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Authors: Salvatore Sauro, Timothy F. Watson, Ian Thompson, Avijit Banerjee

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One-bottle self-etching adhesives applied to dentine air-abraded using Bioactive glasses containing polyacrylic acid: an in vitro μTBS and confocal microscopy study.

Salvatore Sauro, Timothy F Watson, Ian Thompson, Avijit Banerjee,

Biomaterials, Biomimetics & Biophotonics, King's College London Dental Institute, King's Health Partners at Guy's Hospital, London.

Short Title: self-etching adhesives bonded to Bioglass air-abraded dentine

*Corresponding Author:

Dr. Salvatore Sauro
Biomaterials, Biomimetics & Biophotonics
King's College London Dental Institute
Floor 17, Tower Wing
Guy’s Hospital
London, SE1 9RT, England, UK
Email: salvatore.sauro@kcl.ac.uk
ABSTRACT

Objectives: The aim of this study was to test the microtensile bond strength (µTBS) of two “simplified” self-etching adhesives bonded to air-abraded dentine using experimental bioactive glass powders containing polyacrylic acid.

Methods: Sound dentine specimens were air-abraded using a pure Bioglass 45S5 (Bioglass) powder or two Bioglass powders containing different concentration of polyacrylic acid (PAA: 15 wt% or 40 wt%). The bonding procedures were accomplished by the application of two self-etching adhesives (CS3: Clearfil S3 Bond; Kuraray, Osaka, Japan or GB: G Bond; GC Ltd, Tokyo, Japan). The resin-bonded specimens were cut in beams (0.9 mm²) and the µTBS testing was performed after 24 h or 6 months of phosphate buffer solution (PBS) storage. The results were statistically analysed by three-way ANOVA and Student–Newman–Keuls test used (α = 0.05). Further bonded-dentine specimens were used for the confocal microscopy interfacial characterisation and micropermeability analysis.

Results: The CS3 adhesive system achieved higher µTBS than those attained in the specimens bonded with GB both after 24 h and 6 months of PBS storage. The CLSM analysis performed after 6 months of PBS storage indicated severe micropermeability within the bonded-dentine interfaces created using GB applied onto dentine air-abraded with Bioglass/PAA-15 and Bioglass/PAA-40. Conversely, CS3 exhibited no dye penetration (micropermeability) at the resin-dentine interface.

Conclusion: It is possible to affirm that air-abrasion procedures performed using pure Bioglass or Bioglass containing 15 wt% of PAA do not interfere with the immediate bonding performance of self-etching adhesives. However, the durability of the bonded-dentine interfaces created subsequent air-abrasion procedures using bioactive glasses will depend also upon the chemical composition of the self-etch adhesive systems.
INTRODUCTION

Contemporary concepts of minimally invasive cavity preparation coupled with the advent of the latest generation of self-etching adhesive systems have influenced radically the modern philosophy for the operative management of dental caries \(^1,^2\). A minimally invasive approach to caries excavation should result in the selective removal of the heavily bacteria-contaminated, denatured, caries-infected dentine, preserving as much caries-affected dentine as possible for potential remineralisation by bioactive restorative materials \(^3-^5\).

Conventional hand and rotary instrumentation are used routinely in clinical practice for caries excavation. However, due to their lack of inherent tissue selectivity and therefore reliance on operators’ clinical variability, alternative and more selective methods are still required for the ideal minimal cavity design with the removal of infected dentine only \(^1,^4-^7\).

Air-abrasion has been reported to be a promising technique to achieve ideal minimally invasive cavity preparation. Nevertheless, the choice of powders to perform selective caries removal may affect the quality and durability of the adhesive bond between the dentine and the adhesive/composite restoration, achieved using self-etching adhesives \(^1,^3\). It has been shown that air-abrasion performed using sodium bicarbonate or crystalline cellulose affects the bonding performance of self-etching adhesives bonded to air-abraded dentine \(^8\).

The bioactive calcium-sodium phosphosilicate (Bioglass 45S5) may be used as a substitute for alumina powder in air-abrasion systems \(^9,^{11}\). Although Bioglass 45S5 has a Young’s modulus (35 GPa) and Vickers’ hardness (458 VHN) which is lower than that of alumina (380 GPa and 2300 VHN, respectively) it is still similar to the hardness of mineralised dentine \(^12,^{13}\).

