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Subcutaneous tissue response and osteogenic performance of calcium phosphate nanoparticle-enriched hydrogels in the tibial medullary cavity of guinea pigs

REVISED MANUSCRIPT

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ABSTRACT

In the current study, oligo(poly(ethylene glycol) fumarate) (OPF)-based hydrogels were tested for the first time as injectable bone substitute materials. The primary feature of the material design was the incorporation of calcium phosphate (CaP) nanoparticles within the polymeric matrix in order to compare the soft tissue response and bone forming capacity of plain OPF hydrogels with CaP-enriched OPF hydrogel composites. To that end, pre-set scaffolds were implanted subcutaneously, whereas flowable polymeric precursor solutions were injected in a tibial ablation model in guinea pigs. After 8 weeks of implantation, histological and histomorphometrical evaluation of the subcutaneous scaffolds confirmed the biocompatibility of both types of hydrogels. Nevertheless, OPF hydrogels presented a loose structure, massive cellular infiltration and extensive material degradation compared to OPF-CaP hydrogels that were more compact. MicroCT, histological and histomorphometrical analyses showed comparable amount of new trabecular bone in all tibias and some material remnants in the medial and distal regions. Particularly, highly calcified areas were observed in the distal region of OPF-CaP treated tibias. These results indicate that CaP nanoparticles agglomerated and left sediment, resulting into a heterogeneous distribution of the mineral phase throughout the hydrogel matrix. This phenomenon can be attributed to either hindered gelation under highly perfused in vivo conditions or a faster degradation rate of the polymeric hydrogel matrix compared to the nanostructured mineral phase resulting into loss of entrapment of the CaP nanoparticles and subsequent sedimentation.

Keywords:

Hydrogels; calcium phosphate nanoparticles; injectable; animal model; bone formation.

1. Introduction

The growing incidence of bone loss and injuries as a result of an increasing elderly population combined with accidental trauma and diseases represent a problem with major social importance and significant economical burden on the health care system. This critical situation has led researchers in the field of regenerative medicine to focus on the development of synthetic biomaterials for bone regeneration, which provide temporary support to missing or damaged tissue while inducing and directing the regeneration of new healthy bone tissue [1].

The selection of appropriate biomaterials for bone repair depends on the clinical application (e.g. load-bearing or non-load-bearing) and requires consideration of several fundamental requirements, including biocompatibility, biodegradability, ability to promote bone formation and, ultimately, to regenerate bone marrow [2,3]. An appealing direction for the treatment of bone defects under non-load-bearing conditions is the application of injectable and readily available (“off the shelf”) biomaterials. From a clinical perspective, the use of injectable biomaterials is attractive as it allows a minimally invasive surgical approach, which reduces both patient discomfort and cost of treatment. In addition, injectable biomaterials provide many advantages over pre-shaped materials (e.g. granules and blocks), including optimal defect filling with direct contact between biomaterial and surrounding tissue [4]. Hydrogels are a class of polymer-based biomaterials which fulfill all the aforementioned requirements. These highly hydrated polymer networks are injectable, biodegradable as well as biocompatible due to their high water content. As a result, hydrogels can act as artificial extracellular matrices (ECM) which provide a temporary three dimensional (3D) environment suitable for cell colonization and eventual tissue regeneration [5]. Originally, hydrogels were mainly considered as fillers for soft tissues. Recently, however, hydrogels are also considered for hard tissue regeneration in the field of orthopedics as well as oral and maxillofacial surgery. To that end, hydrogels are currently functionalized using different strategies, including bioactive agents (e.g. calcium phosphate nanocrystals, bioglasses), enzymes or growth factors [6].

Among synthetic hydrogel-based materials, oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels have been extensively explored for cartilage and bone tissue regeneration applications both in vitro and in vivo [7-10]. Structurally, OPF polymers are composed of two

repeating units, poly(ethylene glycol) (PEG) and fumaric acids, which are alternately linked by ester bonds and can be degraded through hydrolytic cleavage [11]. OPF gelation, i.e. the transition from flowable polymer precursor solution to hydrogel, occurs upon chemical cross-linking of macromers in a 10-15 minute time period and at 37°C [12,13]. These properties will potentially allow clinicians to prepare the material directly in the operating theatre, inject it into the desired site through a small incision, and close the wound after in situ gelation. Moreover, it has been shown that the choice of oligomer length and crosslinking agents offers versatile swelling characteristics as well as

Since bone mainly consists of calcium phosphate nanocrystals (~70 wt%), the incorporation of CaP nanoparticles into polymeric hydrogels is a straightforward approach to stimulate the formation of new bone by the surrounding tissue [15]. Recently, we showed that CaP nanoparticle-enriched OPF hydrogels also have a stimulatory effect on mineralized matrix production by encapsulated rat osteoblast-like stem cells (OBLCs) in vitro [16]. This advantage of OPF-CaP hydrogels suggests their potential for application as a bone substitute material.

The present study was designed to test the biological performance of OPF-based hydrogels, either or not enriched with CaP nanoparticles, in a guinea pig model. To that end, OPF-based hydrogels were ectopically implanted at a subcutaneous location in a pre-set form to assess biocompatibility and injected in the tibial medullary cavity to evaluate osteogenic responses. Specifically, the ease of the surgical procedure and the adequate dimensions of the tibial medullary cavity of guinea pigs for material injection encouraged the selection of this model. Moreover, taking into account the advantage of the rapid endosteal bone formation that occurs following injury to the marrow cavity of tibial bone [17], we hypothesized that CaP-enriched OPF hydrogels would enhance bone formation capacity compared to the plain OPF hydrogels.

