Remineralizing effect of cold plasma and/or bioglass on demineralized enamel

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This study investigated the combining effect of cold plasma and bioglass-phosphoric acid paste on demineralized enamel. Fifty bovine incisors' enamel specimens were challenged by a demineralization solution of pH 4.47 for 72 h. Specimens were divided into five groups: (I) Control, demineralized enamel (C); (II) Demineralized enamel treated with fluoride varnish (F); (III) Cold plasma application to demineralized enamel (P); (IV) Demineralized enamel treated with bioglass paste (B); (V) Application of bioglass paste to cold plasma-treated demineralized enamel (PB). Specimens were then immersed in remineralizing solution for 24 h, before being examined with micro-computed tomography (micro-CT), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS) and cross-sectional micro-hardness measurement. The results showed that; treating demineralized enamel with cold plasma before bioglass application ensued a significant high mineral volume recovery and micro-hardness of demineralized region. It can be concluded that cold plasmas may improve the remineralization of bioglass on demineralized enamel.

Keywords: Demineralized enamel, Cold plasmas, Bioglass, Remineralization

INTRODUCTION

Dental caries and erosion are the results of excessive tooth demineralization. It is suggested that their management should focus on the inhibition of demineralization, early detection and tooth remineralization, before applying a restorative approach that requires removal of tooth tissues¹,². Remineralization of enamel surfaces may restore or even increase the mechanical properties of these regions and their resistance to any further cariogenic or erosive challenges³.

With sufficient concentrations, topical application of fluoride can be effective in protecting enamel from demineralization and enhancing remineralization⁴.

Inspired by the use of bioactive materials for bone repair and regeneration, the potential of bioglass materials for repairing dental tissues is attracting increased interest⁵. Recently, a 45S5 bioglass-containing paste that could release calcium and phosphate ions was shown to be capable of penetrating and remineralizing enamel that had been subjected to an erosive challenge⁶. The bioglass paste formed a calcium phosphate-rich layer on the enamel surfaces within 24 h. This layer showed resistance to abrasion and transformed into hydroxyapatite crystals after 14 days of storage in a remineralizing solution. Bioglass reacts in aqueous environments to release Ca²⁺, Na⁺, and PO₄³⁻. The ionic exchange of Na⁺ with H⁺ or H₂O⁻ at the glass-liquid interface allowed Ca²⁺ and PO₄³⁻ to be released to form a supersaturated ionic reservoir for the enamel apatite.

After the bioglass network dissolved, silanols underwent rearrangement by poly-condensation and served as nucleation sites. The deposition of free calcium and phosphate together with undissolved bioglass particles formed a calcium phosphate-rich layer on the enamel surface⁶.

Cold plasmas are gaseous media generated at low pressure and have been intensively studied for the past two decades⁷. Lately, there has been increased interest in cold plasmas application in biomedical field because they can interact with subjects physically, chemically and biologically without causing damage. The possible plasmas applications include modification of surface properties such as the electrochemical charge and amount of oxidation, as well as attachment of surface-bound chemical groups. Cold plasmas can also specifically and precisely modulate properties such as hardness, resistance to physical abrasion, wettability, and affinity towards specific molecules⁸,⁹.

In dentistry, cold plasmas have been used for endodontic treatment, tooth bleaching, adhesion and caries treatment with partial successes¹⁰. In contrast to laser beams, which propagate linearly and are prone to being scattered, cold plasma can penetrate into irregular cavities and fissures. Moreover, it kills only pathogens in bacterial plaque without damaging the oral tissues.

Yang et al.¹¹ investigated the bactericidal effect of a non-thermal atmospheric-pressure plasma brush on S. mutans and L. acidophilus, which are major pathogens in dental caries. Their results indicated that cold plasmas were effective in deactivating oral bacteria in culture after 5 min of application, this might play some roles in prevention or treatment of dental caries. It was also found that the cold plasma treatment improved primer infiltration compared to a control¹². One study revealed that atmospheric-pressure plasmas increased the surface energy and surface wettability of enamel, and as a result its bond strength with sealants, potentially serving as a

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Plasma surface modification was reported as a possible method for aided mineral re-crystallization process. It can be used to produce nano-sized crystals with improved internal quality and dissolution rate, compared to conventional size crystalline products, however further research is needed for process optimization\textsuperscript{14-16).

