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The effects of ethanol on the size-exclusion characteristics of type I dentin collagen to adhesive resin monomers

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ABSTRACT

During dentin bonding with etch-and-rinse adhesive systems, phosphoric acid etching of mineralized dentin solubilizes the mineral crystallites and replaces them with bound and unbound water. During the infiltration phase of dentin bonding, solvated adhesive resin comonomers are supposed to replace all of the unbound collagen water and polymerize into copolymers. A recently published review suggested that dental monomers are too large to enter and displace water from tightly-packed collagen molecules. Conversely, recent work from the authors' laboratory demonstrated that HEMA and TEGDMA freely equilibrate with water-saturated dentin matrices. However, because adhesive blends are solvated in organic solvents, those solvents may remove enough free water to allow collagen molecules to come close enough to exclude adhesive monomer permeation. The present study analyzed the size-exclusion characteristics of dentin collagen, using a gel permeation-like column chromatography technique, filled with dentin powder instead of Sephadex beads as the stationary phase. The elution volumes of different sized test molecules, including adhesive resin monomers, studied in both water-saturated dentin, and again in ethanol-dehydrated dentin powder, showed that adhesive resin monomers can freely diffuse into both hydrated and dehydrated collagen molecules. Under these *in vitro* conditions, all free and some of the loosely-bound water seems to have been removed by ethanol. These results validate the concept that adhesive resin monomers can permeate tightly-bound water in ethanol-saturated collagen molecules during infiltration by etch-and-rinse adhesives.

Statement of Significance

It has been reported that collagen molecules in dentin matrices are packed too close together to allow permeation of adhesive monomers between them. Resin infiltration, in this view, would be limited to extrafibrillar spaces. Our work suggests that monomers equilibrate with collagen water in both water and ethanol-saturated dentin matrices.

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1. Introduction

The distribution of bound and unbound (free) water in completely demineralized dentin matrices was found to be about 25 wt% and 75 wt%, respectively [1]. Water that is tightly bound to dentin matrices should be regarded as structural water. It is bound so tightly that it does not behave like regular free water [2]. That is, tightly-bound water cannot be evaporated at atmospheric pressure and body temperature. It cannot diffuse into water-free but water-miscible solvents like ethanol [1]. Presumably, when adhesive monomers infiltrate into demineralized dentin, they diffuse over and around tightly-bound water [1].

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Fortunately, three-quarters of the total water in dentin is unbound and can be removed by evaporation and/or chemical dehydration using water-miscible, water-free organic solvents [1].

If only half of the unbound water is removed during the 60–90 s used by most dentists to bond resins to dentin, then up to half of the collagen matrix will remain infiltrated by water rather than resin. That water is used by the endogenous proteases of dentin to slowly hydrolyze collagen fibrils, solubilizing insoluble collagen and causing decreases in resin-dentin bond strength over time [3,4]. The more free water that can be removed from demineralized dentin, the more adhesive resin monomers can be taken up by the matrix during resin infiltration. The key to creation of ideal resin-dentin bonds is to replace all unbound water with water-free, but water-miscible solvents like ethanol [5] that can solvate adhesive resin monomers. The most tightly bound is the first layer that directly hydrogen bonds to collagen [1]. The second layer is firmly bound and is covered by the least tightly bound water on the outside of collagen. When adhesive monomers infiltrate into the matrix, they can only interact with the outermost, loosely bound water.

In a study on the limitations to resin bonding at the nanometer scale [6], the authors argued that the packing density of hydrated collagen molecules in the collagen fibrils of demineralized dentin is so dense, there is insufficient space for the uptake of adhesive monomers like 2-hydroxyethyl methacrylate (HEMA) or triethylene glycol dimethacrylate (TEGDMA). They implied that collagen molecules were covered with bound, structural water that is necessary for the stability of collagen, and that there was too little space available between collagen molecules for resin uptake. A recent ^{1}H NMR report indicated that resin infiltration of ethanol-rinsed demineralized dentin by EXCITE® (Ivoclar-Vivadent, Schaan, Lichtenstein, an adhesive containing 50 vol% ethanol) removed free water but not all bound water [7]. This suggests that solvated adhesive resin monomers can diffuse down to the nanoscopic level in collagen fibrils where bound water resides, and that it may remove some loosely-bound water, but leaves most of the tightly-bound water in place. Those authors [7] speculated that bound water holds intermolecular spaces between collagen molecules open for adhesive resin monomer uptake. This suggests that adhesive resin monomers, especially water-insoluble dimethacrylates, may partially permeate between collagen molecules by diffusing over collagen's most peripheral, loosely bound water that was partially replaced by ethanol.