Nonetheless, there is no information published to the authors’ knowledge on the effect of the “bioactive” smear layer produced during air-abrasion procedures using specific Bioglass powders, on the performance of self-etching adhesives bonded to air-abraded dentine.

Thus, the aim of this study was to test the microtensile bond strength (µTBS) after 6 months PBS storage of two self-etching adhesives bonded to dentine, air-abraded using pure Bioglass 45S5 or two Bioglass powders containing polyacrylic acid (PAA: 15 wt% or 40 wt%). The interfacial
characterisation and micropermeability of the bonded interfaces were evaluated using confocal microscopy (CLSM). The null hypothesis to be tested was that the air-abrasion using pure Bioglass 45S5 or two Bioglass powders containing PAA do not influence the µTBS and the ultra-morphology of the resin-bonded dentine interface after 24 h or 6 months PBS storage.

MATERIAL AND METHODS

Specimen preparation

Caries-free human molars, extracted for surgical reasons under a protocol approved by an institutional review board (ref. 10/H0721/55) were used in this study. The teeth were stored in deionised water (pH 7.1) at 4 °C for no longer than 1 month. Coronal dentine specimens were prepared by sectioning the roots 1 mm beneath the cemento-enamel junction (CEJ) using a hard tissue microtome (Isomet 11/1180; Buehler, Coventry, UK) equipped with a diamond embedded blade (XL-12205; Benetec, London, UK). The smear layer-covered middle coronal dentine was exposed by removing the occlusal enamel with a parallel cut and by a polishing treatment protocol performed using a 180-grit SiC paper for 1 min under continuous water irrigation. The specimens were divided into experimental groups and subgroups according to Table 1.

Experimental design: dentine pre-treatment and bonding procedures

The dentine specimens were air-abraded using PAA-containing or PAA-free Bioactive glasses (Sylc Bioglass 45S5; OSspray, London, UK) with a particle size of 30-60-90 µm: i) Bioglass control: pure Bioglass 45S5; ii) Bioglass/PAA-15: Bioglass 45S5 in combination with 15 wt% of PAA powder with a particle size <70 µm (Polyacrylic acid - MW 1800; SIGMA-ALDRICH, St. Gillingham, UK); iii) Bioglass/PAA-40: Bioglass 45S5 in combination with 40 wt% of PAA (SIGMA-ALDRICH, UK). The use of the Bioglass powder in air-abrasion devices using certain operating parameters (i.e. air pressure > 400 MPa) causes a cutting effect and permits Bioglass particles to embed in the dentine surface and within dentine tubules. The polyacrylic acid was included to increase the probability for Bioglass
particles embedding in dentine and modulate the cutting efficacy with respect to selective caries removal (2). A pilot study was performed to decide the minimum (15 wt%) and maximum (40 wt%) concentration of PAA to be included within the Bioglass composition to modulate the cutting efficacy of the powder. The air-abrasion system used to deliver the Bioactive glasses was (AQUACUT Quattro; VELOPEX International, London, UK) equipped with a hand-piece nozzle diameter of 0.6 mm working at an air pressure of 5 bar (500 MPa) for 1 min at 1 cm distance from the dentine surface (powder flow rate: ~2.0 g/min). The pH of the dentine surface immediately after each air-abrasion procedure was evaluated using a professional contact pH electrode (InPro 4260SG; Mettler-Toledo, Leicester, UK) (Table 1).

The bonding procedures were accomplished by the application two self-etching adhesives containing either a phosphoric functional monomer (Clearfil S3 Bond; Kuraray, Osaka, Japan) or a carboxylic functional monomer (G Bond; GC Ltd. Tokyo, Japan), (see Table 1) to the alternatively air-abraded dentine surfaces as per the manufacturers’ instructions. The adhesives were light-cured for 20 s with a light-curing unit (Optilux VLC; Demetron, Research, CT, USA) with a blue light source, (470 nm, 600 mW/cm²). A flowable resin composite (Filtek Supreme XT; 3M ESPE, St. Paul, USA) was then layered in 1 mm increments, each photo-cured for 40 s, so creating a 5 mm build-up.

