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Nano-Structured Gelatin/Bioactive Glass Hybrid Scaffolds for the Enhancement of Odontogenic Differentiation of Human Dental Pulp Stem Cells

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Abstract

Tooth decay is one of the most common chronic disorders throughout the world. Regenerating decayed dentin/pulp structure requires the design of novel scaffolding materials that mimic the architecture of natural dental extracellular matrix (ECM) and provide suitable environments for the attachment, proliferation, differentiation, and biomineralization of dental pulp stem cells (DPSCs). In this work, we developed an approach to prepare three-dimensional (3D) nano-fibrous gelatin/silica bioactive glass (NF-gelatin/SBG) hybrid scaffolds that mimic the nano-structured architecture and chemical composition of natural dental ECM. This approach involved the combination of a thermally induced phase separation, sol-gel, and porogen leaching process, and synthesized hybrid scaffolds possessing natural ECM-like architecture, high porosity, well-defined pore size and interconnectivity, and improved mechanical strength. An *in vitro* cell culture study showed that human DPSCs had a significantly higher proliferation rate on NF-gelatin/SBG scaffolds compared to NF-gelatin scaffolds under the same conditions. Furthermore, the integration of SBG into the hybrid scaffold significantly promoted the differentiation and biomineralization of the human DPSCs. The alkaline phosphatase (ALP) activity and expressions of marker genes for odontogenic differentiation (Col I, ALP, OCN, DSPP and DMP-1) were all significantly higher in the NF-gelatin/SBG than in the NF-gelatin group. Those results were further confirmed by hematoxylin and eosin (H&E) and von Kossa staining, as evidenced by greater ECM secretion and mineral deposition in the hybrid scaffold. In summary, the biomimetic NF-gelatin/SBG hybrid scaffolds provide an excellent environment for the growth and differentiation of human DPSCs and are promising candidates for dentin/pulp tissue regeneration.

1 Introduction

Dental caries, also known as tooth decay, is one of the most common chronic disorders throughout the world.¹ If left untreated, the disease can lead to pain, infection and tooth loss, all of which cause physical and mental suffering and compromise the patient's self-esteem and quality of life. Currently, root canal therapy is the most widely used method for the treatment of dental caries. This method involves the removal of the necrotic tissue and replaces it with synthetic materials that are bio-inert and incapable of restoring the biological functions of the lost dental tissues. Furthermore, endodontically-treated teeth become devitalized, brittle and susceptible to post-operative fracture and other complications.²

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Dentin and pulp regeneration using a tissue engineering strategy represents a promising approach to replacing damaged dental structures and restoring the functions of the compromised dentin/pulp.³ In this approach, one of the main components is a scaffold, which plays a pivotal role in the success of dentin/pulp regeneration. The scaffold serves as an artificial extracellular matrix (ECM) and as a temporal template for tissue regeneration.⁴⁻⁶ Ideally, it should be biodegradable, biocompatible, promote cellular interactions and tissue development, and possess proper mechanical properties. In an attempt to regenerate dentin and pulp, several types of scaffolds have been tested with dental pulp stem cells (DPSCs) both *in vitro* and *in vivo*.⁷⁻¹⁰ However, all those studies regenerated only a limited number of dental tissues, and none of them has been used in clinical trials. Thus, there remains the challenge to develop a suitable scaffold that can provide the proper microenvironment for DPSC adhesion, proliferation, and differentiation.

Since a scaffold is an artificial ECM, it is beneficial for it to mimic certain advantageous features of natural ECM. In the body, the ECM is a nano-structured three-dimensional (3D) network. Consequently, several techniques using synthetic biomaterials have been developed to mimic such nano-structured architecture.¹¹⁻¹⁴ In our previous study, we prepared a biomimetic nanofibrous gelatin (NF-gelatin) scaffold that mimicked both the chemical composition and physical architecture of natural collagen.¹⁵ The NF-gelatin possessed a large surface area ($>32 \text{ m}^2/\text{g}$), abundant porosities ($>96\%$), good mechanical properties, and nanofibrous pore wall structures. The *in vitro* results further showed that the NF-gelatin scaffold provided better microenvironments for cell adhesion, proliferation, and differentiation than conventional gelatin counterparts.¹⁵

