Ultramorphological Characterization of the Resin Dentin Interface – An *in vitro* Analysis of Nanoleakage Patterns of Dentin Adhesives

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Aim and Design: *The requirements of an effective dentin adhesive system include the ability to thoroughly infiltrate the collagen network and partially demineralized zone, to encapsulate the collagen and hydroxyapatite crystallites, to produce a well polymerized durable hybrid layer with high bond strengths. Microleakage and marginal percolation are the most detrimental factors thwarting the success of any restoration . However, the presence of leakage pathways, called nanoleakage have been observed in the hybrid layer, even in the absence of microleakage. The nanoleakage patterns of four dentin adhesives (Prime & Bond NT, AdheSE, Clearfil S3 bond and Fuji Bond LC) was compared using Scanning electron microscopy and Energy Dispersive Spectroscopy, by the silver nitrate penetration method.*

Results and conclusion: *The results indicated that etch and rinse adhesives showed the maximum nanoleakage followed by the self etch systems. The glass ionomer based bonding agents showed the least nanoleakage. It is extremely important to take nanoleakage as a consideration while choosing dentin adhesives, to ensure success of the restoration.*

Keywords: *Bonding, nanoleakage, hybrid layer, etch and rinse, self etch, glass ionomer*

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INTRODUCTION

The fundamental objective of resin dentin bonding is to create adhesion between two dissimilar surfaces namely, mineralized tooth structure and the restorative material, and to seal the dentinal tubules against the entry of bacteria and their toxins. A successful union will also prevent both inward and outward flow of fluid. This percolation is termed “microleakage” and refers to the ingress of oral fluids and flora. It is considered responsible for marginal staining, recurrent caries and post operative sensitivity, which may jeopardize the long term performance of restorative materials.[1] Variations in the chemical and morphological characteristics of teeth, especially dentin, and the constant presence of surface contamination and moisture, pose a significant challenge in achieving adequate adhesion. A good compatibility between the adhesive and the adherent is an important factor for success of the restoration.

Conventional dentin adhesive systems characteristically bond via resin penetration and entanglement of exposed collagen in the demineralized dentin zone. This distinct zone is called the “hybrid layer or resin reinforced inter-diffusion or inter penetration zone.” The quality of hybridization of dentin is the benchmark of good bonding, according to Nakabayashi *et al.*[2] This zone of hybridized dentin is considered to behave like an impermeable membrane that reduces the risk of microleakage.

Dentin adhesive systems are currently available as three step, two step and single step systems, depending on how the cardinal steps of etching, priming and bonding to tooth structure are accomplished. Two step systems are further subdivided into single bottle self priming adhesives that require a separate etching step and two bottle self etching primers that require an additional bonding step.[3] The recently introduced adhesive systems combine these procedures as a single step application. These systems are available as either single bottle or two bottle systems. The application of self etching adhesive systems does not require removal of the smear layer and smear plug, thereby incorporating the smear layer in the hybrid layer. Recently, resin modified glass ionomer based bonding systems have been

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introduced, which have both micromechanical and chemical bonding to dentin.

Microleakage has been demonstrated along the dentinal wall when using dentin adhesive systems. Sano and others suggested the existence of a leakage pathway through a porous zone at the hybrid layer – adhesive interface without gap formation. This penetration reveals the lack of perfect seal, but is not microleakage in the classical sense. It represents permeation, laterally, through micron to submicron spaces, less than 50 nm in size, in the base of the hybrid layer that have not been filled with adhesive resin or which were left when poorly polymerized resin was extracted by oral or dentinal fluids. To distinguish this leakage from typical microleakage, they suggested the term "nanoleakage." [4, 5]

The studies regarding the nanoleakage patterns of the different generations of adhesive systems are not yet conclusive. The present study was undertaken to analyze the ultramorphological characteristics of the resin dentin interface by evaluating the nanoleakage patterns of four dentin bonding agents using the Scanning Electron Microscope.