Overall, four experimental groups were generated:

**Group 1.** The dentine specimens were abraded using 180 grit SiC abrasive paper (1 min) under continuous irrigation, followed by a water rinse (20 s), air-drying (2 s) and adhesive/composite placed as described previously (negative control group).

**Group 2.** The dentine specimens were air-abraded with 100% Bioglass under continuous H₂O shroud (1 min), rinsed with water (20 s), dried and adhesive/composite placed as described previously.

**Group 3.** The dentine specimens were air-abraded with Bioglass (85 wt%) and PAA (15 wt%) under continuous H₂O shroud, rinsed with water (20 s) and adhesive/composite placed as described previously.
Group 4. The dentine specimens were air-abraded with Bioglass (60 wt%) and PAA (40 wt%) under continuous H$_2$O shroud, rinsed with water (20 s) and adhesive/composite placed as described previously.

The intact specimens were stored in phosphate buffer solution (PBS) at 37 °C for 24 h or 6 months, depending on the experimental group. The composition of the PBS (in g/L) was CaCl$_2$ (0.103), MgCl$_2$$\times$6H$_2$O (0.019), KH$_2$PO$_4$ (0.544), KCl (30) and HEPES (acid) buffer (4.77); the pH was 7.4. All the chemicals used to formulate the PBS were purchased from SIGMA-ALDRICH, St. Gillingham, UK.

**Micro-tensile bond strength test (µTBS)**

After 24 h or 6 month PBS storage, the resin-bonded specimens from each group (Table 1) were sectioned using a hard tissue microtome (Buehler, UK) in both X and Y directions across the bonding interface obtaining beams with cross-sectional areas of 0.9 mm$^2$. The beams obtained peripherally including enamel were excluded from the µTBS test. 50% of beams (n ~30/group) suitable for µTBS were tested immediately after 24 h or 6 months of PBS storage. The µTBS test was performed using a customised microtensile jig on a linear actuator (SMAC II; SMAC Europe, Horsham, UK) with LAC-1 (high speed controller single axis with built-in amplifier) and LAL300 linear actuator (stroke length of 50 mm; peak force of 250 N; displacement resolution of 0.5 mm). Bond strength data was analysed statistically using three-way ANOVA including interactions between factors, using µTBS as a dependent variable, and dentine surface treatment, adhesive system and PBS-storage time as independent variables. Post hoc multiple comparisons were performed using the Student–Newman–Keuls test. Statistical significance level was set at $\alpha = 0.05$. Modes of failure were classified as percentage of adhesive (A), mixed (M) or cohesive (C) failures when the failed bonds were examined at 50x magnification using stereoscopic microscopy.

**Confocal microscopy evaluation (CLSM)**

Further resin-bonded dentine specimens (Table 1) were prepared as described previously using the same self-etching adhesives doped with 0.1 wt% rhodamine B powder (SIGMA-ALDRICH, UK).
The roots of each specimen were cut using a hard tissue microtome as previously described and the pulp chambers filled with 1 wt% aqueous fluorescein dye solution for 3 h after 24 h or 6 months of PBS storage. Subsequent to the PBS storage, the specimens were rinsed copiously with water in an ultrasonic bath for 2 min. The specimens were sliced vertically into 1 mm slabs using a slow-speed water-cooled diamond saw (Benetec, UK) and polished using 1200 grit silicon carbide paper for 30 s followed by a further ultrasonic bath (1 min). The micropermeability along the interface was examined using a confocal laser scanning microscope (CLSM Leica SP2; Leica, Heidelberg, Germany) equipped with a 63/1.4 oil immersion lens using a 488 nm argon ion laser illumination for fluorescein excitation or a 568 nm krypton ion laser excitation for rhodamine B. CLSM reflection and fluorescence images were obtained with 1 µm z-step to section optically the specimens to a depth of up to 20 µm below the surface. The z-axis scan of the interface surface was pseudo-coloured arbitrarily by two independent operators for improved exposure and compiled into both single and topographic projections using Leica SP2 CLSM image-processing software (LAS-AF; Leica, Germany). The configuration of the system was standardised and used at the same settings for the entire investigation. Each resin-dentine interface was investigated in totality and five optical images were randomly captured. Micrographs representing the most common features of micropermeability observed along the resin-dentine interface were captured and recorded.

