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Polypeptide-catalyzed Biosilicification of Dentin Surfaces

ABSTRACT

In situ formation of mineral particles by biocatalysis would be advantageous for occluding dentin tubules to reduce permeability or for sealing of material-tooth interfaces. One approach would require that the peptide-catalyst remain functional on the dentin surface. Based on recent observations of retained activity on other surfaces, we hypothesized that poly(L-lysine) (PLL), an analog of the protein catalyst responsible for silica formation in primitive marine species, would remain functional on dentin. PLL was applied to dentin discs along with a pre-hydrolyzed silica precursor, tetramethyl orthosilicate (TMOS). Discs were analyzed microscopically (scanning electron microscopy, SEM) and chemically (x-ray photoelectron spectroscopy, XPS). The treated discs, but not the negative controls, exhibited partial distinct coating whose XPS survey was consistent with that of silica, demonstrating that the polypeptide was required and retained its mediating activity. Peptide-catalysts that mediate mineral formation can retain functionality on dentin, suggesting a wide range of preventive and treatment strategies.

KEY WORDS: silica, morphology, dentin, tubules, permeability.

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INTRODUCTION

Restorative dentistry procedures create an interface, primarily with dentin, that can become susceptible to the common clinical problems of microleakage, sensitivity, and secondary caries. Dentin bonding is effective at reducing these problems, but the systems are technique-sensitive, and questions of the resins' long-term stability remain (Manso *et al.*, 2008). Additional sealing of the tubules with mineral would be intuitively desirable. Similarly, desensitizing agents act by occluding tubules through either transport of intact particles or precipitation of minerals such as oxalates (Pashley and Galloway, 1985; Yiu *et al.*, 2005) and calcium phosphates (Imai and Akimoto, 1990). In the proposed clinical applications, the conceptual advantage of a catalyzed reaction is the potential to strategically immobilize the catalyst or precursor so that subsequent introduction of the other solution yields mineral *in situ*—within the tubules, for example—or at an otherwise-inaccessible interface. Additionally, a catalyzed reaction should allow for control of reaction rate and particle morphology and properties.

Recent discoveries concerning the biomineralization process in primitive marine species may hold promise for the development of a synthetic technology appropriate for dentistry. Cell-secreted proteins responsible for diatom (Kroger *et al.*, 1999) and sponge (Shimizu *et al.*, 1998) biomineralization have been isolated and shown to remain active *in vitro*. Simplified synthetic cationic macromolecules, including poly(L-lysine) (PLL), that contain primary and secondary amine groups, are structurally similar to the functional regions of the natural proteins and have also been shown to promote silicification (Rodriguez *et al.*, 2004; Patwardhan *et al.*, 2005). The mechanisms and reaction parameters that influence biomimetic mineralization have been recently reviewed (Xu *et al.*, 2007).

As a complementary approach to protein mediation of hydroxyapatite mineralization (Kirker-Head, 2000; Moradian-Oldak, 2001; Tay and Pashley, 2008), the silica system may offer several advantages for sealing dentin tubules or interfaces. The silicification reaction is faster, may be more robust, and we anticipate that the formed material could have sufficient integrity for sealing, stabilizing, or reinforcing purposes (Advincula *et al.*, 2009). The silica system has been studied in solution (Rodriguez *et al.*, 2004, and references therein), providing some information on the effects of reaction conditions on important characteristics such as the rate of silicification and morphology of the formed silica. A few studies have examined biocatalyzed silicification on surfaces (Coradin *et al.*, 2005; Advincula *et al.*, 2006; Pogula *et al.*, 2007), but there are no reports of these peptide-catalysts being applied to surfaces similar to dentin or even its components. Protein-catalysts can be immobilized on a surface by simple adsorption and maintain their activity, but most have a significant reduction of functionality (Nicolini, 1997; Cao, 2005a), and optimized methods for immobilizing biocatalysts remain elusive (Cao, 2005b). Therefore, while a rationale exists for the use of PLL on dentin, no data exist to show that it would be effective. Accordingly, we tested the hypothesis that PLL would maintain its functionality and promote silica formation on a dentin surface.

