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Slanted orientations of dentine tubules on remineralized dentine surfaces

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Abstract Dentine carious lesions can be remineralized under optimal conditions, while the surface characteristics of the caries-attacked area may play an important role in the remineralization process. To understand such a surface mechanism, we examined the microstructures of the remineralized area pretreated with different methods. It was found that dentinal tubules on the remineralized surface orientated differently from intrinsic dentine tubules, with the specific alignment angle determined by different surface treatments. Various surface treatments included in this study were 37% phosphoric acid treatment (the etched group), 37% phosphoric acid etching followed by the application of 10% sodium hypochlorite treatment (the deproteinized group), and untreatment (the control group). These findings are helpful for understanding the non-restorative repair of dentine lesions and the remineralization process of the caries-affected dentin surface.

1 Introduction

Dental caries are common oral, infectious and communicable diseases involved in demineralization and remineralization of dentine when caries lesions extending into dentine. Deep into dentine, the caries pores become supersaturated to apatite formation and the crystal growth of apatite crystallites contributes to remineralizing the partially dissolved hard tissue [1]. It was implied that the new formed crystals were not induced by nucleation of organic matrix but the residual crystals in the partially demineralized tissue [2, 3]. Opposite views urged that macromolecules might be responsible for mineral induction during dentine remineralization [4]. For example, collagen and dentine matrix protein 1 (DMP1) could co-modulate the crystal nucleation and growth processes, in which apatites deposited at the collagen-DMP1 bound sites in the presence of calcium and phosphate ions [5]. Collagen matrix in dentine can guide the tissue remineralization through interfibrillar and intrafibrillar apatite deposition [6].

Dentine is a hydrate biological composite with distinct and variable morphology. The peritubular dentine is largely apatites and lines the lumen of each tubule. The intertubular dentine is a composite that consists mainly of two distinct materials: a collagen matrix and an apatite crystal reinforcement. During caries attacking on dentine, as well as etchants infiltrating into dentine, the residual tissues would vary in the component of organic matrix and inherent crystals. Some surface treatment reagents would specifically react with the dentine tissue and produce a functionalized surface. The conditioning of teeth affects surface topography and may affect the retention of dental restorations [7]. With phosphoric acid (H_3PO_4) treatment, peritubular dentinal hydroxyapatite is rapidly etched and tubule orifices are opened; while the intertubular matrix morphology remains relatively stable [8]. As a well-known nonspecific proteolytic agent capable of removing organic materials, sodium hypochlorite (NaOCl) has been used to remove collagen fibril network on dentine surface [9, 10]. The specific aim of this study was to investigate the effect of functionalized dentine surfaces, by modifying with different reagents mentioned above, on the remineralization

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behavior. In addition, transverse microradiography (TMR) has been used as a standardized method in studies on the remineralizing process of dentine for decades, but failed to observe the remineralized/unaffected dentine interface. In the present study, we used atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM) to observe the two-dimensional microstructure of dentine surface and subsurface.

2 Materials and methods

2.1 Sample preparation and surface treatments

The human premolars extracted for orthodontic treatment were debrided of residual soft tissue using a dental curette and disinfected by immersion for 24 h in 0.2% thymol solution for further use. Teeth previously extracted with informed donor consent were used in this study with approval of the Ethics Review Board in Tongji Hospital of Huazhong University of Science and Technology. Nine dentine disks with 2 mm thickness were created in mid-coronal dentine of non-carious premolar teeth using a diamond saw under water irrigation. Each dentine surface was abraded with 240-, 320-, 400-, 600-, 800-, 1000- and 1200-grit abrasive paper respectively to create a uniform flat surface, and then polished to mirror flatness with 0.25 μm aluminum oxide powders. The smear layer was removed with ultrasonic treatment (38 kHz, 100 W) for 15 min three times [11]. The samples were then stored in deionized water prior to further treatment.

