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ORIGINAL ARTICLE

Preparation and properties of calcium-silicate filled resins for dental restoration. Part II: Micro-mechanical behaviour to primed mineral-depleted dentine

ANDREA CORRADO PROFETA

Department of Restorative Dentistry, Biomaterials Science, Biomimetics and Biophotonics (B3) Research Group, King's College London Dental Institute, Guy's Dental Hospital, London, UK

Abstract

Objective. Evaluating microtensile bond strength (μTBS) and Knoop micro-hardness (KHN) of resin bonded-dentine interfaces created with two methacrylate-based systems either incorporating Bioglass® 45S5 (3-E&RA/BG) or MTA (3-E&RA/WMTA). ***Materials and methods.*** Solvated resins (50% ethanol/50% co-monomers) were used as primers while their neat counterparts were filled with the two calcium-silicate compounds. Application of neat resin adhesive with no filler served as control (3-E&RA). μTBS, KHN analysis and confocal tandem scanning microscopy (TSM) micropermeability were carried out after 24 h and 10 months of storage in phosphate buffer solution (DPBS). Scanning electron microscopy (SEM) was also performed after debonding. ***Results.*** High μTBS values were achieved in all groups after 24 h of DPBS storage. On the contrary, solely the specimens created using 3-E&RA/BG and 3-E&RA/WMTA agents showed no significant reduction in terms of μTBS even after 10 months in DPBS; similarly, they did not restore the average superficial micro-hardness to the level of sound dentine, but maintained unchanged KHN values, and no statistical decrease was found following 10 months of DPBS storage. The only statistically significant changes occurred in the resin–dentine interfaces bonded with 3-E&RA that were subjected to a reduction of both μTBS and KHN values with ageing. In terms of micropermeability, adverse results were obtained with 3-E&RA while 3-E&RA/BG and 3-E&RA/WMTA demonstrated a beneficial effect after prolonged DPBS storage. ***Conclusion.*** Calcium-silicate filled composite resins performed better than a current etch-and-rinse adhesive and had a therapeutic/protective effect on the micro-mechanical properties of mineral-depleted resin–dentine interfaces. ***Clinical significance.*** The incorporation of calcium-silicates into dental restorative and bonding agents can create more biomimetic (life-like) restorations. This will not only enable these materials to mimic the physical characteristics of the tooth structure, but will also stabilize and protect the remaining dental hard tissues.

Key Words: *resin bonded-dentine interface, biomechanical properties, Bioglass® 45S5, mineral trioxide aggregate, etch-and-rinse systems*

Introduction

Current concepts of resin/dentine adhesion imply that chemicals are applied before bonding to alter the structure of dentine and favour resin infiltration [1]. Subsequently, resin hybridization should restore biological and mechanical properties to approximate those of the original undemineralized dentine.

Unfortunately, despite significant improvements in the adhesives systems, the resin/dentine bonded interface formed by a mixture of collagen organic matrix, residual hydroxyapatite crystallites and resin monomers still remains the weakest area of composite restorations [2].

The discrepancy between the etching depth and the adhesive system penetrating capacity make the collagen-rich zone underlying the hybrid layer susceptible to the formation of pathways in which oral fluid and endogenous proteolytic enzymes concur to degrade each component of the resin–dentine bonds [3].

Recently, progressive removal of water by apatite deposition emerged as a viable strategy to address the fundamental issue of replacing mineral that is iatrogenically depleted during the acid etching phase [4,5]. This

Correspondence: Andrea Corrado Profeta, BDS PhD, Department of Restorative Dentistry, Biomaterials Science, Biomimetics and Biophotonics (B3) Research Group, King's College London Dental Institute, Floor 17, Tower Wing, Guy's Dental Hospital, Great Maze Pond SE1 9RT, London, UK. Tel: +44 020 7188 1824. Fax: +44 020 7188 1823. E-mail: andrea.profeta@kcl.ac.uk

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should lead to a more durable form of tissue engineered dentine that is capable of preserving its organic components and maintain adhesive strength after ageing.

Remineralization of incompletely resin-infiltrated collagen matrices can be promoted by the use of biomimetic, ion-releasing materials, e.g. glass-ionomer cements [6] or resin-based calcium-phosphate (CaP) cements [7].

Bresciani et al. [8] revealed significantly increased Knoop hardness along the interface of resin-bonded dentine, confirming the capacity of materials containing CaP cements to promote remineralization of caries-affected residual dentine.

Conventional calcium-silicate compounds, including calcium/sodium phosphosilicates, such as Bioglass® 45S5 (BG), and certain calcium-silicates composed primarily of Portland Cement Type I like Mineral Trioxide Aggregate (often referred to as MTA) [9] are known to release ions in aqueous solution and induce deposition of carbonated hydroxyapatite (HCA). Whereas BG has been successfully used for dentine hypersensitivity [10,11] and MTA is already employed in dentistry for different endodontic applications [12], the development of aesthetic restorative materials containing calcium-silicate based micro-fillers with self-healing effects on the mineral-depleted areas within the bonded-dentine interface remains an important clinical target to accomplish [13].

Therefore, the purpose of this study was to appraise the effectiveness of two experimental adhesives, either incorporating BG or MTA, in improving the micromechanical characteristics of resin-dentine interfaces created with a three-step, etch-and-rinse bonding technique. This aim was accomplished by evaluating microtensile bond strength (μTBS) after 24 h or 10 months of storage in phosphate-containing solutions (Dulbecco's Phosphate Buffered Saline, DPBS). Scanning electron microscopy (SEM) fractography on the de-bonded specimens and Knoop micro-hardness (KHN) analysis of the area that was considered to represent the resin–dentine interface (hybrid layer and its surroundings) were executed. Confocal tandem scanning microscopy (TSM) micropermeability analysis was also performed after filling the pulp chamber with 1 wt% aqueous Rhodamine-B.

