Tailored Three-Dimensionally Printed Triply Periodic Calcium Phosphate Implants: A Preclinical Study for Craniofacial Bone Repair

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Supporting Information

ABSTRACT: Finding alternative strategies for the regeneration of craniofacial bone defects (CSDs), such as combining a synthetic ephemeral calcium phosphate (CaP) implant and/or active substances and cells, would contribute to solving this reconstructive roadblock. However, CaP’s architectural features (i.e., architecture and composition) still need to be tailored, and the use of processed stem cells and synthetic active substances (e.g., recombinant human bone morphogenetic protein 2) drastically limits the clinical application of such approaches. Focusing on solutions that are directly transposable to the clinical setting, biphasic calcium phosphate (BCP) and carbonated hydroxyapatite (CHA) 3D-printed disks with a triply periodic minimal structure (TPMS) were implanted in calvarial critical-sized defects (rat model) with or without addition of total bone marrow (TBM). Bone regeneration within the defect was evaluated, and the outcomes were compared to a standard-care procedure based on BCP granules soaked with TBM (positive control). After seven weeks, de novo bone formation was significantly greater in the CHA disks + TBM group than in the positive controls (3.33 and 2.15 mm³, respectively, P = 0.04). These encouraging results indicate that both CHA and TPMS architectures are potentially advantageous in the repair of CSDs and that this one-step procedure warrants further clinical investigation.

KEYWORDS: bone tissue engineering, bioceramics, calcium phosphates, 3D printing, bone marrow, calvaria

1. INTRODUCTION

The ability to repair large and critical-sized craniofacial bone defects (CSDs) in both pediatric and adult populations today remains limited. The number of patients living with craniofacial osseous deficiencies will continue to grow because cranioectomy remains to be the clinical care standard in treating an entire age range of patients with traumatic head injuries, stroke, and resection of tumors. Despite the well-known limitations associated with autologous bone graft (BG) transplantation (e.g., damaging healthy bone, resorption, morbidity, and infection), it remains to be the most preferred technique in repairing skull defects, especially in growing patients (where synthetic nonvital implants are contraindicated) and compromised wound beds (poor soft tissue coverage, previous radiotherapy, or infection).1−4

To contribute to solving this reconstructive roadblock, the search for innovative solutions based on synthetic materials serving as ephemeral scaffolds for the growth of new bone has

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been extensive. Synthetic calcium phosphate bone substitutes (BSs) such as hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and calcium phosphate cement (CPC) have demonstrated considerable potential for the regeneration of CSDs given their biocompatibility and osteoconductive features but are often insufficient. Experimentally, many attempts to enhance the regenerative capacity of BSs to induce or augment cranial repair have been made with the addition of expanded stem cells, growth factors, or cytokines, alone or in a combination (i.e., tissue engineering strategies). While tissue engineering might have a bright future in the reconstructive field, significant drawbacks such as the lack of reliable efficacy, high costs, potential side effects of the synthetic bioactive substances (e.g., ectopic bone formation), potential safety risks (e.g., tumors), and ethical issues hinder its current implementation in clinical cases. Safer alternatives to these processed substances (e.g., ectopic bone formation), potential safety risks, high costs, potential side effects of the synthetic bioactive substances based on dispensable scaffolding. 

Another way of improvement lies in the optimization of synthetic scaffolds to enhance biological response. Indeed, both scaffold architecture and composition are known to modulate cell behavior directly and indirectly and thus could significantly affect CSD repair once implanted; the success of the clinical procedure is mainly determined by the ability of the implant to keep endogenous and exogenous cells alive and functional. This requires the scaffold to have micron-scale pores and roughness (<10 μm, ideally >1 μm) for osteogenic cell adherence and bone formation as well as a macroscopic porous network (>100 μm) for cell colonization, mass transport, and blood vessel guidance. Triply periodic minimal surfaces (TPMSs) are a promising tool for designing the macroscale pore architecture of biomaterials. These open periodic porous structures, which have zero mean curvature, show higher intrinsic features than those of conventional semirandom porous architectures (e.g., salt-leached scaffold), such as the surface-volume ratio and permeability, both playing a critical role in the conduction of chemical (e.g., nutrients) and biochemical (e.g., cytokines) species, cells, and tissues.

Ideally, scaffold biodegradation and bone formation should also occur concurrently at a matching rate. However, clinically used calcium phosphates in the pure phase (e.g., HA) or as biphasic calcium phosphates (BCP) display limited in vivo degradation, that is, low solubility (chemical property) and resorbability (from cellular activity). Ionic substitutions or insertion within the apatite lattice (e.g., carbonate and silicate) have been shown to modulate the biodegradation of the implant, coupled with stimulation of bone and vascular ingrowth. Specifically, the interesting potential of carbonated hydroxyapatites (CHA) has been emphasized for years as their in vivo behavior, especially their biodegradation rate, can be tailored by the amount of carbonate ions present in both phosphate (B-type) and hydroxide (A-type) sites of their lattice.