Three groups were involved in this study: no treatment served as the control group; 37% H_3PO_4 treatment for 40 s as the etched group [12]; 37% H_3PO_4 treatment for 40 s followed by 10% NaOCl treatment for 120 s as the deproteinized group [10, 13, 14]. Three of the nine samples were randomly assigned into each group and rinsed by deionized water after surface treatments.

2.2 Remineralization

Two samples from each group received remineralization after surface treatments. Another sample without remineralization was used as a control. The remineralizing solution consists of 0.7 mM $CaCl_2$, 4 mM KH_2PO_4, 0.2 mM $MgCl_2$, 30 mM KCl, 20 mM HEPES, Ca/P molar ratio = 0.175, pH was adjusted to 7.0 with 1 M sodium hydroxide. The exposed dentine surfaces were immersed into the remineralizing solution for 1 h at 37°C. The solution was continuously stirred and samples remained static. Immediately afterwards, the samples were carefully washed using deionized water to remove the remaining salt and dried prior to observation [11].

2.3 AFM and FESEM observations

One remineralized sample and one control sample without remineralization in each group were characterized by atomic force microscopy (AFM). The exposed surface was prepared as slides approximately 5 mm × 4 mm × 2 mm (height). A digital Picoscan TM MI atomic force microscope (TM MI AFM, MI Co, USA) was used to image the surface character. Briefly, each sample was placed in the specimen chamber of the AFM and a force of less than 10^{-7} N was put on the sample with a Si_3Ni_4 tip mounted on a cantilever. A laser beam and photodiode detector was used to sense the deflection of the cantilever as the tip scanning over the sample surface. The in-plane resolution of the AFM is 0.1 nm dictated by the radium of curvature of the tip and the vertical resolution is 0.01 nm [15, 16].

Another remineralized sample in each group was fixed in polyester rings with self curing acrylic resin (Shanghai Dental Material Factory, Shanghai, China). The remineralized subsurface was exposed by splitting the sample into two parts with a chisel from distal-mesial direction. The specimens were adhered to observation table and gold-coated using a sputter coater system. The morphology of samples was observed using a field emission scanning electron microscopy (FESEM, Sirion 200, FEI, USA). When necessary, the element was analyzed by energy dispersed X-ray (EDX) which went with the FESEM observation.

3 Results

The surface properties of dentine under different surface treatments varied in all AFM images (Fig. 1). Phosphoric acid treatment enlarged the dentinal tubules (Fig. 1c). While phosphoric acid followed by NaOCl treatment removed intertubular dentine, resulting in an amorphous structure without any discernible features (Fig. 1e). After remineralization, needle-like fine crystals deposited at the polished dentine surface in the control group, while smooth and bulk crystals deposited at the demineralized dentine surface in the etched group. In these two groups, the remineralized dentinal tubules became declined to inherent dentinal tubules (Fig. 1b, d). Much differently, it was found smooth and plate-like crystals on the deproteinized dentine surface after remineralization (Fig. 1f).

From the FESEM observations with different magnification (Fig. 2), it was appeared that, both in the control group and phosphoric acid group, the remineralized dentinal tubules were rearranged to be slanted in contrast to the inherent dentinal tubules. In the deproteinized group, the deposited crystals were covered on the treated surface and inserted into the intrinsic dentinal tubules (Fig. 2g, h). The

