidelines for Care and Use of Laboratory Animals* (EEC Directives 86/609/CEE of 24.11.1986). The implantation protocol has been approved by the local ethics committee on animal research (Lariboisiere/Villemin, Paris, France). Animals were preoperatively given analgesics, anaesthetized and then prepared as previously described [28]. Each sample was implanted aseptically in the back in space created between the muscles and the aponeurotic layer (six implants were implanted per animal). The soft tissues at the implantation sites were closed with interrupted non-resorbable sutures. Six weeks post-implantation, the animals were sacrificed via injection of lethal doses of sodium pentobarbital (Sanofi–Aventis, Paris, France). The implants were then retrieved *en-bloc* and fixed in 10% phosphate-buffered formalin before analysis as described below.

2.8. Histomorphometric analysis

Retrieved implants were processed for undecalcified histology following previously established protocols [35,36] and as previously described for titanium [37]. After embedding in methyl methacrylate, each sample was then cut into 6 or 7 sections (500 μm each) using a diamond. These sections were then grounded to a thickness of 100 μm and stained using Stevenel blue and van Gieson Picrofuschin for histological analysis [37]. Three unadjacent sections from each specimen were selected for histomorphometry analysis. Histological examination was performed under an optical microscopy (Nikon Eclipse TE2000-U, Nikon, France) equipped with a numeric camera (DXM1200F, Nikon). Bone was quantified on whole sections using the NIS-Elements BR 2.30 software (Nikon). Three parameters were quantified in each section: (1) Bone area (%) which is the surface of bone (stained in red) normalized over the "available area" (calculated by subtracting the titanium scaffold surface from the whole surface delineated around each specimen); (2) Bone-scaffold contact (%) which is the available titanium scaffold perimeter in contact to bone normalized over the titanium scaffold perimeter length and (3) Tissue-scaffold contact (%) which is the available titanium scaffold perimeter in contact to (both bony and soft) tissue normalized over the titanium scaffold perimeter length; for consistency, the percentages of both bone-scaffold contact and tissue-scaffold contact were quantified only inside the pore channels of the titanium scaffolds tested.

2.9. Statistical analysis

Numerical results were reported as average ± standard error of the mean. Data from histological findings are presented as box-and-whisker plots. When comparing data between more than two conditions, the Kruskal–Wallis one-way analysis test was used. The nonparametric Mann–Whitney *U* test was used to analyze data from two independent conditions. For all analyses, differences were accepted to be statistically significant at $p \leq 0.05$. Statistical analyses were conducted using the Statgraphics centurion version XV.2 (Statpoint, Inc., Herdon, VA, USA).

3. Results

3.1. PEM-film deposition on porous titanium substrates

The principle of biofunctionalization of the titanium substrate is shown in Fig. 1A. The biomimetic film deposited was formed via layer-by-layer assembly using PLL as a polycation and HA as a polyanion. Next, the film was covalently cross-linked using the carbodiimide chemistry to create amide bonds between the carboxylic groups of HA and the ammonium groups of PLL [38]. To modulate the crosslink extent of the films, the carbodiimide concentration was adjusted at 10, 30 and 70 mg/mL; the obtained films were noted in the following, film@EDC*x* ($x = 10$, 30 and 70). Finally, the film was exposed to a solution of BMP-2. The growth factor was incorporated by passive diffusion into the film [27].

Planar titanium substrates and custom-made 3D porous substrates made of Ti–6Al–4V (TA6V) (Fig. 1B and Fig. S1) were coated with the biomimetic film and examined at the macro and micro-scales using scanning electron microscopy (SEM) (Fig. 1B′, C, C′) and fluorescence macroscopy, respectively (Fig. 1D, D′). SEM revealed the roughness of the bare titanium substrate (Fig. 1B′) and the smoother surface after PEM coating (Fig. 1C, C′). The coating was visible both inside the pore channels (Fig. 1C) as well as on the outer part (surface) of the implant (Fig. 1C′). This observation was confirmed by atomic force microscopy (AFM) imaging (Fig. S1). Indeed, film AFM measurements of film thickness on planar Ti substrates revealed that film thickness was of 6.1 ± 0.5 μm in wet state and 1.1 ± 0.2 μm in dry state. In addition, uniform fluorescence of the film ending with a final layer of PLL^{FITC} was observed on the top of the implant and inside the channels by fluorescence macroscope (Fig. 1D, D′). Together, SEM, fluorescence macroscopy and AFM images provided evidence that the films deposited onto the porous titanium implants were homogeneous.

3.2. Incorporation of BMP-2 in PEM-coated TA6V substrates

To determine the BMP-2 loading capacity of PEM films cross-linked to different extents (film@EDC10, 30 and 70), we employed fluorescently-labeled BMP-2. Indeed, we showed previously that the bioactivity of carboxyfluorescein modified BMP-2 is similar to that of unlabeled BMP-2 [27]. The retention capacity of the film after loading BMP-2 at two different concentrations and its release kinetics over 7 days were investigated (Fig. 2). The initial adsorbed amounts (Γ_i) and final amounts (Γ_F) of BMP-2 remaining in the films after extensive washes are given in Fig. 2A. Unbound and/or loosely bound BMP-2 was mostly desorbed during the first 2 h (corresponding to the initial burst release), after which a plateau value was observed with no significant changes over 7 days (Fig. 2B). Overall, the initial amounts of BMP-2 incorporated in the film (before washing) ranged between 4.2 and 19 μg/cm^2, depending on the cross-linking extent and the BMP-2 initial concentration in solution. The film@EDC10 incorporated the highest amount of BMP-2. In this case, the percentage of weakly bound BMP-2 represented about 62–77% of the initial amount compared to 27–31% for the film@EDC30 and 7–11% for the film@EDC70. As a consequence, at the end of the release kinetics, the amounts of BMP-2 retained within the various films tested ranged from 1.4 to 14.3 μg/cm^2. The sequestered amount, Γ_F, increased with the cross-linking extent of the film (approximately a 3-fold increase in case of film@EDC10 versus the film@EDC70). Furthermore, increasing the BMP-2 concentration from 20 to 100 μg/mL led to a 3–4 fold increase in Γ_F. It should be noted that the release kinetics of BMP-2 was not affected by drying the film and subsequent rehydration (Fig. S2).