The null hypotheses to be tested were that the inclusion of calcium-silicate micro-fillers within the composition of the experimental bonding agent induces (i) no effect on the bond strength durability, (ii) micro-hardness and (iii) micropermeability of demineralized 'poorly resin-infiltrated' areas of the resin–dentine interface.

Materials and methods

Experimental bonding systems and calcium-silicate fillers

A resin co-monomer blend representing the formulation of a typical three-step, etch-and-rinse adhesive was used in this study. It was prepared from commercially available monomers [14] and included a

Table I. Chemical composition (wt%) and application mode of the experimental adhesive systems used in this study. To obtain dental bonding systems with chemical affinity to Calcium (Ca^{2+}), an acidic functional monomer (PMDM) was included [14]. A binary photoinitiator system CQ/EDAB made the neat resin light-curable.

Adhesive systems	3-E&RA/BG	3-E&RA/WMTA	3-E&RA
Primer	20.21 wt% Bis-GMA, 14.25 wt% HEMA, 15.54 wt% PMDM, 50 wt% EtOH		
Bond	24 wt% Bis-GMA 17.25 wt% HEMA 18 wt% PMDM 0.15 wt% CQ 0.6 wt% EDAB 40 wt% BG	24 wt% Bis-GMA 17.25 wt% HEMA 18 wt% PMDM 0.15 wt% CQ 0.6 wt% EDAB 40 wt% WMTA	40 wt% Bis-GMA 28.75 wt% HEMA 30 wt% PMDM 0.25 wt% CQ 1.0 wt% EDAB
Bonding procedure	1. Human third molars were used to prepare standardized dentine surfaces that were conditioned with 37% phosphoric acid solution (H_3PO_4; Aldrich Chemical) solution for 15 s. 2. Copious rinse with deionized water → gently air-drying for 2 s to remove the excess of water and leave a wet reflective substrate → application of two consecutive coats of the ethanol-solvated primer → air-drying for 5 s at maximum stream power. 3. Application of a layer of bond adhesive within a period of 20 s → gently air-drying for 2 s → light-curing for 30 s. 4. Resin composite build-up and light-curing.		

Bis-GMA, 2,2-bis [4(2-hydroxy-3-methacryloyloxy-propyloxy)-phenyl] propane (Esstech, Essington, PA); HEMA, hydrophilic 2-hydroxyethyl methacrylate (Aldrich Chemical, Gillingham, UK); PMDM, 2,5-dimethacryloyloxyethyloxycarbonyl-1,4-benzenedicarboxylic acid (Esstech); EtOH, absolute ethanol (Sigma-Aldrich); CQ, camphoroquinone (Aldrich Chemical); EDAB, 2-ethyl-dimethyl-4-aminobenzoate (Aldrich Chemical); BG, Bioglass® 45S5 (SYLC, OSspray Ltd, London, UK); WMTA, hydrated, white ProRoot MTA (Lot No. 08003395; Dentsply Tulsa Dental, Tulsa, OK).

50 wt% ethanol-solvated resin mixture used as primer (Table I).

Two different fillers were incorporated by mixing neat resin (60 wt%) and filler (40 wt%) for 30 s on a glass plate to form an homogeneous paste prior to the bonding procedures: (1) BG with particle sizes <20 μm (SYLC, OSspray Ltd, London, UK) and (2) a hydrated Portland Cement Type I (WMTA). The latter was obtained by mixing 1 g of white ProRoot MTA (WMTA, Lot No. 08003395; Dentsply Tulsa Dental, Tulsa, OK) with 0.35 mL of sterile water for 1 min on a glass slab by using a stainless steel spatula [15]. Subsequent to the setting time (24 h/~ 37°C), the cement was ground in an agate ball mill as well as being sieved to obtain a 20–30 μm-sized, hydrated calcium-silicate filler. A generic label for the two experimental dentine bonding systems has been proposed to refer to their main components (3-E&RA/BG and 3-E&RA/WMTA). Application of the neat resin adhesive with no filler served as a control group (3-E&RA).

Teeth preparation and bonding procedures

Thirty intact caries-free sound molars were stored in deionized water (pH 7.1) at 4°C and used within 1 month after extraction under a protocol approved by the institutional review board of Guy's Hospital (South East London ethical approval 10/H0804/056). The surgical treatment plan of any of the involved patients (20–40 years old), who had given informed consent for use of their extracted teeth for research purposes, was not altered by this study. All teeth were cleaned with a prophy cup under a slow speed for 15 s and randomly assigned to three groups (listed in Table I):

- 3-E&RA/BG,
- 3-E&RA/WMTA, and
- 3-E&RA.

Each tooth was prepared by exposing a flat mid-coronal dentine surface using a slow-speed, water-cooled diamond saw (IsoMet 1000, Buehler Ltd., Lake Bluff, IL). Dentine was polished with wet 600-grit SiC abrasive paper for 60 s to create a standardized and more clinically relevant smear layer [16].

After the application and light-curing of the adhesives (Table I) performed for 30 s using a quartz-tungsten-halogen lamp (600 mW/cm^2, Optilux VLC; Demetron, CT), crowns were restored with three 2-mm-thick increments of resin composite (Filtek Z250; 3M-ESPE, St Paul, MN). Each increment was irradiated for 40 s.

