NMR Study on the Adhesion Efficacy of Experimental Phosphonic Acid Monomers

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Three experimental self-etching primers — consisting of N-methacryloyl- \( \omega \)-aminoalkyl phosphonic acid (NM\( \omega \)P) with different methylene chain lengths and N-methacryloyl glycine (NM Gly) — were formulated. The influence of methylene chain length in NM\( \omega \)P derivatives on the chemical nature of calcium salts was examined following their application to tooth components. Bond strengths of experimental self-etching primers created with these monomers to enamel and dentin were also investigated. Nuclear magnetic resonance spectroscopy showed that NM\( \omega \)Ps decalcified tooth components with formation of calcium salts, which changed from calcium hydrogen phosphate to calcium phosphonate with increase in methylene chain length within the NM\( \omega \)P structure. Disparity in calcium salt formation was related to increases in bond strength to enamel from 18 to 24 MPa. However, bond strength to dentin remained unchanged (22 MPa). The relative dependency of bond strength on monomer methylene chain length was probably attributable to the sites where these NM\( \omega \)P calcium salts had deposited on the bonding substrates.

Keywords: Self-etching primer, Acid-base interaction, \(^{13} \text{C} \) NMR

INTRODUCTION

The application of self-etching primers, consisting of acidic and hydrophilic methacrylate resin monomers, enhances bonding at the resin-tooth interface. To understand the bonding mechanism, the decalcification of tooth components by acidic monomers utilized in self-etching primers have been examined using electron spectroscopy for chemical analysis (ESCA) and nuclear magnetic resonance (NMR) techniques. These studies reported that acidic monomers generated an acid-base interaction with calcium via decalcification of the apatitic phase in dental hard tissues. As for the exact nature of reactant residues produced by these acidic monomers, it has yet to be clarified.

Adhesives are directly applied to conditioned tooth surfaces where the reactant residues of acidic monomers have precipitated. Thus, it is anticipated that these residues may affect the bonding efficacy and durability of resin to the conditioned tooth surface. With regard to this surmise, Inoue et al. examined the solubility of the calcium salts produced by the interaction of 10-methacryloyloxydecyl dihydrogen phosphate (MDP), 2-methacryloyloxyethyl phenyl hydrogen phosphate (phenyl-P), and 4-methacryloyloxyethoxycarbonylphthalic acid (4-MET) with hydroxyapatite in water. The authors concluded that the chemical characteristics of calcium salts created by these acidic monomers affected the long-term durability of their resin-dentin bonds.

In this study, we designed experimental N-methacryloyl- \( \omega \)-aminoalkyl phosphonic acid-N-methacryloyl glycine (NM\( \omega \)P-NMGly) primers by adding a synthesized acidic monomer, NM\( \omega \)P, to aqueous solutions of NMGly. Then, the following aspects were examined: the effect of methylene chain length in NM\( \omega \)P derivatives on the chemical structure of reactant residues, and the effect on resin-tooth bond strength as a result of these experimental phosphonic acid-derived primers. The null hypotheses tested were that alteration in methylene chain length in NM\( \omega \)P has no effect on (1) chemical nature of reactant residues, and (2) bond strengths of resin to enamel and dentin created by the experimental self-etching primers.

MATERIALS AND METHODS

Three NM\( \omega \)P derivatives with different methylene chain lengths were synthesized by the condensation reaction of methacrylic chloride with the amine of \( \omega \)-aminoalkyl phosphonic acid. Numbers depicting the different methylene chain lengths were — 1:
N-methacryloyl-1-aminomethyl phosphonic acid (NMMP); 2: N-methacryloyl-2-amoethyl phosphonic acid (NMEP); 3: N-methacryloyl-3-aminopropyl phosphonic acid (NMPP). NM Gly was also synthesized by a condensation reaction between methacrylic chloride and the amine of glycine. The NMωP and NM Gly products were recrystallized using ethanol and ethyl acetate or ethyl acetate and hexane solvent system, respectively. Each crystallized compound was confirmed using the 1H and 3C NMR techniques.