Overall, these results indicated that the amount of BMP-2 incorporated in the PEM films can be tuned over a large range depending on both the extent of film cross-linking and on the initial BMP-2 concentration.

3.3. Shelf-life of dry PEM films containing BMP-2

In view of using PEM coatings in a clinical setting – *i.e.*, implants coated with BMP-2 loaded on PEM films – the option to store them

Table 1
Experimental design of TA6V scaffolds used per group for the *in vivo* study. The table indicates the cross-linking degree, the presence or not of BMP-2 in the coating, and whether the PEM films have been dried (D) and/or γ-irradiated (I).

Group ($n = 8$)	Cross-linking of PEM coating	Presence of BMP-2	PEM film dried	Irradiated
Bare Ti	–	–	–	–
EDC10	EDC10	–	–	–
Bare Ti + BMP-2	–	+	–	–
EDC10 + BMP-2	EDC10	+	–	–
EDC10 + BMP-2 – D	EDC10	+	+	–
EDC10 + BMP-2 – D + I	EDC10	+	+	+
EDC30 + BMP-2 – D	EDC30	+	+	–
EDC30 + BMP-2 – D + I	EDC30	+	+	+

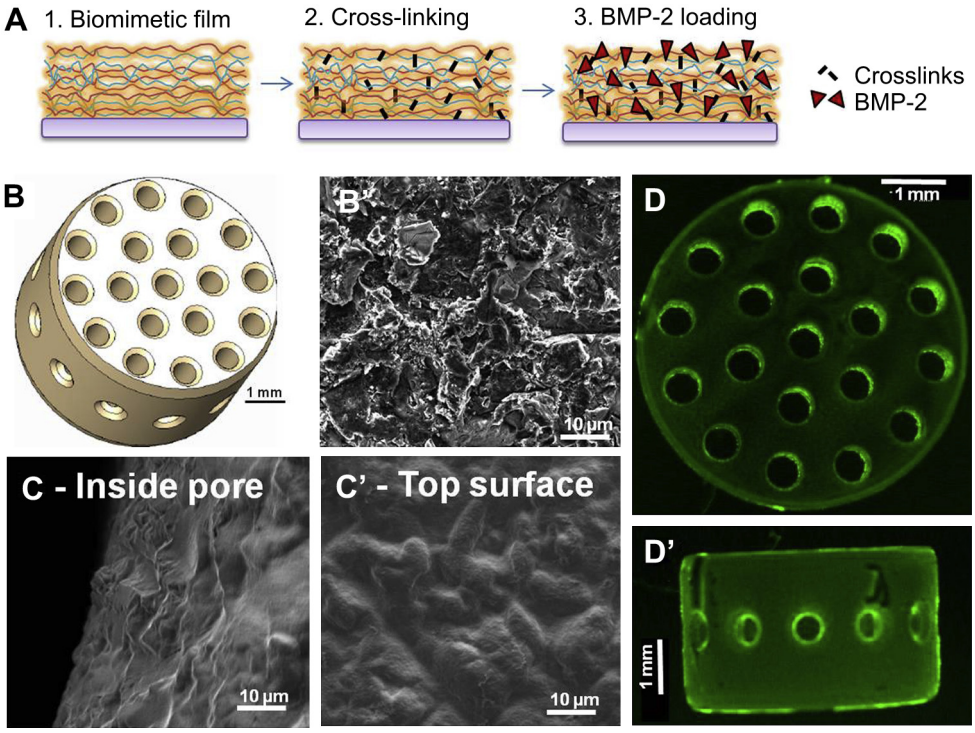

Fig. 1. Polyelectrolyte multilayer film coating of titanium substrates: (A) three steps of the bioactive film preparation onto a material substrate: 1) film deposition by layer-by-layer assembly onto the substrate, 2) cross-linking, and 3) BMP-2 loading. (B) Schematic of porous TA6V scaffold with 500 µm pore size. (B′) SEM micrograph of the uncoated TA6V surface imaged at the implant surface. SEM micrographs of $(PLL/HA)_{24}$ film-coated surface of the TA6V scaffold either (C) inside a pore channel or (C′) at the outer surface. Observation of a PEM-film deposited on the TA6V scaffold by a macroscope: (D) top view and (D′) side view.

in dry state while preserving the bioactivity of the protein would be advantageous. In order to assess the shelf-life of the BMP-2 coated implants, the long-term stability of the PEM films and the *in vitro* bioactivity of the loaded BMP-2 were both investigated upon storage in a dry state for several months. To this end, the coated titanium implants were thoroughly washed with water, air-dried, and stored for up to 12 months. The PEM stability was observed via infrared spectroscopy (Fig. 3A). The spectra of the dry PEM films cross-linked at different extents revealed the characteristic bands of HA (polysaccharide rings and carboxylic group) and PLL (amide I, II and III bands) (Fig. S3). The specific signature of the cross-linking was also visible with a decrease in the carboxylic peak and a concomitant increase of amide I, II and III bands. Interestingly, storage of the dried film@EDC30 over 12 months did not cause noticeable changes in the infrared spectrum (Fig. 3A). Only a minor change (less than 3%) was noted for the major amide I band, possibly due to the different levels of hydration of the films. Similar spectra were obtained for the film@EDC10 and EDC70 (data not shown). Overall, these data provide evidence that the PEM films were stable when stored under dry conditions for at least 12 months without noticeable chemical changes.

