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Dentin interaction with universal adhesive containing isopropanol solvent studied by solid-state NMR spectroscopy

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Abstract

Objectives

This study investigated the chemical and structural changes in the mineral phase and collagen of dentin during application of a mild universal adhesive. Particular attention was paid to the role of isopropanol and changes in water molecules.

Methods

In vitro application of the mild universal adhesive on dentin with two established etching modes (self-etch and etch-and-rinse) was studied using solid state nuclear magnetic resonance spectroscopy.

Results

It was evidenced that the etch-and-rinse mode leads to a decrease of the inorganic apatite and a reorganization of the residual mineral phase with a low amount of adhesive phosphate monoesters calcium salt formed, compared to the self-etch mode. In contrast, the adhesive interacts very similarly to the level of dentin collagen in both protocols, with a strong decrease in the amount of the free water molecules induced by the presence of isopropanol as the adhesive solvent, but without significant changes in the initial collagen structure. For both modes, the adhesive acrylates monomers remain mobile and can infiltrate the collagen.

Significance

Understanding the molecular interactions between dentin and adhesive solutions is a major challenge for designing products that lead to the formation of ideal dentin resin hybrid layer. Notably, one point considered essential is the presence of unbound water which, over time, is associated with a hydrolytic degradation of the organic matrix. Isopropanol, as an adhesive solvent, leads to a decrease in the amount of the less stable water molecules while the water molecules strongly attached to the collagen are retained, thus preserving the collagen structure.

Keywords

Dentin, adhesive, solid state NMR, water molecules, isopropanol, chemical organisation

1. Introduction

The formation of dentin resin hybrid layer is well known but is still a difficult challenge, as it is dependent on many factors related to the dentin substrate and the adhesive system, notably its solvent [1]. The hybridization process requires exposing the network of collagen fibrils through an etching step with acids that demineralizes the dentin. The universal dental adhesives developed can be applied with two different strategies called the “etch-and-rinse” or “self-etch” modes [2,3]. For the “etch-and-rinse” mode, the etching step is performed by application of a phosphoric acid solution followed by water rinsing. For the “self-etch” mode, the etching is realized by acidic functional monomers, most often carboxylate and/or phosphate groups, present in the adhesive [4]. After demineralization, the adhesive system can infiltrate into the collagen matrix of the dentin. However, adhesive infiltration is incomplete due in large part to the difficulty of removing residual water [5,6]. That water is associated

with a hydrolytic degradation of the organic matrix through activation of proteases that decreases the resin-dentin bond strength over time [7,8]. The problem of water residues is therefore fundamental to create a stable resin-dentin hybrid layer. Two main types of water molecules in the dental matrix have been described: bound and unbound water. Unbound (or free) water accounted for 75% of the total water in demineralized dentin, while bound water only accounted for 25% of the total [9]. The bound water layers are important components sustaining the stability of the collagen fibrils by maintaining the helical structures with the hydrogen bonding within collagen [10]. Water linked to the dentin matrix can be considered as structural water and cannot be evaporated at body temperature. Free water can be removed by evaporation and/or dehydration, notably by using water-miscible organic solvents [11]. Thus, a key to creating ideal resin-dentin bonds is the removal of the free water layers, which allows increasing the amount of resin monomers absorbed by the collagen [6,12]. The adhesive solvent is considered as one of the important factors of stability [13,14]. In particular, the ethanol wet-bonding concept was introduced [15]. Ethanol allows collagen water displacement and penetration of the hydrophilic resins inside the dentin matrix. Ethanol works mainly by mixing with unbound water through the rearrangement of the collagen monomers [6]. During application, interfibrillar water and ethanol evaporate together, leaving behind a monomer rich composition that avoids collagen collapsing and can limit its hydrolytic degradation.

In this work, in vitro application of a mild universal adhesive whose solvent is isopropanol on dentin, is compared according to the two adhesive strategies: self-etch or etch-and-rinse modes. For this purpose, we used a set of solid-state NMR experiments previously applied for the characterization of the main components of dentin, notably water [16]. We investigated the chemical and structural changes of the dentin mineral and collagen phases during the etching steps and adhesive infiltration. We were also interested in the behaviour of the adhesive functional monomers after adhesive infiltration. Particular attention was paid to the role of isopropanol and changes for water molecules and to the structural changes in collagen molecules when they shrink in contact with isopropanol solvent.

2. Materials and methods

2.1 Sample preparation

Human non-carious, unerupted third molars extracted for prophylactic reasons from 18- to 25-year-old patients were used to make dentin particles. No impacted teeth were included in the number. Specimens were prepared immediately after extractions.

To obtain dentin particles (Dentin), roots were sectioned, pulp was removed and enamel eliminated with diamond bur under irrigation. The dentin blocks obtained were crushed at room temperature and at high-pressure using a 13 mm diameter anvil placed in a hydraulic press (P/O/Weber, Remshalden, Germany) under pressures near 50 GPa. The resulting powder was sieved and collected, only fragments smaller than 50 μm were kept [17,18]. They were stored in small tightly closed vials to prevent dehydration. Etched dentin (Et-dentin) powder was prepared by adding 35 % phosphoric acid for 15 sec to the dentin particles. Following etching, the powder was rinsed for 30 sec with water and dried by swabbing with filter paper to absorb moisture without overdrying. This protocol is required to avoid contraction and collapse of the demineralized collagen matrix by maintaining it in its hydrated state [19].

The bonding system (Prime&Bond active™, Dentsply Sirona GmbH, Konstanz, Germany) was mixed directly into the normal or etched dentin powder (Dentin_Adhe and Et-Dentin_Adhe). Typically, 80 mg of dentin powder was mixed with 20 mg of adhesive solution for 30 sec and the wet powder was transferred directly and packed into zirconia rotors for solid state NMR experiments. This ratio was chosen after several trials to obtain the best compromise between the NMR signal intensities of the adhesive interacting with dentin and the free adhesive. The rotor was tightly closed with the rotor caps after about 2 min.

The composition of the adhesive solution (from scientific manual) is: Phosphoric acid modified acrylate resin - PENTA (dipentaerythritol pentacrylate phosphate) and MDP (10-methacryloyloxydecyl dihydrogen phosphate), bi- and multifunctional acrylate, isopropanol, water, initiator and stabilizer. Its pH value is 2.63 [20].

The coding name and composition of the samples used in this study are summarised in the Table 1.

Table 1 - Overview of the samples used in this study.		
Coding name	Sample	Composition
Dentin	Dentin	Dentin particles smaller than 50 μm
Et-dentin	Etched dentin	Dentin particles etched with 35% phosphoric acid, rinsed and dried
Adhe	Prime&Bond active™ Adhesive	Phosphoric acid modified acrylate resin, dipentaerythritol pentacrylate phosphate, 10-methacryloyloxydecyl dihydrogen phosphate), bi- and multifunctional acrylate, isopropanol, water, initiator, stabilizer
Dentin_Adhe	Dentin + Adhesive	80 mg of dentin particles mixed with 20 mg of adhesive
Et-dentin_Adhe	Etched dentin + Adhesive	80 mg of etched dentin particles mixed with 20 mg of adhesive

2.2 Solid-state NMR

Solid state NMR experiments were recorded on a Bruker Avance 400 III HD spectrometer operating at a 9.4 T magnetic field. Samples were packed into 4 mm or 1.3 mm zirconia rotors. The rotors were spun at 10 or 60 kHz (ν_r) at 298 K. ^{31}P MAS (Magic Angle Spinning) and CPMAS (Cross-Polarization MAS) were done with recycle delays of 300 s and 3 s, respectively and a contact time of 5 ms (spinning frequency of 10 kHz). ^{13}C MAS and CPMAS have been performed with recycle delays of 3 sec and 2 sec, respectively and a contact time of 2 ms (spinning frequency of 10 kHz). ^1H MAS performed with the DEPTH pulse sequence and ^1H MAS with rotor synchronous dipolar filtering of 6 ms and spin echo excitation (DF-SE) [21] were acquired with a recycle delay of 10 s (spinning frequency of 60 kHz). $^1\text{H}/^{31}\text{P}$ double cross polarization (DCP) MAS [22] were performed with a first CP transfer of 6 ms, a second CP transfer between 2 ms and a recycle delay of 3 s (spinning frequency of 60 kHz). $^1\text{H}/^{31}\text{P}$ HETCOR experiment was carried out with a contact time of 2 ms and with frequency switched Lee-

Goldburg homonuclear decoupling during ^1H evolution at a spinning frequency of 10 kHz. The 2D ^1H - ^1H double-quantum–single quantum (DQ-SQ) MAS and single Quantum–single Quantum (SQ-SQ, NOESY) experiments were recorded with a recycle delay of 3 s at a spinning frequency of 60 kHz. The DQMAS experiment were performed with 2 periods recoupling using the back-to-back (BABA) sequence. The NOESY experiment were achieved with a mixing time of 2.85 ms using the radio frequency-driven recoupling (RFDR) pulse sequence to recouple ^1H - ^1H dipolar interactions [23]. Chemical shifts were externally referenced to TMS for ^1H and ^{13}C , to 85% H_3PO_4 solution for ^{31}P .