Hydroxyapatite (HAP 200, Taihei Chemistry, Osaka, Japan) was used as a model for enamel in this study for two reasons: it is crystallized—a feature that closely resembles enamel, and its Ca/P ratio of 1.69 is similar to that of enamel.28 Dentin particles were prepared from bovine crown dentin using an air turbine with a diamond bur under water cooling. They were then obtained by decanting off the water coolant, rinsed with water, and air-dried for one day at 20°C.

**Determination of pKa values of hydroxy groups in NMωPs**

A quantity of 1.5 mmol of each NMωP was dissolved in 2 ml of 20 mass% of deuterium oxide aqueous solution. Hydrochloric acid or sodium hydroxide was subsequently added to each NMωP solution to vary the pH value. After measuring the pH values of each NMωP solution, 1H NMR spectrum of each NMωP solution was measured as a function of the pH value of NMωP solution at 25°C using an EX 270 spectrometer (JEOL, Tokyo, Japan). Chemical shifts of the NMR peak attributed to ω-methylene carbon bonded to phosphorus in the NMωP derivatives were determined by varying the pH value of each NMωP solution. These experiments were conducted once. For the NMR observations, a 45° pulse was used with accumulation of 5000 scans and a repetition time of 3.8 seconds. Resolution of the chemical shift for 1H NMR peak was 0.009 ppm. Hexamethylene disiloxane (HMDSO) was used as an external reference.

The pKa values of two hydroxy groups bonded to phosphorus in each NMωP derivative were determined from the inflexion points on the titration curve corresponding to the chemical shift of ω-methylene carbon peak.

**Decalcification of tooth components by NMωP-NMGly primers**

A quantity of 5 mol% of NMGly was dissolved in 20 mass% deuterium oxide aqueous solution. Three NMωP-NMGly primers were then prepared by dissolving 0.7 mmol of each NMωP derivative in 1.00 g of NMGly solution.29 A quantity of 60 mg of hydroxyapatite or dentin powder was suspended in 600 mg of NMGly primer or the respective NMωP-NMGly primer, and these suspensions were vibrated for two minutes. The pH value of each suspension was then measured. Following which, 1H NMR spectrum of the hydroxyapatite or dentin suspension was observed with the EX 270 spectrometer. Chemical shifts of the NMR peaks assigned to ω-methylene carbon in NMωP and to ω-carbonyl carbon for carboxylic acid in NMGly were determined, before and after the addition of tooth components. The NMGly primer was used as a control. These experiments were conducted twice.

**Bond strength measurement**

Flat bovine enamel or dentin surfaces were produced by polishing sequentially with 100-grit and 600-grit silicon carbide papers under water irrigation. A polyethylene ring with an internal diameter of 3.8 mm and a depth of 2.0 mm was mounted on each surface. The enamel or dentin surface inside the ring was conditioned with NMGly primer, respective NMωP-NMGly primer, or Mega Bond Primer (Kuraray Medical Inc., Tokyo, Japan) for 30 seconds, and air-dried for 15 seconds. Next, Clearfil SE Bond (Kuraray Medical Inc.) was applied to the conditioned enamel or dentin surface, air-dried for three seconds, and light-activated for 10 seconds using Quick Light (UL-1, Morita, Tokyo, Japan). Finally, Clearfil APX (Kuraray Medical Inc.), a light-cured hybrid composite, was placed on the cured adhesive and light-activated for 30 seconds.

After removing the polyethylene ring, the bonded specimens were stored in water at 37°C. After 24 hours, the specimens were mounted in a universal testing machine (DCS-2000, Shimadzu, Kyoto, Japan). Tensile bond strengths of resin to enamel or dentin (N=10) were measured at a constant loading speed of 2 mm/min. The NMGly primer was also used as a control in these experiments. Bond strength data for enamel or dentin were individually analyzed by one-way analysis of variance (ANOVA) and post hoc Fisher’s protected LSD test at α=0.05.

