J Mater Sci: Mater Med (2007) 18:2093–2099
DOI 10.1007/s10856-007-3148-9

Multinuclear magnetic resonance studies on the chemical interaction of a self-etching adhesive with radicular and coronal human dentin

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Received: 20 November 2005 / Accepted: 21 August 2006 / Published online: 12 June 2007

Abstract This study presents evidence at molecular level for the chemical interaction between human dentin from different tooth regions and a monomer with phosphate groups, incorporated in the formulation of a simplified adhesive system. Because dentin was observed as a powder, previous verification was obtained for an eventual collagen denaturation due to the grinding process. The presence of chemical bonds involving coronal (CD) or radicular dentin (RD) was investigated using multinuclear magnetic resonance (MR) techniques. Narrow signals were identified in the carbon magic angle spinning (MAS) spectra of CD and RD treated with the adhesive, which were assigned to methylenic groups in methacryloyloxydecyl dihydrogen phosphate (MDP) bound to hydroxyapatite Ca^{2+}; ^{1}H spectra of the adhesive components and treated dentin, in ethanol, support this conclusion. ^{31}P MAS spectra obtained from both dentin regions present additional shielding and broadening effects subsequent to application and photopolymerization of the adhesive, which were higher for CD. Multinuclear MR studies provided evidence for the interaction of the adhesive with dentin, which involves hydroxyapatite and is stronger for CD than for RD, but no direct proof was obtained on bonding to collagen.

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Introduction

Bonding of composite resins to dentin, mainly composed of collagen and hydroxyapatite, is achieved because the bonding agents penetrate the dentin structure being physically and, eventually, chemically absorbed. Self-etching, self-priming adhesives are used on dry dentin and they bond well to smear layer-covered dentin [1] or to ground enamel [2, 3]. The impact of the moisture condition of dentin is reduced by the presence of water, or ethanol, in the adhesive formulation [4]. A two-step self-etching adhesive, to be used with resin based restorative materials, involves the application of the etching and the bonding solutions in two steps, without mixing. It was found that the self-etching primer of a specific two-step adhesive did not dissolve the smear layer but it etched through the smear layer to demineralise the dentin subsurface to a depth of 0.5 μm [5]. This adhesive contains 10-methacryloyloxydecyl dihydrogen phosphate (MDP), a phosphoric acid monoester (Fig. 1), at low concentration (25–30%) as compared with acid concentrations in other adhesives [5]. Considering that mild self-etch adhesives demineralize dentin only partially, it was hypothesized that the residual hydroxyapatite may serve as a receptor for chemical

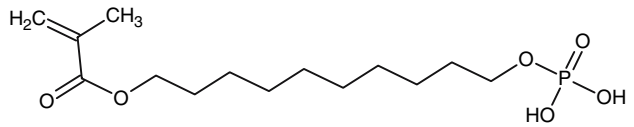

Fig. 1 Chemical structure of methacryloyloxydecyl dihydrogen phosphate (MDP)

interaction with the functional monomer and, subsequently, contribute to adhesive performance in addition to micromechanical hybridization [6].

Recently, was evaluated the influence of pH of 2-hydroxyethylmethacrylate (HEMA) solution on the bond strength of resin to etched dentin [7]; higher bond strengths were obtained with more acidic primer solutions and this finding was explained by the formation of an hydrogen-bond between the ester carbonyl HEMA group and the undissociated carboxylic acid of the collagen functional group, which was favoured at lower pH values. Moreover, further improved bond strength was obtained with a MDP-based primer, which was related to the undissociation of the carboxylic acids of collagen functional groups, due to the presence of MDP, and to the subsequent hydrogen-bond formation with HEMA [7]. However, this explanation requires further investigation because it must be taken into account that, due to the presence of hydroxyapatite, which releases calcium and phosphate ions and thus limits the H^+ penetration of adhesives, dentin is a highly effective solid buffer against acids.

Nuclear magnetic resonance (NMR) spectroscopy has been used to investigate chemical interactions between dentin major components and adhesives. For example, carboxylic acid reactivity with the calcium phosphate in hydroxyapatite, which is a convenient model for enamel, was studied by ^{13}C NMR in order to evaluate the efficiency of *N*-methacryloyl glycine as a monomer for a self-etching primer and the fact that the signal of CO group was shifted to lower magnetic field provided evidence for acid–base interactions between the carboxylic group in the monomer and the Ca cation in bovine teeth components or in hydroxyapatite [8].

