3D-printed scaffold combined to 2D osteoinductive coatings to repair a critical-size mandibular bone defect

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ABSTRACT

The reconstruction of large bone defects (12 cm³) remains a challenge for clinicians. We developed a new critical-size mandibular bone defect model on a minipig, close to human clinical issues. We analyzed the bone reconstruction obtained by a 3D-printed scaffold made of clinical-grade polylactic acid (PLA), coated with a poly-electrolyte film delivering an osteogenic bioactive molecule (BMP-2). We compared the results (computed tomography scans, microcomputed tomography scans, histology) to the gold standard solution, bone autograft. We demonstrated that the dose of BMP-2 delivered from the scaffold significantly influenced the amount of regenerated bone and the repair kinetics, with a clear BMP-2 dose-dependence. Bone was homogeneously formed inside the scaffold without ectopic bone formation. The bone repair was as good as for the bone autograft. The BMP-2 doses applied in our study were reduced 20- to 75-fold compared to the commercial collagen sponges used in the current clinical applications, without any adverse effects. Three-dimensional printed PLA scaffolds loaded with reduced doses of BMP-2 may be a safe and simple solution for large bone defects faced in the clinic.

1. Introduction

To date, autologous bone graft remains the major clinical solution to treat extensive bone loss and trauma [1], but it is hampered by several drawbacks, including limited availability, pain for the patient, additional healing time, and donor site morbidity. Tissue engineering using synthetic scaffolds, bioactive factors, and/or stem cells offers alternative therapeutic strategies and holds promise for bone regeneration [2], but the repair of large bone defects (around 5 cm³) remains challenging [3]. In particular, for large bone defects, a structural synthetic scaffold may not be enough to support complete regeneration. Ceramics, notably composites of hydroxyapatite (HAP) and tricalcium phosphate (TCP) are the most biomimetic scaffolds [4], but are brittle and exhibit some variable biodegradability. Besides, they induce a basic level of bone formation [5]. Metals such as titanium are interesting for their mechanical properties [6] but can yield to stress shielding and are not biodegradable. The use of polymers has expanded, in view of their versatility, tunable mechanical properties, and biodegradability. To date, polycaprolactone (PCL) and polylactic acid (PLA) derivatives are the most widely used in bone tissue engineering [7].

Very interestingly, recent developments in additive manufacturing enable the design of custom-made three-dimensional (3D) architectured scaffolds that can be adapted to the defect size [8] and are easier to implement from a regulatory perspective [9]. Polymers are particularly

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well-suited for additive manufacturing of scaffolds [10]. They can be manufactured in the form of filaments and 3D printed using several techniques, including fused deposition modeling (FDM) [2,11]. The 3D architecured scaffold plays the role of a space filler that should be mechanically stable enough to enable bone ingrowth inside the pores of the scaffold. However, for large defect areas, a structural scaffold may not be enough to support complete regeneration.

In these cases, stem cells or exogenous factors can be added to the scaffold to enhance regeneration [12]. Using stem cells in combination with scaffolds appears to have potential because of their secretion of factors [13], but is more complicated to set up because different steps are required to harvest the cells from the patients, expand them in culture, and finally implant them back into the patient. As an alternative to stem cell implantation, the use of growth factors aims to recruit stem cells directly to the site of implantation. So far, bone morphogenetic protein 2 (BMP-2) has been the most widely studied clinically approved protein because of its ability to directly target BMP receptors at the cell surface and trigger stem cell differentiation in bone [14,15]. BMP-7 has also been used in combination with TCP/PCL scaffolds [5]. The challenge is to optimize the dose of BMPs to avoid possible side-effects [12,16] that can lead to inflammation and ectopic bone [17]. Recently, a BMP/activin A chimera (BV-265) has been developed with increased receptor binding [18]. Used in a composite matrix made of HAP granules and collagen I, BV-265 improved bone repair in non-primate bone defect models, and allowed a 30-fold decrease in dose compared to BMP-2.

As an alternative to the incorporation of BMPs inside a carrier, surface coatings appear interesting [19,20] in that they enable to decouple the 3D scaffold architecture from the two-dimensional (2D) osteoinductive coatings. In our previous study [21], we showed that it is possible to repair a critical-size femoral bone defect in rats by combining a polymeric scaffold (3D hollow tube) with an osteoinductive surface coating using a polyelectrolyte film coating as a BMP-2 carrier.

An important step toward the clinical translation of a tissue-engineered construct is its preclinical testing on critical-size bone defects in large animals [2], which are needed to repair large bone volume defects (typically bone defects with a volume above 5 cm³). To date, preclinical models still need to be improved [22] and are limited when it comes to the clinical translation of results [3,22]. Less than 13% of the studies are performed in large animals (sheep, goat, dog, and pig) and the vast majority do not use skeletally mature large animals [3].