A

$[BMP\text{-}2]_i$	20 µg/mL			100 µg/mL		
Type of PEM film	Γ_i (µg/cm^2)	% released	Γ_F	Γ_i	% released	Γ_F (µg/cm^2)
Film@EDC10	4.2 ± 0.3	62 ± 4	1.4 ± 0.2	18.9 ± 0.8	77 ± 6	4.1 ± 1.2
Film@EDC30	4.1 ± 0.2	27 ± 2	2.9 ± 0.1	13.6 ± 0.2	31 ± 2	9.3 ± 0.3
Film@EDC70	3.9 ± 0.1	7 ± 2	3.7 ± 0.2	16.1 ± 2.4	11 ± 2	14.3 ± 2.1

B

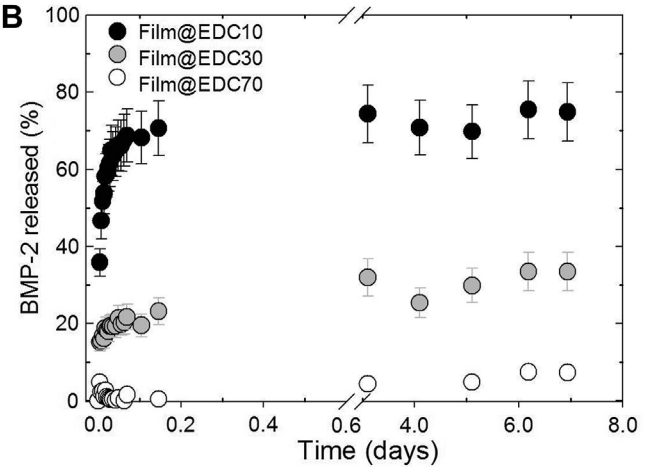

Fig. 2. Quantification of BMP-2 loaded in and released from PEM films. The amounts of BMP-2 loaded in (PLL/HA) films made of 24 layer pairs and cross-linked@EDC10, EDC30 and EDC70 were compared. (A) Summary table of the initial (Γ_i, in µg/cm^2) and final (Γ_F) BMP-2 amounts adsorbed on the three PEM films tested using initial BMP-2 concentration in solution at 20 or 100 µg/mL. (B) Release kinetics of BMP-2 from each type of PEM film tested in a Hepes–NaCl buffer as a function of time over 7 days.

The *in vitro* bioactivity of BMP-2 was assessed using the pluripotent C2C12 myoblasts as cell model [39]. Alkaline phosphatase (ALP) activity of cells seeded on BMP-2-loaded films cross-linked to the various extents tested in the present study was assayed after 3 days of cell culture [27]. The bioactivity of BMP-2 sequestered into either dry films or wet films (no drying step) was similar (Fig. 3B). A systematic decrease of ALP activity was observed when the cross-linking extent increased, with the most cross-linked films (film@EDC70) exhibiting the lowest ALP bioactivity (Fig. 3B). Of note, when bare TA6V substrates were dipped into BMP-2 loading solution (similar procedure to that of BMP-2 loading in PEM films), there was no adsorption of the growth factor and thus no ALP activity.

The BMP-2 bioactivity was then determined after storage of the PEM film-coated substrates in dry state from 2 h to one year (Fig. 3C). At those times, all substrates were rehydrated and the