Table 1. Composition of the materials used in this study

Prime & Bond NT (Dentsply Caulk, USA)	Di and Tri methacrylate resins, Functionalized amorphous silica, PENTA, Photo initiators, Stabilizers, Cetylamine hydrochloride, Acetone (LOT NO.0605001364)
AdheSE (Ivoclar Vivadent, Liechtenstein)	PRIMER (LOT NO.J09134): Dimethacrylate, Phosphonic acid acrylate, Water, Stabilizers. BONDING AGENT (LOT NO.J05130): Dimethacrylates, HEMA, Silica, Initiators, Stabilizers.
Clearfil S^3 bond (Kuraray Medical Co., Japan)	10 – MDP, Bis GMA, HEMA, Hydrophobic dimethacrylates, di camphoroquinone, ethanol, water, silanated colloidal silica (LOT NO.00036A)
Fuji Bond LC (GC Corporation, Tokyo)	CONDITIONER (LOT NO.0510271): 20 % polyacrylic acid LIQUID (LOT NO. 0511011): HEMA, UDMA, TEGDMA,Polyacrylic acid, Distilled water. POWDER (LOT NO.0511091): Aluminosilicate glass

MATERIALS AND METHODS

40 non carious freshly extracted mandibular third molars stored in physiological saline containing 0.1 % thymol were used for this study. The methodology was reviewed and accepted by the Ethical Committee and Institutional Review Board of the University.

After cleaning all the teeth ultrasonically, the occlusal enamel was removed by transverse sectioning using a slow speed diamond disc under running water, to expose a flat dentin surface. The dentin surfaces were wet ground with 180 grit silicon carbide paper for 60 seconds. The teeth were randomly divided into four groups ie, ten specimens in each group. The specimens were bonded with one of the dentin bonding systems (Table 1), following the manufacturer's instructions. The treatment groups were the following: Group A – Prime & Bond NT (Dentsply Caulk, DE, U.S.A); Group B – AdheSE (Ivoclar Vivadent, Liechtenstein); Group C – Clearfil S^3 Bond (Kuraray Medical Co., Tokyo, Japan); Group D – Fuji Bond LC (GC Corporation, Tokyo)

The flat bonded surfaces were then restored with a 2mm thick layer of universal composite (Filtek Z 250, 3M ESPE, MN, U.S.A) and light cured for 20 seconds. Light curing was done using a halogen light curing unit (Astralis, Ivoclar Vivadent). The teeth were then stored in water for 24 hours at room temperature.

Preparation of tracer solution

A 50 weight % solution of ammoniacal silver nitrate was used as the tracer following the silver staining protocol proposed by Tay *et al.*[6] The solution was prepared by dissolving 25 grams of silver nitrate crystals in 25 ml of 28 % ammonium hydroxide in the presence of ambient laboratory light. This created a suspension of black silver particles. Additional 28% ammonium hydroxide solution was used to titrate the black solution, with magnetic stirring at room temperature, until the solution became clear, as the ammonium ions complexed with the silver into diamine silver ions. This solution was diluted to 50 ml with distilled water to achieve a 50 weight % solution that was transferred to an amber colored bottle for storage before use.

The specimens were coated with two layers of fast setting nail varnish, except 1 mm away from the bonded interfaces and immediately immersed in the ammoniacal silver nitrate solution for 24 hours. The silver stained teeth were rinsed thoroughly in distilled water and placed in photo developer solution for 8 hours under a fluorescent light to facilitate reduction of the silver ions into metallic silver particles within the potential voids along the bonded interfaces as well as in the polymerized resin layer.

The teeth were thoroughly washed and the root surfaces below the cemento – enamel junction were removed. The specimens were then longitudinally sectioned into two, polished with 600 grit silicon carbide paper and demineralized with 37% phosphoric acid for 1 minute and 2% sodium hypochlorite for 2 minutes. They were ultrasonically cleaned, air dried, mounted on aluminium stubs, sputter coated with platinum and observed with a Scanning Electron Microscope (JSM – 6390A Analytical SEM, JEOL, Tokyo, Japan), with an accelerating voltage of 25 kV, operated in the secondary electron mode, at 1500 x magnification. In addition, whole area silver detection was carried out by an Energy Dispersive X – Ray Spectroscope with ZAF correction (JEOL EDS Detector). Initial energy spectra analyses were performed to determine the elemental composition of the whole area. Line scan analysis across the resin composite – adhesive – superficial dentin interface was also performed. One reading was carried out for each of the ten specimens per group. The penetration of silver ions into the hybrid layer, and the adhesive layer was evaluated and graded, based on the criteria proposed by Yuan and Tagami.[7]

Table 2. Evaluation of nanoleakage location of the four adhesives.

