International Journal of Adhesion & Adhesives ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

International Journal of Adhesion & Adhesives

journal homepage: www.elsevier.com/locate/ijadhadh

Strategies to stabilise dentine-bonded interfaces through remineralising operative approaches – State of The Art

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ARTICLE INFO

Keywords:
Remineralisation
Glass-ionomer cements
Bioglass
Dentine
Biomimetic
Bioactive resin-dentine interface
Degradation
Bonding durability

ABSTRACT

Dental adhesive systems have improved considerably over the last ten years, although shortcomings such as post-operative sensitivity, premature reductions in bond strength, interface and marginal degradation, and biocompatibility are still considered important issues with such materials. Enzymatic degradation of collagen fibrils within the hybrid layer and hydrolysis of polymers are the major factors thought to destabilise the resin-dentine interface. However, "smart" resin-based materials that can interact therapeutically with dental hard tissues and reduce the degradation of the resin-dentine interface via remineralisation of the mineral-depleted dental hard tissues can improve the durability of resin-dentine bonds. Moreover, as the resin-dentine interfaces produced by contemporary adhesives are characterised by low mechanical properties, therapeutic remineralising bonding approaches may also contribute to strengthening of hybrid layers, producing more gradual gradients of stiffness that prevents localised stress concentrations. This review attempted to bring together a number of seemingly unrelated events, to show how they may contribute to improvements in the durability of resin-dentine bonds. Innovative new approaches to remineralise the resin-dentine interface may protect hybrid layers from different types of degradations over time, and have a therapeutic role in caries prevention. Recent investigations have revealed that the air-abrasion technique performed with bioactive glass 45S5 (BAG) is capable of creating a therapeutic bioactive smear-layer-covered surface for bonding procedures. BAG can react with body fluids, evoking hydroxyapatite (HAP) precipitation and remineralisation of dentine at the bonded interface, especially when used in combination with fluoride-releasing materials such as glass ionomer cements (GIC) and resin-modified glass ionomer cements (RMGIC). The remineralising potential of these therapeutic approaches is potentiated in the presence of a calcium-sequestering agent such as poly (acrylic acid). However, GIC-based materials as well as calcium silicate cements are not able to restore the mechanical properties of dentine. Thus, experimental adhesive systems containing (30–50 wt%) ion-releasing fillers with advanced remineralising properties and matrix metallo-proteinases (MMP) inhibitors have been developed and used in combination with resin primers containing Ca-sequestering polyanion acids such as poly(aspartic acid) (PASA) or poly(acrylic acid) (PAA) and biomimetic analogues of collagen phosphoproteins such as sodium trimetaphosphate to remineralise resin-dentine interfaces. This biomimetic approach is able to evoke a "bottom-up" remineralisation that restores the original stiffness (i.e. Young's Modulus) of water-rich/resin-poor dentine-bonded interfaces. The next step will be the commercialisation of resin-based materials such as flowable composites and "smart" adhesive systems containing biomimetic reagents that can remineralise and prevent degradation of resin-dentine bonds to enhance their clinical longevity.

1. Overview and basic coverage

It is important to define the terms that will be used in this review. Remineralisation is meant to describe a process of restoring the biomechanical properties of dental hard tissues (i.e. enamel and dentine) that have lost mineral and then, later, regained that mineral. A good example would be remineralisation of acid-etched enamel. The common use of 32–37% phosphoric acid on enamel solubilises several microns of enamel crystallites in a non-uniform manner. This roughened enamel surface contains millions of nano- and micro-sized irregularities that can create mechanical retention when bonded with adhesive resins. The "chalky" appearance of non-resin-infiltrated acid-etched enamel

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http://dx.doi.org/10.1016/j.ijadhadh.2016.03.014
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slowly disappear over days to weeks as salivary ionised calcium and phosphates remineralise enamel by epitaxial crystals deposition using the residual apatite seed crystallites to grow more "enamel" layer by layer [1].

Remineralisation of completely demineralised dentine collagen fibrils is far more difficult because the original demineralising event often removes all of extra-fibrillar and intra-fibrillar apatite crystallites. If one demineralise dentine using ethylenediaminetetraacetic acid (EDTA) for a short time, only some, but not all, of the extra-fibrillar apatite crystallites are removed. When exposed to appropriate level of ionised calcium and trivalent phosphates, those surfaces may regain mineral by epitaxial deposition [2].

The use of 32–37% phosphoric acid to demineralise dentine, removes all crystallites from dentine collagen; no extra-fibrillar or intra-fibrillar apatite minerals remain to serve as residue patterns for nucleation of new crystals. If one exposes such dentine to supersaturated solution of Ca^{++} and PO_4^{3-}, the structure will take up mineral, but the mineral will be on the collagen fibrils rather than "in" them. This is usually due to the Ca/P crystals growing too rapidly. In order to reinstate the mechanical properties of dentine collagen fibrils, specific Ca/P compounds such as amorphous calcium phosphate must fill the nanometric-sized gap regions in demineralised dentine collagen [3].

Most authorities agree that these crystals begin in the gap region of the fibrils that are only 40 nm long. If any Ca/P crystals are larger than 40 nm, they may not "fit" into demineralised collagen. This is the case of most contemporary remineralising techniques used to remineralise dentine collagen at the bonding interface (i.e. glass ionomer cements and calcium silicate cements) [4]. Conversely, the most recent research suggests that amorphous (non-crystalline) calcium phosphate enters collagen fibrils in a biomimetically stabilised "fluidic" state. The biomimetic substances that can restrict the size of the Ca/P are generally polyanionic compounds such as PAA, PASA and other poly(carboxylic acids). These all bind ionised calcium to lower its functional concentration and size, while it slowly infiltrates nanometre-sized water-filled spaces within the collagen fibrils.

The presence of nanometre-sized apatite crystallites inside collagen fibrils can only be observed by high-resolution transmission electron microscopy (TEM). Measurements of mineral density using technique such as X-ray density or energy-dispersive X-ray spectroscopy (EDX) only show mineral deposition; too often, that mineral is on and not in collagen fibrils. Such remineralisation cannot re-establish the stiffness of mineralised dentine back to its original values (18-22 GPa). Nano-indentation of resin-dentine interfaces provides important, quantitative information of local hardness and stiffness [5,6]. In multilayer composites, when layers have very different degree of stiffness, application of stress to those composites creates high stress concentrations where the difference in stiffness are greatest. When dentine surfaces are acid-etched to completely demineralise them, the stiffness of the mineral-free water-saturated demineralised dentine matrix was reported to be as lower as 134 KPa [7]. However, even if water-saturated dentine matrix could be perfectly infiltrated with adhesive resins, the stiffness of these polymerised resins is only 3–4 GPa [6]. Thus, one would expect stresses to concentrate at the top of the adhesive and hybrid layer covered with stiff resin composite (10–15 GPa) and at the bottom of the hybrid layer were water-filled demineralised matrices meet the underlying mineralised dentine (18–21 GPa).

Ideal multi-layered composites exhibit a "smooth" gradient of stiffness that precludes localised stress concentrations. When resin-bonded dentine has been "back-filled" with apatite minerals, those regions of the hybrid layers have stiffness values close to 18 GPa, while resin infiltrated regions only 3–4 GPa [5,6]. The ideal dentine hybrid layer would be 2–3 μm of partially demineralised collagen fibrils, held open by infiltration of a 3–4 GPa adhesive resins covered by a flowable resin-based composite that contains calcium and phosphates, along with biomimetic molecules like polyanions acid. The ion-releasing flowable composite would slowly release polyanion-stabilised-calcium and phosphate ions (i.e. amorphous calcium-phosphate), that would slowly replace water in the resin-infiltrated collagen with apatite crystals [8]. This would increase the stiffness of the resin-infiltrated dentine to 10–15 GPa. As the therapeutic resin composite releases its filler contents, the resin-dentine interface would ultimately become filled with apatite crystals so that the stiffness of the completely remineralised dentine can reach values of 18–20 GPa [5,6]. The end result would be collagen fibrils that regained their original stiffness and were re-fossilised so that even their endogenous proteases would be inactive due to remineralisation of the collagen to which they are bound [9].

2. Background and introduction

Dr. Buonocore [10] introduced the first fundamental concepts for the adhesion of resin-based materials to hard dental substrates such as enamel. Essentially, he recommended the use of 85% ortho-phosphoric acid to chemically condition the enamel, thereby forming microporosities in which liquid resin would permeate, creating nano- and micro-resin projections into enamel after polymerisation [11]. It is generally agreed that enamel bonding is predictable and successful when etched with phosphoric acid [2]. This is mainly due to the unique composition of enamel, which is made of very stiff hydroxyapatite crystals (~96 wt%), and 1–2 wt% of enamel-specific developmental proteins known as enamelins bound to hydroxyapatite crystals [12,13]. Enamel contains no collagen, and the only metalloproteinase identified in enamel is MMP-20 [9].

Conversely, dentine comprises the bulk of the tooth. It is made of 50–70 vol% calcium-deficient and carbonate-rich hydroxyapatite [HAP: $Ca_{10}(PO_4)6(OH)_2$]. The crystal size in dentine (5 nm × 30 nm × 100 nm) is smaller than in enamel, with less calcium than stoichiometric HAP, but also contains 4–5% of carbonates. Dentine also contains 30% of organic component which is represented by: i) 90% type I collagen with a very minimal amounts of type III and V collagen, and 10% water-rich non-collagenous proteins (NCPs) such as proteoglycans and glycosaminoglycans; ii) 20% of the volume of the dentine is made of free (e.g. intratubular fluid) and bound water [14]. Non-collagenous proteins are able to bind selectively to different surfaces of the forming apatite crystals, guiding their growth in certain directions and allowing various crystal shapes, (e.g. needle-shaped and plate-like crystals). Indeed, HAP crystals are closely packed and incorporated with and between the collagen fibrils [15,16].

One of the most characteristic aspects of dentine is the presence of long microscopic tubules of about 0.8–2.5 μm in their inner diameter, that are filled with the odontoblast cytoplasmatic process (Fig. 1A) and intratubular fluid. Transdentinal fluid movement accounts for extreme sensitivity of exposed dentine surfaces, due to the outward seepage of pulpal fluid (Fig. 1B) [17]. The difference in intrinsic moisture between the superficial dentine and deep dentine may be responsible for lower resin-dentine bond strengths in deep dentine due to the insolubility of some dimethacrylate monomers such as bis-GMA (bisphenol-A-diglycidil dimethacrylate), TEGDMA (triethyleneglycol-dimethacrylate) and UDMA (urethane-dimethacrylate) in water-saturated dentine [18,19]. The lumen of the tubules is surrounded by a dense collar of hypermineralised peritubular dentine (PTD), while dentine between the tubules is known as intertubular dentine (ITD). The amount of mineralised collagen fibrils in ITD, and other non-collagenic proteins oriented orthogonal to the tubule long axis is about 30 wt%, while much smaller amount of collagen,

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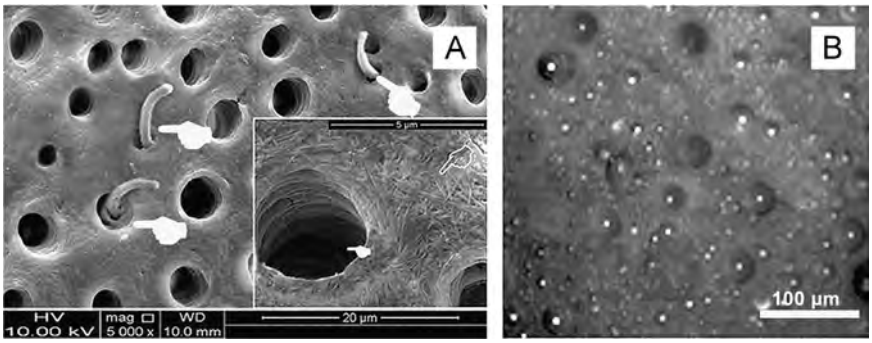

Fig. 1. SEM micrograph and TSM images of dentine. A: SEM micrograph showing some remaining odontoblast processes inside dentinal tubules (pointer). In the higher magnification image in the insert, it is possible to see open tubules (white pointer) and exposed collapsed collagen fibrils (pointer). B: Tandem scanning microscopy (TSM) image (× 20/0.8 NA) of dentine specimens under simulated pulpal pressure of 20 cm H_2O which induced the formation of water droplets as dentinal fluid transudated from the dentinal tubules. (All the images in this figure are original and never published before).

(~10 wt%) can be found in the PTD [20]. The intrinsic wetness of dentine as well as the collagen phase in dentine decreases the surface energy making successful resin bonding difficult [11]. This is, in part, responsible for the reduction in durability of resin-dentine bonds compared to resin-enamel bonds [2,6], although water is extremely necessary during bonding procedures to avoid collapse of demineralised collagen fibrils [21].