**RESULTS**

**pKa values of NMωP monomers**

Figure 1 shows the 1H NMR spectra of the three NMωP derivatives. Detected 1H NMR peaks were assigned to the carbons constituting the NMωP derivatives. No unassigned NMR peak was detected in the 1H NMR spectra. As for the NMR peak attributed to the ω-methylene carbon bonded to phosphorus in the NMωP structure, it was split into two distinct peaks.

Figure 2 shows the pH dependency of the chemical shifts of the right peak (higher field) of split ω-methylene carbon peak when the pH values...
The shift of detected right peak of split a-methylene carbon in NMa)P derivative in the presence of dentin.

**Fig. 1** $^1$H NMR spectra of N-methacryloyl-$\omega$-aminoalkyl phosphonic acid (NM$\omega$P) derivatives with different methylene chain lengths and peak assignments for the NMR peaks of the NM$\omega$P. Deuterium oxide was used as an NMR solvent. Upper spectrum: N-methacryloyl-1-aminomethyl phosphonic acid (NMMP); middle spectrum: N-methacryloyl-2-aminomethyl phosphonic acid (NMEP); lower spectrum: N-methacryloyl-3-aminopropyl phosphonic acid (NMPP).

The $pK_a$ and $pK_{ai}$ values determined for the individual hydroxy groups of the NM$\omega$P derivatives, as well as the shift differentials of a-methylene carbon peak caused by the dissociation of one or two hydroxy groups in NM$\omega$P, are summarized in Fig. 2.

**Effects from the addition of tooth components**

Table 1 shows the pH values of NM Gly and NM$\omega$P-NMGly primers before and after the addition of the NM$\omega$P solutions were varied. When the pH value of NM$\omega$P solution was increased by the addition of sodium hydroxide, the a-methylene carbon peak shifted to a lower field, reflecting the dissociation of hydroxy group in the NM$\omega$P structure through an acid-base interaction with the sodium ion. The titration curve exhibited three stages. Chemical shifts in the lower stage of the a-methylene carbon peak (35.74 ppm for NMMP, 25.54 ppm for NMEP, and 22.30 ppm for NMPP) were attributed to the NM$\omega$P species where the dissociation of both hydroxy groups were completely inhibited (i.e., $-\text{CH}_2-P(O)\text{(OH)}_2$). Chemical shifts in the middle stage (36.83 ppm for NMMP, 26.97 ppm for NMEP, and 23.34 ppm for NMPP) were attributed to the species where one of the two hydroxy groups had dissociated (i.e., $-\text{CH}_2-P(O)(\text{OH})\text{(O)}$). Chemical shifts in the upper stage (38.24 ppm for NMMP, 28.06 ppm for NMEP, and 24.22 ppm for NMPP) were attributed to the species where both hydroxy groups had completely dissociated (i.e., $-\text{CH}_2-P(O)(\text{O})$).

The $pK_{ai}$ and $pK_{ai}$ values determined for the individual hydroxy groups of the NM$\omega$P derivatives, as well as the shift differentials of a-methylene carbon peak caused by the dissociation of one or two hydroxy groups in NM$\omega$P, are summarized in Fig. 2.

**Fig. 2** Titration curves of the chemical shifts of the right peak of split a-methylene carbon peak of each NM$\omega$P derivative by varying the pH value of NM$\omega$P solution.

- : Chemical shift of detected right peak of split a-methylene carbon in NM$\omega$P derivative when the solution's pH value was varied.
- : Chemical shift of detected right peak of split a-methylene carbon in NM$\omega$P derivative in the absence of tooth components.
- : Chemical shift of detected right peak of split a-methylene carbon in NM$\omega$P derivative in the presence of hydroxyapatite.
- : Chemical shift of detected right peak of split a-methylene carbon in NM$\omega$P derivative in the presence of dentin.
In addition, the chemical shifts of the right peak of split $\alpha$-methylene carbon peak of each $\text{NM}_\omega\text{P}$ derivative, as well as the carbonyl carbon peak of carboxylic acid in $\text{NM}_\text{Gly}$, are also shown. The relationship between $pH$ value and chemical shift of $\alpha$-methylene carbon peaks, before and after addition of tooth component to $\text{NM}_\omega\text{P}$-$\text{NM}_\text{Gly}$ primers, are further depicted in Fig. 2.