2. Materials and methods

2.1. Materials

Oligo(PEG fumarate) with PEG nominal molecular weight of 10,000 g/mol was synthesized as previously described [18]. Poly(ethylene glycol) diacrylate (PEGDA, nominal molecular weight 3400) was obtained from Glycosan Biosystem (Salt Lake City UT, USA). *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased from Fluka (Buchs, Switzerland). Ammonium persulfate (APS) and phosphate-buffered saline pH 7.4 (PBS) were purchased

from Sigma-Aldrich (St. Louis MO, USA). Fumaryl chloride, phosphoric acid (H_3PO_4) and calcium hydroxide ($\text{Ca}(\text{OH})_2$) were acquired from Acros (Pittsburgh PA, USA).

2.2. CaP nanoparticles preparation

A homogeneous suspension of nano-sized calcium phosphate (CaP) particles was prepared according to a wet-chemical precipitation process as previously described [16]. In order to mimic the physiological condition, H_3PO_4 (3.56 M) and $\text{Ca}(\text{OH})_2$ (5.92 M) were prepared in PBS, and the precursors compounds were used at a stoichiometric Ca/P ratio of 1.67 to obtain a final solid content of ~ 0.3 g/ml (30% w/v). This method resulted in stable needle-shaped hydroxyapatite nanoparticles with an average length of 76 nm and a diameter of 19 nm, and minor traces of monetite as a secondary CaP phase, as confirmed by XRD and TEM [16].

2.3. Experimental groups

In this animal study, a total of 3 experimental groups were used. Table 1 shows the number of subcutaneous scaffolds and injection into the tibial medullary cavities for each experimental group. Specifically, a total of 32 scaffolds were prepared and 16 hydrogel injections were performed. Sham-operated group received neither subcutaneous scaffolds nor material injection in the tibial medullary cavity.

The composition of OPF-based hydrogel scaffolds is depicted in Table 2 and synthesis was carried out as previously described by Kinard and co-workers [18]. In the same paper, cross-linking methods for OPF hydrogels have been thoroughly described. Briefly, OPF hydrogels can be formed by radical polymerization in the presence of either a thermally induced or ultraviolet-induced radical initiator. In addition, cross-linking molecules are used in OPF hydrogel fabrication to reduce cross-linking time and provide suitable handling characteristics for injectable applications. In the current study, OPF polymers were combined with PEGDA cross-linkers, and hydrogels were formed by radical polymerization in the presence of thermally induced radical initiators, i.e. the water-soluble redox pairs ammonium persulfate/N,N,N',N'-tetramethylethylenediamine (APS/TEMED). In view of that, the temperature was a key factor in controlling the gelation time.

For pre-set scaffolds, OPF and PEGDA were dissolved either in PBS (CaP-free) or CaP suspension (also in PBS, polymer/CaP ratio: 50/50) in order to prepare OPF and OPF-CaP hydrogels, respectively. The mixtures were shaken at room temperature for 30 minutes to

reach complete dissolution of polymers. Subsequently, filter sterilized (0.2 μ m filter) redox initiators, APS and TEMED, were added in equal concentration of 0.3 M. Immediately, 30 μ l of the polymer mixtures were injected into pre-sterilized Teflon molds ($\varnothing$ 6 mm, 1 mm thickness) and incubated for 15 minutes at 37°C. After gel formation, the hydrogels were transferred to 24-well plates and rinsed in sterile double-distilled water (ddH₂O) for 2 days. For sterilization, the hydrogels were soaked in 70% ethanol for 3 days followed by immersion in sterile PBS for 3 days, changing the buffered solution once a day under sterile conditions, as previously described [9].

For injectable hydrogel formulations, OPF and PEGDA were sterilized prior to use via exposure to ultraviolet light for 3 hours and freeze-dried overnight following an established technique [19]. The two components were then transferred in a 2.5 ml syringe under sterile conditions. In the operating room just prior to surgery, either sterilized PBS (for OPF) or CaP suspension (for OPF-CaP) was added and the mixtures were incubated at 37°C during the entire time of surgery in order to reach complete dissolution of polymers. When the tibia bone was ready for injection, sterile APS and TEMED, were added and the hydrogel solution was immediately injected.

2.4. Animal model and surgical procedure

The tibial marrow ablation model is an established model to study endosteal bone formation followed by bone marrow regeneration in mammals, such as rats, rabbits and guinea pigs [17,20-23]. In this study, the choice for the medullary cavity of guinea pigs for material injection was based on three preliminary facts: (1) two small holes are sufficient as an easily accessible site for injection; (2) the procedure ensures complete filling of the cavity using injectable materials, i.e. the outflow of the hydrogel material from the distal hole warrants complete filling; and (3) surgical procedures and material injection are reproducible, owing to a limited amount of surrounding soft tissues which enables easy manipulation and accessibility. Female animals were used because mature males are known to be aggressive toward unfamiliar adult males [24].