Designing and producing implants with a controlled composition and architecture, tailored to the craniofacial defect to repair, is now possible with the development of additive manufacturing technologies and associated software. Authors proposed a flexible manufacturing process based on the impregnation of wax molds, produced by additive manufactur-

2. MATERIALS AND METHODS

2.1. MBCP Granules. MBCP ceramic granules measuring 0.5−1.0 mm in diameter were provided by Biomatlante SA (Vigneux-de-Bretagne, France). They were defined as micro-macroporous biphasic calcium phosphate composed of 58% hydroxyapatite (HA) and 42% β-tricalcium phosphate (β-TCP), exhibiting a specific surface area (SSA) of 2.5 m²/g. The total porosity volume has been evaluated between 70 and 75% and consists mainly of macro pores (~30% v/v) and micropores (~70% v/v) ranging from 100 to 500 μm and 0.1 to 1 μm, respectively. It was double-packed and autoclave-sterilized at 121 °C for 20 min.

2.2. Tailored TPMS CaP Disks. 2.2.1. Powder Preparation. HA, apatitic tricalcium phosphate (TCPap), and β-type carbonated hydroxyapatite (CHA) powders were synthesized by a conventional aqueous precipitation method using a fully automated synthesis station. Briefly, a diammonium hydrogen phosphate solution ((NH₄)₂HPO₄, 99%, Merck, Germany, [P] = 1.2 M) mixed, if applicable, with an ammonium hydrogen carbonate solution ((NH₄)₂CO₃, Merck, Germany) was added at 100 mL/min to a calcium nitrate solution (Ca(NO₃)₂, 99%, Merck, Germany, [Ca] = 2.1 M), maintained under stirring (500 rpm). Reagent ratios were calculated according to the following theoretical formula:

\[ \text{Ca}_{10} \left( \text{PO}_4 \right)_6 \left( \text{OH} \right)_2, \quad \text{Ca}_3 \left( \text{PO}_4 \right)_2 \left( \text{OH} \right)_2, \quad \text{Ca}_{10−x} \left( \text{PO}_4 \right)_{6−x} \left( \text{CO}_3 \right)_x \left( \text{OH} \right)_{2−x} \quad \text{with } x = 0.8 \text{ for HA, TCPap, and CHA powders, respectively.} \]

The pH of the suspensions was adjusted to 7.0 (TCPap) or 8.0 (HA and CHA) by the addition of 28% ammonia solution (Merck, Germany) by means of a dosing pump (ProMinent, U.K.) coupled with a pH controller (Mettler Toledo M400, U.K.A.) and a pH electrode (Mettler Toledo Inpro 4800/120/PT100, U.S.A.). The temperature was controlled and regulated automatically at 35 °C (TCPap) or 65 °C (HA and CHA) with an external T-probe connected to a cryothermostat (Huber, Germany). An argon flow (Air Products, 0.1 L/min) was maintained in the reactors to prevent any atmospheric uncontrolled carbonation throughout the synthesis process. After complete introduction of the phosphate solution, the suspension was matured for 20 ± 2 h and finally centrifuged at 4000 rpm for 5 min (ThermoFisher Scientific, Sorvall Legend XF). The wet powder agglomerates obtained were dried at 80 °C for 24 h then ground in absolute ethanol (H₃C₂H₄OH, >99.5%; VWR, Germany) by means of a planetary ball mill (PM400, Retsch, Germany) with zirconium oxide jars and balls and finally sieved at 25 μm (Russellfinex, Belgium).

Lastly, HA, TCPap, and CHA powders were heat-treated to reduce their surface area as to transform TCPap into β-TCP according to the following general reaction:

\[ \text{CA}_3(\text{PO}_4)_2(\text{OH})_3 \rightarrow 3\text{CA}_3(\text{PO}_4)_2 + \text{H}_2\text{O} \]

TCPap and HA powders were thus heat-treated at 900 and 1000 °C for 2 h under air (ramp of 4 °C/min; Nabertherm, Germany), and CHA powder was heat-treated at 900 °C for 5 h under CO₂ (P_CO₂ = 1 atm, ramp of 5 °C/min; Nabertherm, Germany). The SSA of 4.8 ± 0.1, 4.3 ± 0.2, and 4.7 ± 0.1 m²/g were finally achieved. These values were determined on powders, tubes fired at 200 °C for 8 h, by means of the Brunauer−Emmett−Teller (BET) five-point method using N₂ adsorption isotherms (Micromeritics ASAP 2010, Germany).

