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Bioprinting-based PDLSC-ECM Screening for in vivo Repair of Alveolar Bone Defect using Cell-laden, Injectable and Photocrosslinkable Hydrogels

Yufei Ma†‡, Yuan Ji†‡, Tianyu Zhong‡§, Wanting Wan‡§, Qingzhen Yang†‡, Ang Li§, Xiaohui Zhang‡§*, Min Lin‡§*

†The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi’an Jiaotong University, No. 28 Xianning West Road, Xi’an 710049, P.R. China
‡Bioinspired Engineering and Biomechanics Center (BEBC), Xi’an Jiaotong University, No. 28 Xianning West Road, Xi’an 710049, P.R. China
§Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi’an Jiaotong University, No. 98 Xiwu Road, Xi’an 710004, Xi’an 710049, P.R. China

Corresponding Authors

*E-mail: minlin@mail.xjtu.edu.cn (M.L.).

*E-mail: xiaohuizhang@mail.xjtu.edu.cn (X.H.Z.).
**ABSTRACT:** Periodontitis is an inflammatory disease worldwide that may result in periodontal defect (especially alveolar bone defect) and even tooth loss. Stem cell-based approach combined with injectable hydrogels has been proposed as a promising strategy in periodontal treatments. Stem cells fate closely depends on their extracellular matrix (ECM) characteristics. Hence, it is necessary to engineer an appropriate injectable hydrogel to deliver stem cells into the defect while serving as the ECM during healing. Therefore, stem cell-ECM interaction should be studied for better stem cell transplantation. In this study, we developed a bioprinting-based strategy to study stem cell-ECM interaction and thus screen an appropriate ECM for *in vivo* repair of alveolar bone defect. Periodontal ligament stem cells (PDLSCs) were encapsulated in injectable, photocrosslinkable composite hydrogels composed of gelatin methacrylate (GelMA) and poly(ethylene glycol) dimethacrylate (PEGDA). PDLSC-laden GelMA/PEGDA hydrogels with varying composition were efficiently fabricated via a 3D bioprinting platform by controlling the volume ratio of GelMA-to-PEGDA. PDLSC behavior and fate were found to be closely related to the engineered ECM composition. The 4/1 GelMA/PEGDA composite hydrogel was selected since the best performance in osteogenic differentiation *in vitro*. Finally, *in vivo* study indicated a maximal and robust new bone formation in the defects treated with the PDLSC-laden hydrogel with optimized composition as compared to the hydrogel alone and the saline ones. The developed approach would be useful for studying cell-ECM interaction in 3D and paving the way for regeneration of functional tissue.

**KEYWORDS:** 3D bioprinting, cell-ECM screening, PDLSCs, injectable hydrogel, alveolar bone repair
1. INTRODUCTION

Periodontitis is one of the most common oral diseases that may cause alveolar bone defect and eventually tooth loss, affecting ~15% of adults worldwide.\textsuperscript{1,2} Although present clinical treatments (e.g., periodontal flap surgery and root planning conditioning) demonstrate therapeutic effects on controlling periodontal inflammation and preventing further periodontitis progression, such therapies cannot repair the damaged alveolar bone and thus cannot recover the functionality of the diseased teeth.\textsuperscript{3} Previously, bone graft and guided tissue regeneration methods have been developed for periodontal treatments. However, such strategies strongly depend on the defect shape or size.\textsuperscript{4,5} Recently, stem cell transplantation via injection has been demonstrated as a promising therapeutic method. Accumulating evidence has shown that periodontal ligament stem cells (PDLSCs) can facilitate the formation of new alveolar bone and functional periodontal ligament in damaged periodontal tissue under the proper stimulations.\textsuperscript{6-8} Nonetheless, injection of PDLSC suspension has several limitations for direct clinical application. One is potential issue of cell membrane damage caused by mechanical forces during injection and the other is the lack of a three-dimensional (3D) matrix post-transplant, resulting in a low cell retention and poor cell survival.\textsuperscript{7,9} Therefore, more advanced strategies are needed to promote the defect repair.

To address these challenges, injectable hydrogels acting as structural and functional microenvironments have been extensively explored. Compared to traditional and preformed scaffolds, injectable hydrogels can fill defect with any size or shape without the need for additional surgical procedure and facilitate to formulate with cells by mixing.\textsuperscript{10} To avoid undesirable diffusion of cells and hydrogel precursors to the surrounding tissues, the hydrogel
should be rapidly crosslinked after injection. Hence, photocrosslinkable hydrogels, such as gelatin methacrylate (GelMA), aminoethyl methacrylate-functionalized alginate and chitosan, poly(ethylene glycol) dimethacrylate (PEGDA) and poly(vinyl alcohol) methacrylate, have attracted increasing attention in cell transplantation via injection. GelMA is the modified naturally-derived polymer that possesses the binding sites in all polymer chains, which is beneficial to cell adhesion, spreading and proliferation. PEGDA is synthetic polymer that is composed of the specific structural repeating units, which is in favor of tuning its physicochemical properties. After UV radiation, GelMA and PEGDA can be crosslinked to form hydrogels in the time scale from tens of seconds to several minutes. Therefore, taking advantages of these two photocrosslinkable hydrogels, PDLSCs can be transplanted via a fast-formed 3D composite hydrogel with excellent biocompatibility and tunable physicochemical properties.

It is known that stem cell fate is affected by the microenvironment provided by the injected hydrogels post-transplantation. As the ECM mimics, the injected hydrogels have significant effects on stem cell migration, proliferation, differentiation, and cell-cell communication. However, the influence of the ECM characteristics on PDLSC fate remains unclear. Hence, it is necessary to investigate PDLSC-ECM interaction and screen an appropriate ECM for PDLSC transplantation in periodontal therapy. Recently, various techniques have been developed to study cell-ECM interaction, such as microarray, photo-patterning, soft lithography, microfluidic and bioprinting. However, these methods are associated with some limitations. For instance, most of the microarray platforms and soft lithography techniques are based on two-dimensional (2D) substrates, which cannot closely mimic the native cell microenvironment as reflected by the
significant difference in cell behaviors (e.g., adhesion, spreading, proliferation and differentiation) when cultured in 2D vs. 3D.\textsuperscript{24, 25} Although photo-patterning and microfluidic techniques have been explored to fabricate cell-laden hydrogels for cell-ECM screening in 3D, they have limited control over the composition, microstructure and mechanical property of the ECM in a single plate. Currently, the rapid development of the bioprinting shows great advantages, such as fast cell encapsulation, flexible 3D manufacture, precise position control and high-throughput manner in fabricating various cell-laden hydrogels,\textsuperscript{23, 26} which can be a promising candidate for studying cell-ECM interaction and screening the appropriate ECM in 3D.