**RESULTS**

**Micro-tensile bond strength test (μTBS)**

Dentine surface treatments, adhesive system and PBS storage time influenced the μTBS results (p < 0.01). Interactions between factors were also significant (F =98.315; p < 0.05). Mean (±SD) μTBS of the resin-bonded dentine tested in this study are shown in Table 2. The CS3 self-etching adhesive containing 10-MDP (phosphoric functional monomer) achieved higher μTBS compared to the GB adhesive system containing 4-MET (carboxylic functional monomer) in all the dentine pre-treatment groups both after 24h and 6 months of PBS storage.
The GB adhesive bonded to dentine air-abraded using Bioglass or Bioglass/PAA-15 achieved higher (p<0.05) µTBS results compared to those observed in the control group 1 (SiC-treated dentine). Bioglass and Bioglass/PAA-15 bonded-dentine failed prevalently in mixed and cohesive mode (Table 2). The µTBS results obtained from group 4 (Bioglass/PAA-40) were comparable (p>0.05) to those attained in the control group, where the adhesive was applied on smear layer-covered SiC-treated dentine (group 1). PBS storage (6 months) induced a statistical drop (p<0.05) in the µTBS values within all dentine pre-treatment groups and bonded-dentine specimens failed prevalently in an adhesive and mixed mode (Table 2).

The CS3 adhesive exhibited a different behaviour in terms of µTBS results both after 24h and 6 months of PBS storage. Although no statistical significance (p>0.05) was observed, CS3 applied both onto Bioglass and Bioglass-PAA air-abraded dentine obtained higher µTBS results after 6 months of PBS storage than those achieved in the control PBS storage (24 h). The most common failure mode observed in all dentine treatment groups bonded using CS3 was mixed and cohesive both after 24h and 6 months of PBS storage (Table 2). Only the µTBS results obtained in group 4 (Bioglass/PAA-40) were significantly lower (p<0.05) than those attained in the CS3-bonded SiC-treated dentine (group 1). CS3 applied on Bioglass/PAA-40 air-abraded dentine failed mainly in mixed mode; adhesive mode failure was also attained in some beams particularly after 6 months PBS storage (Table 2).

**Confocal microscopy evaluation (CLSM)**

The CLSM interfacial characterisation and micropermeability analysis performed after 24 h and 6 months PBS storage showed important features regarding the application of self-etching adhesives on differently air-abraded dentine.

The bonded-dentine interface created by GB applied on sound smear layer-covered dentine (Figures 1A) showed fluorescein dye penetration (i.e. micropermeability) and water sorption through the entire thickness of the adhesive layer (Figure 1A-1). This adhesive layer was also characterised by the presence of resin tags within the dentine tubules (Figure 1A-2). Similarly, CS3 applied on a sound
smear layer-covered created a bonded-dentine interface characterised by micropermeability corresponding with the inter-diffusion layer (IDF), (Figures 1B). The adhesive layer was affected by marked water sorption (Figure 1B-1) and presented evident resin tags (Figure 1B-2).

The application of GB on Bioglass air-abraded dentine created a bonding interface affected by micropermeability both corresponding to the inter-diffusion layer (Figure 1C) and along the entire thickness of the adhesive layer (Figure 1C-1); this latter layer showed few and short resin tags (Figure 1C-2). Conversely, the bonded-dentine interface created using CS3 on Bioglass air-abraded dentine showed uniform dye accumulation at the resin-dentine interface (Figure 1D) and water sorption through the entire thickness of the adhesive layer (Figure 1D-1). Also in this case, the presence of Bioglass particles inside the dentine tubules was responsible potentially for the formation of an adhesive layer with very short or no resin tags (Figure 1D-2). The application of GB or CS3 on both Bioglass/PAA-15 (Figure 2A) and Bioglass/PAA-40 (Figure 2B) air-abraded dentine created a bonding interface affected by micropermeability only at the resin-dentine interface (Figures 2 A-D) with no sign of water sorption within the adhesive layer (Figures 2E and 2F).

