Immobilization of quaternary ammonium based antibacterial monomer onto dentin substrate by non-thermal atmospheric plasma

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INTRODUCTION

Secondary caries is one of the primary clinical causes in dental restorative failures. When bonding to a prepared dental cavity, composite restoration with no antibacterial function provides a potential for microbial growth, which occurs not only on the tooth surfaces or tooth-composite margins, but also in the resin/dentin interfaces. Since their introduction in the 1990s, quaternary ammonium methacrylates (QAMs), synthesized through a modified Menschutkin reaction, have been popularly investigated for the development of antibacterial dental resins. Dimethylaminohexadecyl methacrylate (DMAHDM), a newly synthesized QAM with an alkyl chain length of 16, was proved to be the optimal monomer with effective antibacterial function, reasonable bond strength and comparable cytotoxicity, after comparing different chain lengths (6–18) of the dimethylaminodecyl methacrylates. Generally, QAMs exhibit strong inhibitory effect before polymerization; however, after polymerization or co-polymerization with other monomers, QAMs’ antibacterial effect is dramatically diminished due to the limited movement within resin matrix. Therefore, contact inhibition is considered as a main antibacterial effect of the QAMs. When incorporated into composite, polymerization shrinkage and resulting micro-leakage may result in detachment of QAMs from tooth substrates, in which bacteria/caries exist/occur. In the recent years, QAMs have also been incorporated into primers/adhesives to make them in contact with tooth substrates during bonding; however, there are no/little chemical interactions between methacrylate monomers and bonding substrates. The contact or bonding between current primers/adhesives and tooth structure is mainly through micro-mechanical interlocking, which is much weaker in comparison with chemical interactions. At the dentin/adhesive interface, there is exposed collagen at the bottom of the interface due to poor adhesive penetration/polymerization. In addition, there are residual bacteria and incomplete removal of bacteria in caries-affected dentin substrates that clinicians usually bond to. Thus, there are concerns about sustainable antibacterial performance as well as dentin bond durability due to no/poor direct binding of QAMs to tooth structure. Therefore, a method, which could directly immobilize an antibacterial agent onto the dental substrates under clinical conditions without interfering its inhibitory effect, is urgently needed.

Non-thermal atmospheric plasma (NTAP) has been introduced to the field of dentistry in recent years, which contains highly reactive particles such as electrons, electronically excited neutrals, radicals, and ultraviolet (UV) photons etc. produced at room temperature. As a non-invasive technique, NTAP is widely used in dental surface modification, adhesive bonding/composite restorations, cavities/root canal disinfection, tooth bleaching etc. Advantages derived from the NTAP technique include improving surface hydrophilicity of different dental substrates, adhesive penetration...
into dentin\textsuperscript{32}, polymerization\textsuperscript{33}, and HEMA grafting onto dentin collagen\textsuperscript{34}, thus enhancing the resin-dentin bonding\textsuperscript{35,36}. To date, no antibacterial monomer has been used in the previous NTAP dental applications.

In this proof-of-concept study, DMAHDM as an antibacterial agent was firstly immobilized onto the dentin bonding substrate directly by using the NTAP technique, and its antibacterial effects were evaluated. The schematic diagram for this proof-of-concept study is shown in Fig. 1. The hypotheses tested were: (1) DMAHDM could be stably immobilized onto the dentin bonding substrate by NTAP; (2) DMAHDM immobilized onto dentin substrate would provide the surface with a direct, effective and long-lasting antibacterial function.

**MATERIALS AND METHODS**

**Specimen preparation**

Non-carious human third molars, collected from the Oral Surgery Clinic, University of Missouri Kansas City (UMKC) School of Dentistry under an approved protocol, were cut by using a water-cooled diamond saw (Buehler, Lake Bluff, IL, USA). The mid-coronal dentin was sectioned into 4×2×0.7 mm slices, and checked under a light microscope (Nikon Instruments, Eclipse ME600P, Tokyo, Japan) to ensure enamel was totally removed. A dentin bonding substrate with a several-micron-demineralized layer (Figs. 2A–B) was created by soaking each of the slices into 0.5 mol/L EDTA solution (pH=7.4) for 30 min. The resulting slices were autoclaved (Primus, Primus Sterilizer, Omaha, NE, USA) for 20 min at 121°C for sterilization, then stored in Dulbecco’s Phosphate-Buffered Saline (DPBS, 1X, without calcium & magnesium, Sterile. Cellgro, Mediatech, Manassas, VA, USA) at 4°C before use.