Oskar Hagger, the "Father of Modern Dental Adhesives" developed the first adhesive system in 1949 by incorporating dimethacrylate-glycerophosphoric-acid (GPDM) in a liquid cavity sealer (Sevriton Cavity Seal®; Swiss Patent No 278946, 1951) along with a chemically cured resin-based restorative material (Sevriton®) [22–24]. This system was able to create an interaction zone between dentine and Sevriton®. This was first demonstrated by histological examination [25] and subsequently, the presence of a unique interface was confirmed by using TEM analysis that showed these resin-based systems only penetrated few microns (~3 µm) into dentine; Nakabayashi called it the hybrid layer [26], since it was neither resin nor dentine, but a combination of both. Moreover, Eick et al. [27], using scanning electron microscopy (SEM) confirmed that monomers would penetrate into dentine. However, since smear layer covered both sides of failed adhesive bonds, it was concluded that the apparent bond strength was actually a measure of the cohesive forces within the smear layer particles [28]. These types of adhesive systems were used between 1960 and 1970 and were defined as first-generation and second-generation bonding agents. A typical first-generation system contained a functional co-monomer NPG-GMA (N-(2-hydroxy-3-methacryloxypropyl)-N-phenylglycine) that is able to chelate calcium ions within the smear layer and generate bond strength of 2–3 MPa. The second-generation bonding agents usually contained an acidic phosphate-ester monomer such as phenyl-P [2-(methacryloxy)ethyl phenyl hydrogen phosphate] and an hydrophilic resin monomer (HEMA: hydroxyethyl methacrylate) in ethanol solvent. Its mechanism of action was based on electrostatic interactions between the monomers' phosphate groups and calcium ions within the smear layer that could attain bond strength of 5–6 MPa. Such week bonds could not withstand the polymerisation contraction forces (12–15 MPa) that developed when these systems were polymerised [29], and debonded from the cavity margins, causing marginal leakage and permitted bacterial leakage [11,28]

In the 1980s, the third-generation of bonding agents was introduced. These adhesive systems employed a strong acid etchant, usually 37% phosphoric acid, to entirely remove the smear layer and to fully demineralise the underlying dentine matrix to a depth of 8–10 µm. A separate primer was designed to penetrate into the demineralised dentine and dentinal tubules in an attempt to increase the bond strength. However, bond strengths were still below the forces of polymerisation contraction [29,30]. Moreover, the bonding performance of these adhesive systems was usually affected by degradation over time; this caused margins discoloration, microleakage and secondary caries [23,28]. There was concern that etchants were demineralising the dentine matrix deeper than monomers could infiltrate. Moreover, too often, clinicians were over-drying dentine when drying the enamel, causing the collapse of collagen fibrils. In an attempt to improve resin-infiltration of dentine, Kanca [21] introduced the wet-bonding technique.

In the early 1990, a new family of adhesive systems (4th generation) was created which required an etchant, a primer, and an adhesive. The hydrophilic primer could penetrate both etched dentinal tubules and dentine substrate to facilitate the penetration of more hydrophobic adhesives. At that moment in history, there was a substantial revolution in adhesive dentistry. Indeed, the fifth generation of adhesive systems were developed at the end of 1990s, by combining the primer and adhesive into one bottle that simplified the bonding steps from three-steps to two-steps. Some authors call these “self-priming adhesives”.

In the early 2000s, a new class of adhesives was introduced as sixth-generation systems. These were called “self-etching primers and adhesive” and represented a significant step advance in technology. These adhesive systems, the acid-etching step using phosphoric acid was eliminated by the incorporation of sufficient functional acidic monomers into the primers that were able to etch and prime the enamel and dentine simultaneously, followed by the application of a separate solvent-free, relatively hydrophobic adhesive [31,32].

The latest adhesives are known as “all-in-one” and/or “universal” adhesives that combine etchant, primer, and adhesive in a single solution which can be used both with phosphoric acid etching pre-treatment or as a self-etching adhesive [11,32]. Currently, only two main classes of adhesive systems are available for clinical applications; etch-and-rinse adhesives (ERAs) and self-etching adhesives (SEAs). We include the glass ionomer materials in the self-etching category [11,32].

It is curious how Dr. Hagger's concepts have been adopted by current researchers to generate new dental adhesives with longer lasting performance. Today, after many years of accepting that the key to the success of dental adhesives is the micromechanical retention resulting from acid etching of dentine and enamel, modern concepts about dentine adhesion are firmly based on the Dr. Hagger's original conceptions that bonding can be achieved via molecular interactions between adhesives and tooth surfaces [23].

The incomplete infiltration of adhesive systems into demineralised dentine, particularly when ERAs are used, represents a clear impediment to clinical success of resin-based dental restorations [16,33,34]. Most adhesive systems produce very good immediate

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bond strengths, but the long-term strengths are still a cause for concern in restorative and adhesive dentistry [16,35]. Complete infiltration of resin into demineralised dentine would be the ideal situation because acid-etching with phosphoric acid also uncovers and activates endogenous proteases of the dentinal matrix, such MMPs and cysteine cathepsins [9,16,31]. These proteases are hydrolases, so they require water to hydrolyse collagen. However, the complete infiltration and the replacement of all water by resin is seldom achieved, thus creating water-filled nano- and micro-porosities within hybrid layers where collagen fibres remain unprotected, and undergo enzymatic-mediated hydrolytic degradation jeopardising the durability of the resin-dentine bonds [16,31,32].

Degradation of the hybrid layer causes decreased durability of resin-dentine bonds, and continued destruction of tooth structure. This revision will attempt to show how new technologies can contribute to improvements in the durability of resin-dentine bonds. Innovative approaches to remineralisation of the resin-dentine interface may protect hybrid layers from degradation over time.

3. Synopsis on adhesive systems currently used in dentistry

For many years, adhesives systems have been categorised into "generations" in order to schematise the different components (e.g. etchant, primer, and adhesive) of all classes of products. However, the use of such a classification based on adhesive generations is not universally accepted. Moreover, as a great number of new materials are created every year, this type of classification has become rather clumsy and confusing.

Contemporary adhesives are currently classified based on their mode of interaction with dental hard tissues as either etch-and-rinse adhesives (ERAs) or self-etch adhesives (SEAs) [31,32]. ERAs require complete removal of smear layer and complete demineralisation of superficial dentine by 32-40% phosphoric acid etching (Fig. 1A), which is then rinsed, followed by the application of a resin primer, and adhesive (three-step systems) or by a single-bottle self-priming agent (two-step systems) [31]. Conversely, self-etching adhesives only partially remove the smear layer (Fig. 2A and B) and expose a very thin layer of demineralised collagen (Fig. 2C). This is achieved through the use of acidic methacrylate primers containing phosphate or carboxylic functional monomers, such as glycerophosphate dimethacrylate (GPDM), 10-methacryloxydecyl-dihydrogen-phosphate (10-MDP) or 4-methacryloxyethyl-trimellitic-acid (4-META). After allowing the primer to etch for 10–20 s, the primed dentine is not rinsed with water, but the 10-15% water in the primer is evaporated with air, and is then covered with an adhesive and light-cured (two-step systems). The one-bottle or all-in-one adhesives involve application of 1–3 layers a single bottle adhesive that contains acidic-methacrylates, hydrophilic monomers such as HEMA and some cross-linking di-methacrylates such as UDMA, TEGDMA, etc. (all-in-one single-step systems) [31,32].

Although the composition and chemistry of the adhesives differ between the different classes, they all have common basic ingredients such as acrylic-based resin monomers, organic solvents, initiators, inhibitors, and sometimes nano-filler particles [36]. Cross-linking di-methacrylates and functional monomers represent the main components of an adhesive system. The structure of these monomers can be classified in three distinct parts: i) one or more polymerisable methacrylate or acrylates groups; ii) a carbon spacer-chain; iii) and in some cases a functional acidic group. Being part of a large molecule (e.g. bis-GMA), they generally show a hydrophobic behaviour as di-methacrylates are not water-soluble. Such adhesives are usually solvated in ethanol, acetone or HEMA [32,36,37].

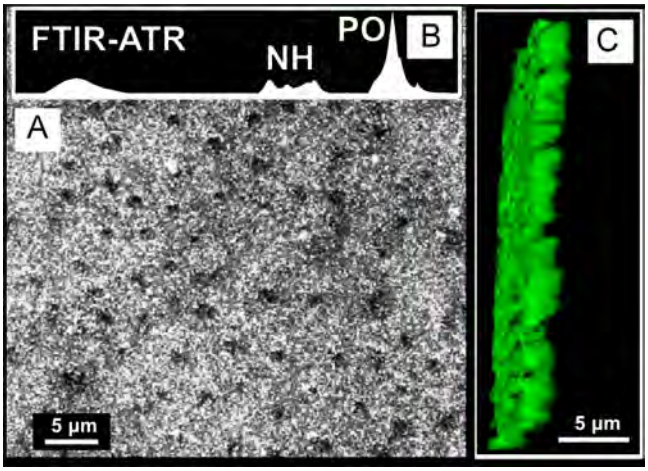

Fig. 2. FTIR analysis and confocal (CLSM) z-stack single projections and topographical reconstructions. A: Confocal image showing the presence of residual smear layer both on the dentine surface and inside the dentinal tubules. These results were confirmed by the FTIR (B) analysis which showed high intensity peaks related to P-O in hydroxyapatite. A very thin layer ($<2\,\mu m$) of demineralised fluorescein-stained dentine collagen could be detected during fluorescence confocal imaging. The smear layer-covered dentine was etched with a primer containing 20 wt% glycerophosphate dimethacrylate (Universal New Extra Bond One, DEI-Italia, Varese, Italy) for 20s, rinsed with acetone (30 s) and deionised water (1 min) (C). Confocal images in reflection and fluorescence mode were performed as described by Sauro et al., [174,177], using sodium fluorescein to stain the exposed collagen. (All the images in this figure are original).

Regarding the carbon spacer-chain, its main function is to keep functional and polymerisable groups well separated, thereby protecting the properties of the monomer and the hydrophilicity of the resulting polymer. Moreover, the polarity of the spacer may influence the solubility of the monomer during aging. For instance, the use of hydrophilic spacers of monomers in an adhesive system can increase water sorption and solubility, while the use of hydrophobic spacers can decrease these values [38,39].

Functional monomers may serve several purposes, such as etching for demineralisation and infiltration of the dental hard tissues, wetting, release of fluoride, and antibacterial effects [40,41]. However, an additional role of some functional monomers is to promote chemical bonding to the calcium of the HAP present in dental substrates [42,42]. The most common functional groups used in commercial adhesives are carboxylic acid-based monomers, (i.e. 4-META) and phosphate-based monomers such as 10-MDP or GPDM. However, many other functional monomers are currently being used or are being developed [36].

The bonding potential of functional monomers is related to their acidity. Indeed, it is generally accepted that if an adhesive system has a very low pH [pH <1], its acid reaction with HAP is excessively aggressive, leaving the dentine almost completely demineralised [40,44]. Thus, the resulting hybrid layer, which no longer contains HAP crystals, is prone to degradation processes similar to those occurring when simplified ERA systems are employed [16,32]. However, comparing the different efficacies of functional monomers in aqueous environments, our results (Fig. 3) demonstrated that the mildly acidic resin-bonding promoted by 10-MDP produces more effective and stable bonds to HAP, Further studies [32,40] suggest that 10-MDP produces more effective and stable ionic bonds to HAP compared to those other types of functional monomers (i.e. 4-META, Phenyl-P) currently employed in adhesive formulations. One more important research outcome to highlight is that 10-MDP may inhibit secondary caries [45]. This conclusion is derived by the presence of an acid-base resistant “brushite-rich” dentine zone created at the base of hybrid layer formed by self-etching adhesive containing 10-MDP. Indeed, this