Conservative dental treatment can sometimes be painful due to heat generation with drilling. In contrast, the temperature increase in the pulp of a tooth was found to be only $\sim 2.3^\circ C$ during cold plasma treatment of the enamel surface, that does not cause discomfort to patients, because it does not induce thermal damage\textsuperscript{17,18).

Given the aforementioned potential benefits of cold plasmas in oral application, it was thought that they might have a synergistic effect with bioglass paste on tooth remineralization. This study, therefore, assessed the effect of applying bioglass paste on plasma treated demineralized enamel by testing the hypothesis that treating demineralized enamel surface with cold plasma before bioglass application would improve its remineralization.

**MATERIALS AND METHODS**

**Specimen preparation**

1. Specimen cutting, grouping and demineralization

Fifty non-carious bovine incisors were used in this study: 25 for micro-computed tomography ($\mu$CT), and 25 for scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and hardness measurement. Bovine enamel was used as a substitute for human enamel due to their similar physicochemical properties, such as composition, density, hardness and tensile strength\textsuperscript{19}.

The recently extracted bovine teeth were obtained from a slaughterhouse, stored in a refrigerator and used within a month from extraction. They were first sectioned to remove their buccal surfaces using a water-cooled diamond saw (IsoMet Low Speed Saw, Buehler, Lake Bluff, IL, USA), giving one enamel specimen per tooth. They were then fixed in acrylic resin molds and ground using water-cooled silicon carbide discs with sequentially 600-, 1200-, 1800- and 2400-grade papers (Buehler) to obtain flat enamel surfaces, which helped to create more consistent and reproducible artificial enamel demineralization. The specimens were then flushed with distilled water in an ultrasonic cleaner (Cole-Parmer, Model 08849-00, Vernon Hills, IL, USA) for 30 min to remove all surface debris. Four positioning holes ($\sim 0.8$ mm) were made by a carbide bur (SSW FG-330 ISO # 500, Lakewood, New Jersey, USA), one adjacent to each corner. These holes helped to locate the demineralized region during $\mu$CT examination. Two layers of nail varnish (Lot TV14078, Revlon Miami, Florida, USA) were applied to cover most of the enamel surfaces, leaving a window of $4\times 4$ mm for demineralization (Fig. 1). Each specimen was challenged by 15 mL of demineralization solution containing 2.2 mM CaCl$_2$, 2.2 mM NaH$_2$PO$_4$, and 50 mM acetic acid with the pH adjusted to 4.47 at $37^\circ C$ with stirring (150 rpm)\textsuperscript{20}.

A pilot demineralization study was conducted for 24, 48, 72 and 96 h, and the demineralized enamel areas were examined using $\mu$CT. The 72 h demineralization was chosen for the main study because it was able to produce $\sim 150$ $\mu$m deep of demineralized enamel on the surface (Fig. 2). In contrast, demineralized areas produced with the shorter durations were harder to analyze because of the lower signal-to-noise ratios; while those produced with the longer duration had large-scale erosion.

As shown in Table 1, specimens were randomly assigned to five groups ($n=10$): (C) Control group with...
no treatment; (F) Treatment with fluoride varnish as a conventional non-invasive treatment mode for comparison; (P) Cold plasma application; (B) Treatment with bioglass paste; (PB) Treatment with cold plasma, followed by application of bioglass paste. After treatment, the specimens were immersed in a remineralizing solution for 24 h.

2. Fluoride gel application
A fluoride varnish containing 5% Sodium Fluoride (031716211, DuraShield, Sultan Health Care, Hackensack, NJ, USA) was applied, using the provided brush, to the demineralized surfaces for 5 min, as recommended by the manufacturer. The (F) group was used as a standard remineralization group for comparison.