Here, we designed a 3D polymeric scaffold made of clinical-grade PLA combined with an osteoinductive surface coating containing a tunable dose of BMP-2 to repair a critical-size bone defect in minipig mandibles [23]. The clinical-grade poly(L-lactide) (PLA, Poly-Med, Inc, Lactopore®) 100 M Monofilament 1.75 mm) were fabricated by FDM (3DXP - One). PLA filaments of ~400 μm in diameter and 200 μm in height were deposited following a +45°/-45° pattern with an inter-spacing distance of ~2 mm. The melting temperature for PLA 3D printing was 190 °C. Scaffolds had a porosity of 85%, with fully interconnected pores. After their fabrication and before their coating with polyelectrolyte films, the scaffolds were stored away from moisture in a desiccator with a silica gel.

To render the scaffolds osteoinductive, they were coated with polyelectrolyte films made of 24 alternating bilayers of poly(L-lysine) (PLL, Sigma, France) and hyaluronic acid (HA, Lifecore, USA), as previously described [21]. Briefly, the polyelectrolyte films were deposited layer-by-layer with a DR3 dip-coating robot (Riegler & Kirstein GmbH). A first layer of poly(ethylenimine) (PEI, Sigma-Aldrich, France) was deposited by hand using a concentration of 5 mg/mL, followed by alternated layers of HA at 1 mg/mL and PLL at 0.5 mg/mL using the robot. The film crosslinking level was controlled by incubating the coated scaffolds in 30 or 70 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma, France). The film crosslinking level has an effect on the film stiffness, as shown in previous studies [23], EDC30 films being softer than EDC70 films. After UV sterilization of the film-coated scaffolds, BMP-2 (InductOs, Medtronic) was postloaded in the polyelectrolyte films at increasing loading concentrations of 20, 50, or 110 μg/cm³, as previously described [21,24]. Finally, the osteoinductive scaffolds were rinsed, dried, and stored away from moisture in a desiccator with a silica gel until implantation.

2.2. Characterization of polyelectrolyte films and quantification of BMP-2 loading

Fluorescence microscopy and scanning electron microscopy (SEM) were used to characterize the film coating on the scaffolds. The effective coating of the air-dried polyelectrolyte films on the PLA scaffolds was assessed, after scratching the film with a needle, by SEM imaging with an FEI-Quanta 250 SEM-FEG in high vacuum at 15 keV using the Everhart-Thornley detector, as previously described [21,25]. Regarding fluorescence microscopy, the film-coated scaffolds were labeled with PLLFITC, as previously described [26]. They were imaged using a Leica Macrofluo (Z16 Apo) fluorescence system using a 0.8X objective [25] and a Zeiss LSM 700 confocal microscope with a 10X objective to assess the homogeneous coating of the film.

The quantification of BMP-2 initially loaded in the polyelectrolyte film (deposited at the bottom of a 96-well microplate) was performed using a micro bicinchoninic acid assay (µBCA) test for low BMP-2 concentration (BMP20), whereas Nanodrop (Thermo Fisher) was used for higher concentrations. The concentration of BMP-2 in the loading solution was measured initially and then after 1 h and a half of incubation with the film-coated scaffold. The loaded amount, corresponding to the difference between these two values was also expressed as μg of protein per volume of scaffold (μg/cm³). The quantity of BMP-2 effectively loaded onto the scaffolds was then deduced from this quantification (Table 1). The percentage of BMP-2 release in vitro after several washes with a physiological buffer (HEPES-NaCl) was determined by fluorescence spectrometry using BMP-2 carboxyfluorescein (BMP-2CF).
isoflurane. Animals were placed in supine position, the mandibular area was shaved and prepared with an iodine scrub. The mandibular body was exposed via a submandibular approach, leaving the periosteum on the bone. Bone resection was made with an oscillating saw, creating a full-thickness bone defect of $4 \times 3 \text{ cm}^2$, including the periosteum, and penetrating the mandibular nerve canal. This resection was standardized using a phantom (an uncoated implant) to delimit the perimeter of resection. Two titanium reconstructive plates (Stryker 2.8 system, Freiburg, Germany) and twelve 2.7 mm diameter screws were used to stabilize the mandible by triangulation, before insertion of the implant in the defect (Stryker Leibinger GmbH & Co, Freiburg, Germany).

The implant was fixed on the titanium plate using 2/0 nylon stitches (Fig. 1). When a bone graft was used (positive control group), it was harvested from the iliac bone and fixed on the plate using two screws. The wound was closed in three layers without drainage. Both sides of the mandible were operated on in the same way.