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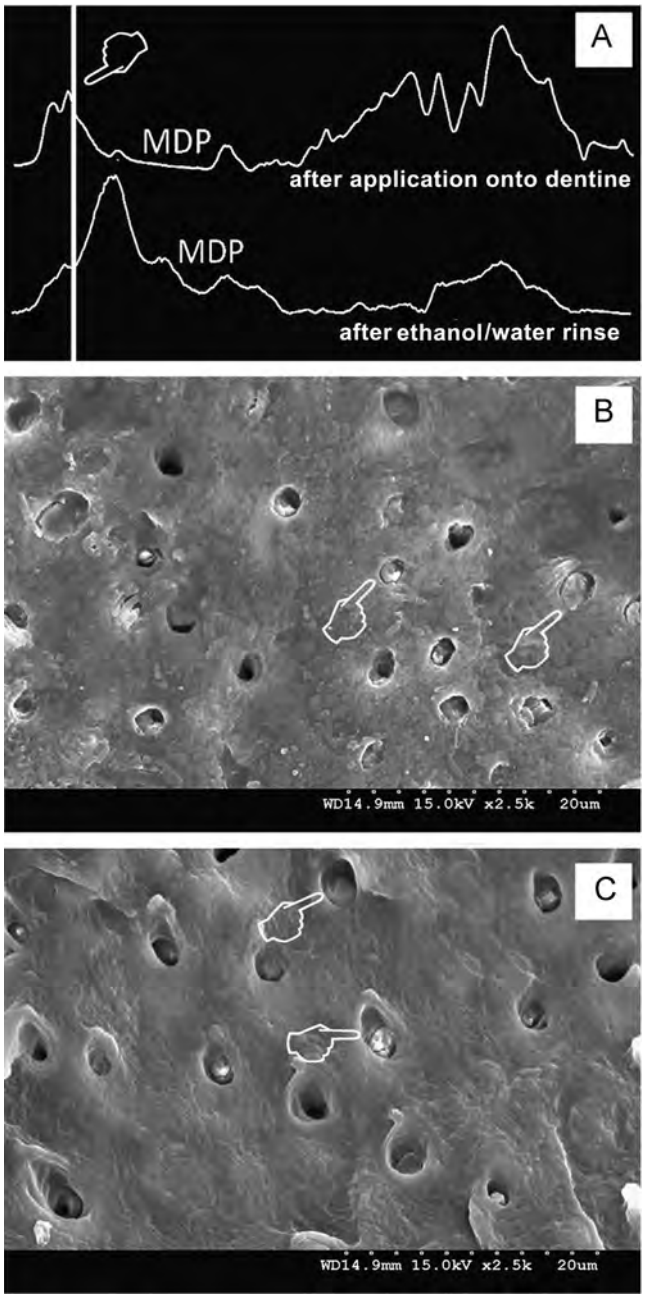

Fig. 3. FTIR and SEM image attained following the protocol suggested by Feitosa et al., [38,39]. A: The FTIR spectra show that 10-MDP bonded to the dentine surface (pointer) before and after challenge (1 min in distilled water and 30 s in absolute ethanol) due to its strong chemical affinity to calcium in dentine. B: Scanning electron microscopy (SEM) micrograph depicting the chemical interaction of the 10-MDP with dentine surface. Note the presence of residual smear plugs inside the tubules below the dentine surface (Pointers). C: SEM micrographs taken from a dentine specimen treated with 10-MDP and subsequently rinsed with water/ethanol challenge. In accordance with the FTIR analysis, the surface appears still covered by the functional monomer and the dentine tubules, although slightly wider, are still partially obliterated by smear plugs (Pointers). (All the images in Fig. 3 are original and never published before).

zone contains densely packed crystals resistant to demineralising solution (pH 4.5) and sodium hypochlorite, [41,45]. However, further studies are still ongoing to elucidate the mechanism of formation of this "ultra-resistant" dentine layer.

Controversy remains regarding how much chemical bonding contributes to the total retention of resin-dentine bonds. Some suggest chemical bonding might only contribute 10%, while others argue that it contributes more to retention. Chemical bonding is probably high on very flat surfaces that are close to each other, but it is more difficult to demonstrate in typical microscopically rough enamel and dentine surfaces [31]

4. Pathways for hybrid layer degradation

Over the last few decades, dental materials have evolved and improved, particularly in the field of restorative dentistry, especially adhesive systems and resin composites. However, the longevity of dentine adhesive restorations still remains a challenge as such failures lead to recurrent caries (e.g. secondary caries), and represent the main reason for replacement of direct restorations [46,47]. Indeed, more than 50% of restorations being replaced have been reported to be caused by formation of secondary caries [46,48]. The longevity of amalgam is over 20 years, while adhesive/composite restorations are estimated to endure in the patients' mouth less than 7 years [49,50]; the failure rate of posterior resin composite restorations after 7 years can be 50% greater than that of high-copper content amalgams [51,52].

The issue about the longevity of adhesive composite restorations is related to two main mechanisms which have been identified to contribute to resin-dentine hybrid layer degradation: i) Intrinsic or proteolytic degradation of the organic matrix; ii) Extrinsic or hydrolytic degradation of the resin matrix. Both mechanisms are interlinked and occur simultaneously, decreasing the durability of resin-dentine bonds.

4.1. Proteolytic degradation of the collagen matrix

As stated previously, most of the organic substance in dentine is type I collagen and the remaining portion of organic substance (~10 vol%) is comprised of non-collagenous proteins, including proteoglycans, phospholipids and matrix proteases. Recently, endogenous dentinal proteases, especially matrix metalloproteinases (MMPs) and cysteine cathepsins, have been identified and these are receiving a great deal of attention because of their potential role in hybrid layer degradation [9,16].

As recently reported by Sabatini and Pashley [53], human MMPs are a family of enzymes that are divided into 6 groups based on their structural homology and their substrate specificity: collagenases (MMP-1, MMP-8, MMP-13, and MMP-18); gelatinases (MMP-2, and MMP-9); stromelysins (MMP-3, MMP-10, and MMP-11); transmembrane (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25); other (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-27, and MMP-28)". These endopeptidases are dependent on calcium and zinc, and upon activation, these proteases can break down nearly all proteins within the extra-cellular matrix (ECM) [54].

These enzymes play many important physiological roles including angiogenesis, tissue remodelling and dentinogenesis [55], but are also involved in dentinal caries, pathological degradation of periodontal ligament (periodontal disease), and resin-dentine hybrid layers [42,56]. It has been demonstrated through immunohistochemical analysis that MMP-2 and MMP-9 are intrinsic proteases of the organic collagen matrix in dentine, and thus may potentially play a role in the destruction of the dentine collagen [57]. MMPs, in their typical natural state, are present as inactive zymogens in order to protect their connective tissue environment. A change in chemical morphology is necessary in order for them to become activated. Inhibitors of metalloproteinases (TIMPs) are just one mechanism that has been postulated to control the functional activity of MMPs, as they bind naturally to MMPs, when they are in their active form, inhibiting their hydrolysis and proteolysis activities [9,32].

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Sulkala et al., [58] confirmed that both phosphoric acid and mild acids, such as acidic resin monomers, can inhibit or displace TIMPs and thus allow MMPs to become active. However, more studies are required to completely understand the inhibition of TIMPs as it may help prevent hybrid layer degradation. On the other hand, Nishitani et al., [59] provided evidence of pathological collagenolytic and gelatinolytic activity, by MMP gelatinases (MMP-2 and MMP-9), within dentine that was previously treated using ERAs and SEAs adhesives to partially demineralise dentine. The same study also indicated that 37% phosphoric acid used in ERAs had a pH low enough to temporarily inactivate some of the endogenous MMPs. A later study demonstrated that this was due to the very high PO_4^{3-} content of phosphoric acid that forms $CaHPO_4$ when it reacts with mineralised dentine. This calcium phosphate covers collagen and MMPs with a fine precipitate that slowly solubilise over several days to weeks, allowing the activated proteases to attack collagen fibrils [60]. Many etch-and-rinse adhesives are able to solubilise this fine precipitate and reactivate the MMPS due to the presence of acidic functional monomer within their composition [61].

Self-etching primers can also activate these enzymes [46]. Indeed, Mazzoni et al., [62] showed using zymography analysis that significant gelatinolytic activity could be seen within the resin-dentine hybrid layer when using 2-step ERAs adhesives, suggesting that the activation of the dentinal enzymes may occur via a two-step process. This process starts with the initial demineralisation and exposure of collagen fibrils, and then the acidity of bonding systems may cause secondary enzyme activation. That study also provided more evidence that the proteolytic and gelatinolytic activity that destroys collagen fibrils, begins at the bottom of the hybrid layer, in particular if it is incompletely infiltrated by resin. Hence, as the bottom half of the hybrid layer degrades, the MMPs spread into the upper regions of the resin-bonded dentine, increasing the porosities within the hybrid layer [33] (Fig. 4A and B).

Cysteine cathepsins have also been suggested to cause hybrid layer degradation. They are a group of 11 human proteases with "broad substrate specificity", but normally present as inactive zymogens [9,32,63]. The activation of these papain-like enzymes can occur in conditions of slightly acidic, but this can also occur in alkaline conditions (pH 11–13). Tersariol et al., [63] identified cysteine cathepsin B in human dentinal tubules using DNA microarrays. Cysteine cathepsins have also been shown to have proteolytic activity that positively correlates with MMPs and they also, in the same way as MMPs, can be activated after the use of a low-pH adhesive [53,64]. It is therefore reasonable to suggest that the activation of cysteine cathepsins may result in the degradation of the resin-dentine hybrid layer [65,66].

There is a current consensus that collagen degradation occurs more in ERAs since the use of phosphoric acid etchants demineralise dentine more deeply and more completely, leaving collagen fibrils exposed, and making them more susceptible to proteolytic degradation by the endogenous dentinal enzymes (Fig. 4B) [67]. With the use of milder SEAs, degradation still occurs but is less when compared to ERAs, because the use of the milder acidic primer does not totally expose the collagen fibrils and leaves small smear plugs within the dentinal tubules [43,68,69] (Fig. 2A). It is also noteworthy that the demineralisation and resin-infiltration occurs simultaneously when using SEAs [43].

4.2. Hydrolytic degradation of the resin matrix

As previously mentioned, the immediate bonding performance of most adhesive systems currently in use is excellent. Conversely, the main drawback of these systems, in particular those with "simplified" composition, is their poor durability. An adequate penetration of the adhesive into the demineralised dentinal matrix and the formation of a "high quality" and durable hybrid layer are compulsory requirements to ensure long-term bond strength [11,16].

The most commonly used bonding technique for ERAs is "water-wet bonding". This method allows the dentinal surface to remain wet with water that prevents the collapse of the demineralised dentinal matrix that occurs after extensive air drying [17,69]. However, it has been suggested that excess water would also compete with hydrophilic resin monomers in the penetration into the dentinal matrix, thus contributing to incomplete infiltration of the resin [34,70]. The volume of water remaining within the demineralised dentine is difficult to control, and excess residual water can cause phase separation [71,72] and "water blisters" [34,47,73]. Unfortunately, even the inclusion of small amounts of water may culminate in nano-phase separation of the adhesive components in the form of water-trees between the polymerised hydrophilic and hydrophobic resin phases; this may increase uptake water and jeopardise the mechanical properties of the polymerised adhesives [68,74,75]. No dimethacrylate monomers can infiltrate into water-filled spaces in hybrid layers [31].

Hydrolysis of resinous polymers and subsequent elution of the degraded products represent a major factor thought to destabilise the resin-dentine interface [68,75]. Unlike proteolytic degradation of the hybrid layer, hydrolysis of polymerised resin matrix occurs in SEAs and ERAs and is related to water sorption occurring within the synthetic polymers. Loss of covalent bonds between the

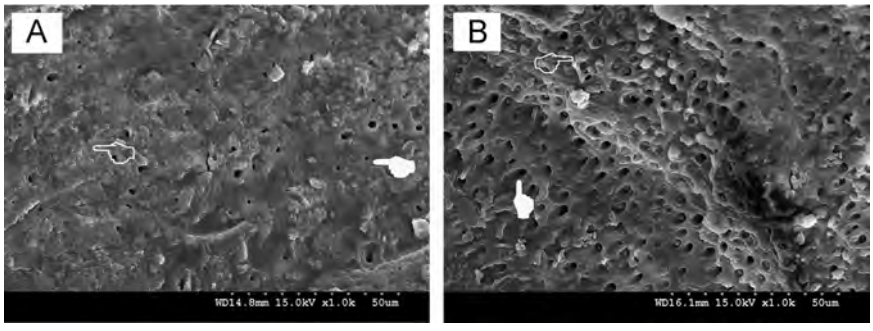

Fig. 4. Fractographic SEM analysis performed after micro-tensile debonding of specimen created with self-etching adhesives under simulated pulpal pressure for 6 months. A: SEM micrograph of a fractured dentine specimen previously bonded with a self-etching adhesive containing 20 wt% glycerophosphate dimethacrylate (Universal New Extra Bond Two, DEI-Italia, Varese, Italy) and submitted to microtensile bond strength test after 6 months under simulated pulpal pressure. Note that the dentine surface remains covered by polymerised resin (open pointer) and only a few tubules are partially exposed. There was limited degradation of the interface. B: SEM micrograph of a fractured dentine specimen previously bonded with a self-etching (pH 0.8), Adper Prompt (3M ESPE. St. Paul, MN, USA) and subsequently submitted to microtensile bond strength test after 6 months under simulated pulpal pressure. Note that the fracture occurred at the bottom of the hybrid layer where the dentine surface contains many wide funnelled tubules (closed white pointers). Very few tubules remained occluded by resin tags (open pointer). (All the images in this figure are original and never published before).