In the present study, we utilized a bioprinting-based strategy to efficiently generate the 3D cell-laden hydrogels with varying biomaterial composition to study PDLSC-ECM interaction and thus screen an appropriate ECM for \textit{in vivo} repair of alveolar bone defect. By tuning the volume ratio of injectable and photocrosslinkable hydrogels, the composite hydrogels (GelMA and PEGDA) with varying composition was efficiently fabricated by this bioprinting platform. The relationship between physical characteristics of GelMA/PEGDA hydrogels and their material composition was accessed. Subsequently, GelMA/PEGDA hydrogels serving as the ECM mimics were used to encapsulate PDLSCs with the aid of the bioprinting platform, and cell behaviors (e.g., cell viability, proliferation and differentiation) of PDLSCs encapsulated in the composite hydrogels with varying composition were systematically investigated \textit{in vitro}. Finally, PDLSC-laden GelMA/PEGDA hydrogels with optimized composition were injected into the alveolar bone defects in SD rat model, and the defect repair capacity was evaluated \textit{in vivo}. This bioprinting-based strategy may be not only helpful for screening an appropriate ECM of PDLSCs
and promoting repair of alveolar bone defect, but also fit for other cell-ECM screening studies and
benefit of functional tissue regeneration.

2. EXPERIMENTAL SECTION

2.1 PDLSC isolation, culture and characterization

Rat PDLSCs were isolated and cultured according to the previously published protocols,\textsuperscript{27} and all
procedures were approved by the Ethics Committee in Xi’an Jiaotong University. Briefly, periodontal ligament tissues obtained from healthy Sprague-Dawley (SD) rats were gently collected and rinsed with PBS three times. Then the obtained tissue were digested in 3 mg/mL collagenase type I (Sigma-Aldrich, USA) and 4 mg/mL dispase (Sigma-Aldrich, USA) with continuous shaking for 1h at 37°C. Cell suspensions were gained by filtering the mixture via a 40-µm cell strainer (Thermo Fisher Scientific, USA). The obtained cell suspensions were centrifuged and washed with PBS. Subsequently, cells were seeded, cultured with α-MEM containing 15% fetal calf serum (Gibco, USA), 100 U/mL penicillin streptomycin (Sigma-Aldrich, USA) and 2 mM glutamine (Sigma-Aldrich, USA), and then incubated in 5% CO\textsubscript{2} at 37°C. Limiting dilution method was used to obtain the PDLSCs. To identify the PDLSCs, a flow cytometer was used to exam the expression of the conventional surface markers including CD29, CD31, CD45 and CD90.

2.2 Bioprinting-based strategy to fabricate the ECM mimics using injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying composition

To fabricate injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying
composition in a high efficiency, a customer-built bioprinting platform was set up on the basis of our previous work. For such a pressure-assisted and valve-based bioprinting platform, the volume of printed droplet could be mediated via precise control of valve opening duration or inlet pressure.\textsuperscript{28} In our study, the diameter of nozzle orifice was 150 µm and inlet pressure was constant (50 kPa). Hence, the droplet volume could be varied by regulating valve opening duration (\textit{e.g.}, from 1 ms to 5 ms) via a signal generator. Bioprinting platform was integrated with two valves that connected with independent bio-ink reservoir. One of the bio-ink reservoirs was loaded with 5\% (w/v) GelMA containing 2-hydroxy-2-methylpropiophenone (TCI, China) as the photoinitiator at a concentration of 0.5\% (w/v). GelMA was synthesized according to our published protocols\textsuperscript{23}. The other bio-ink reservoir was loaded with 5\% (w/v) PEGDA (Sigma-Aldrich, USA) containing the photoinitiator at same concentration. To fabricate GelMA/PEG hydrogels with gradient composition, a microarray of GelMA droplets with increasing droplet volume was first printed. Then a microarray of PEGDA droplets with decreasing droplet volume was printed upon the existing GelMA microarray, leading to the formation of a microarray composed of GelMA/PEGDA droplets with uniform volume and gradient composition (droplet volume: 15 µL; volume ratios of GelMA-to-PEGDA: 1/4, 2/3, 3/2, 4/1 and 5/0). The GelMA/PEGDA droplets were then exposed to UV light (2.9 mW/cm\textsuperscript{2}) for 30 s, resulting in the formation of GelMA/PEGDA hydrogels with varying composition. The volume ratio of 0/5 was not included because 5\% (w/v) PEGDA alone did not polymerize to form the hydrogel.\textsuperscript{16}

2.3 Physical properties characterization of injectable and photocrosslinkable GelMA/PEGDA hydrogels
Microstructure characterization: GelMA/PEGDA hydrogels with varying composition were frozen at -20°C and then lyophilized at -60°C and 10 Pa for 2 days until all ice was sublimed. Next, the freeze-dried hydrogels were cut to expose their cross sections and thus surface-coated via sputter coating with Au for scanning electron microscopy (SEM) examination. The microstructure and pore size of the composite hydrogels were observed via LEO-1530 SEM (LEO company, Germany), fitted with a field-emission source operating at an accelerating voltage of 10 kV.

Swelling characterization: GelMA/PEGDA hydrogels with varying composition were prepared and incubated in phosphate buffered saline (PBS) at 37°C for 24h. Then the samples were removed from PBS, blotted dry with filter paper and weighed ($W_s$). These weighed hydrogels were lyophilized overnight and weighed to obtain the dry weight ($W_d$). Swelling ratio of each composite hydrogel was obtained according to the following equation.

$$\text{Swelling ratio} = \frac{(W_s - W_d)}{W_d} \times 100\%$$

(1)

Characterization of mechanical property: Composite hydrogels with different volume ratios of GelMA-to-PEGDA were incubated in PBS at 37°C overnight. Then they were lightly blotted dry with filter paper and compressed by using a BOSE ELF 3200 dynamic mechanical analyzer (BOSE, USA) with compression rate of 1 mm/min. The compressive modulus of each sample was calculated as the slope in the linear region of the stress–strain curve between 0 and 10% strain. Three replicates were measured for each GelMA/PEGDA hydrogel composition.
Degradation performance: GelMA/PEGDA hydrogels with varying composition were rinsed with PBS and then placed in 1.5 mL eppendorf tubes. These hydrogels were freeze-dried and their initial weights ($W_i$) were measured. Subsequently, the dried composite hydrogels were rehydrated in PBS overnight and incubated in 1 mL PBS containing collagenase type II at the concentration of 0.2 U/mL. To maintain constant enzyme activity, the collagenase solution was replaced every 3 days. At designed time points (0.5, 1, 3, 5, 7 and 14 days), the composite hydrogels were taken from enzyme solutions, rinsed with PBS twice, lyophilized and weighted to obtain their remaining weights ($W_r$). Three replicates were measured for each hydrogel composition. Meanwhile, appearance of GelMA/PEGDA hydrogels was recorded via a camera at different time points. The percentage of remaining mass after enzymatic degradation was calculated as following equation.