Different μ-tensile bond strengths of a MDP-based self-etching adhesive to different regions of dentin were measured in vitro [9]. Aiming to explain if those findings are related to chemical interactions, we report here an investigation of chemical bonding of the adhesive system to collagen or to hydroxyapatite, present in coronal and in radicular human dentin, and to synthetic hydroxyapatite, using solid state high-resolution NMR (^{13}C and ^{31}P), which is a non-destructive technique, and liquid state NMR (^{1}H,^{13}C and ^{31}P).

Materials and methods

Materials

The self-etching adhesive used in the present study was Clearfil SE Bond, CSEB (Kuraray Co. Ltd., Osaka, Japan, Batch No. 41111). The chemical composition and the relative concentration of the major compounds [10] are shown in Table 1. Hydroxyapatite was purchased from Aldrich (USA) and used as received.

Sample preparation

Three human sound molar teeth were polished, to remove the cement and most of the enamel, and sectioned in order to obtain fragments with exposed surfaces of coronal (CD), cervical or radicular (RD) dentin regions. After the NMR spectrum acquisition of both CD and RD fragments, fragments from the same dentin region were mixed and crushed in a ball mill (MM200, Retsch, Germany), in order to obtain fine powders with an average particle size of about 50 μm. A compressed layer of each powdered sample was then treated with the adhesive, following the instructions of the manufacturers (only coronal and radicular dentins were used in adhesive–dentin interaction studies). The specimens were irradiated (~470 nm, 500 mW/cm^2) with a visible-light source (Optilux 401, Demetron Research Corporation, Danbury, CT, USA) to induce photopolymerization. All the samples were stored at 5 °C prior to the NMR observations.

NMR liquid- and solid-state studies

A Bruker MSL 300 P spectrometer was used to observe ^{1}H, ^{13}C and ^{31}P resonances at 300.13, 75.47 and 121.44 MHz, respectively. ^{1}H and ^{13}C spectra were obtained from the adhesive components in ethanol-d_6 and from suspensions, also in ethanol-d_6, of the photopolymerized adhesive and of powdered dentin treated with the adhesive, subsequently photopolymerized; TMS was the internal reference for the chemical shifts (0 ppm) and the accuracy of the ^{1}H and ^{13}C peak positions was 0.001 and 0.03 ppm, respectively. ^{13}C (with ^{1}H cross-polarization, CP, and decoupling) and ^{31}P NMR solid-state MAS spectra were acquired from all the samples at spinning rates of 4.2, 6 or 8 kHz; 4 or 7 mm o.d. zirconia rotors were used at spinning speeds respectively higher or lower than 6 kHz. The external references for ^{13}C and ^{31}P chemical shifts (δ) were glycine (CH_2, 41.4 ppm) and 85% phosphoric acid (0 ppm), respectively. The ^{31}P spectra were acquired using one-radio frequency pulse sequence (Bloch decay), with a pulse duration of 2 μs and a relaxation delay always longer than 20 s. Deconvolution of

Table 1 Composition of the adhesive used in the present study, according to the manufacturers, and relative concentration (in mol, underlined) of the major compounds obtained from [10]

Component	Composition
Primer	10-Methacryloyloxydecyl dihydrogen phosphate (MDP) (0.6), 2-hydroxyethyl methacrylate) (HEMA) (1.0), hydrophilic dimethacrylate, D,L-camphorquinone (CQ), *N*,*N*-diethanol-*p*-toluidine (DET), water (40) (pH = 2.0, [5])
Bond	MDP (0.04), HEMA (1.0), bis-GMA (0.75), hydrophobic dimethacrylate, CQ, DET, silanated colloidal silica

the overlapping signals was performed using the Bruker software Win-NMR. The accuracy of the ^{13}C and ^{31}P peak positions was 0.24 and 0.11 ppm, respectively. All the spectra were acquired at room temperature (about 22 °C).