3. Results

Dentin and Etched dentin (Et-dentin) comparison

The ^{31}P MAS spectrum of Dentin and Et-dentin are very different (Fig. 1a,b). That of Dentin shows the characteristic signal at ~ 3.2 ppm of phosphate groups of carbonated apatite, composed of a crystalline hydroxyapatite (HAp) core cover by amorphous calcium phosphates (ACP) [24-26]. This ^{31}P resonance displays dipolar correlations with H atoms at 0.0 ppm (HAp's hydroxyls) and between 5.5 to ~ 14 ppm (H_2O molecules and HPO_4^{2-} ions in ACP) in 2D $^1\text{H}/^{31}\text{P}$ HETCOR experiments (Fig. 2a). The apatitic signal is still present in a very low amount in Et-dentin, but several new, sharper and more intense signals could be observed. These signals were assigned from the literature [27,28] and 2D $^1\text{H}/^{31}\text{P}$ HETCOR experiment (Fig. 2b). The main ^{31}P resonance at ~ 1.6 ppm that correlates mainly with H atoms at 4.6, 6.3, 10.1 ppm in the HETCOR spectrum corresponds to the di-calcium phosphate di-hydrate or brushite species ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD). The second species with ^{31}P resonances at ~ 0.2 ppm and ~ -4.0 ppm (correlations with H atoms at 5.9, 9.8 and 11.9 ppm) was identified as monocalcium phosphate monohydrate ($\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$, MCPM). The third species with the lowest ^{31}P resonances at ~ 2.7 ppm and ~ -0.2 ppm (correlations with H atoms at 6.8 and 11.1 ppm) was assigned to octacalcium phosphate ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$, OCP).

In conclusion, the ^{31}P MAS data of Et-dentin show a strong diminution of the carbonated apatite species after etching with phosphoric acid. Much of the apatite dissolved by the phosphoric acid has probably been removed by the rinsing step carried out afterwards. However, we evidenced that part of the dentin mineral phase can be preserved after etching in the form of different hydrated calcium phosphate phases.

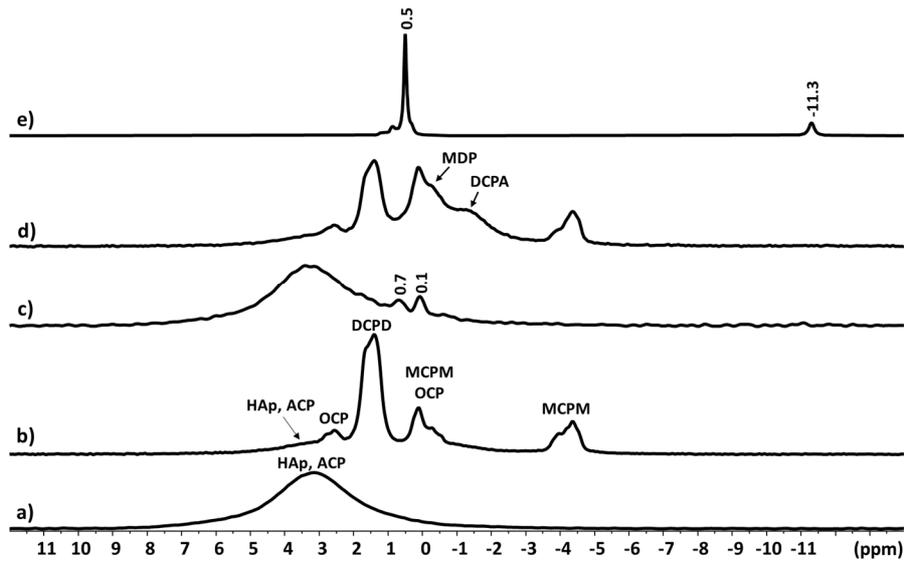
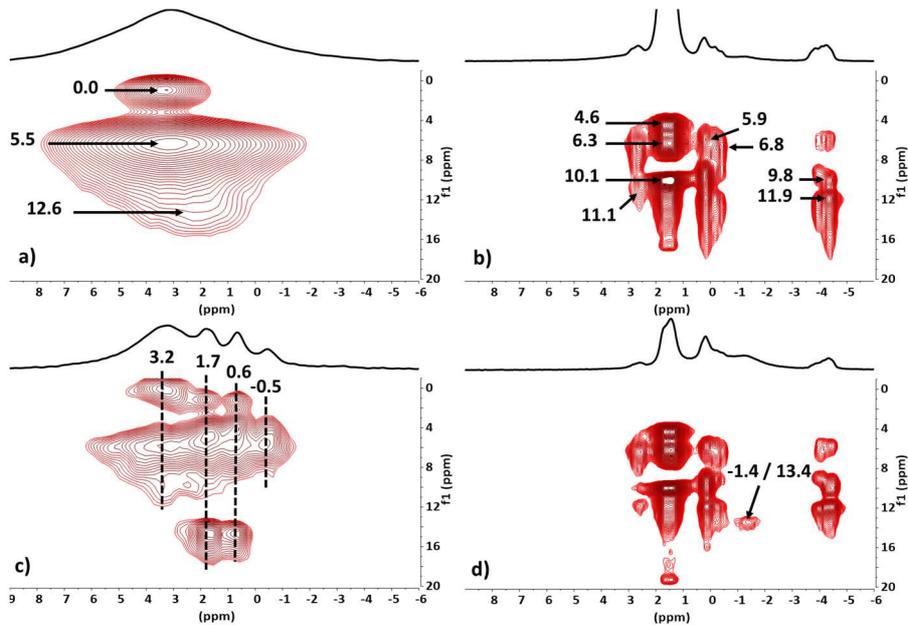


Fig. 1. ^{31}P MAS of (a) Dentin, (b) Et-dentin (Etched dentin), (c) Dentin_Adhe, (d) Et-dentin_Adhe and (e) Adhe (Adhesive).

Contrary to ^{31}P data, the ^{13}C CPMAS spectra (Fig. 3a,b) are very similar for Dentin and Et-dentin. These spectra are dominated by the signals from collagen proteins [24,29]. The ^{13}C signals of the Et-dentin sample are only slightly broader than the ones of the Dentin sample. This can indicate a higher structural disorder for the Et-dentin collagen associated to the changes in the mineral phases but also possibly to a slight dehydration [30]. Moreover, we observe the disappearance of the ^{13}C signal of citrate ions (at 179.9 and 75.5 ppm) [31] and small changes in the signal of the carboxyl groups (178-164 ppm) that can potentially be associated to the relative quantity of carboxylate ions CO_3^{2-} linked to the apatitic phase [32].