The CLSM analysis performed after 6 months of PBS storage indicated different micropermeability in the bonded-dentine interfaces created using GB applied on Bioglass/PAA-15 (Figures 3A) and Bioglass/PAA-40 (Figures 3B) when compared to the 24h analysis. The fluorescein dye penetration was localised mainly at the resin-dentine interface and in part of the adhesive layer (Figures 3-A1 and 3-B1). All the bonded-dentine interfaces created using CS3 exhibited no evident differences when compared to those stored in PBS for 24 h. Fluorescein dye penetration was observed both at the resin-dentine interface and within the adhesive layer in Bioglass group (Figures 3C and 3-C1) and Bioglass/PAA-40 group (Figures 3D and 3D1).

**DISCUSSION**

The common criteria used by dental clinicians to guide caries excavation and cavity preparation (i.e. hardness and the colour of the tissue) are influenced by the operator’s subjective judgement. The
lack of selective operative procedures to remove caries-infected dentine may compromise the strength of the remaining tooth structure and the pulpal health due to clinical consequences caused by variations in the size of the cavities. Air-abrasion can be used operatively for the preparation of a noise, vibration and pain-free cavity with rounded internal and cavo-surface angles. Moreover, Paolinelis et al., showed the air-abrasion performed using Bioglass 45S5 can prepare both sound and carious dentine. The authors also demonstrated that Bioglass was able to cut carious dentine in a slower rate than alumina powder, so reducing the probability to over-prepare the cavity. However, although a number of innovative materials have been advocated to achieve a more selective caries removal, no information is available on the effect of pure and PAA-containing Bioglass 45S5 used within air-abrasion systems on the performance of self-etching adhesives bonded to air-abraded dentine.

The null hypothesis that air-abrasion performed using pure Bioglass 45S5 or two (15%-40%) PAA-containing Bioglass 45S5 powders do not influence the µTBS and the ultra-morphology of the resin-bonded dentine interface after 24 h or 6 months of PBS storage must be partially rejected.

The results of this in vitro study showed that GB applied on a dentine surface air-abraded with Bioglass or Bioglass/PAA-15 produced µTBS values statistically higher (p<0.05) than those attained on SiC-abraded dentine (control) after 24 h of PBS (Table 2). When GB was applied on Bioglass/PAA-40 air-abraded dentine, the µTBS values were lower than Bioglass or Bioglass/PAA-15 but not different statistically from those attained on SiC-abraded dentine (p>0.05). It is well documented that 4-MET (4-methacryloxy-ethyl-trimellitate) has both excellent adhesion promoting and demineralising qualities due to the two carboxylic groups attached to the aromatic group backbone, providing acidic, wetting properties and establishing an ionic bond with calcium in hydroxyapatite. Therefore, it is possible that the functional monomer (4-MET) contained within the composition of the G-Bond (GB) adhesive system may have established a greater number of Ca/4-MET ionic bonds both with the dentinal hydroxyapatite and with the Bioglass 45S5 embedded on the dentine surface and within the dentine tubules.
In terms of CLSM ultra-morphology results (PBS, 24 h), the only distinctive difference was observed in the adhesive layer of GB applied on Bioglass air-abraded dentine, characterised by very short or absent resin tags created (Figure 1 C-2) due to the obliteration of the dentine tubules by Bioglass. However, only the resin-dentine interfaces created by GB applied on Bioglass/PAA-15 or Bioglass/PAA-40 air-abraded dentine showed limited micropermeability between the dentine and adhesive without compromising the adhesive layer (Figure 2A and 2B). This absence of dye diffusion through the adhesive layer may have been induced by the ability of the multiple pendent carboxylic acid groups present in the Bioglass/PAA and in the 4-MET accumulating at the resin-adhesive interface, binding water so preventing water sorption into the adhesive layer.

Six month PBS storage outcomes offered distinctive information both in terms of µTBS and confocal microscopy. A statistical drop (p<0.05) in µTBS values was observed in all dentine treatment groups although those obtained in specimens created by applying GB on Bioglass or Bioglass/PAA (15% or 40%) were statistically higher (p <0.05) than those µTBS values attained on SiC-abraded dentine (Table 2). A possible reason for this statistical µTBS reduction may be attributed to the relatively high solubility of the Ca/4-MET salts generated by 4-MET over a PBS storage period of 6 months. On the other hand, the higher µTBS results obtained in the specimens air-abraded with Bioglass or Bioglass/PAA might be due to the bioactive and protective properties of the calcium-sodium phosphosilicate (Bioglass 45S5) within the bonding interface.