Twenty-two adult female Dunkin-Hartley guinea-pigs (age: 7 month; mean body weight: 1 kg) were included in this study. The guinea pigs were housed in three separate groups, with free access to food and water, and subjected to daily low-stress handling 1 month prior to surgery for reasons of accustoming [25]. The study was approved by the Animal Ethics Committee of Nijmegen, the Netherlands (DEC 2009-159) and national guidelines for animal

experimental work were obeyed. All surgical procedures were performed under general anesthesia and sterile conditions according to a previously described method [26]. Briefly, a full-thickness cortical defect (1.8 mm Ø) was performed in both distal (D) and proximal (P) sites of the right tibial diaphysis (Fig. 1a). After bone marrow evacuation (Fig. 1b), hydrogel solution, loaded in a 2.5-mL syringe connected to a catheter needle (BD Venflon™ Pro 1.8 x 45mm, Becton Dickinson), was injected into the ablated tibial medullary cavity. The proximal hole served as entrance for injection and the distal one as evacuation site for hydrogel material to ensure complete filling (Fig. 1c). After injection, gelation was allowed for 15 minutes and confirmed by visual changes in hydrogel color and palpation of excess hydrogel material at the evacuation site. In sham-operated group control, the ablated medullary cavity was left empty to study the effect of ablation on bone regeneration.

For the subcutaneous implantation of pre-set hydrogels, the dorsum of hydrogel-treated guinea pigs was shaved and disinfected with povidone iodine. Paravertebrally, two longitudinal incisions of about 15 mm were made through the full thickness of the skin, one on each side of the vertebral column. Subsequently, lateral to the incisions a subcutaneous pocket was created by blunt dissection with scissors. Preset hydrogels were placed in these pockets and the wounds were closed with resorbable sutures. No scaffold implantation or skin incisions were performed in sham-operated group. Postoperatively, antibiotics and analgesics were administered according to the established protocol. Animals were housed again in three separated groups with extra bedding to cushion the impact generated by walking and jumping, and given water and chow *ad libitum*. Moreover, animals were physically examined at regular intervals with focus on body weight, infections or adverse reactions. At 8 weeks post-implantation, all animals were euthanized.

2.5. Processing of explanted subcutaneous scaffolds

Immediately after euthanasia, subcutaneous implants with surrounding tissue were retrieved. The specimens were fixed in 10% neutral buffered formalin solution for 2 days and dehydrated through graded series of ethanol. Subsequently, each scaffold was cut in half and embedded in paraffin.

2.5.1. Histological and histomorphometrical analysis

Six sections (6 μm) of each specimen were stained with haematoxylin and eosin (HE). Histological evaluation on all sections was performed using previously reported scoring methods [27]. Briefly, the parameters were fibrous capsule quality and fibrous capsule thickness, as a measure of cellular composition and maturity, and number of fibroblast layers surrounding the scaffolds, respectively (Table 3). The histological evaluation was done in four random fields along the scaffold–tissue interface using an optical microscope (Axio Imager Microscope Z1, Carl Zeiss Micro imaging GmbH, Göttingen, Germany) at a magnification of 40X by two blinded evaluators.

2.6. Processing of explanted tibia

Treated tibias were harvested, excess tissue and epiphyses were removed, and the specimens were fixed in 10% neutral buffered formalin solution for 2 days and dehydrated in gradual series of alcohol from 70 to 100%. All samples were examined by microcomputed tomography (microCT), and histological and histomorphometrical analyses. In addition, six non-treated (left) tibias were retrieved and subjected to the same surgery and hydrogel injection ex vivo, in order to validate the animal model including assessment of the initial uniformity and quality of filling throughout the entire medullary cavity. These tibias were evaluated by microCT and histological analysis.

2.6.1. Microcomputed tomography (microCT)

MicroCT was performed on tibias to quantify the 3D microarchitecture of new bone within the medullary cavity. The specimens were wrapped in parafilm to prevent drying during scanning. Then, all tibia were scanned at an energy of 100 kV and intensity of 98 μA with a resolution of 15 μm using an aluminium filter (1 mm; Skyscan-1072 X-ray microtomograph, TomoNT version 3N.5, Skyscan[®], Belgium). Each 3D image data set consisted of approximately 800 slice images with 16-bit gray level. Cone-Beam reconstruction of cross-section images from tomography projection images was performed (NRecon[®], version 1.5.1.4, Skyscan), after which further analysis was performed by CT Analyzer (version 1.4, Skyscan[®], Belgium). The volume of interest (VOI) was specified as the cylindrical area delimited by cortical bone and included within the two small holes (excluded). In this area, cavity volume (CV, mm^3) and bone volume (BV, mm^3) were used to calculate the bone volume to cavity volume ratio, expressed as a percentage (BV/CV, %).

2.6.2. Histological and histomorphometrical analysis

After performing microCT imaging, all tibias were cut in 2 parts, i.e. a proximal (P) and a distal (D) part, which were alternately embedded in methyl methacrylate (MMA) or paraffin for histological analyses as depicted in Fig. 2.

Half of the specimens ($n = 11$ proximal parts (P); and $n = 11$ distal parts (D)) were dehydrated in gradual series of alcohol and embedded in methylmethacrylate (MMA). The temperature was controlled by placing the jars in a water bath at room temperature to prevent bubble formation in the samples. After polymerization in MMA, non-decalcified sections (10 μm thickness) were prepared in the transversal direction perpendicular to the main axis of the cavity using a diamond blade saw microtome. Six histological sections, three close to the distal (d) or proximal (p) hole and three close to the medial part (m), were made for each half tibia and were stained with methylene blue and basic fuchsin.