When NMR observations of $\text{NM}_\omega\text{P}$-$\text{NM}_\text{Gly}$ primers were performed prior to addition of tooth components, the $\alpha$-methylene carbon peaks of the $\text{NM}_\omega\text{P}$ derivatives were detected at a lower field, rather than at the lower stage of the titration curves (open triangle, Fig. 2).

Addition of tooth components to the $\text{NM}_\omega\text{P}$-$\text{NM}_\text{Gly}$ primers allowed for the lower-field shift of the $\alpha$-methylene carbon peaks of $\text{NM}_\omega\text{P}$ derivatives and the carbonyl carbon peak of $\text{NM}_\text{Gly}$’s carboxylic acid (Table 1; open square for hydroxyapatite and black square for dentin in Fig. 2). The shift difference of $\alpha$-methylene carbon peak from the lower stage in the titration curve was approximated to the shift differential of $\text{NM}_\omega\text{P}$ species where one or two hydroxy groups had completely dissociated. In contrast, the lower-field shift of the carbonyl carbon peak of carboxylic acid in $\text{NM}_\text{Gly}$ was limited when compared with that of $\text{NM}_\text{Gly}$ primer, as a control. It should be noted that there were no changes in the intensity of the NMR peaks assigned to $\text{NM}_\omega\text{P}$ and $\text{NM}_\text{Gly}$, since $\text{NM}_\omega\text{P}$ and $\text{NM}_\text{Gly}$ formed water-soluble calcium salts.

Effect of methylene chain length in the $\text{NM}_\omega\text{P}$ structure on tensile bond strength

Table 2 shows the mean bond strengths of the experimental primer-adhesives to enamel and dentin. The mean bond strengths to enamel and dentin conditioned by Mega Bond Primer were 20 MPa and 21 MPa, respectively. Application of $\text{NM}_\text{Gly}$ primer to enamel resulted in a decrease in mean bond strength.
strength by 7 MPa when compared with that obtained from Mega Bond Primer. However, addition of NMωP derivatives to NMGLy primer resulted in significant increases in bond strength (p<0.05). By increasing the methylene chain length of the spacer group in NMωP structure, the bond strength in enamel increased from 18 to 24 MPa. On the other hand, the mean bond strength to dentin remained unchanged at approximately 22 MPa (p>0.05), even when NMωP derivatives were added to NMGLy primer.

DISCUSSION

In this study, we designed experimental self-etching primers consisting of methacrylamide monomer, since amide bonds are hydrolytically more stable than ester bonds in conventional methacrylate monomers. When NMωP-NMGLy primers interacted with the tooth components, the α-methylene carbon peaks of NMωP derivatives shifted to a lower field. These peaks approximated those of the respective monomers upon the dissociation of one or two of their hydroxy groups. The chemical shift of these peaks for NMMP and NMEP was closer to that of the respective species where one of the two hydroxy groups had an acid-base interaction with sodium. In contrast, the chemical shift of the α-methylene carbon peak for NMPP was closer to that of the NMPP species where two hydroxy groups had an acid-base interaction with sodium. The NMR results clearly demonstrated that: (1) NMωP in NMωP-NMGLy primers decalcified tooth apatite until one or two hydroxy groups of the NMωP species formed calcium salts, and (2) the predominant calcium salts formed differed among the NMωP derivatives, with NMMP and NMEP producing calcium hydrogen phosphate [$\text{CH}_2\text{P(O)(OH)}\text{Ca}^+]$salts and NMPP producing calcium phosphate [$\text{CH}_2\text{P(O)Ca}^+]$. Based on these NMR results, we had to reject the first null hypothesis that alteration in methylene chain length in NMωP has no effect on the nature of reactant residues. This difference in the chemical structure of their calcium salts was probably caused by an increase in methylene chain length in the NMωP structure. Conversely, the addition of NMωP derivatives to NMGLy primer resulted in a decrease in the lower-field shift of the carbonyl carbon peak of carboxylic acid in NMGLy. This decrease was probably due to the formation of calcium salts by the phosphonic acid in NMωP in preference to the carboxylic acid in NMGLy. It could therefore be concluded that the predominant species which had precipitated on the tooth surface were NMωP calcium salts, when tooth surfaces were conditioned by NMωP-NMGLy primers.

