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Characterizing the collagen stabilizing effect of crosslinked chitosan nanoparticles against collagenase degradation

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ABSTRACT

Antibacterial and chelating properties of chitosan has been widely studied for various dental applications.

Objective. To characterize the interaction between chitosan-nanoparticles (CSnp) and collagen, and understand their stabilizing effect against collagenase degradation for dentin matrix stabilization.

Methods. Phase-1: a single Type I collagen-fibril model was used to study the interaction with CSnp along with carbodiimides crosslinking treatment. Degradation of the crosslinked fibrils was studied with bacterial collagenase enzyme and monitored using Fourier Transform Infrared (FTIR) spectroscopy, turbidity measurement (400 nm), ninhydrin assay and Atomic Force Microscopy (AFM). Interaction of CSnp with collagenase and Type I collagen, were evaluated using SDS-PAGE, and proteolytic cleavage potential of a synthetic peptide. Phase-2: degradation of dentin collagen crosslinked with/without CSnp was evaluated using FTIR, ninhydrin assay and Scanning Electron Microscopy (SEM). Glutaraldehyde crosslinking was used as a positive control.

Results. Both native collagen-fibrils and dentin collagen after crosslinking showed higher resistance to collagenase degradation, as observed in turbidity measurements and FTIR spectra. AFM images showed the interaction of CSnp with single collagen-fibril and crosslinked collagen resisted collagenase degradation up to 54 h. The collagen and collagenase both formed complexes with CSnp resulting in thickening of bands and reduction in collagen degradation. CSnp treated collagenase showed significantly reduced cleavage of the fluorescent peptides. Dentin collagen was coated with CSnp following crosslinking with significant increase in resistance to collagenase degradation.

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Significance. Crosslinked CSnp on collagen stabilized and enhanced the resistance of dentin matrix against bacterial collagenase degradation due to non-specific interaction with both collagen and collagenase.

1. Introduction

The structural framework of dentin that is provided by the inorganic and organic fraction, is crucial to maintain the mechanical integrity of teeth [1]. Clinical observations have shown varying degrees of dentin collagen degradation depending upon the years of function [2]. In addition, caustic chemicals such as sodium hypochlorite (NaOCl), chelating agent such as ethylenediamine tetra-acetic acid (EDTA), and high pH medicaments such as calcium hydroxide ($Ca(OH)_2$), when used for treatment purposes are known to induce surface changes on root dentin [1,3–5]. These disease (non-iatrogenic) and treatment-mediated (iatrogenic) processes, results in compromised physical and mechanical characteristics of dentin, which increases the risk of fracture in root-filled teeth [2,3,6]. Clinical studies have highlighted that approximately 11% of the extracted root-filled teeth show signs of vertical root fractures [7].

Type I collagen is the major structural protein comprising up to 90% of the organic fraction of dentin [8]. Exposed collagen from dentin matrix following disease or iatrogenic procedures is susceptible to degradation. Proteases from bacteria, and saliva or host derived matrix metalloproteinases are known to degrade dentin collagen with time [2,9,10]. Degradation of dentin occurs due to the dissolution or denaturation of the organic proteins and proteoglycan molecules, which bonds the collagen network with the hydroxyapatite crystals in dentin [6,11]. The degradation of collagen on root canal dentin surface may lead to interfacial failures at the restoration–dentin interface, leading to microbial penetration and restorative failures in root filled teeth [12]. Any treatment attempt to stabilize the ultrastructure of dentin collagen and neutralize the effect of bacterial collagenase on dentin collagen will significantly improve the mechanical integrity of root filled teeth [1,13]. Thus, crosslinking has been applied to improve the mechanical and biological stability of dentin collagen [14–17].

Crosslinking methods such as chemical, physical or photocrosslinking will induce intra- and intermolecular crosslinks in collagen, and have been successfully used in tissue engineering to stabilize biological tissues and artificial constructs [18,19]. Glutaraldehyde has been shown as an effective crosslinking agent to increase the tensile properties and stiffness of demineralized dentin [14,16]. However, glutaraldehyde is highly cytotoxic due to the reaction by-products, limiting their application *in vivo* [20]. Another chemical crosslinker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of N-hydroxysuccinimide (NHS) are known to stabilize collagen by the formation of additional crosslinks [14,18]. They are non-toxic, leave no residues following treatment and forms amide-type crosslinks with collagen [21]. A recent study has demonstrated increased resistance to fatigue cracks growth in resin–dentin interface following carbodiimide conditioning even after aging for 6 months in artificial saliva [22]. These crosslinking techniques have also been employed to incorporate natural biopolymers into collagen scaffold. These crosslinked biopolymers would act as fillers that further enhanced the mechanical characteristics of the scaffold [14,23,24].

Chitosan is a hydrophilic biopolymer (2-amino-2-deoxy-β-D-glucopyranose) with large number of free hydroxyl and amino groups that can form crosslinks with other reactive molecules. The free reactive groups in chitosan can interact to form chemical bonds with collagen [24,25]. The chitosan incorporated and crosslinked dentin collagen displayed superior mechanical properties as compared to crosslinking alone, as well as resisted degradation by bacterial collagenase [14,26]. In addition, chitosan nanoparticles exhibited excellent antibiofilm properties and also inhibited bacterial adherence on dentin [27]. In spite of all these interests, the specific role of chitosan-nanoparticles (CSnp) in combination with chemical crosslinking to stabilize collagen ultrastructure and its ability to inhibit bacterial collagenolytic activity has not been fully characterized. Crosslinking CSnp on dentin collagen in situ, prior to endodontic procedures or adhesive restorations could provide several advantages such as resistance to interfacial degradation, prevent bacterial recolonization at the interface and improve mechanical properties of dentin matrix.

The purpose of this study was twofold: (1) to characterize the interaction and stabilizing effect of CSnp crosslinking on type I collagen and effect of CSnp on collagenase enzyme activity. (2) To study the effect of CSnp crosslinking on dentin collagen towards bacterial collagenase degradation. The null hypothesis is that CSnp crosslinking does not have any effect on the degradation of Type I collagen.

2. Materials and methods

All the chemicals were purchased from Sigma Aldrich (St. Louis, USA) unless mentioned otherwise. CSnp was synthesized based on a previously published protocol [28,29]. Bovine type I collagen (Purecol, 3 mg/mL) fibrils were assembled in 200 mM sodium phosphate buffer (pH 7.3) [30]. Collagenase from *clostridium histolyticum* with an activity of 125 CDU/mg solid (P/N C-0130) was prepared in 50 mM HEPES buffer (pH 7.4) containing 0.36 mM $CaCl_2$. EDC and NHS were used for chemical crosslinking [24].

2.1. *Native type I collagen fibril model*

2.1.1. *Effect of crosslinking and CSnp treatment on collagen*

Native collagen fibrils were synthesized as described in the literature [30]. Briefly, a reaction buffer containing 150 μL of water, 500 μL of 200 mM Na_2HPO_4 and 250 μL of 400 mM KCl