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Glutaraldehyde-induced remineralization improves the mechanical properties and biostability of dentin collagen

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ABSTRACT

The purpose of this study was to induce a biomimetic remineralization process by using glutaraldehyde (GA) to reconstruct the mechanical properties and biostability of demineralized collagen. Demineralized dentin disks (35% phosphoric acid, 10 s) were pretreated with a 5% GA solution for 3 min and then cultivated in a calcium phosphate remineralization solution. The remineralization kinetics and superstructure of the remineralization layer were evaluated by Raman spectroscopy, transmission electron microscopy, scanning electron microscopy and nanoindentation tests. The biostability was examined by enzymatic degradation experiments. A significant difference was found in dentin remineralization process between dentin with and without GA pretreating. GA showed a specific affinity to dentin collagen resulting in the formation of a cross-linking superstructure. GA pretreating could remarkably shorten remineralization time from 7 days to 2 days. The GA-induced remineralized collagen fibrils were well encapsulated by newly formed hydroxyapatite mineral nanocrystals. With the nano-hydroxyapatite coating, both the mechanical properties (elastic modulus and hardness) and the biostability against enzymatic degradation of the collagen were significantly enhanced, matching those of natural dentin. The results indicated that GA cross-linking of dentin collagen could promote dentin biomimetic remineralization, resulting in an improved mechanical properties and biostability. It may provide a promising tissue-engineering technology for dentin repair.

1. Introduction

Dentin is a collagenous mineralized tissue consisting of a collagen matrix and hierarchically arranged apatite nanocrystals [1,2]. This mineralized collagen matrix is of major significance for the functions of dentin by supporting the surrounding enamel, reinforcing its mechanical properties and preserving its biological properties [3]. However, it can be demineralized by some biochemical procedures and dental operations, such as decalcification due to bacteria in caries and acid-etching in resin-dentin bonding procedures [4]. The demineralized collagen matrix exhibits much weaker mechanical properties and can easily be degraded by host-derived endogenous matrix metalloproteinase (MMPs) and cysteine cathepsins, which represent the critical barriers to future progress in dental adhesion [5,6,7]. Biomimetic dentin remineralization, as an effective approach to improve the biomechanical properties of the collagen matrix and to protect it from biodegradation, has become a potential tissue-engineering technology for treating the caries-affected dentin and improving the resin-dentin bonding durability [8,9,10].

The sophisticated assembly process of natural dentin, an organic-inorganic compound, is regulated by acidic non-collagenous proteins (NCPs), e.g. the dentin matrix protein 1 (DMP1) and dentin phosphophoryn (DPP), which can induce and regulate dentin biomineralization by working either as nucleators or inhibitors [11,12]. In contrast to the classic ion-mediated crystallization, current remineralization technologies, which are based on the non-classical crystallization pathway theory, try to pursue a biomimetic approach for remineralization [13,14]. Recent studies have focused on the ability of NCP analogues, like polyacrylic acid (PAA) [15], polyaspartic acid (pAsp) [16] and synthetic peptides [17], to mimic the functional domains of natural proteins in order to reproduce the structural hierarchy of ordered apatite deposition in the collagen matrix. For instance, Tay and Pashley have studied the biomimetic remineralization of acid-etched dentin disks by applying PAA and PVPA acids to a Portland cement–phosphate-containing fluid (Portland cement–PCF) system to regulate apatite nucleation and growth [9,18]. The key function of the NCP analogues is to stabilize and control the dimensions of the amorphous calcium phosphate (ACP) nanoprecursor particles so that the intermediate phase can penetrate into the microfibrillar spaces within the collagen fibrils for the subsequent transformation-based hydroxyapatite (HA) crystallization [18,19].

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Currently, despite the recent progress concerning successful biomimetic remineralization of the demineralized dentin by utilizing the cooperative effect of PAA and L-glutamic acid [20,21], the process remains too time-consuming for clinical applications. Therefore, an efficient way to promote the remineralization process is highly desirable for the future development of dental operation practices.

Glutaraldehyde (GA) is a gold-standard cross-linking agent that has been widely accepted in the biomedical field and is considered to improve tissue function, especially the structural integrity and bio-stability of collagen-based biomaterials, such as heart valves, vascular grafts, and artificial skin implanted in vivo [22,23,24,25]. However, some studies have also shown that GA has a deteriorative effect on the calcification of bio-prosthesis materials, which can compromise its function and is the predominant cause for the clinical failure of implanted bio-prostheses [26,27]. It has been revealed that the collagen-based aldehyde-treated tissue has an affinity towards calcium ions, which can act as mineralization centers for calcium phosphate precipitation [28,29]. This precipitation is undesirable in soft-tissue implants but can be an advantage for the remineralization of collagenous mineralized tissues, such as dentin. In dentistry, GA is the main composition of Gluma dentin desensitizer, and 5.0% concentration has less cytotoxic effect [30,31]. Low concentrations of GA (around 5%) have already been used as a collagen cross-linking agent to enhance the stability of the resin-dentin interface and improve bond durability [32,33,34]. However, its potential effect on the biomimetic remineralization of dentin collagen requires further study.

Inspired by the functional characteristics of GA, the experiments were designed to combine the results from our previous studies with the GA-based modification of dentin collagen [20,35]. Our objective was to investigate the effect of GA-induced cross-linking on the remineralization of demineralized dentin collagen by observing the time-dependent remineralization process and studying the micro-morphological changes on the remineralized dentin surface (collagen fibrils), and the biomechanical and biological properties of the remineralized dentin collagen were investigated.

