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Non-thermal atmospheric plasma brush induces HEMA grafting onto dentin collagen

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Abstract

Objective—Non-thermal atmospheric plasma (NTAP) brush has been regarded as a promising technique to enhance dental interfacial bonding. However, the principal enhancement mechanisms have not been well identified. In this study, the effect of non-thermal plasmas on grafting of HEMA, a typical dental monomer, onto dentin collagen thin films was investigated.

Methods—Human dentin was sectioned into 10-um-thick films. After total demineralization in 0.5 M EDTA solution for 30 min, the dentin collagen films were water-rinsed, air-dried, treated with 35 wt% HEMA aqueous solution. The films were then subject to plasma-exposure under a NTAP brush with different time (1–8 min) / input power (5–15 w). For comparison, the dentin collagen films were also treated with the above HEMA solution containing photo-initiators, then subject to light-curing. After plasma-exposure or light-curing, the HEMA-collagen films were rinsed in deionized water, and then examined by FTIR spectroscopy and TEM.

Results—The FITR results indicated that plasma-exposure could induce significant HEMA grafting onto dentin collagen thin films. In contrast, light-curing led to no detectable interaction of HEMA with dentin collagen. Quantitative IR spectral analysis (i.e., 1720/3075 or 749/3075, HEMA/collagen ratios) further suggested that the grafting efficacy of HEMA onto the plasma-exposed collagen thin films strongly depended on the treatment time and input power of plasmas. TEM results indicated that plasma treatment did not alter collagen's banding structure.

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Significance—The current study provides deeper insight into the mechanism of dental adhesion enhancement induced by non-thermal plasmas treatment. The NTAP brush could be a promising method to create chemical bond between resin monomers and dentin collagen.

Keywords

non-thermal plasmas; plasma-induced grafting; dental monomer; dentin collagen; FTIR; TEM

1. Introduction

A dentin adhesion process using bonding agents generally involves functional hydrophilic monomers to facilitate diffusion of the bonding resin into demineralized dentin. Among such “wetting monomers”, 2-hydroxyethyl methacrylate (HEMA) is one of the most widely used. HEMA has a low molecular weight and ethanol-like hydrophilic portion, which allow it to infiltrate into the network of dentin organic matrix (collagen) and prevent collagen from collapse [1–3]. This process, meanwhile, provides a method for HEMA (along with other adhesive monomers) to mechanically entangle the collagen matrix after polymerization, thus potentially benefits the resin/dentin bonding [4–6]. Furthermore, studies also showed that HEMA might exhibit affinity to dentin through some type of chemical or physical interactions [7–9] with dentinal collagen to promote adhesion.

Despite of extensive use, HEMA presented some obvious problems in dentin bonding applications. For example, HEMA was disclosed to be one of the major components released from adhesive resin, which likely induced cytotoxic effect [10–12]. The leaching may occur during the setting period of the adhesive resin or later when the resin is degraded [13]. This fact possibly suggests that the affinity of HEMA to dentin (or dentin collagen), if any, might be rather weak. The leaching of HEMA may also be related to the poor polymerization capacity of HEMA [14], since unpolymerized monomers are easier to be released to surrounding environment with oral fluids. In addition, the release and suboptimal curing of HEMA might lead to enhanced water uptake and hydrolysis in the hybrid layer [15–17]. All of these factors would consequently undermine the long-term stability of the adhesive/dentin interface.

Non-thermal atmospheric plasma technique has been recently proven to be an “effective” and “clean” approach for materials surface modification [18–20]. This technique combines exceptional chemical reactivity with a relatively mild, non-destructive character resulting from a cold gas phase. Depending on plasma chemistry and gas composition, the highly reactive plasma species react with, clean, or etch surface materials. Meanwhile, the modification is limited to the outmost layer that allows the bulk properties be kept unchanged [19, 21]. Such great advantages have made non-thermal atmospheric plasmas a promising technique in dentistry such as adhesion enhancement of dental restorations. Recent studies [21, 22] have reported the significant improvement of the adhesive/dentin bonding induced by using non-thermal atmospheric plasma (NTAP) brush. However, the exact mechanism is not well understood. Especially, investigations are needed to obtain deeper insights into plasma-induced interactions of the individual components of adhesive and dentin, such as HEMA and collagen. In this study, dentin collagen thin films were prepared, and the effect of NTAP brush on interaction of HEMA with dentin collagen was

investigated. The null hypothesis tested was that plasma treatment would not enhance the interaction of HEMA with dentin collagen.