To measure the size inclusion or exclusion characteristics of insoluble type I collagen, Toroian et al. [8] filled 25 × 1.25 cm diameter columns with mineralized bone powder to conduct gel permeation-like experiments. In gel-permeation column chromatography, a column is filled with hydrated polymeric beads, where more than half the total water of the column resides inside the beads, while the rest resides outside the beads. When large molecules such as albumin are applied, they are too large to enter the polymer beads, hence they elute from the column quickly in the first few collected fractions (Fig. 1). Small molecules such as glucose or phosphate easily penetrate the hydrated beads. Their elution is delayed because they equilibrate with intrabead free-water. Since there was little free water in mineralized bone, none of the small (or large) tracers were delayed in their transit through the column. They all eluted from the column rapidly in the void volume. However, when they completely demineralized the bone powder, large amounts of water (60–70 vol%) replaced the original mineral volume. Some of that water was bound, but much of it was unbound. When they applied tracer molecules of increasing size to columns filled with demineralized bone powder, they found that large molecules, such as albumin (67 kDa), were too large to permeate into "collagen water" and were eluted quickly. Intermediate-size molecules like calcitonin (5.7 kDa) were delayed somewhat in their elution, while small molecules like glucose (180 Da) and phosphate were even more delayed in their elution, indicating that they equilibrated with what is presumed to be unbound collagen water.

That study was repeated on dentin collagen by Takahashi et al. [9]. Their results were similar to those of Toroian et al. [8], but they used dentin powder instead of bone powder, and included the dental adhesive resin monomers 2-hydroxyethyl methacrylate (HEMA) and triethyleneglycol dimethacrylate (TEGDMA) in their small molecular tracers. The authors showed that water-saturated, completely demineralized dentin powder equilibrated with HEMA and TEGDMA, and that both monomers were delayed in their elution, indicating that those monomers equilibrated with collagen water. This suggests that there may be sufficient space between collagen molecules for resin uptake. However, adhesive resin monomers are solvated in ethanol, not water. When applied to dentin, these solvents may remove intracollagen free water by chemical dehydration and allow the collagen molecules to come closer together, excluding monomer uptake. Thus, the experiments of Takahashi et al. [9] need to be repeated on columns of demineralized dentin powder that are equilibrated with ethanol, to determine if dimethacrylates such as TEGDMA or bisphenol A glycidyl dimethacrylate (BisGMA) can enter the ethanol-solvated volume of dehydrated dentin matrix collagen, or if they are excluded. Accordingly, the purpose of the present work was to determine if TEGDMA and BisGMA, as examples of small, relatively hydrophobic adhesive dimethacrylate monomers, can equilibrate with ethanol within collagen molecules in demineralized dentin powder. The null hypothesis tested was that TEGDMA and BisGMA cannot equilibrate with ethanol-solvated dentin collagen.

2. Materials and Methods

2.1. Materials

Blue Dextran (2 × 106 Da) was obtained from Sigma/Aldrich (St. Louis, MO, USA), and was used as an example of a relatively large water-soluble tracer. HEMA (130 Da), TEGDMA (286 Da) and BisGMA (512 Da) were obtained from ESSTECH (Essington, PA, USA).

2.2. Creation of dentin powder

Extracted bovine incisors were obtained from a local abattoir. Six hundred freshly extracted bovine incisor teeth, which were stored in 0.9% NaCl containing 0.02% sodium azide at 4 °C to prevent bacterial growth, were used in the present study. Dentin devoid of enamel, cementum and pulpal tissues were prepared from those teeth using dental burs with a high speed hand piece with copious air–water spray. Using an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling, the dentin specimens were cut into four equal-sized fragments. The resulting tooth fragments were dehydrated in acetone for 20 min and placed in liquid nitrogen for 15 min. The frozen dentin fragments were then reduced to dentin powder in a Wiley Mini Mill (Thomas Scientific, Model 3383-L10, Swedesboro, NJ, USA). The resulting powder was passed through a series of stacked sieves (Nos. 18, 30, 50, 140; Cole-Palmer, Vernon Hills, IL, USA). The powder that passed through 300 μm sieves but was retained on 106 μm sieves, was used to fill the column. The mineralized dentin powder was kept frozen at −80 °C until use, to prevent degradation of the collagen component.

2.3. Gel filtration procedures

A 1 × 30 cm glass column was filled with 25 cm^3 of mineralized dentin powder and equilibrated with 20 mM Tris buffer (pH 7.4)

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