2.2.2. Manufacturing Process. Macroporous disk-shaped bioceramic implants were produced by a method detailed elsewhere based on
the impregnation of wax molds with the latter being built layer by layer with a drop-on-demand 3D printer (3Z Studio, Solid scape, Multistation, Dinard). The molds were designed as the negative structure of the intended implant and printed with a layer thickness of 25 μm. Once printed and cleaned, molds were impregnated with a ceramic powder suspension (hereafter called slurry). After drying overnight at room temperature, the green bodies were cleaned of all excess dried slurry using a surgical blade (Swann-Morton, U.K.). Then, they were heat-treated in a debinding furnace (Carbolite, U.K.) up to 500 °C to eliminate the wax mold and organic adjuvants and finally sintered either at 1100 °C for 2 h under air (ramp of 4 °C/min) or at 1050 °C for 2 h under CO₂ (P CO₂ = 1 atm, ramp of 5 °C/min) to obtain the biphasic calcium phosphate (BCP: 60% HA and 40% β-TCP (w/w)) or the CHA bioceramics, respectively.

Slurries were prepared by blending 71.7% (w/w) powdered CHA or a powder blend of BCP: 60% HA and 40% β-TCP (w/w), 27.7% (w/w) pure water, and a 0.6% (w/w) dispersing agent (polyacrylate) excess dried slurry using a surgical blade (Swann-Morton, U.K.). Then, each mold was impregnated with an organic binder (1.9% (w/w), pure water, and a 0.6% (w/w) dispersing agent (polyacrylate) excess dried slurry using a surgical blade (Swann-Morton, U.K.). Then, the molds were cured at 180 °C in a poupinel dry heat sterilizer for 30 min.

2.2.3. Design of the Macroporous Bioceramic Disk Implants. Implants were designed to perfectly match the geometry of a rat calvarial defect 5.5 mm in diameter (Figure 1) using ScanIP software

Simpleware (U.K.). The thickness of the implant was chosen not to exceed the height of the rat parietal bone, ~1 mm (Figure 2A). Regarding its computer-aided design (CAD) model, the intended bioceramics should display a gyroid structure wherein a 300 μm sphere can freely move for a total macroporosity of 40% (Figure 2B). For informative purposes, Figure 2C also illustrates the link between the maximum size of a sphere that can go through the entire gyroid structure depending on the size of the gyroid’s fundamental unit and its porosity.

2.2.4. Characterization of the Macroporous Bioceramic Disk Implants. The crystalline phases of the samples were identified by means of a Bruker D8 Advance θ/θ X-ray diffractometer (XRD) with a Lynx-Eye XE-T Detector (with a 2.93° aperture angle) using Cu Kα radiation and operating at 40 kV and 20 mA. XRD patterns were collected over the 2θ range of 10–120° at a step size of 0.01° and counting time of 0.2 s per step. The crystalline phases were identified with Bruker Diffrac.EVA 4.0 software (Bruker AXS, Germany) and the PDF004+ database. Rietveld method refinement was achieved with DIA FRAC.TOPAS v.5.0 software (Bruker AXS, Germany) using the Pawley algorithm and the following fundamental parameters for HA and β-TCP structures: PDF no 00-009-0432, space group P63/m (176), and PDF no. 00-009-0169, space group R3c (167), respectively. The XRD patterns were also used to determine the HA/β-TCP phase ratio of the BCP bioceramics according to a standard procedure.

Ground bioceramics were analyzed by Fourier transform infrared (FTIR) spectroscopy using a Bruker VERTEX 70 spectrometer (Bruker Optics, France) equipped with a monolithic diamond ATR crystal (Quartz ATR diamond, Specac, U.S.A.). The spectra, obtained by signal averaging of 64 successive scans, were recorded from 4000 to 400 cm⁻¹ at a resolution of 2 cm⁻¹. A curve-fitting analysis in the ν CO₂ (900–840 cm⁻¹) and ν C (1600–1300 cm⁻¹) carbonate domains of the FTIR spectra (dedicated in-house method34) was performed by means of OriginPro 2018b software (OriginLab, U.S.A.). Carbon content in CHA bioceramics was also determined by an elemental analyzer using an infrared detector (LECO CS-444, U.S.A.).

Morphometric analyses of the bioceramics produced were carried out at various scales. Each bioceramic was first imaged using a Nanotom S X-ray computed tomography system (Phoenix, AZ, U.S.A.) with a voltage of 80 kV (tungsten target), integration time of 750 ms, and 3.5 mm voxel resolution. For reconstruction of the volume data, a proprietary implementation based on the Feldkamps cone beam-reconstruction algorithm was used. VG Studio software (Volume Graphics, Heidelberg, Germany) was used for the 3D visualization of the volume data and the data set was exported in a .DICOM format for image analysis.

Once imported into Simpleware, the .DICOM images allowed the reconstruction of a 3D model of the bioceramic, which was exported in a .STL format. After manual gross superimposition of the bioceramic model with its original CAD design, the .STL files were imported in CloudCompare freeware (EDF R&D, France) for further comparison. A dedicated algorithm allowed for the fine superimposition of the two models. The “cloud to mesh” algorithm was used to compare the 3D-printed bioceramic to its CAD model, the latter serving as the reference.