In the current study, we aimed to develop a biomimetic gelatin/bioactive glass hybrid scaffold for dentin/pulp regeneration. Because collagen (type I) is the major organic component of a natural dentin matrix, we chose gelatin as the scaffolding substrate to mimic the chemical composition of collagen fibers in dentin matrices. To simulate the physical architecture of collagen fibers, we developed a thermally induced phase separation (TIPS) method to fabricate nanofibrous gelatin.¹⁵ Compared to other biomimetic techniques, the TIPS method has the advantages of readily integrating a well-defined pore size and pore geometry in the 3D scaffold.⁴ In order to enhance the odontogenic differentiation of DPSCs in the scaffold, we further incorporated silicate bioactive glass (SBG) into the NF-gelatin via a sol-gel process. SBG is a widely accepted bioactive material with excellent bone-bonding properties.¹⁶ A number of studies have indicated that SBG stimulates the growth and osteogenic differentiation of human primary osteoblasts.¹⁷⁻¹⁹ However, to date, the effect of SBG on human DPSCs is relatively unknown. We hypothesize that the release of soluble ions (e.g., Si^{4+}) from the degradation of SBG will lead to favorable intracellular and extracellular responses promoting odontogenic differentiation of DPSCs. In this work, we first synthesized the biomimetic NF-gelatin/SBG scaffold by combining a TIPS, sol-gel, and porogen leaching process. The adhesion, proliferation, migration, differentiation, and biomineralization of human DPSCs on the hybrid scaffold and the control group (NF-gelatin only) were then examined for a total of 4 weeks' culture time *in vitro*. Our results show that NF-gelatin supports human DPSC growth, and the incorporation of the SBG into the hybrid scaffold significantly enhanced the odontogenic differentiation of human DPSCs.

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 obtained from Aldrich Chemical (Milwaukee, WI), and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) was purchased from Pierce Biotechnology (Rockford, IL). Tetraethyl

orthosilicate (TEOS), triethylphosphate (TEP), calcium nitride, citric acid, and all other reagents were ordered from Thermol Fisher Scientific (Fair Lawn, New Jersey).

2.2 Preparation of 3D NF-gelatin/SBG hybrid scaffolds

The NF-gelatin/SBG scaffolds were fabricated using a combination of the TIPS, sol-gel, and porogen leaching processes. A silica sol precursor was prepared according to a method similar to that reported by Lei et al.²⁰ Briefly, TEOS (3.13 g) and TEP (0.18 g) were mixed in ethanol to form a 10 ml solution. Calcium nitride (CN) (2.13 g) and citric acid (CA, 0.04 g) were dissolved in 2.5 ml deionized water, and ethanol was added to the aqueous solution to reach a total volume of 5 ml. The CN-CA solution was slowly added to the TEOS-TEP solution, and the mixture was continuously stirred at room temperature for 5 h.

A gelatin solution was prepared separately by dissolving 1 g gelatin in a 10 ml water/ethanol (50/50, v/v) mixture at 50°C for 1h. Subsequently, a predetermined amount of the silica sol prepared as above was added to the gelatin solution, and this mixture was stirred at 50°C for another hour to form a uniform solution. The solution (0.2 ml) was then cast onto a paraffin sphere assembly prepared as we described previously.⁶ The gelatin/silica solution in the paraffin assembly was phase-separated at -80°C for 12 h. Next, the gelatin/silica gel/paraffin sphere assembly composites were immersed in 20 mL of 1,4-dioxane for solvent exchange three times (fresh 1,4-dioxane was replaced at every 8 h) and freeze-dried for three days. After cutting the assembly composites into 1.5 mm thick slices, the composites were soaked in 50 mL hexane to leach out the paraffin spheres. Hexane was changed every 12 h six times to remove any residual paraffin in the scaffold. Cyclohexane (10 ml) then was used to exchange the hexane in the scaffold for 4 h. Finally, the hybrid scaffold was freeze-dried for 24 h.¹⁵ For comparison, a NF-gelatin control sample was prepared using the same method except that no silica sol was added to the gelatin solution.

2.3 Chemical crosslinking of 3D NF-gelatin/SBG hybrid scaffolds

Since the gelation temperature of gelatin (approximate 32°C) is below cell culture temperature (37°C), the NF-gelatin/SBG hybrid scaffold must be crosslinked to improve its thermal and mechanical stabilities prior to its tissue engineering application. Chemical crosslinking of the NF-gelatin/SBG hybrid scaffolds with EDC and NHS was carried out in MES buffer (pH 5.3, 0.05 M) at 4°C.¹⁵ To maintain the microstructure and prevent the swelling of the gelatin matrices in water, acetone/water (90/10, v/v) solvent mixture was chosen instead of pure water. After crosslinking for the designated number of times, the reaction was stopped by adding 0.1 M glycine, and the scaffolds were washed with distilled water at 37°C three times, then freeze-dried for 24 h and stored in a desiccator for later use. In this study, all the scaffolds were chemically crosslinked for 8 h unless specified otherwise.