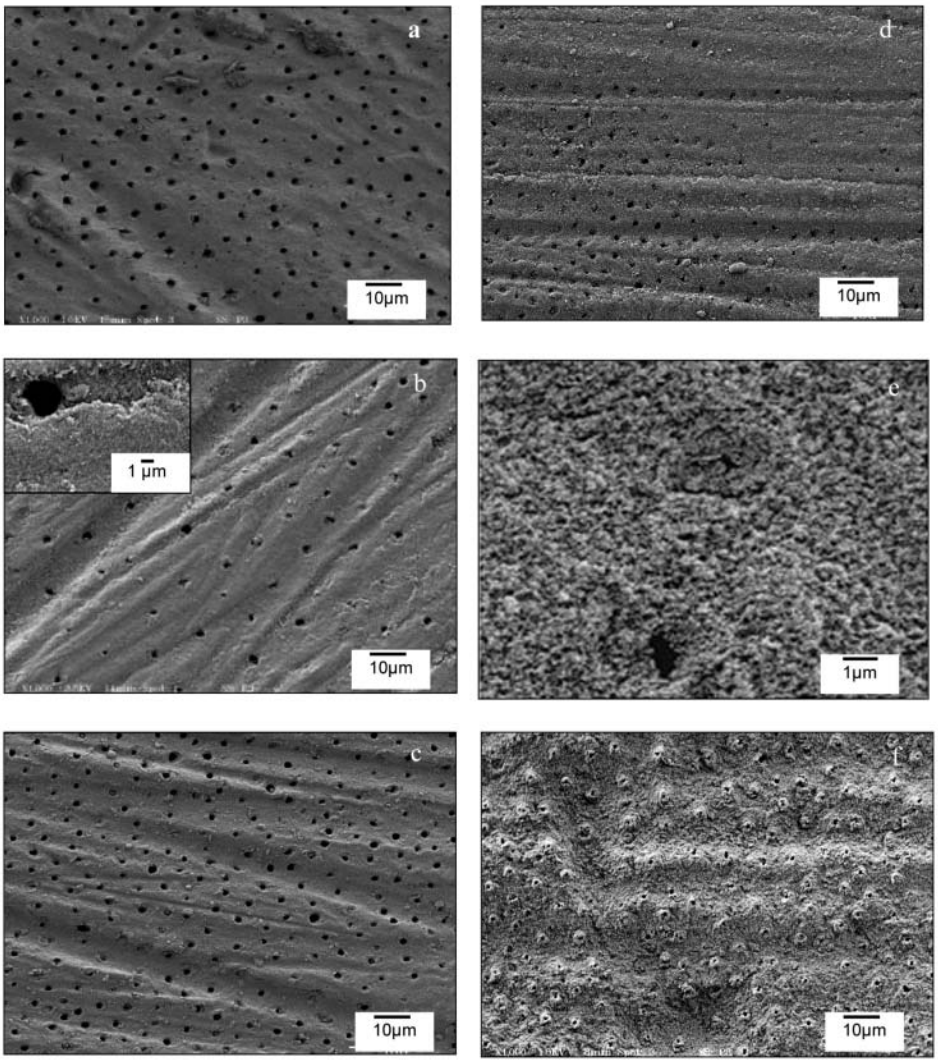

Figure 1. SEM of dentin surfaces: **(a)** smear-layer-free surface with open, exposed tubules after polishing and treatment with EDTA and NaOCl; **(b)** treatment with only TMOS; **(c)** treatment with only PLL; **(d,e)** treatment with PLL catalyst and TMOS precursor; **(f)** surface coating following permeability test.

MATERIALS & METHODS

Silicification and Analysis of Dentin Discs

Unidentified extracted human, non-carious molar teeth from informed, consenting individuals in the School of Dental Medicine clinics and private practices in the area were donated following a procedure approved by the University of Connecticut Health Center IRB. The teeth were cleaned and stored in 0.2-0.5% sodium azide solution until use. After being washed with water, the teeth were sectioned perpendicular to the long axis just inside the DEJ with a slow-speed diamond saw (Isomet 1000, Buehler, Ltd., Lake Bluff, IL, USA) at 300 rpm and polished through 320-grit metallurgical paper to obtain discs nominally 0.5 mm thick. We removed smear layers by immersing the discs in 17% EDTA (5 min), rinsing them in water, immersing them in 5.25% NaOCl (5 min), and finally rinsing them again under running water for 30 min.

We prepared a 100-mM silicic acid, $Si(OH)_4$, precursor solution by mixing 1 M tetramethyl orthosilicate (TMOS, Aldrich, Milwaukee, WI, USA), previously hydrolyzed in 1 mM HCl, with ethanol at a ratio of 1:10. The polypeptide catalyst solution was 1 mM (based on the manufacturer's structural information) of 30- to 70-kDa poly(L-lysine)-HBr (PLL, Aldrich) prepared in water. The biosilicification reaction was conducted by first immersion of the smear-free dentin discs in the silicic acid solution for 5 min, followed by rinses in ethanol, then water (pH 7.0), and drying. Next, the discs were immersed in the PLL solution for at least 30 min, followed by a water (pH 11.0) rinse. Finally, the silicic acid immersion, rinses, and drying were repeated. In total, 13 discs were prepared, including 7 receiving the TMOS/PLL/TMOS (silicification) treatment, and 2 discs each of 3 control groups of TMOS (silicic acid) only, PLL only, and untreated.