$$\text{Mass remained (\%) } = \frac{W_r}{W_i} \times 100\% \quad (2)$$

2.4 Bioprinting-based strategy to study PDLSC-ECM interaction and screen the optimized ECM composition

A bioprinting-based strategy was explored to investigate PDLSC-ECM interaction and screen the optimized ECM composition (Fig. 1A). Pneumatic power source was used to eject cells, avoiding strong external stimulus (e.g., temperature higher than 37°C and high shear force). PDLSCs were suspended in 5% (w/v) GelMA containing 0.5% (w/v) photoinitiator at a cell density of $1 \times 10^6$ cells/mL, which served as the first bio-ink. Then, PDLSCs were suspended in 5% (w/v) PEGDA containing 0.5% (w/v) photoinitiator at a cell density of $1 \times 10^6$ cells/mL, which served as the second bio-ink. Similar to 2.2, a microarray of PDLSC-laden GelMA droplets with increasing droplet volume and a microarray of PDLSC-laden PEGDA droplets with decreasing droplet
volume were printed in turn, resulting in the formation of a microarray composed of PDLSC-laden GelMA/PEGDA droplets with uniform volume and varying composition (droplet volume: 15 µL; cell density: $1 \times 10^6$ cells/mL; volume ratios of GelMA-to-PEGDA: 1/4, 2/3, 3/2 and 4/1). Upon UV exposure (2.9 mW/cm$^2$, 30 s), PDLSC-laden GelMA/PEGDA composite hydrogels with different composition were obtained.

![Figure 1. Schematics of bioprinting-based strategy for screening the optimized ECM of PDLSCs and in vivo repair of alveolar bone defect.](image)

(A) Two kinds of injectable and photocrosslinkable hydrogels including GelMA and PEGDA were used to encapsulate PDLSCs. Such cell-laden GelMA and PEGDA were printed sequentially to obtain a PDLSC-encapsulated hydrogel microarray with varying hydrogel composition by tuning the volume ratio of GelMA-to-PEGDA. The ECM composition was optimized through ECM characterization and study on PDLSC-ECM interaction in vitro. (B) Finally, a PDLSC-laden, injectable and photocrosslinkable hydrogel with optimized composition was injected and in situ UV crosslinked in alveolar bone defect to investigate the defect repair capacity in vivo.

2.5 PDLSC behavior characterization

*Cell viability:* PDLSCs were encapsulated in GelMA/PEGDA hydrogels with varying composition. After 3 days of culture, a live/dead assay was performed by calcein AM/ethidium homodimer-1
(Life Technologies, USA) according to the protocol suggested by the manufacturer. PDLSC-laden hydrogels were removed from the culture medium and incubated in 200 µL prepared live/dead dye solution (0.6 µL calcein AM, 2 µL ethidium homodimer and 1 mL PBS) for 20 min at 37°C in the dark. Subsequently, PDLSC-laden hydrogels were rinsed with PBS and imaged using an Olympus IX 81 fluorescence microscope (Olympus, Japan). Total number of live and dead cells was quantitatively determined via Image-Pro Plus software and the cell viability was calculated by dividing the number of live cells with the total cell number and the obtained results were converted into corresponding % values.

Cell proliferation: PDLSC proliferation in composite hydrogels was quantitatively analyzed via cell count kit-8 assay (CCK-8, Dojindo, Japan). After 1 and 7 days of culture, the medium containing 10% (v/v) CCK-8 was changed and PDLSC-laden hydrogels were incubated in it at 37°C for 4h. Subsequently, optical density of reaction solution (100 µL) was measured at 450 nm through a micro-plate reader (Bio-Rad Laboratories, USA). Three replicates were measured for each hydrogel composition.

Cell spreading: To evaluate PDLSC spreading in GelMA/PEGDA hydrogels with varying composition after 7 days of culture, rhodamine-labeled phalloidin and DAPI (Thermo Fisher Scientific, USA) were used to stain F-actin and cell nuclei. First, PDLSC-laden hydrogels were rinsed by PBS twice and fixed by 4% paraformaldehyde for 20 min. Next, they were permeabilized via 0.1% Triton X-100 for 20 min and subsequently blocked in BSA solution for 20 min. Then, PDLSC-laden hydrogels were orderly incubated in rhodamine-labeled phalloidin and
DAPI solutions at 37°C for 15 min and 5 min, respectively. Finally, PDLCS-laden hydrogels were imaged through an Olympus IX 81 fluorescence microscope (Olympus, Japan), and the cell spreading area were analyzed via Image-Pro Plus software.  

2.6 PDLSC differentiation characterization

*Alkaline phosphatase (ALP) activity assay:* PDLSCs were encapsulated in GelMA/PEGDA hydrogels with different composition and cultured in growth medium for 1 day. Then growth medium was changed to osteogenic medium (containing 10 mM β-glycerophosphate disodium, 10^{-7} M dexamethasone and 50 μg/mL ascorbic acid) for further osteoinductive culture. At day 7 and day 10, PDLSC-laden hydrogels were rinsed and cut into pieces. Such cell-laden hydrogel fragments were collected and lysed on ice using RIPA lysis buffer (Thermo Fisher Scientific, USA) for 25 min. Next, the lysates were harvested, centrifuged at 10,000 rpm for 12 min at 4°C and analyzed by an ALP Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instruction. The ALP activity was normalized by the content of total protein determined by a BCA Assay Kit (Nanjing Jiancheng Bioengineering Institute, China). Three replicates were evaluated for each hydrogel composition.

*Real-time polymerase chain reaction (RT-PCR):* PDLSC-laden GelMA/PEGDA hydrogels with different volume ratios were cultured in osteogenic medium for 14 days. Subsequently, the total RNA of PDLSCs encapsulated in each hydrogel was extracted by a RNA Isolation Kit (QIAGEN, Germany) according to the protocol suggested by the manufacturer. The extracted RNA was then reverse-transcribed by using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientif
Scientific, USA). Finally, the prepared cDNA was mixed with SYBR Green PCR Master Mix (Life Technologies, USA) and loaded in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA) to analyze the expression levels of osteogenesis relative proteins including Runx 2, OC (osteocalcin) and OPN (osteopontin). The sense and antisense primers of these target genes and GAPDH serving as a housekeeping gene were designed (Table 1). Three replicates were measured for each hydrogel composition.