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polymers can also occur via salivary esterase due to the addition of water to the ester bonds [11,74,76,77]. Subsequent to the elution of degraded monomers, it is possible to have a decrease of the mechanical properties of the resin-dentine interface which causes an increase in water uptake. This water can then reduce the frictional forces between the polymer chains by inducing polymer swelling and plasticisation of the resin matrix [32,75,78].

An important factor with this type of degradation is related to the chemical components within the composition of the different adhesives [32,76,77]. The increased demand for simpler adhesive protocols has led to the formulation of adhesive systems with fewer application steps such as 1-step SEAs and 2-step ERAs adhesives. It is well known that the new fewer-step adhesives exhibited lower bond strength after aging and less predictable clinical results, compared to the other more complex protocols. New "all-in-one" adhesives are too hydrophilic and allow excessive water sorption that lead to hydrolytic degradation of the resin matrix [47,76,79]. Indeed, manufacturers have also incorporated high concentrations of hydrophilic (i.e. HEMA) and/or functional carboxylic and phosphate monomers (i.e. 4-META) to make these adhesives even more compatible for bonding to intrinsically moist, acid-etched dentine. Moreover, ester linkages typically present in methacrylate-based monomers can be susceptible to degradation induced by several esterases present in body fluids (e.g. saliva and intratubular fluid) [76–78]. The ability of esterases to attack ester bonds in poly-methacrylates can be reduced by incorporating a bulky side chain next to the ester bond [74].

It has been generally accepted that 3-step ERAs and 2-step SEAs adhesives are the "gold standard" in dental adhesion. One reason for their success is that the primer is separated from the adhesive components which allows placement of a solvent-free hydrophobic adhesive layer over the hydrophilic primer. This seems to reduce water sorption at the resin-dentine interface [80–83]. Moreover 2-step SEAs have been shown to be less technique-sensitive and therefore may be more recommendable than 3-step ERAs adhesives when dealing with extensive dentine cavity preparation [32,77,80,84]. Unfortunately, none of these "passive" adhesive systems can compensate for the degradation of the resin-dentine interface with mineral precipitation which can fill the micro- and nano-porosities within the hybrid layer as well as inhibit and fossilise the endogenous dentine proteases [9,16,66]. Conversely, this would be possible when using materials and/or bonding procedures that can evoke mineral uptake within the bonded-dentine interface, especially in presence of "smart" bioactive/biomimetic ion-releasing resin-based materials [5,6,8,10]

5. Therapeutic bonding strategies and stability of resin-dentine interface

As previously stated, modern adhesive systems are currently classified as: "etch-and-rinse adhesives" (ERAs), "self-etch adhesives" (SEAs) or "glass inomer-based materials" (GICs). This latter class of materials include classic GICs and RMGICs that can be also used for bonding purpose [32,75].

The main difference between these adhesives is their degree of "invasiveness". Generally, ERAs adhesive systems induce substantial modification of the bonding substrate (e.g. total demineralisation of the substrate via acid etching), which exceeds that of SEAs and RMGIC that intensively interact with the dental hard tissues modifying the smear layer and only partially exposing collagen fibrils (Fig. 5). RMGIC are also identified as self-adhesive materials; they are the only true 'self-adhesive' materials as they can bond to dentine micromechanically, through infiltration of the collagen network previously exposed by using a (10%)-PAA pretreatment, in combination with chemical bonding obtained by

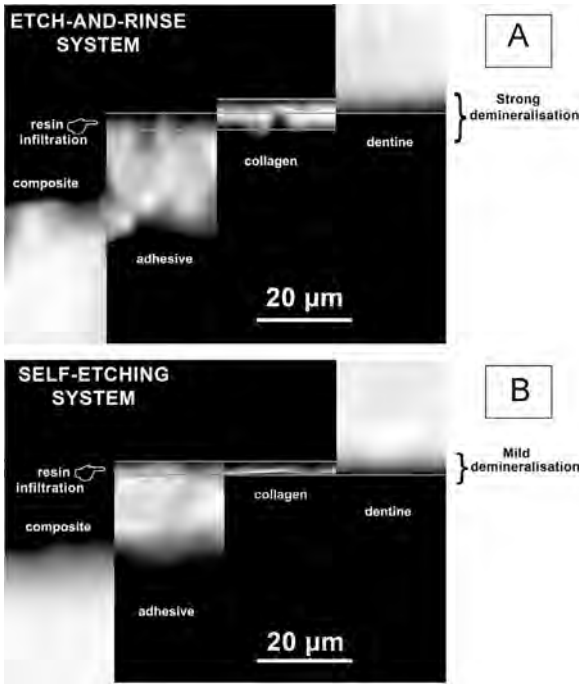

Fig. 5. Raman spectroscopy images performed along resin-dentine interfaces created with: A: etch-and-rinse or B: self-etching adhesive following the protocol of specimens preparation and imaging of Almahdy at al., [175]. A: Images obtained after cluster analysis of the Raman data which produced four independent spectral components representing 1) mineral in sound dentine, 2) protein content (collagen), 3) adhesive components , 4) composite components along the resin-dentine interface created with an etch-and-rinse adhesive. The distribution of the 4 Raman components along the resin-dentine interface shows a thick (8–10 μm) layer of demineralised collagen only partially infiltrate by the adhesive; the bottom part remained poorly resin-infiltrate (pointer). B: Images obtained after cluster analysis of the Raman data which produced four independent spectral components representing 1) mineral in sound dentine, 2) protein content (collagen), 3) adhesive components, 4) composite components along the resin-dentine interface created with an self-etching adhesive. The distribution of the four Raman components along the resin-dentine interface shows a very thin layer < 2 μm) of demineralised collagen completely infiltrated by the adhesive (pointer). (All the images in this figure are original and never published before).

ionic interaction of carboxyl groups from the acid with calcium ions of remaining HAP crystals [83,85].

The good chemical bonding of glass ionomers to dental tissues along with their ability to release specific ions (e.g. fluoride) at the bonded interface, made this adhesive material the main therapeutic bonding cement, that has both antibacterial and remineralising properties, currently available in clinical dentistry [32,83,85]. Furthermore, GIC-based materials are biocompatible and have thermal expansion coefficients that match that of dentine [86].

Further ion-releasing therapeutic approaches, such as the pretreatment of dental substrates using BAG in air-abrasion devices, are currently used in restorative dentistry to create a "bioactive smear layer" within the interface, which can be incorporated within RMGIC and SEAs during bonding procedures, and remain "therapeutically" available at the bonding interface (Fig. 6). The ion-release ability of such bioactive glasses (e.g. 45S5) favours remineralisation and protection of the bonded interface [87,88].

Several experimental resin-based ion-releasing materials have been investigated and advocated as future innovative adhesive systems to remineralise and protect the hybrid layer [8,89–91]. These specific therapeutic goals can be achieved through different mechanisms for dentine remineralisation: i) Top-down remineralisation of collagen matrix; ii) Biomimetic extra- and intra-fibrillar remineralisation of collagen matrix [8,92].

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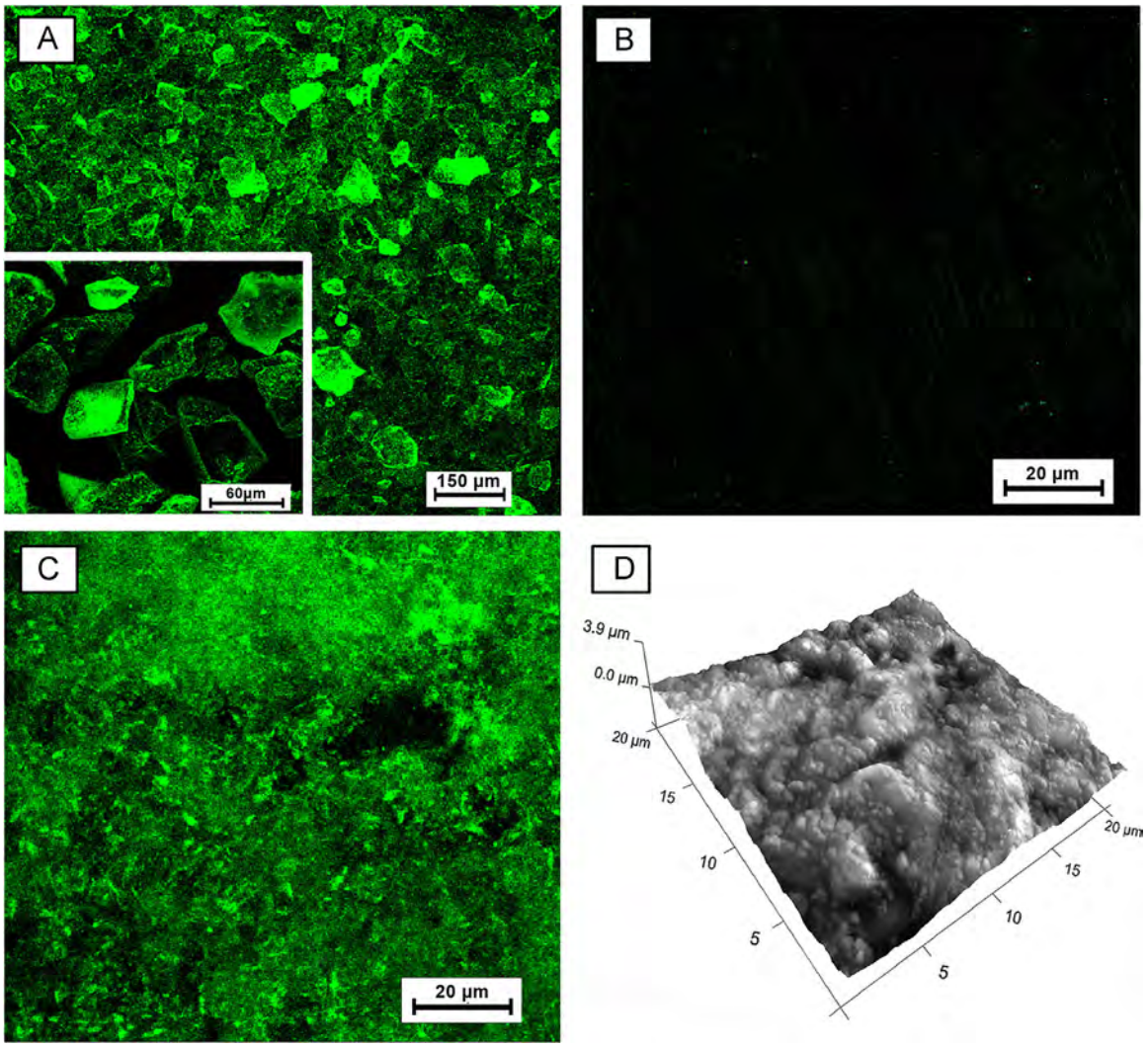

Fig. 6. CLSM projections and AFM images of bioactive glass before and after air-abrasion on sound dentine. A: CLSM 3D projections of BAG 45S5 stained using a FITC-silane solution were prepared by mixing 0.265 ml of pure (3-amino-propyl)trimethoxysilane (APS) and 0.05 g of fluorescein isothiocyanate isomer 1 (Sigma-Aldrich, London, UK) diluted in 5 ml ethanol as a co-solvent [176]. Note irregular bioglass particles with a size between 30 and 100 μm. B: CLSM 3D projections of sound dentine abraded with SiC paper. No clear fluorescence signal is emitted from the dentine surface. C: CLSM 3D projections of dentine air-abraded using Aquacut Quattro (VELOPEX International, UK) with BAG 45S5 (Sylc, OSspray). Note the presence of clear bioglass-rich (bioactive)-smear layer covering the dentine surface. D: AFM image of dentine air-abraded with BAG 45S5. Note a rough dentine surface covered by bioglass-rich (bioactive)-smear layer. (All the images in this figure are original and never published before).

As described before, dentine possesses an intrinsic wetness due to the presence of free water in dentinal tubules. Water is also bound to collagen with variable degrees of affinity to the triple helices of the collagen molecules and their polar chains [14]. In dentine mineralisation, water is necessary to provide an aqueous environment for calcium-phosphate (Ca/P) precursors to infiltrate the gap regions of collagen fibrils and initiate nucleation and growth of extra- and intra-fibrillar apatite crystallites [71–74]. However, top-down mineralisation might induce a relatively rapid mineral precipitation within the resin-dentine interface. Mineralisation results in dehydration of matrix collagen; thus, while the demineralised dentine is being remineralised, the exposed collagen fibrils may be less susceptible to enzymatic degradation as these crystallites displace water from collagen. Free water is required for MMP activity [8,92].