3. Cold plasma application
A plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, NY, USA) was used in this study to activate the surfaces

Table 1  Experimental groups and their treatments

| Treatments                  | Groups | Group I Control | Group II Fluoride | Group III Plasma | Group IV Bioglass | Group V Plasma/Bioglass |
|-----------------------------|--------|-----------------|-------------------|------------------|-------------------|-------------------------|
| Demineralization            | +      | +               | +                 | +                | +                 | +                       |
| Plasma application to enamel| −      | −               | +                 | −                | −                 | +                       |
| Bioglass application        | −      | −               | −                 | +                | +                 | +                       |
| Fluoride varnish application| −      | +               | −                 | −                | −                 | −                       |
| Remineralization solution immersion | +      | +               | +                 | +                | +                 | +                       |

(+) Applied and (−) not applied

Fig. 2  Micro-CT images of enamel specimen (a) at baseline, after (b) 48 h, (c) 72 h, and (d) 96 h of demineralization.
of specimens in Groups P and PB, with oxygen being used as the gas source. The minimum vacuum pump speed was set at 1.4 m³/h and the final total pressure lower than 200 mTorr. The duration of plasma treatment was 5 min.

A pilot study was conducted to determine the activity of the enamel surface before and after cold plasma treatment. Sessile-drop contact-angle measurements were performed on enamel specimens using a contact angle analyzer (DM-CE1, Kyowa Interface Science, Saitama, Japan) with appropriate software (FAMAS, Kyowa Interface Science). Deionized water was used as the wetting liquid with a drop volume of 2 μL. The results showed that the surface contact angle was greatly reduced from 76° to 9° after plasma treatment, but this effect diminished rapidly with time. Thus, any further treatment was done within 20 s of completing the plasma application.

4. Bioglass application
Point one grams of 45S5 bioglass powder; composition and procedure are mentioned in Table 221) (44-μm average particle size, containing 24.5 wt% Na₂O, 24.4 wt% CaO, 6 wt% P₂O₅, and 45 wt% SiO₂) was mixed with a spatula on a glass slab with 0.2 mL of 50 wt% phosphoric acid (Fisher Scientific Company, Fairlawn, New Jersey, USA) for 1 min to form a paste 22). This slurry paste was applied to the demineralized specimens of Groups B and PB using a micro-brush (Disposable Applicators, Size M-silver Lot: 185522, 3M ESPE, St. Paul, MN, USA) with a rotational rubbing motion for 1 min. The approximate thickness of the paste was nearly 2 mm.

5. Adhesive application
One-step self-etching adhesion promoter (Adper Easy Bond, 3M ESPE) was placed passively over bioglass paste (groups B and PB) to protect it. The adhesive was then cured using a light-curing device (Optilux VCL 401, Demetron Research, Danbury, CT, USA) with a power density of 550 mW·cm⁻² for 10 s.

6. Storage of specimens
All specimens were stored individually in glass containers containing 15 mL of a remineralization solution for 24 h at 37°C. The remineralization solution comprised of 1.5 mM CaCl₂, 0.9 mM NaH₂PO₄, 130 mM KCl, and 20 mM cacodylate buffer, with the pH adjusted to 7.0 with stirring (150 rpm)²². At the end of the storage period, the adhesive covering the bioglass paste was gently removed with a tweezer; any remaining fluoride varnish was removed by using an alcohol swap. The specimens were then rinsed with deionized water, and sonicated for 5 min before being examined.

µCT measurement and evaluation
1. Specimen mounting and scanning
For each specimen, an index (Fig. 1) was made using an orthodontic self-cured resin (Dentsply, Caulk, Milford, DE, USA) to attach it to the platform of the µCT unit (HMX-XT 225, X-tek System, UK). The index was attached to a mounting jig with two metal pins that allowed the specimen to be positioned inside the µCT chamber in a reproducible way. This minimized the shifts between scans taken at different times for ease of comparison. During scanning, a piece of moist cotton was placed on the specimen to prevent it from cracking through drying. Scanning was performed using X-rays produced with a tube voltage of 90 kV and a tube current of 90 μA. A total of 720 projections, with 4 frames per projection, were acquired.

