# MMP-Inhibitory Effect of Chlorhexidine Applied in a Self-etching Adhesive

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**Purpose:** To evaluate the MMP-inhibitory effect of chlorhexidine when incorporated in the primer of a two-step self-etching adhesive (Clearfil SE Bond).

**Materials and Methods:** Powdered dentin made from human teeth was treated with Clearfil SE Bond primers containing chlorhexidine of different concentrations for 20 s or for 2 min. The collagenolytic activity of the dentin powder was assayed using fluorescein-labelled collagen.

**Results:** Untreated dentin powder contains a low but measurable level of intrinsic activity, which was significantly inhibited by 0.05% chlorhexidine. Treating dentin powder for 20 s with SE Bond primers containing chlorhexidine (0.5%, 1.0%, and 2.0%) reduced the collagenolytic activities by 15.6%, 44.9%, and 56.7% respectively. When treated for 2 min, only SE Bond primer containing 2.0% chlorhexidine could inhibit the collagenolytic activity by 41.1%.

**Conclusion:** When incorporated in a two-step self-etching adhesive primer (Clearfil SE Bond primer), chlorhexidine can partially maintain its inhibitory effect on MMPs.

**Keywords:** chlorhexidine, matrix metalloproteinases, self-etching adhesives, bond degradation, collagenolytic activity.

*J Adhes Dent 2010; 12: xx-xx.* *Submitted for publication: 17.12.08; accepted for publication: 16.11.09.*

It has been widely stated that resin-dentin bonds obtained with contemporary adhesive systems can deteriorate over time.[2,5,9,11,13] Several in vivo studies have provided morphological evidence of hydrolytic degradation of collagen matrices in aged dentin hybrid layers.[12,15,24,27] For etch-and-rinse adhesives, a decreasing gradient of resin monomer diffusion within the acid-etched dentin[32] results in incompletely infiltrated zones along the bottom of hybrid layers that contain denuded collagen fibrils.[1,14] For self-etching adhesives, incomplete resin infiltration was also observed as nanoleakage within hybrid layers,[23] despite the ability of these adhesives to etch and prime simultaneously. The subsequent resin elution from hydrolytically unstable polymeric hydrogels within the hybrid layers further leaves the collagen fibrils unprotected and vulnerable.[31] These unprotected collagen fibrils can auto-degrade over time,[12,24] even in the absence of bacterial enzymes,[22] thereby compromising the durability of the resin-dentin bonds. Pashley et al[22] proved that the auto-degradation of these denuded collagen fibrils occurs by the slow action of host-derived matrix metalloproteinases (MMPs).

MMPs are a class of zinc- and calcium-dependent endopeptidases capable of degrading all extra-cellular matrix components. Human dentin contains at least MMP-2, MMP-8 , MMP-9 and MMP-20.[17,18,25,26] They are trapped within the mineralized dentin matrix during tooth development[3] and play strategic roles in tooth development[3] and dentinal caries.[29] The majority of MMPs are produced as latent zymogens (pro-MMPs) which are proteolytically or non-proteolytically activated via the cysteine-switch mechanism.[20,30] It has been shown that simplified etch-and-rinse adhesives[19] and self-etching adhesives[21,28] are capable of re-

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leasing and activating endogenous MMPs during dentin bonding, which are thought to be responsible for the manifestation of thinning and disappearance of collagen fibrils from incompletely infiltrated hybrid layers in aged, bonded dentin.

The activity of MMPs can be suppressed by protease inhibitors,[22] indicating that MMP inhibition may be beneficial in the preservation of hybrid layers. This has been demonstrated in recent studies in which the application of chlorhexidine, known to have a broad-spectrum MMP-inhibitory effect,[10] significantly improved the integrity of the hybrid layers created by a simplified etch-and-rinse adhesive.[4,7,8] However, whether chlorhexidine can be applied in self-etching adhesives to preserve dentin bonding is unclear. We employed chlorhexidine to improve the bond durability of a two-step self-etching adhesive (Clearfil SE Bond). To simplify the bonding procedure, chlorhexidine was added directly into the primer of Clearfil SE Bond. It has been confirmed that the addition of chlorhexidine to Clearfil SE Bond primer has no adverse effect on the immediate resin-dentin bond strength when the chlorhexidine concentration in the adhesive primer is lower than or equal to 1.0%[34] and that chlorhexidine can preserve the dentin bond for at least one year as long as the concentration of chlorhexidine in the primer is higher than or equal to 0.1%.[33] Nevertheless, the exact effect of chlorhexidine on MMPs when incorporated in Clearfil SE Bond primer is still unclear and has to be evaluated.