Results and discussion

^{13}C spectra

In order to study by NMR the dentin-adhesive chemical interaction, it was convenient to expose large dentin surfaces, which was achieved by grinding separately dentin fragments from the three different dentin regions (coronal, cervical and radicular). Aiming to detect eventual protein structural changes in each dentin region, due to the powdering process, the spectra obtained from tooth fragments were compared with those recorded from different ground dentin regions. Figure 2a–f shows the ^{13}C CP/MAS spectra obtained from coronal and radicular dentin fragments and the spectra obtained from powdered coronal, cervical and radicular dentin, respectively. Pro C_β and Pro $C\gamma$ signals are well resolved in all the spectra and this may be considered as an indication that no solid denatured collagen is present in the observed samples [11]. Similar spectra were obtained from fragments of different dentin regions (Fig. 2c, d). However, some differences may be noticed when comparing these spectra with those recorded from ground dentin (Fig. 2a, b, e, f); for instance, higher resolved signals were obtained for Ala C_β (CH_3) and Glu C_α (CH) groups in the spectra obtained from the tooth fragments. These findings may be explained by Ala and Glu appearing as residues with slightly different conformations in the observed teeth, leading to different electronic environments and, consequently, to the observed line broadening. Figure 2e, f shows the MAS spectra obtained from cervical ground dentin; evidence for higher mobility of Ala C_β (CH_3) groups was gained by comparing spectra obtained with the same spinning rate (6 kHz) but with different contact times: 200 μs (Fig. 2e) and 50 μs (Fig. 2f). In fact, because the CP NMR technique promotes a magnetization transfer from ^1H to ^{13}C nuclei during a certain time (contact time) signals from carbons with lower multiplicity and/or in more rigid groups are enhanced and the acquisition of spectra with different contact times allows obtaining further spectral discrimination. In Fig. 2e, ^{13}CO signals are observed at 173.6 ppm (with the highest intensity) and at 170.3, 177 and 168 ppm (less resolved signals). By further decreasing the contact time to 50 μs, only one ^{13}CO resonance is recorded, which has chemical shift 173.6 ppm, and a single Ala C_β (CH_3) signal is observed at 17.9 ppm (Fig. 2f). The signal at 19.6 ppm in Fig. 2c is also from Ala C_β CH_3 group, in a different conformation, while other differences in the signal intensities of Fig. 2e, f lead to a confirmation of the previous spectral assignment [11]. The intensity of the signal at 59.4 ppm (Pro C_α and Hyp $C_{\alpha,\delta}$) is lower in Fig. 2c spectrum. The signals at 49.5 and 48.1 ppm were assigned to Ala C_α and Pro C_δ [11]; it is observed that the intensity of

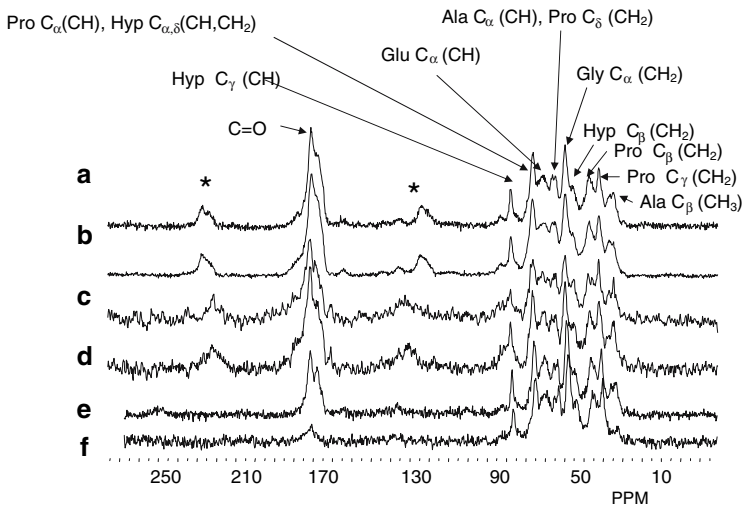

Fig. 2 $^{13}CP/MAS$ spectra, obtained with a spinning rate of about 4 kHz, relaxation delay 10 s and contact time 2 ms, from: (**a**) coronal dentin fragment, (**b**) radicular dentin fragment, (**c**) ground coronal dentin and (**d**) ground radicular dentin. $^{13}CP/MAS$ spectra (**e**) and (**f**) are from cervical ground dentin and were recorded with 6 kHz MAS rate and a contact time of 200 μs or 50 μs, respectively. *spinning side bands

Ala C_α is lower in the spectrum of the Fig. 2f, as expected for a carbon with a lower multiplicity.