2. Materials and methods

2.1 Dentin collagen film / HEMA solution preparation

Ten extracted non-carious human third molars were collected after the patients' informed consent under a protocol approved by the University of Missouri-Kansas City adult health sciences IRB. The teeth were stored in 0.9% w/v phosphate buffered saline (PBS) containing 0.002% sodium azide at 4 °C before use. The occlusal one-third to one-half of the crown was removed using a water-cooled diamond saw (Buehler Ltd, Lake Bluff, IL, USA) until all enamel was removed (as determined by reflective light microscopy). Four additional cuts in the occlusal-apical direction were performed to remove all side walls of the enamel. The remaining dentin block was then sectioned into thin film with thickness of 10 µm by means of a tungsten carbide knife mounted on a microtome (SM2500S, Leica, Nussloch, Germany). 15 to 20 10-µm-thick slices/films were obtained from each tooth. The obtained dentin slices were fully demineralized by immersing into 0.5 mol/L EDTA solution (pH=7.4) for 30 min to obtain dentin collagen films (The complete demineralization was assured by using FTIR, in which the disappearance of the phosphate peak at 1070 cm⁻¹ was monitored.). The collagen films were washed in deionized water and transferred onto Mylar films (22 mm×22 mm×0.25 mm, Fisher Scientific, Pittsburg, PA, USA), air-dried and stored in a desiccator.

The HEMA solutions used for plasma-exposure were either neat HEMA (Acros Organics, Morris Plain, NJ, USA) or 35 wt% HEMA in deionized water. For comparison, a three-component photoinitiator system (0.5 wt% camphorquinone (CQ), 0.5 wt% ethyl 4-dimethylaminobenzoate (EDMAB) and 1.0 wt% diphenyliodonium hexafluorophosphate (DPIHP) was added to the above solutions to provide light-curing capability. Photoinitiator concentration was calculated based on pure HEMA monomer. These initiator components were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Non-thermal atmospheric plasma (NTAP) brush

The non-thermal atmospheric plasma brush (Figure 1) employed in this study was fabricated by Nanova, Inc (Columbia, MO, USA). The detailed information about this device can be found in previous publications [23, 24]. Compressed argon gas (ultra high purity) was used as the plasma gas supply. A MKS mass flow controller (MKS Instruments Inc., Andover, MA, USA) was introduced to adjust the argon gas flow rate (2000 sccm). A glow discharge by the direct current power source (Model 1556C, Power Designs Inc, Westbury, NY, USA) was ignited between the two electrodes in a walled, Teflon chamber. One of the electrodes was attached to a ballasted resistor which controlled the discharge current. The other electrode was grounded for electrical safety. The formed plasma discharge could be blown out of the chamber to create a brush-shaped non-thermal plasma jet, which was generated in about 2 s after turning on the power. The prepared specimens (HEMA solutions and dentin collagen films) were placed under the plasma nozzle (5–6 mm from bottom of the nozzle)

for treatment with varying input power (5, 10 and 15 W) and treatment times (1, 2, 4, 6 and 8 min).

2.3 Plasma-exposure and light-curing of specimens

The prepared HEMA solutions were subjected to plasma-exposure and light-curing. For plasma-exposure, the glass slide substrates were pre-treated by plasma brush for 60 s in order to improve the surface hydrophilicity. A small volume (2 μ L) of neat HEMA without photoinitiator was then cast on the glass slide and cured by the plasma brush with different input powers and treatment times. For light-curing, a small volume (2 μ L) of HEMA solution with photoinitiator was cast on the diamond crystal top-plate of the ATR accessory (of the FT-IR spectrometer), and covered by a Mylar film. A 40 s-exposure from a conventional dental light polymerization unit (Spectrum Light, DENTSPLY, Milford, DE, USA) emitting 550 mW/cm² was applied. The distance from the top of the Mylar film to the distal end of the light guide was kept at ~2 mm.