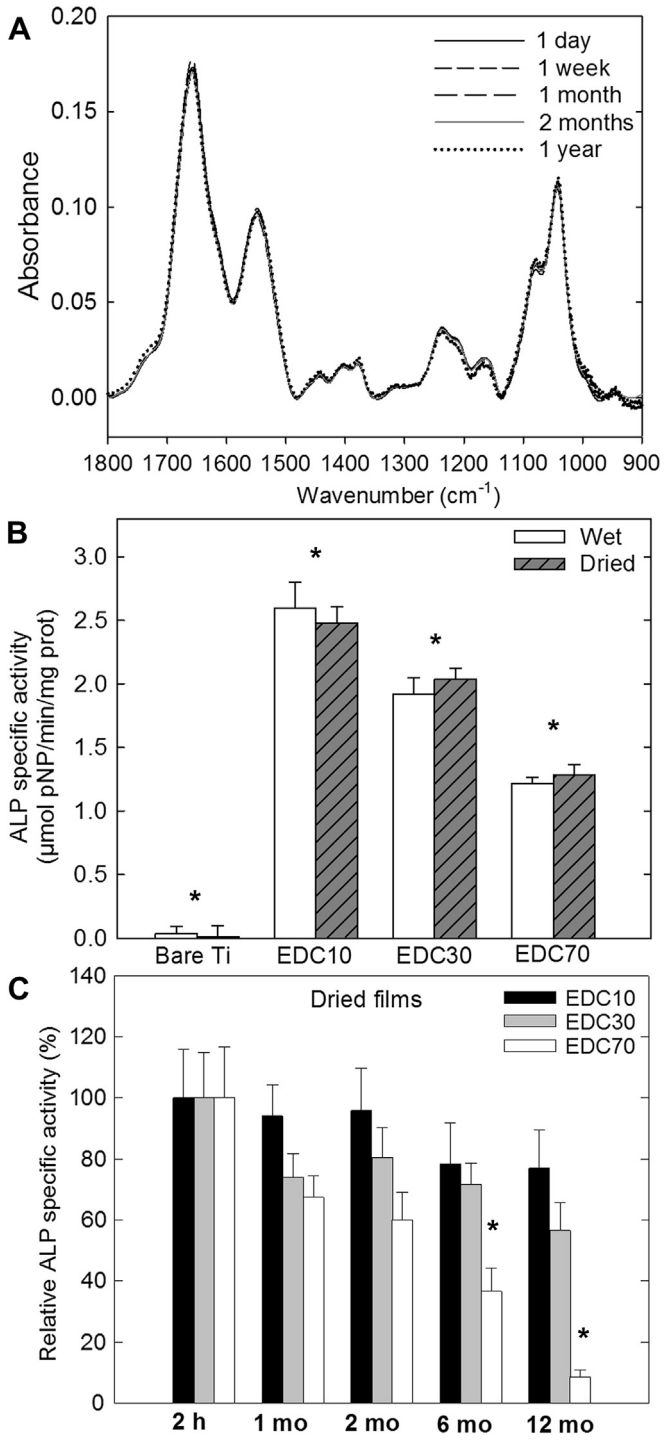

Fig. 3. Shelf-life of BMP-2 containing-PEM films in dry state. (A) Comparison of FTIR spectra of film@EDC30 in dry state obtained at different time points over one year. (B) ALP activity of C2C12 myoblasts cultured for 3 days on either bare or on PEM film-coated Ti substrates loaded with BMP-2 at 20 µg/mL (films@EDC10, 30 and 70). ALP activity was assessed for C2C12 cells cultured on films that were either let hydrated (wet) or air-dried for 2 h. (C) Kinetics of ALP activity of BMP-2-loaded PEM films stored in dry state for up to 1 year. Data were normalized over the values obtained with dried films stored for 2 h.

BMP-2 bioactivity was assayed. A weak, but not statistically significant, decrease in the bioactivity of BMP-2 loaded on both the film@EDC10 and film@EDC30 was noted after storage for one year. In contrast, a significant decrease ($p < 0.01$) of ALP activity was obtained when the C2C12 cells were cultured on the film@EDC70 stored up to one year. In this case, only 38% of the initial ALP activity remained after 6 months and only 9% after one year. Thus, the preservation of the activity of BMP-2 sequestered into PEM films that are stored in dry state depended on the film cross-linking extent. Of note, bioactivity was also preserved when films were submitted to accelerated aging for 4 days (relative humidity of 75%) at 37 °C and 50°(Fig. S4). All together, these findings provided evidence that implants coated with BMP-2-containing PEM films can be stored in dry state while preserving the growth factor bioactivity for at least one year for film@EDC10 and EDC30.

3.4. Film stability and preservation of BMP-2 bioactivity upon sterilization

The stability of the PEM films to γ-irradiation at 25 and 50 kGy, typical doses used in sterilizing metallic implants [40], was assessed using AFM imaging (Fig. 4A) and FTIR analysis (Fig. 4B, B′). The topographic analysis showed that the film@EDC30 at the two irradiation doses tested exhibited similar roughness as the control (*i.e.*, non-irradiated) film surface (2.5 ± 1.5 nm) (Fig. 4A, A′). The infrared spectra revealed that the films were stable after irradiation (Fig. 4B). By plotting the difference between the FTIR spectra of the various cross-linked PEM films after irradiation versus that of the films prior irradiation, a small decrease of the COO^- peak at 1611 cm^{-1} and a very small increase in the ester bond at 1736 cm^{-1} were observed (Fig. 4B′). Quantitative analysis (Fig. 4C) revealed that the decrease of the COO^- peak depended both on the extent of the cross-linking and on the γ-irradiation dose. In fact, this decrease was more pronounced when the extent of cross-linking was lower and when the irradiation dose was higher. These results suggested that exposure of the PEM film@EDC10 to γ-irradiation induced an additional cross-linking of this film. In contrast, the higher cross-linked films were barely affected.

We next tested the *in vitro* bioactivity of the BMP-2 loaded films after γ-irradiation (Fig. 4D). A dose-dependent decrease of the ALP activity was noted for cells cultured on all PEM films. The ALP activity signal decreased to ~50% and to ~20% of its initial value after irradiation with the 25 and 50 kGy doses, respectively. However, that, in these cases, the ALP activity remained significantly higher than that obtained from cells cultured on bare material surfaces with adsorbed BMP-2 (control) that showed absolutely no ALP signal (Fig. 3B).

All together, these data provided evidence that the chemical stability of the PEM films post-exposure to γ-irradiation depended on the extent of cross-linking of the film. The preservation of the bioactivity of BMP-2 sequestered in PEM films depended on the irradiation dose.

3.5. Osteoinductive performance of the PEM-coated TA6V scaffolds in vivo

In order to determine the osteoinductive potential of the porous TA6V scaffolds coated with BMP-2 loaded-containing PEM films, these materials were implanted intramuscularly in the back of rats. For these *in vivo* studies, substrates coated with the PEM film@EDC10 and film@EDC30, which exhibited the highest bioactivity upon storage and γ-irradiation *in vitro*, were used. The eight experimental conditions, including three different controls (bare Ti, bare Ti with adsorbed BMP-2 and Ti with film@EDC10 in the absence of BMP-2) are listed in Table 1. Based on the results obtained in Fig. 2, we estimated the total BMP-2 dose delivered *in vivo* to be between 15.5 ± 0.4 µg and 21.5 ± 0.9 µg, taking into account the total available surface of the Ti scaffold (1.14 cm^2).

