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Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp

Rong Kuang [a,b], Zhanpeng Zhang [c], Xiaobing Jin [a], Jiang Hu [a], Songtao Shi [f], Longxing Ni [b,*], Peter X. Ma [a,c,d,e,*]

[a] Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI 48109, USA
[b] State Key Laboratory of Military Stomatology, Department of Operative Dentistry and Endodontics, School of Stomatology, Fourth Military Medical University, Xi'an 710032, People's Republic of China
[c] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA
[d] Macromolecular Science and Engineering Center, University of Michigan, Ann Arbor, MI 48109, USA
[e] Department of Materials Science and Engineering, University of Michigan, Ann Arbor, MI 48109, USA
[f] Department of Anatomy and Cell Biology, School of Dental Medicine, University of Pennsylvania, 240 S. 40th Street, Philadelphia, PA 19104, USA

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ABSTRACT

Dental pulp infection and necrosis are widespread diseases. Conventional endodontic treatments result in a devitalized and weakened tooth. In this work, we synthesized novel star-shaped polymer to self-assemble into unique nanofibrous spongy microspheres (NF-SMS), which were used to carry human dental pulp stem cells (hDPSCs) into the pulp cavity to regenerate living dental pulp tissues. It was found that NF-SMS significantly enhanced hDPSCs attachment, proliferation, odontogenic differentiation and angiogenesis, as compared to control cell carriers. Additionally, NF-SMS promoted vascular endothelial growth factor (VEGF) expression of hDPSCs in a 3D hypoxic culture. Hypoxia-primed hDPSCs/NF-SMS complexes were injected into the cleaned pulp cavities of rabbit molars for subcutaneous implantation in mice. After 4 weeks, the hypoxia group significantly enhanced angiogenesis inside the pulp chamber and promoted the formation of ondontoblast-like cells lining along the dentin–pulp interface, as compared to the control groups (hDPSCs alone group, NF-SMS alone group, and hDPSCs/NF-SMS group pre-cultured under normoxic conditions). Furthermore, in an *in situ* dental pulp repair model in rats, hypoxia-primed hDPSCs/NF-SMS were injected to fully fill the pulp cavity and regenerate pulp-like tissues with a rich vasculature and a histological structure similar to the native pulp.

Statement of Significance

Vascularization is key to the regeneration of many vital tissues. However, it is challenging to create a suitable microenvironment for stem cells to regenerate vascularized tissue structure. This manuscript reports a novel star-shaped block copolymer that self-assembles into unique nanofibrous spongy microspheres, which as an injectable scaffold recapitulate the cell–cell and cell–matrix interactions in development. Using a clinically-relevant surgical procedure and a hypoxic treatment, the nanofibrous spongy microspheres were used to deliver stem cells and successfully regenerate dental pulp with a rich vasculature and a complex histologic structure similar to that of the native dental pulp. The novel microspheres can likely be used to regenerate many other vascularized tissues.

1. Introduction

The vitality of the entire tooth is supported by the dental pulp, which is responsible for nutritional supply, dentin production, and tooth sensation [1]. However, dental pulp is vulnerable to infections resulted from mechanical, chemical, thermal or microbial irritants. In addition to unbearable pain, dental pulp infection can lead to irreversible pulp necrosis and interruption of dentin formation, which can result in unclosed apical foraman in young permanent teeth or the formation of large pulp chambers [2]. The current endodontic treatment of irreversible pulp disease, known as root canal treatment (RCT), cannot restore the function of dental pulp

* Corresponding authors at: Department of Biologic and Materials Sciences, University of Michigan, 1011 North University Ave., Room 2211, Ann Arbor, MI 48109-1078, USA (P.X. Ma).
E-mail addresses: nilx2007@gmail.com (L. Ni), mapx@umich.edu (P.X. Ma).

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and thereby results in a permanently devitalized tooth more susceptible to structural failure and re-infection due to coronal leakages or microleakages [3]. Restoring the functions of dental pulp through tissue regeneration may potentially resolve these issues [4–6]. However, a key challenge to functional dental pulp regeneration is to rebuild the complex, highly organized histological structure of the native pulp, which is vascularized and contains several types of cells in different zones (e.g. microvasculature in the central region and odontoblasts in the peripheral lining against the dentinal wall).

Human dental pulp stem cells (hDPSCs) are an excellent cell source for dental pulp regeneration, because hDPSCs are capable of odontogenic, neurogenic and angiogenic differentiations [7,8] to form the key cell types in natural dental pulp. In addition, hDPSCs are advantageous for clinical application because of their easy isolation [7] from third molars, extracted or broken teeth [9–11], and their regenerative capacity after cryopreservation [12]. To fully harness the regenerative potential of the hDPSCs, an advanced cell carrier is needed to effectively induce angiogenesis and direct hDPSCs differentiation to regenerate the entire vascularized dental pulp [7,13–16]. To design cell carriers for dental pulp regeneration, structural features at multiple scales have to be considered. At the nano-scale, nano-fibrous (NF) structure can mimic the fibrous structure of extracellular matrix (ECM) and promote hDPSCs attachment, proliferation, and odontogenic differentiation [17,18]. At the micro-scale, a porous structure with interconnected pores is required for efficient cell seeding/ingrowth, mass transfer, and vasculature formation [19–21]. At the macro-scale, cell carriers should be injectable to enable cell delivery into the small, irregularly-shaped dental pulp cavity using a minimal invasive procedure [22,23].