The plasma-exposure and light-curing of the prepared dentin collagen films were conducted after they were pre-treated with 35 wt% HEMA aqueous solutions (without and with photoinitiator, respectively), and gently air-dried for 15 s. After air-drying, the collagen films were still wet, but no free, excess liquid could be observed on the film surfaces. The collagen films treated with HEMA without photoinitiator were then plasma-exposed with different input powers and treatment times. The collagen films treated with HEMA containing photoinitiator were covered by Mylar films and light-cured for 40 s at the light intensity of 550 mW/cm². The plasma-exposed and light-cured HEMA and dentin collagen film specimens were then measured by FT-IR spectrometer. After measurements, each of the dentin collagen film specimens was immersed in 30 ml deionized water and placed on a rotational shaker (a speed of 60 r/min) for 5 min. After air drying, the collagen films were examined again using FT-IR spectrometer, or employed for TEM specimen preparation and measurements. As control, some of the dentin collagen films treated with HEMA solutions but without plasma-exposure or light-curing, were also subjected to FT-IR measurements.

2.4 Attenuated total reflectance Fourier transform infrared (ATR/FT-IR) spectroscopy

The FT-IR spectra of the above specimens were collected using a Fourier transform infrared spectrometer equipped with an ATR attachment (Spectrum One, Perkin-Elmer, Waltham, MA, USA) at a resolution of 4 cm⁻¹ and scan time of 32. The specimens were cast or pressed on the crystal top-plate of the ATR accessory. The ATR crystal was diamond with a transmission range between 650 and 4000 cm⁻¹. At least 3 FT-IR spectra were collected for each specimen group.

2.5 Transmission electron microscopy (TEM)

Collagen film specimens were fixed in 2.5% glutaraldehyde (in sodium cacodylate buffer) for 2 hr and post-fixed in 1% osmium tetroxide for 1 hr. Then they were dehydrated and embedded in Embed-812 resin. Sectioning was performed with diamond knife (Diatome, Biel, Switzerland) on EM UC7 ultramicrotome (Leica Microsystems, Inc., Buffalo Grove, IL). Sections were stained with phosphotungstic acid. Micrographs were collected at 80 kV accelerating voltage with CM12 TEM (FEI, Hillsboro, OR) equipped with SC1000

ORius® digital camera (Gatan, Pleasanton, CA). At least 3 collagen film specimens of each treatment group were examined with TEM.

3. Results

Representative FTIR spectra of uncured, light-cured, and plasma-exposed collagen films (after treated with HEMA) are shown in Figure 2. The spectra of HEMA (I) and non-HEMA-treated collagen film (II) are also presented for reference. Spectral comparison of the HEMA-treated collagen films before and after water rinse (III and IV) disclosed that for uncured and light-cured collagen films, the characteristic IR bands of HEMA at 1720 (C=O) and 1152 cm^{-1} (C-O) disappeared (or almost disappeared) after rinse (Fig. 2A, 2B). In contrast, these two bands could be clearly detected after rinse for plasma-exposed collagen films (Fig. 2C). IR band at 749 cm^{-1} (-CH₂), which is related to the polymerized HEMA, also displayed similarly as the light-cured and plasma-exposed specimens were compared. More characteristic bands of HEMA at 3450 (OH) and 2950 cm^{-1} (C-H) could be found in the higher wavenumber region of representative IR spectrum for the plasma-exposed specimens after rinse (Fig. 2D) (spectra of light-cured specimens not shown).

In order to quantify the amount of HEMA in the plasma-exposed collagen films, band ratios of 1720 and 749 cm^{-1} to 3075 cm^{-1} (N-H of collagen, as internal standard) were calculated and are shown in Figure 3. The results indicated that both 749 cm^{-1} /3075 cm^{-1} and 1720 cm^{-1} /3075 cm^{-1} ratios increased consistently with the plasma treatment time at the input power of 5 W. As for input powers of 10 and 15 W, 749 cm^{-1} /3075 cm^{-1} and 1720 cm^{-1} /3075 cm^{-1} ratios increased with the plasma treatment time from 0 to 4 min, but exhibited a decreasing trend as the plasma treatment time further increased to 8 min. Within the limitations of the current study, the optimal plasma-treatment condition to achieve the highest 749 cm^{-1} /3075 cm^{-1} and 1720 cm^{-1} /3075 cm^{-1} ratios appears to be at the input power of 10 W with the treatment time of 4 min.