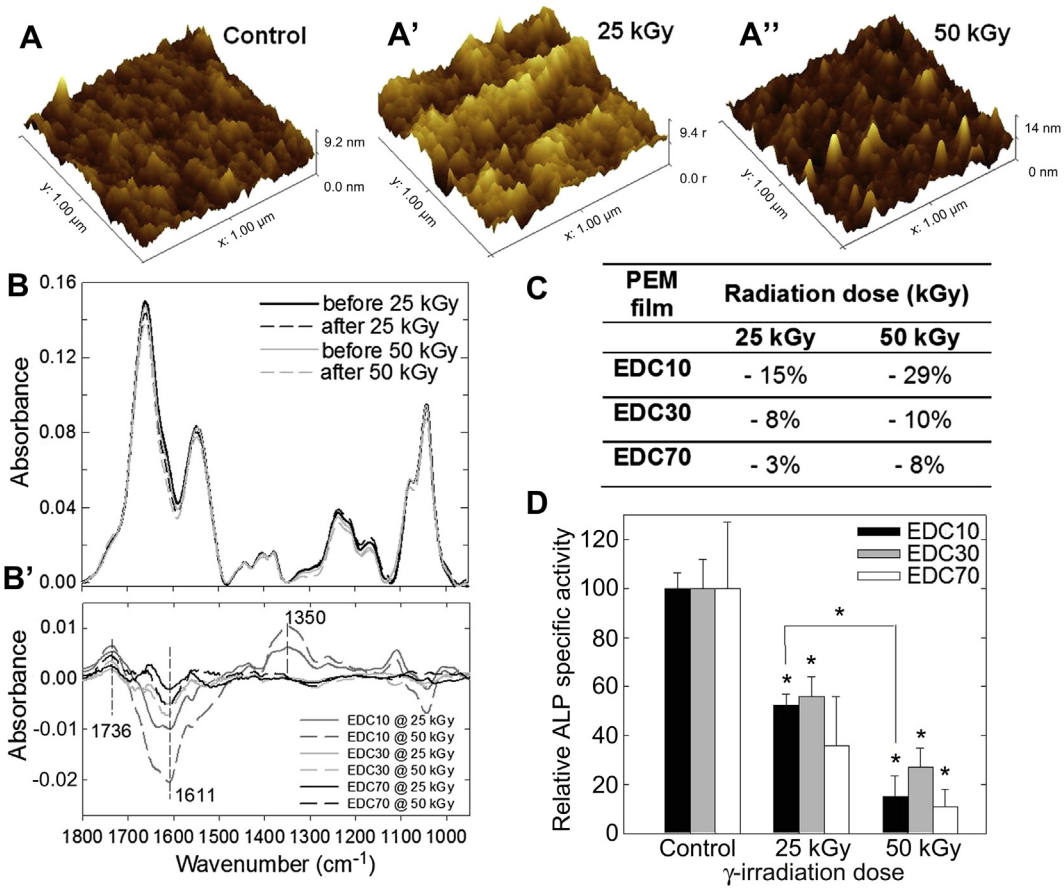

Fig. 4. Effect of γ-irradiation on PEM film stability and BMP-2 bioactivity. AFM topographic images of (A) PEM film@EDC30 before and after γ-irradiation at either (A′) 25 kGy or (A″) 50 kGy doses. (B) FTIR spectra of a PEM film@EDC30 before and after γ-irradiation at 25 and 50 kGy. (B′) Difference between the FTIR spectra acquired before and after γ-irradiation at 25 and 50 kGy. (C) Summary of the percent of decrease of the COO^- peak at 1611 cm^{-1} measured for the three different films at two different irradiation doses. (D) ALP activity of C2C12 cells cultured on BMP-2-loaded PEM that had been air-dried and γ-irradiated at either 25 or 50 kGy.

The explanted specimens were examined using micro-CT. The strong scatter effects from the metallic implant (causing the so-called inherent halation artefacts), however, rendered quantitative measurements of the new bone formation invalid. For this reason, bone formation was only quantified by histology. Representative histological images from sections of both bare and of PEM-coated scaffolds loaded with BMP-2 as well as from one pore channel from each type of scaffold tested are shown in Fig. 5A and A′, respectively. A first global examination of the explants revealed, as expected, that in the absence of BMP-2, no bone was observed in both the bare and film@EDC10 coated substrates (0/8 scaffolds). In addition, bare Ti with adsorbed BMP-2 did not lead to noticeable bone growth. Low and inconsistent bone deposits (3/8 scaffolds contained bone) were visible as spots inside the porous scaffold. This bone tissue was preferentially located in the junction of two orthogonally transversal pore channels (in 1 out of 4 channels). It was solely in the cases where BMP-2 was loaded in PEM films that large and consistent bone growth was observed (Fig. 5A). The bone formation was found to occur inside all the 2 mm length-pore channels of these samples (Fig. 5A′).

We noted that the quality of the formed bone in BMP-2 loaded scaffolds was similar, whatever the cross-linking extent of the PEM film and the treatment (drying and γ-irradiation). Predominance of woven bone with areas of some lamellar bone, proven by the presence of collagen strands, was observed (Fig. 5B and C). Both types of bone tissue contained embedded osteocytes and osteoblasts laying down osteoid tissue. Such observations were consistent with active new bone formation (Fig. 5C–D). Notably, the bone tissue was surrounded by vascularized marrow spaces containing adipose tissue (Fig. 5B). In addition, there was no inflammation in any section examined. Neither giant cells nor osteoclasts were observed indicating absence of bone tissue remodelling. Interestingly, bone tissue lined along the surfaces of PEM-coated Ti scaffolds regardless of both the type of PEM film tested and their pre-implantation treatment (air-drying and/or γ-irradiation) (arrows in Fig. 5B and D).