Bond strength to dentin increased from 18 MPa to 24 MPa with increase in methylene chain length in the NMωP structure. However, increase in methylene chain length in NMωP did not affect dentin bond strength. This difference probably arose from the dissimilar locations at which NMωP calcium salts were deposited on enamel and dentin. The bonding resin monomer was directly applied over the NMωP-NMGLy primer-conditioned enamel surface, which should be covered with precipitated NMωP calcium salts. As a result, a hybridized bonding layer at the resin-enamel interface was formed, which consisted of the bonding agent and water-soluble NMωP calcium salt. We speculated that the chemical characteristics of these NMωP calcium salts would affect the mechanical property of the bonding layer. Indeed, an increase in enamel bond strength with an increase in methylene chain length was probably due to the corresponding increase in hydrophobicity of the NMωP calcium salts. This was because the solubility of NMωP calcium salts decreased with increase in methylene chain length.

The bond strength to dentin remained unchanged, although NMωP calcium salts were also precipitated on the conditioned dentin surface. Two explanations could be proffered for this finding: (1) NMωP calcium salts were dispersed throughout the entire demineralized collagen network instead of accumulating only on the surface, as was the case of enamel; and (2) dentin bonding of adhesive resin monomer was associated with the hydrogen bonding between NMGLy species and dentinal collagen — which was exposed by NMωP etching. Therefore, the second hypothesis that changes in the methylene chain length of NMωP derivatives have no effect on the bond strengths of enamel and dentin bonds created by NMωP-NMGLy primers had to be rejected for enamel, but not for dentin.

Clinically, self-etching primers are usually applied to smear layer-covered tooth substrates. However, if a NMωP-NMGLy primer were to contain sufficient amount of NMωP to decalcify the inorganic apatitic phase in the smear layer and the underlying intact tooth substrate, the NMωP-NMGLy primer would provide noticeably high bond strength. This was because as NMωP decalcified the apatitic phase, it paved the way for NMGLy to permeate to the intact tooth surface through the smear layer. However, additional study on the hydrolytic durability of the hybridized bonding layer created by these NMωP-NMGLy primers would be required, because this layer might also contain other debris such as bacteria.
CONCLUSIONS

Three experimental self-etching primers — consisting of NMωP with different methylene chain lengths and NMGly — were developed. This was done with a view to investigating the effect of methylene chain length in NMωP derivatives on the chemical nature of calcium salts, as well as its effect on the bond strengths of resin to enamel and dentin.

NMR analysis data showed that NMωP decalcified tooth components with formation of calcium salts, which changed from calcium hydrogen phosphate to calcium phosphate with increase in methylene chain length within the NMωP structure. Disparity in calcium salt formation was related to increases in bond strength to enamel from 18 to 24 MPa, reflecting an increase in the hydrophobicity of NMωP calcium salt by increasing the methylene chain length. However, bond strength to dentin remained unchanged at approximately 22 MPa. This difference probably arose from the dissimilar locations at which NMωP calcium salts were deposited on enamel and dentin. This could be due to the dispersion of NMωP calcium salts throughout the entire demineralized collagen network instead of accumulating only on the surface, as was the case of enamel.

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