Figure 3a–c shows the spectra obtained from ground radicular dentin, from the photopolimerized MDP-based self-etching adhesive (CSEB) and from ground radicular dentin treated with CSEB and subsequently photopolymerized. Comparing these three spectra, it is clearly observed that the signals at δ 31.4 and 27.3 ppm in the spectrum of ground radicular treated dentin are practically absent in the spectrum of the photopolymerized adhesive. These are well-resolved resonances from MDP methylenic groups which appear about 1 ppm low-field shifted from the corresponding spectral positions obtained for the primer in ethanol-d_6. Furthermore, in the presence of dentin, signals at δ 55.8 (quaternary carbon in polymeric methacrylate moieties) and at δ 16.7 ppm (assigned to methyl groups) become less intense, while signals from carbonyl groups in unreacted methacrylate groups are much more intense: 168.0 (which was confirmed to be assigned to more mobile groups) and 170.5 ppm (resonance not identified in the spectrum of the polymer). By comparing these data with a spectrum obtained from the self-etching primer in ethanol-d_6 (not shown here) it was concluded that these resonances are assigned to unreacted monomers in the primer (Table 1). In this spectrum, the intensity of the signal at 170.5 ppm is comparable to the intensity of the other carbonyl resonances but in the spectrum of treated dentin suspensions, also in ethanol-d_6, a very small resonance was observed at 170.5 ppm (not shown here). However, 170.5 ppm is not the expected chemical shift for the MDP carbonyl group (about 167 ppm) unless the group is involved in hydrogen bonding. Table 2 shows the ^{13}C chemical shifts of the carbonyl groups identified in the spectra of the photopolymerized adhesive in order to be compared with δ values obtained for the carbonyl resonances of radicular dentin and radicular treated dentin. Figure 3d–e shows the spectra obtained from coronal dentin treated with the adhesive system; the acquisition mode of the spectrum 3e was selected in order to favour the observation of carbons in more rigid groups and/or with lower multiplicities. It is worth noting that, in spectrum 3e, the signals at 31.2 and 27.1 ppm remain intense while, for example, the resonances at about 126, 64 and 19 ppm practically vanished. Similarly to radicular treated dentin data, the signals at 31.2 and 27.1 ppm are assigned to MDP CH_2 groups (separated at least by three chemical bonds from the oxygen atoms) while the other signals are from unreacted methacrylate moieties (~126 and 19 ppm) and CH_2–O fragments (~64 ppm). The signals at 31.2 and 27.1 ppm were not identified in the spectrum of treated coronal dentin suspensions in ethanol-d_6, which is questionable due to the low spectral signal to noise ratio (not shown here). These observations let us to conclude that the narrow resonances identified at 31.2 and 27.1 ppm in the solid state spectra of treated dentin are mainly from unreacted MDP molecules which must be strongly immobilized at the adhesive-dentin interface in order to allow ^{1}H–^{13}C cross-polarization to be effective over the short contact time selected for the acquisition of the spectrum shown in Fig. 3e. However, at this stage we can only hypothesize that this restricted motion is not due to monomer-collagen hydrogen bond interactions, like previously reported on etched dentin primed with HEMA or dentin treated with CSEB primer studies [7], but is explained by an ionic interaction of the MDP phosphoric group with calcium cations; this interaction, certainly enhanced in the presence of ground dentin, would contribute to inhibit photopolymerization by restricting MDP diffusion. The overlapping of dentin and adhesive ^{13}C signals do not allow conclusive findings about the type of chemical interactions and further evidence will be given next, which was provided from ^{31}P and ^{1}H NMR data. Overall, no significant differences were found between the spectra obtained from different treated dentin regions.

Table 2 ^{13}C chemical shifts (ppm) of the carbonyl groups identified in the spectra of photopolymerized CSEB, radicular dentin and radicular dentin treated with CSEB

Photopolymerized CSEB[a]	Radicular dentin	Treated radicular dentin
–	181.12	181.79
178.89	–	177.69
–	174.60	175.27
–	171.94	170.45
168.03	–	168.03

[a] The chemical shifts in ethanol-d_6 are: 170.5 and 168.7 ppm for the primer and about 168 ppm (two resonances) for the bond