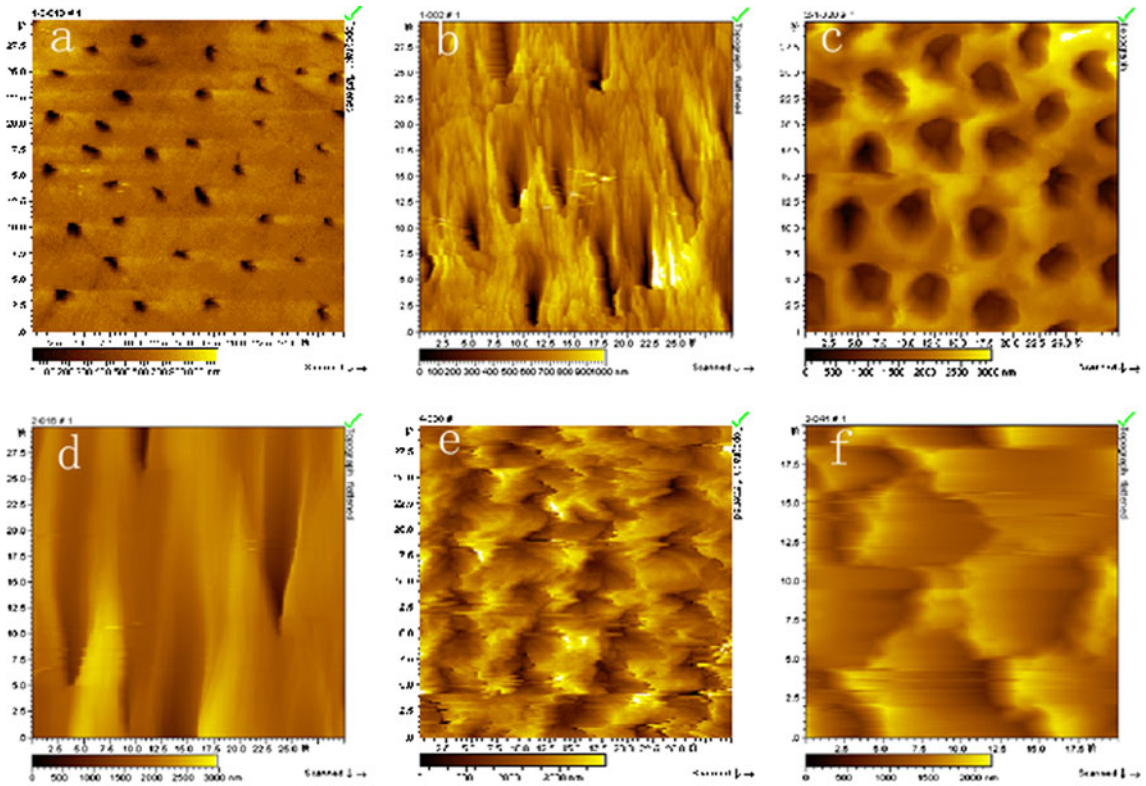

Fig. 1 A series of representative AFM images for surface treatments and subsequently remineralization. They indicate the polished dentine surface (**a**), enlarged dentine tubules in the etched group (**c**) and amorphous structure in the deproteinization group (**e**). After remineralization, needle-like fine crystals, smooth crystals and plated-like crystals on the polished surface (**b**), the etched surface (**d**) and the deproteinized surface (**f**) are showed respectively

covered deposits were mostly detached from the surface and evidenced to be Ca–P composite by EDX (Fig. 2i). The remineralized subsurface was obviously distinct from the unaffected dentine surface in all groups.

4 Discussions

The current study provides the first evidence of changeable dentinal tubule orientation relying on surface characteristics. It was found that dentine had a tendency for remineralization since intrinsic dentine behaves a higher crystallization Ca/P (1.57) than the remineralizing solution (Ca/P = 0.175) whose chemical composite is similar to human saliva [11]. The phosphoric acid etchant removed dentine minerals and kept a remnant of collagen matrix [10], afterwards, bulk and smooth crystals were found in the remineralized surface in contrast to the fine and needle-like crystals in the control group. The dentine tubules in the two groups mentioned above were tilted to the observed planes. In the deproteinized group, the collagen of etched dentine was subsequently removed by 10% NaOCl for 120 s, and the underlying mineralized dentine was left [10, 14]. The deposited crystal significantly altered its orientation and was covered on the dentine surface. Obviously, the organic matrix determines the surface characteristics and plays an important role in crystal deposition.