μTBS and SEM fractography of the failed bonds

Once the build-up was completed, the 10 dentine-bonded teeth of each group were sectioned under water irrigation using a slow-speed diamond saw (Accutom 50, Struers A/S, Ballerup, Denmark) in X and Y directions to obtain beams with a cross-section of 0.9 mm^2. A digital caliper (Thermo Fisher Scientific Inc., Pittsburgh, PA) with an accuracy of 0.01 mm was used to measure the sides of the bonding interface so that the bonding area could be calculated. By excluding peripheral beams showing the presence of residual enamel, 16 beams were selected from each tooth, totalling 160 resin–dentine specimens per bonding material. For each group, half of the beams (n = 80) were tested after 24 h while the remaining half (n = 80) following 10 months of storage in DPBS solutions (Oxoid, Basingstoke, Hampshire, UK) at 37°C. The beams were individually attached to a stainless steel notched Geraldeli's jig [17] with cyanoacrylate glue (Zapit, Dental Ventures of America,Corona, CA) and then submitted to a tension load using a Shimadzu Autograph (Shimadzu AG-IS, Tokyo, Japan) universal testing machine at 1 mm/min crosshead speed. The peak load at fracture and the bonding surface area of the specimen were registered and bond strength data were calculated in MPa.

For each of the adhesives, μTBS values of beams from the same restored tooth were averaged and the resultant mean bond strength (mean-MPa) was used as one experimental unit for the statistical analysis. Mean-MPa data were analysed using a repeated-measures ANOVA and Tukey's post-hoc test for pairwise comparisons (α = 0.05). The mode of failure was classified as a percentage of adhesive (occurring at the dentine–adhesive interface), mixed (presence of composite and dentine at the interface) or cohesive (in dentine) when the failed bonds were analysed by two observers under a stereo microscope (Leica M205A; Leica Microsystems, Wetzlar, Germany) at ×30 magnification.

For each group, five representative de-bonded specimens, depicting the most frequent failure modes, were selected for ultramorphology analysis of the fractured surface (SEM Fractography). They were dried overnight and mounted on aluminium stubs with carbon cement, then sputter-coated with gold (SCD 004 Sputter Coater; Bal-Tec, Vaduz, Liechtenstein) and examined using a scanning electron microscope (SEM) (S-3500; Hitachi, Wokingham, UK) with an accelerating voltage of 15 kV and a working distance of 25 mm at increasing magnifications from ×60 to ×5000.

KHN analysis

A further three teeth for each group were prepared and bonded in the same manner as described above. Every restored tooth was sectioned across the adhesive interface using a water-cooled slow-speed diamond saw (Accutom 50) to obtain four 2 mm-thick resin-dentine slabs (n = 12 per group). Following 24 h

of storage in DPBS, each slab was treated in an ultrasonic water bath for 2 min and polished using ascending 500-, 1200-, 2400- and 4000-grit wet SiC abrasive papers. Between each polishing step the slabs were cleansed in an ultrasonic bath containing deionized water for 5 min.

The Knoop micro-hardness evaluation (Duramin-5, Struers A/S, DK-2750 Ballerup, Denmark) was performed using a 25 g load and 15 s dwell time to produce indentations with long diagonals of a suitable size for accurate measurements in relation to the thickness of the hybrid layer, while minimizing surface damage.

To minimize errors caused by tilting and to avoid the introduction of stresses during the micro-hardness testing, each section was mounted and supported on a glass slide using green-stick compound and a parallelling device. The metal chuck containing the dentine slices was clamped onto the stage of the testing machine and the surfaces were oriented perpendicular to the diamond indenter axis.

Each surface received 15 indentations performed immediately after polishing to provide a more uniform surface for reading and to improve the precision of the indentations. These were arranged in three widely separated straight lines starting from the hybrid layer and placed perpendicular to the resin–dentine interface. As a result, any interference of the deformation areas caused by neighbouring marks was avoided. Five measurements were executed along each line, every 30 μm up to 115 μm in depth for sufficient hardness data to be subjected to statistical analysis [8,18] (Figure 1). The dentine surface was covered with a wet tissue paper for 1 min after each indentation to avoid dehydration of the surface [19]. The length of the long diagonal of each indentation was determined immediately to avoid possible shrinkage caused by mechanical recovery of the tooth surfaces with a resolution of 0.1 μm (Duramin-5 software; Struers). The main criteria for accepting an indentation were clearness of outline and visibility of its apices.

The values obtained were converted into Knoop Hardness Numbers according to the following formula:

where P = applied load in g and d = length of the longest diagonal in μm.

After 10 months of DPBS storage, hardness measurements were executed again from the same sections not far away from the first indentations. In total, 540 data points were obtained, 270 after 24 h and 270 after the prolonged ageing period, respectively.

Micro-hardness values of the slabs obtained from the same tooth were averaged and just one value per tooth was used in the statistical analysis. Mean (± SD) KHN numbers were treated with two-way analysis of variance (ANOVA) to determine differences between materials and the effect of ageing. Subsequent one-way ANOVA was performed to assess differences between materials for the two different storage times separately. Post-ANOVA contrasts were performed using a Bonferroni test for multiple comparisons.

TSM micropermeability analysis

Six further dentine-bonded specimens were prepared as previously described for each group and then allocated to two sub-groups (n = 3/sub-groups) based on the period of storage in DPBS (24 h or 10 months). Following the ageing period, the roots of each specimen were cut using a hard tissue microtome and the pulpal tissue was carefully removed from the exposed pulp chamber of each crown segment, without crushing the pulpal-dentine walls using thin tissue forceps. Each pulp chamber was filled with 1 wt% aqueous Rhodamine B (Rh-B: Sigma-Aldrich) dye solution for 3 h. The specimens were then rinsed copiously with

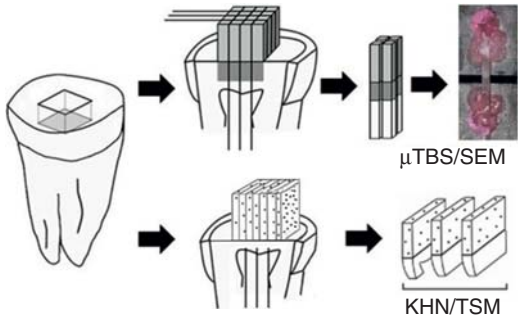

Figure 1. Schematic of sample preparation and study procedures. Composite-tooth matchsticks were prepared for microtensile bond strength (μTBS) testing and scanning electron microscopy failure (SEM) analysis. This schematic also illustrates how composite-tooth slabs were prepared for Knoop micro-hardness (KHN) analysis and confocal tandem scanning microscopy (TSM) micropermeability.