To examine for the presence of reaction products and their morphology, we sputter-coated 2 of the TMOS/PLL/TMOS-treated discs and 1 from each control group (Hummer X, Analtech Ltd., Newark, DE, USA) with approximately 12 nm of gold/palladium and examined them with scanning electron microscopy, SEM (JEOL JSM-6320F, Tokyo, Japan). We used x-ray photoelectron spectroscopy, XPS (VG Escalab MK II and Phi Multiprobe System with 15-255GAR Analyzer and 04-548 Dual Anode X-ray Source) (Escalab Mark II, VG Scientific Ltd., East Grinstead, Sussex, UK; PHI Multiprobe System with 15-255GAR Analyzer and 04-548 Dual Anode X-ray Source, Physical Electronics, Chanhassen, MN, USA), to determine the surface composition of 1 TMOS/PLL/TMOS disc and 1 disc from each control group. We selected XPS to identify the presence of silicon, determine its bonding state, and limit analysis to the outermost layer of material. The VG XPS was equipped with a non-monochromated Mg x-ray source operated at about 150 Watts, with pass energies of 60 eV and 25 eV for surveys and high-resolution scans, respectively. The Phi system was similarly equipped and operated at about 200 Watts, with pass energies of 100 eV and 50 eV for surveys and high-resolution scans, respectively. The C 1s peak at a binding energy (BE) of 284.8 eV was used as the charge-correction reference.

Permeability of Silicified Dentin Discs

Because one potential application of biosilicification could be sealing of dentin tubules, dentin permeability was evaluated

according to a well-established perfusion procedure (Pashley, 1986). The permeability of 4 TMOS/PLL/TMOS-treated discs was measured 3 times before and after silicification. Differences were compared with a matched-pair statistical test (Prizm 4, GraphPad Software, San Diego, CA, USA). Following permeability testing, each disc was dried, sputter-coated, and examined with SEM.

RESULTS

SEM Examination

SEM confirmed that polishing and the EDTA/NaOCl treatment produced the intended smear-free dentin surface with patent tubules (Fig. 1a). The dentin disc treated with TMOS showed only a sparse, thin coating of material (Fig. 1b), whose morphology was sometimes distinct, but more often was featureless (insert). Discs receiving only PLL showed no deposited material (Fig. 1c) and appeared similar to the untreated surface (Fig. 1a). The dentin discs treated with TMOS precursor and PLL catalyst solutions (TMOS/PLL/TMOS) were covered with a layer of material clearly visible with SEM (Fig. 1d). The coating appeared to consist of uniform agglomerated particles less than 1 μm in diameter (Fig. 1e). In most cases, the coating formed a continuous bridge over the tubules, although it was not uniform over the entire surface of the dentin discs. Approximately one-quarter of the surfaces remained uncoated.