BONDING AGENT	ADHESIVE LAYER			HYBRID LAYER			RESIN TAGS		
	-	±	+	-	±	+	-	±	+
Prime & Bond NT	0	1	9	0	0	10	0	1	9
AdheSE	1	8	1	0	9	1	0	8	2
Clearfil S3 Bond	1	8	1	0	8	2	1	7	2
Fuji Bond LC	7	2	1	8	2	0	9	1	0

n = 10 samples per group
- = no nanoleakage
± = slight nanoleakage
+ = distinct nanoleakage

(-): No leakage (Score 0), (±): Slight leakage (Score 1) and (+): Distinct leakage (Score 2). Data were statistically analyzed by Chi square test with statistical significance set at p = 0.05 (SPSS 11.5 for Windows, SPSS Inc, Chicago, USA).

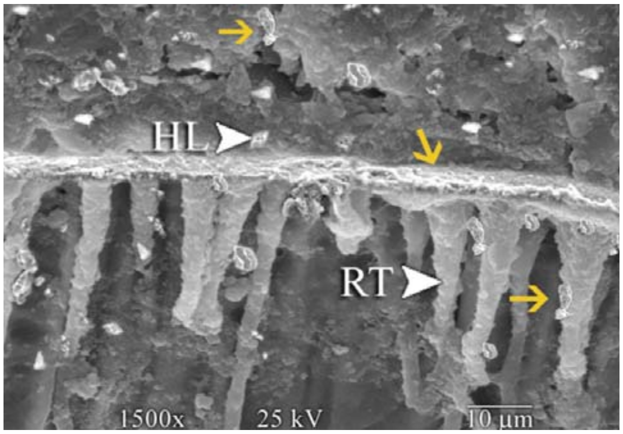

Figure 1. Nanoleakage pattern of Prime & Bond NT (Group A)

RESULTS

The Scanning Electron Microscopic secondary electron images clearly revealed the penetration of silver particles within the adhesive layer, hybrid layer and the resin tags. EDS analysis permitted identification of the silver particles, thus excluding both false positive and false negative identifications.

The typical nanoleakage patterns at the resin – dentin interface and the corresponding EDS spectra, for each of these adhesive systems are illustrated in Figures 1–8. The results of evaluation of nanoleakage locations of the four adhesive systems are summarized in Table 2.

GROUP A – PRIME & BOND NT showed dense silver deposits almost entirely within the hybrid layer, which was around 7– 8µ thick and quite homogeneous. The EDS analysis confirmed the presence of dense deposits of silver in these regions. The resin tags were numerous and dense. Some specimens also showed dense silver deposition at the base and lower half of the hybrid layer and within the resin tags. The adhesive layer showed specks of silver deposits. Almost all the specimens showed silver deposition within the adhesive layer (Figures 1, 2).

GROUP B – AdheSE showed moderate silver deposits within the adhesive layer, hybrid layer and resin tags. However, the density of silver deposits was much lesser than in Group A. The hybrid layer was around 3–5µ thick. The resin tags were numerous, but lesser when compared to Group A. SEM images did not show distinct silver penetration. EDS analysis and subsequent area mapping and line scan analysis confirmed this result. The silver deposits were seen to be distributed along the entire length of the base of the hybrid layer (Figures 3, 4).

GROUP C – CLEARFIL S^3 BOND exhibited a different kind of nanoleakage. The silver deposits were very minimal, which was confirmed with EDS. Much of this silver was found at the top surface of the hybrid layer. The hybrid layer was very thin (about 1–3µ) with few resin tags. Most of the