The remaining specimens ($n = 11$ distal parts (D); and $n = 11$ proximal parts (P)) were decalcified for 3 days. Each half tibia was cut in two halves, i.e. proximal (p) and medial (m) side for the proximal parts (P), and medial (m) and distal (d) side for the distal parts (D)), which were subsequently embedded in paraffin. Twelve transversal microtome sections (six distal (d) or proximal (p), and six medial (m); 6 μm thickness) were made and stained with Hematoxylin and Eosin (HE) and Elastica-Von Gieson (EVG) stain.

The light microscopical evaluation of all sections was done using an optical microscope (Axio Imager Microscope Z1, Carl Zeiss Micro imaging GmbH, Göttingen, Germany) and consisted of an assessment of material remnants and a complete morphological description of the tissue response to different treatments, including primary bone remodeling and marrow restoration. Histomorphometrical analysis was conducted for at maximum six different EVG-stained cross sections, each section containing two halves (6p and 6m, or 6d and 6m), resulting in a total of twelve measurements per specimen. The region of interest (ROI) was identified by drawing a line around the endosteal border of cortical bone (i.e. the border adjacent to the medullary cavity), and the percentage of newly formed bone within the ROI was scored using a computer-based image analysis technique (The Leica[®] Qwin Pro-image analysis system, Wetzlar, Germany). The measurements of all sections for each samples were averaged.

2.6.3. EDS analysis

MMA sections of OPF-CaP treated tibia (50-100 μm thick) at the proximal, medial and distal positions at 8 weeks of injection as well as a representative section of an OPF-CaP treated tibia *ex vivo* (used as reference) were processed for elemental mapping using a diamond saw. The elemental analysis was carried out using a Scanning Electron Microscope (Philips XL30, Eindhoven, the Netherlands) equipped with an Energy Dispersive Spectrometer (EDS, EDAX, AMETEK Materials Analysis Division, Mahwah NJ, USA). To improve the surface conductivity of the samples, a thin gold layer was deposited on the samples using a common sputtering instrument (Cressington 108A, Watford, England, UK). EDS analysis provided information on the distribution of elements of interest (Ca, P, O and C) and the atomic fraction of each element in the analyzed area. The atomic fraction of each element was presented as percentage with respect to other elements of interest (i.e. $\text{Ca} + \text{P} + \text{O} + \text{C} = 100\%$). The associated error for all the EDS analyses was calculated to be less than 10%.

2.7. Statistical analysis

Statistical analysis was performed with GraphPad InStat version 3.06 (GraphPad Software, San Diego CA, USA), using one way ANOVA combined with a post-hoc Tukey-Kramer Multiple Comparisons Test to detect statistically significant differences at a significance level (p-value) of $p < 0.05$. Results are presented as means $\pm$ standard deviations.

3. Results

3.1. Macroscopic observation on experimental animals

Two guinea pigs in OPF group were euthanized after 6 days due to clear evidence of stress and respiratory problems, resulting in loss of four OPF implanted subcutaneous scaffolds and two OPF-treated tibias. All the remaining animals, after a short-term weight loss, regained average weight and normal movement within 1 week post-surgery. At implant retrieval, no clinical signs of inflammation or infection (i.e. swelling or redness) were observed upon visual inspection of the treated hind limbs and in the dorsum of the animals. Table 4 depicts the number of subcutaneous scaffolds and tibias that were retrieved after the experimental period, and included for microCT and histological/histomorphometrical analyses. All implanted scaffolds could be retrieved and used for histological analysis. During processing

of the tibias, one medial side (m) of sham group, one proximal side (p), one medial side (m) and two distal sides (d) of OPF-CaP group were damaged and could not be used for histological/histomorphometrical analyses.

3.2. Subcutaneous scaffolds

3.2.1. Descriptive histology and histomorphometrical analysis

Gross observation of HE-stained sections of subcutaneous scaffolds after 8 weeks of implantation showed a loose structure for plain OPF (Fig. 3a), and a compact structure for OPF-CaP hydrogels (Fig. 3d). At higher magnification, examination of the internal structure revealed a substantial cell infiltration (i) throughout the entire OPF scaffolds (Fig. 3b) and a dense mass without cells for OPF-CaP scaffolds (Fig. 3e). A thin fibrous capsule (Fc) surrounding OPF hydrogels was composed by elongated fibroblasts with oval nuclei and all orientated in the same direction (Fig. 3c). Differently, OPF-CaP hydrogels were surrounded by a thin fibrous capsule formed by fibroblasts with oval nuclei (Fc) adjacent to an equally thin capsule layer of cells with round nuclei (i) directly in contact with the hydrogel scaffold (H) (Fig. 3f).

The results of the histomorphometrical analyses are presented in Fig. 3g. Based on the scoring system used for the qualitative (Fig. 3g-i) and quantitative (Fig. 3g-ii) evaluation of fibroblast capsule surrounding the scaffolds, a fibrous tissue, not dense, resembling connective tissue and consisting of approximately 5 fibroblast layers was observed surrounding both hydrogel types.