DMAHDM (kindly provided by Dr. H.K. Xu, University of Maryland School of Dentistry) was dissolved in ethanol at room temperature with different mass fractions. The 5 wt% or 30 wt% DMAHDM (5%D or 30%D) was used to verify immobilization/grafting onto dentin substrate, while only 5%D was used for antibacterial measurements, as at this concentration, sufficient antibacterial potency and reasonable cytotoxicity were reported according to Li’s study\textsuperscript{12,16}).

*Streptococcus mutans* (ATCC, 25175) were used in this study. Single colony of *S. mutans* was transferred to 2 mL Brain Heart Infusion (BHI) broth (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C and 5% CO\textsubscript{2} to an optical density (OD) of 0.6 (3×10\textsuperscript{8} cell/mL) at 600 nm, then stored in 60% glycerol at −80°C for future use.

**NTAP treatment**

The NTAP brush employed in this study was manufactured by Nanova (Columbia, MO, USA). The device setup information could be found in previous publications\textsuperscript{37,38). Briefly, the compressed argon gas (ultra-high purity) was used as plasma gas supply. The argon gas flow rate of 2,000 sccm was regulated by a MKS mass flow controller (MKS Instruments, Andover, MA, USA). A glow discharge by the direct current power source (Model 1556C, Power Designs, Westbury, NY, USA) was ignited between the two electrodes in a Teflon chamber. The brush-shaped plasma jet was kept at a fixed distance from the specimens (5–6 mm from bottom of the nozzle) for treatment with an input power of 10 W\textsuperscript{34) and a treatment time of 30 s (clinically acceptable).}

The surface of the above sterilized dentin slices was treated with the prepared DMAHDM solutions by the plasma brush. Briefly, the dentin substrates were...
gently air-dried for 15 s before the treatment to remove the excess liquid. Prior to the plasma treatment, 2 μL of 5 or 30 wt% DMAHDM solution was applied onto the substrates, which then were gently air-dried for another 15 s. All of the procedures were performed in a biologically safe hood. The following groups were tested:

1. DS: Blank control, no treatment;
2. DS+5%D (or 30%D): with 5 or 30 wt % DMAHDM solution applied, no plasma exposure;
3. DS+5%D (or 30%D)+P: with 5 or 30 wt % DMAHDM solution applied, plasma exposure (10 W, 30 s);

After treatment, all the specimens (including blank control) were immersed in the DPBS for rinsing out free/un-mobilized monomer from dentin substrates. According to the pilot study, rinsing for 4–6 times (each for 30 min) was enough to remove the un-mobilized or free monomer to an acceptable concentration that is below an effective level which impacts the bacteria. Within the pilot study, the specimens were rinsed for 10 times in BHI broth, and the extracts of each rinse were used to incubate bacteria (20 μL S. mutans suspension, OD600=0.2 (1×10^8 cell/mL) and 180 μL extract in BHI broth were added into 96-well plate, incubated for 24 h at 37°C and 5% CO2) and evaluate the antibacterial effect of the eluents, which decreased with the number of rinse. After 4–6th rinse, there was no inhibitory effect of the eluents. Therefore, in this study, rinsing for at least 7 times was used to ensure the specimens were free of un-immobilized monomer that impacts the bacteria. Followed by rinsing, 5-min vortex (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA) and 5-min ultrasonic bath (Cole-Parmer, Vernon Hills, IL, USA) in the autoclave-sterilized-centrifuge tubes were applied. These dentin specimens were then collected for scanning electron microscopy (SEM) and attenuated total reflectance Fourier transformed infrared (FTIR) spectroscopy examinations.

**Characterization of DMAHDM-immobilized-onto dentin substrates**

To investigate the surface morphology of the dentin substrates after different treatments, specimens were subjected to SEM assessment after rinsing and oscillation. The dentin slices were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h, and subsequently dehydrated in the graded ethanol series. After air-dried for overnight and sputter coated with gold-palladium, specimens were examined at a variety of magnifications using a Philips XL 30 SEM (Eindhoven, the Netherlands) at 5 kV. Three specimens from each group were examined.

