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Influence of fluoride on the mineralization of collagen via the polymer-induced liquid-precursor (PILP) process

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ABSTRACT

Objective. The polymer-induced liquid-precursor (PILP) mineralization process has been shown to remineralize artificial dentin lesions to levels consistent with those of native dentin. However, nanoindentation revealed that the moduli of those remineralized lesions were only ~50% that of native dentin. We hypothesize that this may be due to the PILP process having been previously optimized to obtain high amounts (~70 wt%) of intrafibrillar crystals, but without sufficient interfibrillar mineral, another significant component of dentin.

Methods. Fluoride was added to the PILP-mineralization of collagen from rat tail tendon at varying concentrations to determine if a better balance of intra- versus inter-fibrillar mineralization could be obtained, as determined by electron microscopy. Nanoindentation was used to determine if fluoridated apatite could improve the mechanical properties of the composites.

Results. Fluoride was successfully incorporated into the PILP-mineralization of rat tail tendon and resulted in collagen-mineral composite systems with the mineral phase of hydroxyapatite containing various levels of fluoridation. As the fluoride concentration increased, the crystals became larger and more rod-like, with an increasing tendency to form on the fibril surfaces rather than the interior. Nanomechanical testing of the mineralized tendons

Abbreviations: μ-CT, micro-x-ray computed tomography; ACP, amorphous calcium phosphate; AFM, atomic force microscopy; DTA, differential thermal analysis; EDS, energy dispersive x-ray spectroscopy, often coupled with electron microscopy to determine what elements makeup an area of a specimen; EDTA, ethylenediaminetetraacedic acid, frequently used as a calcium chelator; GPa, gigapascal; kDa, kilodaltons, 1 kilodalton = 1 kg/mol; MPa, megapascal; pAsp, poly(aspartic acid); PBS, phosphate-buffered saline solution; PILP, polymer-induced liquid-precursor; ppm, parts per million; SAED, selected area electron diffraction; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TGA, thermogravimetric analysis, usually involves the measurement of sample weight as ambient temperature is increased; wt.%, weight percentage; XRD, x-ray diffraction.

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revealed that fluoride addition did not increase modulus over PILP mineralization alone. This likely resulted from the separated nature of collagen fibrils that comprise tendon, which does not provide lateral reinforcement and therefore may not be suited for the compressive loads of nanoindentation.
Significance. This work contributes to the development of minimally invasive approaches to caries treatment by determining if collagen can be functionally mineralized.

1. Introduction

Because of the short longevity of commonly used restorations, researchers are exploring biomimetic methods to restore less damaged areas of carious lesions to their natural state. While some success has been achieved for enamel repair, dentin remineralization poses a unique challenge given the large component of organic matrix [1–9]. In our prior report, when the polymer-induced liquid-precursor (PILP) process was used to remineralize artificial dentin caries, full apatitic mineral density recovery was obtained as determined by micro-x-ray computed tomography analysis (μ-CT) and similar nanostructures to native dentin were observed via transmission electron microscopy (TEM). However, nanoindentation analysis revealed that the modulus and hardness were not fully restored at the outer portions of the lesions, resulting in values ∼50% of those of native dentin [10]. In the PILP process, anionic polypeptides (such as polyaspartic acid, pAsp) are placed in solution with supersaturated levels of calcium and phosphate. The acidic polypeptide sequesters ions to induce or stabilize nanodroplets of a liquid-like, amorphous calcium phosphate (ACP) precursor phase. These liquid-like precursors infiltrate collagen fibrils, perhaps by capillary action or via the Gibbs–Donnan effect [11,12]. After infiltration, the precursor solidifies into ACP and finally crystallizes into hydroxyapatite, resulting in large amounts of aligned, intrafibrillar mineral [11,13,14]. We hypothesize that we were unable to restore the mechanical properties due to preferential infiltration of PILP droplets into the interior of collagen fibrils, wherein the spatial confinement leads to very small, intrafibrillar crystals that may not provide sufficient hardness. Both intra- and interfibrillar crystals are present in native dentin and are believed to augment the mechanical properties [15,16].

The work presented in this paper is to evaluate the effectiveness of adding fluoride to the PILP mineralization of type I collagen. We used rat tail tendon as a model system because it consists of relatively pure (∼97%) and well-aligned type I collagen and our group has shown that it can be readily mineralized via the PILP process [17]. Given the larger dimensions of fluorapatite crystals, we hypothesized that these crystals would be unable to grow within the collagen fibrils and force them to associate more with the exterior surfaces of the fibrils, creating more inter- and/or extrafibrillar mineral than when no fluoride is present. In this paper, we use the terminology "intrafibrillar mineral" to refer to mineral that resides within the collagen fibrils, "interfibrillar mineral" as that which is located between the fibrils, and "extrafibrillar mineral" when describing crystals that do not appear to be templated by the fibrils (i.e., superficial spherulites).

Fluorapatite has a lower solubility product (8.6×10^{-61}) than hydroxyapatite (2.35×10^{-59}) and is thus less likely to dissolve in aqueous/mildly acidic environments [18–22]. This lower solubility is the reason why fluoride is used in enamel remineralization. Fluoride induces remineralization of partially demineralized enamel, so it was of interest here to see if that would also be the case for dentin. Fluorapatite is also harder and stiffer than hydroxyapatite and tends to create more rod-like crystals than hydroxyapatite, which is more plate-like [23–28]. This study was designed to examine the effect of fluoride on the PILP system, which we hypothesized would increase mechanical properties of the resultant composite by both increasing the amount of interfibrillar mineral relative to intrafibrillar mineral and also by having a stiffer mineral component present within the composite material.

2. Materials and methods

2.1. *Sample preparation*

Tendons were extracted from tails of 12–18 week old Sprague Dawley rats and cleaned by centrifugation at 4400 rpm for 5 min in PBS two to three times, replacing the supernatant after each centrifugation cycle. The specimens were then placed in 4 M NaCl (Fisher Scientific) solution for 5 min and then placed in a 0.5 M Tris buffer solution containing 0.5 M ethylenediaminetetraacetic acid (EDTA, Fisher) for 72 h under constant stirring [29]. The tendons were then rinsed in deionized water for 1.5 h, replacing the water every 30 min while continually shaking. They were rinsed for an additional 24 h in deionized water and then stored in PBS with 0.5 mg sodium azide (Fisher) at 4 °C until use. Just before addition to the mineralization solutions, pieces 1.5″ long were cut with a degreased razor blade.

2.2. *Mineralization*

Specimens were mineralized in 50 mM Tris buffer with 0.9% NaCl, 0.02% sodium azide, 4.5 mM $CaCl_2$, 2.1 mM K_2HPO_4, 50 μg/mL 27 kDa pAsp (200-mer, Alamanda Polymers), and 0, 0.5, 1, 2, 5, 25, 50, 100, or 200 ppm F (powdered NaF, Acros Organics). Tris buffer solution containing 0.9% NaCl and 0.02% sodium azide was prepared. This solution was divided in half and $CaCl_2$ (9 mM) was added to one half while K_2HPO_4 (4.2 mM) and NaF was added to the other. The solutions were filtered

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