However, using biomimetic remineralisation approaches, based on the use of phosphoprotein biomimetic analogues such us polytrimetaphosphate and PAA or PASA, the water-filled defects within resin-dentine interfaces are first infiltrated by amorphous calcium-phosphate nano-precursors, which penetrate the resin-sparse water-rich regions of the hybrid layer within the inter-fibrillar nano-porosities, and then promote the remineralisation of water-rich/resin-poor collagen scaffold, that lead to inhibition and fossilisation of dentine proteses and re-establishment of the mechanical properties of demineralised dentine [9,16,93].

5.1. Glass ionomer and resin-modified glass ionomer cements

Glass ionomer (GICs) and resin-modified glass ionomer cements (RMGICs) are considered self-adhesive materials that are currently used as fluoride-releasing materials for therapeutic (remineralising) restorations and to create a bonding interface with good durability [32,87,94]. Wilson and collaborators [86] developed the first GIC in the late 1960 at the Laboratory of the Government Chemist (LGC - London) as a substitute for dental silicate cements. Although several formulations are currently available on the market, GIC-based materials are all made of PAA, alkenoic copolymers, ion-leachable fluoroaluminosilicate fillers (FAS) and water. Resin-modified glass-ionomer cements (RMGICs) contain methacrylate-based components (e.g. HEMA, TEGDMA, UDMA), vinyl-modified polyalkenoic acid (VPA) and photo-activators such as camphorquinone and tertiary amines co-initiators [95,96], which have been subsequently

incorporated in order to permit to be light-cured. Their advantages include improved mechanical strengths and lower early-moisture sensitivity [97]. Moreover, rapid setting of RMGICs occurs *via* free-radical photo-polymerisation followed by an acid–base reaction between the polycarboxylic acids and the fluoroaluminosilicate glass. However, due to the hydrophilic nature of HEMA and PVA present within the composition of RMGICs, these materials exhibit increased water sorption and solubility after prolonged water storage. These processes result in hygroscopic expansion and plasticisation of the resin matrix of RMGICs [98].

These 'self-adhesive' materials (GICs and RMGICs) are able to bond to dentine micromechanically, through infiltration of a collagen network previously exposed by means of pre-conditioning the dentine with 10% PAA, along with chemical bonding obtained by ionic interaction of carboxyl groups of polyalkenoic acids with calcium [85,94,95]. Indeed, PAA-treated dentine is generally free from smear-layers and is characterised by a very thin layer of partially demineralised collagen fibrils (0.1–1 μm). Glass-ionomer components diffuse into the demineralised dentine and establish a micromechanical bond via creation of thin hybrid layers, and simultaneously the functional carboxylic groups present in PAA and PVA bind the calcium of the remaining HAP along the collagen fibrils and within the front of demineralisation/etching in dentine [85,86].

The ability of GICs to provide a shallow but uniform hybrid layer is considered convenient in terms of immediate bonding performance, as well as long-term durability [94,99]. This is due to the mild acidity of these materials which only demineralise the extra-fibrillar spaces around collagen fibrils, leaving intra-fibrillar apatite crystals around endogenous proteases and single fibrils that can be morphologically distinguished within the hybrid layer during analysis via ultra-resolution imaging [100,101].

An amorphous 'gel phase' of calcium polycarboxylate salts has been identified at the GIC-dentine bonding interface. However, it is important to consider that GICs and RMGICs contain relatively high-molecular-weight polycarboxyl-base polymers (MW: 8000-to-15,000) that are excluded from infiltration of phosphoric-acid-decalcified dentine [85,87]. Indeed in H_3PO_4-etched dentine the collagen network can remain unprotected by mineral or polymer and thus exposed to hydrolytic degradation. Such aggressive dentine pre-treatments should not be adopted when using GIC-based materials [32,75,99], because their polyalkeonic polymers are excluded from permeating into dentine collagen [102].

Despite some aesthetic disadvantages of GICs, the immediate bonding performance, in terms of microtensile bond strength (MTBS) to dentine, is appropriate for clinical application, but it may decrease significantly after prolonged water aging [75,99,102]. Takahashi et al., [103] reported that the MTBS of GIC restoration *in vivo* may drop up to 50% after 1 year. However, it seems that this degradation process was correlated to a reduction of the material properties rather than issues regarding the bonding interface. Conversely, RMGICs are a viable alternative for Class V restorations [104–107], as the durability of RMGICs exceeds five years under *in vivo* conditions. These results were comparable to those attained when using 'gold standard' multi-step adhesives such as three-step ERAs and two-step SEAs [108,109]. These unique outcomes can be essentially attributed to the low modulus of elasticity of RMGICs compared to classic GICs [110]. Clinically, the dentine-bonded interface created using RMGICs seems very resistant to the challenging conditions of the oral environment. De Munck et al., [94] correlated MTBS data to failure analysis through SEM and TEM, indicating that dentine pre-treatment with polyalkenoic acid conditioner may create durable bond strength after 4 years of water storage due to a combined micro-mechanical and chemical bonding created at the dentine-bonded interface [99]. In an in vitro study, Sauro et al., [87] showed that the bonded-dentine interface created using GC Fuji II LC (GC, United Kingdom, Newport Pagnell, UK) applied to smear layers (no PAA etching) showed no dye permeation after 6 months of storage in artificial saliva (AS) when tested for both nanoleakage and micropermeability. These findings were attributed to important ultramorphological changes at the bonded dentine interface induced by the application of the RMGIC on smear layer-covered dentine. On the contrary, the 10% PAA-etched dentine bonded with RMGIC showed a shallow interdiffusion layer (IDL: ~ 2μm) within the bonding interface affected by slight micropermeability and nanoleakage that reduced even more after prolonged water storage. In our recent studies (unpublished results – S. Sauro) there was no significant reduction ($p = > .05$) of MTBS after 6 months of storage in artificial saliva, and no micropermeability within the dentine-bonded interface created with an

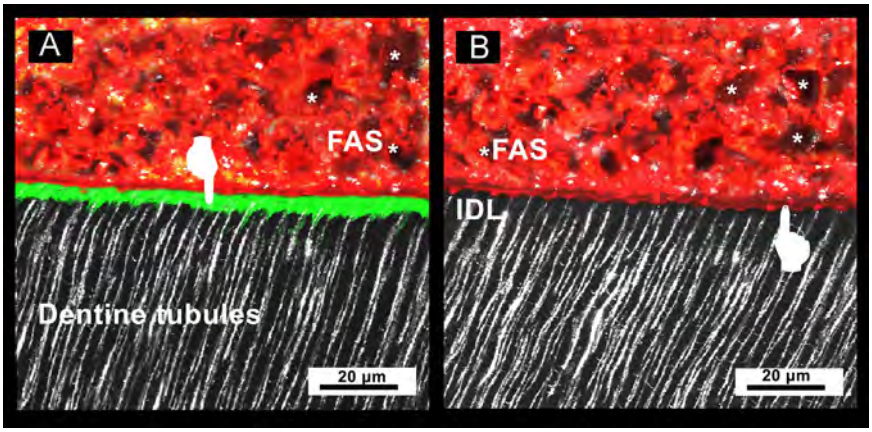

Fig. 7. Confocal laser scanning microscopy (CLSM) projections showing the interfacial characterisation and micropermeability of RMGIC bonded–dentine specimens after storage in AS for (A): 24 h or (B): 6 months. A: The CLSM projection image (reflection/fluorescence) shows the interfacial characteristics of the RMGIC–dentine interface created by application of the resin-modified glass ionomer cement onto dentine after PAA dentine pre-treatment immediately after placement. After 24 h of AS aging, note a layer free of fluoro-alumino silicate (FAS*) filler particles located between the dentine and the interdiffusion layer (IDL). Dentinal tubules contain fluorescein dye used for micropermeability assessment. This anatomical feature may indicate the accumulation of HEMA and the presence of a reactive silica gel layer created by the reaction of the polycarboxylic acids and the FAS particles (pointer). B: The CLSM projection image (reflection/fluorescence) of the interfacial characteristics of the RMGIC–dentine interface created by application of the resin-modified glass ionomer cement to dentine after PAA dentine pre-treatment after, 6 months of AS aging. Note a thin layer free of fluoro-alumino silicate (FAS*) located between the dentine and the interdifusion layer (IDL) that is free of fluorescein dye (no sign of micropermeability), (pointer). The specimen preparation and imaging procedures were performed as described by Sauro et al., [87] (All the images in this figure are original and never published before).

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experimental RMGIC applied on 10% PAA pre-treatment dentine (Fig. 7). This type of RMGIC contains a radiopaque, high ion-releasing, polycarboxylate zinc-doped calcium-phosphosilicate glasses which may assist with mineral precipitation within the bonding interface. Further studies are on-going to better understand the therapeutic properties of such a Bioglass-doped RMGIC, as well as those of the GIC-based adhesive systems such as Riva Bond LC (SDI Australia).

Dentine specimens bonded with GC Fuji II LC, with and without 10% PAA pre-treatment showed a significant drop in MTBS after 6 months of storage in AS [87]. It was hypothesised that the reduction in MTBS could be attributed to the hydrolytic degradation of the resin matrix at the IDL, along with enzymatic collagen degradation as a result of PAA accumulation within the PAA-etched dentine surface and inside the dentinal tubules which may have caused a "delayed" demineralising effect even after several days in AS storage and activation of endogenous dentine proteases (MMPs and cathepsins) at the bottom of the interdiffusion layer [85,87].

In view of such contrasting *in vivo* and *in vitro* results, further improvements in the mechanical properties of the current GICs are required before they can be used for the restoration of posterior teeth in extensive class I and II [95]. Moreover, the use GIC-based materials may not represent "the ideal" therapeutic approach to biomimetically remineralise the resin-dentine interface, although GICs and RMGICs specifically developed for dentine remineralisation have the ability of inducing crystal growth within the micro-porosity at the bonding interface after long-term water storage [111]. It has recently been demonstrated that GIC-based materials fail to remineralise completely apatite-depleted dentine due to a lack of nucleation of new apatite [112] even when biomimetic remineralising analogues were employed during the aging period [113]. Hence, further innovative ion-releasing resin-based materials should be developed for dental application in combination with biomimetic analogues, in order to attain an "ultimate" therapeutic bonding strategy able to achieve remineralisation and a more reliable approach to re-establish the elastic modulus values of the demineralised collagen within the hybrid layer, and long-lasting bonded-dentine interfaces [89–92].

5.2. Alternative bonding approaches based on bioactive dentine pre-treatments

The contemporary philosophy in atraumatic restorative dentistry is based on the preparation of minimally invasive cavities in order to preserve as much sound dental tissues as possible. However, these types of treatments should be performed using therapeutic restorative approaches that protect the resin-dentine interface from degradation processes and prevent the re-occurrence of secondary carious lesions [114]. All of these operative procedures are in accordance with the model "prevention of extension" which seeks to minimise removal of natural tooth, thus preserving the overall biomechanical properties of the tooth, by combining prevention, remineralisation, and minimal replacement of natural dental structure, with synthetic adhesive materials such as resin adhesive/composites [106,117].

In general, dentine caries lesions may be potentially arrested using specific therapeutic restorative approaches which can seal the caries tissue from fermentable carbohydrates, and halt the evolution of the lesion, by changing the cariogenic environment. One of these methods has been described by Bjørndal et al., [115], and it is based on a atraumatic restorative treatment (ART) which requires a step-wise excavation approach. The first step in this "alternative" treatment is the removal of caries-infected tissue (i.e. necrotic dentine) from the peripheral lesion and from the base of the cavity; however, in case of deep lesions, soft and wet caries-infected tissue is often left on this latter part of the cavity in order to avoid pulp exposure. Subsequently, the base of the cavity may be first lined with a calcium hydroxide-based medication and then restored using temporary restorative materials. After about 12-to-20 months, the temporary restorative materials can be removed and the residual dentine tissue is hard enough to restore with proper resin composite restoration. Nevertheless, the success of this technique is not based on the proper *in situ* remineralisation of the carious tissue left inside the cavity during selective excavation, but rather it is due to the release of dentine growth factors by $Ca(OH)_2$ that causes pulpal dentinogenesis. This leads to the formation of reparative dentine along those walls of the pulp chamber in proximity of the lesion, and to a process of hypermineralisation underneath the front of demineralisation of the caries lesion (i.e. formation of sclerotic dentine) which impedes the invasion of bacteria and the progression of the lesion toward the pulp [116].