The ceramics were also examined by scanning electron microscopy (SEM, JEOL JSM-6500F, U.S.A.) by applying a gold coating (approximately 10 nm) using the sputtering technique (Quorum, 6300 ES).
Q150R ES, U.K.). The porosity at the samples’ surfaces was quantified from SEM images using ImageJ freeware (National Institutes of Health, Bethesda, MD, U.S.A.). Additionally, the minimum ($x_{F,\text{min}}$) and maximum ($x_{F,\text{max}}$) Feret diameters of the micropores as well as the three morphological factors, aspect ratio (AR), sphericity (S), and roundness (R), were evaluated ($N = 3$ SEM images analyzed/sample; $N = 2$ samples/macroporous bioceramic).26,35

Finally, the ceramic SSAs were determined as described previously using the BET five-point method (five ceramics/triplicate measurements).

2.3. Animals. Twenty-four adult inbred Lewis 1A-haploype RT1a rats were obtained from a certified breeding center (Janvier Labs, LeGenest-Saint-Isle, France) and acclimatized for two weeks to the conditions of the local vivarium. Animal experiments were conducted according to the European directive 2010/63/EU and approved by the French Ministry of Research and Education (APAFIS 18616-V1) and Pays de la Loire Ethics Committee (France).

2.4. Bone Marrow Harvesting. Three rats were specifically designated as TBM donors. The animals were anesthetized using inhaled isoflurane (Forene; Abott, Rungis, France) and sacrificed via intracardiac overdose of sodium thiopental (Nedonal; Rhône-Merieux, Lyon, France). The TBM was isolated from femurs, humeri, and tibias for extemporaneous grafting. Briefly, the ends of each bone were cut, and 1 mL of the TBM mixed with saline was obtained through an intramedullary bone flush procedure performed with a 26-gauge needle. After pooling, the TBM was seeded into the CaP biomaterial, which was immediately implanted in the calvarial defect.

2.5. Implant Preparation and Study Groups. The animals were randomly assigned to one of the following groups: BCP granules, BCP granules + TBM, BCP disk, BCP disk + TBM, CHA disk, CHA disk + TBM, and empty defect. The groups of empty defect and BCP granules + TBM were used as the negative and positive control, respectively. Study was performed on $N = 6$ samples/group.

2.6. Surgery. All procedures were performed under general anesthesia and lasted 20 min. After local subcutaneous xylocaine injection (0.1 mL, 0.1%), a 2 cm longitudinal incision was made on the head of each rat from the forehead to the neck. The skin and periosteum were lifted. Subsequently, critical-sized parietal defects (5.5 mm) were created bilaterally using a circular trephine (Komet Medical, Lemgo, Germany) under a saline solution infusion. Each animal received two randomly assigned implants ($N = 6$) as detailed in Table S1. The skin was then closed with nonabsorbable sutures (Ethylon 5.0, Ethicon). Immediate postoperative analgesia was provided through subcutaneous injection of buprenorphine hydrochloride (Buprecare; Animalcare, Dunnington, U.K.) and maintained for two days. Seven weeks after implantation, the animals were sacrificed by sodium thiopental overdose.

2.7. Microcomputed Tomography Analysis. Qualitative analysis of total mineral and newly formed bone contents was performed at the time of necropsy using the SkyScan-1272 high-resolution 3D X-ray
micro-computed tomography (micro-CT) system for small-sample imaging (Bruker, Belgium). The scanner was equipped with a 20 to 100 kV (10 W) X-ray source and an 11-megapixel X-ray detector. Each sample was placed on a holder with the sagittal suture oriented parallel to the X-ray detector and scanned using a 0.5 mm aluminum filter, 18 μm isotropic voxels, 0.71° rotation step, and frame averaging of 4. For 3D reconstruction (NRecon software, Bruker) without smoothing, the ring artifact correction, beam hardening correction, and absorption coefficient were set to 4, 20%, and from 0.005 to 0.1, respectively. Standard 3D morphometric parameters (CTAn software, Bruker) were determined in the region of interest (5.5 mm circle; 100 cuts) and put on the defect. Representative 3D images were created using CTvox software (Bruker) for each implant to assess bone formation in control and treated animals. The overall augmented contour was evaluated in the 3D reconstructed view and calculated in CTAn. Boundaries were set to standardize the region of the augmented volume to be analyzed. Bone volume (BV; mm3) was calculated as the volume occupied by bone within the region of interest.

2.8. Histology. To observe the newly formed bone and osteoblastic cells, double staining was performed. In short, specimens were fixed for 24 h in 4% paraformaldehyde and then dehydrated through a graded series of ethanol treatments. Nondecalciﬁed bone specimens were inﬁltrated and embedded in glycol-methyl-methacrylate (GMMA; Technovit 9100, Kulzer, Germany). For each sample, a cranio-caudal section was performed at the maximum diameter of each implant using a circular diamond saw (SP1600; Leica, Wetzlar, Germany) and serial 5 μm sections were cut using a hard tissue microtome (Polycut SM 2500; Leica, Wetzlar, Germany). The sections were stained with Goldner’s trichrome and hematoxylin–eosin–safran and then examined using a light microscope (Leica-DM 4000 B, Wetzlar, Germany).