Aftercare included Morphone 0.2 mg/kg injected subcutaneously according to clinical symptoms, a Fentanyl patch (50 μg/h) changed every 3 days for a period depending on the residual pain, antibiotics (Amoxicillin and Clavulanic acid 12.5 mg/kg) intraorally for 15 days, and Meloxicam 0.4 mg/kg for 3 days. The animals were followed up for 13 weeks in individual boxes. A veterinarian clinically evaluated the animals every day, three times per day during the first 2 days after surgery and once per day after. Analgesia was adapted according to symptoms. The animals were weighed once a week. Special attention was given to re-feeding and weight evolution. A complete blood analysis was performed before surgery, immediately after surgery, and once a week until euthanasia to assess an eventual general inflammatory reaction, or liver or kidney complications due to BMP or due to the resorption of the film and scaffold. Complete blood count, haptoglobin, and protein electrophoresis were measured to assess inflammation and hemostasis, whereas aspartate aminotransferases (ASAT), alanine aminotransferases (ALAT), alkaline phosphatase (ALP), gamma-glutaminate transferase (GGT), and bilirubinemia were used to assess hepatic function. Serum creatinine and urea were quantified to assess renal function.

To optimize bone regeneration and the operating techniques, we performed a preliminary experiment in the minipig mandibles. We thus screened six conditions on three minipigs ($n = 1$): two crosslinking levels EDC30 and EDC70, two BMP-2 initial loading concentrations of 20 and 110 μg/cm$^3$, and two negative controls: i) empty defect to assess the critical size of the defect, and ii) film-coated scaffold (EDC70) without BMP-2. Each implant was randomized, implanted, and analyzed in a blind manner using computed tomography (CT) scans, the poly-electrolyte films being macroscopically indistinguishable. All analyses of experimental groups were also made in a blind manner.

In a second main experiment, two conditions were further studied in larger groups to perform statistical analysis (EDC30 was used for all groups). The lowest BMP-2 dose was increased from 20 to 50 μg/cm$^3$ to reach better bone regeneration ($n = 6$) and the highest BMP-2 dose was kept (110 μg/cm$^3$; $n = 5$). Another negative control was added (EDC30 film-coated scaffold without BMP-2), and bone autograft (BG; $n = 4$) was added as a positive control (see Table 2 for all experimental conditions).

After the last CT scan (day 91), the animals were euthanized by Pentobarbital injection. The area around the mandible was examined attentively to notice eventual complications (fistulas, collection, inflammation, etc.).

Using the same submandibular approach, the titanium plates and screws were removed. On each side, a full-thickness sample of bone was removed using a surgical guide (Fig. 1) to get a margin of 1 cm native bone all around the initial implant. The bone pieces were fixed in 4% neutral-buffered formalin (Sigma, France) for 1 week at $4^\circ$C.

2.4. CT scan and micro-CT analysis

Assessment of bone formation was made by CT scan after 30, 50, and 90 days in the preliminary experiment and after 16, 30, 51, and 91 days in the main experiment. These scans were acquired using a helicoidal BrightSpeed 16 scanner (General Electric) under gaseous anesthesia.

First, a CT-scan score was defined by the authors considering four criteria: the percentage of filling of the porous implant (F), the homogeneity of the newly formed bone (H), the ability to distinguish between cortical and cancellous bones (D), and the amount of ‘ectopic’ bone (bone outside the implant) (E). Each criterion was evaluated in a blind manner by four clinicians using a score between 0 and 4 (0 being the lowest grade and 4 the highest). Then, the global score (S) was calculated by each clinician and for each CT scan made during the follow-up period.

**Table 1**

| Targeted BMP-2 loading (μg/cm$^3$) | Total BMP-2 loaded (μg/implant) | Volumetric dose (μg/cm$^3$) |
|---------------------------------|---------------------------------|------------------------------|
| Preliminary experiment          |                                |                              |
| BMP20 ($n = 2$)                 | 20                             | 240                          | 20                           |
| BMP110 ($n = 2$)                | 110                            | 870                          | 72.5                         |
| Second experiment               |                                |                              |                              |
| BMP50 ($n = 6$)                 | 50                             | 326 ± 80                     | 27                           |
| BMP110 ($n = 5$)                | 110                            | 1000 ± 64                    | 83                           |

The total volume of the scaffold was 12 cm$^3$ and its effective surface measured by $\mu$CT was 144 cm$^2$. We targeted a BMP-2 loading (unit mass per unit of scaffold volume in μg/cm$^3$). The total amount of BMP-2 effectively loaded was calculated for each implant and reported in ‘mass per volume of implant’ (μg/cm$^3$).
More weight was given to the criteria F and H related to bone growth. Finally, the global score was represented as the mean score ± standard deviation (SD) of the four scores given by each clinician independently.

Second, a quantitative analysis of bone growth was performed using the software InVesalius 3 (Centro de Tecnologia da Informação Renato Archer, CTA). The volume of interest (VOI) was defined as the total volume of newly formed bone inside and around the bone defect. BG was used as a positive control. Based on the visual observations, we decided to segment and define two types of bones: poorly and highly mineralized.

To quantify these two fractions, the VOI was segmented using a global threshold, namely between 230 and 629 HU for poorly mineralized bone and values >630 HU for highly mineralized bone. The volumes of the newly formed bone were expressed in cm³. For the quantification of the bone volume inside and around the bone defect. BG was adjusted to the volume of the scaffold. Then, the volume of bone inside this new VOI was subtracted from the total bone volume formed.