3.3. Tibias

3.3.1. Model validation

Fig. 4a shows a representative image of the anatomy and MMA sections of the internal structure at different regions of a guinea pig tibia. The anatomy of the tibia resembles an irregular inverted cone, with a trapezoid shape of the cross-section in the proximal part (~5.0 mm central axis), an oval shape of the cross-section in the middle part (~4.0 mm Ø), and a circular shape of the cross-section in the distal part of the tibia (~3.0-3.5 mm Ø). The thickness of cortical bone surrounding the cavity varies from ~0.4-1.0 mm in the proximal

side up to ~1.0-1.6 mm in the distal part where the cavity diameter is smaller. The cavity space is occupied by bone marrow.

In view of model validation, two small holes (1.8 mm Ø) close to the proximal (P) and distal metaphysis (D) were made *ex vivo* at a distance of about 2 cm from each other (Fig. 4b). Light microscopy examination of MMA-embedded tibias filled with OPF-CaP hydrogel *ex vivo* proved the ability of the material to completely fill the tibial medullary space and to adapt to the irregular shape of the cavity when initially injected (Fig. 4b-i). Nevertheless, 2D and 3D microCT imaging of same samples showed that the material was hardly detected due to the low radiodensity relative to the threshold limit of the system (Fig. 4b-ii).

Fig. 4c shows microCT analysis related to the radiopacity value within the VOI of sham, OPF and OPF-CaP-treated tibias *ex vivo*, and to the cavity length (mm) and volume (mm³) between the proximal and distal holes of treated tibias *in vivo*. For all these parameters, similar values were measured for all groups ($p > 0.05$), i.e. ~21 mm for the cavity length and ~81 mm³ for the cavity volume.

3.3.2. Bone tissue response

3.3.2.1. MicroCT

Fig. 5a shows the microCT results at 8 weeks. The VOI in microCT analyses is schematically depicted in Fig. 5a-i. Different from *ex vivo* treated tibias, the 2D grayscale cross-sectional images of OPF-CaP-treated tibias after 8 weeks showed areas of increasing radiopacity from the proximal to the distal region. The grey color was converted to green color to visualize the areas of radiopacity within the VOI in the 3D-reconstructed models (Fig. 5a-ii). Analysis of microCT scan gray values showed comparable results between the groups (sham $30.6 \pm 10.3\%$; OPF $22.1 \pm 9.6\%$; OPF-CaP $27.2 \pm 10.8\%$) (Fig. 5a-iii).

3.3.2.2. Descriptive histology and histomorphometrical analysis

The histological and histomorphometrical results at 8 weeks are reported in Fig. 5b. The ROI in histological and histomorphometrical analyses are schematically depicted in Fig. 5b-i. HE-stained histological sections of sham, OPF and OPF-CaP treated tibias at the proximal, medial and distal regions showed different scenarios between the groups and even between the same group (sections separated by dotted lines), as described in detail below (Fig. 5b-ii).

Sham In the proximal region, either solely fatty marrow or bone trabecular islands with varying thickness were evident throughout the marrow space. The medial area was remodeled into relatively long and dense trabeculae containing a moderate number of fat cells. At the distal site, a very thick callus tissue bridging the cortical bone containing connective tissue and few cellular marrow was observed.

OPF The proximal region was comparable to sham-treated specimens, i.e. either only filled with mature marrow or thin trabecular islands surrounded by cellular marrow. In the medial and distal region, three out of six marrow cavities showed OPF hydrogel (H) within the medullary bone; the other three tibias showed only thin trabeculae disconnected from each other, highly cellular marrow and no material remnants.

OPF-CaP The proximal region was comparable to proximal regions in sham and OPF-treated tibias. The material was no longer detected. In the medial and distal region of three out of eight OPF-CaP treated tibias, hydrogel (H) remnants were present, surrounded by immature and cellular marrow. In addition, some distal regions showed distinct cell-free calcified areas (dark purple color in the boxed region). All the other samples (5/8) did not show hydrogel material remnants, but rather a thickening of trabeculae, extending from the cortical border, matched with a gradual depletion of fatty cells in the marrow.

The newly formed bone, including the new bone in direct contact to the surrounding cortical bone and the trabecular structures within the medullary cavity, was measured within the ROI. The total amount of new bone was comparable between the sham ($27.7 \pm 16.9\%$), OPF ($14.0 \pm 7.8\%$) and OPF-CaP ($13.8 \pm 9.5\%$) (Fig. 5b-iii). Specifically, all groups showed a higher percentage of newly formed bone in the medial region and the distal region compared to the proximal regions. However, only sham showed statistical differences ($p < 0.05$) between the distal ($41.3 \pm 23.8\%$) and proximal ($8.7 \pm 10.6\%$) regions.

3.3.2.3. EDS analysis

The amount of phosphorous (P) and calcium (Ca) in arbitrary regions within the medullary cavities of OPF-CaP treated-tibias after 8 weeks was observed to be different from the composition of the ex vivo-OPF-CaP treated tibia (Figure 6a-i). Importantly, the relative amounts of P and Ca were lowest in the proximal region and highest in the distal region (Fig. 6a-ii).