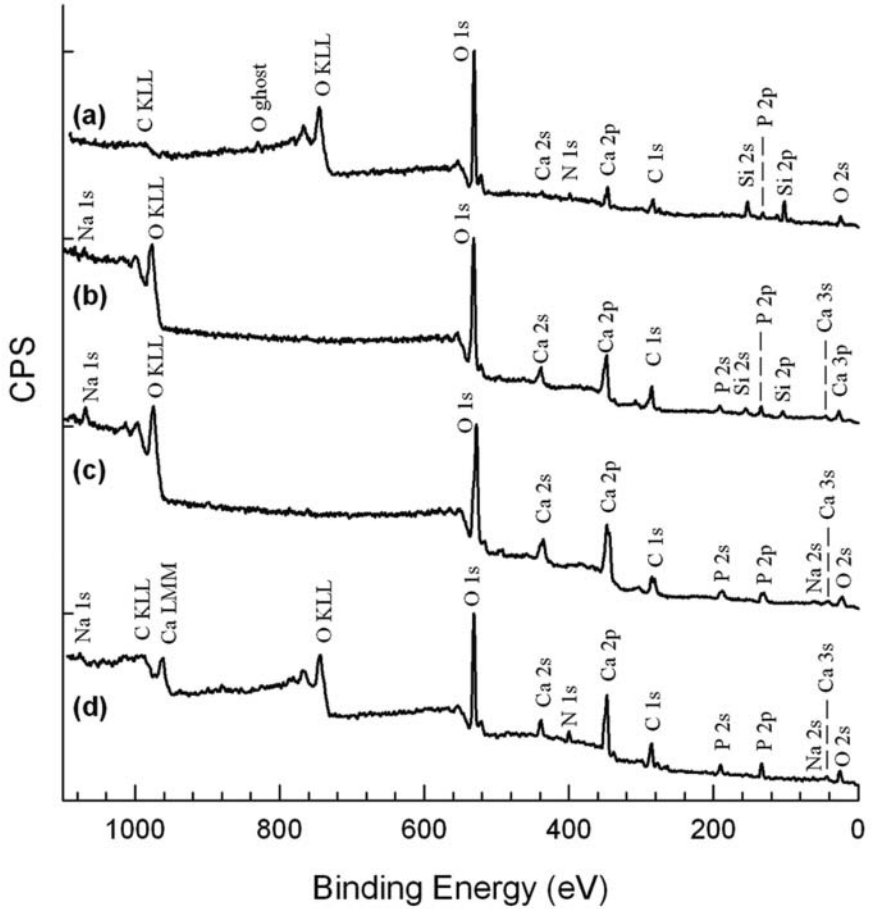

Figure 2. XPS survey of dentin surfaces **(a)** treated with TMOS/PLL/TMOS, **(b)** treated with TMOS only, **(c)** treated with PLL only, and **(d)** untreated, smear-layer-free. The Si 2p peak in **(a)** is in the expected position for SiO_2, 103.4 eV, while the Si 2p peak in **(b)** is consistent with sub-oxides (101.3 eV). The Ca and P peaks are in the expected locations for dentin.

XPS Analysis

The XPS surveys of the TMOS/PLL/TMOS, TMOS-only, PLL-only, and untreated control dentin discs are shown in the binding energy region of 0-1100 eV (Fig. 2). The relative atomic compositions are shown in the Table. The large C peak at 284.8 eV is due to atmospheric contamination (adventitious C), but there is also a smaller C peak at 287.5 eV associated with type I collagen (Eugenio *et al.*, 2005).

For all discs surveyed, the Ca 2p and P 2p peaks at approximately 346 and 132eV, respectively, appeared in the expected locations for dentin (Eugenio *et al.*, 2005). These peaks were present in the TMOS/PLL/TMOS-treated discs because the formed coating is mesoporous and did not cover the entire dentin surface (Fig. 1d), while the XPS spot size was relatively large, 3 mm.

Si was detected on the TMOS/PLL/TMOS and TMOS-only discs. The Si 2p peak in the TMOS/PLL/TMOS survey was located at 103.4 eV, as expected for silica (Awazu and Shimizu, 2000; Kasten *et al.*, 2003). The Si 2p peak in the TMOS-only survey was located at 101.3 eV, indicating a sub-oxide (partially condensed TMOS) and not SiO_2 (Himpsel *et al.*, 1988). Additionally, the Si atomic percent, and Si/O and Si/Ca ratios were higher in the TMOS/PLL/TMOS than in the TMOS-only survey. These results are consistent with SiO_2 formation on the TMOS/PLL/TMOS disc and a sub-oxide, silicic acid, or residual TMOS on the TMOS-only disc. No Si was detected on either the PLL-only or the untreated dentin discs.

Table. Relative Atomic Percentages of Elements in TMOS/PLL/TMOS, TMOS Only, PLL Only, and Untreated Samples Calculated from the XPS Analysis

Element	TMOS/PLL/TMOS	TMOS Only	PLL Only	Untreated
Oxygen	46.5	46.8	44.5	38.3
Carbon	17.7	26.3	25.3	29.4
Nitrogen	2.9	0.0	0.0	4.7
Calcium	5.7	12.3	16.4	14.8
Phosphorus	4.9	5.3	12.0	11.1
Silicon	22.4	8.5	0.0	0.0
Sodium	0.0	0.9	1.9	1.6

Permeability of Silicified Dentin Discs

The mean hydraulic conductance (permeability) of the 4 TMOS/PLL/TMOS-treated dentin discs was reduced from 1.40 ± 2.40 x 10^{-3} in the smear-free condition to 0.56 ± 0.97 x 10^{-3} μL/(cm^2-min-cmH_2O) following silicification, but the difference was not statistically significant ($p = 0.325$). Normalizing the smear-free conductance of each disc to 100%, the mean ± SD silicified value was 35.0% ± 41.3% and $p = 0.051$. SEM examination following permeability testing (Fig. 1f) showed more coating material on peritubular dentin matrix than on intertubular matrix, suggesting that the coating was preferentially bound to more highly mineralized peritubular dentin. The preferential binding of silica to peritubular dentin has been previously reported (Addy *et al.*, 1985).