Third, micro-computed tomography (µCT) imaging was performed on fixed mandibular explants after 90 days, using high-resolution scanner (vivaCT 40, ScancoMedical, Switzerland), as previously described [21]. The acquisition parameters were set at 70 kV with an intensity of 114 mA, a 100-ms integration time, and an isotropic voxel size of 76 μm. The VOI was defined as the volume of the initial bone defect in the mandible (4 × 3 × 1 cm³). Bone volume was determined after segmentation using threshold values (438–2730 mg HA/cm³) and Gaussian filter (sigma 0.8, support 1). Bone mineral density (BMD, mg HA/cm³) and the ratio of bone volume divided by total VOI (BV/TV) were calculated.

2.5. Bone homogeneity score

The implant of well-defined dimensions (4 cm × 3 cm × 1 cm, total volume TV of 12 cm³) was taken as the region of interest (ROI). It was separated into 10 slices of equal thickness along each axis (X, Y, and Z). For each slice, the bone volume ratio (BVr = BV/TV) was calculated as the volume of bone inside one slice (BV) divided by the volume of the slice of interest (corresponding to TV/10). This quantification was done for each axis (Fig. SI 9): the SD of BVr was calculated and the homogeneity score (HS) was defined as the sum of the three SDs over X, Y, and Z axes.

2.6. Histology and histomorphometry

The specimens were dehydrated in a graded series of alcohol and embedded in methylmethacrylate. Three slices in the XZ plane were cut with a laser microtome (TissueSurgeon, LLS ROWIAK GmbH, Hannover, Germany) [27] and stained with Sanderson’s rapid stain and Van Gieson’s staining. Bone appears in pink/orange, whereas mesenchymal tissue appears in blue or yellow. Slice thickness was 10–100 μm. Sections were imaged using a slide scanner (Aperio AT2, Leica Biosystems Imaging, Inc). Histological examination was performed by a pathologist using an Olympus BX51 microscope with transmitted or polarized light. The histomorphometry analysis was performed in a blind manner by three independent operators. The operators gave a visual approximation of the bone area to total area ratio since a systemic analysis was not possible because of the high differences in staining between the different sections, named hereafter S1, S2, and S3.

2.7. Statistical analysis

OriginPro (OriginLab, Excel (Microsoft Office), and R for Mac OS X (R Foundation for Statistical Computing, CRAN) were used for all analyses. Data were expressed as mean ± SD. Non-parametric data were presented by median and interquartile range. Differences between groups were assessed by analysis of variance and Bonferroni post-hoc analysis or Student’s t-test. Differences between groups at p<0.05 (*) and <0.01 (**) were considered as significant.

3. Results

3.1. 3D architectured scaffolds combined with an osteoinductive surface coating to repair a critical-size mandibular bone defect

Parallelipedal scaffolds (4 × 3 × 1 cm³, for a total volume of 12 cm³) were made of clinical-grade PLA and custom-fabricated using FDM. The PLA filaments of ~400 μm in diameter were deposited following a +45°/−45° pattern with an interspacing of 2 mm, resulting in a scaffold porosity of 85% with fully interconnected pores (Fig. 1A) to enable the transport of fluids and nutrients in the core of the scaffold. This choice has been made after testing in laboratory several types of scaffold architectures (data not shown). The variations concerned their trabecular thickness from 1 to 2.5 mm, porosity from low to high porosity, and geometry. Their compression resistance, surface state, and fixation possibilities (suturing) were qualitatively tested. We also reasoned based on our preliminary study using film-coated poly(lactic-co-glycolic) acid PLGA hollow cylinders that showed that 3 mm diameter cylinders enabled bone formation inside the empty space of the cylinder [21].

As an implantation site, a large critical-size minipig mandibular bone defect (12 cm³; Fig. 1B) was chosen because the pig mandible mimics the human mandible [28,29]. This was a full-thickness defect on the basilar anterior location was chosen because it has been shown that the extent of defect regeneration from spontaneous healing was significantly less in the posterior than in the anterior mandibular defects [30]. Buccal and vestibular periosteum were removed around the defect to avoid spontaneous ossification. In addition, the animals were mature, older than 24 months, without any remaining growth potential. This age was selected as our previous preliminary study on four minipigs aged 6–8 months had shown some spontaneous ossification of the defect spreading from the edges (data not shown). Thus, choosing animals older than 24 months ensured that bone maturation had occurred and that new bone formation would not result from the endogenous bone formation resulting from the animal growth. The implant was inserted in the defect and fixed using nylon stitches on the two thick titanium plates used to stabilize the mandible by triangulation; 12 screws fixed the plates (Fig. 1B).

The homogeneous coating of the scaffold by the polyelectrolyte film was visualized by fluorescent labeling of the film with PLLFITC and imaged using a fluorescence microscope (Fig. 2A) and confocal microscopy (Fig. 2B). As observed by SEM after scratching the film using a needle, the polyelectrolyte film fully coated the PLGA filaments (Fig. 2C).

