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Poly (fumaroyl bioxirane) maleate: A potential functional scaffold for bone regeneration

Yi Ding [a,1], Yun Su [a,1], Ziyin Lv [b,1], Hao Sun [a], Xiaoping Bi [a], Linna Lu [a], Huifang Zhou [a], Zhengwei You [b], Yadong Wang [c], Jing Ruan [a,*], Ping Gu [a,*], Xianqun Fan [a,*]

[a] Department of Ophthalmology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, People's Republic of China
[b] Biomaterials and Tissue Engineering Laboratory, College of Chemistry & Chemical Engineering and Biotechnology, Donghua University, Shanghai, People's Republic of China
[c] Departments of Bioengineering, Chemical Engineering, Surgery, and the McGowan Institute, University of Pittsburgh, 3700 O'Hara Street, Pittsburgh, PA 15261, USA

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ABSTRACT

Proper scaffolds combined with mesenchymal stem cells (MSCs) represent a promising strategy for repairing bone defects. In a previous study, poly (fumaroyl bioxirane) maleate (PFM), a newly developed functional polymer with numerous functional groups, exhibited excellent biocompatibility and enhanced the alkaline phosphatase (ALP) activity of osteoblasts in vitro. Here, to provide further and comprehensive insight into the application of PFM in bone tissue engineering, we investigated the osteoinductive potential of PFM cultured with rat adipose-derived mesenchymal stem cells (rADSCs). The results showed that PFM resulted in greater proliferation of rADSCs and that the PFM substrate had stronger osteoinductivity than PLGA and the control, as indicated by the significant upregulation of osteogenesis-related genes, proteins and calcium mineralization in vitro. Next, PFM was combined with rADSCs to repair a critical-sized calvarial defect in rats. Compared to the PLGA scaffold, the PFM scaffold significantly promoted new bone formation and exhibited excellent effects in repairing rat calvarial defects. In conclusion, PFM possesses strong osteoinductivity, which could markedly enhance bone regeneration, suggesting that PFM could serve as a promising and effective optimization method for traditional scaffolds in bone regeneration.

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1. Introduction

Bone defects arising from trauma, tumours, or skeletal diseases are common issues and lead to many clinical and social problems that often require bone reconstruction [1]. Bioactive scaffold-based tissue engineering has been a widely investigated and applied strategy for bone regeneration [2–4]. As a key component, scaffolds play critical roles in supporting cells in proliferation, osteo-differentiation, and bone formation in vivo [5,6].

For decades, research regarding novel three-dimensional biocompatible scaffolds has been the focus of the field of bone tissue engineering [7,8]. Conventional scaffolds, such as biodegradable synthetic polymers (e.g., Poly(ε-caprolactone) (PCL) and PLGA) [9–11], ceramic materials (e.g., β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA)) [12–14], metal materials (e.g., titanium alloy) [15] and natural materials (e.g., silk fibroin) [16,17] have been applied for bone regeneration in experiments but have had several disadvantages, including low mechanical strength, hydrophilia, inappropriate degradation rates, and poor biocompatibility and osteoinductive properties. Sufficient mechanical strength is an essential characteristic of an ideal scaffold for bone defects, and suitable hydrophilia allows the sufficient transfer of metabolites and nutrients [18,19]. Furthermore, a controlled degradation rate can balance the speed between scaffold degradation and new bone regeneration, which is better for bone regeneration [20]. Recent work has demonstrated that osteoinductive properties can be promoted by modifying the surface of the scaffold, such as by introducing hydroxyl and carboxyl functional groups, especially for synthetic polymers [21–23].

Poly (fumaroyl bioxirane) maleate (PFM), a newly developed bioactive polymer that contains quantities of hydroxyl, carboxyl, and alkenyl groups, may overcome the disadvantages of conventional bone graft materials [24]. The extension functional groups on PFM could serve as cellular recognition sites, which may enhance cell adhesion, proliferation, differentiation, and meanwhile benefit tissue formation and growth [22,25]. A previous study has shown that the carboxyl functional

* Corresponding authors at: Department of Ophthalmology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Zhizaoju Road No. 639, Shanghai 200011, People's Republic of China.
E-mail addresses: drjruan@163.com (J. Ruan), guping2009@126.com (P. Gu), xianqun_fan@126.com (X. Fan).