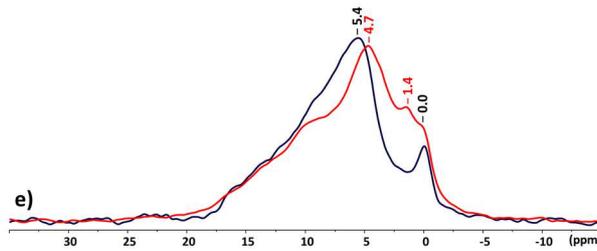


Fig. 2. 2D $^1\text{H}/^{31}\text{P}$ HETCOR of (a) Dentin, (b) Et-dentin, (c) Dentin_Adhe and (d) Et-dentin_Adhe ; Horizontal trace: ^{31}P CPMAS spectra. (e) $^1\text{H}/^{31}\text{P}$ DCP of Dentin (black) and of Dentin_Adhe (red).

The ^1H MAS spectrum of Dentin and Et-dentin also shows some differences (Fig. 4a,b). First, some new ^1H resonances are detected at 10.1 and 11.9 ppm. These correspond to the hydroxyl P-OH groups of the calcium phosphate DCPD and MCPM species observed in the $^1\text{H}/^{31}\text{P}$ HETCOR experiment. Secondly, the ^1H resonance at 0.0 ppm of hydroxyapatite's hydroxyls is absent due to the strong reduction of the amount of the apatitic phase. Lastly, the broad signal of water molecules at 5.0 ppm for Dentin shifts to high frequency at 6.4 ppm for Et-dentin. This is the result of an acidification of the water phases. The other ^1H signals are mostly related to the collagen proteins and should not change too much according to the ^{13}C results. These differences are even more pronounced in ^1H MAS NMR experiments performed with a long dipolar filtering time of 6.0 ms that allows for the edition of signal with weak H-H dipolar couplings (mobile species or isolated H atoms) (Fig. 4c,d). The appearance of new signals at 11.9 and 10.1 ppm and the disappearance of the signal at 0.0 ppm are clearly seen. For Dentin, several other resonances are detected notably at 1.2, 4.8 and 7.6 ppm. They have been previously assigned to mobile hydroxyl groups, water molecules and collagen amide hydrogens [16]. For Et-dentin, a pool of mobile water is also observed at ~ 6.0 ppm with also several sharp resonances in the spectral range between 6.5 to 1.2 ppm. These resonances are probably related to different kinds of mobile hydroxyl groups but could not be assigned with certainty. However, their quantities are quite low as can be deduced from the ^1H MAS spectrum.

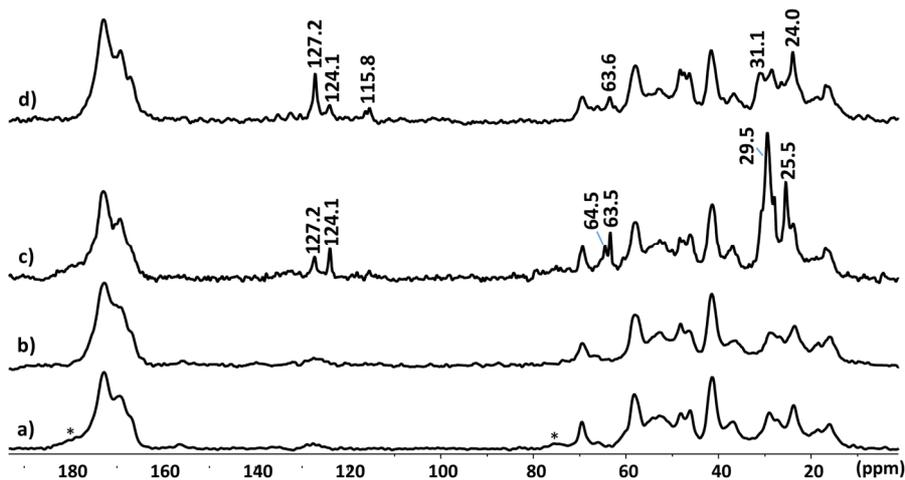


Fig. 3. ^{13}C CP MAS of (a) Dentin, (b) Et-dentin, (c) Dentin_Adhe and (d) Et-dentin_Adhe. * Citrate ions.

Finally, the Dentin and Et-dentin were compared through the ^1H NOESY-RFDR NMR experiment (Fig. 5). For Dentin, the main set of correlations involved dipolar transfer of magnetization (due to spatial proximity) between the H of collagen (NH, H α , H β , H γ ...). Dipolar correlations could also be observed between H of water molecules

(~ 5.0 ppm) and H of collagen associated with good hydration of collagen fibrils. For Et-dentin, similar dipolar correlations could be detected between the H of collagen and H of water molecules (~ 6.4 ppm). This confirms the results observed by ^{13}C NMR and indicates that the collagen fibrils in Et-dentin are still well hydrated. Furthermore, strong correlations are also seen between H of water molecules and hydroxyl groups of the mineral phase evidencing a spatial proximity between them.

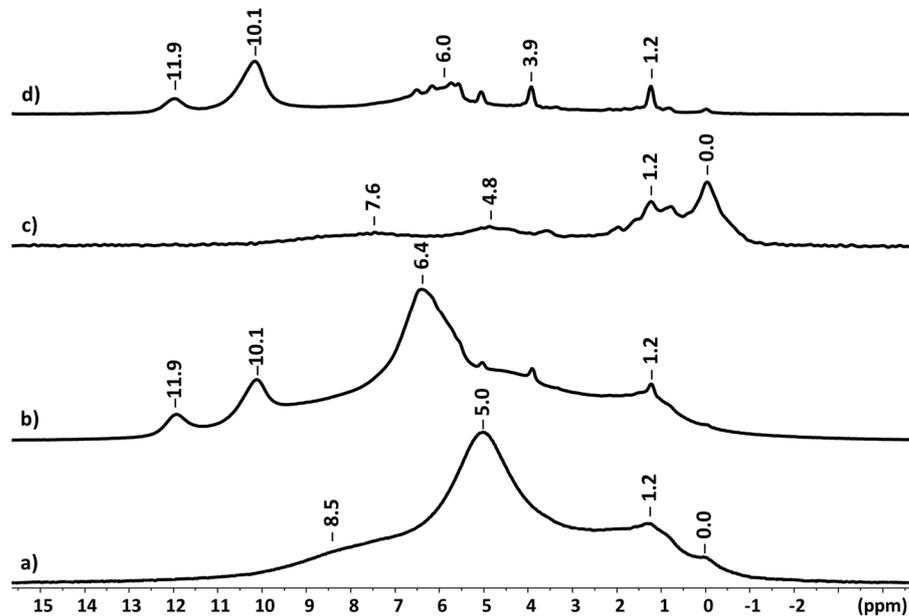


Fig. 4. ^1H MAS (a, b) and ^1H MAS with 6 ms dipolar filtering time (c,d) of Dentin (a,c) and Et-dentin (b,d).

Adhesive (Adhe)

The Prime&Bond activeTM (Dentsply Sirona) universal adhesive is a solution containing several components. It is primarily composed of an acrylate resin (bifunctional acryl crosslinker), phosphoric acid esters PENTA (dipentaerythritol pentacrylate phosphate) and MDP (10-methacryloyloxydecyl dihydrogen phosphate), isopropanol and water (solvents). In order to compare the NMR spectra of the adhesive with its mixtures with Dentin and Et-dentin under the closest possible conditions, the pure adhesive solution was placed in a NMR MAS rotor. ^{31}P , ^{13}C and ^1H MAS spectrum of Dentin are shown in Fig. 1e, 6a and 7a. Several signals around 0.5 ppm were detected in the ^{31}P spectrum corresponding to the phosphate monoester moieties of MDP and PENTA. An additional resonance at -11.3 ppm can also be seen assignable to a pyrophosphate species. ^{13}C MAS spectrum presents a set of majority signals at 166.7, between 134 and 115 ppm and between 50 and 46 ppm associated with the acrylate resin. Two intense signals corresponding to isopropanol are detected at 63.2 and 24.6 ppm. Numerous unassigned weak signals are also observed corresponding to other minor constituents of the adhesive including the phosphoric acid esters. However, the MDP molecules can be identified by its alkyl chain signal at 29.0 ppm. ^1H spectrum displays also mainly the alkene signals of the acrylate resin between 6.6 and 4.9 ppm, isopropanol signals at 3.8 and 1.0 ppm and a water signal at 4.7 ppm. Again, numerous unassigned weak signals are also detected, notably in the 4.2-2.9 ppm range (alkane H connected to electronegative nuclei like oxygen and nitrogen) and in the aliphatics range (2.0 - 0.6 ppm).