Histomorphometric analysis (Fig. 6) confirmed these observations. The bone formation areas were similar in all PEM-coated scaffolds that contained BMP-2. They were of the order of 10–30% as compared to 0.7% for BMP-2-adsorbed bare scaffolds ($p < 0.001$) (Fig. 6A). In addition, the bone-implant contact was similar in all BMP-2/PEM-coated scaffolds and varied between 7 and 27% (Fig. 6B). Similarly, the surface of TA6V scaffold in contact with the embedding host tissue did not also statistically varied between the different BMP-2/PEM groups. However, all the BMP-2 PEM films were significantly different from the control groups. Indeed, a very high tissue-implant contact (mean between 55 and 73%) was noted (Fig. 6C).

All together, these results provided evidence of the following: (i) the BMP-2 containing-PEM films coated onto TA6V scaffolds promoted the new bone formation; (ii) the degree of PEM film cross-linking did not affect the BMP-2 mediated bone induction; and, more importantly that (iii) drying and γ-sterilization treatments did not affect the osteoinductive potential of BMP-2 loaded within the PEM films as well as the osteo-conductive property of the PEM-coated scaffolds.

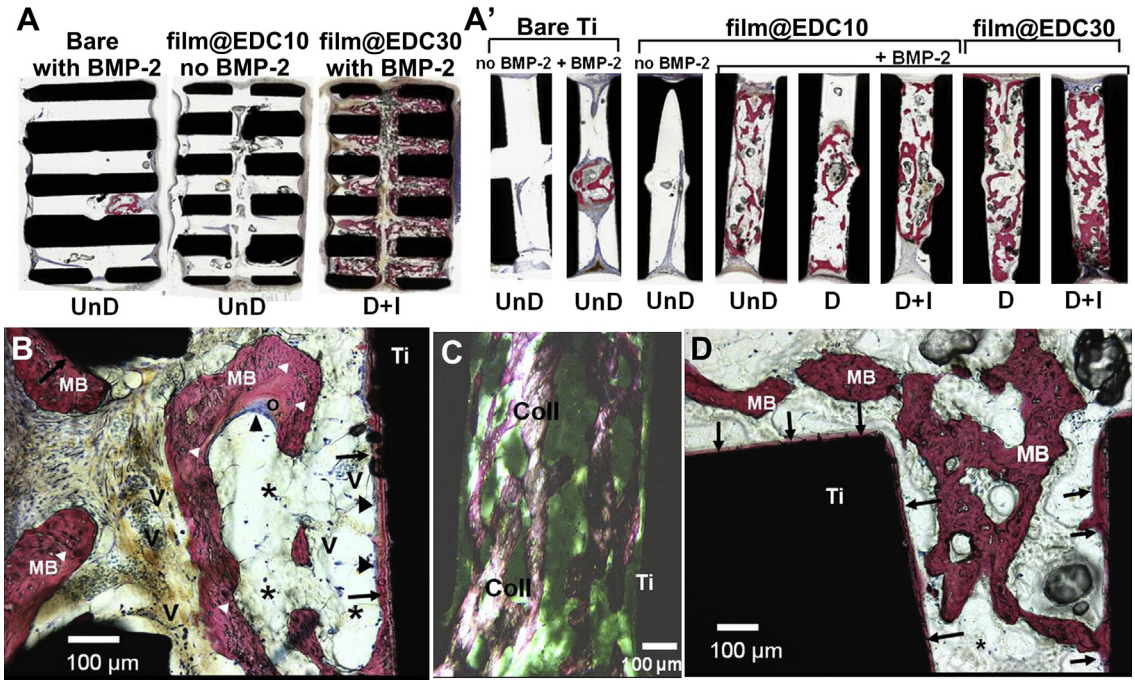

Fig. 5. Ectopic bone formation mediated by BMP-2-loaded PEM films coated on TA6V scaffolds 6 weeks post-implantation. (A) Representative histological cross-sections of the TA6V scaffolds that were either adsorbed with BMP-2 (left image), coated with film@EDC10 (no BMP-2) (middle image) or coated with film@EDC30, loaded with BMP-2, and then dried and irradiated (right image). (A′) Representative histological cross-sections of one pore channel of each type of scaffold tested (see Table 1 for the detail of the 8 different experimental conditions): TA6V scaffolds were coated with either PEM film@EDC10 or film@EDC30, then loaded with BMP-2. These scaffolds were either undried (UnD), dried (D), or both dried and γ-irradiated at 25 kGy (D + I) before implantation. Bare scaffolds either unloaded or loaded with BMP-2 as well as scaffolds only coated with film@EDC10 were used as controls. Histological analysis of tissue sections of scaffolds coated with BMP-2-containing dried film@EDC30 (B and C). Woven bone was present inside the channels (B) and lined on the film-coated surface (arrows in B). The presence of embedded osteocytes and osteoblasts laying down osteoid tissue is consistent with active new bone formation: (Ti) TA6V scaffold; (MB) mineralized bone; (v) blood vessel; (black arrowhead) osteoblasts; (white arrowhead) osteocytes; (o) osteoid; (*) bone marrow-like tissue; (Coll) collagen birefringence. Black arrow indicates apposition of bone at the Ti implant surface. (C) Corresponding cross-polarized light micrograph showing representative birefringence of collagen strands. (D) Histological analysis for film@EDC30 that has been dried and irradiated (same symbols as for (B)).

4. Discussion

Although BMP-2 is recognized as an important molecule for bone regeneration, its supraphysiological doses currently used in clinical practice have raised serious concerns about cost-effectiveness and safety issues [13,41]. Thus, there is a strong motivation to engineer new delivery systems or to provide already approved materials with new functionalities. Immobilizing the growth factor onto the surface of implants would reduce protein diffusion and increase residence time at the implantation site. To date, modifying the surfaces of metal materials, such as titanium or titanium alloys, at the nanometer scale to render them osteoinductive remains a challenging approach [16].