Reconstruction of the volumetric specimen image with the microradiographs was performed using the software CT Pro 3D (Nikon Metrology, Brighton, MI, USA). The reconstructed volume was then post-processed using the software VGStudio Max (Version 2.1.3, 64 bit, Volume Graphics, Charlotte, NC, USA). Each specimen was scanned three times: before demineralization as a baseline for comparison, after 72 h of demineralization, and after 24 h of remineralization treatment. The reconstructed image had a resolution of 2,048×2,048 pixels with an isotropic voxel size of 25 μm.

The µCT images were analyzed by making comparisons between the base line attenuation coefficient, those obtained after demineralization and after remineralization. Changes in the mineral content

| Materials          | Composition                  | Procedures                                      |
|--------------------|------------------------------|-------------------------------------------------|
| 45S5 bioglass      | Weight percent               | Mix 0.1 g of 45S5 bioglass with 0.2 mL of phosphoric acid |
|                    | 45% SiO₂                     |                                                 |
|                    | 24.5% Na₂O                  |                                                 |
|                    | 24.4% CaO                   |                                                 |
|                    | 6% P₂O₅                     |                                                 |
| Adper Easy Bond    | HEMA, Bis-GMA, Methacrylated phosphoric esters, 1,6 hexanediol dimethacrylate, Methacrylate functionalized Polyalkenoic (Vitrebond Copolymer), Finely dispersed bonded silica filler with 7 nm primary particle size, Ethanol, Water, PI, Stabilizers | Apply adhesive for 20 s, dry for 5 s, then light cure for 10 s |
| 3M ESPE, St. Paul, MN, USA |                                                     |                                                 |

HEMA=2-hydroxyethyl methacrylate; Bis-GMA=bisphenol glycidyl methacrylate; PI=photoinitiator
of the treated regions were calculated. For this purpose, the linear attenuation coefficients of the specimens were converted to mineral density values assuming that the material absorbing the X-rays was calcium hydroxyapatite.

2. Establishing relationship between mineral density and attenuation coefficient

Eleven cylindrical hydroxyapatite (HA) tablets with density of 1.03–1.53 g/cm$^3$, a diameter of 4 mm, and a thickness of 2 mm were vertically stacked using an acrylic holder and scanned with the same μCT system and scanning parameters as described above. Reconstruction of the volumetric images of the tablets with the microradiographs was again performed using the software CT Pro 3D (Nikon Metrology).

Extraction and post-processing of the attenuation coefficients were carried out using the software ImageJ (Version 1.47d, National Institutes of Health (NIH), Bethesda, MD, USA). To minimize the beam-hardening effect, measurement of the attenuation coefficient was confined to a central area of 50×50 pixels within the vertical mid-plane of each HA tablet. Therefore, a total of 2,500 measurements of the attenuation coefficient were extracted from each tablet and the average value calculated. The averaged attenuation coefficients of all the tablets were then plotted against their densities to establish the relationship between the two variables.

The through-thickness density profiles of the exposed region obtained from the baseline, demineralization and remineralization scans were used to calculate the percentage of mineral volume recovery for each specimen using the following equation:

\[
\% \text{ of Mineral Volume Recovery} = \left( \frac{\text{integrated area under the remineralized curve} - \text{integrated area under the demineralized curve}}{\text{integrated area under the baseline curve} - \text{integrated area under the demineralized curve}} \right) \times 100
\]

SEM/EDS surface examination

Specimens (5 from each group) for SEM, EDS, and hardness measurement were embedded in a clear acrylic resin (Dentsply) and then cut into two halves horizontally through the center of the treated area with a diamond disc under water irrigation.