### Table 1. Sequence of primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx 2</td>
<td>CCTCAGTGATTTAGGGCGCA</td>
<td>GGTGTGGTAGTGAGTGGTG</td>
</tr>
<tr>
<td>OC</td>
<td>ATTTGGCCCTACCTCCATC</td>
<td>TCTGAAAGCCGATGTC</td>
</tr>
<tr>
<td>OPN</td>
<td>TCTAGGCACGTACCAAGCAC</td>
<td>TCTGATGGCTTTCGTTGGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTCCTGTTCGACAGTCAGCC</td>
<td>TTCCTGCCTCAGGCTTG</td>
</tr>
</tbody>
</table>

**Alizarin red S staining:** After 21 days of osteoinductive culture, PDLSC-laden GelMA/PEGDA hydrogels with varying composition were rinsed twice, fixed in 4% paraformaldehyde for 20 min, and stained by Alizarin red S (Sigma-Aldrich, USA) at 37°C for 30 min. Subsequently, such PDLSC-laden hydrogels were imaged through a phase-contrast microscope (Leica, Germany) when they were rinsed totally. To further quantify the retention of Alizarin red S, the stained PDLSC-laden GelMA/PEGDA hydrogels were incubated in 500 µL of 10% (w/v) cetylpyridinium chloride in disodium hydrogen phosphate solution at 37°C for 10 min. Finally, the optical density was determined by a microplate reader (Bio-Rad Laboratories, USA) at 570 nm.

### 2.7 In vivo repair of alveolar bone defect

Thirty three-month-old SD rats (230-250 g) were purchased from the Animal Experimental Center of Xi’an Jiaotong University. All animals in our study were treated and used according to the
guidelines and regulations for the use and care of animals at Xi’an Jiaotong University. The brief work process of in vivo repair study was as follow (Fig. 1B). The unilateral alveolar bone defects (length× width×height: 4×3×2 mm) in each of the animals were prepared by using a dental round bur under physiological saline irrigation according to the protocols published previously. The defects were randomly assigned to the three groups: (1) saline group (n=10), (2) hydrogel-treated group (n=10), and (3) PDLSCs/hydrogel-treated group (n=10). The sterilized saline, GelMA/PEGDA hydrogels with optimized composition as well as PDLSC-laden GelMA/PEGDA hydrogels with same composition (cell density: 1×10^6 cells/mL) were injected into the defects. Upon UV exposure via an OmniCure®S2000 UV spot curing system (2.9 mW/cm², 45 s), in situ photopolymerization was performed in hydrogel-treated group and PDLSCs/hydrogel-treated group, respectively. Five animals in each group were sacrificed after 3 and 6 weeks of healing, respectively. After sacrifice, harvested specimens were prepared for micro-CT analyses and histologic studies.

Micro-computed tomography (Micro-CT) analysis: After 3 and 6 weeks of healing, five harvested specimens in each group were analyzed by using a SkyScan1076 high resolution micro-CT scanner (Bruker, USA) for evaluation of the newly regenerated bone in the defects. The specimens were scanned at 110 kV and 160 µA at a spatial resolution of 20 µm (voxel dimension). 3D images were produced by using a reconstruction software (Volume Graphics, USA). For the analysis of alveolar bone defect healing, the region of interest (ROI) (length × width × height: 3 mm × 1.5 mm × 1 mm) was positioned in the center of the initial defects. The amount of new bone formation was characterized through analyzing the bone volume fraction (bone volume/ total...
volume, BV/TV) for each specimen via VG-studio software (Volume Graphics, USA).

Histological studies: After 6 weeks of healing, five used specimens for micro-CT analyses were decalcified in 5% formaldehyde containing 10% EDTA, at 4°C for 6 weeks. Following a completed decalcification, the specimens were dehydrated in an ascending series of ethanol, embedded in paraffin and sectioned into slices with 6-µm thickness. The randomly selected sections from each group were stained with hematoxylin-eosin (HE) and Masson’s trichrome (Solarbio, China) according to the manufacturer’s instruction. Images were obtained using an Olympus optical microscope (Olympus, Japan).

2.8 Statistical analysis

Experimental results were described as means ± standard deviation (SD). Comparisons between different groups were estimated by one-way analysis of variance (ANOVA). Differences were considered to be statistical significance and extreme significance at the level of $p < 0.05$ and $p < 0.01$, respectively. All statistical analyses were performed with the use of SPSS software (version 11.5; SPSS Inc, Chicago, IL).

3. RESULTS AND DISCUSSION

3.1 Screening physical properties of the injectable and photocrosslinkable hydrogel

By tuning the volume ratio of two precursor solutions of GelMA and PEGDA (e.g., 5/0, 4/1, 3/2, 2/3 and 1/4) via a bioprinting platform, the two polymer solutions could be mixed and formed the homogeneous solutions with varying composition (Fig. S1). Since 5% (w/v) PEGDA alone could
not polymerize as reported previously, the 0/5 sample was not included in the present study. To check the injectability, we injected the GelMA/PEGDA precursor solutions with volume ratios ranging from 5/0 to 1/4 through a conventional 27-gauge needle into designed molds with varied shapes (e.g., triangle, round, square, star and hexagon), mimicking different shaped and sized alveolar bone defects. Upon UV exposure, we observed that they all crosslinked and formed hydrogels with the shape of the molds, suggesting their photocrosslinkability (Fig. S2).

Microstructure of hydrogel, especially its pore size, is an important parameter for engineering the ECM. We first characterized the microstructure of GelMA/PEGDA hydrogels. SEM images illustrated an obvious correlation between the volume ratio of GelMA-to-PEGDA and pore size (Fig. 2A). Pore sizes of the hydrogels were 55.7±10.1 µm, 96.1±15.5 µm, 111.3±20.8 µm, 160.0±26.4 µm and 248.9±35.5 µm, corresponding to ratios of 1/4, 2/3, 3/2, 4/1 and 5/0, respectively. Previous studies demonstrated that cells could not spread within the 3D microstructure if the pore size of the biomaterials was smaller than the cell size (~10 µm). All of our fabricated hydrogels have pore size larger than 50 µm, providing enough space for cell spreading.
Figure 2. Physical property characterizations of the GelMA/PEGDA hydrogel-based ECM with varying composition. (A) Microstructure. SEM images of hydrogels (a) and their pore sizes (b). (B) Swelling ratio. (C) Compressive modulus. (D) Degradation property. Mass retention (a) and representative photographs of morphology change (b) during in vitro degradation of GelMA/PEGDA hydrogels with different composition. Scale bar: 50 µm; * indicates $p < 0.05$; ** indicates $p < 0.01$.

Since swelling ability is related to hydrophilicity and thus predicts the degradation rate of
We further evaluated the swelling behavior of hydrogels by incubating the hydrogels in a PBS solution (Fig. 2B). We observed that the swelling ratio of GelMA/PEGDA hydrogels increased significantly from ~750% to ~950% as the volume ratio of GelMA-to-PEGDA changed from 1/4 to 4/1 ($p < 0.05$). Particularly, swelling ratio of the 5/0 hydrogel (i.e., pure GelMA) was ~1200%, significantly higher than GelMA/PEGDA hydrogel with the volume ratio of 4/1 ($p < 0.01$). This is likely due to the fact that the hydrogels with larger pore size induce more swelling compared to hydrogels with smaller pore size. The larger pore size not only increases the rate and amount of water penetration, but also might facilitate the degradation rate of GelMA/PEGDA hydrogels.