A FTIR spectrometer (Spectrum One, Perkin-Elmer, Waltham, MA, USA) equipped with a universal attenuated total reflectance (ATR) attachment (Perkin-Elmer) was used in this study. After air-dried for overnight, the infrared spectra of specimens from the above three treatment groups as well as DMAHDM monomer were collected at a resolution of 4 cm⁻¹, scan time of 128, and scan range of 650–4,000 cm⁻¹. At least 3 specimens per group were randomly selected for FTIR measurements.

**Contact inhibition of dentin substrates immobilized with DMAHDM**

Frozen S. mutans suspension was recovered/diluted, incubated to OD600=0.2 (1×10^8 cell/mL). To test the
contact inhibitory effect of the DMAHDM-immobilized-dentin substrate, S. mutans suspension (OD=0.2) was diluted (1:100) into fresh BHI broth. Then, 20 µL of the diluted solution was dropped onto each of the dentin surfaces from three different groups in 24-well plate. The specimens were covered by sterilized polyester films, incubated for 2 h at 37°C and 5% CO₂. Then 1 mL BHI broth was added into each well and the specimens were continued to incubate for a total of 24 h.

1. Effect of saliva pellicle
Saliva protein films were reported to have an attenuated function of the antibacterial properties for QAMs\textsuperscript{18,19,39}). Human saliva was collected from an investigator who had no active caries or periodontal problems, and not taken any antibiotics within the past 3 months. The saliva was prepared by using a centrifuge for 15 min at 12,000×g to remove debris, and then annealed for 30 min at 60°C to kill bacteria\textsuperscript{40}). Each of rinsed dentin slices from three different treatment groups was soaked in 1 mL of filter-sterilized (0.2 µm cellulose acetate membrane, Nalgene, Rochester, NY, USA), bacteria free saliva for 2 h at 37°C to develop pellicles. Pellicle-covered specimens were rinsed with the DPBS, followed by using the same contact inhibitory test as the above.

2. Effect of aging process
Since long-lasting antibacterial properties of QAMs were necessary\textsuperscript{11}), aging treatment was performed to ensure stability of the NTAP-induced DMAHDM immobilization. After rinse for 7 times, each of the dentin slices from three different groups was stored in 2 mL of the DPBS solution for 1 month, with the solution changing every 48 h\textsuperscript{41}). The same contact inhibitory test was performed.

After 24 h incubation, each of the dentin slices (rinse only/saliva pellicle treated/aging) was transferred into a centrifuge tube with 0.5 mL DPBS solution. The tubes were then vortexed for 5 min and ultra-sonicated for another 5 min. This process was repeated for 3 times. The bacterial suspensions obtained were serially diluted, and spread onto the BHI agar plates for colony-forming unit (CFU) analysis. Ten replicates were tested for each group.

Confocal scanning laser microscopy (CSLM) measurement
CSLM (Leica CTR 6500, Wetzlar, Germany) was employed to confirm the DMAHDM-immobilized-dentin’s contact inhibitory effect\textsuperscript{42}). Live/dead BacLight bacterial viability kit (L7007, Invitrogen Molecular Probes, USA) was used to stain the bacterial cells. According to the instructions of the manufacturer, the SYTO9 taken up by live cells emitted green fluorescent signals, and the propidium iodide taken up by dead cells emitted red fluorescent signals. Three spots of dentin slice randomly selected from each group were chosen to observe direct contact inhibitory effect. The fluorescence intensity of each image at green or red channel would be proportional to the number of bacteria with integral cytoplasmic membranes or compromised membranes, respectively\textsuperscript{41}). The ratio of the intensities (green/red or live/dead) was used as an index for bacterial membrane damage. Live/dead cells were analyzed by Image J (Image Processing and Analysis in Java, National Institutes of Health, Bethesda, MD, USA). Three duplicates of each group were prepared.