In the present study, our results provided evidence that the coating of titanium implants with BMP-2-loaded polyelectrolyte multilayer films conferred the implant surface with osteoinductive properties, promoting local bone formation activity. In addition, bone formation was also found to occur inside the 500 μm wide pore channels. Several strategies have already been attempted by other research groups to either graft or adsorb BMP-2 on the titanium surface using inorganic, organic, or hybrid organic/inorganic coatings [14,16,17]. Large amounts of BMP-2 (specifically, 5–8 μg/cm^2) can be adsorbed or grafted onto roughened Ti surfaces treated with chromosulfuric acid [42]. Proof of successful subsequent osteo-induction of such treated implants was obtained in a gap-healing model in the trabecular bone of the distal femur condyle in sheep. Inorganic coatings based on HAP are also very popular because of the high affinity of BMP-2 for HAP. In this case, the growth factor was either directly adsorbed onto or incorporated into the crystal latticework and is delivered as the inorganic layer undergoes degradation [21,23,43]. Biomimetic coatings based on biopolymers are also currently developed in view of their similarities with natural tissues. For example, collagen coatings of dental screws enhanced the bone-implant contact and peri-implant bone formation in dogs 3 months post-implantation [24]. Addition of BMP-2, however, did not increase the peri-implant bone formation in implants [24]. Other studies used BMP-2 immobilized on chitosan- [25] and on heparin-grafted titanium [26]. So far, effective osteo-induction in these cases was only shown *in vitro*. Due to the variation of findings between different studies, there is still a need for an optimal and stable carrier on the implant surface to provide sufficient retention of BMPs at the repair site. In addition, none of the above mentioned studies investigated the shelf-life duration and sterilization of the coating.

The results of the present study using cross-linked PEM films loaded with BMP-2 showed that various amounts of BMP-2 can be loaded in such films depending on the cross-linking extent and initial BMP-2 concentration (Fig. 2). An advantage of the process tested is that film deposition, and then BMP-2 loading, were both performed in mild conditions (only salt solutions, no solvent) using an automated dipping machine. The BMP-2 amounts loaded varied between 4 and 19 μg/cm^2 before rinsing of the films, and between 1.5 and 15 μg/cm^2 after the first release phase. The highly cross-linked PEM film (film@EDC70) trapped and retained more protein. BMP-2 bioactivity *in vitro*, however, was higher on less cross-linked PEM films (film@EDC10 and EDC30). This result may be explained by the different levels of hydration and/or smaller pore size in highly cross-linked films, causing either reduced accessibility to the BMP-2 molecules or partial denaturation of part of BMP-2 molecules.

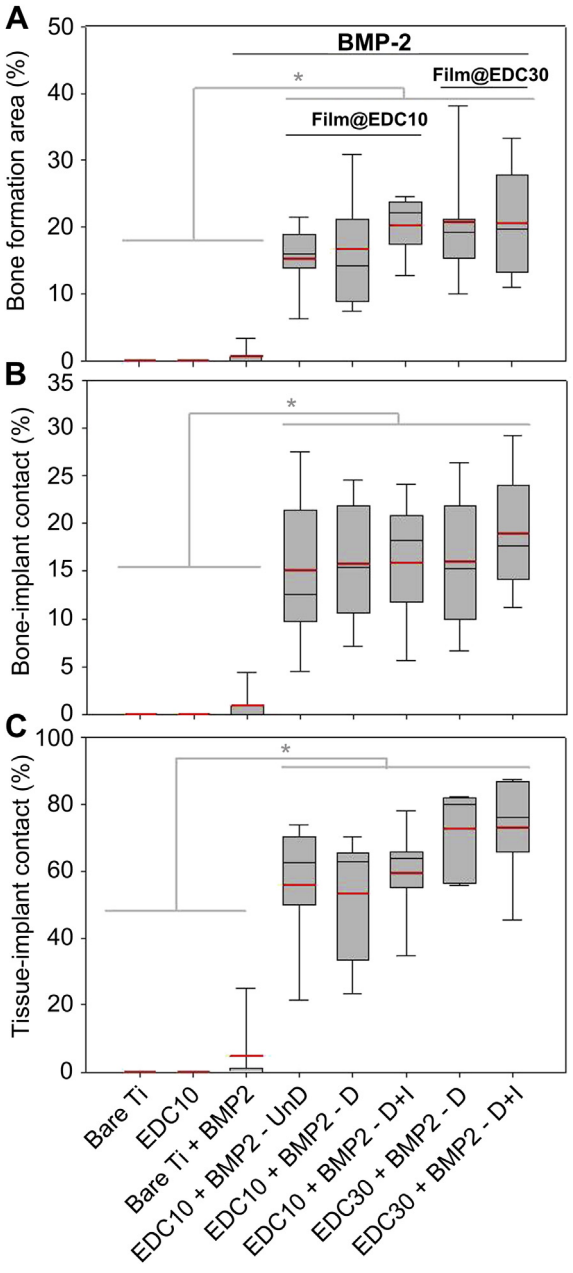

Fig. 6. Osteoinductive performance of the PEM-coated TA6V scaffolds. Box-and-whisker plots of (A) bone formation area given as a *% of the "available area" (by subtracting the titanium scaffold surface from the whole surface)*, (B) bone-implant contact, given as the *% of TA6V scaffold perimeter in contact to bone normalized over the Ti scaffold perimeter length* and (C) tissue-implant contact in each group of TA6V scaffolds tested determined by histomorphometric analysis. $^{*}p < 0.05$.