Figure 4 shows representative TEM micrographs of the collagen films with different treatment (with or without HEMA) and curing (with light or plasma) conditions. The result revealed no significant difference in the collagen matrix feature among the uncured (Fig. 4A), light-cured (Fig. 4B), and non-HEMA-treated, plasma-exposed (Fig. 4C, 4D) specimens. However, as the HEMA-treated, plasma-exposed group (Fig. 4E, 4F) was also compared, obvious distinction could be discerned: numerous bright spots appeared in the areas of the plasma-exposed side of the collagen films. TEM micrographs collected from the other side of collagen films showed no presence of the bright spots (images not shown here). Moreover, collagen films with the longer plasma treatment time (4 min, Fig. 4F) exhibited even wider region of such bright-spot feature.

Representative FT-IR spectra of light-cured and plasma-exposed collagen films (both were HEMA-treated and after water rinse) in the wavenumber regions of amides A, I, II, and III are shown in Figure 5. The spectrum of uncured collagen films in these regions demonstrated similar feature to that of light-cured ones (not shown here). Spectral comparison between the light-cured and plasma-exposed groups disclosed significant IR band shifts or changes. For example, after plasma-exposure in the presence of HEMA, the

amide A was shifted from 3314 to 3304 cm^{-1} (Fig. 5A), the amide II was split from one band at 1543 cm^{-1} to two bands at 1547 and 1536 cm^{-1} (Fig. 5B), and the amide III was shifted from 1234 to 1237 cm^{-1} (Fig. 5C). However, no obvious band shift or change was observed in the amide I region.

4. Discussion

Non-thermal plasmas are partially ionized gases that contain electronically excited atoms, molecules, and ionic and free-radical species. These highly reactive particles can cross-link or react rapidly to form various chemical functional groups on the substrates [19, 25, 26]. This feature of non-thermal plasmas provides unique opportunity to enhance the affinity of dental adhesive components (such as HEMA) with collagen fibrils through chemical bonding approaches. In the present study, the characteristic bands of HEMA were clearly discerned from the IR spectra of the plasma-exposed collagen films after water rinse (Fig. 2C, 2D-IV). In contrast, such IR bands were absent in the spectra of uncured (Fig. 2A-IV) and light-cured (Fig. 2B-IV) samples. The results evidently indicated that the plasma treatment could enhance the presence of HEMA within dentin collagen films, or in other words, the interaction of HEMA with dentin collagen matrix. This observation can be further demonstrated by the morphological evidences obtained from TEM observations. As compared to those of the uncured (Fig. 4A) and light-cured collagen films (Fig. 4B), the TEM images of the plasma-exposed (with HEMA-treatment) samples (Fig. 4E, 4F) displayed apparent presence of numerous bright spots. Since such morphological feature was not detected from the non-HEMA-treated, plasma-exposed collagen films (Fig. 4C, 4D), it is likely that the bright spots were associated with HEMA incorporated within the plasma-exposed collagen films.

The enhanced HEMA interaction with dentin collagen induced by plasma treatment was further elucidated by a more detailed spectral analysis. Spectral comparison of light-cured and plasma-exposed collagen films (both were HEMA-treated and after rinse, see Fig. 5) revealed significant IR band shifts or change with amides A, II and III. These IR band alterations were most likely related to the dentin collagen surface modification induced by plasmas, and the subsequent (or simultaneous) chemical interaction with HEMA. As mentioned above, argon plasmas can produce various reactive species (such as free radicals) that may react and be incorporated into the dentin surface, especially the organic components (collagen) of dentin surface [21, 22]. From these activated sites, the grafting polymerization of HEMA could be initiated. Consequently, IR bands shift or change with the amide groups of collagen would be expected due to the formed chemical (covalent) bonds. Our currently observed spectral changes induced by the plasma and HEMA treatment (Fig. 5) were different from the reported work that employed only plasma treatment (amide II shifted from 1543 to 1533 cm^{-1}) [21]. This suggested that HEMA was actively involved in the chemical interaction with collagen through grafting in this study. The current results rejected the null hypothesis that plasma treatment would not enhance the interaction of HEMA with dentin collagen. Our result also revealed that amide I band displayed no significant change (Fig. 5B) after the plasma and HEMA treatment, which indicated an intact collagen triple helix structure (or bulk collagen matrix) [27] despite of the collagen surface grafting of HEMA.