2. Materials and methods

2.1. Specimen preparation

Eighty recently extracted non-carious human third molars were obtained with the patients' consent under a protocol approved by the ethical committee of the First Affiliated Hospital of the Zhejiang University College of Medicine. The teeth were stored at 4 °C in a 0.1 wt% thymol solution (Sigma-Aldrich, St. Louis, MO, USA) containing 0.02 wt% sodium azide (Sigma-Aldrich, St. Louis, MO, USA), which was added to inhibit bacterial and fungal contamination (pH 7.4). Dentin stubs were prepared by parallel cutting ~2 mm below the cement-enamel junction and perpendicular to the longitudinal axis with a water-cooled slow-speed Isomet diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA). Enamel and pulp exposure was avoided for all specimens. The obtained dentin stubs were then cut into two different types of samples: dentin disks with dimensions of 10.0 mm × 8.0 mm × 1.0 mm, and dentin beams with dimensions of 10 mm × 0.8 mm × 0.8 mm, as illustrated by Fig. 1. The surface of each specimen was further polished under triply deionized water using 600-grid and 2000-grid silicon carbide paper to create a smooth surface layer. A ~3 μm thick demineralized collagen matrix layer was produced on the surface of the dentin by etching with a 35% phosphoric acid (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 10 s. The specimens were divided into different experimental groups, as shown in Table 1.

2.2. GA pretreatment and Raman investigation of the dentin disks

The GA solution was prepared by diluting a 25 vol% aqueous solution of GA (Grade II, Sigma-Aldrich, St. Louis, MO, USA) with deionized water to a concentration of 5% v/v. The acid-etched dentin disks were randomly divided into two groups. 54 acid-etched dentin disks were immersed in deionized water for 3 min (untreated group). The other 54 acid-etched dentin disks were immersed in the 5% GA solution for 3 min (GA cross-linking group). All treated dentin disks were thoroughly rinsed with deionized water, and then the excess water was removed using blotting paper.

Raman spectra (inVia Reflex, Renishaw, Gloucestershire, England) of samples from both the control group and the GA-pretreated group (each group containing 6 dentin disks) were recorded to confirm the chemical modification of the dentin collagen by the GA. The spectrometer was operated with monochromatic radiation emitted by a He–Ne laser (532 nm wavelength) at 50 mW, and the laser beam was focused using an optical lens with a magnification factor of 100 (Nikon). Spectra in the wavelength range from 800 to 1800 cm^{-1} were recorded using the following parameters: a 2400 grating, 25 mV laser power and 5 consecutive scans with an accumulation time of 3 s each at each site. The Raman-shift frequency was calibrated using a silicon wafer as reference. Spectral mappings were performed at positions corresponding to 1 μm intervals across the surface of the dentin disks using the computer-controlled stage and averaged over five consecutive scans. No post-processing of the data was performed.

2.3. Biomimetic remineralization

The remineralization solution was prepared by mixing the phosphate-containing solution and the calcium-containing solution (Table 2). The pH value of the remineralization solution was stabilized at 7.4 ± 0.1 using a 1 M HCl solution and a 1 M NaOH solution (both obtained from Aladdin, Shanghai, China). The phosphate-containing solution (12.0 mM) was prepared using Na_2HPO_4 (Sigma-Aldrich, St. Louis, MO, USA). The calcium-containing solution was prepared by dissolving a calculated amount of NaCl (Aladdin, Shanghai, China) and Tris (AMRESCO, Ohio, USA) in an aqueous solution of 20.0 mM $CaCl_2 \cdot 2H_2O$ (Aladdin, Shanghai, China). A calculated amount of PAA (average Mw: 1800; Sigma-Aldrich, St. Louis, MO, USA) was added to the calcium solutions as ACP stabilizer. After mixing 25 ml of the calcium solution

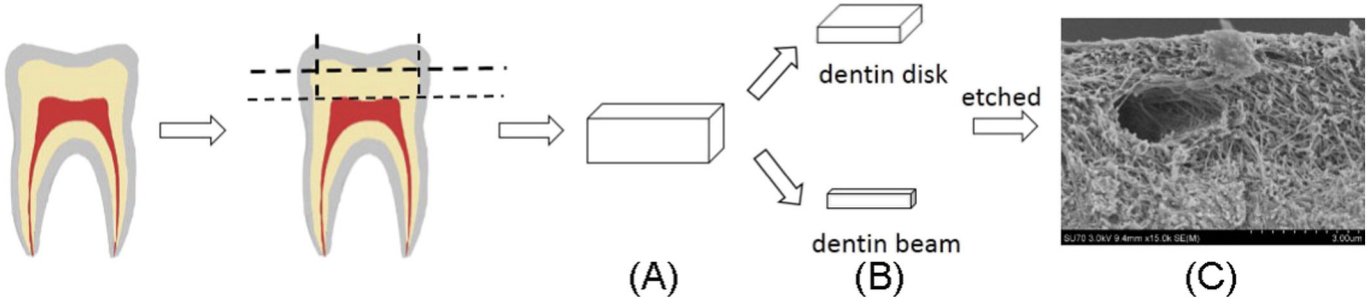

Fig. 1. Schematic illustration of the specimen preparation process: (A) dentin stubs obtained from sectioning crowns, (B) dentin disks (10.0 mm × 8.0 mm × 1.0 mm) prepared for further examination and dentin beams (10 mm × 0.8 mm × 0.8 mm) for the enzymatic degradation analysis, (C) a 3 μm thick layer of demineralized dentin collagen on the surface after etching.