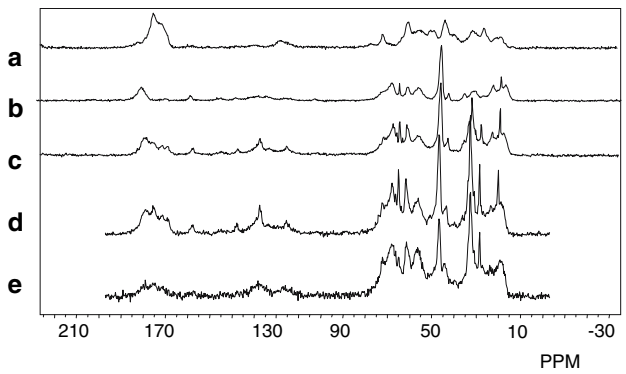

Fig. 3 $^{13}CP/MAS$ spectra, recorded with 4.2 kHz MAS rate and 2 ms contact time, from: (**a**) radicular dentin, (**b**) photopolymerized CSEB, (**c**) radicular dentin treated with CSEB and (**d**) coronal dentin treated with CSEB. Spectrum (**e**), also from coronal dentin treated with CSEB, was acquired with a contact time of 200 μs. Radicular and coronal dentins were as powders

It must be pointed out here that carbonate (a common substitution ion in hydroxyapatite) gives signal at about 170 ppm [12] and carbonate could be involved in the binding of the adhesive (as substitutions are more likely near the surface of a crystal in general); however, this would imply a shift of the carbonate resonance in the presence of the adhesive, which was not observed, at least with the experimental conditions used in this study.

^{13}C NMR solution studies were recently reported on the addition of hydroxyapatite or dentin to the self-etching primer and the subsequent decrease of the intensity of carbon resonances assigned to MDP was explained by the presence of an acid–base interaction of this monomer with calcium cations [13]. According to the present study, ^{13}C NMR solution data is insufficient to clarify this important issue.

^{31}P spectra

In the MAS spectrum obtained from cervical ground dentin (not shown) only a single resonance was identified (δ 3.42 ppm and full width at half maximum, FWHM, 347 Hz). It is known that dentin insoluble collagen contains the covalently attached phosphoprotein (phosphophoryn) but the corresponding very broad ^{31}P signal, which was already obtained from completely demineralized dentin and assigned to phosphate groups in the matrix-associated phosphoprotein [14] (about 7 ppm referred to inorganic orthophosphate), was not resolved in the spectra obtained from dentin samples prepared for the present study. A single signal (δ = 2.98 ppm) was observed for synthetic hydroxyapatite.

The following resonances were observed in the ^{31}P spectra (not shown here) of the two liquid components of the MDP-based self-etching adhesive: one signal at 0 ppm for the primer and two narrow signals for the bond at 13.36 and 0 ppm with the integrals 37 and 63%, respectively. The signal at 0 ppm is assigned to the MDP phosphate group; the resonance at 13.36 ppm, which is not assigned here, is very intense and, consequently, it is unlikely to be from a product of MDP-DET amine acid–base reaction. This signal is most probably due to ^{31}P directly bound to aliphatic groups; we hypothesize that it is from the hydrophobic dimethacrylate (Table 1).

The resonance identified at 13.36 ppm in the spectrum obtained from the liquid sample is not observed in the ^{31}P MAS spectrum of the photopolymerized adhesive system (not shown here); in this case, beyond the intense signal at δ 0.11 ppm, a broad, much less intense signal is displayed at about –11 ppm.

Figure 4 shows the MAS spectra of coronal and radicular dentin, both treated with the self-etching adhesive system, which were obtained with a spinning rate of 8 kHz.

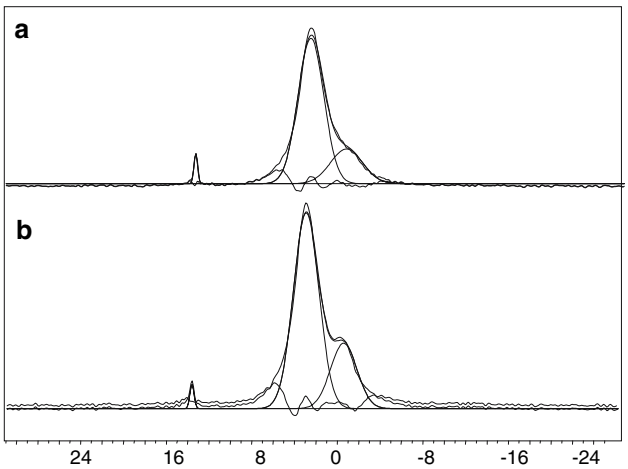

Fig. 4 ^{31}P MAS spectra, obtained with 8 kHz MAS rate and 20 s relaxation delay, from the following powdered dentin regions treated with CSEB: (**a**) Coronal and (**b**) Radicular. Simulated individual peaks, composite spectra and residues are also displayed