2.4 Characterization of 3D NF-gelatin/SBG scaffolds

2.4.1 Surface morphology examination, elemental analysis, and porosity calculation—The surface morphology of the scaffolds was examined using scanning electron microscopy (SEM, JEOL JSM-6010LA) with an accelerating voltage of 10 kV. The porosity of the scaffold was calculated as we reported previously.¹⁵ Measurements of the elemental contents and distribution on the surface of the scaffolds were made using the energy dispersive X-ray spectroscopy (EDS) associated with the SEM. The scaffolds were coated with gold for 120s using a sputter coater (SPI-Module Sputter Coater Unit, SPI Supplies/Structure Probe, Inc.). During the gold coating process, the gas pressure was kept at 50 mTorr and the current at 20 mA.

2.4.2 Attenuated total reflection Fourier transform Infrared (ATR-FTIR)

spectroscopy—The ATR-FTIR spectra of NF-gelatin/SBG, gelatin, and SBG were obtained with a Nicolet iS10 FTIR spectrometer from Thermo Fisher Scientific (Madison, WI). A range of 500 to 4000 cm^{-1} was scanned at a resolution of 1 cm^{-1} , and the signals were averaged.

2.4.3 X-ray diffraction (XRD) analysis

—A Rigaku Miniflex diffractometer (Woodlands, TX) with Cu K α radiation and a Ni filter ($\lambda = 1.5406 \text{ \AA}$) was used to carry out XRD to characterize the NF-gelatin/SBG samples as well as gelatin and SBG controls. The diffractometer was operated at 40 kV and 110 mA with a 2θ range of 10° – 60° at a scanning speed of $0.05^\circ \text{ sec}^{-1}$.

2.4.4 Mechanical test—The compressive moduli of the scaffolds in a dry status were measured using a mechanical tester (TestResources, Shakopee, MN) as reported previously.^{4, 15} All the samples were circular discs (17 mm in diameter and 1.5 mm in thickness) and were chemically crosslinked for 8 h. The specimens were compressed at a crosshead speed of 0.5 mm/min, and the stress vs. strain curve was recorded. The modulus was calculated as the slope of the linear portion of the stress-strain curve, and the averages and standard deviations were reported (n=3).

2.4.5 In vitro degradation evaluation—The NF-gelatin and NF-gelatin/SBG samples (diameter = 17 mm, thickness = 1.5 mm) were immersed in 5 ml of phosphate-buffered saline (PBS, pH 7.4) containing 60 $\mu\text{g/ml}$ (16 units) of collagenase at 37°C .²¹ At pre-designated time intervals, the samples were stopped by addition of 0.2 ml of 0.25 M EDTA (Sigma, USA) solution. The samples were removed from the PBS solution, washed with distilled water and freeze-dried to constant weights. The remaining weight of each sample was measured. For each time point, the average value and the standard deviation were reported (n=3).

2.4.6 In vitro release of silicon ions from NF-gelatin/SBG scaffolds—The NF-gelatin/SBG hybrid scaffolds (diameter = 17 mm, thickness = 1.5 mm) were immersed in a simulated body fluid (SBF) buffer with a pH value of 7.25 and incubated at 37°C as we described previously.⁴ At designated time points (1, 3, 7, 14, and 21 days), the samples were centrifuged to collect the supernatant, which was replaced with equal amounts of fresh SBF buffer. The silicon ion concentration was measured using the ammonium molybdate method reported by Wang et al.²²

2.5 In vitro biocompatibility evaluation

2.5.1 Cell culture and seeding onto NF-gelatin/SBG scaffolds—The human DPSCs used in this study were a gift from Dr. Songtao Shi, School of Dentistry, University of Southern California. The thawed DPSCs (passage 2) were cultured in ascorbic acid-free α -modified essential medium (α -MEM) (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 at 37°C . The culture medium was changed every 2 days. DPSCs of passages 4–6 were used for all cellular studies.

The scaffolds (NF-gelatin/SBG and NF-gelatin) were soaked in 70% ethanol for 30 min, and a low vacuum (approximately 30 torr) was applied to remove any air bubbles in the scaffolds. The scaffolds were washed three times (30 min each) with PBS to remove the residual ethanol. After that, they were washed twice with α -MEM containing 10% FBS (2 h each). Human DPSCs (5×10^5) were seeded onto each scaffold. The cell-scaffold constructs were cultured in α -MEM supplemented with 10% FBS for 24 h before being transferred to

12-well plates, each containing 2 ml medium. For the differentiation of DPSCs, “odontogenic” medium (containing 50 µg/mL ascorbic acid, 5 mM β -glycerophosphate, and 10 nM dexamethasone) was used. All cell-scaffold constructs were cultured at 37°C on an orbital shaker (Orbi-shaker™CO₂, Benchmark, USA) in an incubator with 5% CO₂. The culture medium was changed every other day.

