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

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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. Microcomputed tomography and histological and histomorphometrical analyses showed comparable amounts 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, which indicate 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 in loss of entrapment of the CaP nanoparticles and subsequent sedimentation.


## 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 healthcare systems. 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 fulfil all the aforementioned requirements. These highly hydrated polymer networks are injectable and biodegradable as well as biocompatible due to their high water content. As a result, hydrogels can act as artificial extracellular matrices (ECMs) which provide a temporary three-dimensional (3-D) 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

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**Table 1**
Experimental groups used in this study.

| Group | No. of subcutaneous samples | No. of injection |
|---|---|---|
| 1) Sham | – | – |
| 2) OPF | 16 | 8 |
| 3) OPF–CaP | 16 | 8 |

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 min 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 tailorable structural and mechanical properties of resulting OPF-based hydrogels [13,14].

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 $mol^{-1}$ 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 (PBS) pH 7.4 were purchased from Sigma–Aldrich (St Louis, MO, USA). Fumaryl chloride, phosphoric acid ($H_3PO_4$) and calcium hydroxide ($Ca(OH)_2$) were acquired from Acros (Pittsburgh, PA, USA).

### 2.2. CaP nanoparticles preparation

A homogeneous suspension of nanosized 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 $Ca(OH)_2$ (5.92 M) were prepared in PBS, and the precursor compounds were used at a stoichiometric Ca/P ratio of 1.67 to obtain a final solid content of ~0.3 g $ml^{-1}$ (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 three 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. The 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 in 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 min to reach complete dissolution of polymers. Subsequently, filter sterilized (0.2 μm filter) redox initiators, APS and TEMED, were added in equal concentrations of 0.3 M. Immediately, 30 μl of the polymer mixtures were injected into pre-sterilized Teflon molds (∅ 6 mm, 1 mm thickness) and incubated for 15 min at 37 °C. After gel formation, the hydrogels were transferred to 24-well plates and rinsed in sterile double-distilled water ($ddH_2O$) for 2 days. For sterilization, the hydrogels were soaked in 70% ethanol for 3 days followed by immersion in sterile PBS for 3 days; the buffered solution was changed 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 h 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 regen-