One half of each slab was assigned for cross-sectional micro-hardness measurement and the other for SEM and EDS assessments. The cut surfaces were polished by a Buehler Isomet II polishing machine using 600-2400 ascending grades silicon carbide papers, followed by 1-μm and then 0.3-μm Alumina abrasive (40-6321 016, Buehler), before being sonicated for 30 min in distilled water. SEM images were obtained of the treated region for one of the halves using a scanning electron microscope (TM 3000, Hitachi High Technologies, Japan) with the following operational parameters: accelerating voltage of 15 kV, charge-reduction mode, 10-mm working distance, and 2,000× magnification. Elemental maps were obtained using EDS across the treated region for the following elements: phosphorus, calcium, sodium, silicon and fluorine.

Micro-hardness measurements

The other half of each cut specimen was used to measure the micro-hardness in the treated region at 30, 50, 70, 100 and 200 μm from the outer enamel surface. Hardness was measured at room temperature using a Micro Surface Vickers Hardness Tester (Buehler Micromet II, Buehler) and a diamond pyramid micro-indenter with a 136° angle between the opposing faces. A load of 25 g was used for 10 s at the outer edge of the treated region to avoid cracking the specimen. The Vicker’s hardness number (VHN) was obtained using the following equation:

\[
\text{VHN} = 1854.4 \frac{P}{d^2}
\]

where $P$ is the applied load in grams and $d$ is the average length of the indentation measured in μm. Fifteen indentations were made at each depth and averaged to obtain a micro-hardness vs. depth profile.

Statistical analysis

The % mineral volume recovery calculated from 5 specimens per group was analyzed using the non-parametric Kruskal-Wallis test followed by pairwise comparisons to test the significance of difference between the groups ($p<0.05$). Micro-hardness values at different depths between the groups were analyzed by one-way ANOVA followed by LSD post-hoc test using the Statistics software SPSS (Version 20, IBM, Chicago, IL, USA).

RESULTS

Micro-CT

1. Relationship between HA mineral density and attenuation coefficient

The densities ($\rho$) of the eleven HA tablets ranged from 1.03 to 1.53 g cm$^{-3}$, and the corresponding attenuation coefficients ($\mu$) ranged from 116.96 to 170.98 cm$^{-1}$. Figure 3 shows that, within these ranges of values, the attenuation coefficient is linearly related to the HA mineral density ($R^2= 0.9519$) with

\[
\mu = 113.32\rho - 3.8932
\]

where $\rho$ is the density of the material in g cm$^{-3}$.
It was found that the linear relationship between attenuation coefficient and density quiet holds for higher values of density\textsuperscript{27}.

Figure 4 shows representative enamel mineral density profiles of specimens at baseline, after demineralization and remineralization for the different treatment groups. With regard to the percentage of mineral volume recovery, all treatment groups showed a significant difference from the control group (C), which had a mean value of 19.5% (Table 3). The highest mineral volume recovery among the treated groups after 24 h of remineralization was 32.8% in the PB group (treated with plasma then bioglass paste, and protected by adhesive prior to storage in the remineralization solution), and the lowest was 22.1% in the F group (treated with fluoride then stored in remineralizing solution). The other groups’ mineral volume recoveries were 25.9% (P, 5-min plasma application only), and 29.8% (B, treated with Bioglass paste then protected by adhesive). The (PB) group was significantly different from the (F, B and P) groups. The fluoride group was insignificantly different than plasma group, while showed a significant difference than bioglass group.

**SEM and EDS**

Representative SEM images of the specimens’ cross-sections at baseline, after demineralization and after remineralization for the different treatment groups are shown in Fig. 5. The “baseline” image shows the usual
Table 3  Results from non-parametric Kruskal-Wallis statistical test followed by pair wise post hoc comparison test showing median, mean, standard deviation (SD) and (minimum, maximum) values of volume recovery percentage of control and study groups (n=5) and their p values

| Groups         | Median volume recovery (%) | Mean volume recovery (%)* | ±SD   | (Min, max) |
|----------------|---------------------------|--------------------------|-------|------------|
| Control        | 19.5                      | 18.4a                    | 3.4   | (13.5, 22.3) |
| Fluoride       | 22.1                      | 23.7b                    | 2.8   | (21.2, 27.1) |
| Plasma         | 25.9                      | 25.1b                    | 3.1   | (20.7, 28.8) |
| Bioglass       | 29.8                      | 29.8c                    | 1.7   | (27.9, 32.0) |
| Plasma/Bioglass| 32.8                      | 33.0d                    | 2.4   | (30.9, 36.8) |