[1] These authors contributed equally to this work.

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group in extracellular matrix (ECM) is the key factor of ossification. Thus, the carboxyl functional group on PFM may promote bone regeneration by imitating the structure of natural bone and providing mineralization sites [26,27]. Such functional groups may also flexibly bind peptides and proteins to achieve certain biological effects. In addition, the modification of the crosslinking degrees, including catalysing condensation reactions of the functional groups and radical addition reactions of alkenyl groups, allows for highly adjustable physiochemical properties of PFM, such as stiffness, toughness and degradation rate. Furthermore, PFM is made using a newly developed synthetic method called acid-induced epoxide ring-opening polymerization. The entire fabrication process is based on commercially available reagents and can be easily expanded [28].

Mesenchymal stem cells (MSCs) have multiple differentiation potential and significant clinical potential for the treatment of bone defects [29]. Of the various sources of tissues, such as adipose tissue, bone marrow, and umbilical cord blood [30,31], adipose tissue and bone marrow derived mesenchymal stem cells (ADSCs and BMSCs) are two major species of seed cells that have been widely used for tendon [32], cartilage [33], bone regeneration [34,35] and wound healing [36]. Compared with BMSCs, ADSCs lack HLA-ABC expression, meaning that ADSCs are less likely to be attacked by the host's immune system [37]. Moreover, ADSCs could be derived from the waste of liposuction or doubled eyelid surgery, which makes ADSCs more accessible in clinical practice [38]. Thus, ADSCs are ideal seed cells for bone regeneration.

In the present study, we investigated the influence of PFM on rat ADSCs (rADSCs) via cell viability, cell proliferation, osteo-differentiation in vitro, and the capacity for bone reconstruction in a rat critical-sized defect model in vivo. Based on these evaluations, we verified the utility of the promising PFM polymer in bone tissue engineering both in vitro and in vivo.

2. Methods and materials

2.1. Polymer synthesis and characterization

PFM was synthesized via acid-initiated epoxide ring-opening polymerization according to previous reports [24]. Briefly, equivalent amounts of 2,2-bioxirane (Sigma Aldrich, 97%) and fumaric acid (Mallinckroft, 99.5%) were reacted in the presence of 0.6 mol% bis (tetrabu-tylammonium) sebacate in *N*,*N*-dimethylformamide (DMF, Acros, 99.8%) at 85 °C for 26 h. Then, the purified and freeze-dried resultant poly (fumaroyl bioxirane) (PFB) was mixed with 2 equimolar amounts of maleic anhydride (Alfa Aesar, 99%) at 80 °C for 4.5 h. Finally, after freeze-dried for 60 h, PFM was obtained.

For polymer characterization, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectrum and attenuated total reflectance-Fourier transformed infrared (ATR-FTIR) spectrum was recorded using a Bruker Avance 600 NMR and a Thermo Nicolet IR iS 10 spectrometer respectively. Universal Analysis 2000 software from TA instruments was used to record the glass transition temperature (Tg). The static air–water contact angle of the PFM slab (thickness = 1.3 mm) was determined using a KSV Instruments Theta Lite Optical Tensiometer TL100. Three measurements were performed and averaged.

2.2. PFM substrate preparation for cell culture

PFM substrate was prepared as previously reported [24]. Briefly, PFM was coated on the surfaces of tissue-culture-treated polystyrene (TCPS). A 0.2-μm filter was used to filter the 2,2,2-trifluoroethanol solution of polymer (1 g/L), and the solution was then added to 24-well (80 μl/well) or 96-well (20 μl/well) TCPS plates. After the solvent evaporating in air under a vacuum overnight, the plates were sterilized and washed with gentle shaking before use. PLGA and no substrate were used as the control.