The purpose of this study was therefore to investigate the exact effect of chlorhexidine on MMPs when incorporated in the primer of Clearfil SE Bond.

## MATERIALS AND METHODS

### *Preparation of Self-etching Adhesive Primers Containing Chlorhexidine*

A two-step self-etching adhesive, Clearfil SE Bond (Kuraray; Osaka, Japan), was used for this study. Different amounts of 20 wt% chlorhexidine digluconate (Mingfeng; Quzhou, Zhejiang, China) were added directly to the Clearfil SE Bond primer to prepare mixtures containing three different concentrations of chlorhexidine: 0.5 wt%, 1.0 wt%, and 2.0 wt%.

### *Preparation of Mineralized Dentin Powder*

Forty extracted unerupted human third molars or premolars scheduled for extraction as part of orthodontic treatment were used in the study, and were collected with the patients' informed consent obtained under a protocol approved by the Ethics Committee for Human Studies, Peking University, China. The patients' ages ranged from 14 to 25 years. The teeth were stored in 0.9% NaCl containing 0.02% sodium azide at 4°C for no more than one month. Dentin pieces (crown and root) were harvested after grinding away enamel and cementum, removing pulpal soft tissues, and dehydrating in acetone. Pieces of dried dentin were reduced to fine powder by freezing the dentin pieces in liquid nitrogen and triturating them collectively in a stainless steel mixer mill (Model MM400, Retsch; Newtown, PA, USA) for 6 min at 30 Hz. The fractured frozen dentin was then sieved through a 125-µm screen but retained on a 60-µm sieve. This fine mineralized dentin powder was kept dry and frozen until use.

### *Dentin Powder Treatment*

The mineralized dentin powder was divided into 10 lots of 320 mg each. One lot of powder was retained as the control group (group 1). The other nine lots of dentin powder were treated with 0.05% chlorhexidine digluconate, SE Bond primer, or SE Bond primers containing different concentrations of chlorhexidine.

#### Group 2 – 0.05% chlorhexidine digluconate for 2 minutes

0.8 ml of 0.05% chlorhexidine digluconate was added to one lot of dentin powder and stirred continuously for 2 min. The reagent-dentin powder mixture was then diluted with deionized water to a volume of 10 ml and spun down with a centrifuge (Megafuge 1.0 R, Heraeus; Hanau, Germany) at 1500 rpm for 2 min. This was repeated three times. The supernatant was discarded and the precipitate was re-suspended in 10 ml of acetone and re-centrifuged. This procedure was repeated twice and the precipitate was left to air dry in a Drierite-containing desiccator at 25°C for 24 h.

#### Groups 3 and 7 – SE Bond primer for 20 seconds or 2 minutes

Another two lots of dentin powder were each treated with 0.8 ml of Clearfil SE Bond primer for 20 s (group 3) or for 2 min (group 7). The etching reaction was terminated by extracting the adhesive monomers with 100% acetone. The latter was added to the adhesive-treated dentin powder to reach a volume of 10 ml. The dentin powder/acetone suspension was centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the extraction procedure was repeated three times. After the final rinse, the precipitate was left to air dry in a Drierite-containing desiccator for 24 h.

#### Groups 4 to 6 – SE Bond primers containing chlorhexidine (0.5%, 1.0% or 2.0%) for 20 seconds

Three additional lots of dentin powder were treated for 20 s with 0.8 ml SE Bond primers containing chlorhexidine of different concentrations: 0.5%, 1.0% or 2.0% (groups 4, 5, and 6, resp). Extraction of the adhesives and retrieval of the dentin powder followed the procedures previously described.