2.5.2 DNA assay—To determine the proliferation of DPSCs on the scaffolds, the cell-scaffold constructs were homogenized in 1 ml 1×DNA assay buffer (Sigma, St. Louis, MO) using an ultrasonic homogenizer (Virsonic60, Virtis, NY). One milliliter cell lysis buffer was added, and the samples were incubated at 37°C for 1 h. The cell lysis was spun down at 5000 g for 5 min. The total DNA was quantified using a fluorescence assay (QuantiFluor™, Promega, CA) with Hoechst 33258 dye, according to the manual (Sigma).

2.5.3 Confocal laser scan microscopy observation—After 3 days' culturing, the cell-scaffold constructs were rinsed in PBS and fixed in 2.5% glutaraldehyde for 30 min. The constructs were then stained with 0.5% phalloidin for 1h and observed under the confocal laser scan microscope (TCS SP5, Leica, Buffalo, USA).²³ Images were taken using an A-plan apochromat 63× objective (0.9 N.A.). The 633 nm HeNe (50% power) laser was used to excite the Alexa Fluor® 633 Phalloidin (absorption 573-645 nm).

2.5.4 Alkaline phosphatase (ALP) activity quantification—ALP activity was detected using a SensoLyte™ pNPP ALP Assay Kit (AnaSpec, CA, USA) according to the manufacturer's protocol. Briefly, the cells on the scaffold were homogenized in 1 ml of the lysis buffer supplied with the kit. The lysates were centrifuged at 10,000 g and 4°C for 15 min. The supernatant was collected for ALP assay using p-nitrophenyl phosphate as a phosphatase substrate and the ALP supplied by the kit as a standard. The absorbance was measured at 405 nm, and the amount of ALP in the cells was normalized against total protein content.

2.5.5 Hematoxylin-eosin (H&E) staining and von Kossa staining—After 4 weeks' culture, the cell-scaffold constructs were fixed in 4% paraformaldehyde (PFA) at 4°C for 1 h. The samples were sequentially washed under water, dehydrated in ethanol, cleared in xylene and embedded in paraffin. The specimens were cut in the lateral direction into alternating 5µm-thick sections and stained with H&E for histological examination. In the von Kossa staining procedure, the specimens were rinsed with PBS and then fixed with 4% PFA. The von Kossa method for calcium kit (Polysciences, Germany) was used to stain the calcium deposits according to the protocol provided by the manufacturer.

2.5.6 Real-time polymerase chain reaction (PCR)—The total RNA was extracted using Trizol (Invitrogen), and the first-strand cDNA was reversely transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) from each sample. The human-specific primers were designed and synthesized as follows: collagen (1) I (sense 5'-AAAAGGAAGCTTGGTCCACT-3'; antisense 5'-GTGTGGAGAAAGGAGCAGAA-3'), ALP (sense 5'-CCACGTCTTCACATTTGGTG-3'; antisense 5'-AGACTGCGCCTGGTAGTTGT-3'), dentin matrix protein 1 (sense 5'-TGGGGATTATCCTGTGCTCT-3'; antisense 5'-TACTTCTGGGGTCACTGTGCG-3'); osteocalcin (sense 5'-ACTGTGACGAGTTGGCTGAC-3'; antisense 5'-CAAGGGCAAGAGGAAAGAAG-3') and dentin sialophosphoprotein (sense 5'-TTAAATGCCAGTGGAACCAT-3', antisense 5'-ATTCCCTTCTCCCTTGTGAC-3'). Glyceraldehyde-3-phosphate dehydrogenase (sense 5'-GAGTCAACGGATTGGTCGT-3'; antisense 5'-GACAAGCTTCCCGTTCTGAG-3') and beta-actin (sense 5'-AAACTGGAACGGTGAAGGTG-3'; antisense 5'-TTTTTAGGAGGGCAAGGGACT-3').

was used as an internal control. The conditions for real-time PCR were as follows: denaturation at 95°C (10 min); 45 cycles at 95°C (15 sec), 55°C (1 min, annealing) followed by a final dissociation step. The qRT-PCR reactions were performed using power SYBR Green QPCR Master Mix (Applied Biosystems) and the Bio-rad Real-Time PCR Detection System (Bio-rad, USA). The reactions were measured 3 times.