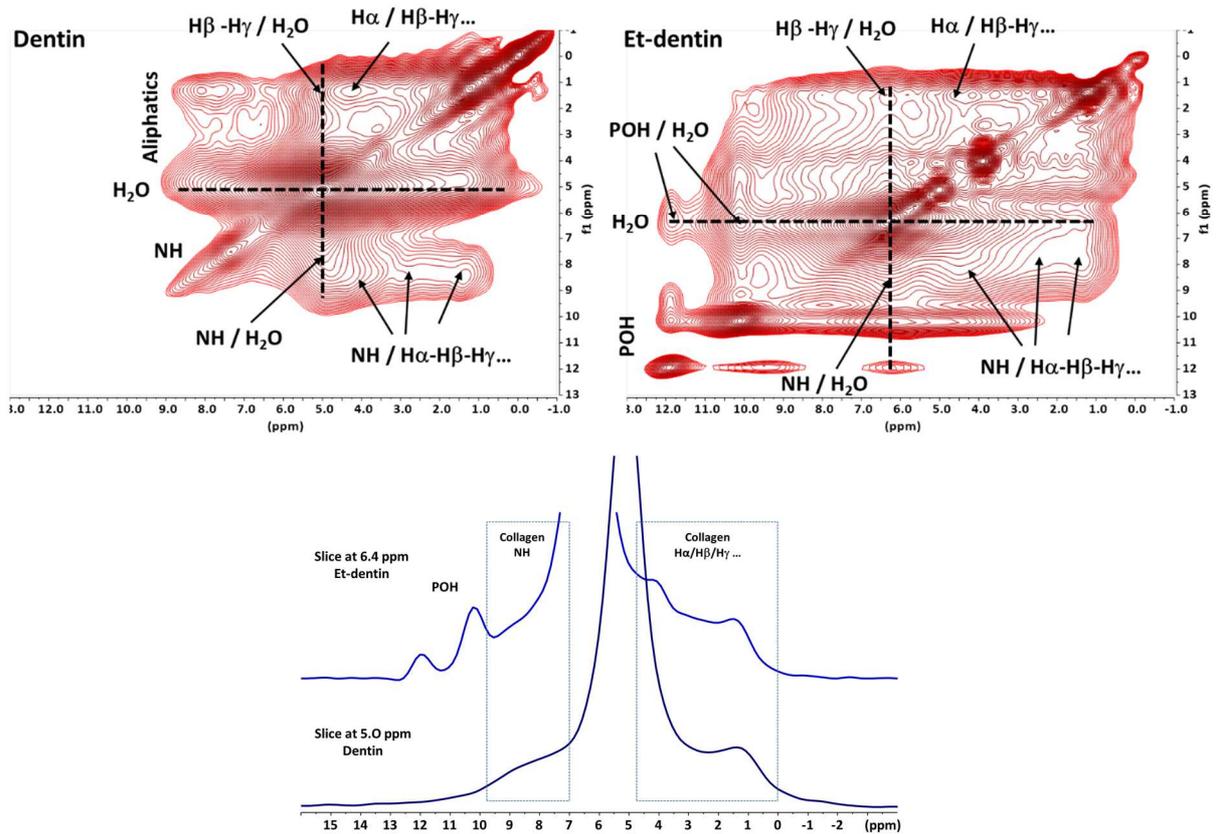


Fig. 5. (top) 2D ^1H NOESY-RFDR and (bottom) slices taken at H_2O frequency of Dentin and Et-dentin.

Dentin – adhesive: self-etch mode (Dentin Adhe)

The ^{31}P MAS spectra of dentin mixed with the adhesive (self-etch mode), with direct- or cross-polarisation are shown in Fig. 1c and Fig. 2c. The ^{31}P MAS spectrum of Dentin_Adhe shows mainly the broad dentin apatite signal at ~ 3.2 ppm but also two other sharp signals at 0.1 and 0.7 ppm. These last two signals can be assigned to the phosphate monoester moieties of the adhesive with two different environments. The ^{31}P CPMAS spectrum of Dentin_Adhe displays also mainly the apatite resonance and three other resonances at 1.7, 0.6 and -0.5 ppm. These last signals were also observed in the ^{31}P MAS spectrum but with very weak intensity. As the cross-polarisation scheme only evidences species with low mobility, these results indicate that most of the adhesive phosphate monoesters keep a liquid behaviour while a small part become more rigid by interacting with dentin components. These ^{31}P CPMAS resonances have already been described for dentin demineralized by the acidic monomer MDP by Yokota and Nishiyama [33]. The signals at 1.7, 0.6 and -0.5 ppm were assigned to di-calcium salts of MDP. The signals of mono-calcium salts are usually observed at lower frequencies (below -1.3 ppm).

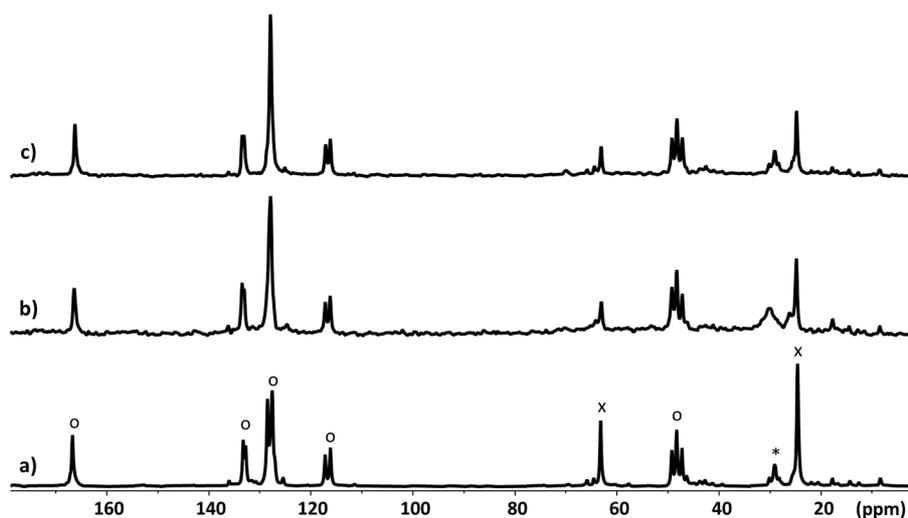


Fig. 6. ^{13}C MAS of (a) Adhe, (b) Dentin_Adhe and (c) Et-dentin_Adhe. o: acrylate resin, x: isopropanol, *: CH_2 of MDP alkyl chain.

Additional information could be obtained with the 2D $^1\text{H}/^{31}\text{P}$ HETCOR experiment (Fig. 2c). The ^{31}P resonance of apatite (at 3.2 ppm) still displays the correlations with OH^- groups (at 0.0 ppm) and with H_2O molecules and HPO_4^{2-} ions (between 5.5 to ~ 14 ppm). The ^{31}P CPMAS resonances at 0.6 and 1.7 ppm showed very similar correlations. They correlate with H atoms at 1.2 and ~ 4.0 ppm that can be attributed to central CH_2 and to CH_2O group of the MDP alkyl chain confirming that these signals belong to the phosphate monoester species. However, unexpectedly, these resonances of di-anionic phosphate groups (di-calcium salts) also correlate strongly with H atoms in the 5 - 7 and 14 - 17 ppm range that correspond to hydroxyl groups of water and P-OH groups. These results suggest that either the di-calcium MDP salts are absorbed at the amorphous calcium phosphate surface of apatite or form a composite phase containing H_2O molecules and phosphate ions. The last ^{31}P CPMAS resonance at - 0.5 ppm shows the most intense dipolar correlations with H atoms in the 5-6 ppm range assignable to hydroxyl groups of water molecules. It displays additional weak correlations with CH_2 groups and HPO_4^{2-} ions. This signal also belongs to a phosphate monoester species but with a different organisation than the species at 0.6 and 1.7 ppm. The immobilisation of some MDP molecules is confirmed by the appearance of a CH_2 signal at ~1.4 ppm in the 1D $^1\text{H}/^{31}\text{P}$ double cross polarization (DCP) NMR experiment providing ^{31}P -filtered ^1H NMR spectrum of rigid species (Fig. 2e). This experiment also evidences a shift of the water signal in interaction with the mineral phase from 5.4 to 4.7 ppm.