FESEM combined with AFM observation in this study evidenced that the dentinal tubular orientation became slanted to inherent dentinal tubular in the remineralized dentine surface. According to the shadowcasting of declined dentinal tubule with respect to the observing plane, the slanted angle of dentine tubular orientation was determined as:

$$\mathrm{Sin}\ \alpha = r/d$$

Where in the shadowcasting plane, α represents the slanted angle with respect to the observing plane (perpendicular plane or parallel plane), d notes the length of entire dentine tubule and r indicates the width of dentinal tubule, which is also equal to the diameter of the dentinal tubule (Fig. 3a).

The slanted angles calculated from the AFM and FE-SEM photographs were showed in the Table 1. The slanted angle in perpendicular observation plane did not show significant difference between the control group and the etched group. However, it was significantly lower in the etched group than the control group in parallel observation

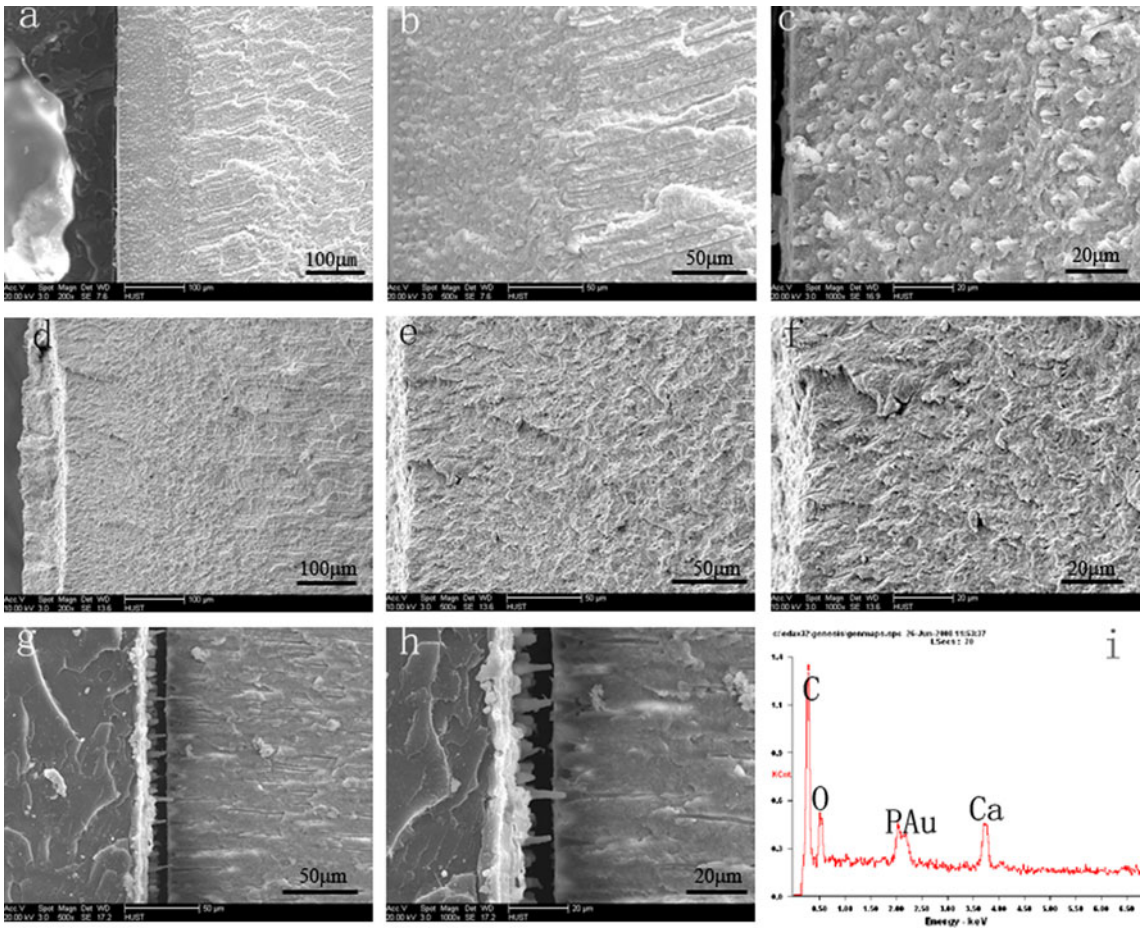

Fig. 2 FESEM observations of remineralized subsurfaces. In the control group (**a**, **b** and **c**) and the phosphate acid etching group (**d**, **e** and **f**), the dentinal tubules on the remineralized subsurface become obviously slanted in contrast to inherent dentinal tubules. In the deproteinization group, the deposited crystals are covered on the dentine surface and partially inserted to the dentinal tubules (**g** and **h**). The covered deposition is detached from the surface and evidenced to be Ca–P composite by EDX (**i**)