The amount of BMP-2 initially loaded in the polyelectrolyte film and the percentage of BMP-2 released in vitro were determined by fluorescence
spectrometry using fluorescently labeled BMP-2 (Fig. 2D and E). This in vitro release study allowed having a general idea of the initial release, though the in vivo behavior may be different on the long term. The amount of BMP-2 adsorbed in the film increased with the initial concentration of BMP-2 in the loading solution before reaching a plateau. This plateau was reached faster for the EDC70 film (Fig. 2D), meaning that the EDC30 film was able to adsorb more BMP-2 than the EDC70 film. The amount of BMP-2 released from the film was higher for the EDC30 film: the maximum percentage of BMP-2 released from the film was ~50% for EDC30 films, compared to ~20% for EDC70 films (Fig. 2E). It thus appears that EDC30 films could adsorb and then release more BMP-2 than more crosslinked films.

### 3.2. A first preliminary experiment to validate the mandibular bone defect model and select the film coating conditions

Six different conditions ($n = 1$ for each condition) were initially screened to validate the bone defect model and optimize the selection of the film crosslinking level and BMP-2 loading. Two film crosslinking levels (EDC30 and EDC70) and two concentrations of BMP-2 (BMP20 and BMP110) were tested (Table 2). We chose these BMP-2 doses following a literature analysis, indicating that the studies were previously conducted in the range from 0.03 to 3 mg/cm$^3$ [16,44–47] and based on our previous studies in rat and rabbit [21,48]. Our aim was to reduce the BMP-2 dose compared to what was usually done. These concentrations, expressed in μg of BMP-2 per cm$^2$ of scaffold, were the targeted ‘volumetric’ concentrations of BMP-2. Knowing the effective surface of the scaffold (144 cm$^2$) and the amount of BMP-2 loaded in the polyelectrolyte film (Fig. 2D), the concentration of BMP-2 in the loading solution (in μg/mL) into which the scaffold was dipped was defined. The amounts of BMP-2 that were effectively loaded in the film-coated 3D scaffolds were quantified (Table 1). Two negative controls were added: an empty defect without any implant, and a defect with a film-coated implant (crosslinking level EDC70) but without BMP-2.

The animals were in good health. There was no postoperative infection, implant failure, or sign of blood disorder. All the surgical procedures...
were uneventful and there were no surgical complications. For one implant, it was necessary to recut the anterior border of the defect to improve the fit of the implant. All the titanium plates were stable and fixed to the native bone (no loosening). During explantation, it was impossible to macroscopically identify the implant from bone reconstruction or scar tissue. The complete blood analysis performed on three different minipigs did not reveal any abnormality (Fig. SI 1). We concluded that the scaffold with or without the film and/or BMP-2 did not cause a general inflammation, a hepatic reaction, or a renal function impairment.

CT scans were acquired during the follow-up period (Fig. SI 2). For each acquisition, a CT-scan score was given in a blind manner and independently by four clinicians (Fig. 3A and B). Bone grafts were not scored because bone homogeneity and cortical/cancellous differentiation would not change during the study and because they did not produce ectopic bone. All BMP groups exhibited bone regeneration, regardless of the crosslinking level of the film and the BMP-2 loading concentration, whereas the negative controls did not show any bone formation (Fig. 3A and B). The CT-scan scores were plotted as a function of time and fitted an exponential function. The CT-scan score was used to calculate a plateau value (Bmax) and a characteristic time to reach the plateau (t), by fitting an exponential function to the experimental data [21]. For the EDC30 films (Fig. 3A), the scores steadily increased before reaching Bmax, which was higher for the BMP110 than for BMP20 (4.8 ± 0.4 vs. 1.4 ± 0.0, respectively). t was approximately 2.6 times faster for the lower dose than for the higher dose (21 ± 2 days vs. 55 ± 10 days). In contrast, for the EDC70 films, the exponential fit to the data was poor for the highest BMP-2 concentration, whereas the negative controls did not show any bone formation (Fig. 3B). For the lower BMP-2 dose, Bmax was at 3.3 ± 0.4 and t was 25 ± 10 days. These data indicate that bone repair is BMP-2 dose-dependent with EDC30 films but not with EDC70 films where scores are similar for BMP20 and BMP110 conditions. With the two film...
films with a BA/TA ratio of 1.5 ± 1% for BMP20 and 40.8 ± 1.4% for BMP110 (Fig. SI 6F). Surprisingly, BA/TA significantly decreased with the BMP-2 dose for the EDC70 films (25.8 ± 3.8% for BMP20 vs. 12.5 ± 1.5% for BMP110).

Altogether, our results established the critical size of the mandibular bone defect, the difference in bone repair kinetics depending on the BMP-2 dose, and the influence of the film crosslinking level on the amount of newly formed bone. A clear BMP-2 dose-dependence was evidenced for the EDC30 films. This first preliminary experiment allowed to optimize our film coating parameters for the second main experiment.