DISCUSSION

Results were consistent with the catalyzed or at least mediated formation of silica (SiO_2) on the dentin surface. While not uniform over the entire dentin discs, the coatings produced by the TMOS/PLL/TMOS treatment were clear and distinct. The position of the XPS Si peak from the TMOS/PLL/TMOS samples was consistent with silica. We therefore conclude that the poly(L-lysine) remained active on dentin and mediated formation of SiO_2. Additionally, the dentin itself did not appear to catalyze the reaction within the time frame studied. The TMOS-only disc had a residual coating of TMOS, silicic acid, or a sub-oxide silicate. The formation of a sub-oxide is not unexpected, since hydrolyzed TMOS will condense to silicate compounds given sufficient time and/or temperature.

The catalytic control demonstrated here implies that the rate and location of the reaction and potentially the morphology of the formed mineral could be manipulated. For example, 'flowing' a precursor solution into the dentin tubules, followed by application of a catalyst, could allow formation of mineral within the tubules, a useful strategy if sealing is the desired outcome. The formulations and application sequence would depend on the dentin pre-treatment, although preliminary studies (not reported) applying PLL first appeared to produce similar results. Alternatively, biocatalyzed mineralization might be applicable to forming particles within a dentin-bonding hybrid layer, potentially reinforcing or stabilizing this zone. Since it has been shown that comparable biocatalysts can remain active within a polymer (Brott *et al.*, 2001), a dental resin could potentially carry the polypeptide catalyst for subsequent formation of particles, a layer, a composite, or even a mineral gradient. In all of these examples, the principle is that particles need not be transported, but could be formed *in situ*, as is done in nature (Calvert and Rieke, 1996).

While the experiments demonstrated the ability to form silica on a dentin surface and bridge the tubules, the variability in results indicates that more control of the parameters is necessary. For example, the silica coating was not uniform over the dentin discs. It is not clear if the TMOS or PLL did not adsorb on these regions, if the dentin surface in these areas inhibited the reaction, or possibly the silica was formed, but was washed off by the water or ethanol rinses. For this to be understood, future studies would need to investigate the complex mechanism of binding to the surface, and biomolecule stability and conformational distortion (Kasemo, 2002). Additionally, an alternative mediator could be considered, such as polyethyleneimine (PEI), which is known to catalyze silica directly from alkoxides without pre-hydrolysis (Jin and Yuan, 2005). PEI could produce a thicker silica coating by alternating layers of PEI and precursor (Patel, 2008).

While biocatalyzed mineral may not need high mechanical properties for the suggested applications, at least minimal mechanical integrity and adhesion to dentin or resin will be necessary. The decreased permeability, while not statistically significant, suggests that the silica blocked the flow of water during the test. Morphologies including plates, fibers, and dendrites have been reported in solution (Rodriguez *et al.*, 2004), so control is possible and might lead to more effective sealing. Catalyzing mineral deeper within the tubules may be a necessary strategy. There was evidence of this effect, and further studies are planned.

Silicification was studied primarily as a model system for catalyzed mineralization in dentistry. The silica system might be useful for clinical applications, but concerns with the toxicity of the alkoxysilanes would need to be addressed. The toxicity of TMOS and TEOS stems from their low solubility in water and alcohol release (Gill and Ballesteros, 2000), but it may be possible to mediate these effects (Avnir *et al.*, 2006).

Alternatively, biocatalysis of other mineralization systems could be considered. Ti-OH and Zr-OH precursors formed upon hydrolysis interact with positively charged or functionalized macromolecules or polypeptides, as found with the silica system. Polyacrylic acid and poly(vinyl alcohol) are capable of such interaction and have been used to form self-supporting TiO_2/PVA, TiO_2/PAA, and ZrO_2/PVA composite films (Hashizume and Kunitake, 2006). Of course, it would be most desirable to use the hydroxyapatite system. The associated catalytic/templating proteins were identified in the 1980s, and the concept was proposed (Slavkin *et al.*, 1985). Indeed, a recent study has demonstrated the feasibility of forming apatite with protein analogs within a fluid system (Tay and Pashley, 2008). As demonstrated here, other mineralization systems may be capable of an intermediate outcome, which, while not representing formation of native tooth structure, could form mineral for occluding tubules and/or reinforcement of dentin-bonding resins.

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