Statistical analysis
Statistical analysis was performed using SPSS 23.0 (IBM SPSS, Armonk, NY, USA). The normality of distribution and homogeneity of variances was tested by Shapiro-Wilk and Levene’s tests, respectively. One-way ANOVA was employed for parametric test, followed by Tukey’s and Dunnett T3 post hoc tests. Kruskal-Wallis test was employed for nonparametric test, followed by pairwise comparisons. The significance level was set at 0.05.
RESULTS

Representative SEM micrographs of the dentin substrates after different treatments are shown in Fig. 2. A several-micron-de-mineralized layer (Figs. 2A, B) was created by EDTA for 30 min, which simulated a typical dentin bonding substrate. The SEM results revealed almost no difference in the surface morphology of the dentin substrates among the control (non-treated, Figs. 2C, D), 5% D (no plasma exposure, Fig. not shown), and 30% D (no plasma exposure, Figs. 2E, F) groups. Without the plasma treatment, DMAHDM had been rinsed off from the dentin substrates. However, after the plasma treatment, obvious distinction could be discerned in both the 5% D+P and 30% D+P groups (Figs. 2G–J): DMAHDM was immobilized onto the substrate, and over half of the dentinal tubules were covered by DMAHDM, especially for the 30% D+P group.

Representative FTIR spectra of the dentin substrates after different treatments are presented in Fig. 3. The spectrum of DMAHDM (Fig. 3A) is shown for reference. Characteristic IR bands at 2,925 cm$^{-1}$ (-CH$_2$), 1,720 cm$^{-1}$ (C=O) and 1,455 cm$^{-1}$ (-CH$_2$) were indicated in the spectrum of the DMAHDM. Among the 5% D groups (Fig. 3B), without plasma treatment, there is no difference between the spectra of the control (DS) and 5% D, indicating complete rinse-off of DMAHDM from the substrate. With the plasma treatment, the IR bands (Fig. 3B-DS+5% D+P) at 2,925 cm$^{-1}$ and 1,720 cm$^{-1}$ could be distinguished, indicating immobilization of DMAHDM onto the substrates. In the 30% D groups (Fig. 3C), the IR band at 2,925 cm$^{-1}$ of the plasma-treated group (Fig. 3C-DS+30% D+P) displays significant higher absorbance, and the bands at 1,720 cm$^{-1}$ and 1,455 cm$^{-1}$ become more evident. No difference was observed between the spectra of the control and 30% D without plasma treatment, which was consistent with SEM observations.

To evaluate the amount of DMAHDM immobilized onto the dentin substrate$^{19}$, the band ratios of 2,925 cm$^{-1}$ and 1,455 cm$^{-1}$ to 1,635 cm$^{-1}$ (N-H of collagen, as internal standard) for the 5% D and 30% D groups were calculated and are presented in Fig. 4. Both ratios showed an increasing trend for the 5% D group (Figs. 4A, B) after plasma treatment, but not statistically different from non-treated controls. However, for the 30% D group, the ratios were significantly higher after plasma treatment than the non-treated controls (Figs. 4C, D).

Contact inhibitory results of DMAHDM immobilized dentin substrates are shown in Fig. 5-rinsed. CFU of the 5% D+P (plasma treatment) group was significantly different from that of other two groups (control and 5% D), which was approximately 4 log lower than that of the non-plasma treatment groups. The strong inhibitory effects were minimally affected by saliva pellicle treatment and aging process (Fig. 5-saliva treated and -aging). CFU of the control and 5% D groups showed no difference when effects of the rinse-only, saliva treatment and aging were compared. However, CFU of the 5% D+P (plasma treatment) group was slightly higher when specimens were treated with saliva pellicle (Fig. 5).

Representative CSLM images of the contact inhibition examination are shown in Figs. 6A–C. The number of green live cells was decreased in the order from the control (Fig. 6A), 5% D (Fig. 6B), 5% D+P (Fig. 6C).
6C) groups, whereas, the number of red dead cells was increased sequentially. The live/dead cell ratios are shown in Fig. 6D. The ratios of the 5%M+P group were significantly lower than those of the other two groups, which was consistent with the CFU results.

**DISCUSSION**

Since introduction as an antimicrobial agent, quaternary ammonium salts have gone through various application processes from “simple use as an additive” to “immobilization onto material surfaces”, aiming to gain a long-term antibacterial function. Chemical surface immobilization approach, plasma surface treatment and layer by layer deposition are the three main techniques for rendering surfaces with permanent contact-active antimicrobial function. Yet it has also been certified that the inhibitory effects of the antibacterial components such as QAMs are decreased after incorporation with resin and polymerization. A technique, that could immobilize these components without diminishing or even possibly enhancing their antibacterial functions, is much desired.