2.3. Cell isolation, culture and inductive osteogenesis

All research was approved by the Animal Research Committee of Shanghai Ninth People Hospital, affiliated with the Shanghai Jiao Tong University School of Medicine. rADSCs were isolated as previously reported [2]. Briefly, 8-week-old male Sprague Dawley (SD) rats (Shanghai Animal Experimental Center, China) were sacrificed to obtain fat pads of inguinal area. Fat pads were minced and 0.1% collagenase solution (Sigma-Aldrich Corp, USA) was used to digest tissues. Then, the digested tissues were incubated at 37 °C with gentle shaking for 12 h and resuspended in alpha-Minimum Essential Medium (a-MEM, Invitrogen, USA) containing 10% foetal bovine serum (FBS, Gibco) and 100 units/ml of penicillin and streptomycin (Invitrogen) before incubation at 37 °C in an atmosphere with 5% CO_2 [39]. For inductive osteogenesis, rADSCs were cultured on PFM substrates at a density of 5.0×10^4/cm^2. After incubation for 24 h, the culture medium was changed to osteogenic inductive medium supplemented with 10% FBS (Gibco), 100 units/ml of penicillin and streptomycin (Invitrogen), 10 mM b-glycerophosphate (Sigma), 50 mg/ml ascorbic acid (Sigma) and 100 nM dexamethasone (Sigma-Aldrich). All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Live/Dead assay and lactate dehydrogenase (LDH) assay

Cell viability was assessed by Live/Dead assay (Invitrogen, UK), and LDH assay for quantification following the manufacturer's instructions. Briefly, after incubation of rADSCs on PFM, PLGA or without substrate at a density of 3.0×10^4/cm^2 for 3 days. 2.5 μl/ml of 4 μM ethidium homodimer-1 (EthD-1) and 1 μl/ml of 2 μM calcein AM were mixed into 1 ml of PBS. Each sample was incubated with 200 μl of the mixed solution for 15 min, then, the samples were washed and viewed under a Nikon Eclipse E600 fluorescence microscope with 494 nm (green, calcein) and 528 nm (red, EthD-1) excitation filters. The quantification of cell viability was evaluated the release of lactate dehydrogenase (LDH). The level of released LDH was detected by LDH cytotoxicity assay detection kit (Beyotime, China). The absorbance at 490 nm was measured by the microplate reader within 1 h.

2.5. CCK-8 and BrdU assays

The influence of PFM, PLGA or no substrate on rADSC proliferation was evaluated using cell counting kit-8 (CCK-8) assays and 5-bromo-2-deoxyuridine (BrdU) assays as previously reported [40]. For CCK-8 assays, the cells were incubated at a density of 3.0×10^4/cm^2 in 96-well plates, and 10 μl of CCK-8 solution was added to each well (Dojindo Molecular Technologies, Japan) for 4 h at 37 °C. The absorbance at 450 nm in wavelength was measured. For BrdU assays, the cells were incubated in 24-well plates, and BrdU (Sigma) was added to each well at a final concentration of 10 mM for 10 h at 37 °C. After being fixed in 4% PFA (Sigma) and permeabilized with 0.3% Triton X-100 (Invitrogen), the cells were incubated with anti-BrdU monoclonal antibody (1:200, Santa Cruz, USA) at 4 °C overnight. Then, 546-goat anti-mouse secondary antibody (1:800, Alexa Fluor, USA) was added to 24-well plates and Hoechst (Invitrogen) was used to counterstain the cell nuclei. rADSC proliferation was recorded and evaluated under a fluorescence microscope (Olympus BX51, Japan).

2.6. Quantitative real-time PCR

The mRNA expression of several inflammatory-related genes, including Interleukin-1 (IL-1), Interleukin-6 (IL-6), Caspase3, and osteogenic differentiation-related genes, including Runt-related transcription factor 2 (RUNX2), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), osterix (OSX), alkaline phosphatase (ALP), collagen 1A (COL1A), were quantified by real-time polymerase chain reaction (PCR). PCR was performed as previously described [41]. Briefly,

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