The presence of Bioglass within the resin-dentine interface may have induced the release of a silicic acid Si(OH)₄ and a subsequent poly-condensation reaction within the thin, demineralised, resin-impregnated collagen layer which interfered with the ability of MMPs to bind the fibrils in specific sites and execute a collagenolytic and gelatinolytic activity. Moreover, the demineralised collagen fibrils may chemosorb the Si(OH)₄ via electrostatic, ionic and/or hydrogen bonding, which condensates into a porous SiO₂-rich layer and serve as a template for apatite precipitation. The sodium (Na⁺) and hydrogen cations (H⁺ or H₂O⁺) exchange and due to the rapid release of Ca²⁺ and PO₄⁻³ from Bioglass, may have contributed, together with the Si(OH)₄ condensation and the apatite
precipitation, also to the inhibition of MMP activity within the Bioglass-air-abraded bonded dentine interface (Table 1)\(^\text{32, 33}\).

The µTBS results obtained from the specimens created using CS3 were significantly higher (\(p>0.05\)) than those obtained by GB in all dentine treatment groups. When CS3 was applied on SiC-abraded, Bioglass or Bioglass/PAA-15 air-abraded dentine (Table 2), no statistical difference in the µTBS results (\(p>0.05\)) was detected. On the other hand, the specimens created by applying CS3 on the dentine surface air-abraded with Bioglass/PAA-40 produced lower µTBS values statistically than those obtained upon SiC-abraded, Bioglass-air-abraded and Bioglass/PAA-15 air-abraded dentine groups (\(p<0.05\)). Nonetheless, after 6 months of PBS storage there was no statistical drop of the µTBS in any of the tested groups. In terms of micropermeability results, no obvious change was observed between the resin-dentine interfaces aged for 24 h and 6 months of PBS storage.

A possible explanation for the results achieved using CS3 compared to GB both after 24h and 6 months of PBS storage may be the presence of the 10-MDP (10-methacryloyloxydecyl dihydrogenphosphate) functional monomer within the CS3 adhesive. This functional monomer is a long carbonyl chain (with lower hydrophilicity compared to 4-MET) acid molecule characterised by the dihydrogenphosphate group, which dissociates in water and forms strong and less dissoluble ionic bonds with calcium (Ca-salts)\(^\text{25, 26}\). Despite the absence of statistical differences after prolonged PBS storage, the high affinity of polyacrylic acid contained in the Bioglass/PAA-40 powder, to absorb water and the presence of ethanol as a solvent for the adhesive system may have influenced negatively its bonding ability. Indeed, it is possible that the vapour pressure of the ethanol solvent was not sufficient to ensure adequate evaporation of the water trapped by the PAA on the air-abraded dentine surface after the bonding procedure\(^\text{26, 34, 35}\). The incomplete evaporation of the water within the resin-dentine interface may have affected also the degree of resin polymerisation\(^\text{36, 37}\), increased the nanoporosities, so jeopardising the bonding ability and the durability of the bonding interface\(^\text{27, 38, 39}\).

In conclusion, it is possible to affirm that air-abrasion procedures performed using pure Bioglass or PAA-containing Bioglass do not interfere with the immediate bonding performance of self-etching
all-in-one adhesive systems formulated with specific functional monomers such as 10-MDP or 4MET. Nevertheless, since the ability of PAA to absorb water, the use of the Bioglass/PAA-40 caries-removal powder would be optimised by following this with bonding procedures where adhesive systems containing high vapour pressure solvents such as acetone are used. However, the durability of the resin-dentine interface will depend also upon the chemical nature and the hydrophilicity of functional monomers contained in the adhesive systems selected for the restorative procedures.

Further experimental studies are in progress to compare the ability of pure Bioglass or PAA-containing Bioglass to remove selectively the caries-infected dentine. A comparison of the influence of these caries removal approaches on the performance of self-etch and etch & rinse adhesive systems must be investigated.

REFERENCES


**FIGURE 1:** CLSM images showing the interfacial characterisation and micropermeability after 24h of PBS storage of the sound and Bioglass air-abraded bonded dentine investigated in this study.