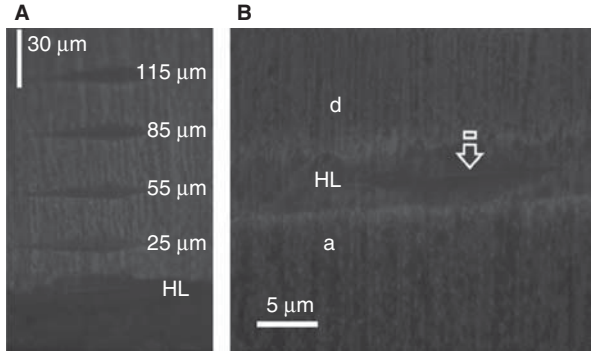

Figure 2. Optical images obtained during the KHN test along the resin–dentine interface. (A) Picture illustrating how the five measurements (indentations) were taken along each line every 30 μm up to 115 μm in depth. (B) At high magnification it is possible to observe that the first indentation was performed exactly on a hybrid layer (HL; arrow) located between the adhesive resins (a) and the dentine surface (d).

water in an ultrasonic bath for 2 min, sliced vertically into 1 mm slabs using a slow-speed water-cooled diamond saw (Labcut, Agar Scientific, Stansted, UK) and polished using 1200-grit silicon carbide paper for 30 s followed by a further ultrasonic bath (1 min) [20].

The micropermeability along the interface was examined using a confocal tandem scanning microscope (TSM: Noran Instruments, Middleton, WI) in both the fluorescence and reflection modes. Reflection and fluorescence images were recorded using a AndoriXon^{EM}, EMCCD camera (Andor Instruments, Belfast, Northern Ireland), digitized and image processed or reconstructed with suitable computer hardware and software (iQ, AndoriXon^{EM}, Andor Instruments).

To measure the micropermeability of the bonded interface, all the specimens were examined using a ×100/1.4 NA oil immersion objective with a 10× ocular and phototube in the fluorescence mode with 546 nm excitation and 600 nm long-pass filters and in the reflection mode. Since peripheral dentine located close to the dentine–enamel junction has a low dentine permeability due to its reduced density of dentinal tubules [21], only the resin-bonded dentine surfaces located at least 1 mm from the enamel were analysed in order to avoid under-estimating micropermeability [22]. Each resin–dentine interface was investigated in totality and three representative images were recorded 1 mm from the dentine–enamel junction. One image was taken from the centre of the bonded interface and two additional images in proximity of the pulpal horns were obtained from each site [22]. These images were intended to be representative of the most common features observed in each specimen.

Results

μ*TBS and SEM fractography of the failed bonds*

The interaction between bonding system vs DPBS storage was statistically significant for the 3-E&RA/BG and 3-E&RA/WMTA groups ($p = 0.001$); no significant reduction of the μTBS values was observed after 10 months of DPBS ageing ($p > 0.05$). On the other hand, significant μTBS reductions were observed in the 3-E&RA group ($p < 0.05$) after prolonged storage in DPBS (10 months). The μTBS results (expressed as Mean and SD) and modes of failures obtained for each bonding system are summarized in Table II.

All the tested materials showed high μTBS values after 24 h of DPBS storage with failures occurring mainly in cohesive mode. In contrast, important changes in the μTBS were observed after 10 months of storage in DPBS. Notably, the resin–dentine specimens of the 3-E&RA/BG and 3-E&RA/WMTA groups maintained high μTBS values ($p > 0.05$) (25.09 ± 7.44 and 28.3 ± 4.5 MPa, respectively) and they debonded prevalently in cohesive mode (44% and 59%, respectively) while the μTBS of specimens in the 3-E&RA (no filler) showed a significant decrease ($p < 0.05$) (17.98 ± 5.12) and failed mostly in adhesive mode (70%).

Table II. Mean and standard deviation (± SD) of the μTBS values (MPa) obtained for each experimental bonding system.

	μTBS, mean ± SD (*n* of tested/pre-failed beams) % Failure [A/M/C]	
Bonding system	24 h	10 month
3-E&RA/BG	27.11 ± 3.12 A1 (80/0) [0/11/89]	25.09 ± 7.44 A1 (77/3) [15/41/44]
3-E&RA/WMTA	30.9 ± 4.4 A1 (80/0) [5/32/63]	28.3 ± 4.5 A1 (6/74) [35/6/59]
3-E&RA	28.92 ± 4.15 A1 (80/0) [0/3/97]	17.98 ± 5.12 B2 (75/5) [70/8/22]

For each horizontal row: values with identical numbers indicate no significant difference after 24 h and 10 monthd of DPBS storage ($p > 0.05$).
For each vertical column: values with identical capital letters indicate no significant difference between the different bonding systems ($p > 0.05$).
Premature failures were included in the statistical analysis as zero values and are indicated in parentheses (for instance 75/5 means that there were five premature failures and 75 testable beams). The modes of failure are expressed as percentages in the brackets [Adhesive/Mix/Cohesive].