2.9. Immunohistochemistry. Immunohistochemistry (IHC) was performed to evaluate the vessel formation in biomaterials and the empty defect. Sections measuring 5 μm were cut from GMMA-embedded blocks and then stained. A rabbit polyclonal anti-CD 31 antibody (Abcam 28364, dilution of 1:100) was used for endothelial staining and vessel visualization. A negative control was performed without the primary antibody CD31. Quantitative analysis of the vessel formation was performed by a pathologist using a light microscope (Leica-DM 4000 B, Wetzlar, Germany); the vessel count was performed for each reconstruction including 10 fields per defect with a ×40 magniﬁcation.

2.10. Statistical Analysis. Each result was expressed as the mean ± standard deviation (SD) of six samples. A one-way ANOVA followed by a post hoc test (Fisher’s protected least significant difference) was performed. P values of <0.05 were considered to be statistically signiﬁcant.

3. RESULTS

3.1. Analysis of the Macroporous Disk-Shaped Bioceramics. Figure 3 shows the XRD patterns and FTIR spectra of BCP and CHA ground bioceramics, centered on the ranges of 10 ≥ 2θ ≥ 46° and 3620 ≥ ν ≥ 3540 cm−1 and 1600 ≥ ν ≥ 1350 cm−1 and 1200 ≥ ν ≥ 500 cm−1, respectively, as well as images of the macroporous disk-shaped bioceramics. Both the diffractogram (Figure 3A) and the FTIR spectrum (Figure 3B) of the BCP sample exhibited the characteristic diffraction lines or bands of HA and β-TCP phases (PDF nos. 00-009-432 and 00-009-169, respectively). No other crystalline or amorphous phase was detected. The a and c lattice parameters of the HA (a = 9.422 Å and c = 6.882 Å) and β-TCP (a = 10.437 Å and c = 37.426 Å) phases detected in the BCP sample are equivalent to values reported in the ICDD PDF card nos. 00-009-432 (a = 9.418 Å and c = 6.884 Å) and 00-009-169 (a = 10.429 Å and c = 37.380 Å), respectively. Moreover, the percentages by mass of crystalline phases of HA and β-TCP in BCP implants were assessed to be 55.9 ± 0.2% and 44.1 ± 0.2%, respectively.

The XRD pattern of the CHA implant (Figure 3A) is typical of a hydroxyapatite structure (PDF no. 00-009-432). Lattice parameters a and c are equal to 9.455 and 6.890 Å, respectively. Both are signiﬁcantly higher than those of HA (ICDD PDF card no. 00-009-432) as commonly observed when carbonate ions simultaneously substitute for phosphate (B-sites of the apatite structure) and hydroxide (A-sites of the apatite structure) ions in the HA lattice.37,38

The infrared spectrum exhibits only the characteristic bands of AB-type carbonated hydroxyapatites (Figure 3B),31,39 conﬁrming that the chemical composition of CHA implants can be illustrated with the following general formula:

\[
\text{Ca}_{10-x} (\text{PO}_4)_{6-x} (\text{CO}_3)_x (\text{OH})_2 - x - z (\text{CO}_3)_z \quad (1)
\]

with 0 ≤ x (B-type carbonate ions) ≤ 2 and 0 ≤ z (A-type carbonate ions) ≤ 2 − x.

The carbonate content of the CHA implants was evaluated to be 5.48 ± 0.05% w/w. The amounts of A-type and B-type carbonate ions, investigated by a curve-ﬁtting method, were evaluated to be 0.78 ± 0.19% and 4.70 ± 0.23% w/w, respectively.

Figure 3C–E shows pictures and X-ray tomography images of the BCP and CHA macroporous disk-shaped bioceramics. Their ﬁnal dimensions are equivalent and were assessed to be Φ = 5.44 ± 0.04 mm and h = 827 ± 41 μm (n = 24) and Φ = 5.44 ± 0.05 mm and h = 843 ± 38 μm (n = 18). Moreover, the CloudCompare comparison revealed that both BCP and CHA bioceramics were very similar to their initial CAD model with a distribution of the deviation centered on 0 μm with most of the deviations between −40 and +40 μm (Figure S1).

The SEM images of the BCP and CHA bioceramic macro- to microstructures (Figure 3F,G) clearly reveal the printing orientation of the mold parallel to the surface of the disks as well as submicropores. As described in Table 1, the concentrations of submicropores at the surface of the BCP and CHA bioceramics are approximately 10 and 14%, respectively. This small diﬀerence in the concentration generates a diﬀerence less than 1 m2/g between the two types of macroporous disks (see Table 1). The size (x/sub/min ≈ 0.7 μm and x/sub/max ≈ 1.3 μm) and morphology of the submicropores are similar; the latter can be deﬁned as domino-shaped micropores surrounded with a low sphericity.