The chemical shifts of the individual components obtained by signal deconvolution were: 13.82, 3.28, 0.06 ppm. The resonances at about 0 and 13 ppm were assigned to the adhesive and the relative intensities and the FWHM of these signals depend on the treated dentin region, as shown in Table 3. Accordingly, the signal at about 0 ppm was narrower and more intense for the adhesive in the presence of radicular dentin. When the spectra were run with shorter inter RF pulse delay (not shown here) the signal at 13.8 ppm became the most intense one, demonstrating that this resonance is from more mobile molecules present in the treated dentin (with a shorter spin-lattice relaxation time), not the signal at about 0 ppm, which must then be assigned to bound MDP phosphate groups.

These data show that the relative intensities and FWHM of the adhesive signals depend on the type of dentin which was treated. The observation of shielding and broadening of ^{31}P MAS signals, which were more important for CD, point to the presence of MDP phosphate group-dentin interactions and, because higher Ca^{2+} concentration is expected in this more mineralized dentin region, agree well with the presence of MDP–Ca^{2+} interactions. Further evidence for this explanation was obtained from ^{31}P studies on synthetic hydroxyapatite–adhesive interaction, which are presented next.

Table 3 Intensity and FWHM ratios of the ^{31}P signals observed at about 0 and 13 ppm in the MAS spectra obtained from coronal and radicular dentin, both treated with CSEB, at a spinning rate of 8 kHz

Dentin	Intensity ratio	FWHM ratio
Coronal	1.1	9.1
Radicular	2.7	6.5

Hydroxyapatite consists of Ca^{2+} ions surrounded by PO_4^{3-} and OH^- ions; following an acid attack some calcium ions are leached out and coordinate with free water. HAP is a convenient model for enamel because its Ca/P ratio and crystallitinity are similar to the enamel. In order to get further evidence for the interaction of the adhesive with hydroxyapatite in dentin, we have observed the ^{31}P MAS spectra of synthetic hydroxyapatite and hydroxyapatite treated with the adhesive. Figure 5a–c shows the ^{31}P spectra obtained from hydroxyapatite and from the adhesive system mixed with increasing amounts of hydroxyapatite. While the ^{31}P MAS spectrum of hydroxyapatite shows a single resonance at 3.0 ppm (FWHM = 170 Hz), the spectra of treated hydroxyapatite display the following signals: 13.6, 3.0, 0.2 and –11 ppm (this signal is only visible in spectrum b). It may be observed that, while the FWHM of the resonance at 0.2 ppm increased by increasing the amount of hydroxypatite, the width of the signal at 13.6 ppm remains unchanged; this fact can only be explained by the presence of MDP phosphate group-Ca^{2+} interactions and agrees well with previous X-ray photoelectron spectroscopy data reported on the interaction of MDP with synthetic hydroxyapatite [6].

^1H solution spectra

Figure 6 shows the ^1H spectra obtained from the following samples in ethanol-d_6: (a) primer, (b) bond, (c) photopolymerized adhesive suspensions and (d) suspensions of radicular dentin treated with the adhesive. The chemical shifts of the centres of the multiplets obtained for the methylenic protons adjacent to the carboxylate and to the phosphoric acid groups of MDP are shown in Table 4. The chemical shifts of the centres of the multiplets assigned to the methylenic groups of HEMA are also shown. These

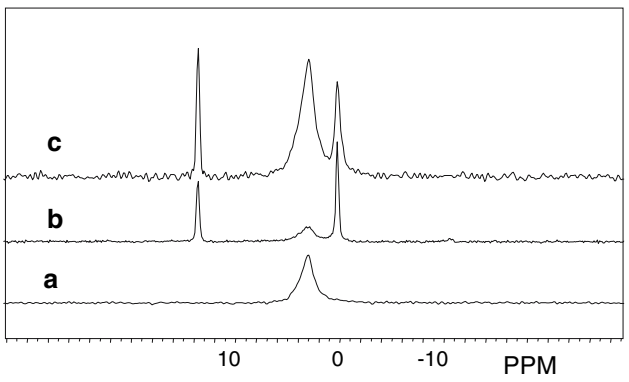

Fig. 5 ^{31}P spectra, recorded with 8 kHz MAS rate and 20 s relaxation delay, from synthetic hydroxyapatite (**a**) and from synthetic hydroxyapatite treated with increasing, cumulative, amounts of CSEB (**b** and **c**)