plane. Previous studies have showed that the surface layer of caries lesion is the preferred site for mineral deposition comparing with the subsurface; therefore, the crystal formation in the subsurface may be different from the surface [17, 18]. In this model, although the slanted angle varied slightly between the surface and subsurface, it did not present significant difference, which was similar to the finding in enamel [19]. Considering the relationship between the crystal growth and dentinal tubule orientation, the process of crystal deposition, which might result in slanted angle of dentinal tubule orientation, was inferred as Fig. 3b.

The remineralized depth determined by the distinct interface between the remineralized and unaffected area in FESEM observation was also summarized in the Table 1. It was found that phosphoric acid treatment improved the mineralizing depth whereas deproteinization decreased the mineralizing depth. In the enamel remineralization, it was described that the extent of remineralization of subsurface lesion immersed in the acidic solution was greater than that immersed in the neutral solution, which suggested that acid etching might improve the ion infiltration [19]. The decreased remineralized depth in the deproteinization group may be related to the partial removal of collagen. Etching followed by NaOCl treatment could completely remove the collagen matrix [20], and the decreased collagen scaffold might limit the remineralized depth.

In the remineralized subsurface, crystals were deposited in the demineralized lesion (etched group) or partially in intrinsic dentine (control group). We cannot make a judgement that whether the slanted dental tubular is a result of crystal epitaxial growth or crystal deposition in the dentinal collagen network. Epitaxial mineral deposition was only observed in the deproteinization group, with weak bonding force between the deposition–dentine interfaces, so that the deposits were detached from the dentine surface for the condensation of self-curing resin. In addition, the precipitated minerals were distinctly inserted into the