^{13}C MAS spectrum of Dentin_Adhe looks quite similar to that of Adhe alone (Fig. 6b). The main set of resonances associated with the acrylate resin are only slightly broadened due to the strong heterogeneities of the sample. It indicates that the acrylate monomers keep a liquid behaviour in the presence of dentin with no evidence of strong interaction between them. The main difference is a notable intensity decrease of the isopropanol signals at 63.2 and 24.6 ppm. Collagen signals in the ^{13}C CPMAS spectrum of Dentin_Adhe are also very similar to Dentin ones (Fig. 3c). They evidence that the structure of the collagen strands is not affected by the presence of the adhesive. Moreover, several new signals could be detected. Those at 29.5 and 64.5 ppm can be attributed to central CH_2 and to CH_2O group of the MDP alkyl chain confirming the presence of MDP with low mobility. The resonances at 25.5 and 63.5 ppm correspond to isopropanol molecules with low mobility that interact with some dentin

components. The signals at 124.1 and 127.2 ppm are characteristic of alkene carbons and can be tentatively assigned to the ones of the rigid phosphate monoester species.

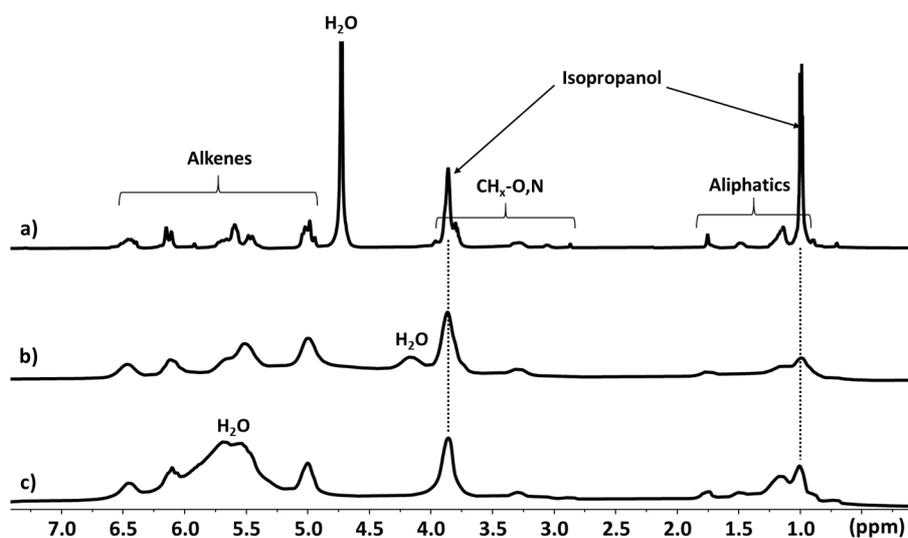


Fig. 7. ^1H MAS of (a) Adhe, (b) Dentin_Adhe and (c) Et-dentin_Adhe.

The ^1H MAS spectrum of Dentin_Adhe mainly shows the sharp resonances of the adhesive components that retain a liquid behaviour (Fig. 7b). The salient information that can be learned from this experiment is the sharp decrease of the water signal compared to that of isopropanol. Interestingly, the residual free water signal in Dentin_Adhe also has a chemical shift of 4.2 ppm, which is lower than that usually observed for water clusters (4.7 ppm). It suggests weaker hydrogen bonds for the free water molecules in Dentin_Adhe than in water clusters.

To obtain more information on the species with low mobility, 2D ^1H DQ-MAS were performed to identify pairs of spatially close hydrogens. The DQ-MAS spectrum of Dentin_Adhe shows similar DQ dipolar correlations between the H of collagen as for Dentin (Fig. 8). Concerning the ^{13}C CPMAS result, it confirms that the structure of the collagen strands is very similar with or without the presence of the adhesive. Also, the DQ correlations assigned previously to structural water molecules strongly bound to collagen and ACP [16] are still clearly detected evidencing that these water molecules are not affected by the presence of the adhesive. In contrast, the signal of the interfacial water molecules (between collagen fibrils or between collagen and the mineral phase) is strongly impacted by the presence of the adhesive. This signal shifts to low frequency from 5.0 to 4.5 ppm with a notable intensity decrease as it can be observed in DQ slice taken at 9.1 ppm (Fig. 8 bottom). Moreover, some signals assignable to isopropanol are detected in the DQ-MAS spectrum of Dentin_Adhe (DQ correlations at 3.8/9.1 and 1.0/6.0), indicating spatial proximities between isopropanol molecules with water or collagen molecules. It can be concluded that the adhesive solution infiltrates at the interfaces around the collagen fibrils and mixes with the interfacial water molecules initially present.

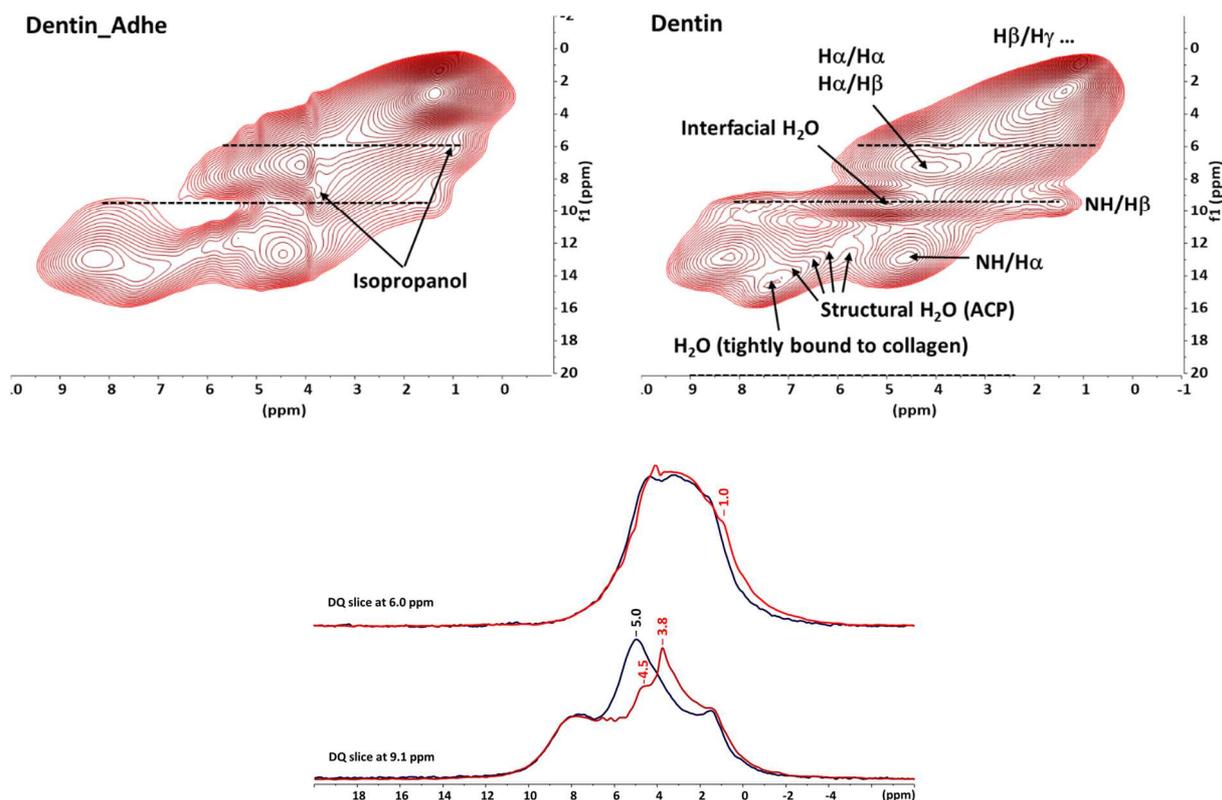


Fig. 8. (top) ^1H DQ MAS of Dentin and Dentin-Adhe. (bottom) slices in the DQ dimension at 6.0 and 9.1 ppm of Dentin (black) and Dentin_Adhe (red).