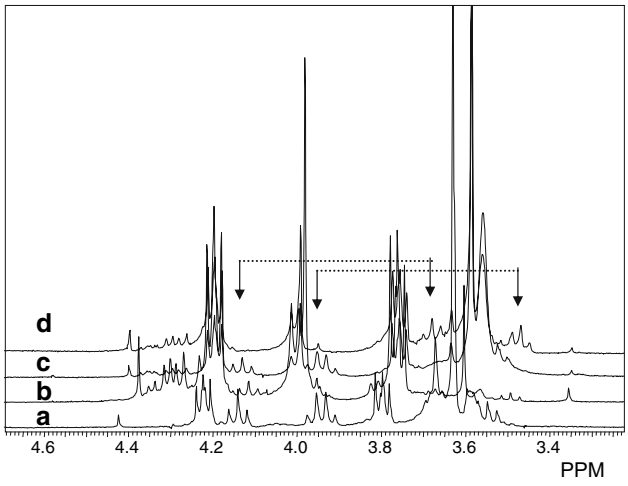

Fig. 6 ^1H spectra of the following samples in ethanol-d_6: (**a**) Clearfil primer, (**b**) Clearfil bond, (**c**) Photopolymerized CSEB and (**d**) Radicular dentin treated with Clearfil components, subsequently photopolymerized. Both (**c**) and (**d**) were as suspensions

results demonstrate that HEMA protons have the same electronic environment in samples (b, c) and (d) but, the observed displacement to low field in the spectrum of the primer (a) is consistent with a more effective stabilization of hydrogen bonds due to a higher concentration of H^+ and water in the primer solution and agrees well with the previous reported low-field shift obtained for a ^{13}CO, which was recorded at 170.5 not at about 167 ppm, as previously mentioned. On the other hand, a shift to high field is observed on the resonances of MDP in spectrum (d), as compared with the other spectra (labelled with arrows). This result is in agreement with an ionic interaction of PO_4^{2-}, in MDP, with Ca^{2+} leached out from dentin and do not confirm HEMA-collagen hydrogen bond interactions, a previously proposed explanation for the observations on etched dentin primed with HEMA or dentin treated with the MDP-based CSEB primer [7]. An ionic interaction of 4-MET, derived from 4-methacryloyloxyethyl-trimellitate anhydride, with calcium was also demonstrated due to

Table 4 ^1H chemical shifts (ppm) of multiplet centres from some methylenic groups in Clearfil primer (a) and bond (b), in ethanol-d_6, and of photopolymerized CSEB (c) and of radicular dentin treated with CSEB (d) suspensions in ethanol-d_6

Monomer	Methylenic group	Sample			
		a	b	c	d
HEMA	–OCO–C*H*$_2$–	4.22	4.20	4.19	4.19
	–OCO–CH$_2$–C*H*$_2$–	3.80	3.76	3.76	3.76
MDP	–OCO–C*H*$_2$–	4.14	4.11	4.13	3.68
	–C*H*$_2$–O–PO$_3$H$_2$–	3.94	3.95	3.94	3.48

upfield shift of trimellitic proton signals with increased phosphate concentration [15].

Conclusions

Multinuclear MR studies provide evidence at molecular level for the interaction of dentin with MDP, incorporated in a self-etch adhesive formulation, which involves hydroxyapatite and is stronger for CD than for RD. Higher intense monomer signals were identified in ^{13}C and ^{31}P solid state MAS spectra of treated dentin, as compared with the corresponding copolymer spectra, showing that a less efficient light curing of the adhesives takes place in the presence of dentin. Particularly for MDP, this polymerization inhibition may be explained by molecular diffusion restrictions due to the phosphate group-dentin chemical bonding.

No evidence was found for monomer-collagen hydrogen bond interaction, although Ala and Glu appear as particularly highly mobile residues and consequently more exposed to chemical interactions.

Quantitative determination of the ^{13}C–^{31}P distance will be carried out on dentin samples using Rotational Echo DOuble Resonance (REDOR) in order to study the interaction organic phase—mineral phase (like previously reported on bones, e.g. [16]) and to gain further insight on mineral phase-adhesive interaction.

Acknowledgements This work was supported by the Portuguese Foundation for Science and Technology/FEDER (POCTI/33193/99) and by CICYT/FEDER MAT2004-06872-C02; RED CYTED VIII.J.

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