### 3.3. The second main experiment shows that the BMP-2 dose influences the bone repair kinetics and the amount of highly mineralized bone

In view of the results of the preliminary experiment showing that BMP20 lead to only little bone formation and EDC70 induced lower bone regeneration, we decided to pursue the next study with only EDC30 films and increased the lowest BMP-2 dose from 20 to 50 μg/cm³. The experiments were next repeated with more minipigs per condition (Table 2) to assess quantitatively the effect of BMP-2 dose. The highest dose BMP110 was kept (n = 5) and BMP50 was added (n = 6). BGs were added as a positive control (n = 4), and a film-coated scaffold without BMP-2 as a negative control (EDC30 film). An additional earlier time point (D16) was also added for the CT-scan acquisitions.

Once again, there was no surgical complication. In two cases, the BG was in two pieces because of the small size of the iliac bone, but in all cases, the defect was completely filled. In two cases (BG), a small serous collection was found around the implant, and in one case (high dose of BMP-2), a small suppurated collection with a cutaneous fistula appeared at D90.

![Fig. 4. Representative 3D reconstructions of the CT scans showing the kinetics of bone regeneration for four representative conditions: negative control (Ctrl -), film-coated scaffold without BMP-2 in the film), film-coated scaffold with a low BMP-2 dose (BMP50) and a high BMP-2 dose (BMP110), and BG. Scale bar is 4 mm.](image)

![Fig. 5. Quantitative analysis of the kinetics of bone formation followed by CT for EDC30 films loaded with two BMP-2 doses. The film-coated scaffolds were loaded with BMP-2 at 50 (n = 6) and 110 μg/cm³ (n = 5) and their bone regenerative capacity was compared to bone autograft (BG; n = 4).](image)

(A-C) Box plot representations of the total bone volume (A), poorly mineralized (B), and highly mineralized bone volumes (C) as a function of the BMP-2 dose BMP50 vs. BMP110 in comparison to BG. (D) CT-scan scores as a function of time and corresponding exponential fits to the data (colored lines) for EDC30 films; corresponding plateau value (Bmax), characteristic time (τ) deduced from the fits, and fit quality R are given in the table. (E) % of bone outside the implant (named “ectopic bone”) as a function of time for BMP50 and BMP110. *p<0.05; **p<0.01.
The amount of bone progressively increased when increasing the BMP-2 dose, and the newly formed bone entirely filled the pores of the 3D scaffold in a homogeneous manner for the highest dose. There was no sign of excessive ectopic bone formation, even at the highest dose. This was also visible on the 3D reconstructed μCT images (Fig. 7A and Fig. SI 7). The mean bone volume was higher for BMP110 (7.6 cm³) than for BMP50 (4.8 cm³) (Fig. 7B), and was also higher than for the BG reference but not significantly. In addition, bone regeneration was more dispersed with BMP50, with a variation coefficient of 34% compared to 13% for BMP110. When plotting all the experimental bone volumes as a function of the BMP-2 total dose per implant, a linear correlation was found (Fig. 7C), confirming the BMP-2 dose-dependence of bone regeneration. BMD was not significantly different for the different doses but was significantly higher for BMP50 than for BG (Fig. 7D). Furthermore, the amount of bone grown outside of the implant did not depend on the BMP-2 dose (Fig. SI 8), which confirmed what was found on CT scans. Finally, the HS of bone inside the scaffold was similar for BMP50 and BMP110 (Fig. 7E and Fig. SI9) and showed the homogeneous reconstruction of bone using the implants. These results showed that the 3D-printed PLA scaffolds coated with osteoinductive coatings and loaded with BMP-2 lead to a full bridging of the critical-size mandibular defect, with performances similar to BG. The best performances were achieved in the BMP110 group. The quantitative results of the CT scan analysis and the μCT analysis were compared to the CT-scan score (Fig. SI 10). The linear correlation between the quantification of the new bone volume using μCT acquisitions and the CT-scan score had a regression coefficient $R^2 = 0.82$ (Fig. SI 10B). This confirmed the usefulness of scoring by clinicians, which is a simple method to implement, leading to interesting results.

The histological examination (Fig. 8) revealed that when no BMP-2 was present, only mesenchymal tissue (m) was formed (Fig. 8A and B). With BMP50, the amount of new bone was low and mesenchymal tissue was visible (Fig. 8C and D). The presence of mature bone with a characteristic Haversian structure in BMP110 implants was evidenced (Fig. 8E-G). Imaging under polarized light (Fig. 8G) allowed a better visualization of the Haversian canals (highlighted by asterisks *) and the connections between osteocytes. Furthermore, the interface between the host bone and the newly formed bone was visible (Fig. 8H-J with dashed lines in Fig. 8I and J), since the host bone had a more lamellar structure than the newly-formed bone (Fig. 8J). Some bridges were visible between the two types of bones (white arrows in Fig. 8J), which may contribute to increasing the mechanical resistance of the newly formed bone. In some cases, especially for BMP110, the difference between native and new bone was not even distinguishable (data not shown). In the case of BG, host bone and grafted bone were in direct contact (Fig. 8K-M) or separated by mesenchymal tissue and new bone showed a Haversian structure (Fig. 8L and M). The bone area over total area (BA/TA in %) was quantified based on these images (Fig. 8N). In agreement with the CT and μCT quantifications, more bone was formed for BMP110, whose median value was similar with BG. The homogeneity of the newly formed bone inside the 3D architecture implant was quantified (Fig. 8O). Here again, bone formation was similar for the three sections of the sample, proving the homogeneity of bone formation. Finally, no local inflammation occurred because of the implant according to the absence of inflammatory cells and PLA degradation had not occurred yet (Fig. 8). These histological results confirmed the results from CT and μCT analyses showing that bone regeneration is BMP-2 dose-dependent. Moreover, BMP110 lead to similar bone formation as BG and new bone formed in this experimental group was mature, as assessed by the presence of Haversian canals.