The mapping of elements (Fig. 6b) within the medullary cavities showed that P and Ca were hardly detected in the ex vivo-treated tibia as well as in the proximal region of in vivo-treated

tibia after 8 weeks. Differently, large accumulation of P and Ca were evident in the medial and distal regions of in vivo-treated tibia after 8 weeks.

4. Discussion

The present study aimed to evaluate the soft tissue response and osteogenic performance of plain OPF hydrogels and OPF hydrogels enriched with CaP nanoparticles, using both subcutaneous implantation of pre-set scaffolds and injection of hydrogel precursor solutions into the tibial medullary cavity of guinea pigs. Incorporation of CaP nanoparticles was explored as a mimic of the inorganic phase of bone, for which we hypothesized that OPF-CaP hydrogels would outperform plain OPF hydrogels in terms of tissue response and bone forming capacity. The results of this study indicated similar biocompatibility for both types of pre-set OPF-based scaffolds, although OPF-CaP maintained a dense structure and reduced cellular infiltration as well as material degradation compared to OPF scaffolds. The tibial marrow ablation model in guinea pigs represented a simple and reproducible surgical technique and allowed an adequate volume ($\sim 81 \text{ mm}^3$) for application of hydrogels in injectable formulation. At 8 weeks following ablation, new trabecular bone was formed in all tibias, but the bone remodeling and bone marrow restoration varied between the different regions within the medullary cavity. Importantly, hydrogel remnants were still present in only few tibias and, for OPF-CaP group, highly calcified areas were found in the distal region as a result of aggregation and sedimentation of CaP nanoparticles upon implantation.

Previous work using OPF-based hydrogels demonstrated the biocompatibility of these materials and their degradation products [9,12,28]. Similarly, in this study, both types of OPF-based hydrogels evoked a mild tissue response after 8 weeks of implantation. Nevertheless, OPF scaffolds presented a loose structure combined with a remarkable number of infiltrating cells and pronounced degradation, as a result of a high water content ($\sim$ over 90% of w/v). Differently, the dense structure of OPF-CaP scaffolds was maintained by both the presence of homogeneously distributed CaP nanoparticles throughout the polymeric matrix and reduced water content (approximately two times less than OPF hydrogels [16,29].

While earlier studies used pre-formed OPF-based hydrogels to test the regenerative potential in osteochondral and bone defects [9,12,28], the current study was the first attempt to evaluate the in vivo performance of these materials as injectable formulation.

The literature describes the use of injectable bone substitute materials in animal models either for bone defect repair or bone augmentation. Specifically, bone defects are generally created

by drilling into or removing parts of intact bone tissue, whereas bone augmentation procedures are performed to obtain an increased amount of high quality bone. Although a few models in small animals (e.g. mouse and rat calvaria, epiphysis or diaphysis) are clinically feasible for injectable bone substitute materials, none of those allows injection of substantial amounts of materials. For instance, full thickness or partial craniotomies are not suitable for injectable hydrogels as they might penetrate inside the brain or leak out of the defect after scaffold swelling. Similarly, the diaphysis or epiphysis of long bones of mice and rats is not suitable for direct injection of in situ crosslinkable materials due to too small diameter incisions, too small diameter needles and too low cavity volume [30]. Consequently, the tibial marrow ablation model in guinea pigs was considered to be the smallest animal that would offer a reservoir with adequate capacity for material injection (~1 ml of hydrogels). As new bone formation was induced in a space (i.e. the medullary cavity) where bone tissue is not normally present, this model served to test the potential use of OPF-based hydrogels for bone augmentation application. Specifically, mechanical emptying of the medullary cavity of a tubular bone triggers endosteal bone formation [17,31] because of the osteogenic potential of the endosteum, i.e. a thin layer of connective tissue surrounding the bone marrow spaces [32]. After marrow removal, the blood clot fills the diaphyseal medullary cavity and releases several osteogenic growth polypeptides, such as insulin-like growth factor (IGF-I), and osteogenic growth peptide (OGP), which mediate the replacement with primary trabecular bone (the so-called initial intramedullary osteogenic phase) [33,34]. The formation of bone progresses centripetally, i.e. from the cortical endosteal surface towards the center of the medullary cavity, until reaching almost complete filling with transient trabecular mesh at the peak of the osteogenic phase. Subsequently, the bone trabeculae are subjected to osteoclastic resorption and replacement by new marrow, which is identical to the original tissue that was removed (the so-called second osteogenic phase) [35,36]. In rats, the entire process is completed within 35 days [2]. Not surprisingly, in this study consistent amounts of new bone were formed in the sham group and the process was not complete after 8 weeks, as guinea pigs have substantially larger long bones and lower metabolic activity compared to rats.

Little is known about the impact of bone graft materials on the formation of primary bone within the marrow cavity. According to a previous study, chemical composition and structural properties of bone graft materials affect both the rate and extent of primary bone formation, and influence the quality and rate at which marrow regeneration occurs [2]. As mentioned above, the present study was initiated to test for the first time OPF-based hydrogels in injectable formulations in vivo. Similar to the pre-set scaffolds, the excellent tolerability of

such materials was confirmed in the tibia as no signs of prolonged inflammatory response nor leg swelling were observed. Histological evaluation showed new bone formation in all tibias and presence of material remnants in only few specimens. More surprisingly, unexpected dark and dense regions were observed in the distal tibias of OPF-CaP group as indication of heterogeneously calcified areas, suggesting the aggregation and sedimentation of CaP nanoparticles. This hypothesis was corroborated by EDS analysis which attested the presence of much higher amounts of phosphorous and calcium, the major components of CaP minerals, in the distal regions compared to the medial and proximal regions.