* Groups with different superscripts have statistically significant differences (p<0.05)

\[ p \]

| Groups         | Control  | Fluoride | Plasma | Bioglass | Plasma/Bioglass |
|----------------|----------|----------|--------|----------|-----------------|
| Control        | —        | 0.047    | 0.016  | 0.009    | 0.009           |
| Fluoride       | —        | —        | 0.602  | 0.009    | 0.009           |
| Plasma         | —        | —        | —      | 0.028    | 0.009           |
| Bioglass       | —        | —        | —      | —        | 0.047           |
| Plasma/Bioglass| —        | —        | —      | —        | —               |

Fig. 5  Cross-sectional scanning electron microphotographs of specimens after remineralization: (a) baseline, (b) Control, (c) Fluoride, (d) Plasma (e) Bioglass, (f) Plasma/Bioglass.

keyhole prism appearance with intact prism sheaths and negligible inter-prism porosity (Fig. 5a). Specimens from the control group (C), with no special treatment showed a top porous layer of ~30 μm deep with destruction around the enamel prisms’ peripheries (Fig. 5b). The group treated with fluoride (F) prior to storage in the remineralization solution showed some remineralization which reached a depth of ~5 μm from the top surface (Fig. 5c). The group with 5-min plasma application (P) showed a surface layer, ~15 μm deep, of irregular and porous minerals deposited (Fig. 5d). Specimens treated with the Bioglass paste (B) showed remineralized enamel (~10
μm deep) with more homogenous and denser mineral content that obliterate the surface porosities found in the fluoride- and plasma- groups (Fig. 5e). The group treated with plasma and bioglass paste (PB) showed similar results as bioglass group (B), but with finer and denser mineral deposits that went deeper into the demineralized region (Fig. 5f).

Chemical analysis of the “baseline” enamel using EDS showed that it had predominantly Oxygen (O), Carbon (C), Calcium (Ca), and Phosphorus (P). The atomic Ca/P ratio was found to be approximately 1.70 (Table 4). The same ratio rose to 1.75 for the control demineralized group. The fluoride-treated (F) group showed a superficial layer that was rich in calcium and phosphorus, with trace amount of fluoride. The atomic Ca/P ratio for this group was found to be 1.87. The newly formed layer in the plasma-treated group (P) was also rich in calcium and phosphorus, the atomic Ca/P ratio there being 1.92. Apart from being rich in calcium and phosphorous, chemical characterization of the (B) and (PB) groups also showed trace amounts of sodium and silica. The atomic Ca/P ratios for these two groups were found to be 1.93 and 1.76 respectively. The Ca and P atomic percentages were slightly higher in the (PB) group than the (B) group, meanwhile, Na and Si percentages were slightly lower.

### Micro-hardness

Table 5 shows the Vicker’s micro-hardness numbers (VHN) in g·μm$^{-2}$ for the control and experimental groups. One-way ANOVA followed by LSD post-hoc showed that all the remineralization methods had a significant effect on the cross-sectional micro-hardness at depths of 30, 50, 70 and 100 μm ($p \leq 0.05$) when compared with the control group. The range of hardness values was 134.2–175.3 at 30-μm, 180.5–221.0 at 50-μm, 200.4–232.6 at 70-μm, 220.5–238.0 at 100-μm and 242.1–252.5 at 200-μm depth. The effect became insignificant at 200 μm as intact enamel was reached. The VHN for (PB) group were statistically significantly higher than (B) group at 30 and 50 μm. However, the difference between these two bioglass groups (PB and B) was insignificant at 70–200 μm. There was also no significant difference between Groups (B, F and P) at all depths except at 70 μm. Further, there was no significant difference between Groups (F and P) in VHN at all depths.

### DISCUSSION

Dental caries is still routinely being treated by drilling out the decay and inserting filling materials such as resin composite and glass ionomer cement (GIC). While the latter can inhibit further demineralization and promote
remineralization of the surrounding tooth tissues\(^{29}\), it is better if we can remineralize caries in the first place using, for example, bioglass.