2.6 Statistical analysis

Data were reported as means $\pm$ standard deviations ($n = 3$). The statistical analysis was carried out using the Student's t-test for differences among groups, and a value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Characterization of the NF-gelatin/SBG hybrid scaffolds

The NF-gelatin/SBG hybrid scaffolds were prepared by a process involving TIPS, sol-gel, and porogen leaching steps. As shown in Figure 1, the hybrid scaffolds were porous structures (porosity $96.5 \pm 0.2\%$) with macropores ranging from 250–420 μm , which were determined by the sizes of paraffin sphere templates and were considered to be the optimal sizes for osteoblastic and odontoblastic cell adhesion, proliferation and differentiation.²⁴ The incorporation of a small amount of SBG (10% wt in the scaffold) into the scaffolds did not affect the pore size or porosity of the hybrid scaffold (Figure 1A–C). Each pore was interconnected with other pores in the scaffold, and the pore walls were composed of nanofibers (Figure 1D–I). Higher magnification further indicated that these nanofibers were between 50 to 500 nm, which is the same range as natural collagen fibers (Figure 1G–I).²⁵ As the ratio of the SBG in the SBG/NF-gelatin increased, the average diameter of the fibers increased, and the average fiber length decreased (Figure 1G–I).

In the ATR-FTIR spectra, the NF-gelatin/SBG scaffolds display the characteristic peaks of both the gelatin and the SBG controls, as indicated by dashed lines in Figure 2. Specifically, the peaks at 1542 cm^{-1} (dashed line A) and 1240 cm^{-1} (dashed line B) are assigned to the amide II (N–H bend and C–H stretch) and amide III (C–N stretch) vibrations of gelatin, respectively.²⁶ The band at 1123 cm^{-1} (dashed line C) is attributed to the asymmetric stretching vibration of Si–O, while 818 cm^{-1} (dashed line D) is associated with the bending vibration of Si–O in the SBG.²⁷ The presence of SBG was further confirmed by EDS analyses. As shown in Figure 3, the hybrid scaffold appeared as strong peaks assigned to Si and Ca. In addition, the EDS mapping showed that Si and Ca elements were uniformly distributed throughout the entire NF-gelatin/SBG scaffold (Figure 3C–D). In the XRD spectra, the NF-gelatin/SBG appeared only as broad peaks, suggesting an amorphous phase of the SBG in the hybrid scaffolds (Figure 4).

The mechanical properties of the NF-gelatin/SBG scaffolds were examined and are shown in Figure 5. Under a low concentration of SBG in the hybrid scaffold, the compressive modulus increased with the amount of SBG. For example, the compressive modulus of the NF-gelatin/SBG scaffold with 5% (wt/wt) of SBG was $388 \pm 41\text{ kPa}$, which is 35.2% higher than that of the NF-gelatin control ($287 \pm 39\text{ kPa}$). The mechanical strength of the hybrid scaffold decreased with higher SBG concentration ($>5\%$ wt/wt). However, the compressive modulus was still as high as $256 \pm 46\text{ kPa}$ when the SBG concentration was 10% (wt/wt) in the hybrid scaffold. Since the NF-gelatin/SBG hybrid scaffolds with 5% (wt/wt) of SBG had the best mechanical strength, excellent nanofibrous architecture, and similar porosity to that of NF-gelatin control, they were used for the rest of the study, unless specified otherwise.

3.2 In vitro degradation of the hybrid scaffolds

The *in vitro* degradation of the scaffolds was performed in PBS with 60 µg/ml collagenase at 37°C; the results are shown in Figure 6. Overall, the hybrid scaffolds had faster degradation rates than did the gelatin control scaffolds. When the scaffolds were crosslinked with EDC/NHS for 30 min, the NF-gelatin/SBG and NF-gelatin lost their entire weight at 1 h and 4 h, respectively (Figure 6 A-B). As the crosslinking time increased to 1 h, the hybrid scaffold completely degraded at 7 h, while the NF-gelatin control lost more than 94% of its original weight at 24 h (Figure 6 C-D). When the crosslinking time was further increased to 8 h, less than 12.7% and 11.3% of their weight was lost from the hybrid scaffold and gelatin control, respectively, after 24 h incubation in the medium (Figure 6 E-F).

3.3 Silicon ion release from the hybrid scaffolds

The release of silicon ion from the hybrid scaffold (5% wt/wt SBG in the scaffold) was examined and is shown in Figure 7. No apparent burst release was observed on the first day, and Si⁴⁺ was released at an average concentration of 28.3±1.6 mg/L for the first 3 days. After that, the Si⁴⁺ was released at a constant lower rate, and approximately 93% of Si⁴⁺ was released at the end of 4 weeks. Other concentrations of SBG (2%, 7%, and 10% wt/wt) in the hybrid scaffolds were also tested and had similar release profiles (data not shown).