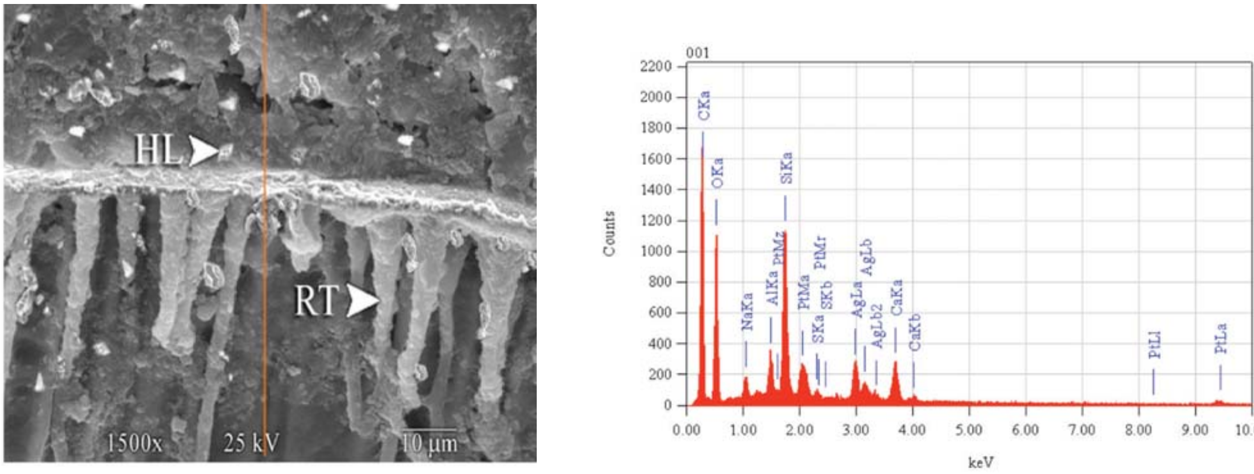

Figure 2. EDS spectrum for Prime & Bond NT (Group A)

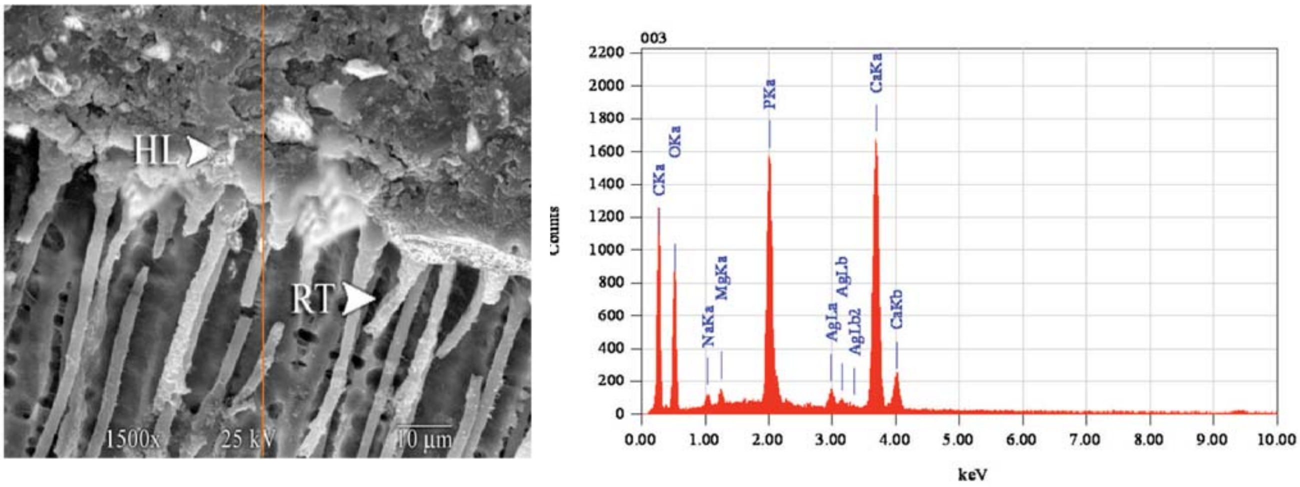

Figure 4. EDS spectrum for AdheSE (Group B)

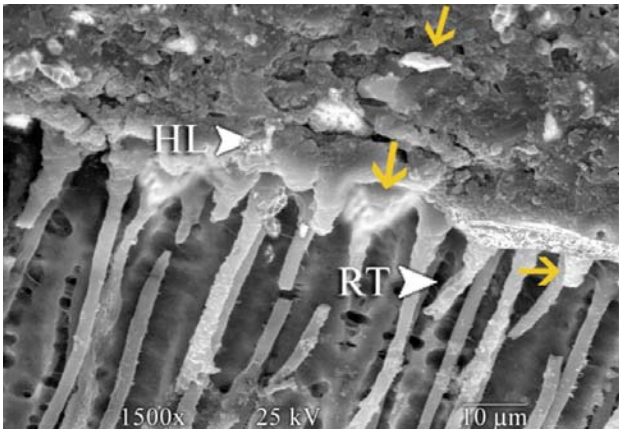

Figure 3. Nanoleakage pattern of AdheSE (Group B)

