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Complete pulpodentin complex regeneration by modulating the stiffness of biomimetic matrix

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ABSTRACT

Dental caries is one of the most prevalent chronic diseases in all populations. The regeneration of dentin-pulp tissues (pulpodentin) using a scaffold-based tissue engineering strategy is a promising approach to replacing damaged dental structures and restoring their biological functions. However, the current scaffolding design for pulpodentin regeneration does not take into account the distinct difference between pulp and dentin, therefore, is incapable of regenerating a complete tooth-like pulpodentin complex. In this study, we determined that scaffolding stiffness is a crucial biophysical cue to modulate dental pulp stem cell (DPSC) differentiation. The DPSCs on a high-stiffness three-dimensional (3D) nanofibrous gelatin (NF-gelatin) scaffold had more organized cytoskeletons and a larger spreading area than on a low-stiffness NF-gelatin scaffold. In the same differentiation medium, a high-stiffness NF-gelatin facilitated DPSC differentiation to form a mineralized tissue, while a low-stiffness NF-gelatin promoted a soft pulp-like tissue formation from the DPSCs. A facile method was then developed to integrate the low- and high-stiffness gelatin matrices into a single scaffold (S-scaffold) for pulpodentin complex regeneration. A 4-week *in vitro* experiment showed that biomineralization took place only in the high-stiffness peripheral area and formed a ring-like structure surrounding the non-mineralized central area of the DPSC/S-scaffold construct. A complete pulpodentin complex similar to natural pulpodentin was successfully regenerated after subcutaneous implantation of the DPSC/S-scaffold in nude mice for 4 weeks. Histological staining showed a significant amount of extracellular matrix (ECM) formation in the newly formed pulpodentin complex, and a number of blood vessels were observed in the pulp tissue. Taken together, this work shows that modulating the stiffness of the NF-gelatin scaffold is a successful approach to regenerating a complete tooth-like pulpodentin complex.

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1. Introduction

The regeneration of dental tissues using a scaffold-based tissue engineering strategy represents a promising approach to replacing damaged dental structures and restoring their biological functions [1,2]. This strategy has been exploited extensively for dentin/pulp tissue regeneration in recent years [3–8]. In a natural tooth, the dentin is a mineralized tissue surrounding the central chamber that is filled with a highly vascular connective pulp tissue. Embryologically, histologically, and functionally, dentin and pulp are closely related and are considered together as an inseparable unit termed "pulpodentin" [9]. Clinically, during a root canal treatment to remove the necrotic pulp tissue and disinfect the tooth chamber, the dental instrument is rigid and difficult to completely adapt to the complex shape of the root canal. Consequently, artificial elongation and straightening of the course of the canal are inevitable, leading to the loss of dentin and increased risk of tooth fracture [10–12]. Therefore, successful dentin/pulp regeneration should include both dentin and pulp tissues in order to fully restore the biological and mechanical functions of the diseased tooth.

Since human dental pulp stem cells (DPSCs) were identified in 2000 [13], they have been widely accepted as a potentially suitable cell source for dentin/pulp regeneration because DPSCs are easily obtained from pulp tissue of extracted teeth that are generally discarded in the clinic. A number of experiments have confirmed that DPSCs can differentiate to form dentin and pulp tissues under appropriate conditions [6,8,13–20]. Anatomically, dentin is a mineralized hard tissue, while pulp is a soft tissue, indicating that

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DPSCs are surrounded by two distinct microenvironments (niches) during the organogenesis. However, none of the scaffolds currently used for DPSCs mimic the pulpodentin microenvironments [8,13–17]. Consequently, the regenerated pulp/dentin complex is either the pulp tissue alone or the pulp tissue with a thin layer of bone-like dentin lining along the surfaces of the tooth slice [6,8,18–20]. Furthermore, the scaffolds used for pulp regeneration are often injectable hydrogels (e.g., collagen gels) with weak mechanical strength which causes dramatic contraction of the cell/scaffold constructs after *in vivo* implantation [1,21]. To date, a rational scaffolding design to regenerate a complete tooth-like pulpodentin complex has not been achieved.

Matrix stiffness is now recognized as an important biophysical cue to modulate stem cell adhesion, proliferation, migration, and differentiation [22–28]. For example, human mesenchymal stem cells (MSCs) cultured on polyacrylamide gels under the same serum condition differentiate into neuron-like cells on soft matrices ($E \sim 0.1$–1 kPa), myoblasts on moderately stiff matrices ($E \sim 8$–17 kPa), and osteoblasts on rigid matrices ($E \sim 25$–40 kPa) [29]. Similarly, matrix stiffness directs adult neural stem cell differentiation into different lineages [30]. Those results suggest that modulation of scaffolding stiffness is a feasible approach to controlling stem cell differentiation and neo tissue formation. In fact, a recent publication has shown that high mechanical strength of three-dimensional (3D) scaffolds promoted bone regeneration by promoting endochondral ossification of MSCs [31]. Compared to these widely studied stem cells, the response of DPSCs to matrix stiffness is largely unknown.