3.4 DPSCs adhesion, proliferation, and migration on the hybrid scaffolds

The same number of DPSCs (5×10⁵ cells/scaffold) was seeded onto both the hybrid scaffold and the NF-gelatin control. During the 3-week culture period, the number of DPSCs on the NF-gelatin/SBG was always significantly higher ($P < 0.05$) than that on the NF-gelatin (Figure 8). At 1 week, the amount of DNA on the hybrid scaffold (4584±72 ng/scaffold) was more than 32.1% higher than that on the gelatin control (3471±48 ng/scaffold). At the end of 3 weeks, the amount of DNA on the hybrid scaffold (14801±165 ng/scaffold) was 60.1% higher than that on the control (9246±201 ng/scaffold). These results indicated that the DPSCs on the NF-gelatin/SBG had a higher proliferation rate than on the NF-gelatin.

The migration of DPSCs inside the scaffolds was examined using confocal microscopy, and the images were taken on the plane 500 µm below the surface of the scaffold. As shown in Figure 9, the DPSCs migrated inside both the hybrid and control scaffolds three days after cell seeding. The DPSCs (red staining) were spindle-shaped with numerous pseudopodia interconnected with the scaffolds (non-specific green staining), indicating that these DPSCs were healthy and had closely interacted with the scaffolds. Meanwhile, more DPSCs migrated inside the NF-gelatin/SBG scaffold (Figure 9B) than in the NF-gelatin control (Figure 9A), which is consistent with the DNA measurements.

3.5 DPSC differentiation on the hybrid scaffolds

The expression of ALP activity on both the hybrid and the control scaffolds increased with time for the first 3 weeks. At each time point (1, 2, and 3 weeks), the NF-gelatin/SBG group always showed significantly higher ALP levels ($P < 0.05$) compared to the NF-gelatin group (Figure 10). At 4 and 5 weeks, the ALP activity decreased for both groups. However, the NF-gelatin/SBG group had a higher ALP expression than the NF-gelatin group, indicating that NF-gelatin/SBG enhanced DPSC differentiation.

The H&E staining showed that more DPSCs penetrated into the central region of the NF-gelatin/SBG scaffold than in the NF-gelatin scaffold after 4 weeks of culture in the odontogenic medium (Figure 11). Furthermore, the DPSCs on the NF-gelatin/SBG scaffold secreted a higher amount of ECM compared to the NF-gelatin scaffold. After 4 weeks of cell culture, von Kossa staining revealed a higher amount of mineral deposition in the NF-gelatin/SBG group than in the control group (Figure 12). Expressions of marker genes for

odontogenic differentiation (Col I, ALP, OCN, DSPP and DMP-1) were examined using RT-PCR (Figure 13). The expression levels of all these genes were significantly higher in the NF-gelatin/SBG than in the NF-gelatin. Specifically, the expressions of DMP-1 and Col I on the hybrid scaffold group were more than 17-fold and 12-fold higher than on the control group, respectively. These results showed that the incorporation of the SBG into the NF-gelatin significantly enhanced the differentiation of the DPSCs.

4. Discussion

The success of dentin/pulp regeneration depends on the development of suitable scaffolding materials as carriers for DPSCs. In this study, we developed a 3D NF-gelatin/SBG hybrid scaffold that mimicked the chemical composition and nano-structured architecture of dental ECM, possessed high porosity and pore interconnectivity, and had excellent mechanical stability. This *in vitro* study further showed that the integration of SBG into the hybrid scaffold significantly enhanced human DPSC proliferation and differentiation.

Among all approaches to scaffolding fabrication, the integration of inorganic components into biodegradable polymer substrates is one of the most widely used methods, because it combines the synergetic properties of each component, leading to better biological and mechanical properties of the hybrid scaffold than the individual components. Directly mixing polymer with inorganic particles (e.g., hydroxyapatite) is a simple approach to synthesizing composite scaffolds. In fact, several gelatin-based hybrid scaffolds have been developed using this method.²⁸⁻³⁰ However, directly mixing inorganic particles with polymer solution generally forms a heterogeneous mixture (particles dispersed in a polymer solution), which therefore results in an uneven distribution of the composition within the composite and limited mechanical strength. In the present study, we used a homogenous solution composed of gelatin and the bioactive glass precursor solution to prepare NF-gelatin/SBG hybrid scaffolds. After the TIPS, sol-gel, and porogen leaching process, a composite scaffold with uniform distribution of the SBG throughout the hybrid was obtained and confirmed by EDS measurement (Figure 3). The mechanical strength of the NF-gelatin/SBG hybrid scaffold (SBG = 5% wt/wt) was also significantly higher than that of the NF-gelatin (Figure 5). A high amount of SBG (SBG > 5% wt/wt) in the hybrid scaffold did not contribute to the overall mechanical properties, probably due to its interference with the phase separation of the gelatin solution during the scaffold formation. Further study is needed to illustrate the mechanism of how SBG affects the TIPS process of the hybrid scaffold.