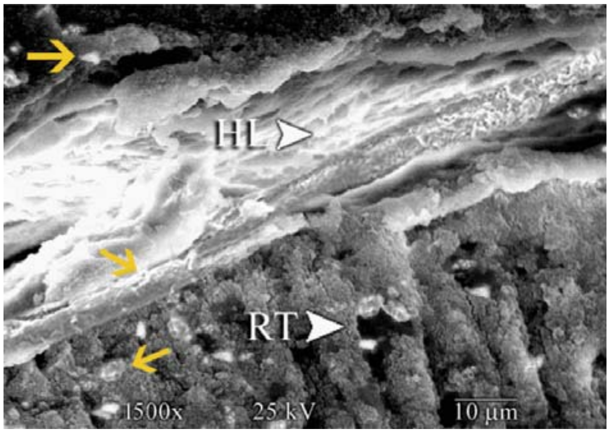

Figure 5. Nanoleakage pattern of Clearfil S3 Bond (Group C)

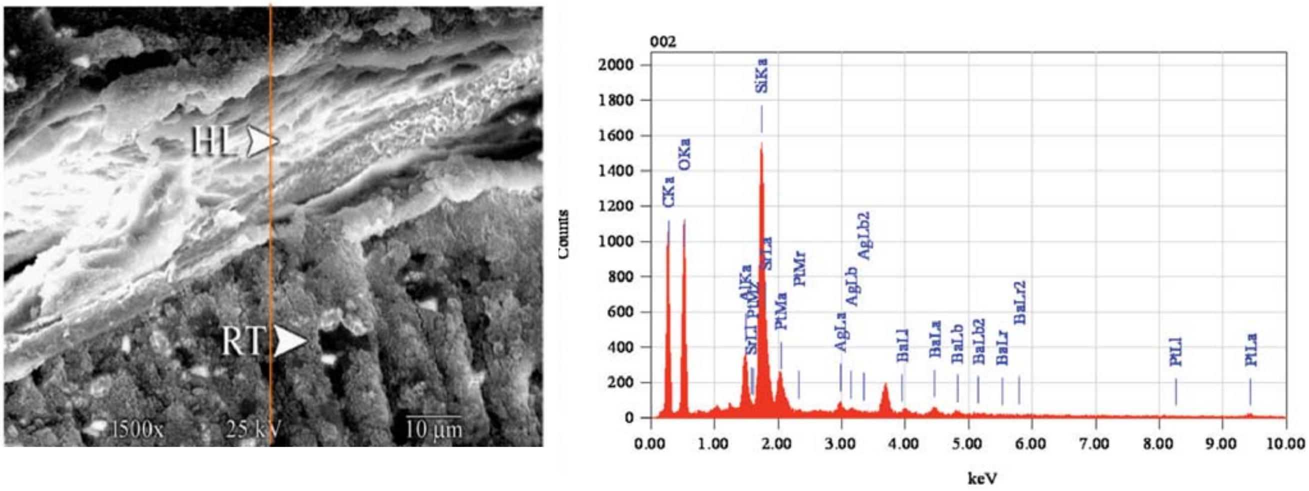

Figure 6. EDS spectrum for Clearfil S3 Bond (Group C)

specimens showed a moderate degree of nanoleakage at the adhesive layer and resin tags. The silver deposition was discontinuous and diffuse (Figures 5, 6).

GROUP D – FUJI BOND LC demonstrated minimal silver deposits, which were globular but intermittent. Very few specimens showed mild nanoleakage within the adhesive layer and hybrid layer. These deposits were seen as occasional specks of silver. The resin dentin interface was quite different from what was observed for the other specimens in that there was almost no distinct hybrid layer formation. The silver deposits were sparingly observed both by the secondary electron images and EDS analysis. The silver deposits were also observed as streaks, along the composite – adhesive interface. The adhesive layer also demonstrated some filler particles, which was confirmed by EDS analysis (Figures 7, 8).

Statistical analysis of the results with Chi square test showed significant difference in the occurrence of nanoleakage between Group D (Fuji Bond LC) and the other groups.

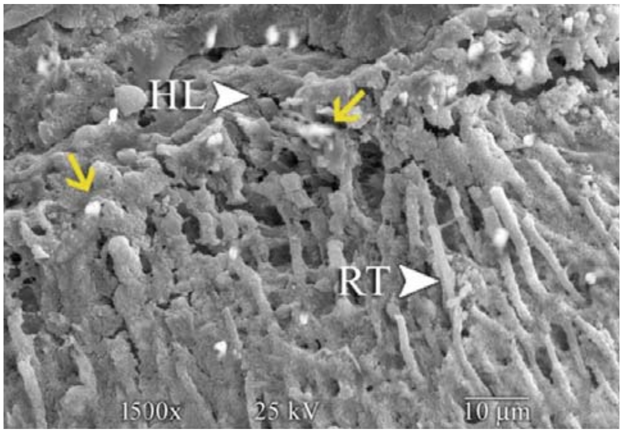

Figure 7. Nanoleakage pattern of Fuji Bond LC (Group D)