Our results were in accord with those reported by other investigators. For example, Milly \textit{et al.}\(^{29}\) showed that surface treatment with bioactive glass and polyacrylic acid enhanced the remineralization of artificial enamel white spot lesions, as demonstrated by the improved mechanical properties, higher phosphate content and morphological changes within the lesions. Also, by mixing bioglass with phosphoric acid and applying the resulting paste to eroded enamel surfaces, Bakry \textit{et al.}\(^{5}\) were able to improve the micro-mechanical properties of the eroded enamel within a short time.

During this study, low-pressure plasma was the only available type. However, atmospheric-pressure plasma jet exhibits many characteristics of a low-pressure glow discharge. In the jet, the gas temperature ranges from 25–200°C, charged-particle densities are \(10^{11}–10^{12} \text{ cm}^{-3}\), and reactive species are present in high concentrations. Although ions and atoms concentrations are lower in a low-pressure plasma, the impingement rate of these species on a substrate may be the same, since the flux to the surface increases with decreasing pressure. It appears that the atmospheric-pressure plasma jet exhibits the greatest similarity to a low-pressure glow discharge. Since the jet source may be scaled to treat large areas non restricted to vacuum, it could be used in a number of materials and chair-side applications\(^{29}\).

It is possible that the acidic paste helped open up diffusion channels within the enamel, increased the release of Ca and P ions from the bioglass, and facilitated their diffusion into deeper enamel layers to enhance remineralization\(^6\). Phosphoric acid was chosen in this study to form a paste with bioglass, because it is commonly used in dental clinics as an etchant.

It is worth mentioning that bioglass is a highly biocompatible material when tested on pulp cells\(^{31}\) and it can safely be used for longer periods, should full mineral recovery be attempted. Contrariwise, NaF varnish is not recommended for prolonged use on teeth, as it might diffuse to the gingival or periodontal tissues and cause adverse effects. In fact, it was found to be toxic to the oral mucosal fibroblasts \textit{in vitro} by its inhibition of mitochondrial function, protein synthesis, and depletion of cellular ATP\(^{32}\).

Placing and curing a layer of adhesive over the bioglass paste allowed it to stay in contact with the enamel substrate for 24 h without being washed off. It has been shown that the bioactive reaction takes at least 2 h to complete\(^{22,23}\). The application of a hydrophilic HEMA; self-etching adhesion promoter coat, did not seem to limit the diffusion of water and ions from the remineralizing solution through the resin coat layer to the bioglass and enamel interface. As self-etching adhesives are characterized by their relatively high water uptake\(^{33}\).

From the EDS results (Table 4), it is noticed that the atomic percentages of both Ca and P are slightly higher in the plasma/bioglass group than the bioglass group, meanwhile, Na and Si atomic percentages are slightly lower. This might be attributed to the formation of Ca, P rich surface layer within the silica gel structure, when the surface is stabilized and Ca/P ratio approaches unity, the leaching is retarded. It can be seen that the Si content gradually decreases as the content of Ca and P increase. It is suggested that the initially formed calcium phosphate is amorphous and that the crystallization is initiated within the silica gel\(^{34}\).

Ten Bosch and Angmar-Månsson\(^{35}\) in a detailed review of quantitative methods to determine mineral changes, recommended the use of radiographic methods to quantify mineral loss in whole teeth due to the ability of X-rays to travel through matter without destroying the specimens. \(\mu\)CT does not require the preparation of thin sections for scanning and it enables three-dimensional longitudinal experiments to be conducted, thereby overcoming the disadvantages of microradiography\(^{36}\). The \(\mu\)CT data collected in this study allowed quantitative comparison between the different treatment groups through the percentage of mineral volume recovery. The mineral density profiles obtained from the \(\mu\)CT images for the bioglass-treated groups corresponded well with the cross-sectional SEM images, which showed the top 30 μm of the remineralized layer to be homogenous and dense, with the porosity almost totally obliterated.