NTAP contains a room-temperature mixture of highly reactive particles, including electrons, electronically excited neutrals, radicals, and UV photons, etc. These highly reactive particles could interact with nearly any substrates rapidly to form different surface chemical/functional groups. This NTAP behavior has induced grafting of HEMA onto collagen and thus improved chemical interactions between HEMA monomer and collagen fibrils. In this study, NTAP was applied to test if it could be used to immobilize a QAM (DMAHDM)

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**Fig. 5** *S. mutans* CFU results of DMAHDM treated dentin’s direct contact inhibitory tests (rinse only as well as effect of saliva pellicle and aging, n=10). The y-axis is in log scale. * shows significant difference with DS, ** shows significant differences between DS+5%M and DS+5%M+P. Statistical differences are indicated as different letters in DS+5%M+P groups (p<0.05). No statistical difference between DS and DS+5%M groups (p>0.05).

**Fig. 6** CSLM images of contact inhibition (A–C) examination, and the live/dead cell ratios (n=3) comparison (D). * shows significant difference with DS. ** shows significant differences between DS+5%M and DS+5%M+P (p<0.05). The fluorescence intensity ratio (green/red or live/dead) was used as an index for bacterial membrane damage. The lower the ratio, the more severe the damage is.
onto the dentin substrate within a clinically acceptable treatment time (30 s).

SEM micrographs showed that the affinity of DMAHDM to the dentin substrate had been greatly improved by the plasma treatment/exposure, in which most of the dentinal tubules and surface were penetrated/covered by DMAHDM (Figs. 2 G–J). Some tubules were open as the un-immobilized DMAHDM might have been rinsed off. Contrarily, after rinsing, DMAHDM could hardly be retained on the dentin surface and in tubules for the non-plasma-treatment group (Figs. 2E, F). IR spectral analysis was carried out for further chemical confirmation of the structural distinctions. The alkyl (-CH₃, 2,925 cm⁻¹, 1,455 cm⁻¹) and carbonyl (C=O, 1,720 cm⁻¹) bands are associated with the presence of DMAHDM. As shown in Fig. 3, the bands at 2,925 cm⁻¹ and 1,455 cm⁻¹ could be apparently discriminated and the C=O band was clearly noticeable in the spectra of the plasma-treated dentin substrate. In contrast, the above bands were absent or not obviously visible from the spectra of blank dentin substrate (DS) and DMAHDM applied without plasma after rinsing (Figs. 3B, C). The existence of these characteristic bands indicated the presence of the DMAHDM on the substrate. Therefore, the IR spectral analysis confirmed that the plasma treatment could enhance the chemical interaction of DMAHDM with dentin substrate.

The band ratios of 2,925 cm⁻¹/1,635 cm⁻¹ and 1,455 cm⁻¹/1,635 cm⁻¹ (DMAHDM/collagen ratios) were calculated to assess the relative degree of DMAHDM immobilization onto dentin substrate induced by plasma treatment. The 1,720 cm⁻¹ (C=O) band illustrated the presence of DMAHDM on the substrate of the 5%D plasma treatment group, although the difference in the band ratios among the 5%D groups (a lower DMAHDM mass fraction) was not significant (Figs. 4A, B). However, the 30%D plasma-treatment group showed the highest in both band ratios, indicating more DMAHDM was immobilized onto the dentin substrate, significantly higher than other groups (Figs. 4C, D). With the morphological observation and detailed IR analysis, DMAHDM was proved to be immobilized onto the dentin substrate. Therefore, the 1st hypothesis was accepted.