There was no significant difference between Groups B and C, except for the density and pattern of deposition within the hybrid layer. On comparison, Group D (Fuji Bond LC) showed the least nanoleakage amongst all the groups tested. Group A (Prime & Bond NT) showed the maximum silver deposits, while Group B (AdheSE) and Group C (Clearfil S^3 Bond) showed moderate degrees of nanoleakage.

DISCUSSION

There is evidence to support the hypothesis that potential leakage pathways within the hybrid layer and the adhesive resin layer may contribute to slow fluid flow across a dentin – adhesive joint, which may allow the penetration of bacterial products and dentinal or oral fluids along the interface resulting in hydrolytic breakdown of either the adhesive resin or collagen within the hybrid layer, thereby compromising the stability of the resin – dentin bonds.[6, 7]

The term nanoleakage was first quoted as being the impregnation of silver grains within the porosities of the hybrid layer that was not properly filled with adhesive resin. Sano described this different pattern of leakage as nanoleakage. A second mode of nanoleakage, termed the "reticular mode" has also been described to occur in bonded interfaces[6] wherein, silver grains were identified not only in the porosities of the hybrid layer, but also within the hybrid layer. These delicate, branching channels of nanovoids are speculated to be morphological manifestations of the water treeing phenomenon, which is probably responsible for water induced deterioration of polymers during aging.[8, 9]

Absorption of water into the polymer triggers chemical degradation, followed by elution of resin from the hybrid layer or adhesive layer. The exposed collagen is degraded by matrix metalloproteinases in dentin or human saliva.[10] Nanoleakage is an important indicator to judge a material's sealing ability and enhance the quality of hybrid layer. The silver nitrate penetration patterns vary with regard to the

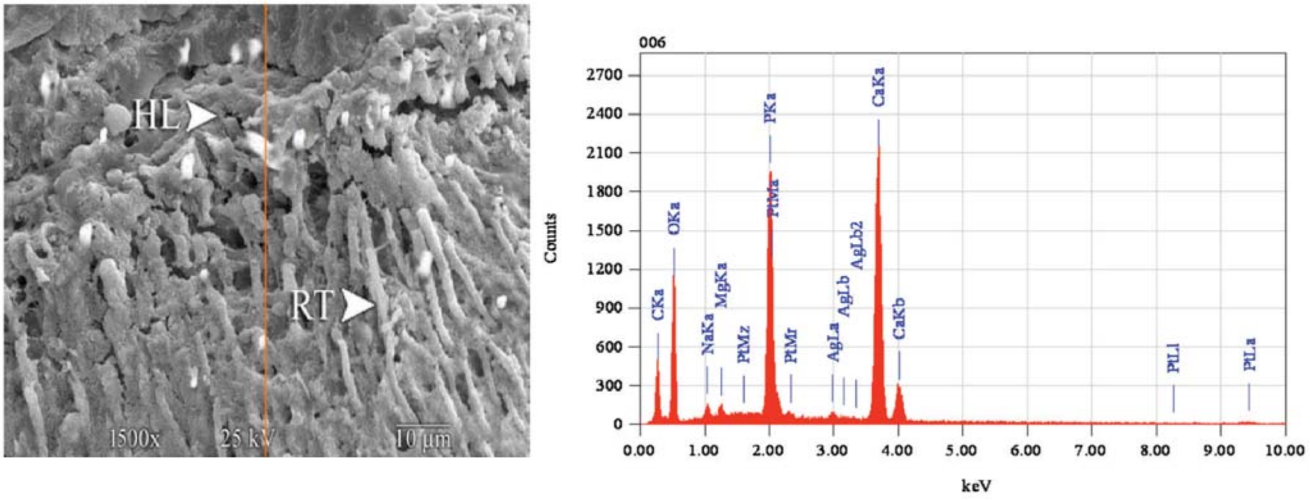

Figure 8. EDS spectrum for Fuji Bond LC (Group D)

adhesive systems and their chemistry.[11] However, current knowledge about this phenomenon and comparison of nanoleakage of different bonding systems is limited.