#### Groups 8 to 10 – SE Bond primers containing chlorhexidine (0.5%, 1.0%, or 2.0%) for 2 minutes

Another three lots of dentin powder were treated for 2 min with 0.8 ml SE Bond primers containing chlorhexidine of different concentrations: 0.5%, 1.0%, or 2.0% (groups 8, 9, and 10, resp). Extraction of the adhesives and retrieval of the dentin powder followed the procedures previously described.

***Assay for Functional Collagenolytic Activity***

The functional collagenolytic activity of dentin powder was assayed according to the protocol described by Tay et al,[28] using the EnzChek gelatinolytic/collagenolytic assay kit (E-12055, Molecular Probes; Eugene, OR, USA) that were supplemented with type I bovine soluble skin collagen-fluorescein conjugate (Cat. D-12060; Molecular Probes). The fluorescence of the collagen substrate is internally quenched and is only released when it is cleaved enzymatically into highly fluorescent, low molecular weight peptides. The fluorescent cleavage products were read in a 96-well fluorescent plate reader (Synergy 4, Bio-Tec; Winooski, VT, USA), operated at an absorption maxima of 495 nm and a fluorescent emission maxima of 515 nm. Because the enzyme activity was very low, the reactions were run for 64 h at 37°C prior to performing fluorescence measurements. All assays included a series of control collagenase standards as well as reagent blanks.

***Calibration of Fluorescence Intensity with a Control Collagenase Standard***

To ensure that the fluorescent reading could be employed for linear quantification of enzymatic activities, a calibration assay was first performed using different concentrations of a purified collagenase standard from Clostridium histolyticum. A linear regression analysis was performed between the fluorometer readings from the series of Clostridium standards and their corresponding concentrations at $\alpha = 0.05$.

***Reagent Blanks***

To accommodate for the time-dependent increases in background fluorescence of the reagent blanks, 20 µl of the fluorescein-conjugated collagen were added to 180 µl of the Tris-$CaCl_2$ buffer in 4 wells. Readings were taken after 64 h incubation of the 96-well plates at 37°C.

***Fluorescence Assay of the Different Groups of Dentin Powder***

Four aliquots of 50 µg of dentin powder from each of the ten groups were introduced into the wells of a 96-well plate (n = 4). 180 µl of the Tris-$CaCl_2$ buffer were added to each well. To accommodate for the background fluorescence of the apatite, an initial reading was performed before the addition of the fluorescein-conjugated collagen.

Enzyme activation with 4-amino-phenylmercuric acetate (APMA) was not done, since it has been reported that there is no difference in enzyme activity with or without activation.[19,21,28] Twenty µl of the fluorescein-conjugated collagen was then added to each well. The 96-well plate was incubated at 37°C for 64 h before the final fluorescence readings. The net increase in fluorescence from each dentin powder-containing well at 64 h was calculated by subtracting the background fluorescence of the dentin powder from that well and the mean background fluorescence of the reagent blanks at 64 h.

One-way ANOVA and Tukey's multiple comparison test were used to compare the collagenolytic activities of different groups. Statistical significance was pre-set at $\alpha = 0.05$.

## RESULTS

Untreated dentin powder contains a low but measurable level of intrinsic activity of 1013 relative fluorescent units (RFU), which was significantly inhibited by 0.05% chlorhexidine to near-zero levels. Treating dentin powder with SE Bond primer for 20 s or 2 min significantly increased its collagenolytic activities (5154 RFU and 6313 RFU, respectively). Compared to group 3, treating dentin powder for 20 s with SE Bond primers containing chlorhexidine (0.5%, 1.0%, and 2.0%) reduced the collagenolytic activities by 15.6%, 44.9%, and 56.7%, respectively ($p < 0.05$). When treated for 2 min, only SE Bond primer containing 2.0% chlorhexidine could inhibit the collagenolytic activity significantly (compared to group 7, by 41.1%, $p < 0.05$). There was no statistical difference of collagenolytic activities when dentin powder was treated for 2 min with SE Bond primer without chlorhexidine or with SE Bond primers containing 0.5% or 1.0% chlorhexidine ($p > 0.05$). Treating dentin powder with SE Bond primers containing different concentrations of chlorhexidine for 2 min resulted in higher collagenolytic activities compared with those treated for only 20 s ($p < 0.05$) (see Fig 1).