Band ratios of $1720\text{ cm}^{-1}/3075\text{ cm}^{-1}$ and $749\text{ cm}^{-1}/3075\text{ cm}^{-1}$ were used to measure the degree of HEMA grafted onto collagen after plasma treatment. As mentioned above, IR band at 749 cm^{-1} is specifically associated with the polymerized HEMA, whereas 1720 cm^{-1} is a general band indicative of the presence of HEMA. The current data disclosed that the two band ratios of $1720\text{ cm}^{-1}/3075\text{ cm}^{-1}$ and $749\text{ cm}^{-1}/3075\text{ cm}^{-1}$ exhibited quite similar trend as a function of plasma treatment time and input power. This result most likely suggested that the grafting of HEMA onto collagen and its polymerization took place concurrently. The amount of HEMA grafted onto collagen strongly depended on the polymerization performance of HEMA. The results shown in Fig. 3 also revealed that for the 10 and 15 W input powers, the band ratios of $1720\text{ cm}^{-1}/3075\text{ cm}^{-1}$ and $749\text{ cm}^{-1}/3075\text{ cm}^{-1}$ turned to decrease as the plasma treatment time was longer than 4 min. Fundamentally plasma treatment can lead to the surface change in two ways: modification and etching, which compete with each other [21]. For the plasma-treated collagen films, modification of the treated surface could introduce new functionalities, which as a result contributed to the significant grafting of HEMA. However, if the film surfaces underwent prolonged exposure (such as 6 or 8 min in the current study, Fig. 3C–F), some collagen fibrils in the surface area (which served as the supporting substrate of grafted HEMA), or even the grafted HEMA might be etched away. This might explain the observed descending trend of the band ratios of $1720\text{ cm}^{-1}/3075\text{ cm}^{-1}$ and $749\text{ cm}^{-1}/3075\text{ cm}^{-1}$ at the treatment time longer than 4 min.

Current finding also suggested that the uncured and light-cured collagen films showed no detected presence of HEMA. This is different from reported investigations [7–9] that concluded HEMA could be directly absorbed onto the collagen matrix as a result of chemical or physical interactions. Xu. et al[9] examined the absorption characteristics of HEMA onto collagen by using FT-Raman spectroscopy. Their data indicated that HEMA could likely react with functional groups of collagen to form new bonds (such as C-N), thus contributed to the HEMA/collagen interaction and HEMA absorption. A ^{13}C NMR study conducted by Nishiyama et al. [7] suggested that the formation of hydrogen bonding between the ester carbonyl group in HEMA and the dentinal collagen might have been responsible for the observed HEMA absorption. The retention of HEMA in demineralized dentin was also observed by Sharrock et al. [8]. They interpreted the phenomenon according to a salvation mechanism, in which HEMA entangled collagen fibrils from both outside and inside by dissolving into the peptidic polymer network. The discrepancy in observations between the present and previously reported studies can be explained as following. Firstly, the present study employed very thin dentin films ($10\text{ }\mu\text{m}$ in thickness) to prepare collagen films, in contrast to bulk dentin disks or crushed dentin particles ($\sim 50\text{--}100\text{ }\mu\text{m}$ in particle size) in the previously reported studies. The relatively smaller size and more ordered tubule orientation (universally perpendicular to film surface according to SEM images, data not shown) of the specimens in the current study might have facilitated an easier wash-away of HEMA from the collagen matrix. Secondly, to demineralize the dentin disks or particles, stronger acids such as phosphoric acid or nitric acid were typically utilized in the previous studies, as compared to the mild EDTA in the current study. It is possible that the strong acids would react with collagen[7], and generate active sites within collagen matrix to induce interaction with HEMA. In addition, the grinding or crushing procedure used to

prepare dentin particles might have also led to cleavage of collagen fibrils[28], which also offered reactive portions to the collagen to interact with HEMA.