Etched dentin – adhesive: etch and rinse mode (Et-dentin Adhe)

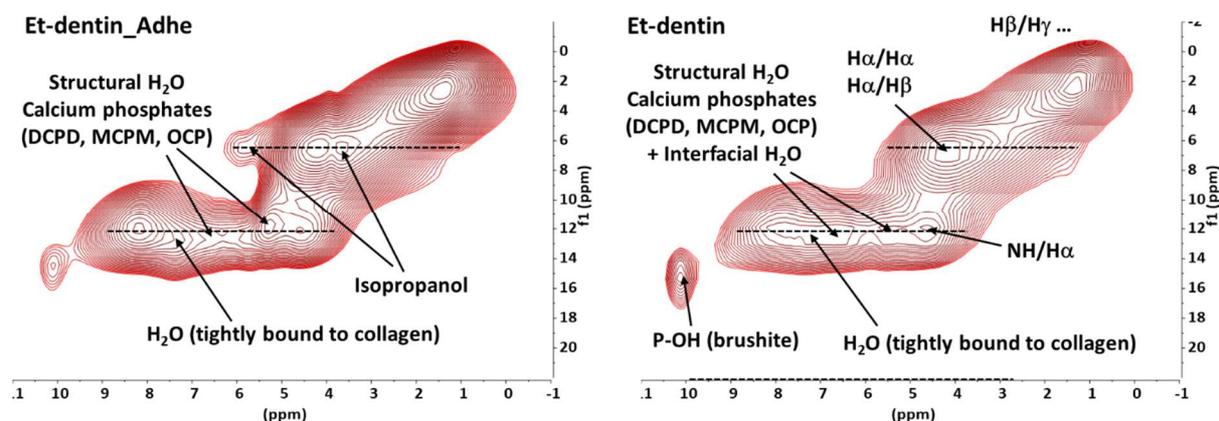
The ^{31}P MAS spectra of Et-dentin mixed with the adhesive (etch-and-rinse mode), with direct- or cross-polarisation are shown in Fig. 1d and Fig. 2d. The ^{31}P MAS spectrum of Et-dentin_Adhe presents the same HAP, ACP, DCP, MCPM and OCP signal observed for Et-dentin. Two additional signals can also be detected. The first one around 0 ppm, underneath signals of MCPM and OCP, can be assigned to the phosphate monoester moieties of the adhesive. The second one at ~ -1.4 ppm was assigned with the help of the 2D $^1\text{H}/^{31}\text{P}$ HETCOR experiment (Fig. 2d). The ^{31}P CPMAS signal at -1.9 ppm for Et-dentin_Adhe displays a main dipolar correlation with hydroxyl signal of P-OH groups at 13.4 ppm and was assigned from the literature [27,28] to the di-calcium phosphate anhydrous or monetite species (CaHPO_4 , DCPA). The appearance of monetite may in fact be related to the decrease in the amount of brushite (DCPD) observed on the ^{31}P MAS spectra. It indicates a partial dehydration of the mineral phase. In the ^{31}P CPMAS and HETCOR experiment, no clear signal of di-calcium MDP salts could be observed for Et-dentin_Adhe as opposed to what had been found for Dentin_Adhe. If these salts are present in Et-dentin_Adhe, they are in very low quantity and their signals are masked by the other phosphate species.

^{13}C (CP) MAS results for Et-dentin_Adhe are very similar to that of Dentin_Adhe. The ^{13}C MAS (Fig. 6c) mainly features the mobile acrylate resin resonances and those of isopropanol with reduced intensity. The ^{13}C CPMAS shows collagen signals quite similar to Et-dentin ones (Fig. 3d). However, some Et-dentin_Adhe collagen resonances are slightly broader and could indicate a slight dehydration of the collagen fibrils in the presence of the adhesive. Resonances of rigid isopropanol at 24.0 and 63.6 ppm are also detected for Et-dentin_Adhe as for Dentin_Adhe. A weak signal at 31.1 ppm can be attributed to central CH_2 of rigid MDP alkyl chain. This signal is

much weaker than for Dentin_Adhe and confirms the ^{31}P CPMAS result. It suggests that the phosphate monoester moieties can only interact with the calcium ions of the apatite phase (HAp, ACP, present in small quantity in Et-dentin_Adhe) but not with those of the other calcium phosphate phases (DCPD, MCPM, OCP and DCPA). The alkene signals at 124.1 ppm also have a reduced intensity compared to that of Dentin_Adhe and can be assigned to the rigid phosphate monoester species. In contrast, the signal at 127.2 ppm is quite intense and corresponds to rigid alkene carbons not clearly identified. Some weak non-identified alkene carbons are also detected around ~ 116 ppm.

The ^1H MAS spectrum of Et-dentin_Adhe as for Dentin_Adhe shows mainly the sharp resonances of the adhesive components that retain a liquid behaviour (Fig.7c). Again, a sharp decrease of the water signal compared to that of isopropanol is observed. Furthermore, the residual free water signal in Et-dentin_Adhe has a chemical shift of 5.6 ppm, an intermediate value between that of water signals in the adhesive (4.7 ppm) and in Et-dentin (6.4 ppm). It indicates a fast exchange between the mobile water molecules present in Et-dentin and those introduced through the adhesive solution.

The DQ-MAS spectrum of Et-dentin_Adhe shows similar DQ dipolar correlations between the H of collagen than for Et-dentin and Dentin (Fig. 9 top) in agreement with the ^{13}C CPMAS result. As for Dentin_Adhe, the DQ correlation at $\sim 7/14$ ppm of the structural water molecules strongly bound to collagen are still observed. These signals are, however, less resolved due to the presence of signals associated with water molecules linked to the different calcium phosphate species (DCPD, MCPM and OCP) with chemical shifts in the 6-7 ppm range (identified in the $^1\text{H}/^{31}\text{P}$ HETCOR). Also for Et-dentin, the interfacial water molecules have a chemical shift of 6.4 ppm in the same spectral region as the structural water molecules. However, with the help of DQ slices (Fig. 9 bottom), some differences could be observed between Et-dentin_Adhe and Et-dentin. In the DQ slice at 11.9 ppm, the signal of interfacial water at 6.4 ppm shows an intensity decrease as for Dentin_Adhe. Some new DQ correlations are also detected (notably the ones at 3.7/6.8 and 6.0/6.8) that can be associated to isopropanol molecules with low mobility close to water or collagen molecules. These results are similar to Dentin_Adhe ones and indicate adhesive solution infiltration around the collagen fibrils.



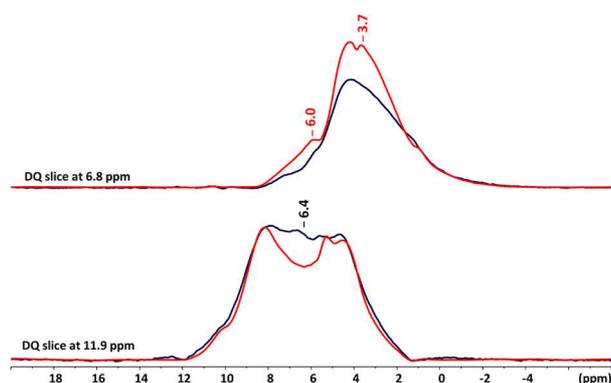


Fig. 9. (top) ^1H DQ MAS of Et-dentin and Et-dentin_Adhe. (bottom) slices in the DQ dimension at 6.8 and 11.9 ppm of Et-dentin (black) and Et-dentin_Adhe (red).

The NMR experiments used in this study with their purposes and main results are summarized in Table 2.