## DISCUSSION

Since treating dentin powder for 20 s with SE Bond primers containing chlorhexidine (0.5%, 1.0%, and 2.0%) reduced the collagenolytic activities by 15.6%, 44.9%, and 56.7%, respectively, we can draw the conclusion that chlorhexidine can partially maintain its inhibitory effect on MMPs when incorporated in Clearfil SE Bond primer.

It is interesting to note that when the dentin powder was treated for 20 s with SE Bond primer containing chlorhexidine of different concentrations, the collagenolytic activities decreased significantly; however, when the dentin powder was treated for 2 min with SE Bond primer containing 0.05% or 0.1% chlorhexidine, the collagenolytic activity was almost the same as the dentin powder that was treated with SE Bond primer without chlorhexidine ($p > 0.05$). It has been previously proposed that a calcium-chelating mechanism is involved in the inhibitory properties of chlorhexidine on MMPs,[10] since MMPs require metal ions (namely, calcium and zinc) for their catalytic activity. Hence, calcium ions could competitively inhibit chlorhexidine and reverse the inhibitory effect of chlorhexidine on these MMPs. It has been reported that adding calcium chloride to the assay mixtures that contained 0.03% chlorhexidine almost completely prevented the inhibition of MMP-9 activity.[10] In our present study, when the dentin powders were treated for 2 min, more calcium ions were released from the underlying mineralized dentin compared with those treated for 20 s. These excessive calcium ions might play a role in preventing the inhibitory effect of chlorhexidine on MMPs. This could be the reason why there was no difference of collagenolytic activities when dentin powder was treated for 2 min with SE Bond primer or with SE Bond primers containing 0.5% or 1.0% chlorhexidine. This might also explain why chlorhexidine could only partially maintain its inhibitory effect on MMPs

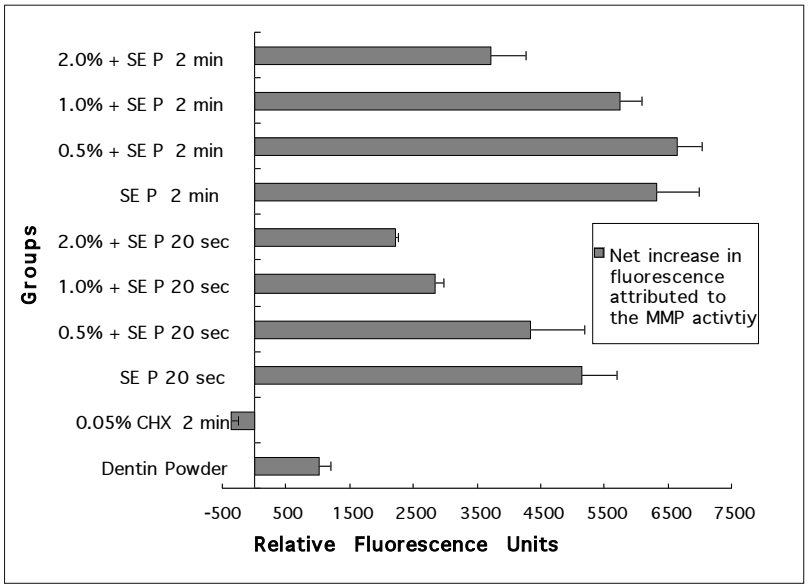


**Fig 1** Net increase in fluroscence (in relative fluorescence units; RFU) from each of the ten groups, representing the quantitative collagenolytic activities of the untreated and treated dentin powder. Group designations: group 1 (Dentin Powder), untreated mineralized dentin powder (positive control); group 2 (0.05% chlorhexidine [CHX] 2 min), dentin powder treated with 0.05% chlorhexidine digluconate for 2 min (negative control); group 3 (SE P 20 sec), dentin powder treated with 0.8 ml SE Bond primer for 20 s; group 4 (0.5% CHX + SE P 20 sec), dentin powder treated with 0.8 ml SE Bond primer containing 0.5 wt% chlorhexidine for 20 s; group 5 (1.0% CHX + SE P 20 sec), dentin powder treated with 0.8 ml SE Bond primer containing 1.0 wt% CHX for 20 s; group 6 (2.0% CHX + SE P 20 sec), dentin powder treated with 0.8 ml SE Bond primer containing 2.0 wt% CHX for 20 s; group 7 (SE P 2 min), dentin powder treated with 0.8 ml SE Bond primer for 2 min; group 8 (0.5% CHX + SE P 2 min), dentin powder treated with 0.8 ml SE Bond primer containing 0.5 wt% CHX for 2 min; group 9 (1.0% CHX + SE P 2 min), dentin powder treated with 0.8 ml SE Bond primer containing 1.0 wt% CHX for 2 min; group 10 (2.0% CHX + SE P 2 min), dentin powder treated with 0.8 ml SE Bond primer containing 2.0 wt% CHX for 2 min.