In addition to physical cues, angiogenic factors are required to induce and promote blood vessel formation, which is critical to tissue vitality and pulp histological organization [24–27]. Resembling the hypoxic condition in dental pulp cavity [28], culturing cells under a low oxygen tension *in vitro* activates transcriptional factor HIF-1 [29,30] and increases vascular endothelial growth factor (VEGF) expression [31–34]. HIF-1α binds to the VEGF gene promoter and recruits other transcriptional regulators, thereby enhancing VEGF gene expression [35]. However, thus far, such studies were conducted in 2D culture plates [36]. Little is known about the hypoxia effect on hDPSCs in 3D culture, especially on microspheres.

In this work, a series of star-shaped block copolymers was synthesized for the first time and was fabricated into novel nanofibrous spongy microspheres (NF-SMS), which integrated the desired synthetic NF architecture and interconnected micro-sized pores into injectable microspheres. We then investigated the effect of hypoxic treatment on hDPSCs seeded on the newly developed NF-SMS in a suspension culture, and examined their hypoxia-induced VEGF gene expression. Furthermore, we evaluated the injectable, hypoxia-primed hDPSCs/NF-SMS complexes for dental pulp regeneration in both a subcutaneous tooth implantation model and an *in situ* pulp regeneration model.

2. Methods

2.1. Synthesis of SS-PLLA-b-PLYS

Star-shaped poly(L-lactic acid)-block-poly(L-lysine) (SS-PLLA-b-PLYS) was prepared (supplemental data, Fig. S1). Briefly, poly(amidoamine) dendrimer with 16 surface hydroxyl groups (PAMAM-OH, generation 2, Sigma–Aldrich) was used to initiate the ring opening polymerization of monomer L-lactide (Sigma–Aldrich) under vacuum at 120–130 °C to synthesize star-shaped poly(L-lactic acid) (SS-PLLA). The hydroxyl end groups of SS-PLLA were then converted into amino groups to form SS-PLLA-NH_2. SS-PLLA-NH_2 was used to initiate a second ring-opening polymerization of lysine N-carboxyanhydride with a carbobenzyloxy protecting group (Z-LYSNCA). SS-PLLA-NH_2 and Z-LYSNCA were dissolved in dry dimethylformamide and the solution was stirred at 30 °C for 72 h with nitrogen purged throughout the reaction. The product SS-PLLA-b-PLYS(Z) was then dissolved in trifluoroacetic acid and treated with hydrogen bromide/acetic acid (volume ratio = 1:3) solution under nitrogen at 0 °C for 1 h to remove the protecting group to obtain SS-PLLA-b-PLYS. Detailed characterization of the materials can be found elsewhere [37].

2.2. Preparation of nanofibrous spongy microspheres (NF-SMS)

NF-SMS were produced from SS-PLLA-b-PLYS through a "reversed" emulsification process. Briefly, the polymer was dissolved in THF at 50 °C with a concentration of 2.0% (wt/v). The polymer solution was quickly added into glycerol (50 °C) under rigorous mechanical stirring (speed 7, MAXIMA, Fisher Scientific Inc.). Five minutes later, the mixture was quickly poured into liquid nitrogen. After 10 min, ice/water mixture was added to exchange solvent for 24 h. The spheres were then sieved and washed with distilled water five times to remove glycerol residue. The spheres were then lyophilized for 2 days.

2.3. Preparation of nanofibrous microspheres (NF-MS)

NF-MS were prepared following a previously described emulsification procedure [22]. PLLA was dissolved in THF at 50 °C with a concentration of 2.0% (wt/v). Under rigorous mechanical stirring (speed 7, MAXIMA, Fisher Scientific Inc.), glycerol (50 °C) was slowly added into the polymer solution, and the stirring continued for 5 min. The mixture was then quickly poured into liquid nitrogen. After 10 min, ice/water mixture was added to exchange solvent for 24 h. The spheres were then sieved and washed with distilled water five times to remove glycerol residue. The spheres were then lyophilized for 2 days.

2.4. Preparation of solid microspheres (S-MS)

Smooth (solid) microspheres were prepared using a conventional solvent evaporation method. The PLLA was dissolved in dichloromethane at a concentration of 2% (wt/v), and added into an aqueous poly(vinyl alcohol) (PVA, M_w = 89,000–98,000, Sigma–Aldrich) solution (2% wt/v). The mixture was then subjected to rigorous stirring (speed 7, MAXIMA, Fisher Scientific Inc.) for 24 h. The spheres were then sieved and washed with distill water five times to remove PVA residue. The spheres were then lyophilized for 2 days.

2.5. Scanning electron microscopy (SEM) observation

The morphology of the polymer microspheres was examined using SEM (Philips XL30 FEG) with an accelerating voltage of 10 kV. The samples were coated with gold using a sputter coater (DeskII, Denton vacuum Inc) with a gas pressure of 50 mtorr and a current of 40 mA.

2.6. Set-up of hypoxia bioreactor culture system

We set up a hypoxia-bioreactor for the hypoxic culture of hDPSCs on microspheres. The system was composed of a gas supply, a spinner flask and a container. After placing the spinner flask in the container, the system was vacuumed and purged with a gas mixture containing 2% O_2 (5% CO_2 and 93% N_2) for three times to

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