The concentration of HEMA aqueous solution was also an important factor to determine the performance of HEMA affinity/grafting to collagen films. In this study, 35 wt% aqueous HEMA solution was employed to treat the dry collagen films before light curing or plasma exposure. The collagen network was generally in a collapsed state after air-drying. Studies have shown that neat HEMA was not able to induce re-expansion of the collapsed collagen network. Instead, it could only attach to the exposed surface of the collagen fibrils [29]. However, utilizing HEMA aqueous solution would be a good way to re-expand the collapsed collagen network, since water plays a critical role in the re-expansion process of demineralized dentin. HEMA concentration of 35 wt% in aqueous solution was suggested to be well suitable to re-expand the collapsed collagen, hybridize the collagen surface, and improve the bond strength[29, 30]. In the current study, treatment of dry collagen films with 35 wt% HEMA solution might have enabled HEMA monomer to infiltrate more thoroughly into collagen network, and to exhibit more intimate contact with the surfaces of collagen fibrils. This would consequently benefit the grafting efficacy of HEMA onto collagen.

In summary, the present study disclosed the remarkable performance of non-thermal atmospheric plasmas in enhancing HEMA interaction with dentin collagen through grafting polymerization. Appropriate selection of plasma treatment conditions could lead to significant HEMA grafting onto dentin collagen. The obtained data also provide deeper understanding on the mechanism of dental adhesion enhancement induced by plasma treatment. However, treatment times chosen in this study are too long to be used in clinical conditions. In the future, reduced treatment times as well as grafting of other monomers onto collagen will be investigated.

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Highlights

- Non-thermal plasmas induce HEMA grafting onto dentin collagen under clinical settings.
- Grafting efficacy of HEMA depends on the treatment time and input power of plasmas.
- A new method to create chemical bond between resin monomers and dentin collagen.

Figure 1.
(A) Schematic diagram and (B) photograph of the experimental plasma brush set-up.

Figure 2. FT-IR spectral comparison of (A) uncured, (B) light-cured, and (C) plasma-exposed groups (input power of 15 W, treatment time of 4 min). (D) showed spectra in the higher wavenumber region of the plasma-exposed group. The spectra from bottom to top in each figure represent (I) HEMA (II) non-HEMA-treated dentin collagen film (III) HEMA-treated collagen film before water rinse, and (IV) HEMA-treated collagen film after water rinse.

Figure 3.

FT-IR band ratios of 749 cm⁻¹/3075 cm⁻¹ (A, C, E) and 1720 cm⁻¹/3075 cm⁻¹ (B, D, F) for HEMA-treated, plasma-exposed collagen films at input powers of 5W (A, B), 10 W (C, D), and 15 W (E, F). All data were expressed as means ± standard deviation (n = 3). Statistical analysis was performed with Statistical Product and Service Solutions (SPSS, Version 21, IBM SPSS, Inc., Chicago, IL) and nominal level of significance at 0.05. The normality of distribution and homogeneity of variances were confirmed with Shapiro-Wilk and Levene's tests, respectively. Comparison of means was performed with one-way ANOVA and Tukey's post hoc test. Statistically different values were indicated by different letters.

Figure 4.

Transmission electron micrographs of (A) non-HEMA-treated, uncured, (B) HEMA-treated, light-cured, (C) non-HEMA-treated, plasma-exposed (10 W, 1 min), (D) non-HEMA-treated, plasma-exposed (10 W, 4 min), (E) HEMA-treated, plasma-exposed (10 W, 1 min), and (F) HEMA-treated, plasma-exposed (10 W, 4 min) collagen films. For (B–F), TEM micrographs were collected from the light cured or plasma-exposed surface side of the collagen films. Arrows may indicate the possible sites (scattered bright spots) of HEMA grafting onto collagen. CM: collagen matrix; ER: embedding resin.

Figure 5. FT-IR spectral comparison of light-cured and plasma-exposed (input power of 15 W, treatment time of 4 min) collagen films (HEMA-treated, after water rinse) at the IR wavenumber region of (A) amide A, (B) amide I, II, and (C) amide III.