<table>
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<tr>
<th>sample</th>
<th>BCP average</th>
<th>SD</th>
<th>CHA average</th>
<th>SD</th>
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<td>amount (s/s)</td>
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<td>0.2</td>
<td>0.5</td>
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<td>0.1</td>
<td>2.3</td>
<td>0.2</td>
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</table>

*Amount (surface/surface), dimension, and morphology of the micropores constituting BCP and CHA macroporous ceramics from SEM image analysis and SSA values; AR is the aspect ratio, R is for roundness, S is for sphericity, and x/sub/min and x/sub/max correspond to the shortest and longest Feret diameters, respectively; SD: standard deviation.*
3.2. Clinical Findings. All animals survived the surgical procedure, 36 defects were healed with biomaterials, and six were left empty. During the healing period, no reconstruction exposure or loss was observed, and no abnormal findings such as inflammation, infection, or separation in the surgical wound were observed.

3.3. Micro-CT Findings. As shown in micro-CT images after sacrifice at seven weeks (Figure 4A), the groups with the BCP and CHA disk presented homogeneous bone formation distributed over the entire surface of the bone defect, conversely to the groups with granules, which systematically presented a partial filling of the defect. An amount of bone grew toward the center of the defect from adjacent host tissues in all groups (disks and granules with or without TBM).

The BCP granule and empty defect groups had the lowest rate of newly formed bone (see Table 2 and Figure 4B), which were significantly lower than for the BCP and CHA disk groups ($p = 0.03$ and $p = 0.04$, respectively). Addition of the TBM prior to implantation systemically improved bone formation, independent of the bone substitute (e.g., 103% increase for granule + TBM). Interestingly, bone content was comparable in the positive control (granule + TBM) BCP and CHA disk groups without TBM. The greatest bone healing was observed in the CHA + TBM group with approximately 55% more bone formed than in the positive control ($p = 0.04$). While not significant, the CHA disk + TBM group also showed greater bone formation than that of the BCP disk + TBM group with the latter displaying results comparable to the granule + TBM group.

3.4. Histologic Findings. Goldner’s Masson trichrome staining is detailed in Figure 5 and confirmed the previous observation on bone regeneration (Figure S2). Notably, abundant bone formation was observed in disk ± TBM groups, regardless of the disk composition, and the BCP granules + TBM groups.

The defect was mainly filled by fibrous tissue in both the BCP granules and empty groups. A small amount of bone was observed, mainly close to the adjacent host bone. Uneven vault reconstruction was observed in the BCP granule group.

In the BCP granules + TBM group, a large amount of bone was observed around the granules close to the adjacent host bone. Uneven vault reconstruction was observed in the BCP granule group. The porous structure of the implant and merging in large bone islands were observed. A homogeneous distribution of a large amount of new bone was detected within the scaffolds in groups combining CaP disks, regardless of their composition, and TBM.

3.5. Bone Volumetric Analysis. Table 2. Bone Volumetric Analysis within the Region of Interest ($n = 6$; mm$^3$)

<table>
<thead>
<tr>
<th>group</th>
<th>mean ± SD</th>
<th>median</th>
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<td>empty</td>
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<tr>
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<td>BCP granule + TBM</td>
<td>2.146 ± 0.975</td>
<td>2.169</td>
</tr>
<tr>
<td>BCP disk</td>
<td>2.515 ± 0.686</td>
<td>2.183</td>
</tr>
<tr>
<td>BCP disk + TBM</td>
<td>2.572 ± 0.543</td>
<td>2.674</td>
</tr>
<tr>
<td>CHA disk</td>
<td>2.258 ± 0.653</td>
<td>2.271</td>
</tr>
<tr>
<td>CHA disk + TBM</td>
<td>3.331 ± 0.303</td>
<td>3.315</td>
</tr>
</tbody>
</table>

$\text{Table showing the mean ± SD and the median of the bone volume in CSDs. Bone formation was significantly lower in the empty (negative control) and BCP granule groups. No statistical difference was observed between BCP granules + TBM (positive control) vs macroporous disks (BCP and CHA) without TBM. CHA + TBM showed a higher rate of bone formation than BCP granules + TBM ($p = 0.0441$).}$

*Significant difference compared to the positive control group (BCP granule + TBM), $p < 0.05$. *Significant difference compared to the CHA disk + TBM group, $p < 0.05$. *Significant difference compared to the BCP granule group, $p < 0.05$. *Significant difference compared to the negative control group (empty defect), $p < 0.05$.

Figure 4. Micro-CT analysis of the critical-sized craniofacial bone defect (CSD) reconstruction. (A) Images of the CSD reconstructions at seven weeks showing calvarial 3D reconstruction, biomaterials + new bone, and newly formed bone alone. CSD repair with BCP granules ± TBM systemically had biomaterial loss and calvarial holes. (B) Graph showing the quantitative analysis of bone volume (BV, mm$^3$) in the region of interest. Empty defect (negative control) and BCP granule groups had the lowest rate of bone formation compared to others ($p < 0.05$). There was no statistical difference between BCP granules + TBM (positive control) and macroporous disks alone, while the CHA + TBM group had significantly higher bone formation than that of BCP granule + TBM ($p = 0.0441$).
with the center of the defect widely colonized by newly mineralized tissue (6/6 of CHA and 4/6 of BCP, respectively).