when added in the SE Bond primer, not like the 0.05% chlorhexidine solution did, which significantly inhibited the collagenolytic activities of dentin powder to near-zero levels. Calcium ions released from dentin powder by SE Bond primer might play the key role. Of course, there might be other explanations. For example, when treating dentin powder for 2 min, more MMPs were exposed, thus more chlorhexidine was needed for their inhibition.

Dentin has been shown to be an excellent buffer for acid.[6] As a result of differences in buffering, the immersion of dentin powder in acidic etchants may overestimate the degree of clinical activation of MMPs that might accompany the application of etchants to solid surfaces.[6] Therefore, during the clinical procedure of bonding, the activities of MMPs activated by SE Bond primer containing chlorhexidine might be lower than the results achieved in our present study. It has been recently reported that chlorhexidine may be released from resin disks in which chlorhexidine diacetate is incorporated.[16] This finding suggests that when applied in the manner of this study, chlorhexidine might be released from cured dentin hybrid layers and continue to inhibit MMPs over time. However, whether the concentrations of released chlorhexidine will exceed the concentrations that are required to inhibit the activities of MMPs is unclear and remains to be determined.

For the successful application of chlorhexidine in self-etching adhesives to preserve dentin bonding, the time, concentration, and method of delivery to be employed for maximal MMP inactivation have to be optimized. It has been reported that the application of 2% chlorhexidine for 10 min before or after the application of Clearfil Liner Bond 2V, a dual-curing two-step self-etching adhesive, resulted in incomplete, but significant reductions (about 50%) of the activated MMP activity,[28] which is very similar to the results obtained in the present study. However, in our study, to simplify bonding procedure, chlorhexidine was incorporated in SE Bond primer directly, and might be released from cured dentin hybrid layers and continue to inhibit MMPs. In addition, the present study tested different treatment times and concentrations of chlorhexidine, indicating that treating dentin powder for 20 s, which is recommended by manufacturer's instructions, is more favorable than for 2 min, and that the chlorhexidine concentrations of 1.0% and 2.0% can result in better outcomes than the concentration of 0.5%.

An important limitation of our study has to be pointed out. During the treatment of the dentin powder, we removed the monomers by dilution and centrifugation. Most of the chlorhexidine is probably discarded together with the supernatant, so that the MMP-inhibitory effect of chlorhexidine might be weakened. Therefore, the exact effect of chlorhexidine in the clinical situation remains a topic of discussion. However, in this in vitro study, we set positive and negative controls. We can at least conclude that when incorporated in Clearfil SE Bond primer, chlorhexidine can inhibit MMPs to some extent.

## CONCLUSIONS

Within the limitations of this study, the conclusion can be drawn that when incorporated in a two-step self-etching adhesive primer (Clearfil SE Bond primer), chlorhexidine can partially maintain its inhibitory effect on MMPs.

## ACKNOWLEDGMENTS

The authors are grateful to Kuraray Co., Ltd. for the generous donation of materials. We are also grateful to Mr. Yuhui Gao (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College) for his assistance in the assay for functional collagenolytic activity.

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**Clinical relevance:** This study proved that when incorporated in Clearfil SE Bond primer, chlorhexidine can partially maintain its inhibitory effect on MMPs. This suggests that when incorporated in Clearfil SE Bond primer, chlorhexidine might preserve dentin bonding by protecting the exposed collagen fibrils in dentin hybrid layers.