Mechanical properties of the ECM greatly affect the behaviors of resident cells, such as regulation of cell migration and spreading, mediation of cell proliferation as well as control of cell differentiation. To access the mechanical property of the composite hydrogels, we characterized the compressive modulus of the hydrogels (Fig. 2C). We found that the stiffness of GelMA/PEGDA hydrogels (i.e., 5/0 hydrogel) was 4.5±2.3 kPa, which was significantly increased to 13.8±1.7 kPa, 17.9±2.3 kPa, 19.2±2.1 kPa and 23.5±2.6 kPa as the volume ratio of GelMA-to-PEGDA decreased to 4/1, 3/2, 2/3, 1/4, respectively. Such increase in stiffness might be associated with not only the increasing crosslinking density of the polymer networks, but also the decreasing pore size of the hydrogel with increasing ratio of PEGDA component (Fig. 2A). As reported previously, the stiffness of hydrogels was inversely proportional to their pore size, which was also observed in our experiment. Hence, all these results revealed that the hydrogel stiffness could be tuned by varying volume ratios of GelMA-to-PEGDA.
Since the degradation speed of hydrogels as ECM mimics should match the pace of neo-tissue formation, we investigated how hydrogel composition mediates degradation behavior. To this end, GelMA/PEGDA hydrogels with varying composition were incubated in collagenase II solution and their degradation behavior was studied by mass-loss analysis over 14 days (time scale for osteogenic differentiation) (Fig. 2D). We observed that the mass loss of all GelMA/PEGDA hydrogels increased with time, with the fastest degradation rate on the first day. Among these hydrogels, the pure GelMA hydrogel (i.e., 5/0 group) degraded fastest, with only ~3% remained after 7 days of incubation. The addition of PEGDA component into hydrogel led to a significant decrease in degradation rate, with hydrogels (1/4, 2/3, 3/2 and 4/1 hydrogels) remained even after 14 days of incubation. This could be attributed to the crosslink formation between GelMA and PEGDA and the addition of a second component (PEGDA) that was not degraded by collagenase, significantly slowing down the GelMA/PEGDA hydrogel degradation compared to the single network hydrogel (the 5/0 hydrogel) ($p < 0.01$). Similar results were also reported that the addition of 15% PEGDA could significantly reduce the degradation rates of composite hydrogels with 5%, 10% and 15% GelMA, respectively as compared to GelMA alone.$^{16}$ Additionally, with more PEGDA component introduced into the hydrogel and the volume ratio of GelMA-to-PEGDA decreased from 4/1 to 1/4, the degradation rate of GelMA/PEGDA hydrogels ($p < 0.05$) decreases. Previous study indicated that the enzymatic degradation of hydrogels was closely correlated with hydrogel stiffness, and degradation rate increased with a decrease in hydrogel stiffness,$^{40}$ which was in agreement with our observation. Although the collagenase levels in our study could not be directly translated to a specific $\textit{in vivo}$ stimulation due to the complexity of the native
microenvironment, similar degradation behaviors might occur in native microenvironment.\textsuperscript{41}

Injectable and photocrosslinkable hydrogels composed of various volume ratios of GelMA-to-PEGDA were fabricated via a 3D bioprinting platform. These composite hydrogels could potentially serve as the ECM to encapsulate PDLSCs for \textit{in vivo} repair of alveolar bone defect. We first characterized their physical properties including microstructure, compressive moduli, swelling as well as degradation performance, considering their important roles in regulating cell behaviors. GelMA/PEGDA hydrogels with varying composition possessed various pore sizes, and their pore sizes could directly determine stiffness and swelling ability, and thus affect their degradation performances. Hydrogel degradation in physiological conditions is beneficial for allowing the hydrogel-based ECM to disappear, thus the newly formed ECM can gradually fill in the degraded portions of the hydrogel, which is crucial to the tissue regeneration.\textsuperscript{29}

Therefore, the engineered hydrogel-based ECM for cell transplantation should be designed to degrade at a rate similar to the rate of tissue formation. It has been reported that the time scale of the formation of new alveolar bone tissue might be varied from tens of days to several weeks.\textsuperscript{2}

The above results indicated that degradation rate of the 5/0 hydrogel (GelMA alone) was too fast, with almost complete degradation by 7 days. Hence, the 5/0 hydrogel would be excluded in the following studies.

3.2 The effect of ECM composition on PDLSC viability, proliferation and spreading \textit{in vitro}

PDLSC-laden GelMA/PEGDA hydrogels with varying volume ratios of GelMA-to-PEGDA (1/4, 2/3, 3/2 and 4/1) were fabricated via a bioprinting platform. We first investigated cell viability of
PDLSCs encapsulated in these hydrogels after 3 days of culture. We found that PDLSCs encapsulated in GelMA/PEGDA hydrogels with varying composition remained viable (Fig. 3A). Our further quantification also confirmed the high cell viability. For instance, PDLSC viability is ~85% in the 1/4 composite hydrogel and ~90% in the composite hydrogels with volume ratio of GelMA-to-PEGDA from 2/3 to 4/1. Meanwhile, there was no significant difference ($p > 0.05$) in cell viability of PDLSCs encapsulated in composite hydrogels with volume ratio ranging from 2/3 to 4/1 (Fig. 3B).

Figure 3. Cell viability, proliferation and spreading of PDLSCs encapsulated in the injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying composition. (A) Representative live/dead fluorescence images of PDLSCs encapsulated in composite hydrogels with different GelMA/PEGDA ratios (v/v) after 3 days of culture. Green fluorescent cells are alive and red fluorescent cells indicate dead cells. Scale bar: 200 µm. (B) Quantification of the staining
of the live and dead cells in the 3D GelMA/PEGDA hydrogels with varying composition by using the Image-Pro Plus software. (C) Cell proliferation of PDLSCs in GelMA/PEGDA hydrogels with varying composition determined via a CCK-8 quantitative analysis. (D) Representative phalloidin/DAPI fluorescence images of PDLSCs encapsulated in hydrogels with different GelMA/PEGDA ratios (v/v) after 7 days of culture. Scale bar: 100 µm. (E) Quantification of the staining of cell spreading area in the 3D GelMA/PEGDA hydrogels with varying composition by using the Image-Pro Plus software. * indicates $p < 0.05$; ** indicates $p < 0.01$.

In addition, the effect of ECM composition on PDLSC proliferation was assessed by using CCK-8 assay. Cell proliferation in composite hydrogels with varying volume ratios was quantitatively evaluated on day 1 and day 7, respectively. As shown in Figure 3C, difference in cell proliferation was observed in GelMA/PEGDA hydrogels with varying composition. For example, the amount of viable cells obviously decreased on day 7 as compared to that on day 1 in the 1/4 hydrogel ($p < 0.05$). Slight decrease and increase in the amount of viable cells were indicated in 2/3 and 3/2 hydrogels, respectively. In particular, PDLSC proliferated significantly after 7-day culture in 4/1 hydrogel ($p < 0.01$).