Generally, anatomical calvarial vault reconstruction was carried out successfully in defects filled with macroporous disks, in contrast to the BCP granule groups. Bone marrow niches were created in groups including the TBM with larger niches when custom-made disk-shaped CaP bioceramics were used.

Goldner staining was compared to HES staining (Figure S3) to evaluate bone maturity and the osteoid band in the samples. No difference was observed between BCP granules and macroporous disks, which for the most part had mature bone at seven weeks.

3.5. Immunohistological Findings. Vessel formation was observed from the edge of the craniectomy in surrounding fibrous tissue and into the scaffolds for all groups. The vessel arrangement showed a high fibrovascular network growing into the macroporous network of the disks or around the BCP granules. No difference in the number of vessels (approximately 10 per field, magnification of ×40) or vessel size was observed (see Figure 6).

4. DISCUSSION

In this study, we aimed to compare one-step and single reconstruction procedures including two types of customized CaP macroporous bioceramics with or without TBM on a rat model.

The design of the macroporous disk-shaped bioceramics was optimized to facilitate handling by the surgeon and enhance...
their osteoconduction and osseointegration compared to a pile of granules. For this purpose, the porosity was set at 40% to prevent erosion of ceramics while avoiding sintering at an excessively high temperature, which in turn would reduce the concentration in micropores. The size of the disks was proved to fit the bone defect volume closely with less than 60 μm of space between the ceramic, centered in the defect, and the parietal bone. A gyroid macroporous structure was accurately patterned in the bioceramics to improve their permeability compared to a standard random macroscopic porous network. A 300 μm macropore size was specifically chosen to create sufficiently confined areas to positively affect tissue growth and blood vessel guidance. Controlling the microporosity of the implants at the submicron scale was also intended to improve their overall osseointegration dynamics. As the microporosity and surface microtopography of the CaP-based implants can be controlled by adjusting the manufacturing parameters, further studies could be performed to determine the relative effect of both the micro- and macroarchitectural features on cell fate.

The BCP macroporous disk-shaped bioceramics are composed of HA (56% w/w) and β-TCP (44% w/w) phases in equal proportion to that of the MBCP granules (58% HA and 42% β-TCP w/w). Assuming eq 1 and the amounts of A-type and B-type carbonate ions in the HA lattice, the CHA bioceramic is a monophasic AB-type carbonated hydroxyapatite that can be described by the following chemical formula:

\[ \text{Ca}_{9.26}\text{(PO}_4\text{)}_{5.26}\text{(CO}_3\text{)}_{3.74}\text{(OH)}_{1.02}\text{(CO}_3\text{)}_{0.12} \]

In our experimental conditions, we considered BCP granules extemporaneously mixed with the TBM as a positive control of bone regeneration as previously described. Indeed, its efficacy in an animal model has been documented in several studies. BCP granules have well-known osteoconductive properties repeatedly described for decades, and the TBM naturally contains a large number of chemotactic and osteogenic factors as well as mesenchymal and hematopoietic progenitor cells promoting bone formation. We previously showed that the mixtures of BCP granules and unprocessed TBM had osteogenic potential in vivo and a positive effect on bone ingrowth. In addition, other studies confirmed enhancement of induced bone formation in ectopic and osseous sites. Thus, we aimed to compare BCP granules + TBM to custom-made disk-shaped bioceramics consisting of a macroporous gyroid structure.

We tested a clinically relevant single-step procedure with no preoperative in vitro procedure such as osteogenic cell isolation. Seven experimental procedures were performed including the negative (defect maintained empty) and positive (BCP granules + TBM) controls.

Comparison of the new bone volume formed in BCP granules and CaP disks demonstrated the importance of the macroscopic shape and porous network of the implant on its osteoconductive properties. Thus, BCP granules had the same rate of new bone growth as that of the empty defect, while BCP disks without any addition of the TBM presented a higher bone volume than that of BCP granules. In addition, CaP disks without the TBM displayed a bone formation equivalent to the positive control (see Figures 4 and 5 and Table 2). This indicates that randomly arranged granules impede spontaneous regeneration of the bone (vault reconstruction), while the gyroid structure guided it.

Osteointegration (i.e., fusion between the defect rim and the scaffold) and bone apposition (i.e., continuum of the mineral between the bone and scaffold) were enhanced by the architecture of the 3D-printed bioceramics whose shape perfectly matched the defect thus allowing for an intimate bone–scaffold contact and whose internal macroporous gyroid structure provides an ideal environment for cell colonization, survival, and physiological metabolism. For instance, the permeability of the gyroid structure is more than 10-fold greater than that with a random pore architecture of comparable porosity and pore size, favoring mass transport (e.g., cells, nutrients, and waste removal). Please refer to the review of Kapfer et al. for more details on the potential of TPMS structure for biological applications. In addition, the micro motion of the disks due to the dura pulsation (nonideal conditions for bone recovery) is very limited in contrast to the BCP granules.