**A:** CLSM image (reflection/fluorescence) of the resin-dentine interface created by GB applied on sound smear layer-covered dentine showing micropermeability (arrow) between dentine (d) and the adhesive layer (a). In the CLSM image captured in fluorescence (Fluorescein excitation/emission only), it is possible to observe an adhesive layer characterised by dye sorption throughout its entire thickness (A1) and by the presence of resin tags (rt) when imaged in rhodamine excitation/emission mode (A2).

**B:** a resin-dentine interface of GB applied on sound smear layer-covered dentine and imaged in reflection/fluorescence showing micropermeability (arrow) between dentine (d) and the adhesive
layer (a). The same specimen imaged in fluorescein excitation/emission shows dye penetration throughout the entire thickness (B1) of the adhesive layer and the presence of resin tags (rt) when imaged in rhodamine excitation/emission mode (B2).

C: CLSM image (reflection/fluorescence) of the resin-dentine interface created by GB on Bioglass air-abraded dentine showing the micropermeability both at the bonding interface between the dentine (d) and the adhesive (a); In this latter layer it is also possible dye uptake (C-1) and nearly devoid of resin tags (C-2).

D: A bonded-dentine interface created using CS3 applied on Bioglass air-abraded dentine and imaged in reflection/fluorescence mode shows fluorescent dye accumulation at the interface between dentine (d) and the adhesive layer (a). In fluorescence mode (fluorescein excitation/emission) clear water sorption within the entire thickness of the adhesive layer can be seen (D-1). In fluorescence mode (rhodamine excitation/emission) only very short or no resin tags can be detected (D-2).

FIGURE 2: CLSM images showing the interfacial characterisation and micropermeability after 24h of PBS storage of the Bioglass/PAA-15 and Bioglass/PAA-40 air-abraded bonded-dentine.

A: CLSM image (reflection/fluorescence mode) of the resin-dentine interface created by GB applied on Bioglass/PAA-15 air-abraded dentine showing an evident micropermeability (arrow) between dentine (d) and the composite (c)/adhesive (a) layer.

B: CLSM image (fluorescence mode) of the resin-dentine interface created by GB applied on Bioglass/PAA-40 air-abraded dentine showing micropermeability in the dentine tubules (t) and an accumulation of fluorescein at the bonding interface situated between dentine (d) and the composite (c)/adhesive (a) layer.

C: Adhesive layer created by GB when applied on Bioglass/PAA-40 air-abraded dentine. It is possible to observe the presence of several resin tags (rt) localised at the bottom of the composite (c)/adhesive (a) layer.
**D:** CLSM image (reflection/fluorescence mode) of the resin-dentine interface created by CS3 applied on Bioglass/PAA-15 air-abraded dentine showing micropermeability (arrow) between dentine (d) and the composite (c)/adhesive (a) layer.

**E:** CLSM image (fluorescence mode) of the resin-dentine interface created by CS3 applied on Bioglass/PAA-40 air-abraded dentine showing micropermeability in the dentine tubules (t) and an accumulation of fluorescein at the interface situated between dentine (d) and the composite (c)/adhesive (a) layer.

**F:** Adhesive layer created by CS3 when applied on Bioglass/PAA-15 air-abraded dentine. It is possible to observe the presence of short resin tags (rt) localised at the bottom of the composite (c)/adhesive (a) layer.

**FIGURE 3: CLSM images showing the interfacial and micropermeability after 6 months of PBS storage.**

**A:** CLSM image (fluorescein/rhodamine B fluorescence mode) of the resin-dentine interface created by GB applied on Bioglass/PAA-15 air-abraded dentine showing micropermeability affecting both the adhesive (a) - dentine (d) interface and part of the adhesive layer (A1).

**B:** CLSM image (fluorescein/rhodamine B fluorescence mode) shows dye accumulation at the interface created by GB applied on Bioglass/PAA-40 air-abraded dentine. In this case, only part of the adhesive layer was affected by dye penetration (B1).