Currently, in modern ART, several methods are available to perform minimally invasive cavity preparation. For instance, air-abrasion performed with bioactive glass (BAG) has been advocated as a technique that can create a bioactive smear-layer-covered surface for bonding procedures (Fig. 6C and D). Indeed, the choice of the abrasive powders for cavity preparation is the key to accomplish this aim [87,88]. Air-abrasion performed using sodium bicarbonate may affect resin bond strength to dentine, due to interference with the degree of polymerisation of adhesive

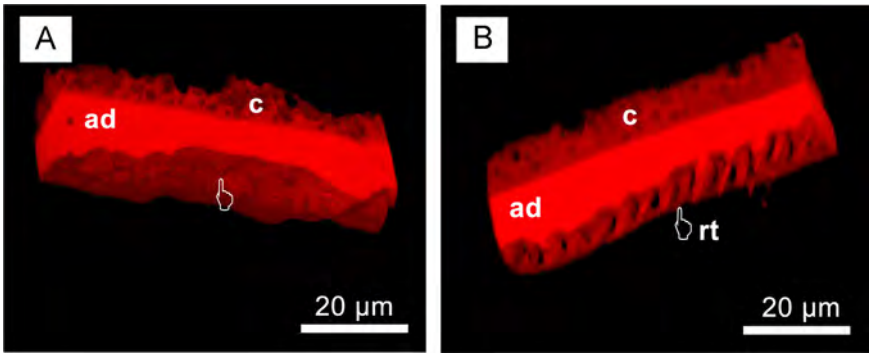

Fig. 8. CLSM 3D projections created following the specimen preparation and imaging procedures described by Sauro et al., [88] using Rodhamine-B to dope self-etching adhesives. A: CLSM projection image (fluorescence – Rhodamine B) of a self-etching adhesive applied on dentine air-abrade using Aquacut Quattro (VELOPEX International) with BAG 45S5 (Sylc, OSspray). Note the bottom part of the adhesive layer (ad) is characterised by an absence of resin tags (rt) or by one few very short (< 2 μm) resin tags (pointer) probably due to BAG-occluded dentine tubules. B: CLSM projection image (fluorescence – Rhodamine B) of a self-etching adhesive applied on smear-layer covered dentine. Note the bottom part of the adhesive layer (ad) contains numerous short (> 2 μm) resin tags (pointer) probably due to BAG-occluded dentine tubules. (All the images in this figure are original and never published before).

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systems. In contrast, air-abrasion with crystalline cellulose (a control abrasive) has no influence on the bonding ability of SEAs applied onto air-abraded dentine [117,118]. Moreover, it seems that air-abrasion performed with alumina (Al_2O_3; 50 μm) does not interfere with the immediate bond strength of SEAs, but showed a significant decrease in bond strength after 3 months of water aging [118,119]. Carvalho et al., [120] showed that air-abrasion performed on dentine using experimental niobophosphate bioactive glass does not interfere with the immediate bonding performance of self-etching and self-adhesive resin-based cements. The same authors stated that further studies are necessary to evaluate the effect of this bioactive glass on the long term bonding performance of self-etching and etch-and-rinse adhesive systems as well as that of self-etching and self-adhesive resin-based cements.

It has been advocated that bioactive glass 45S5 (Sylc, OSspray Ltd., London, UK) or experimental PAA-doped bioactive glass (BAG-PAA) used in an air-abrasion device (Aquacut Quattro, VELOPEX International, UK) may produce a bioactive smear layer with therapeutic properties at the bonding interface that can preserve the integrity of the dentine-bonded interface and the bond strength created when using SEAs or RMGICs [87,88]. Bioactive calcium/sodium phosphate-phyllosilicates such BAG can react with body fluids, producing HAP precipitation and remineralisation of mineral-depleted dentine and enamel [121–123]. BAG can substitute for alumina abrasives, and it has been introduced in operative dentistry for air-abrasion systems as an alternative to traditional hand-pieces. Indeed, the ability of BAG to create cavity preparations with minimal loss of sound tissues is attributed to its unique mechanical properties: i) Young's modulus: 35 GPa; ii) Vickers-hardness: 458 VHN. These values are significantly lower than those of alumina (380 GPa and 2300 VHN), but with a Young's modulus similar to sound dentine and Vickers-hardness similar to enamel [124,125]. Ultraconservative cavities preparation performed with BAG are characterised by rounded internal line angles that can minimise the stress generated by the composite shrinkage along the bonding interface [126].

In an *in vitro* study [88] Sauro et al., applied a simplified one-bottle SEAs (GB; G Bond, GC Ltd. Tokyo, Japan) containing the dicarboxylic functional monomer 4-methacryloxy-ethyl-trimellitate (4-MET), on dentine surfaces air-abraded using BAG alone or a BAG doped with 15 wt/v% PAA (BAG-PA15). This produced (24 h) a bond strength significantly higher than that attained when the same adhesive was applied onto SiC-abraded dentine (control). These outcomes were attributed to the bonding ability of 4-MET, in particular to its carboxylic groups which provide acidity, good wetting proIn aperties and ionic bonds with calcium ions [36,127]. Therefore, it is possible that 4-MET bonded to calcium in apatite during bonding procedures and/or with the calcium of BAG embedded on the air-abraded dentine. The BAG particles obliterated dentinal tubules (Fig. 8) and this may have prevented water diffusion through the dentinal tubules to the resin-bonded interface (reduced water sorption at the hybrid layer). Another all-in-one one-step adhesive (CS3; Clearfil S3 Bond; Kuraray, Osaka, Japan) containing the functional monomer 10-MDP was used in that study. This system was applied on SiC-abraded, BAG 45S5 or BAG-PA15 air-abraded dentine. There were no statistically significant differences in bond strengths between all those groups. However, both in air-abraded (BAG or BAG-PA15) dentine, the bond strengths obtained with CS3 were significantly higher than those obtained with GB.

Hypothetically, the presence of BAG within the "hydrated" resin–dentine interface might induce the release of a hydrated silica $Si(OH)_4$ with a subsequent condensation polymerisation reaction within the thin, demineralised, resin-impregnated collagen layer. This condition may favour the coverage of collagen fibrils with polysiloxane that could block the activity of proteolytic enzymes such as MMPs [128]. Moreover, the $Si(OH)_4$ may bind non-specifically to demineralised collagen fibrils and polymerise into a porous SiO_2-rich layer that might serve as a template for apatite precipitation [129,130]. The sodium (Na^+) and hydrogen ions (H^+) or hydronium ion (H_3O^+) exchanges, and rapid release of Ca^{2+} and PO_4^{3-} from BAG may contribute, together with the $Si(OH)_4$ condensation and the apatite precipitation, to fossilise MMPs within the BAG air-abraded bonded dentine interface, thereby blocking proteolysis by endogenous MMPs [131].

After six months of immersion in phosphate buffer solution (PBS), a significant fall in bond strength was observed in the experimental groups created with GB applied on SiC-abraded or BAG 45S5 air-abraded dentine specimens. However, bonds created using BAG 45S5 or BAG-PAA15 showed significantly higher bond strengths than the other groups. Conversely, no significant drop in bond strength, and no evident micropermeability changes were observed between the resin–dentine interfaces created using CS3. It was hypothesised that the differences were related to the lower hydrophilicity of the functional monomer 10-MDP, due to its long carbon chain and to the dihydrogen phosphate groups that, when dissociated in water, were able to create strong and long-lasting ionic bonds with calcium in dentine and those present in BAG (Fig. 3) [38,39,40,83].

Due to a number of shortcomings associated with the hydrolytic degradation of the resin matrix at resin-dentine interfaces created with simplified adhesive systems, we believe that the use of glass ionomer-based materials are superior restorative materials that can be used during ART as liners or in the laminate technique. Indeed, their use beneath longer wearing restorative materials is an attractive alternative solution to attain restorations with greater bond durability [32,75,94]. We consider glass ionomer-based materials as the only therapeutic alternative to the resin adhesives currently available in operative restorative dentistry. Their fluoride release, moisture tolerance and ability to create stable chemical bonds to dentine (self-adhesive properties) are unique [132,133]. GIC-based materials have antimicrobial properties [134,135] related to their high levels of fluoride release from fluoroaluminosilicate fillers during the initial stages of the acid base reaction [95]. These factors interfere with the metabolism of bacteria, in particular *Streptococcus mutans*, and with their replication and proliferation [136]. GIC-based materials also increase the resistance of dental hard tissue to demineralisation from acid produced by these bacteria [137].

It was also confirmed that glass ionomer cements induce crystal growth in micro-spaces within the interface of the restoration after long-term storage in water [138], and that the chemical composition of these crystals is similar to that of dental hard tissue [4].

Micropermeability is a technique that can be used to determine how well resin tags can seal fluid in the pulp chamber/dentinal tubules from the hybrid layer. Fluorescent dyes are placed in the pulpal chamber under 15–20 cm H_2O pressure. The dyes are transported backwards up the open dentinal tubules and around resin tags if they are not hybridised to the surrounding dentine, and into any water-filled space in the hybrid layer. If the hybrid layer becomes fluorescent within 24 h of creation, but does not become fluorescent after 3–6 months of aging in PBS, it suggests that the dentine tubules and/or the water-filled channels have become occluded over time, perhaps with apatite-like mineral [70] (Fig. 7).

It has been advocated that the operative combination of air-abrasive procedures performed with BAG or PAA-BAG, and subsequent restoration using RMGICs, could be a strategy for performing minimally invasive operative dentistry. The use of such therapeutic materials may protect the bonded interface, increasing the longevity of the restoration [87,88]. Indeed, Sauro et al., [87]

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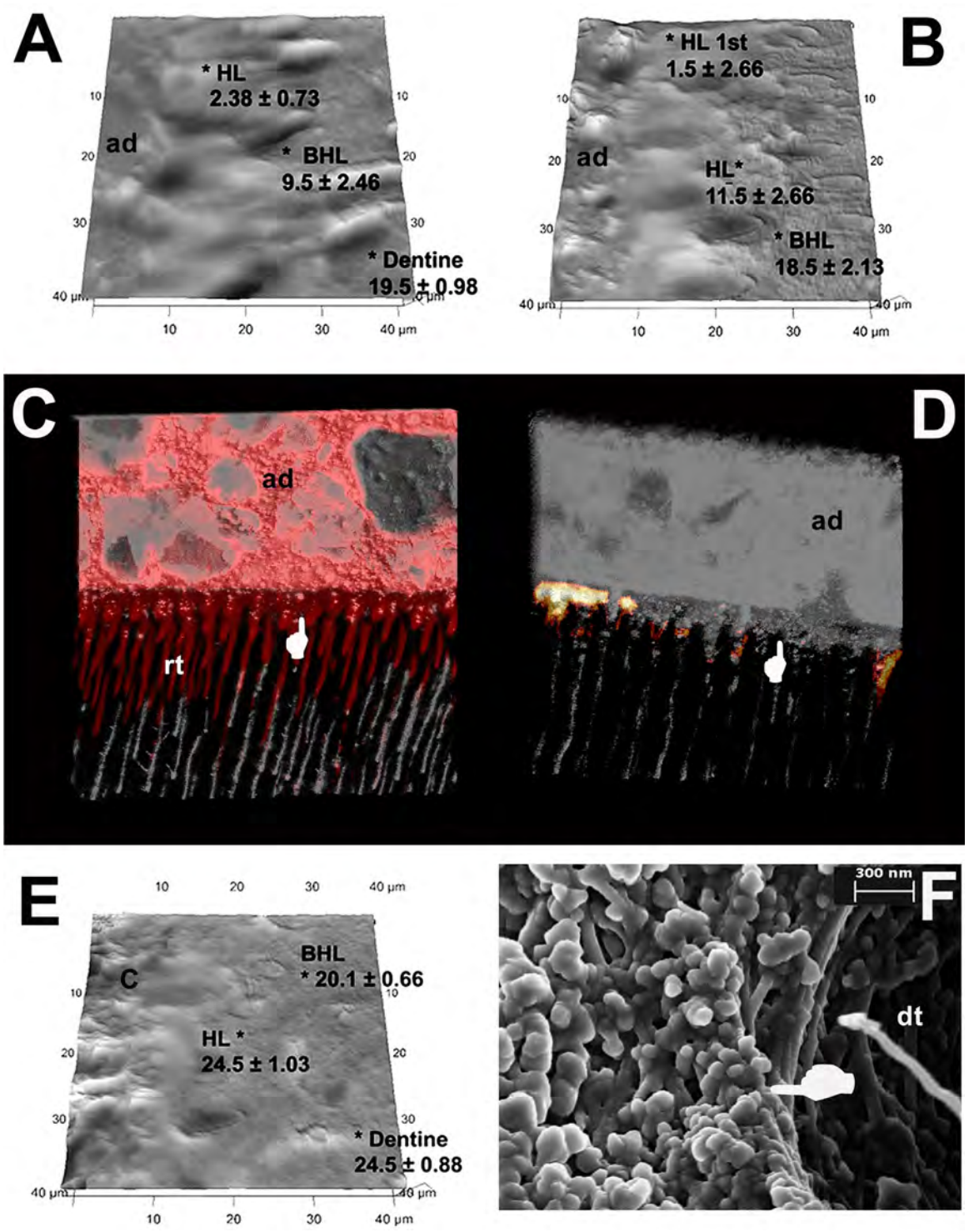