Table 2 - NMR experiments used in this study with their purposes and main results.		
NMR experiment	Purpose	Results
^{31}P MAS	Identification / Quantification of P	Identification and quantification of P of the dentin mineral phases and of the adhesive
^{31}P CP MAS	Emphasis of P with low mobility close to H	Emphasis of P close to H of the dentin mineral phases and of the adhesive P species in interaction with the dentin
$^1\text{H}/^{31}\text{P}$ HETCOR	Provides proximity information between H and P in rigid species	Assignment of the different rigid P species
^{13}C MAS	Identification / Quantification of C	Shows the presence of a large amount of adhesive molecules with a very mobile behaviour
^{13}C CP MAS	Emphasis of C with low mobility close to H	Demonstrates that the collagen structure is little changed by the etching and the adhesive and that some adhesive species interact with dentin.
^1H MAS	Identification / Quantification of H	Shows the presence of a large amount of adhesive molecules with a very mobile behaviour. Monitoring the amount of water and solvent in the samples
^1H MAS with dipolar filtering	Emphasis of H with weak H-H dipolar couplings (mobile species or isolated H)	Emphasis of the mobile H species in the dentin and Et-dentin samples.
^1H DQ MAS	Provides information about two-H proximity in rigid species	Highlights the presence of structural water and allows for an assessment of the amount of interfacial water and isopropanol
^1H NOESY-RFDR	Provides proximity information between H	Observation of H contacts between water and collagen molecules in the dentin and Et-dentin samples.

Abbreviations: H; hydrogen atom; P; Phosphorus atom; C; Carbon atom.

4. Discussion

In this work, the results derived from use of mild universal adhesives on normal dentin or etched dentin are compared to test the equivalence of both procedures, notably to form stable resin dentin hybrid layer. Our results show different situations depending on whether one is interested in the mineral phase or in collagen.

For the mineral phase, the interaction between the adhesive and the dentin or etched dentin is different. It has been shown that acidic monomer, such as MDP, produced calcium salts of MDP (MDP-Ca salts) through the decalcification process of synthesized hydroxyapatite, enamel or dentin [32-36]. These studies have established adsorption of the adhesive phosphate monoesters on the apatite surface and the formation of a layer of MDP-calcium salts. However, the chemical nature of the molecular of MDP-Ca species depend on the experimental conditions and formation of salts composed of mono- and di-calcium MDP monomer and dimer complexes or of CaRPO_4 and $\text{Ca}(\text{RHPO}_4)_2$ phases have been described. The concomitant production of a crystalline phase of di-calcium phosphate di-hydrate (DCPD) was also observed. The formation of MDP-Ca salts is evidenced for the Dentin_Adhe with ^{31}P CPMAS resonances very similar to the ones assigned to molecular di-calcium salts of MDP by Yokota and Nishiyama [32]. Through space dipolar correlations of these ^{31}P resonances with acidic P-OH groups (14-17 ppm) indicated an absorption of the di-calcium MDP salts at the ACP surface or to the formation of a composite di-calcium MDP salt - phosphate phase. When applying adhesive in etch-and-rinse mode, it is noted after etching that the mineral phase is obviously strongly impacted with transformation of apatite into hydrated phosphate calcium DCPD, MCMP and OCP species. No evidence of decalcification for these calcium phosphate phases generated by the etching step was observed after the addition of the adhesive. Only a small quantity of rigid MDP molecules could be evidenced by ^{13}C CPMAS that can be potentially linked to the presence of a small amount of residual apatite phase. So for Et-dentin_Adhe, only a very small quantity of MDP-Ca salts is produced. The main evolution after the addition of the adhesive is a transformation of DCPD (brushite) into DCPA (monetite) due to a partial dehydration of the mineral phase. The transformation of apatite into brushite and monetite was also noted in hybrid layer formation with a phosphoric acid [37,38].

For the collagen phase, the interactions between the adhesive and the normal dentin or etched dentin have many similarities. However, before the addition of the adhesive, the ^1H signal of water molecules of the Et-dentin presents a shift towards high frequency compared to that of Dentin (6.4 ppm versus 5.0 ppm), which is associated with an acidification of the medium following the etching step. Despite the pH decrease, collagen ^1H and ^{13}C NMR signals are only weakly affected, indicating that the collagen structure is not strongly affected by the etching step. This is in line with the fact that dentin collagen is the most cross-linked collagen in the body and these cross-links are responsible for the ability of dentin collagen to be acid-etched during bonding procedures without denaturing its structure [39,40]. After adding adhesive to Dentin and Et-dentin, the collagen ^{13}C signals remain very similar, evidencing that the structure of the collagen strands is also not strongly impacted by the presence of the adhesive solution. Furthermore, the ^1H NMR signal of structural water molecules strongly bound to collagen are still clearly detected in the DQMAS experiment evidencing that the collagen strands remain well hydrated. Other important information that can be obtained for Dentin_Adhe and Et-dentin_Adhe by the ^1H NMR experiments is the sharp decrease and the shift to low frequency of the water ^1H signal after the adhesive addition. The low frequency shifts (from 5.0 to 4.2 ppm and from 6.4 to 6.0 ppm for Dentin_Adhe and Et-dentin_Adhe, respectively) cannot be explained by a pH increase after adhesive addition, as it has a low pH of 2.63. This suggests a weaker stabilization of water due to a decrease in the strength of their hydrogen bonds. In addition, the DQMAS signal of interfacial

water molecules, more strongly bound to collagen, also decrease significantly. These data indicate that for both systems, a rapid evaporation of free, loosely and moderately bound water occurs in presence of the adhesive solution but also that the more strongly collagen bound water are not displaced. Moreover, isopropanol molecules with low mobility that shows dipolar correlations with some dentin components (water and collagen) were evidenced. These results are consistent with studies performed with ethanol as solvent for adhesive [5,8,14,41]. It has been shown that the layers of water strongly bound to collagen (that represent about 25% of total dentin water) are very difficult to remove but that adhesive solution can permeate tightly bound water during infiltration in collagen. Those authors speculated that bound water holds intermolecular spaces between collagen molecules open for adhesive resin monomer uptake. They also demonstrated that water/ ethanol mixing in the non-bound hydration layer is enhanced through the rearrangement of the collagen monomers. The removal of free and loosely bound water is therefore facilitated with ethanol molecules that can potentially replace them. It can be seen that in our system, the isopropanol acts in a similar manner than ethanol and can permeate between collagen molecules. Replacement of all loosely bound or free water with water-free, but water-miscible solvents are considered as a key to creation of ideal resin-dentin bonds [5,11].

When loosely bound water was partially replaced by ethanol, it was shown that adhesive resin monomers, especially water-insoluble acrylates, might partially permeate between collagen strands on the most peripheral parts of the collagen [10]. Our results indicate that acrylate monomers and most of the phosphate monoesters keep a liquid behaviour (sharp ^1H , ^{31}P and ^{13}C NMR resonances) after the addition of the adhesive solution to dentin, etched or not. It shows that these functional monomers are free to diffuse in an isopropanol rich area and so can permeate between collagen strands as isopropanol. It was shown by liquid state NMR that MDP can interact with collagen through hydrophobic interactions between the MDP aliphatic chain and the collagen surface [42]. The ^{13}C CPMAS experiments evidence the presence of rigid MDP alkyl chain for Dentin_Adhe and Et-dentin_Adhe. This signal was assigned to rigid MDP-Ca salts but potentially can also be assigned to MDP in interaction with collagen, especially for Et-dentin_Adhe where the amount of MDP-Ca salts is low. Furthermore, some ^{13}C CPMAS signals in the alkene area (115-130 ppm) are detected. The one at ~124 ppm can be attributed to rigid MDP by comparing its relative intensity with that of the MDP alkyl chain signal in the spectrums of Dentin_Adhe and Et-dentin_Adhe. Signals at ~127 and 116 ppm whose relative intensities are greater in the Et-dentin_Adhe spectrum could possibly be associated with a small quantity of rigid acrylates interacting with collagen.

We show here that solid state NMR experiments enable a fine monitoring of the interactions at the molecular level between adhesive solution and dentin material and can provide important structural and dynamic data on dentin and adhesive components when they are mixed together. It is crucial to obtain such information for optimizing the composition of adhesive solution in order to improve the stability of dentin resin hybrid layer over time. Notably, the study of the behaviour of the dentin water molecules is considered as a key point.