In today’s restorative dentistry, minimally invasive preparation such as incomplete removal of caries-affected dentin to preserve tooth structure is recommended, especially in deep caries. Different types of QAMs have been incorporated into adhesives to generate the contact antibacterial function in the dentin/adhesive interfaces. However, residual bacteria might exist in the affected dentin and the bacterial endotoxin could infiltrate into deep dentin structure in the absence of filtration pressure in a relatively short time period, through which secondary caries or pulpitis would still occur, consequently leading to restoration failures. It has been demonstrated that NTAP itself could “clean or sterilize” the deep dental surfaces according to the studies on disinfection ability of different oral bacteria. With the help of NTAP, the antibacterial DMAHDM immobilized onto the dentin matrix could possibly show multiple inhibitory effects, e.g., at the dentin/adhesive interface and/or inter-tubular peri-tubular dentin regions. In this study, contact inhibition test was employed to verify the antibacterial effects of the DMAHDM-immobilized dentin bonding substrate.

After rinsing, the DMAHDM-immobilized-dentin showed phenomenal antibacterial efficiency in comparison with the control (DS) or non-DMAHDM-immobilized dentin (Fig. 5-Rinsed). The high antibacterial efficiency was kept when dentin substrates were challenged with saliva pellicle treatment and aging process. Saliva protein layers have been demonstrated to have a negative effect on the antibacterial function of QAMs as they were more attractive to the bacteria than to the cationic groups on the QAMs. This was further confirmed by this study that, with the saliva pellicle covered onto the DMAHDM-immobilized dentin, the inhibitory effect was also slightly declined comparing to the rinse-only and aging groups. Despite of this effect, the DMAHDM-immobilized dentin substrate still exhibited remarkable inhibitory effect. Furthermore, the effect was not degraded over time; that is, the same inhibitory trend occurred during the aging process/test (Fig. 5-Aging). With the chemical interaction occurring directly between the substrate and antibacterial components, better and longer inhibitory effects could be projected when comparing with those simple incorporation methods of DMAHDM with either primer, adhesive or composite. However, longer period of inhibition in a clinically relevant environment is needed to confirm the projection.

The positively charged quaternary amine N⁺ of a QAM is prone to coupling with the negatively charged bacterial membrane, which not only alters the balance of essential ions, but also disrupts the cell membrane and causes cytoplasmic leakage. DMAHDM has additional killing effect since its long chain could be inserted into the membrane. This is consistent with Tiller’s study, which also showed very strong antimicrobial activity of polycations when the alkyl chains are long and flexible enough to penetrate into the bacterial membrane. The structural and mechanistic analyses of the antibacterial effects were further displayed by confocal laser microscopic analysis. This technique has been considered to be very useful in discerning the live/dead cells via detecting the integral cytoplasmic membranes (emitting green fluorescence) or damaged membranes (emitting red fluorescence), along with the spatial distribution of the bacterial viability. On the dentin surface of the non-plasma-treated group, more green than red fluorescence was presented (Fig. 6B), as almost all the DMAHAD had been eluted. Contrarily, more red than green was presented (Fig. 6C), as the S. mutans membranes had been dramatically destroyed on the DMAHDM-immobilized-dentin surface. The early-attachment of the bacteria had been chemically damaged by contacting with cationic functional dentin substrate and physically disrupted by the long alkyl chains of DMAHDM. Therefore, further confirmed by the CSLM results, the 2nd hypothesis that DMAHDM...
immobilized onto dentin substrate provided the surface with a direct, effective and long-lasting antibacterial function was accepted. Plasma-induced DMAHDM-immobilization onto dentin substrate was much more effective in inhibition of S. mutans than the non-plasma treatment groups. It was noticed that the difference of contact inhibition between the DS and DS+5%D groups was significant from the CLSM observation (Fig. 6D), but insignificant from the CFU counting method (Fig. 5), which might be due to different measuring resources (directly from specimen surface vs solution) as well as experimental variation in specimens.

In this proof-of-concept study, it was found that NTAPs effectively induced immobilization of a quaternary ammonium methacrylate (DMAHDM) onto dentin bonding substrate within a clinically acceptable treatment time of 30 s, generating an antibacterial surface with remarkable and long-lasting inhibitory function. Further investigations should be performed with respect to the NTAP/DMAHDM’s overall effect when incorporated into actual bonding procedures/existing workflows. For example, by combining DMAHDM with a dental primer or adhesive, more systematic NTAP studies on antibacterial effects of dental restoration under clinically relevant settings are needed. It is expected that highly reactive particles from NTAP should also induce DMAHDM immobilization in presence of other monomers.

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