The combination of gelatin and SBG was recently reported for preparing biomimetic scaffolds for bone regeneration.²⁰ However, there were no macropores (>100 μm) in those scaffolds. Therefore, osteoblasts could grow only on the scaffolding surfaces with very limited thickness.²⁰ Furthermore, the gelatins in the scaffolds were not chemically crosslinked, leading to their fast dissolution in PBS (and culture medium) at 37°C. In the present study, we included a template (paraffin spheres assembly) to generate well-defined macropores (250-420 μm) in the scaffolds. The pore size and interconnectivity of the hybrid scaffold were precisely tailored by the sizes of paraffin spheres and heat treatment time of the paraffin sphere assembly (Figure 1 A-C). Furthermore, we added a chemical crosslinking step in the acetone/water (90/10 v/v) solvent mixture to stabilize the hybrid scaffold. Under this crosslinking condition, the nanofibrous architecture of the hybrid scaffold was well retained after crosslinking (Figure 1 G-H). The crosslinking density of the hybrid scaffold was modulated by the crosslinking time, i.e., a longer reaction time led to a higher crosslinking density. Thus, the degradation of the NF-gelatin/SBG scaffold was readily controlled by crosslinking time, as shown in Figure 6. It should be pointed out that in order to accelerate the *in vitro* degradation process, a relatively high concentration of

collagenase (60 µg/ml) was added to the degradation medium. When placed in PBS only, the hybrid scaffold was very stable and degraded much more slowly than in the PBS with collagenase. To truly reflect the degradation profile of the NF-gelatin/SBG hybrid scaffold in contact with body fluid, an *in vivo* degradation measurement is needed in future studies.

A unique feature of the SBG is its ionic degradation by-products, which are soluble and have been observed to stimulate osteogenesis via activation of the expression of several genes associated with bone formation.¹⁹ Consequently, knowing how to achieve controlled ion release from the SBG is critical to ensure desirable biological responses. The NF-gelatin/SBG scaffolds provided an effective way to control the dissolution of Si⁴⁺ from the hybrids. As shown in Figure 7, the Si⁴⁺ was constantly released from SBG for over 4 weeks, and no apparent burst release was observed on the first day. While more evidence is needed, it is possible that the sol-gel process results in the interactions of polymer-SBG at a molecular level, creating a sustained dissolution of Si⁴⁺ from the hybrid scaffold.³¹

DPSCs are odontogenic progenitor cells from adult human dental pulp and have clonogenic abilities, rapid proliferation rates and multiple differentiation potentials (odontogenic, osteogenic, and adipogenic), providing a suitable cell source for tissue regeneration.³² When DPSCs are used for dentin/pulp tissue regeneration, it is crucial to promote their proliferation rate and enhance their odontogenic differentiation. These goals can be achieved by building an appropriate microenvironment to facilitate cell-material interactions in the scaffolds. SBG is a bioactive material, and its bioactivity is associated with the formation of a crystalline hydroxyapatite surface layer in contact with body fluids.³³ This surface layer is similar to the structure of the inorganic region of bone ECM, therefore facilitating the interactions between osteoblasts and SBG. When SBG was integrated into the NF-gelatin/SBG scaffold, DPSCs on the hybrid scaffold had a significantly higher proliferation rate than on the NF-gelatin control under the same culture conditions (Figure 9). In a separate study, Lei et al. reported that there was no significant difference of MC3T3-E1 osteoblasts proliferation between the gelatin/SBG hybrid matrix and the gelatin control group within 6 days. We believe that the following factors contribute to the difference between the two studies: cell types (DPSC *vs.* osteoblast), scaffold architecture (macropores *vs.* no macropores), scaffold chemical composition (crosslinked gelatin with 5% SBG *vs.* non-crosslinked gelatin with 30% SBG), and testing time points (1-3 weeks *vs.* 2-6 days).

The NF-gelatin/SBG group had a higher ALP expression and stronger von Kossa staining than the NF-gelatin group using the same differentiation medium (Figures 10&12). The expression levels of marker genes for odontogenic differentiation (Col I, ALP, OCN, DSPP and DMP-1) were all significantly higher in the NF-gelatin/SBG than in the NF-gelatin, confirming that the incorporation of the SBG into the NF-gelatin enhanced the differentiation of the DPSCs (Figure 13). Therefore, our *in vitro* data show that the biomimetic NF-gelatin/SBG scaffold provided a better environment to support DPSC proliferation, migration, and differentiation.

Generally, both porosity and ions release from the scaffold influence cellular adhesion, proliferation, and differentiation. However, we only added a low amount of silicon bioactive glass (bioglass/gelatin=5/100) in this study, and it did not significantly change the porosity of the hybrid scaffold. As described above, the hybrid scaffold had a porosity of 96.5±0.2%, which is very close to that of the control NF-gelatin scaffold. SEM images also confirmed that both the NF-gelatin and the NF-gelatin/SBG hybrid scaffolds had the same pore size and very similar porosity (Figure 1a-c). Therefore, the enhanced cell proliferation and differentiation only resulted from the ions release of the SBG in this work. Further study will test the *in vivo* dentin/pulp regeneration of the human DPSCs in the NF-gelatin/SBG hybrid scaffold.