5. Conclusions

In this work, we evidence that important information about the interaction between dentin and dental adhesive can be obtained using solid state NMR experiments. The in vitro use of a mild universal adhesive on dentin was compared for both the adhesive strategy, self-etch or etch-and-rinse modes. The behaviour at the mineral phase is different for the two strategies. With the self-etch mode one retains mainly the apatitic phase, while with the etch-

and-rinse mode there is a reorganization into different stable calcium phosphate phases. This leads to a difference in the amount of adhesive phosphate monoesters calcium salt formed, which is greater in the presence of apatite while the etch-and-rinse protocol limits the amount of available calcium. In contrast, the adhesive interacts very similarly to the level of collagen in both protocols. It leads to a decrease in the amount of the less stable water molecules at the collagen-mineral and collagen-collagen interfaces induced by the presence of isopropanol as the adhesive solvent. The structure of collagen is little affected by this change in solvation. In particular, the water molecules strongly attached to the collagen are preserved. Acrylates monomers remain mobile and can infiltrate the collagen.

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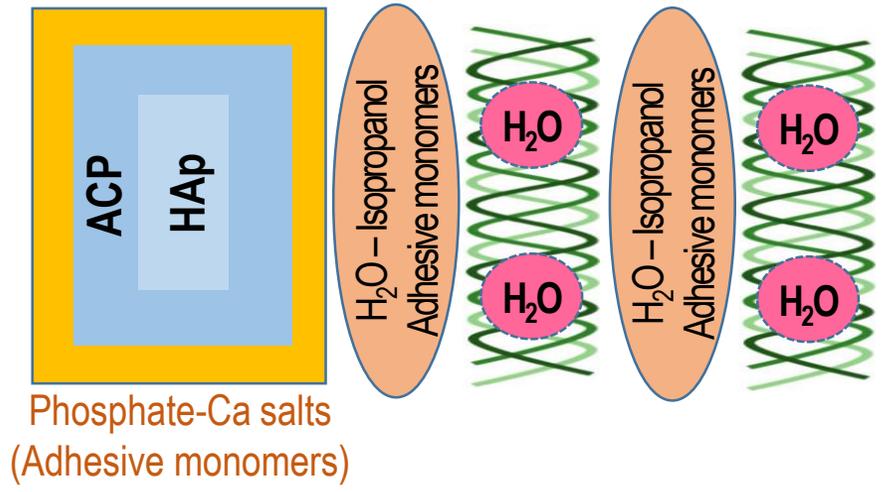
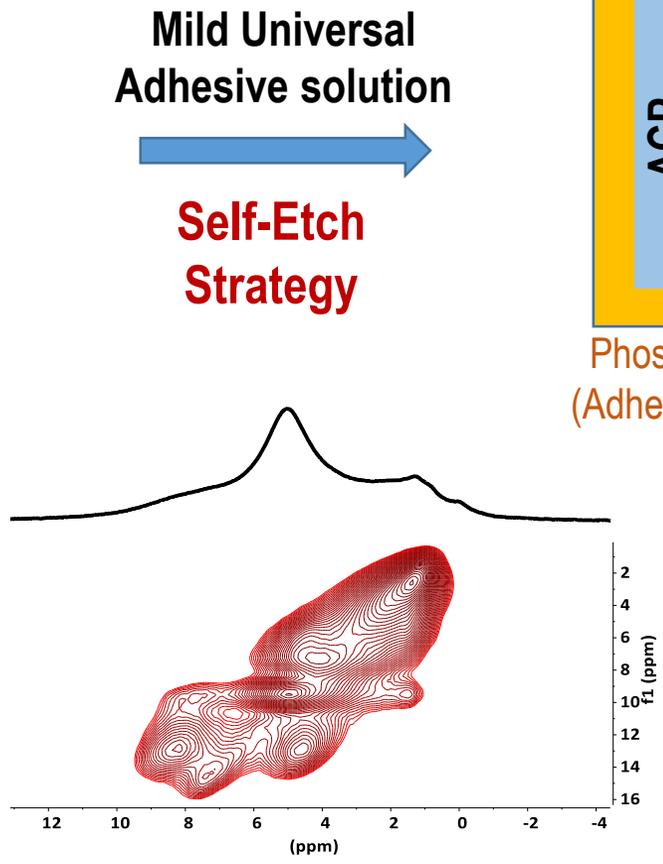
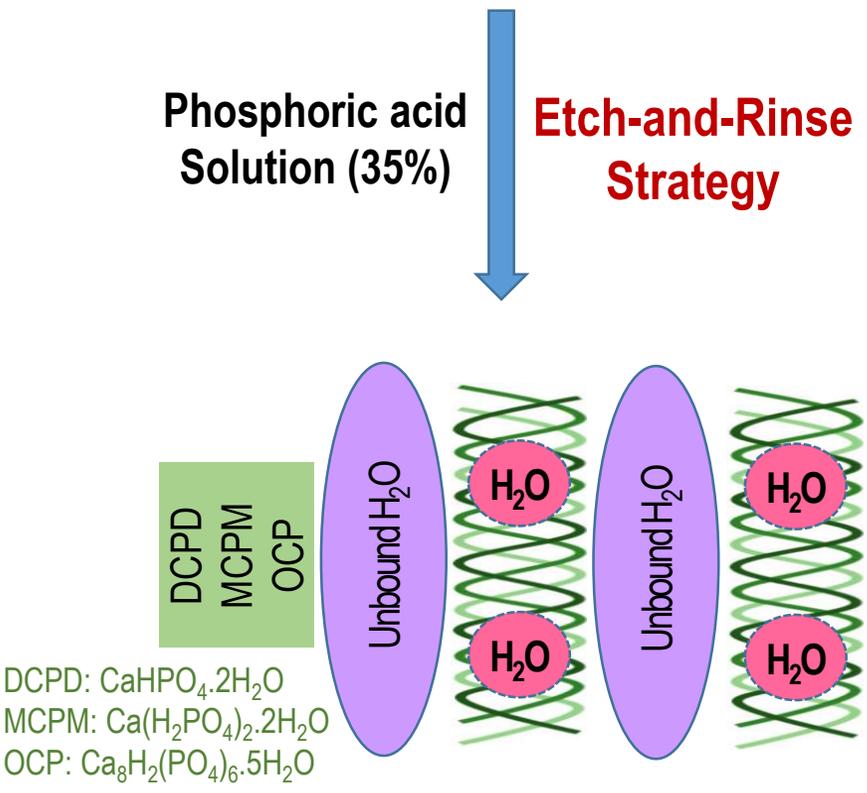
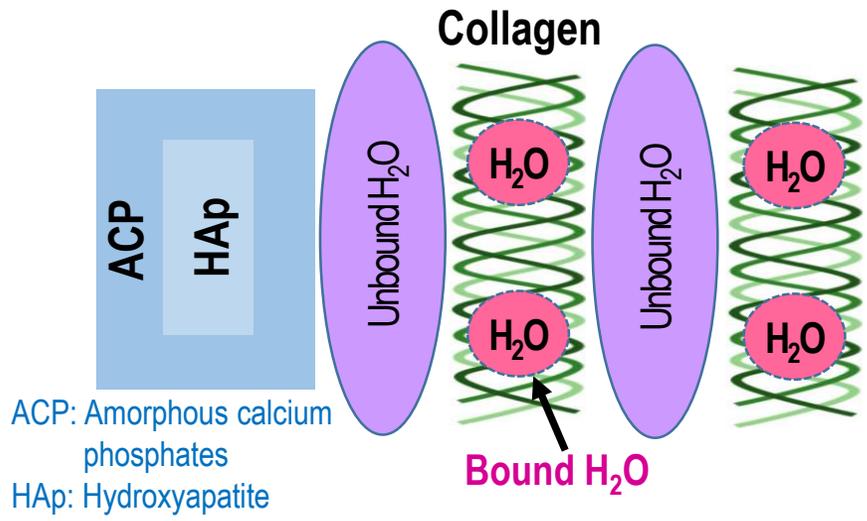
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Solid state NMR Analysis