Summarizing, these findings raised two different assumptions: (i) hydrogel formation from flowable aqueous solutions was hampered in a highly perfused environment, like the medullary cavity [37], which might have caused sedimentation of aggregated CaP nanoparticles in the distal region of the tibias, or ii) degradation of the polymeric hydrogel matrix proceeded faster than degradation of the nanostructured mineral phase resulting into loss of entrapment of the CaP nanoparticles and subsequent sedimentation into the distal parts of the tibia. Both assumptions were supported by the similar amounts of bone formation as measured in sham and hydrogel treated-tibias after 8 weeks. Specifically, the disappearance of OPF-based hydrogels from the medullary cavity, either because of lack of in situ gelation (1st assumption) or too fast degradation (2nd assumption), evoked a comparable outcome for sham and hydrogel-treated tibias. To verify whether the in vivo environment negatively influenced the gelation of OPF-based matrices, a simple additional gelation study was performed. Specifically, the gelation efficiency in both wet and dry environments was tested under both static and dynamic conditions (see Supplementary Information for further experimental details). Under dry conditions, all OPF-based hydrogels formed within 15 min. Figure 7 shows the dry mass percentage of hydrogels formed in a wet environment under static (0 rpm) and dynamic conditions (60, 150 and 300 rpm) relative to the mass of gels formed in a dry environment at static conditions (0 rpm). It was observed that CaP nanoparticles exhibited a stabilizing effect on OPF-CaP hydrogel integrity, while the gelation of OPF hydrogels was considerably compromised by increasing the shaking speed from 0 to a maximum value of 300 rpm. These data suggested that formation of OPF-based hydrogels can be hampered under highly perfused (i.e. wet and highly dynamic) conditions. Since the experimental conditions of this preliminary in vitro study do not reflect the complexity of the physiological environment, it was not possible to correlate the findings of the vitro gelation study to the in vivo study, which warrants the development of a suitable in vitro test that can predict the gelation behavior of hydrogels in vivo.

Two different analytical techniques, i.e. conventional micro-CT and histomorphometry, were used for the quantification of new bone formation within the tibial medullary cavity after 8 weeks of injection. Observation of the results demonstrated that both techniques showed a similar trend. However, regarding the hydrogels-treated tibias, the percentage of bone as measured by micro-CT was higher compared to the histomorphometrical outcomes. This mismatch might be explained by the different principles of the approaches applied [38]. For instance, destructive 2D methods allowed for more accurate measurements due to the ability to discriminate between cortical bone and adjacent newly formed bone, and between trabecular structures and remnants of highly calcified material within the cavity. On the other hand, histological techniques limited the quantification on only a few representative sections of the three distinct areas (i.e. proximal, medial and distal regions of the tibia). Differently, non-destructive 3D analysis examined the entire tibias thus yielding an exhaustive evaluation. However, the indistinguishable gray level values between new bone and the highly calcified area in the distal region of OPF-CaP treated tibias undoubtedly contributed to increase the value of radiopacity in the samples thus to result in an overestimation of bone formation.

5. Conclusion

Biocompatibility and osteogenic capacity of OPF and OPF-CaP hydrogels were investigated through ectopic implantation of preset scaffolds and injection of flowable hydrogel precursor solutions in a tibial ablation model, respectively. Specifically, the results showed a thin and relatively mature fibrous capsule surrounding both types of subcutaneous scaffolds, although the presence of CaP nanoparticles was observed to limit degradation and cellular invasion. Bone formation in the medullary cavity, which is a site predisposed to form bone, demonstrated to be equal for both groups after 8 weeks. However, the presence of highly calcified areas in the distal region of OPF-CaP treated tibias, as a result of CaP nanoparticles sedimentation and agglomeration, suggested incomplete gelation of the injectable hydrogels in vivo. Based on these observations, the high flowability and uncontrolled gelation within the complexity of the physiological environment seemed to be the major critical factors for the application of OPF-based hydrogels in injectable formulation.