Altogether, these data show that bone formation inside the 3D architected scaffold is spatially homogeneous, and that there is a
significant BMP-2 dose-dependent bone formation. BMP-2 mostly influences the formation of mineralized bone and does not induce the formation of ectopic bone.

4. Discussion

Before applying new osteogenic technologies in clinical practice, it is necessary to confirm their effectiveness in preclinical studies using critical-size bone defects in relevant animal models. A critical-size bone defect is a defect that does not spontaneously heal for the duration of the study. Our aim was to create a critical-size bone defect close to clinical situations, particularly in maxillofacial surgery where it is frequently needed to fill defects over 10 cm³. Among the different animal models published, the pig is a very good candidate because the pig mandible mimics the human mandible in size, anatomy, form, and blood supply [29]. The bone physiology of the pig is very close to human bone physiology, with lamellar and Haversian structure. Several studies have reported on a mandibular defect in adult pigs. There is no real consensus on the critical size, it varied from 2 to 10 cm³ depending on the location (tooth-bearing area or not, full-thickness or not, anterior or posterior area, periosteum preservation or not ...) [31]. Otto and coworkers recently proposed a 6 cm³ mandibular defect [32]. We carried out a full-thickness defect on the basilar border of the mandibular angle. The defect measured 3 × 4 cm², in a portion of mandible that was 1 cm thick, for a total defect volume of 12 cm³. To the best of our knowledge, to date, there is no publication dealing with such a volume for a critical-sized bone defect in an animal model [3]. The buccal and vestibular periosteum was removed around the defect to avoid spontaneous ossification, and the age of the animals (24 months) precluded any growth potential as bone maturation had already occurred. The preservation of the alveolar ridge is another advantage of our model. The defect was created far from the teeth, and there was no opening into the oral cavity. This limits the risk for oral advantage of our model. The defect was created far from the teeth, and had already occurred. The preservation of the alveolar ridge is another...
Fig. 8. Histological examination and histomorphometry analysis. (A–M) Representative histological sections to show the structure of the newly-formed bone in the different groups. (A, B) In the absence of BMP-2 in the film, only mesenchymal tissue (m) was formed. (A) Global section of the negative control (film-coated PLA scaffold at EDC30 without BMP-2). (B) Larger magnification of the box in (A) showing mesenchymal tissue around PLA struts. (C, D) With low BMP-2 dose (BMP50), new bone (NB) was formed along with mesenchymal tissue. (C) Global section of the BMP50 condition. (D) Larger magnification of the box in (C). (E–G) With higher BMP-2 dose (BMP110), the formation of new bone was evidenced. (E) Global section of the BMP110 condition where newly formed bone was present in high quantity (orange/pink staining). (F) Larger magnification of the box in (E) where the Haversian structure of newly formed bone was visible (Haversian canals are shown with an asterisk *), (G) and particularly evidenced under polarized light along with both lamellar (LB) and woven bones (WB). (H–M) Representative histological sections to show the contact between host bone and new bone. (H–J) Contact between host bone (HB) and new bone is shown for BMP110 condition with (H) the global section, (I) a larger magnification of the box in (H) showing the contact between host bone and new bone on a transmitted light image evidenced by a dashed line. (J) The HB/NB interface was clearly visible under polarized light, where bony bridges could be observed (white arrows). (K–M) Global section, transmitted and polarized light images for BG. The contact between HB and NB as well as the Haversian structure were evidenced. (N) Quantitative analysis of the bone area over total area (BA/TA). (O) Amount of bone over total area (BA/TA) inside each section of the implant (S1, S2, S3).
bone resorption and bone cyst formation, urogenital events (retrograde ejaculation, bladder retention), and wound complications [17,42,43]. The implication of BMP-2 in carcinogenesis is controversial. For all these reasons, it is important to reduce the dose of BMP-2 and to deliver it locally and progressively. Here, we considerably reduced the amount of BMP-2 with a dose of 240–1,000 μg per 12 cm² defect, which corresponded to 0.02–0.08 mg/cm² (1 mg/cm² is equivalent to mg/mL). This corresponds to a 20-fold (for BMP110) to 75-fold reduction compared to the commercially available collagen sponges, which are approved for a dose of 1.5 mg/mL. We chose these BMP-2 doses following a literature analysis, indicating that the studies were previously conducted in the range from 0.03 to 3 mg/cm² [16,44–47] and based on our previous studies in rat and rabbit [21,46]. Our aim was to reduce the BMP-2 dose compared to what was usually done.