Fig. 9. CLSM, AFM and FEG-SEM images of resin–dentine interface after 24 h or 6 month aging. A: AFM image showing the resin–dentin interface after 24 h of AS storage created with the experimental ion-releasing adhesive applied onto acid-etched dentine. The initial values of elasticity of a sound hybrid layer (HL), bottom of hybrid layer (BHL) and sound dentine are shown digitally. The digital data are nano-indentation derived moduli of elasticity. B: AFM image showing the resin–dentin interface after 90 days of AS storage created with the experimental ion-releasing adhesive applied to acid-etched dentine. Note that neither the hybrid layer (HL) nor the adhesive layer show any sign of degradation, but the presence of the fillers within the adhesive layer (ad). Note the increase of the modulus of elasticity of middle and bottom of hybrid layer (BHL) compared to the same specimen after 24 h AS storage (A). C: CLSM 3D projection image captured after 24 h of AS immersion in reflection/fluorescence showing the resin–dentine interface created with the experimental ion-releasing adhesive applied onto acid-etched dentine Note a strong reflection signal from the filler particles within the adhesive layer (ad) and a clear rhodamine-B stained hybrid layer (pointer) and resin tags. This interface was characterised by intense micropermeability within the hybrid layer (not shown here). D: CLSM 3D projection captured after 90 days of AS immersion in reflection/fluorescence showing the resin–dentine interface created with the experimental ion-releasing adhesive applied onto acid-etched dentine. The resin–dentine interface presents almost no fluorescein micropermeability signal but a good reflection signal, indicting a remineralised hybrid layer (pointer). An intense reflection signal can also be seen within the adhesive layer (ad) and inside the dentine tubules. E: AFM image showing the resin–dentine interface after 90 days of AS storage created with the experimental ion-releasing adhesive applied to the dentine pre-treated with the primer containing polyaspartic acid and sodium trimetaphosphate primer. Note that the indentation-derived moduli of elasticity of the hybrid layer (HL) was completely restored when compared to sound dentine in Fig. 9A. F: SEM image of a debonded specimen dentine after 90 days of AS storage which was created with the experimental ion-releasing adhesive applied to the dentine pre-treated with the primer containing polyaspartic acid and sodium trimetaphosphate primer. Note nanocrystals deposition (pointer) on the collagen fibrils of the intertubular and intratubular dentine (dt). (All the images in this figure are original and never published before).

showed that the creation of a stable RMGIC–dentine interface was only achieved when the bonding procedures were performed on dentine air-abraded with BAG and BAG-PAA, especially if a final etching procedure was carried out with a 10% PAA gel. The RMGIC-bonded specimens stored in PBS for a period of 6 months, revealed important decreases in micropermeability and nanoleakage, when the bonded-dentine interfaces were created on BAG air-abraded dentine. In fact, in these aged specimens, dye diffusion (micro-permeability) along the dentine tubules stopped at the bonded interface. The authors of that article [87] believe that the presence of embedded bioactive glass within the bonded interface may have stabilised the adhesion between dentine and RMGIC, as a result of doping the dentine surface with submicron particles of BAG. This BAG may have induced apatite formation [130,139,140] over the subsequent 6 months in PBS. A possible explanation of these results may be that the 10% PAA fluid using during air-abrasion may have pre-wetted BAG particles during expulsion from the air-abrasion nozzle, facilitating their adhesion to the dentine surface. The use of a PAA fluid, and the further use of PAA etching-gel may also have facilitated subsequent chemical reactions among GIC, BAG, and collagen fibrils [141,142].

Further *in vivo* studies are necessary to confirm these *in vitro* outcomes obtained by combining GIC-based materials and BAG/PAA air-abrasion. Although these treatments may fail to remineralise apatite-depleted dentine owing to a lack of nucleation of new apatite [4,143], it is possible that the air-abrasion procedures performed using a combination of BAG and BAG-PAA fluid, might increase the probability of BAG particles embedding in dentine tubules and the dentine surface. This approach enhances the durability of the RMGIC-bonded dentine when used according to the manufacturer's instructions (i.e. air-abrasion with BAG/10%PAA prior to application of PAA etching gel) [87]. Moreover, the success of BAG/PAA procedures is likely due to the slow release of $Ca^{++}$and PO_4^{3-} from BAG 45S5 along with PAA, a well known Ca^{++} sequestering polyanion that contributes to the formation and maintenance of amorphous calcium phosphate that can infiltrate collagen in a liquid state and remineralise the resin-dentine interface [144,145].

5.3. Experimental therapeutic ion-releasing adhesive systems

Much has been accomplished by improving the formulation of dental adhesive systems, although little attention has been paid by dental material manufacturers to develop innovative “smart” bonding systems that can interact therapeutically with the dental hard tissues. Such a therapeutic bonding approach [31] may represent an alternative way to reduce the degradation of the resin-dentine interface through remineralisation of the mineral-depleted dentine and stabilisation of the bond strength over time, so that dental restorations might have greater longevity [5,90]. The clinical demand for engineered dental tissues requires the development of light-curable adhesives with “controlled” water sorption/solubility properties, so that they can better tolerate dentine moisture and absorb precise amount of fluids (e.g. dentinal fluid and saliva). This should allow the release of particular ions such as Ca^{2+} and PO_4^{-3} [90,128] that can act as precursors of apatite-like minerals (bioactivity) and replace water from the resin-sparse water-rich regions of hybrid layers (micro- and nano-porosities) that are typically found in contemporary resin-dentine interface [113,144]. Mineral uptake and apatite formation can lead to the remineralisation of resin-bonded dentine collagen, protecting the fibrils from enzymatic degradation [66,89].

Innovative “smart” materials should also possess antibacterial properties to combat remaining microorganisms in the bonding substrate, and reduce the risk for secondary caries at the tooth-restoration interface [49,50,128]. Several experimental ion-releasing adhesive systems doped with bioactive micro-fillers such as BAG 45S5 and polycarboxylate zinc-modified bioactive glass have been formulated to accomplish these aims [146]. These “hybrid resin-based” adhesive systems could induce a significant increase of the mechanical properties (nano-elasticity and nano-hardness), along the mineral-depleted areas within the resin-dentine interface (Fig. 9A and B), as well as important reductions of the interfacial porosities after prolonged storage in PBS (Fig. 9C and D). Moreover, a consistent formation of apatite-like mineral was identified using Raman microscopy between one and three months of PBS storage. The control, filler-free, adhesive showed no apatite formation, even after prolonged PBS storage (3 months). The bioactivity of bioglass-doped adhesives at creating apatite, both on the surface of the specimens and in demineralised dentine was confirmed through FTIR-ATR and thin-film XRD analysis [128].

These experimental ion-releasing adhesives [146] were specifically formulated as “simplified” slow release systems that degrade during PBS storage, to provide important delivery of calcium and phosphate ions within the bonded interface [91,92,128]. For this reason, these experimental ion-releasing adhesives showed a significant reduction in bond strength after PBS storage (3 months). However, when the authors performed SEM fractographic analysis, the most prevalent failures were cohesive failures in resin. When the dentine surfaces were exposed, these were shown to be covered by a mineralised resin-dentine matrix [146], similar to the case of bonded interfaces created with GIC cements and submitted to MTBS and SEM fractographic analysis. In contrast, GIC materials generally show tensile bond strengths of only around 5 MPa [147,148].

Osorio and co-workers [131] demonstrated the potential ability of resin-based materials micro-filled with BAG 45S5, or polycarboxylate zinc-modified bioactive glass [89] in inhibiting MMP-mediated collagen degradation via a possible mechanism based on mineral uptake. Indeed, the authors hypothesised the formation of high molecular weight CaP-MMP complexes that immobilised MMP-2 and MMP-9 [149]. However, further possible mechanisms of MMP inhibition have been hypothesised, such as the alkaline pH generated by bioactive glasses during water immersion (release of alkaline elements: Na^+ or K^+ exchanged with H^+or H_3O^+ ions). Since optimum MMP activity occurs at pH 7, that activity would be greatly slowed at pH 10. Moreover, the formation of a SiO_2-rich gel layer on the surface of BAG 45S5 [121,139], the dissolution of Ca^{2+}ions from the glass with their diffusion through the SiO_2-rich layer, and reaction with PO_4^{3-} might lead to the formation of an amorphous calcium phosphate layer which can subsequently converts to HAP. It is well known that such apatite crystals may inhibit MMPs [150]. Likewise in bone regeneration in the presence of Bioglasses, Ca/P complexes are thought to specifically bind to amino acids within exposed collagen [151–153].

In order to improve the durability of bond strength of such ion-releasing adhesives, further experimental three-step etch-and-rinse systems were created by formulating a resin primer containing a carboxylic functional monomer (PMDM; 2,5-dimethacryloyloxyethyloxycarbonyl-1,4-benzenedicarboxylic acid), and an adhesive doped with BAG 45S5 fillers [154] or with experimental Portland cement-based micro-fillers [90]. The use of adhesive systems containing these ion-releasing micro-fillers promoted the creation of a resin-dentine interface with constant bond strength values that were maintained over 6 months of storage in PBS (24 h: 32.2 ± 9.4; 6-months: 30.3 ± 11.5). Conversely, the same adhesive containing no filler (control group), showed a significant reduction in resin-dentine bond strength after aging (24 h: 29.2 ± 9.9; 6-months: 18.5 ± 10.4) [90].

These results were attributed to the use of a separate primer which may have allowed better infiltrated and protected the collagen fibrils within the hybrid layer, as generally happens in multi-

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step self-etch and etch-and-rinse bonding procedures. Moreover, primers containing specific functional monomers may be able to bind the remaining HAP around the collagen fibrils, but more importantly, at the bottom of the hybrid layer where degradation processes seems to begin [33,38,70]. It was also hypothesised that bioactive micro-fillers made of calcium silicates (modified Portland cements) may have reacted with water to release calcium hydroxide that increased the alkalinity of the surrounding environment [155]. That alkaline pH may have slowed the activity of MMPs [66,89]. The interaction between phosphate ions present in the PBS or in the dentine substrate, and the calcium released from the calcium silicate micro-fillers may have enhanced the formation of new mineral deposits [90,91]. Indeed, after immersion in PBS (6 months), the therapeutic mineral deposition within the hybrid layer reduced nanoleakage at the resin-dentine interface. There was clear evidence of remineralisation of the water-rich, resin-poor regions within the hybrid layer by using a special confocal imaging technique based on the use of a Ca^{2+}-chelating fluorophore (xylenol orange) [90]. Subsequent, SEM ultramorphology analysis revealed the presence of mineral crystals within the resin-dentine matrix, after different storage periods in PBS [66,90,91,146].

Specific ions, such as copper and zinc have been advocated as effective MMP inhibitors [143–145] and as antibacterial substances to *Streptococcus Mutans* when incorporated into adhesive resins, with no cytotoxicity to fibroblasts or interference with bond strength to dentine [156]. Indeed, Toledano and co-workers [157] showed that the incorporation of zinc ions, and their subsequent release form adhesive systems, may increase the durability of hybrid layers and the nano-mechanical properties along the dentin-bonded interface by inhibiting dentin MMPs.