In addition to the amount of growth factor loaded and sequestered, packaging, and storage of the devices are important issues that may limit a wide application of biologically functionalized materials [32]. Growth factors present low stability under environmental conditions since they are heat sensitive and tend to be rapidly denatured. For these reasons, growth factors often have to be freeze-dried and stored in solid forms to achieve an acceptable shelf-life [44]. Currently, the shelf-life of BMPs is 2 years for BMP-7 (Osigraft®) and 3 years for BMP-2 (Inductos®) (data from EMEA). Nevertheless, their bioactivity after immobilization onto a substrate may be reduced. Information from literature reports regarding the effects of storage conditions and extent on the bioactivity of growth factors contained in coatings of implants is scarce. Immobilized transforming growth factor beta 1 (TGF-β1) on titanium alloys was shown to remain biologically active for at least 3 weeks upon storage at 4 °C [45]. Insulin growth factor I and TGF-β1 deposited on poly(D,L)lactide materials remained bioactive when stored dry at −20 °C for 5–14 months [46].

To date, there are no data available regarding the shelf-life of BMP-2 immobilized onto implant material surfaces. In the present study, we evaluated *in vitro* the bioactivity of retained BMP-2 by quantification of the alkaline phosphatase activity induction on C2C12 cells and showed that BMP-2 contained in PEM-coated Ti implants remained bioactive *in vitro* for at least 12 months once coated implants had been air-dried. No significant loss of BMP-2 bioactivity was observed for the films cross-linked@EDC10 and EDC30 upon storage while there was a significant decrease for the most cross-linked films@EDC70. This result may be explained by smaller pore size in highly cross-linked film and lower residual water content, thus leading to a lower protection of BMP-2 molecules or to conformational changes leading to protein denaturation. Indeed, we recently showed that BMP-2 secondary structure may be slightly changed upon drying of the PEM-loaded film [34]. Importantly, our *in vivo* results from the rat ectopic model provided evidence that BMP-2 loaded on both weakly cross-linked PEM films (film@EDC10 and EDC30) coated onto Ti implants that had been air-dried prior to implantation remained as osteoinductive as the undried implants.

Finally, prior to their clinical use as implants, medical devices have to be sterilized after packaging and must maintain their functional characteristics as well as their sterility after storage [31,47]. Among the different procedures available to sterilize medical devices, ethylene oxide is not recommended for natural materials. Indeed, sterilization of Ti implants coated with TGF-β1 by ethylene oxide at 42 °C for 12 h resulted in complete inactivation of TGF-β1 [45]. In addition, the doses of ethylene oxide required for sterilization purposes affect adversely the BMP-2 osteo-inductivity [48]. Because steam autoclaving and γ-irradiation are both sterilization processes considered to be safe with respect to chemical contamination, they are commonly used for metal implant materials. However, heat autoclaving is not recommended for heat-sensitive compounds, such as polymers and proteins. In contrast, γ-irradiation is the appropriate sterilization method for heat sensitive materials [40] because the temperature does not noticeably increased during or after the process [49]. For instance, conventional 25 kGy γ-sterilization procedure can be applied to ALP enzyme-grafted biomaterials without noticeable loss of ALP activity [32] Several other literature reports suggested that γ-irradiation is less detrimental for growth factor stability and, therefore, this method has already been applied to BMP-2 using an irradiation dose of 25 kGy [50–53]. Indeed, this dose is recommended by the European Medicines Agency [40]. For the sterilization of orthopedic Ti implants, a higher dose at ~50 kGy is usually used. However, no study has attempted to sterilize BMP-coated implants.

In the present study, we assessed the osteoinductive performance properties of the BMP-2 contained in PEM-coated Ti implants *in vitro* and *in vivo* upon sterilization by γ irradiation. Our data showed that sterilization had a little effect on the structure of PEM film itself, depending on the film cross-linking extent. Film@EDC10 had an infrared signature after γ-irradiation, which was similar to that of a slight additional cross-linking (Fig. 4). Such findings are consistent with literature reports of cross-linking of polymers upon γ-irradiation [50]. Furthermore, we showed that γ-irradiation had a dose-dependent effect on the expression of ALP by C2C12 cells *in vitro*. Regardless of the cross-linking extent, the BMP-2 sequestered in PEM films maintained 50% of its initial bioactivity after γ-irradiation at 25 kGy, while it preserved only 20% of its

initial value at 50 kGy. Interestingly, the *in vivo* studies showed that the osteoinductive potential of BMP-2 contained in PEM-coated Ti implants was fully preserved after implant sterilization at 25 kGy. Similar amounts of new bone tissue were formed in both non-irradiated and irradiated implants. This result suggested that, although some BMP-2 bioactivity may have been lost during the irradiation process, the remaining amount of bioactive BMP-2 was still high enough to induce *de novo* bone tissue.

Altogether, our results provided evidence of the remarkable property of PEM film coatings that both sequester BMP-2 and preserve its full *in vivo* osteoinductive potential upon both storage and γ-sterilization. Although not fully understood, the protective effects of PEM films on the growth factor bioactivity may be attributed to both the high water content in (PLL/HA) films (~90%) and to their porosity, which may provide a "protein-friendly" environment. The biopolymeric PEM film appears to mimic the natural extracellular matrix that preserves the BMP-2 bioactivity.

5. Conclusion

Our results showed that the (PLL/HA) PEM film coating can both sequester BMP-2 and preserve its full *in vivo* osteoinductive potential upon both storage and γ-sterilization. This "off-the-shelf" technology of functionalized implants by layer-by-layer films loaded with BMP-2 opens promising applications in prosthetic and tissue engineering fields. The potential applications of such PEM-coated Ti materials featuring enhanced and/or accelerated osseointegration are numerous in orthopedic, dental and maxillo-facial surgery. In addition, such coatings may be coated on other materials used in bone-related clinical applications, including synthetic polymers. The coating may be translated to use in human since its components, *i.e.*, hyaluronan, poly-L-lysine, and BMP-2, are already approved by regulatory agencies for various biomedical applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.03.067.

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