Besides, we characterized PDLSC spreading in GelMA/PEGDA hydrogels with varying composition. A strong composition-dependent cell spreading was confirmed by fluorescent images (Fig. 3D). For example, PDLSCs in the 4/1 hydrogel spread to form the interconnected networks with other neighboring cells. In contrast, negligible spreading of PDLSCs in 1/4 hydrogel was observed, with most cells remained the isolated and round morphologies. Furthermore, cell-spreading areas were quantified using the Image-Pro Plus software and we observed significantly enhanced PDLSC spreading as the volume ratio of GelMA-to-PEGDA increased from 1/4 to 4/1 (Fig. 3E).
Hydrogels used as the engineered ECM to encapsulate cells for tissue engineering should meet some necessary requirements such as providing structural support, promoting cell spreading, migration and proliferation, and connecting with neighboring cells in 3D. Similar to the native ECM, hydrogels should also have degradability, allowing cells to remodel their microenvironment. GelMA and PEGDA hydrogels are the most widely used biomaterials for biomedical applications including serving as the ECM mimics. One advantage of GelMA is the presence of binding sites on all polymer chains, leading to an improvement in cell adhesion, spreading and proliferation. PEGDA is biologically inert, being both nontoxic and nonimmunogenic and with tunable physicochemical properties. However, when they are used individually, they may suffer from several drawbacks. For example, cells encapsulated in PEGDA hydrogels are generally unable to bind to the hydrogels due to the non-adhesive nature, resulting in the limited cell spreading and proliferation. The disadvantages of GelMA hydrogels are their weak mechanical properties and fast degradation rate. To improve the biocompatibility and physicochemical properties, we fabricated GelMA/PEGDA composite hydrogels as the ECM mimics by controlling volume ratio of these two components via a bioprinting platform. All these results suggest that PDLSC behaviors (e.g., cell spreading and proliferation) were closely related to the ECM composition as reflected by the volume ratio of GelMA-to-PEGDA in composite hydrogels, likely due to the difference in bioactivity between GelMA and PEGDA as described above. Hence, more PEGDA component in composite hydrogels inhibited cell proliferation and spreading in 3D.

3.3 The effect of ECM composition on PDLSC differentiation *in vitro*
To investigate the effect of ECM composition on PDLSC differentiation, we first analyzed alkaline phosphatase (ALP) activity of PDLSCs in composite hydrogels after 7 and 10 days of osteo-induction culture (Fig. 4A). ALP activity of PDLSCs cultured in GelMA/PEGDA hydrogels was normalized and studied at different time points. We found that ALP activity on day 10 was significantly higher than that on day 7 ($p < 0.01$), indicating the osteogenic differentiation of PDLSCs at the early stage. Besides, ALP activity of PDLSCs in GelMA/PEGDA hydrogels obviously increased as the volume ratio of GelMA-to-PEGDA increased from 1/4 to 4/1 on both day 7 and day 10, suggesting a significant influence of hydrogel composition on ALP activity of PDLSCs.
Figure 4. Osteogenic differentiation of PDLSCs encapsulated in the injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying composition. (A) ALP activity of PDLSCs in GelMA/PEGDA hydrogels with different composition after 7 and 10 days of culture. (B) Real-time PCR was performed with PDLSCs cultured in the 3D GelMA/PEGDA hydrogels with varying composition on day 14 after osteo-induction to analyze the expression of (a) Runx 2, (b) OC and (c) OPN. (C) Alizarin red S staining of the PDLSC-laden GelMA/PEGDA hydrogels after 21 days of culture. Microscopic images (a) and the Quantitative result of retention of Alizarin red S (b). Scale bar: 100 µm. * indicates $p < 0.05$; ** indicates $p < 0.01$. 
Expression levels of osteogenesis specific markers, such as Runx 2, OC (osteocalcin) and OPN (osteopontin) in PDLSCs cultured in composite hydrogels for 14 days were subsequently analyzed through RT-PCR (Fig. 4B). These three osteogenesis-related genes showed varied expression levels in GelMA/PEGDA hydrogels with varying volume ratios of GelMA-to-PEGDA. For example, the relative mRNA expression of Runx 2 significantly increased as the volume ratio increased from 1/4 to 3/2, but there is no significant difference ($p > 0.05$) as the volume ratio increased from 3/2 to 4/1. In contrast, the mRNA expressions of OC and OPN were significantly increased in PDLSCs cultured in GelMA/PEGDA hydrogels when the volume ratio increased from 1/4 to 4/1. Hence, these gene expression results indicated that the osteogenic differentiation of PDLSCs could be tuned by engineering the ECM through composition designed.

We further characterized the production of mineralized nodules, a crucial indicator for osteogenesis. PDLSCs in GelMA/PEGDA hydrogels were stained with Alizarin Red S on day 21 in osteo-induction culture (Fig. 4C). We observed that the orange-red precipitates in all GelMA/PEGDA composite hydrogels, especially the network-like mineralized nodules in the 4/1 hydrogel, suggesting that PDLSCs in all groups maintained osteogenesis capability. We then quantified the precipitated orange-red nodules and found a significant positive correlation between hydrogel composition and production of mineralized nodules. The amount of mineralized nodules obviously increased as an increase in the volume ratio of GelMA-to-PEGDA from 1/4 to 4/1.

Stem cells can differentiate into different lineages in response to biophysical cues from surrounding matrices. In detail, stem cells actively remodel their ECM, probe the ECM stiffness
and porosity, and thus undergo the lineage-specific differentiation through integrating different biophysical cues.\textsuperscript{44} For example, Huebsch \textit{et al.} reported that MSC differentiation in physically crosslinked and non-degradable alginate hydrogels was directly guided by hydrogel stiffness.\textsuperscript{45} Although hydrogel degradation was absent, the physically crosslinked hydrogels were mobile enough to enable cellular reorganization of local matrix-bound ligands, cellular traction generation and cell differentiation, with magnitudes and fate dependent on hydrogel stiffness. However, natural ECM is composed of polymers that have both crosslinked and degradable backbones.\textsuperscript{44} Hence, Burdick and colleagues developed covalently crosslinked and degradable hyaluronic hydrogels and found that MSC differentiation in such hydrogels was mediated by degradation-mediated cell traction irrespective of matrix mechanics or cell morphology.\textsuperscript{46} Compared to the non-degradable and covalently crosslinked hydrogels, MSCs encapsulated in degradable and covalently crosslinked hydrogels could interact and degrade the hydrogels, leading to the enhanced osteogenic differentiation. In the present study, the amount of GelMA component increased when the volume ratio of GelMA-to-PEGDA increased from 1/4 to 4/1, resulting in gradually enhanced degradability of GelMA/PEGDA hydrogels. The encapsulated PDLSCs in the 4/1 composite hydrogels spread more, pulled on surrounding matrices, and exhibited more robust cytoskeletal organization and higher cell-generated traction force, finally leading to a stronger osteogenic differentiation compared to that in the other composite hydrogels (\textit{e.g.} 1/4, 2/3 and 3/2 hydrogels). Hence, the 4/1 hydrogel would be chosen for \textit{in vivo} repair of alveolar bone defect in the following \textit{in vivo} study.