Inspired by the anatomic structure of the pulpodentin complex (hard dentin enclosing soft pulp), we hypothesized that scaffolding stiffness is a crucial biophysical cue to modulate DPSC differentiation. Specifically, we postulated that a high-stiffness matrix leads to dentin formation while low-stiffness substrate promotes soft pulp tissue formation from DPSCs. To test this hypothesis, we used a nanofibrous gelatin (NF-gelatin) matrix as the scaffolding material and developed a 3D model to explore how DPSCs interact with matrices of varying stiffness in a 3D environment. NF-gelatin matrix was selected in this study because (1) NF-gelatin matrix mimics the chemical composition and physical architecture of the collagen in dental matrices and provides an excellent microenvironment to support DPSC adhesion, proliferation, differentiation, and neo tissue formation [32,33]; (2) NF-gelatin can be readily fabricated into 3D scaffolds with well-defined macro-, micro-, and nano-structures, offering a unique platform to exploit cell-scaffold interaction in 3D [34–36]; and (3) The stiffness of NF-gelatin can be readily modulated via the crosslinking density without changing other properties of the scaffold. In this study, we first seeded DPSCs into the 3D gelatin scaffold and examined the effect of the scaffolding stiffness on the adhesion, proliferation, differentiation, and biomineralization of the DPSCs. Next, we developed a facile method of integrating the NF-gelatin matrices with two different rigidities into a single scaffold (low stiffness in the center and high stiffness in the periphery). Finally, we seeded DPSCs into this new scaffold and investigated the capability of regenerating a complete pulpodentin complex both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Gelatin was purchased from Sigma Aldrich (St. Louis, MO), N-hydroxy-succinimide (97%) (NHS) and 2-(N-morpholino) ethanesulfonic acid) hydrate (MES) were purchased from Aldrich Chemical (Milwaukee, WI). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) was purchased from Pierce Biotechnology (Rockford, IL), and all other reagents were obtained from Thermo Fisher Scientific (Fair Lawn, New Jersey).

2.2. Preparation of 3D NF-gelatin scaffolds

The 3D nano-structured scaffolds were fabricated by combining a thermally induced phase separation and porogen leaching process as we previously described [34,35]. Briefly, a gelatin solution was prepared by dissolving 1 g of gelatin in 10 ml water/ethanol (50/50, v/v) mixture at 50 °C. The solution (0.2 ml) was cast onto paraffin spheres assembly (250–420 μm), and was phase-separated at −80 °C for 12 h. Next, the gelatin/paraffin spheres assembly composites were immersed in 1,4-dioxane for solvent exchange and freeze-dried. After cutting into a thickness of 1.5 mm and diameter of 5.0 mm, the composites were soaked in 50 ml hexane to leach paraffin spheres. Hexane was changed 6 times every 12 h to remove any residual paraffin in the scaffold. Cyclohexane (10 ml) was then used to exchange hexane in the scaffold for 4 h. Finally, the hybrid scaffold was freeze-dried for 24 h.

2.3. Chemical crosslinking of 3D NF-gelatin scaffolds

Chemical crosslinking of NF-gelatin scaffolds with EDC (5 mmol/L) and NHS (5 mmol/L) was carried out in MES buffer (pH 5.3, 0.05 M) at 4 °C [34,35]. To maintain the microstructure and prevent the swelling of gelatin matrices in water, acetone/water (90/10, v/v) solvent mixture was chosen instead of pure water. After crosslinking for the designated time, the reaction was stopped by adding 0.1 M glycine, and the scaffolds were washed 3 times with distilled water at 37 °C. The scaffolds were freeze-dried for 24 h and stored in a desiccator for later use.

2.4. Coating NF-gelatin matrix with different stiffnesses onto the struts of β-tricalcium phosphate

In the experiment studying how scaffolding stiffness affects DPSC differentiation, we found that the low- and medium-stiffness NF-gelatin scaffold could not withstand the contraction force from the DPSCs and shrank after culturing for a few days. In order to prevent scaffolding shrinkage, we used a β-tricalcium phosphate (β-TCP) as a frame and coated NF-gelatin with different stiffness on the strut surfaces of the β-TCP. The β-TCP scaffolds were prepared using a 3D solid-free-form fabrication technique [37,38]. A Robocad 3.0 (3D Inks, Stillwater, OK, USA) was used for the scaffolding design, in which the in-plane line spacing (from center to center) of the struts was set to 1000 μm and the layer spacing at 250 μm. The resulting β-TCP scaffold was dried in air at room temperature for 48 h and was used as a frame to support NF-gelatin. Paraffin spheres (250–420 μm) were assembled in the line spacing of the β-TCP struts. Gelatin solution was then added into the paraffin assembly to obtain the NF-gelatin and crosslinked for different time periods (30 min, 1 h, and 4 h). SEM examination showed that the obtained 3D NF-gelatin matrices had an average thickness of approximate 100 μm coated on the struts of the β-TCP scaffold.

2.5. Preparation of NF-gelatin scaffolds with low stiffness in the central region and high stiffness in the peripheral region

The non-crosslinked NF-gelatin scaffold (5 mm × 1.5 mm) was concentrically punched to obtain a ring-like scaffold with a 5 mm outer diameter and a 2 mm inner diameter, and a smaller scaffold with a diameter of 2 mm. The ring-like matrix was pre-crosslinked for 4 h, and the 2-mm diameter matrix was added back into the ring-like matrix for co-crosslinking for 30 min. The crosslinking reaction was stopped by adding 0.1 M glycine, and the scaffold