Interestingly, the synergistic effects of both scaffold composition and architecture were discovered in this study; although bone formation within BCP and CHA disks was comparable, it became significantly higher in the CHA + TBM group. While further investigations will be required to precisely understand the underlying mechanisms driving this phenomenon, this indicates that a concomitant modulation of the physicochemical and architectural features of the implant may improve the survival and metabolic activity of endogenous and exogenous cells, thus unveiling new opportunities for the regeneration of the craniofacial bone defect.

Although CaP disks alone are as effective as the positive control, the combination of the AB-type CHA disk with the TBM aspirate, which contribute essential chemotactic and osteogenic factors, was the most efficient procedure in this study. In addition, this combination allowed an even reconstruction of the cranial vault with bridging of the bone defect in contrast to the positive control using BCP granules (see Figure S2), emphasizing the greater potential of tailored TPMS CHA bioceramics than that of BCP granules with or without the TBM aspirate for clinical applications.

To completely avoid autologous harvest, we could use other adjuvants promoting bone formation such as rhBMP-2 that has shown promising results when compared to autologous bone grafts for the craniofacial area. However, rhBMP2 procedures are expensive and can have potentially severe side effects. rhBMP-2 exposes the patient to the risk of wound complication such as breakdown, infection, or significant local swelling with potential acute respiratory failure when it is used too close to the airways. Additionally, bone formation around the bone defect (e.g., ectopic ossification) is commonly described with BTE procedures using BMP. This limits the added value of a custom-made implant except to replace the loading method by soaking BMP-2 in the carrier, leading to the burst release of a supraphysiological dose of BMP-2 (e.g., several milligrams) by grafting of BMP-2 molecules at specific sites at the CaP ceramic surface.

Materials should also enable vascular network formation for bone regeneration. Scaffold porosity is a decisive factor for tissue permeation, angiogenesis, and oxygen diffusion into the materials, which is a key factor for cell viability. Both osteogenesis and angiogenesis processes have to demonstrate a coordinated interplay to allow successful bone healing. Therefore, we designed and manufactured macroporous ceramics with a macroarchitecture including a 300 μm pore size and a gyroid structure to ensure blood vessel growth and good penetration of highly vascular connective tissues. Contrary to our expectations and although we observed greater bone formation with macroporous disks with or without TBM,
no visible difference in vessel formation was observed in this study between tailored disks and granules. However, the large number of vessels in a bone construct is not always predictive of low or high bone formation. For example, a foreign body reaction can imply inflammation processes leading to angiogenesis and vessel formation without new bone formation. In addition, angiogenesis plays a particular key role at the early step during endochondral or immature bone formation. When bone formation was observed in this study, histological exams showed mature and well-vascularized bone at seven weeks. It would be relevant to analyze vessel formation at an earlier stage after material grafted in order to investigate if greater and faster vessel formation is observed with macroporous ceramics.

This study was based on a small animal model with limited bone defect also limiting the size of the macropores constituting the scaffold. Nevertheless, the syngenic Lewis rat is considered a good model to evaluate the bony potential of materials. Among the different animal models of craniofacial bone defects, the critical-size calvarial defect in rats remains to be one of the most widely used because it can be bilaterally, easily, and safely performed. Although the rat calvarial defect is only considered as a preliminary model for testing the clinical relevance of BTE strategies, it has allowed us to examine numerous regenerative approaches simultaneously, which would have been impossible in larger animal models due to cost, ethics, and logistics. In addition, although allogenic TBM was grafted in our experiments, the syngenic rat model (i.e., clone animals) was chosen to highlight the clinical perspective of our procedure using extemporary autologous TBM as it is already applied in clinics. However, it could be assumed that larger bone reconstruction in larger animals or humans using our reconstruction method could also suffer from inadequate oxygen supply due to the greater bone volume to repair. Consequently, sustainable oxygen supply strategies should be developed to increase the O_2 concentration.

5. CONCLUSIONS
The first step toward the regeneration of calvarial CSDs based on 3D-printed CaP disks displaying a TPMS internal architecture was reported in this study. A one-step surgical approach, making full use of a natural, dispersable, and safe bioactive substance (bone marrow) and tailored bioceramics, was successfully implemented and should be seriously considered for further investigation as a relevant alternative to the current gold standards. Safer and cheaper than other promising tissue-engineering strategies based on synthetic active substances (e.g., rhBMP2), the clinical potential of this approach is to be confirmed in large animal models with human-sized relevant craniofacial defects.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.9b01241.

CloudCompare analysis (Figure S1), comparison of the 3D models of the BCP and CHA bioceramics, comparison of bone regeneration between micro CT and IHC (Goldner’s trichome staining) images (Figure S2), magnification of the IHC images (HES and Goldner’s trichome staining) (Figure S3), and animal experiment design for the CSD reconstruction (Table S1) (PDF)

REFERENCES