The SEM analysis of the fractured surfaces belonging to the 3-E&RA/BG and 3-E&RA/WMTA groups (Figures 3 and 4, respectively) were characterized by the absence of both exposed dentine tubules and

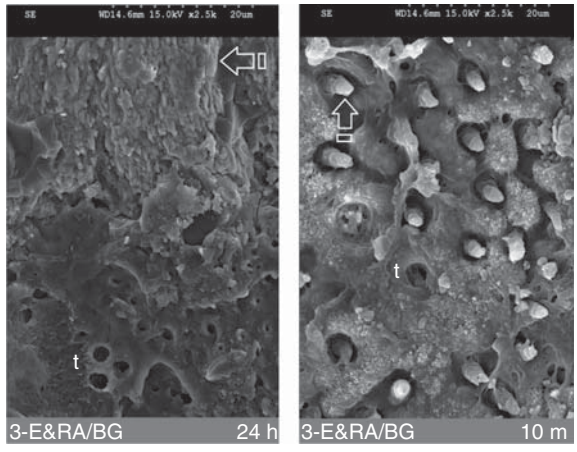

Figure 3. Micrograph of the failure mode (mixed) of the calcium/sodium phosphosilicate-containing adhesive (3-E&RA/BG) bonded to dentine, after 24 h of storage in DPBS. Few exposed dentinal tubules (t) were observed and the dentine surface was well resin-hybridised (arrow). After 10 months of storage in DPBS, the debonded resin–dentine interface showed the presence of mineral crystals embedded within a preserved collagen network (arrow) and inside the dentinal tubules (t).

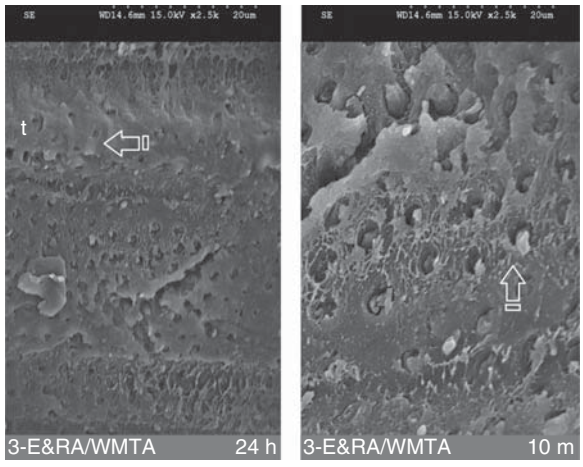

Figure 4. SEM images of an adhesively fractured beam bonded with 3-E&RA/WMTA after 24 h of DPBS storage. Observe the dentine covered by adhesive resin (arrow) without unprotected collagen fibrils and rarely found open dentinal tubules (t). Following 10 months of DPBS storage, the debonded resin–dentine surfaces remained covered with adhesive resin and mineral crystals embedded within a preserved collagen network were vastly encountered (arrow).

collagen fibrils indicating a good hybridization of dentine after 24 h of DPBS storage. Remarkably, the specimens de-bonded after 10 months of DPBS revealed a dentine surface still predominantly covered by residual resin but also mineral crystals embedded within a resin/collagen network.

The 3-E&RA specimens tested after 24 h of DPBS storage and analysed with SEM presented few exposed dentinal tubules but mostly were obliterated by resin tags or covered by resin remnants.

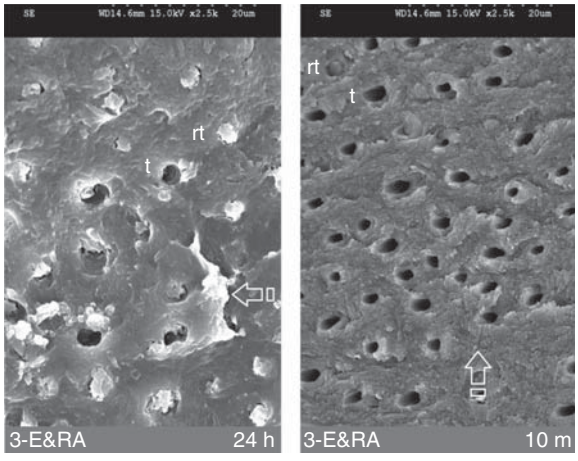

Figure 5. Micrograph of the failure mode (cohesive) of the resin control (3-E&RA; containing no bioactive filler) bonded to etched dentine (37% H_3PO_4) after 24 h of storage in DPBS. It was possible to observe the presence of some exposed dentinal tubules (t), but most remained obliterated by resin tags (rt). No exposed collagen fibrils were visible on the dentine surface and a well resin-hybridized hybrid layer was present (arrow). At 10 months, the resin–dentine interfaces showed only few resin tags (rt) inside funnelled dentinal tubules (t) and no collagen fibrils were visible on the dentine surface (arrow).

Conversely, the surface of the specimens de-bonded after 10 months of DPBS exhibited no collagen fibrils on the dentine surface with rare resin tags and degraded funnelled dentinal tubules (Figure 5).

KHN analysis

The indenter load produced micro-indentations with long diagonals that enabled accurate Knoop micro-hardness measurement. There was no major surface or sub-surface damage evident on all the examined specimens and microscopic inspection revealed no evidence of cracks radiating from the apices of the indentations. The biomechanical properties (surface micro-hardness) were influenced by dentine treatment, position along the resin–dentine interface and storage time. Two-way analysis of variance indicated that there was a statistically significant interaction between materials and the effect of ageing. Statistical comparisons of mean KHN values (± SD) at different depths obtained after 24 h and 10 months of DPBS storage are shown in Table III.

Analysis of the present data showed a statistical reduction of KHN values after prolonged DPBS storage ($p < 0.001$) within the hybrid resin–dentine zones created with the adhesive system containing no filler (3-E&RA) (24 h: 24.4 ± 0.5 KHN; 10 months: 15.9 ± 2.9 KHN). On the contrary, no significant KHN decrease of the average superficial micro-hardness ($p > 0.001$) was observed after 10 months in all the other indentations taken away from the composite resin layer for both groups (Table III, Group 10 months).

The experimental bonding agents of the group 3-E&RA/BG and 3-E&RA/WMTA maintained high KHN values with no statistical difference after the ageing period in all the tested locations ($p > 0.001$). The mean surface micro-hardness values of the resin–dentine regions were 17.5 ± 0.8 (24 h)/17.6 ± 0.3 (aged) for 3-E&RA/BG and 18.8 ± 3.6 (24 h)/ 24.2 ± 1.7 (aged) for 3-E&RA/WMTA. Likewise, all the other KHN values corresponding to 25, 55, 85 and 115 μm in depth did not show a statistical decrease in the mean hardness of the surface after 10 months of DPBS storage (Table III, Group 10 months).