5. Conclusion

Biomimetic NF-gelatin/SBG scaffolds were fabricated via a TIPS, sol-gel, and porogen leaching process. These 3D hybrid scaffolds mimicked the nanofibrous architecture of dentin ECM and possessed high porosity, well-defined pore size and morphology, and improved mechanical strength. The integration of SBG into the hybrid scaffold significantly enhanced the proliferation, migration, differentiation and biomineralization of human DPSCs. The NF-gelatin/SBG hybrid scaffolds, therefore, provide a better environment for DPSCs and are promising candidates for dentin/pulp tissue regeneration.

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Figure 1. SEM micrographs of NF-gelatin and NF-gelatin/SBG hybrid scaffolds. (A, D, G) NF-gelatin scaffolds; (B, E, H) with 5% (wt/wt) SBG in the NF-gelatin/SBG hybrid scaffold; (C, F, I) with 10% (wt/wt) SBG in the NF-gelatin/SBG hybrid scaffold.

Figure 2.
ATR-FTIR spectra of NF-gelatin/SBG, NF-gelatin, and SBG.

Figure 3. Typical EDS spectra of (A) NF-gelatin/SBG hybrid scaffold, and EDS mappings of (B) Si and (C) Ca elements within the NF-gelatin/SBG hybrid scaffold (5% (wt/wt) SBG).

Figure 4.
Typical XRD spectra of NF-gelatin/SBG, NF-gelatin, and SBG.

Figure 5. Compressive modulus of the NF-gelatin/SBG hybrid scaffolds with different SBG concentrations. (* $P < 0.05$ between NF-gelatin/SBG (5% wt/wt SBG) and NF-gelatin).

Figure 6.

Remaining weight of the NF-gelatin/SBG and the NF-gelatin scaffolds with time incubated in 60 $\mu\text{g/ml}$ collagenase degradation medium. (A) NF-gelatin/SBG crosslinked in EDC/NHS for 30 min; (B) NF-gelatin crosslinked in EDC/NHS for 30 min; (C) NF-gelatin/SBG crosslinked in EDC/NHS for 1 h; (D) NF-gelatin crosslinked in EDC/NHS for 1 h; (E) NF-gelatin/SBG crosslinked in EDC/NHS for 8 h; (F) NF-gelatin crosslinked in EDC/NHS for 8 h. The concentration of the SBG was 5% (wt/wt) in the hybrid scaffolds.

Figure 7. Release of silicon ion from the NF-gelatin/SBG hybrid scaffold. The concentration of the SBG was 5% (wt/wt) in the hybrid scaffold.

Figure 8. The proliferation of human DPSCs cultured on NF-gelatin/SBG and NF-gelatin scaffolds. 5×10^5 cells were seeded on each scaffold (* $P < 0.05$ between NF-gelatin/SBG and NF-gelatin).

Figure 9. Confocal images of human DPSCs cultured on NF-gelatin (A) and NF-gelatin/SBG (B) for 3 days. Images were taken on the plane of 500 μm below the surface of the scaffolds. The green colour is the non-specific staining of the gelatin in the scaffold.

Figure 10. ALP activities of human DPSCs cultured on NF-gelatin/SBG and NF-gelatin scaffolds for 5 weeks. (* $P < 0.05$ between NF-gelatin/SBG and NF-gelatin).

Figure 11.

H&E staining images of human DPSCs cultured on NF-gelatin/SBG and NF-gelatin scaffolds for 4 weeks. The human DPSCs were cultured in (A) NF-gelatin; (B) NF-gelatin/SBG; (C) is the higher magnification of (A); (D) is the higher magnification of (B).

Figure 12.

von Kossa staining images of human DPSCs cultured on NF-gelatin/SBG and NF-gelatin scaffolds for 4 weeks. The human DPSCs were cultured in (A) NF-gelatin; (B) NF-gelatin/SBG; (C) is the higher magnification of (A); (D) is the higher magnification of (B).

Figure 13. Relative gene expression (collagen type I [Col I], osteocalcin [OCN], dentin matrix protein 1 [DMP-1], alkaline phosphatase [ALP], and dentin sialophosphoprotein [DSPP]) after human DPSCs were cultured on NF-Gelatin and NF-Gelatin-NCPs scaffolds for 4 weeks. (* $P < 0.05$ between NF-gelatin/SBG and NF-gelatin).