Regarding the difference between EDC30 and EDC70 films in the BMP-2 dose-dependent bone regeneration (Fig. 3), we may hypothesize that it may be due to several reasons: i) the different stiffness of the EDC30 and EDC70 films, ii) the different loading of BMP-2 in the EDC30 and EDC70 films (Fig. 2D), the amount of BMP-2 loaded being higher for the EDC30 films at high BMP-2 concentrations, iii) the higher release for the EDC30 films as shown in Fig. 2E, iv) the fact that BMP-2 internalization by cells is higher for EDC30 than EDC70 [49]. To note, we did not quantify the amount of BMP-2 released in vivo but we know from previous in vitro published studies [21,25] that there is an initial burst release followed by a slower release. For a given BMP-2 loaded concentration, the amount of released BMP-2 is systematically higher for EDC30 films.

We did not notice adverse effects such as local inflammation, swelling, bone cysts, or bone resorption. In three cases, we noticed during the explantation a small seroma of less than 1 mL: two cases for the BG and one case for high-dose BMP-2. The amount of ossification outside the scaffold was small and independent of the BMP-2 dose (Fig. 6).

The visual scoring used by clinicians for the analysis of CT scans is a fine and simple way to evaluate bone regeneration. Although it can be considered as subjective, the number of independent operators (four in our case) and the fact that this is done in a blind manner prevented the risk of a biased view. Furthermore, the results correlated well to the quantitative analysis (Fig. 5I). Another reason for using this score was to be close to the kind of evaluations that are usually conducted in the clinical routine. Similarly, the histomorphometry was conducted in a blind manner by three independent operators (Fig. 8I and J). Although this may result in over-estimation of the amount of new bone formation, it remains a simple and effective way to compare different conditions. Here, a systematic and purely objective evaluation was not possible. Again, the results were in accordance with the previous quantitative analyses (Figs. 5 and 6).

Our study opens perspectives for the clinical translation of these osteoinductive 3D-printed PLA scaffolds. First, the PLA used was clinical-grade and the film components are already approved by the US Food and Drug Administration (FDA) and the European Medicine Agencies (EMA) [21]. 3D printing is a cost-effective solution [10]. It has the potential to produce new medical products with unprecedented structural and functional designs, and its regulatory landscape is rapidly evolving [9]. The fact that the new synthetic graft proposed here is made of a 3D-printed scaffold and a 2D osteoinductive film coating containing BMP-2 offers several modularity possibilities in terms of scaffold design (dimensions, shape, architecture, porosity) and a precise control of BMP-2 dose delivered via the film coating. Regulations regarding the osteoinductive biomolecule, the BMP-2 growth factor, is also less difficult than that of stem cells [9], and is already clinically approved for several indications.

5. Conclusion

In summary, we engineered a 3D-printed scaffold made by FDM and coated with a biomimetic polyelectrolyte film loaded with BMP-2 to repair a new model of critical-size bone defect in minipig mandible. This bone defect model and volume >10 cm³ is equivalent to various clinical applications in humans, showing the translation potential of our technology. The 3D architecture of the scaffold provided a guide for cells to grow inside the volumetric defect while the 2D osteoinductive coating allowed BMP-2 to trigger cell differentiation and bone regeneration. We showed that the BMP-2 dose delivered from the polyelectrolyte film significantly influenced the amount and maturity of regenerated bone with a clear BMP-2 dose-dependence for EDC30. The repair kinetics was also dose-dependent, with a slower kinetics for the high BMP-2 dose. CT scans, μCT acquisitions, and histological examinations proved the formation of mineralized and well-vascularized bone with high BMP-2 doses, whereas lower BMP-2 doses lead to less mineralized bone. In addition, the new bone formed homogeneously inside the scaffold, whatever the BMP-2 dose, and with little ectopic bone formation. This combination of a 3D-printed scaffold with a 2D osteoinductive coating opens perspectives in personalized medicine because 3D printing allows the customization of shape of implants and the biomimetic coating allows the controlled delivery of BMP-2 in space and time.

CRediT author contribution statement

MB: Conceptualization, Formal analysis, Investigation, Methodology, Visualization. CG: Formal analysis, Writing – original draft, Writing – review and editing. Visualization. PM: Investigation. JV: Formal analysis, Investigation, Writing – original draft. VF: Investigation. SM: Formal analysis. JB: Formal analysis. VJ: Formal analysis. GB: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review and editing, Supervision. CP: Conceptualization, Methodology, Writing – original draft, Writing – review and editing, Supervision, Funding acquisition.

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal reasons. They are currently available upon request and will be deposited in a data repository.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2021.100113.

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