TSM micropermeability analysis

Sites of micropermeability within the resin-bonded dentine interfaces had different characteristics between the groups (Figure 6). After prolonged DPBS storage, the results of TSM investigation showed only slight dye penetration along the walls of the tubules and within the confines of the hybrid layer for the two experimental methacrylate-based systems containing conventional calcium-silicate compounds (3-E&RA/BG and 3-E&RA/WMTA).

Table III. The results of the micro-hardness measurements for each bonding system after 24 h and 10 months of DPBS storage.

	Distance				
	I	II	III	IV	V
24 h					
3-E&RA/BG	17.5 (0.8)^{A}	60.4 (3.9)^{B}	60.5 (0.8)^{B}	59.6 (2.6)^{B}	61.9 (0.9)^{B}
3-E&RA/WMTA	18.8 (3.6)^{A}	56.6 (6.5)^{B}	59.4 (4.6)^{B}	60.2 (3.4)^{B}	59.0 (3.0)^{B}
3-E&RA	24.4 (0.5)^{A}	56.7 (4.9)^{B}	61.1 (3.0)^{B}	64.0 (4.0)^{B}	62.8 (4.2)^{B}
10 month					
3-E&RA/BG	17.6 (0.3)^{A}	54.6 (1.2)^{B}	56.5 (2.0)^{B}	58.5 (4.3)^{B}	59.5 (4.5)^{B}
3-E&RA/WMTA	24.2 (1.7)^{A}	62.3 (3.6)^{B}	63.2 (2.4)^{B}	63.6 (2.9)^{B}	63.2 (2.5)^{B}
3-E&RA	15.9 (2.9)^{A*}	55.5 (4.4)^{B}	60.4 (3.2)^{B}	62.4 (3.8)^{B}	63.0 (4.0)^{B}

In each row, the same capital letter indicates no statistical difference between mean KHN values (SD). Asterisk symbol (*) indicates a statistically significant reduction ($p < 0.001$) of mean KHN values (SD) subsequent to 10 months of DPBS storage.
I: Mean KHN values (SD) at the resin–dentine bonded interface. II: Mean KHN values (SD) at the points 25 μm distant from the composite resin layer in the direction of the dentine. III: Mean KHN values (SD) at the points 55 μm distant from the composite resin layer in the direction of the dentine. IV: Mean KHN values (SD) at the points 85 μm distant from the composite resin layer in the direction of the dentine. V: Mean KHN values (SD) at the points 115 μm distant from the composite resin layer in the direction of the dentine.

Conversely, the resin-bonded dentine interfaces obtained after application of the control, neat resin adhesive with no filler (3-E&RA) showed severe Rh-B uptake within the hybrid layers following each ageing period.

Discussion

Restorative dentistry based on biomimetics or imitating nature is concerned with not only the natural appearance and aesthetic aspects of dental restorations, but also the way they work [13]. To copy nature is to understand the mechanics of the tooth, the way it looks and functions and the way every stress is distributed.

Ideally, the resin bonded–dentine interface should provide a secure marginal seal and have the ability to withstand the stresses that have an effect on the bonding integrity of the adhesives, in order to keep the restoration adherent to the cavity walls.

There are enduring challenges in adhesive dentistry due to the incomplete infiltration of wet dentine with resin monomers that yield resin–dentine bonds prone to degradation and loss of mechanical stability [2]. Major concerns have been expressed regarding interfacial ageing caused by absorption of water, hydrolysis of the resin and disruption of the collagen network [23].

At the same time, *in vitro* studies have suggested possible strategies to reduce the flaws inherent in the dentine bonding systems, each one having its own merits and limitations [24].

The development of ion-releasing restorative materials with a biomimetic ability to fill micro- and nano-sized voids by crystal deposition is currently one of the main targets of the dental biomaterial research [8,25,26].

A number of recent investigations have shown that resinous materials filled with particles chemically resembling the inorganic phase of dentine, such as calcium-silicate compounds, may possess bioactivity properties in aqueous environments that contain calcium and phosphate (e.g. saliva, dentinal substrate or phosphate buffered saline solutions) [27,28].

In the present study, BG and MTA micro-fillers (<20 μm) were included within the composition of a representative three-step, etch-and-rinse adhesive in order to create a material with remineralizing action on the water-rich collagen matrices along the resin–dentine interface.

Based on the results obtained in this study, the three null hypotheses must be rejected as the inclusion of calcium-silicate micro-fillers had a therapeutic effect on bond strength, micro-hardness and micro-permeability of the acid-treated resin-infiltrated dentine.

In detail, the two experimental bonding agents containing experimental micro-fillers (3-E&RA/BG, 3-E&RA/WMTA) and the control co-monomer blend (3-E&RA) used to bond the acid-etched dentine produced comparably high μTBS values ($p > 0.05$) following 24 h of storage in DPBS (Table II). Conversely, after 10 months of storage in DPBS a significant decrease in μTBS ($p < 0.05$) was observed for the 3-E&RA group, while the specimens bonded using 3-E&RA/BG or 3-E&RA/WMTA maintained consistent long-term bond strength values ($p > 0.05$). The specimens of the 3-E&RA/BG and 3-E&RA/WMTA groups de-bonded after 10 months of DPBS storage showed, during SEM fractography examination, residual resin presence and newly formed mineral-bodies (Figures 3 and 4, respectively). The SEM analysis also revealed that the