Although all the "smart" materials and bonding approaches discussed so far, may play important therapeutic roles at the resin-dentine interface, few have caused full remineralisation of demineralised dentine matrices to completely recover their original mechanical properties and functionality [2,5,6]. However, a recent investigation [158] has demonstrated that experimental resin-based flowable composite containing Portland cement-based calcium silicates and biomimetic polyanions such as PAA and poly (vinylphosphonic acid) (PVPA) can slowly "back-fill" any water-filled channels (e.g. water trees in adhesives and hybrid layers, and residual water in un-infiltrated hybrid layers) with apatite crystallites in collagen. These crystals displace all free residual water and inactivate all proteases by mineralising the enzymes. Portland cement, and phosphate-containing systems used in the presence of PAA as a stabiliser for amorphous calcium phosphates and PVPA as a biomimetic analogues of collagen-binding matrix proteins can induce complete biomimetic remineralisation of the hybrid layer and increase the durability of such resin–dentin bonds. It has been reported that biomimetic mineralisation of caries-like lesions is also possible when using Portland cements in the presence of a simulated body fluid containing PAA and PVPA or sodium

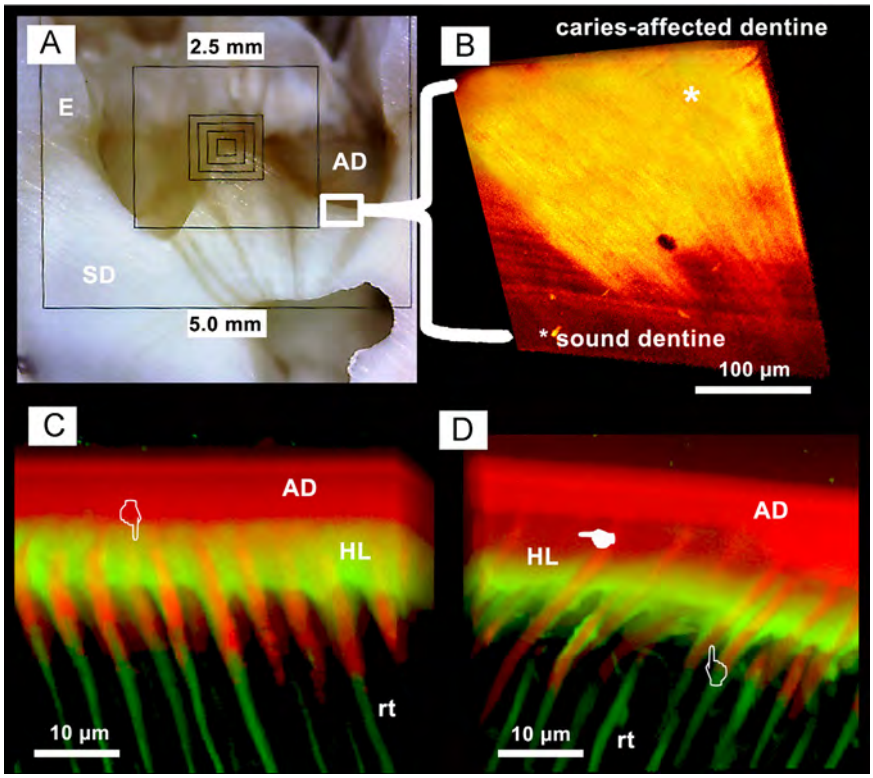

Fig. 10. Stereo microscopy and confocal CLSM images of caries-affected dentine and resin–dentine interface created in sound dentine. A: Image obtained using a using a 30x USB digital-microscope (CY-800B) as described in [146] after minimally invasive cavity preparation in a human molar affected by caries using Aquacut Quattro (VELOPEX International) with BAG 45S5 (Sylc, OSspray). This tooth was then immersed in solution containing 0.05 g of fluorescein isothiocyanate (Sigma-Aldrich, London, UK) diluted in 5 ml ethanol in order to stain the demineralised collagen within the residual caries-affected dentine. B: Note that thickness (> 150 µm) of the residual caries-affected dentine in some specific areas of the prepared cavity. C: CLSM 3D projection captured as described by Sauro et al., [177] showing the resin–dentine interface created with the Scotchbond™ Universal Adhesive (3M ESPE, St. Paul, MN, USA) applied to 37% H3PO4-acid-etched dentine. Note the presence of a hybrid layer (HL) characterised by severe water-filled micro- and nano-porosities that were completely infiltrated by the fluorescein dye (pointer - micropermeability). In this image it is possible to appreciate that common etch-and-rinse adhesive cannot properly infiltrate the demineralised collagen matrix using the water-wet bonding technique. D: However, some parts of the resin–dentine interface created with the universal New Extra Bond II (DEI-Italia, Varese, Italy) applied to 37% H3PO4-acid-etched dentine showed during CLSM analysis that some parts of the hybrid layer (HL) were completely resin-infiltrated and free from fluorescein micropermeability (white close pointer), and only the bottom of the HL was characterised by water-filled micro- and nano-porosities completely infiltrated by the fluorescein dye (open pointer). However, in both cases it was not possible to completely infiltrate the 37% H3PO4 demineralised dentine collagen matrix with resin. (All the images in this figure are original and never published before).

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tripolyphosphate [113,159]. It is important to note that pure Portland-based cements cannot induce biomimetic remineralisation of dentine and re-establish functional biomechanical properties due to their high alkalinity (pH 12), which causes a caustic degradation of collagen fibrils [160,161]. Moreover, Portland-based calcium-silicates cements may contain potential cytotoxic agents such as arsenic ions that are not suitable for clinical application because they also lack radiopacity [162,163]. Nevertheless, these types of cements, have found important applications in dentistry due to their ability to induce minerals precipitation and formation of reparative dentine when used as pulpal capping materials [164,165]. However, this approach requires the presence of phosphate-containing body fluids in order to obtain proper remineralisation of the mineral-depleted dentine [158]. Conversely, the biomimetic remineralisation methods proposed by Pashley and Tay [8] achieved an effective collagen remineralisation *in vitro*, although it cannot be adopted clinically to remineralise carious-affected dentine and/or mineral-depleted interfaces as the biomimetic analogues cannot be dissolved in body fluids. A recent study by Sauro et al., [5] demonstrated that it is possible to functionally re-establish the biomechanical properties (i.e. Young's Modulus) of water-saturated collagen, that remains water-rich and resin-poor within the resin-dentine interface, by using dental adhesives containing ion-releasing micro-fillers applied on acid-etched dentine pretreated with one of the different biomimetic primers containing either sodium trimetaphosphate (TMP) [166] or polyaspartic acid (PASA) [158]. The authors stated that the use of biomimetic primers containing TMP and PASA, in conjunction with the ion-releasing adhesive systems, induced collagen remineralisation. This "bottom-up" remineralisation is opposite to the "top-down" method where one tries to deposit minerals on the moist dentine surface. Some areas that were completely water-filled, became fully remineralised and had stiffness values that were not significantly different from the underlying mineralised dentine [5,167] (Fig. 9E). These remineralised dentine-bonded specimens created with the experimental ion-releasing resin and a primer containing TMP and PASA showed no significant reduction of the MTBS values over 90 days of storage in artificial saliva. Moreover, these specimens exhibited a debonded surface devoid of exposed collagen fibrils, as they were clearly covered by nano-crystals deposition both in the intertubular and intratubular dentine (Fig. 9F).

6. Conclusions

The use of 32–37% phosphoric acid in etch-and-rinse systems or self-etching adhesives removes smear layers and demineralises the underlying dentine. This exposes all proforms of the endogenous proteases of dentine matrices metalloproteinases and cathepsins cysteines, and activates them into functional hydrolases. The rinse water replaces the 65% of the mineral dissolved by phosphoric acid. During resin infiltration, if resin does not replace all residual water, that water will fuel degradation of uninfiltrated collagen [9,16].

Unfortunately, resin infiltration of water-saturated dentine matrices is not uniform, especially after minimally cavity preparation (Fig. 10A), and in thick layers of caries-affected dentine (Fig. 10B). Indeed, some areas of hybrid layers are well infiltrated and exhibit little residual water, while adjacent regions are not properly infiltrated and may contain very little resin, but 30–40% residual water (Fig. 10C and D) [168,169].

It is thought that the resin-sparse, water-rich zones in resin-bonded interfaces degrade over 1–2 years. Their stiffness is so low that they may undergo excessive cyclic strain under normal function leading to fatigue failure [161]. Clinicians have only one chance for resin infiltration. If the average resin–dentine bond contains 30–50% residual water instead of resin, only one "corrective" strategy can be applied to solve that problem. The residual water must be slowly displaced by filling these water-filled voids with nano-sized crystals of apatite generated by smart ion-releasing materials and/or biomimetic remineralising bonding approaches. Remineralisation is a form of dehydration which removes free water so that activated MMPs cannot function. Such "back-filling "of water-filled voids will not occur in well resin-infiltrated portions of hybrid layers because they contain no residual water, but polymerised resin monomers [5]. The remineralising back-fill can even fill water-trees with apatite crystals [8,144].

Unfortunately, current marked aesthetic composite materials have no therapeutic ability to remineralise poorly resin-infiltrated hybrid layers and caries-affected dentine underneath the hybrid layers. Such interfaces are characterised by poor durability/integrity during aging or *in vivo* performance [75,170]. Self-adhesive GIC and RMGIC can bond to dentine micromechanically and induce mineral precipitation at the bonded interface [171], in particular when applied after air-abrasion performed with bioactive glasses [87]. Conversely, Portland-based cements such as ProRoot MTA (DENTSPLY Tulsa, OK, United States) or other quick-setting calcium silicate cements such as Biodentine (Septodont, St. Maur-des-Fossés, France) or ENDOPASS (DEI, Varese, Italy) can cause a caustic degradation of collagen fibrils, which are then mainly replaced by calcium carbonates or by apatite-like crystals only when immersed in phosphate-rich solutions (i.e. body fluids such as saliva and blood) [160,161].

Reliable remineralisation of completely demineralised collagen fibrils should be characterised by intra-fibrillar [3,5,6] and extra-fibrillar mineral deposition with hydroxyapatite crystals orientated in the same direction as those in sound dentine [6,159]. Innovative bioactive/biomimetic strategies that lead to remineralisation of hybrid layers have been demonstrated to be able to restore the modulus of elasticity of mineral-depleted dental collagen structures within bonding interfaces [5] to normal values [6]. Thus, it is time for academic dental researchers and dental industry leaders to cooperate more fully in development of materials with enhanced clinical longevity that can easily be used in dental practice, rather than attempting to improve the formulation of "passive" adhesive systems that can increase the risk for toxicological effects induced by elution of components contained in the light-curable resin-based materials [172,173]. Hopefully in the near future, flowable composite and/or adhesive systems will be developed that can backfill any regions of the hybrid layer that do not fill with resin, with apatite crystallites so that resin-dentine bonds will be self-remineralisable under such conditions and last as long as resin-enamel bonds.

7. Clinical considerations

Currently there is no existing restorative material able to remineralise hybrid layers and completely restore the modulus of elasticity of mineral-depleted dental collagen structures within resin-bonded interfaces though biomimetic apatite formation at intra-fibrillar and extra-fibrillar collagen level. Hence, the first important concept to consider in minimally intervention dentistry is that the restorative materials and techniques currently available are not able to immediately remineralise and protect demineralised collagen fibrils present within remaining caries-affected dentine, as well as those within poorly resin-infiltrated water-filled hybrid layers. In such circumstances, collagen will quickly degrade if not protected through the application of cross-liking [178] anti-MMPs [179] agents such as those commercially available

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based on glutaraldehyde/2-hydroxyethylmethacrylate (GLUMA, Heraeus Kulzer, Hanau, Germany).

Clinicians may create therapeutic restorations that can induce mineral precipitation within resin-dentine interfaces and possibly prevent the re-occurrence of secondary carious lesions [114]. For instance, clinicians may use bioactive glass 45S5 (Sylc, OSspray) in air-abrasion units (Aquacut Quattro, VELOPEX) to perform a final polishing of the cavity dentine, in order to produce a therapeutic "bioactive" smear layer that can protect the bonded interface and preserve the adhesion performance of RMGIC [87] and self-etching adhesives [88].

Especially when dealing with deep cavity lesions close to the pulp chamber, it would also be appropriate to apply quick-setting calcium silicate cements such as Biodentine or ENDOPASS, in combination with a modified step-wise excavation technique, where the final step will require partial removal of the cement (~1–2 mm), followed by the application of an adhesive system and aesthetic composite [115]. The rationale behind the use of such bioactive cements is that, although they can cause a caustic degradation of collagen fibrils in partially demineralised caries-affected dentine, these may also create calcium carbonates and/or apatite-like crystallisation at the interface [160,161] as well as biostimulation of pulpal cells to produce reparative dentine along the walls of the pulpal chamber (Reparative dentine bridge) [180].

Acknowledgement

This work was supported by PEST-1408 and INDI1527 "Programa de Consolidación de Indicadores: Fomento Plan Estatal CEU-UCH 2014-2016" to Prof. Dr. Salvatore Sauro. This work was also supported by NHI/NICR Grant "DE 015306" to David H. Pashley, and by the Faculty of Dentistry at the King Abdulaziz University, Jeddah, Saudi Arabia to David H. Pashley as their highly-cited scholar.

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Please cite this article as: Sauro S, Pashley DH. Strategies to stabilise dentine-bonded interfaces through remineralising operative approaches – State of The Art. Int J Adhes Adhes (2016), http://dx.doi.org/10.1016/j.ijadhadh.2016.03.014

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Please cite this article as: Sauro S, Pashley DH. Strategies to stabilise dentine-bonded interfaces through remineralising operative approaches – State of The Art. Int J Adhes Adhes (2016), http://dx.doi.org/10.1016/j.ijadhadh.2016.03.014

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Please cite this article as: Sauro S, Pashley DH. Strategies to stabilise dentine-bonded interfaces through remineralising operative approaches – State of The Art. Int J Adhes Adhes (2016), http://dx.doi.org/10.1016/j.ijadhadh.2016.03.014

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Please cite this article as: Sauro S, Pashley DH. Strategies to stabilise dentine-bonded interfaces through remineralising operative approaches – State of The Art. Int J Adhes Adhes (2016), http://dx.doi.org/10.1016/j.ijadhadh.2016.03.014