3.4 \textit{In vivo} repair of alveolar bone defect using the PDLSC-laden, injectable and
photocrosslinkable hydrogels with optimized composition

To investigate the repair capacity of alveolar bone defect *in vivo*, we injected saline, GelMA/PEGDA hydrogel with optimized composition and PDLSC-laden GelMA/PEGDA hydrogel with same composition into the alveolar bone defect in SD rats respectively (*Fig. 5*). For this, we first created the alveolar bone defect models with size of 4×3×2 mm (length×width×height), as demonstrated by the 3D reconstructed micro-CT image (*Fig. 5A*). After 3 weeks of healing, we observed that the defect repair was minimal in the saline group, with most defect site remained empty (*Fig. 5C*). In contrast, the hydrogel group and the PDLSC/hydrogel group displayed remarkable mineralized tissue formation within the defect sites (*Fig. 5D, E*). Especially, the PDLSC/hydrogel group showed obvious bone bridging throughout the width of the defect, as seen by the transverse section in *Figure 5E*. After 6 weeks of healing, we observed increased new bone formation in all groups. In particular, the defects that treated with composition-optimized hydrogel and PDLSC-laden hydrogel with same composition healed well. The borders of alveolar bone defects were indistinct and undistinguished from the original alveolar bone (*Fig. 5G, H*). However, the defects treated with the saline still displayed obvious cavity in the center of defect zones (*Fig. 5F*). We further quantified the amount of newly regenerated bone tissue after 3 and 6 weeks of healing by calculating the bone volume fraction of the total tissue volume (BV/TV, %) (*Fig. 5B*). We found that BV/TV was significantly higher in the hydrogel-treated groups compared to the saline-treated groups at 3 weeks (31.2±1.5 vs. 28.7±1.6) and 6 weeks (40.5±4.4 vs. 33.4±4.1), which was further improved to 36.1±3.3 and 60.1±3.6 in the PDLSC/hydrogel-treated groups. Taken together, the PDLSC-laden, injectable and photocrosslinkable GelMA/PEGDA hydrogel with optimized composition could effectively
promote repair of alveolar bone defect \textit{in vivo}.

Figure 5. Micro-CT analysis of the new bone formation in rat alveolar bone defects at 3 and 6 weeks of healing. (A) Micro-CT reconstruction image of alveolar bone defect model after surgery. Scale bar: 1 mm. Representative 3D reconstructed micro-CT images and transverse section images showing the new bone regeneration in the defect for each group (\textit{e.g.}, (C, F) saline group; (D, G) hydrogel-treated group; (E, H) PDLSCs/hydrogel-treated group) at (C, D and E) 3 weeks and (F, G and H) 6 weeks of healing. Red-dotted lines refer to the region of interest (ROI); Scale bar: 1 mm. (B) Quantitative analysis of the regenerated bone volume fraction within the original defects (BV/TV, \%) in different groups at 3 and 6 weeks of healing. * indicates $p < 0.05$; ** indicates $p < 0.01$.

To further evaluate the new bone formation, we performed both HE and Masson’s trichrome staining (Fig. 6). We found that all animals in three groups showed a good healing response, where the injected and treated zones in the defects were stable, without obvious signs of internal inflammation and visible fibrous invasions in our harvested specimens. As shown in these
histological images, the newly regenerated tissue grew from the defect margin toward the central part, which was also confirmed by micro-CT images (Fig. 5). The amount of new bone formation in the defects injected with the GelMA/PEGDA hydrogels encapsulating PDLSCs or not was higher than that in the defects those only received the saline injection. Limited bone tissue regeneration was observed in the saline-treated group (Fig. 6A, D). In contrast, the defects injected with GelMA/PEGDA hydrogels generated new bone tissue that partially filled in the defect zones (Fig. 6B, E). While the PDLSC-laden GelMA/PEGDA hydrogel-treated group exhibited a robust bone tissue formation, and the newly formed bone tissue was continuous and filled almost all defect zones (Fig. 6C, F). Upon closer examination, a number of osteoblasts were noticed in new bone tissues (Fig. 6H). Furthermore, quantitative analysis of the relative osteogenic areas also confirmed that a maximal new bone formation was present in the defect treated with the PDLSC-laden GelMA/PEGDA hydrogel compared to those defects treated with the GelMA/PEGDA hydrogel alone and the saline (Fig. 6G).

Figure 6. Histological study of the newly regenerated bone tissue in rat alveolar bone defects at 6 weeks of healing. Histological sections stained by (A, B, and C) Masson’s trichrome and (D, E and F) HE of the alveolar bone defects treated with the (A and D) saline, (B and E) hydrogel and (C and F) PDLSCs/hydrogel, respectively. Scale bar: 200 µm. (H) The magnified image of the
Formation of new alveolar bone is one of the key processes in the repair of periodontal defect. Stem cell-based strategies, involving the usage of injectable hydrogels for delivering stem cells into the defects and serving as the ECM mimics during healing, have brought a new light in periodontal tissue engineering. Stem cells from oral origin have been proposed first, such as PDLSCs, dental follicle stem cells (DFSCs), dental pulp stem cells (DPSCs), gingival mesenchymal stem cells (GMSCs), stem cells from exfoliated deciduous teeth (SHED) and stem cells from apical papilla (SCAP). These oral stem cells are seeded or encapsulated in biomaterials, which have been proved to repair periodontal tissue without neoplasm formation. Besides, some non-oral stem cells, such as bone marrow stem cells (BMSCs) and adipose-derived stem cells (ASCs) have also shown great potentials in periodontal defect repair. In our study, PDLSCs were encapsulated in injectable and photocrosslinkable GelMA/PEGDA hydrogels, where their composition was first optimized in vitro via a bioprinting-based strategy. Then they were injected into the alveolar bone defects and demonstrated a maximal new bone formation in vivo, which was confirmed by both micro-CT and histological studies. GelMA/PEGDA hydrogels functioned as delivery vehicles carrying PDLSCs and served as the 3D scaffolds that fill the defects and provide space for PDLSC growth. The composition-optimized GelMA/PEGDA hydrogels enhanced the osteogenic differentiation of PDLSCs, and resulted in the best repair of alveolar bone defect treated with the PDLSCs/hydrogel compared to the hydrogel alone and the saline. Similarly, Moshaverinia et al. encapsulated two kinds of dental-derived mesenchymal stem cells (i.e.,
PDLSs or GMsCs) into the injectable alginate-based hydrogels for *in vivo* osteogenesis study.