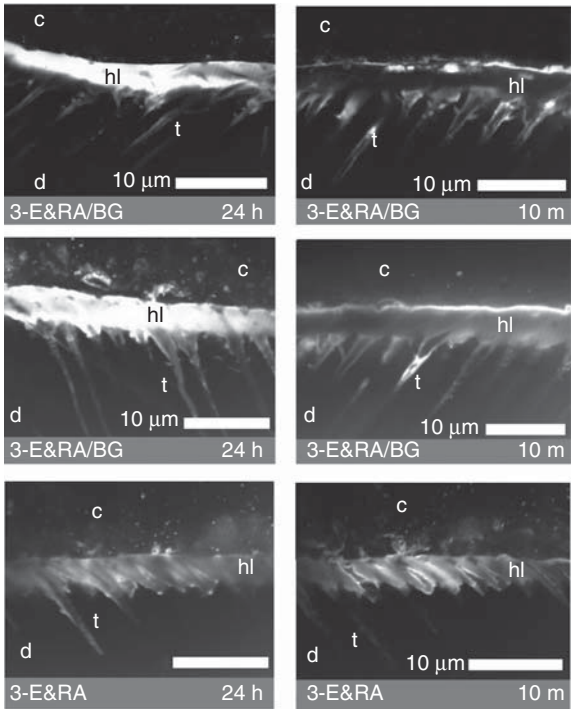

Figure 6. Confocal fluorescence/reflection images of the micropermeability of resin–dentine interfaces created with the three experimental resin bonding systems (3-E&RA, 3-E&RA/BG and 3-E&RA/WMTA): Rh-B infiltrated hybrid layers after 24 h (first column) and after 10 months (second column) of DPBS storage. The scale in all the images is 10 μm. *3-E&RA/BG, upper row*: TSM confocal microscopy showed high dye penetration after 24 h within the hybrid layers (hl) of the resin–dentine interfaces created with the resin bonding systems containing calcium/sodium phosphosilicate (BG). Conversely, slight micropermeability of Rh-B was present only along the walls of the tubules (t) following 10 months of DPBS storage. No fluorescence was detected in the hybrid layer (hl). *3-E&RA/WMTA, central row*: Resin–dentine interfaces created with the 3-step etch-and-rinse adhesive comprising a calcium-silicate Portland-derived micro-filler (WMTA). After 24 h of DPBS storage, it is possible to observe intense Rh-B uptake within the entire resin–dentine interface. The composite layer is detected in reflection mode (c). On the contrary, TSM confocal microscopy showed poor dye penetration limited to the walls of the tubules (t) and slight micropermeability after prolonged DPBS storage within the hybrid layer (hl) of the resin–dentine interfaces created using the same bonding system. *3-E&RA, lower row*: Resin-bonded dentine interfaces obtained after application of the neat resin adhesive with no filler (control group) showed micropermeability of Rh-B along the walls of the tubules (t) and within hybrid layers (hl) after the initial period of DPBS storage. Full Rh-B infiltrated hybrid layers (hl) were also observed after the increased ageing period in the resin–dentine interfaces created with the control resin.

de-bonded dentine surface of the specimens in the 3-E&RA group was well resin-hybridized and characterized by no exposed collagen fibrils after 24 h of DPBS storage. However, these resin–dentine specimens stored for 10 months in DPBS had a fractured surface characterized by 'funnelled' dentinal tubules, indicating degradation of the demineralized peritubular dentine (Figure 5).

These results were also supported by the KHN analysis performed to evaluate regional differences in micro-hardness associated with mineral deposition over time [29]. Indeed, further evidences of the biomimetic potentiality of the experimental bonding agents containing BG and MTA were attained; incorporation of the aforementioned bioactive fillers had an effect on the superficial hardness profile of resin-sparse regions within the bonded interface after prolonged storage in DPBS.

In fact, it appeared that treatment with 3-E&RA/BG and 3-E&RA/WMTA did not result in a statistically significant change in KHN with ageing, as opposed to the reduction of resistance to local deformation in the hybrid resin–dentine zones created with the 3-E&RA bonding systems ($p < 0.001$).

In view of the mechanism of its formation, the resin–dentine interface represents a continuous structure from the hybrid layer (in which decalcified dentine impregnated by resin and that not impregnated by resin are considered to be mix) to the healthy dentine [30]. Because of the complexity of these regions, various methods have been suggested for evaluating the extent of remineralization in dental tissues, mostly based on the determination of changes in mineral content. Although several of these experimental methodologies are able to analyse mineral levels in detail [transverse micro-radiography (TMR), micro-computer tomography (CT), X-ray micro-tomography (XMT), etc.], comparable, objective relative dentine hardness measurements are often undertaken *in vitro* using micro-hardness indenters, resulting in reproducible data sets with minimal damage to the sample [31,32].

Micro-hardness measurements can be correlated with mechanical properties such as modulus of elasticity, fracture resistance [33] and yield strength [34,35]. A positive correlation also exists between Knoop micro-hardness and bond strength and it was proposed that adhesion mechanisms for both enamel and dentine are controlled, to a major extent, by the mineral content of the tooth [36,37].

In addition, Bresciani et al. [8] hypothesized that it may be possible to evaluate the therapeutic ability of bioactive calcium phosphate-based materials in repairing the demineralized intertubular and intrafibrillar dentine collagen by re-establishment of the superficial micro-hardness.

In this study, cross-sectional Knoop micro-indentation measurements provided an average hardness of each surface and gave valuable additional information regarding the behaviour of dentine/restoration interfaces because any variation observed reflected a quantitative difference in mineral content [38–40].

However, many factors may influence the hardness results such as the dentine depth and the relative quantities of the tubular, peritubular or intertubular areas which vary considerably with location [41–43]. Consequently, Knoop test measurements were

obtained from the same specimens and close to previously assessed locations after 10 months of DPBS storage. This was done in order to minimize the effect of the structural variations within the same tooth and to establish a reasonable baseline for evaluation.