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Captions

- Fig. 1** Tibial marrow ablation surgery and hydrogel injection in guinea pigs. (a) Exposed distal and proximal holes; (b) bone marrow (arrow) flushed from the medullary cavity; (c) hydrogel injection through a syringe connected to a catheter needle.
- Fig. 2** Sample processing for histological and histomorphometrical analysis. Specimens were cut in half, i.e. proximal tibia (P) and distal tibia (D), and alternatively embedded in MMA and paraffin. For instance, for tibia 1 the proximal tibia (P1) was embedded in MMA and distal tibia (D1) was embedded in paraffin. For tibia 2, proximal tibia (P2) was embedded in paraffin and distal tibia (D2) was embedded in MMA. For MMA-embedded samples, six sections (3 proximal (p) and 3 medial (m) or 3 medial (m) and 3 distal (d)) were obtained. Paraffin-embedded samples were cut in half and twelve sections (6 medial (m) and 6 distal (d) or 6 proximal (p) and 6 medial (m)) were obtained.
- Fig. 3** (a-d) HE-stained histological sections showing overview of OPF and OPF-CaP subcutaneous scaffolds at 8 weeks (scale bars = 500 μm). (b-e) Internal structure (scale bars = 100 μm) and (c-f) fibrous capsule surrounding OPF and OPF-CaP scaffolds at higher magnification (scale bar = 50 μm). H: hydrogel, i: infiltrated cells, Fc: fibrous capsule. (g) Histological scoring of (i) quality and (ii) thickness of fibrous capsule surrounding OPF and OPF-CaP scaffolds at 8 weeks. Results represent the means $\pm$ standard deviations of 6 scaffolds (n sections=6) per condition.
- Fig. 4** (a) Macroscopic image and MMA-histological sections of the internal structure of a native tibia of guinea pig. (b) Macroscopic image, (i) MMA-histological sections and (ii) 2D and 3D-reconstructed microCT images of a OPF-CaP treated-tibia ex vivo. (c) Radiopacity (%) within the medullary cavity of sham, OPF and OPF-CaP treated tibias ex vivo, and cavity volume (mm^3) and length (mm) of same groups treated in vivo, as determined with microCT. Error bars represent means $\pm$ standard deviation.
- Fig. 5** (a) (i) Schematic representation of the VOI (i.e. the area included between the proximal and distal hole) for microCT analyses; (ii) 2D and 3D-reconstructed microCT images of the medullary cavity of a tibia filled with OPF-CaP hydrogels after 8 weeks and (iii) measurements of radiopacity (%) within VOI of sham, OPF and OPF-CaP treated tibias after 8 weeks. (b) (i) Schematic illustration of the positions of three ROI (proximal, medial and distal) within the medullary cavity for histological and histomorphometrical analysis. (ii) HE-stained sections of sham, OPF

and OPF-CaP tibias at three different regions (scale bars = 1000 μm). Dotted lines discriminates two different conditions belonging to the same group. Boxed region shows highly calcified areas (dark purple color) in the distal region of OPF-CaP treated-tibias (scale bar = 100 μm). (iii) Results of histomorphometrical and statistical analysis of new bone formation (%) displayed for the total of the three zones and for the single zones. *refers to ($p < 0.05$). Error bars represent means $\pm$ standard deviation.

Fig. 6 (a) (i) MMA sections (scale bars = 1000 μm) and (ii) amount of phosphorous (P) and calcium (Ca) in arbitrary regions (black square) within the cavity of OPF-CaP treated tibias ex vivo and after 8 weeks as determined with EDX analyses. (b) Distribution of elements in the representative regions. Carbon (C) and oxygen (O) represent the organic matrix, phosphorous (P) and calcium (Ca) represent the CaP minerals.

Fig. 7 Dry mass of OPF and OPF-CaP hydrogels formed under wet conditions at different shakig speeds (0, 60, 150 and 300 rpm) calculated as the percentage of the control group (i.e. hydrogels formed in dry conditions at 0 rpm). *** $p < 0.001$ related to OPF at 0 rpm. +++ $p < 0.001$ related to OPF-CaP at 0 rpm. Error bars represent $\pm$ standard deviation.

Tibia 1

Tibia 2

Group	n° subcutaneous samples	n° injection
1) sham	-	-
2) OPF	16	8
3) OPF-CaP	16	8

Table 1 Experimental groups used in this study.

	OPF	OPF-CaP
OPF (g)	0.15	0.15
PEGDA (g)	0.075	0.075
PBS (μl)	860	-
CaP suspension (μl)	-	860
0.3 M APS (μl)	70	70
0.3 M TEMED (μl)	70	70
Total volume (μl)	1000	1000

Table 2 Components and relative amounts for CaP-free (OPF) and CaP-enriched (OPF-CaP) hydrogels preparation.

Histological grading scale for capsule quality	Score
Capsule tissue is fibrous, not dense, resembling connective or fat tissue in the noninjured regions	4
Capsule tissue is fibrous but immature, showing fibroblasts and little collagen	3
Capsule tissue is granulous and dense, containing both fibroblasts and many inflammatory cells	2
Capsule tissue consists of masses of inflammatory cells with little or no signs of connective tissue organization	1
Cannot be evaluated because of infection or other factors not necessarily related to the material	0

Histological grading scale for capsule thickness	Score
1-4 fibroblasts	4
5-9 fibroblasts	3
10-30 fibroblasts	2
>30 fibroblasts	1
Not applicable	0

Table 3 Histological grading scale for capsule quality and thickness.

subcutaneous scaffolds			tibias						
Group	n° samples retrieved	histomorphometry	n° samples retrieved	microCT	histology				histomorphometry
					MMA	paraffin			
3) sham	-	-	6	6	3D 3P	3P	3D	3p 5m ^b 3d	
1) OPF	12 ^a	6 ^a	6 ^a	6 ^a	3D 3P	3P	3D	3p 6m 3d	
2) OPF-CaP	16	8	8	8	4D 4P	4P	4D	3p ^b 7m ^b 2d ^b	

^a During the experimental time two animals were euthanized

^b During histological preparation one medial side of sham-tibia, one proximal side, one medial side and two distal sides of OPF-CaP-filled tibia were damaged

Table 4 Number of subcutaneous scaffolds and tibias retrieved for each group and number of samples used for the respective analyses (P:

proximal tibia; D: distal tibia; p: proximal side, m: medial side, d: distal side).