**C:** CLSM image captured in fluorescence mode (fluorescein/rhodamine B) shows a resin-dentine interface created by CS3 applied on Bioglass air-abraded dentine presenting signs of micropermeability both at the adhesive (a) - dentine (d) interface and through the entire thickness of the adhesive layer (C1).
**D:** CLSM image captured in fluorescence mode (fluorescein/rhodamine B) from the resin-dentine interface created by CS3 applied on Bioglass/PAA-40 air-abraded dentine which shows microporosity affecting both the adhesive (a) - dentine (d) interface and the entire thickness of the adhesive layer (D1).
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CONFLICTS OF INTEREST

The authors have no financial affiliation or involvement with any commercial association with direct financial interest in the materials discussed in this manuscript. Any other conflict of interest is disclosed.
Table 2: Microtensile bond strength, number of tested beams and failure mode

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<th>GB: G Bond</th>
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<td>24h</td>
<td>6 months</td>
</tr>
<tr>
<td>SiC-paper (Group 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29.2±7.9 A1</td>
<td>9.4±4.2 A2</td>
</tr>
<tr>
<td></td>
<td>(30/1)</td>
<td>(27/4)</td>
</tr>
<tr>
<td></td>
<td>[91/2/7]</td>
<td>[55/45/0]</td>
</tr>
<tr>
<td>Air-abrasion Bioglass (Group 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.2±5.4 B1</td>
<td>20.1±5.3 B2</td>
</tr>
<tr>
<td></td>
<td>(29/1)</td>
<td>(27/3)</td>
</tr>
<tr>
<td></td>
<td>[93/2/5]</td>
<td>[75/25/0]</td>
</tr>
<tr>
<td>Air-abrasion Bioglass/PAA-15% (Group 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>39.2±4.3 B1</td>
<td>33.8±9.3 C2</td>
</tr>
<tr>
<td></td>
<td>(30/1)</td>
<td>(27/4)</td>
</tr>
<tr>
<td></td>
<td>[89/1/10]</td>
<td>[85/15/0]</td>
</tr>
<tr>
<td>Air-abrasion Bioglass/PAA-40% (Group 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.3±7.3 A1</td>
<td>15.6±3.4 B2</td>
</tr>
<tr>
<td></td>
<td>(30/1)</td>
<td>(27/4)</td>
</tr>
<tr>
<td></td>
<td>[89/1/10]</td>
<td>[85/15/0]</td>
</tr>
</tbody>
</table>

Mean (± S.D.) of µTBS (MPa) to dentine when the two self-etching adhesives were applied after different dentine pre-treatments.

Numbers within parentheses indicate the total number of intact beams/pre-test failed beams.

Numbers within square brackets indicate the percentage mode of failures [Mixed/Adhesive/Cohesive].

Same letter indicates no differences in columns with different dentine treatments maintained in the same storage media.

Same number indicates no differences in rows for different PBS storage time (P > 0.05).
Table 1: Adhesive composition, number of teeth used in each experimental group and pH of the dentine surface after air-abrasion procedures

<table>
<thead>
<tr>
<th>Bonding Systems (Composition and pH)</th>
<th>Dentine pre-treatment and number of teeth for the tests (μTBS/Confocal microscopy)</th>
<th>Dentine pH after air-abrasion procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GB: G Bond</strong></td>
<td>Group 1: SiC-paper (10/6)</td>
<td>~7.5</td>
</tr>
<tr>
<td>(4-Methacryloxy-ethyl-trimellitate, Triethylene glycol dimethacrylate, Urethane di-Camphorquinone dimethacrylates, Acetone. pH ~2.1)</td>
<td>Group 2: Air-abrasion Bioglass (10/6)</td>
<td>~9.2</td>
</tr>
<tr>
<td><strong>CS3: Clearfil S3 Bond</strong></td>
<td>Group 3: Air-abrasion Bioglass/PAA-15 (10/6)</td>
<td>~6.8</td>
</tr>
<tr>
<td>(Hydroxyethyl methacrylate, Bis-phenol A diglycidylmethacrylate, 10 Methacryloyloxy-decyl dihydrogen phosphate, silanated silica di-Camphorquinone, Ethyl alcohol, Water. pH ~2.7)</td>
<td>Group 4: Air-abrasion Bioglass/PAA-40 (10/6)</td>
<td>~6.1</td>
</tr>
</tbody>
</table>