In bonding systems using phosphoric acid, the area 10 μm away from the interface in direction of healthy dentine might be within the decalcified layer not impregnated by resin, resulting in lower hardness [30]. Overall mean KHN values calculated in sound, mineralized dentine adjacent to restorations (25, 55, 85 and 115 μm from the composite resin layer) were in good agreement with those reported by other authors [44–46]. The similarity of reported values is certainly due to the reproducible micro-indentation technique employed [41].

For Knoop hardness, upon unloading, elastic recovery occurs mainly along the shortest diagonal, but the longest diagonal remains relatively unaffected [47,48]. Therefore, the hardness measurements obtained by this method are virtually insensitive to the elastic recovery of the material.

Another chief characteristic of the Knoop hardness test is its sensitivity to surface effects and textures [49,50]. Accordingly, wider impressions of the tool mark were found on all the resin–dentine interfaces, these regions being more elastic and softer than the healthy dentine, and lower KHN values were obtained. Even these observations correlate with previous findings indicating that, in the absence of intrafibrillar mineralization, the hardness and modulus of elasticity are inferior to those of mineralized dentine [51]. In fact, the modulus of elasticity of wet demineralized dentinal matrix is only ~ 5 MPa [52], which is more than 1000 times lower than that of mineralized dentine. This different histological configuration is held accountable for hardness reduction in the resin–dentine interface and, therefore, for the little resistance offered to the testing indenter.

Inert fillers are normally added to reinforce the resin matrix and increase its hydrolytic stability. Amount, size and form of the filler particulate determine physical and mechanical properties of the cured polymer chain such as stiffness and hardness [53].

However, any mechanical reinforcement via static filler-matrix coupling would also have a detrimental effect on surface modification of the bioactive fillers, resulting in a restriction of the ion-leaching process. In this study, dissolution and precipitation events were expected to occur rapidly, owing to the formation of colloidal layers around the ion-leachable particles that gradually replaced the original microfillers [54].

This latter concept may, to some extent, explain why mean KHN values of 3-E&RA at the resin–dentine bonded interface (distance I) appeared higher compared to its filled corresponding versions after 24 h of DPBS storage.

It is also important to consider that the high alkalinity of BG and MTA hydration products provided a caustic proteolytic environment, thereby further degrading the collagenous component of the interfacial dentine and decreasing the resistance to plastic deformation.

This might be initially little desirable, but facilitated the permeation of high concentrations of Ca^{2+}, OH and $CO_3{}^2$ ions (mineral infiltration zone), promoting active mineral deposition and subsequent remineralization of the matrix.

Interestingly, specimens created with the experimental adhesives 3-E&RA/BG and 3-E&RA/WMTA did not restore micro-hardness to the level of sound dentine in these zones, but maintained the same KHN values and no statistical difference reduction was found following 10 months of DPBS storage. The only statistically significant change occurred in the resin–dentine interfaces bonded with 3-E&RA that were subjected to a reduction of KHN values with ageing.

These data are in agreement with Ryou's et al. [5] findings where apatite precipitation and concomitant reduction of water-rich regions within the resin–dentine interface appeared to restrict the collagenolytic and hydrolytic mechanisms responsible for loss of mechanical stability.

Remineralization, defined as restoration of lost mineral content where resin monomers border on decalcified dentine [8], should re-develop the mechanical properties to approximate those of original undemineralized dentine. Even so, the stiffness of resin–dentine interfaces cannot be compared with natural mineralized dentine, as the adhesive resins used to infiltrate the collagen matrices are much more viscoplastic than bioapatites. It is equally suggested that the lack of mechanical reconstitution may be attributed to an heterogeneous arrangement of the newly deposited mineral within the demineralized organic network. In fact, mineralization patterns that differ from the usual organization and orientation of minerals might lead to different mechanical properties of the resultant substrates [8,55].

Although the reincorporation of mineral into the demineralized dentine matrix does not represent a full recovery of its functionality, it still plays a very important role, since the remineralized remnant crystallites in the sub-surface of the tissue may be much more resistant to subsequent acid attacks [56].

Ten months DPBS storage outcomes offered distinctive information also in terms of micropermeability results. TSM results confirmed the presence of a high quality hybrid layer created by the 3-E&RA/BG and 3-E&RA/WMTA bonding system. The reduction of dye diffusion may have been induced by their ability to decrease the distribution of water-rich regions within the hybrid layer [27] via silanols polycondensation and precipitation of Ca-compounds, which probably also prevented hydrolytic and collagenolytic

degradation phenomena responsible for loss of mechanical stability [57].

Strengths of this study include the fact that every effort was made to standardize the experimental process. A thoughtfully planned pilot study was carried out to develop an effective experimental design.

Nonetheless, the relationship between hardness and mineral content remains complex, not yet fully understood and necessitates further detailed investigation [8]. Additional long-term studies and examination of the resin–dentine interface at narrower intervals (i.e. atomic force microscopy nano-indentation) will give us better insight into remineralization dynamics, rate of mineral uptake and hardness modifications induced by novel biomimetic composite resins containing calcium-silicate fillers [58].

Within the limitations of this study, the following conclusions can be drawn:

- The use of a typical three-step, etch-and-rinse adhesive (3-E&RA) showed a statistically significant microtensile and micro-hardness drop in the hybrid layer after 10 months of DPBS storage. Conversely, the resin–dentine interfaces created with biomimetic dental adhesives (3-E&RA/BG and 3-E&RA/WMTA) showed no statistical change in μTBS and KHN values after ageing in DPBS for 10 months.
- The incorporation of calcium-silicates into dental restorative and bonding agents can create more biomimetic (life-like) restorations. This would not only enable these materials to mimic the physical characteristics of the tooth structure, but would have several other benefits including closure of gaps forming at the resin–dentine interface, protection of the remaining dental hard tissues and potentially better bond strength over time (less degradation of bond).

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