The presence of hydrophilic and ionic resin monomers is believed to enhance water sorption and hydrolytic degradation, which in turn facilitates ionic movement within a polymerized resin matrix.[12] Hence, the detection of nanoleakage within the adhesive layer departs from its original definition as sites of incomplete resin infiltration around collagen fibrils of hybrid layers. As already mentioned, adhesive systems are hydrophilic in nature. Although such hydrophilicity is desirable for bonding to a moist substrate like dentin, subsequent water sorption within bonded interfaces is thought to contribute to bond degradation over time.[11]

Traditional evaluation methods such as low power microscopy based microleakage assessments cannot identify the exact location where the microleakage occurs at the resin dentin interface.[13,14] Having agreed that nanoleakage occurs both in the hybrid layer and within the adhesive layer, it was considered that whole field observation, ie., the adhesive layer, adhesive – dentin interface and resin tags is important. Scanning Electron Microscopy (SEM) studies using secondary electron mode, in combination with Energy Dispersive Spectroscopy (EDS) was carried out due to its ability to present distinct images and both sensitive and accurate analysis. EDS has been found to be a very sensitive and accurate method of chemical component detection, to make both quantitative and qualitative analysis on the existence and distribution of various elements.[7]

A 50 wt % ammoniacal silver nitrate solution was used as the tracer. The rationale for using ammoniacal silver nitrate was based on the potential of the diamine silver ion complex to trace water filled regions or water binding polymer domains within the hybrid layer and overlying adhesive layers. Diamine silver ions $[(Ag(NH_3)_2{}^+]$ have two ammonium groups, which compete with water for the hydrogen bonding sites, allowing them to be deposited within these potential spaces. Ag^+ ions have an ionic diameter of 2.5 Å and a single water molecule has a size of 3 Å.[8]

The results of this study certainly showed a qualitative and quantitative difference in the nanoleakage patterns of the four different bonding systems evaluated. So the null hypothesis must be rejected. Prime & Bond NT showed the maximum nanoleakage, while AdheSE and Clearfil S^3 bond showed lesser nanoleakage. The least nanoleakage was found in specimens bonded with Fuji Bond LC.

Prime & Bond NT is a self priming adhesive containing Dipenta erythritol penta acrylate monophosphate (PENTA) and Urethane Di Methacrylate (UDMA). According to the manufacturer, the nanofillers incorporated into the bonding agent are capable of penetrating the spaces between the collagen fibrils and act as additional cross linkers. This is claimed to improve the strength of the adhesive layer and the hybrid layer.

SEM examination of the bonded interface with this material showed silver staining throughout the full thickness of the hybrid layer, indicating the presence of disparities on the depth of resin penetration and dentin demineralization or the presence of water that has not evaporated during polymerization. Defects in resin impregnation and imperfect polymerization of the adhesive resin can create water rich zones that permit silver uptake. Also, during wet bonding, the residual water in the interfibrillar spaces might decrease the polymerization of the bonding resin.[8, 11] The results of our study endorse the results of Sano *et al* [13] and De Munck *et al.*[15]

AdheSE is a two step self etching adhesive system containing a self etching primer and adhesive. The primer contains phosphonic acid acrylates and bis acrylamide, while the adhesive contains HEMA. Basically, three types of self etching adhesives can be distinguished, based on the acidity of the self etching primer: mild (pH ± 2), intermediate (pH ± 1.5) and strong (pH < 1).[3] Besides a higher tendency towards small marginal defects on the enamel side, the mild self etch approach of AdheSE appears to be a clinically reliable, predictable and simplified adhesive technique. Group C - Clearfil S^3 bond is a one step adhesive system, combining etching, priming and bonding in one bottle. The pH of this system is claimed to be higher than that of AdheSE.

In this study, AdheSE and Clearfil S^3 bond did not show distinct silver penetration in the SEM images of most samples. EDS analysis and subsequent line scan identification showed slight silver penetration. The mild etching effect of the self etching primer of AdheSE leaves residual mineral crystals within the hybrid layer and retains the smear plugs in the orifices of the dentinal tubules. This may reduce the outward fluid flow and thereby result in superior dentin sealing due to retained hybridized smear plugs. In the case of Clearfil S^3 bond, the SEM images showed that it usually hermetically capped the modified smear layer along the orifices of the dentinal tubules, without the formation of any smear tags. This structure may even provide a better seal. Studies using XPS wide scan spectra have identified that the residual hydroxyapatite was able to form an intense and stable calcium salt with 10–MDP at the nanometric level. In using such self etch adhesive systems, this twofold bonding mechanism (mechanical and chemical) may be advantageous in terms of restoration longevity. However, the incorporation of increased concentrations of hydrophilic monomers into self etch adhesives may compromise bond durability, as hydrophilicity and hydrolytic stability are antagonistic properties.[16, 17]