Although both alginate hydrogels encapsulating PDLSs or GMsCs exhibited different mineralized bone tissues, the amount of newly generated bone tissue in these dental-derived stem cells/alginate hydrogel groups was much higher than that in alginate hydrogel group (without stem cells) after 8 weeks. Hence, further studies will be focused on bioprinting-based combinatorial screening of the ECM composition and stem cell type for periodontal tissue engineering.

4. CONCLUSIONS

In summary, a bioprinting-based strategy was proposed to study PDLSC-ECM interaction and thus screen an appropriate ECM for *in vivo* repair of alveolar bone defect. By tuning the volume ratio of injectable and photocrosslinkable GelMA and PEGDA, PDLSC-laden GelMA/PEGDA hydrogels with varying composition were efficiently fabricated via a 3D bioprinting platform and upon UV exposure, PDLSC behavior and fate were closely related to the engineered ECM composition, where cell proliferation, spreading and osteogenic differentiation increased with an increase in the volume ratio of GelMA-to-PEGDA. Through PDLSC-ECM interaction study, the optimized ECM (the 4/1 GelMA/PEGDA hydrogel) was selected. Finally, PDLSC-laden GelMA/PEGDA precursor solutions with optimized composition were injected and *in situ* UV crosslinked in rat alveolar bone defects. The robust and maximal new bone formation was observed in the defect treated with the PDLSC-laden hydrogel compared to the hydrogel alone and the saline ones. This bioprinting-based strategy may not only be helpful for screening an appropriate ECM and promoting repair of alveolar bone defect, but also fit for other cell-ECM
screening and benefit of functional tissue regeneration.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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Supporting Information

Fluorescence image of the droplets generated via a bioprinting platform by tuning the volume ratio of FITC and RhB solutions.

Formation of injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying compositions in designed molds.

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Bioprinting-based PDLSC-ECM Screening for in vivo Repair of Alveolar Bone
Defect using Cell-laden, Injectable and Photocrosslinkable Hydrogels

Yufei Ma, Yuan Ji, Tianyu Zhong, Wanting Wan, Qingzhen Yang, Ang Li, Xiaohui Zhang,
Min Lin
Figure 1. Schematics of bioprinting-based strategy for screening the optimized ECM of PDLSCs and in vivo repair of alveolar bone defect. (A) Two kinds of injectable and photocrosslinkable hydrogels including GelMA and PEGDA were used to encapsulate PDLSCs. Such cell-laden GelMA and PEGDA were printed sequentially to obtain a PDLSC-encapsulated hydrogel microarray with varying hydrogel composition by tuning the volume ratio of GelMA-to-PEGDA. The ECM composition was optimized through ECM characterization and study on PDLSC-ECM interaction in vitro. (B) Finally, a PDLSC-laden, injectable and photocrosslinkable hydrogel with optimized composition was injected and in situ UV crosslinked in alveolar bone defect to investigate the defect repair capacity in vivo.

54x35mm (300 x 300 DPI)
Figure 2. Physical property characterizations of the GelMA/PEGDA hydrogel-based ECM with varying composition. (A) Microstructure. SEM images of hydrogels (a) and their pore sizes (b). (B) Swelling ratio. (C) Compressive modulus. (D) Degradation property. Mass retention (a) and representative photographs of morphology change (b) during in vitro degradation of GelMA/PEGDA hydrogels with different composition. Scale bar: 50 µm; * indicates p < 0.05; ** indicates p < 0.01.
Figure 3. Cell viability, proliferation and spreading of PDLSCs encapsulated in the injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying composition. (A) Representative live/dead fluorescence images of PDLSCs encapsulated in composite hydrogels with different GelMA/PEGDA ratios (v/v) after 3 days of culture. Green fluorescent cells are alive and red fluorescent cells indicate dead cells. Scale bar: 200 µm. (B) Quantification of the staining of the live and dead cells in the 3D GelMA/PEGDA hydrogels with varying composition by using the ImagePro Plus software. (C) Cell proliferation of PDLSCs in GelMA/PEGDA hydrogels with varying composition determined via a CCK-8 quantitative analysis. (D) Representative phalloidin/DAPI fluorescence images of PDLSCs encapsulated in hydrogels with different GelMA/PEGDA ratios (v/v) after 7 days of culture. Scale bar: 100 µm. (E) Quantification of the staining of cell spreading area in the 3D GelMA/PEGDA hydrogels with varying composition by using the ImagePro Plus software. * indicates p < 0.05; ** indicates p < 0.01.

173x189mm (300 x 300 DPI)
Figure 4. Osteogenic differentiation of PDLSCs encapsulated in the injectable and photocrosslinkable GelMA/PEGDA hydrogels with varying composition. (A) ALP activity of PDLSCs in GelMA/PEGDA hydrogels with different composition after 7 and 10 days of culture. (B) Real-time PCR was performed with PDLSCs cultured in the 3D GelMA/PEGDA hydrogels with varying composition on day 14 after osteo-induction to analyze the expression of (a) Runx 2, (b) OC and (c) OPN. (C) Alizarin red S staining of the PDLSC-laden GelMA/PEGDA hydrogels after 21 days of culture. Microscopic images (a) and the Quantitative result of retention of Alizarin red S (b). Scale bar: 100 µm. * indicates p < 0.05; ** indicates p < 0.01.
Figure 5. Micro-CT analysis of the new bone formation in rat alveolar bone defects at 3 and 6 weeks of healing. (A) Micro-CT reconstruction image of alveolar bone defect model after surgery. Scale bar: 1 mm. Representative 3D reconstructed micro-CT images and transverse section images showing the new bone regeneration in the defect for each group (e.g., (C, F) saline group; (D, G) hydrogel-treated group; (E, H) PDLSCs/hydrogel-treated group) at (C, D and E) 3 weeks and (F, G and H) 6 weeks of healing. Red-dotted lines refer to the region of interest (ROI); Scale bar: 1 mm. (B) Quantitative analysis of the regenerated bone volume fraction within the original defects (BV/TV, %) in different groups at 3 and 6 weeks of healing. * indicates p < 0.05; ** indicates p < 0.01.

170x168mm (300 x 300 DPI)
Figure 6. Histological study of the newly regenerated bone tissue in rat alveolar bone defects at 6 weeks of healing. Histological sections stained by (A, B, and C) Masson’s trichrome and (D, E and F) HE of the alveolar bone defects treated with the (A and D) saline, (B and E) hydrogel and (C and F) PDLSCs/hydrogel, respectively. Scale bar: 200 µm. (H) The magnified image of the area indicated by red dot line in F. Red arrows point to osteoblasts. Scale bar: 100 µm. (G) Quantitative analysis of the new bone area in HE staining images was carried out using the Image-Pro Plus software. ** indicates p < 0.01. Symbols are new bone (NB), root of tooth (Rt) and periodontal ligament (PDL).

164x87mm (300 x 300 DPI)