Micro-hardness gives information on the mechanical properties (stiffness and strength) of the tooth tissues that are not provided by transverse microradiography (TMR) or \(\mu\)CT. Therefore, it is advisable to combine different methods to analyze enamel remineralization\(^{37}\). The cross-sectional depths at which indentation was performed in this study were based on several pilot studies. Making indentation at depths less than 30 μm usually resulted in cracking the specimen.

The micro-hardness indentation results showed that all the remineralization treatments significantly improved the mechanical properties of the demineralized region. Further, it was found that, close to the surface (30-μm deep), the VHN of the bioglass groups was higher than that of the F group after 24 h of remineralization, although only the bioglass group with prior plasma treatment (PB) had significantly higher VHN values. The micro-hardness results were in accord with Bakry \textit{et al.}\(^{5}\), who also found that eroded enamel specimens treated with bioglass were significantly higher in VHN than those treated with 24 h fluoride at 30- and 40-μm depths.

The newly formed top ~30 μm mineral deposits of both bioglass and plasma/bioglass groups showed the denser most-homogenous mineral deposits, while the Ca/P atomic percentage in the plasma/bioglass group is the closest to normal enamel.

The bioglass paste was able to form a Ca/P rich layer on eroded enamel surfaces within 24 h. XRD analysis of the interaction layer showed the formation of brushite crystals (CaHPO\(_4\)·2H\(_2\)O) on the enamel surface, due to the low pH of 45S5 bioglass-phosphoric-acid gel. This layer showed resistance to abrasion when tested using 6,000 cycles of tooth-brush abrasion under a load of 250...
The profit of using non-thermal or cold plasmas for surface modification is that the surface properties of materials can be selectively boosted while their bulk properties stay unaffected\textsuperscript{38}. Applying plasma alone to the demineralized enamel did not result in a successful remineralization of the tissue. Instead, it only resulted in the deposition of a porous layer of calcium phosphate on the enamel surface. This was probably because of the lack of bioglass remineralizing effect and, the absence of an acid, thus the enamel channels were not opened up for diffusion of the ions into the deeper regions.

Relevant to the current study, cold plasmas could effectively increase the surface hydrophilicity of substrates. It was demonstrated that the contact angles of water on various substrates decreased considerably after 30 s of cold plasma treatment to <5\degree, transforming them to super hydrophilic surfaces. This was in agreement with our pilot study.

Some other surface treatments can also modify enamel hydrophilicity, as well as plasma. For example, it was proven that Er:YAG laser and acid-etch treatment to enamel surfaces can produce high free surface energy values that appeared to be polar, corresponding to hydrophilic interactions\textsuperscript{39}. Er:YAG laser treatments have shown significant protection of enamel demineralization, and carries prevention may be achieved, with a smaller lesion depth observed\textsuperscript{40}. Another study concluded that, CO\textsubscript{2} laser irradiation of dental enamel significantly decreased erosive mineral loss and hardened previously softened enamel in vitro\textsuperscript{41}. It was also found that combining fluoride and argon laser treatment of primary tooth enamel can produce a protective surface barrier against cariogenic attack\textsuperscript{42}. An electron-microscopic observation of laser-irradiated demineralized enamel identified a 100 nm crystalline distinct layer\textsuperscript{43}.

Cold plasmas could effectively increase the surface hydrophilicity of substrates. At the same time, the morphology of the surfaces was not changed, as indicated by SEM\textsuperscript{40}. The hypothesis of this study was that treating demineralized enamel with cold plasma before bioglass application could increase the remineralization effect. It has been shown to be statistically significant in both µCT and micro-hardness measurements. The plasma/bioglass group showed a trend of higher mineral density and better mechanical properties of the remineralized regions, when compared to bioglass group, without prior plasma treatment.

This study concludes that bioglass can remineralize enamel with mineral loss of ~150 μm depth in vitro, and that combining it with surface pretreatment using a cold plasma can potentially increase its effectiveness.

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CONFLICT OF INTEREST

The author declares no conflict of interests.

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