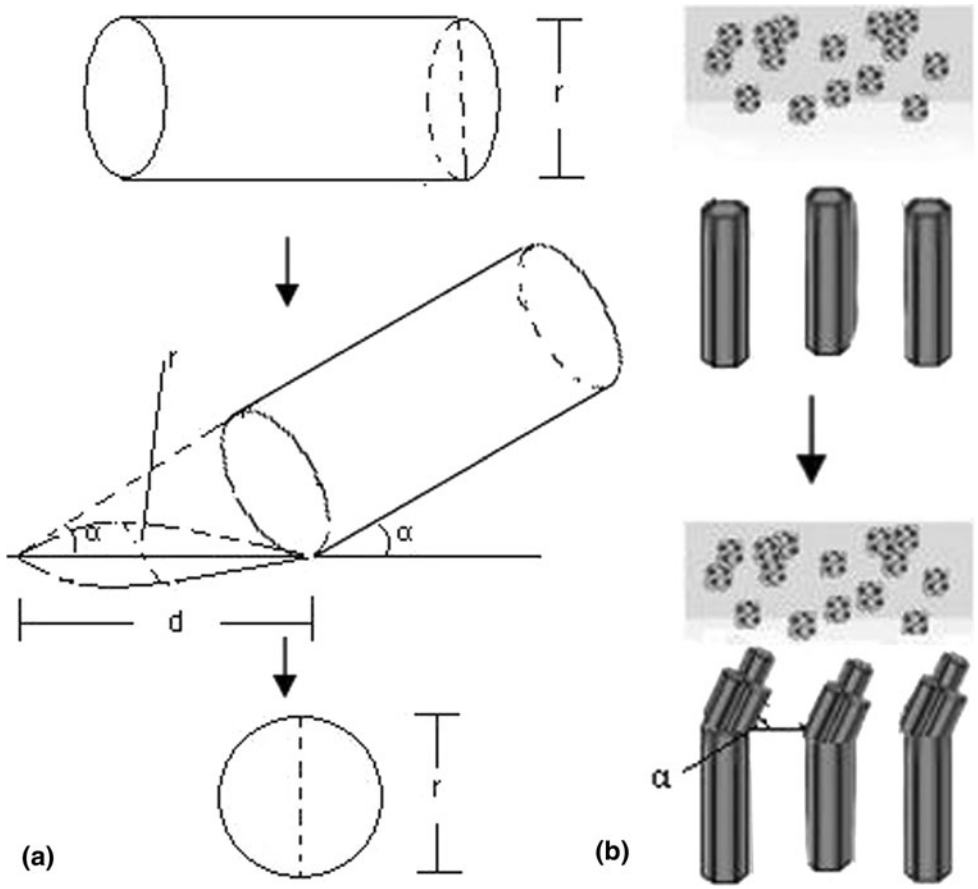

Fig. 3 Schematic of slanted angle calculation and crystal growth model. The dentinal tubule is parallel, perpendicular or slanted in α angle with respect to the observed cutting plane (**a**). The remineralizing solution involving Ca^{2+} and PO_4^- ions has the tendency for apatite deposition. The new formed crystal was presumed to decline to the original crystal orientation in α angle, resulting in slanted dentinal tubular orientations (**b**)

Table 1 Slanted angle of dentinal tubules and the remineralization depth (average ± SD)

Group	Angle (°)		Mineralized depth (μm, $n^{\S} = 6$)
	FSEM ($n^{\S} = 6$)	AFM ($n^{\S} = 3$)	
Control	$16.71 \pm 6.92^{a,*}$	14.74 ± 1.36^{a}	126.3 ± 3.0^{a}
Etched	17.77 ± 3.76^{a}	$<9.79 \pm 1.74^{b,\#}$	243.7 ± 15.4^{b}
Deproteinized	90	90	89.2 ± 18.1^{c}

§ n represents the number of dentinal tubules used for slanted angle calculation

* Groups indicated with the same letters (a, b, c) did not show significant differences under the identical testing conditions by means of one-way ANOVA, $\alpha = 0.05$

The AFM image in phosphate acid treatment group does not fully display the length of dentinal tubule, therefore, the slanted angle should be lower than the calculated value

dentine lumens. It was reported that calcium phosphate precipitation method would result in dentinal tubules occlusion by apatite mineral deposition in the dentinal tubules. In pathological caries-induced transparency area, crystal deposition would obliterate intratubular dentine tubules as a natural defense against caries [21]. Taken together with our data, it indicates that different pretreated schedules of dentine surface would lead to various crystal growth styles including orientation, morphology and remineralized depth.

5 Conclusions

In this study, microstructures of the remineralized area with different treatments are investigated. The following three main conclusions can be drawn: (1) dentinal tubules on the remineralized surface orientate differently from intrinsic dentine tubules, with the specific alignment angle determined by different surface treatments; (2) the crystal deposition process may result in slanted angle of dentinal tubule orientation; (3) the organic matrix determines the surface characteristics and plays an important role in crystal deposition. These findings are helpful for understanding the non-restorative repair of dentine lesions and the remineralization process of the caries-affected dentin surface.

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