There is a tendency for the hydrophobic resin component with the residual water to separate into resin globules and water blisters, in monomer blends that contains a combination of hydrophilic and hydrophobic monomers.[18, 19] This phenomenon, termed adhesive phase separation will hinder the hybrid layer from being an impervious collagen / polymer network, but instead, a porous web, and at the same time, compromises the chemical and mechanical properties of the adhesive layer. Water may be trapped as isohedral water clusters within these reverse micelles via hydrogen bonding, forming thermodynamically stabilized structures that exist physically as areas of increased permeability within the polymerized adhesive.[19, 20] It has been shown that hydrophilic resins absorb more water than hydrophobic resins and those resin-dentin bonds made by self etch adhesives that contain hydrophilic monomers with lower molecular weight and more carboxylic groups deteriorated more rapidly with aging. These factors should ideally result in self etching adhesives exhibiting the maximum nanoleakage.

However, Clearfil S[3] bond has a molecular dispersion technology, wherein the liquids of the hydrophilic and the hydrophobic components are maintained in a homogeneous state at the molecular level, which the manufacturer claims to prevent phase separation. We speculate that this could be another probable reason for the decreased nanoleakage of this material.

However, our study showed that this system also showed a minimal degree of nanoleakage. Single step self etch adhesives etch, prime and bond simultaneously. So it is unrealistic to interpret the presence of silver deposits as being due to incomplete resin infiltration that is caused by diffusion gradient of resin monomers.[21] Water is an important component in these adhesives, providing an ionizing solvent for acidic resin monomers that is responsible for demineralization of dentin. Thus the silver deposits are more likely to represent resin sparse regions in which water was incompletely removed during evaporation of the adhesive solvent. This suggests that the porosity and permeability of the adhesive layer is related to the hydrophilic nature of the resin components.

Water available from either total etch or self etch adhesives or the use of a wet bonding technique may be trapped within resin dentin interfaces. The presence of HEMA, acidic monomers, and dissolved calcium and phosphate ions may lower the vapor pressure of water, making its removal difficult prior to polymerization.[22] Moreover, resins containing carboxyl or hydroxyl functional groups can form salts with solvated alkaline earth metal cations, creating internal defects with bound water within the polymer matrices. Contemporary adhesive systems contain about 35 – 55 volume % HEMA. The use of HEMA – containing adhesives on wet dentin may also create domains of microporous poly (HEMA) hydrogels that permit the movement of water, solvated ions, and polar molecules. This has been speculated to be one of the factors responsible for the nanoleakage phenomenon.[22]

In this study, FUJI BOND LC (Light cure Glass ionomer adhesive), which utilizes basic fluoroaluminosilicate glass (FASG) fillers showed the least leakage among all the groups tested. There was hardly any formation of hybrid layer as the conditioning agent caused very mild demineralization.

Tay *et al* attributed the spotted type of nanoleakage to the leaching of ions from the basic fluoroaluminosilicate glass (FASG) fillers, resulting in partial neutralization of the carboxyl functional groups of remnant surface active resin monomers that were not actively engaged in the formation of the hybrid layer.[23] There are at least two hypotheses for the explanation of nanoleakage seen in this system. These monomers may absorb water, which may be available from either the resin composition, or from the dentin, which may be attributed to the wet bonding technique that was followed.[24]

The occasional silver deposits observed in this study, in the adhesive-composite interface in Group D are speculated to be due to HEMA molecules.[23] Degradation has also been reported to occur within the mixed resin / polyalkenoate matrix as well as in the ion leaching glass particles. The local absorption of water by resin components results in a local swelling of the material, this enhancing water fluxes through the material and consequently accelerating the aging process.

CONCLUSION

Existing potential channels for hydrolytic degradation of resin-dentin bonds, we speculate that the existence of nanoleakage will be of clinical significance and not merely of scientific interest if they are subsequently shown to be self propagating or permit the access of water. Further, extraction of uncured water-soluble monomers and low molecular weight oligomers, through the channels may subsequently result in degradation of resin-dentin bonds and compromise the durability of the restoration.

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