Casein phosphopeptide-amorphous calcium phosphate modified glass ionomer cement attenuates demineralization and modulates biofilm composition in dental caries

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The study investigated the effects of the modified conventional glass ionomer cement (GIC) incorporated with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on biofilm composition of dental caries. Shear bond strength, durability tests, adhesive remnant index (ARI) and scanning electron microscope were used to measure the physical properties. Microhardness and ions release were determined to evaluate anti-demineralization effects; growth of the biofilm and its composition were assessed using MTT assay and Q-PCR assay. All experimental groups exhibited a significant stimulation of ions release, and reduced attenuation of microhardness. Nearly 39% reduction in the bacterial biofilm was observed with 5% CPP-ACP group. The regulation ability is mainly manifested in the inhibition of S. mutans and promotion of S. gordonii. The modified GICs by exhibiting anti-demineralization effects potentially lead to a reduction in the cariogenicity of plaque and can serve as a putative promising remineralization system with both enhanced antimicrobial and remineralization properties.

Keywords: GIC modification, CPP-ACP, Anti-demineralization, Biofilm regulation, Three-species biofilm

INTRODUCTION

Dental caries as a sequel to orthodontic treatment is the most prevalent iatrogenic complication. The increased risk of developing caries is attributed to the prolonged accumulation of a plaque around the fixed appliance (including brackets, archwires, and ligatures) that can be aggravated by inadequate oral hygiene1). White spot lesions (WSLs) develop rapidly around the orthodontic bracket as an early sign of enamel demineralization and can appear on the 4th week after initiating the orthodontic treatment2). The prevalence of these lesions is alarmingly high and reportedly to range from 23–96%3-5). Moreover, without effective control and treatment strategies, this early demineralization may rapidly progress to dental caries. Therefore, it becomes imperative to urgently embark control measures for prevention and reversal of the formation of the WSLs6), particularly, in more susceptible patients undergoing orthodontic treatment.

Development of caries is a multifactorial process characterized by dynamic alterations in the resident microflora of the dental plaque, which can cause an imbalance between the mineral phase of the tooth and the microbial ecology of plaque through the production of acids by the microflora leading to the proliferation of acid-tolerating and acidogenic bacteria and eventual pathogenicity. Numerous studies have been carried out by incorporating antibacterial agents into orthodontic bonding systems; however, showed inconsistent clinical outcomes7,8). Accumulating evidence has suggested that fluoride induces remineralization and suppresses demineralization of dental hard tissue. Besides, a remineralization system should comprise of fluoride, bioavailable calcium and phosphate for effective remineralization. Thus, glass ionomer cement (GIC) as mineral-based materials have been increasingly used as a fluoride-releasing material in dental clinics. It has the capability to create a physicochemical bond with tooth structure and to release fluoride. Nevertheless, as the tooth re-mineralization and the oral fluorides content are limited by both Ca2+ and F− in saliva9), thus low doses of fluoride release may limit the anti-demineralization efficacy of GIC to remineralize early lesions. Therefore, an ideal remineralization system with both enhanced antimicrobial and remineralization properties are highly desirable.

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), a natural protein extracted from milk casein, is a well-recognized re-mineralizing agent. A previous study demonstrated that the modification of glass ionomer cement by incorporating CPP-ACP into the bonding and finishing material could significantly reduce caries activity10). In the present study, the effect of the modified conventional glass ionomer cement on biofilm composition of dental caries was evaluated. The results showed that the modified glass ionomer cement attenuates demineralization and modulates biofilm composition in dental caries.

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agent\textsuperscript{10,11}). CPP-ACP functions by providing bioavailable calcium and phosphate ions, thus allowing suppression of demineralization and induction of the remineralization by these ions at early carious lesions. Furthermore, studies have indicated that CPP-ACP exhibited a controlling potential on plaque and could reduce the growth and adherence of cariogenic bacteria\textsuperscript{22}. Thus, CPP-ACP has been most extensively used as a non-invasive treatment modality against incipient caries lesions\textsuperscript{13-15}. During the past few years, numerous \textit{in vitro}, \textit{in situ}, and \textit{in vivo} studies have been conducted on CPP-ACP modified material to investigate its ion releasing and remineralization capabilities\textsuperscript{12,16-19}, and its antibacterial effect on \textit{Streptococcus mutans}\textsuperscript{20}. However, these studies only focused on investigating its ability to inhibit the \textit{S. mutans} biofilm. As dental plaques around the fixed appliance are complex and difficult to completely eradicate or inactivate, only investigating the inhibitory effects of CPP-ACP modified GIC on \textit{S. mutans} biofilm may be insufficient\textsuperscript{21}. Thus, increased attention should be directed towards regulating the ecology of dental plaque\textsuperscript{22}.

Therefore, the present study was initiated to characterize GIC modified with different concentrations of CPP-ACP. The cytotoxicity, mechanical and physical strength, ability to prevent demineralization, and effects on regulation of microorganism in the three-species biofilm were analyzed.

MATERIALS AND METHODS

\textit{Synthesis of the modified GIC with CPP-ACP}

CPP-ACP was produced using Zraikat’s method as described previously. Briefly, 5 g Casein phosphopeptides (Solorbio, China) was dissolved in 25 mL 0.72 mol/L K\textsubscript{3}PO\textsubscript{4} buffer (pH=7.0), 18.0 mL 1.0 mol/L CaCl\textsubscript{2} solution was added dropwise, 1.0 mol/L KOH was used to maintain the pH value at 7.0 (FiveEasy Plus, Mettler Toledo, Switzerland). After adding CaCl\textsubscript{2}, the solution was adjusted with deionized distilled water (DDW) to 50 mL and stirred at 37°C for 12 h. Three different concentrations of CPP-ACP (1, 3, and 5%, w/w) were prepared by mixing into GIC (GC Fuji ORTHO LC, GC, Tokyo, Japan)\textsuperscript{16,19}. GIC disks were prepared using a hollow tube of diameter 0.5 cm with different CPP-ACP modified GICs representing three different experimental groups including 1% group, 3% group and 5% group and the conventional GIC group.

\textit{Teeth collection and disposition}

The study protocol was approved by the West China Hospital of Stomatology Institutional Review Board, Sichuan University (IRB Reference Number WCHSIRB-D-2017-121). Informed written consent was obtained from each patient. Surgically extracted premolars for orthodontic purpose without caries or WSLs were collected and soaked in 1% thymol solution to inhibit microbial growth and stored at 4°C for later use. Teeth specimens were embedded in acrylic resin (A90800, A91000, Goral, South Korea) with the exposed enamel surface.

\textit{Cell culture}

Human oral keratinocyte cells (HOKCs) was obtained from JENNIO Biological Technology (Guangzhou, China). HOK cells were routinely cultured in Dulbecco’s Modified Eagle Medium (DMEM; HYclone) supplemented with 2% fetal bovine serum (FBS; Gibco, USA), 1% (v/v) antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO\textsubscript{2}. All experiments were carried out in serum-free DMEM.

\textit{Cytotoxicity test}

The GIC disks from the conventional group and three experimental groups were sterilized and immersed in 15 mL serum-free DMEM at 37°C for 24 h, respectively. After 24 h of incubation, the extract-containing DMEM medium was diluted at 2\textsuperscript{10}-fold and cells were treated with four different concentrations of extract-containing DMEM medium. After 24 h, CCK8 assay was performed according to the manufacturer’s instructions (CCK8, Dojindo, Japan) at 37°C for 2 h. The absorbance of suspension was measured at 450 nm with the spectrophotometer (Multiskan GO, Thermo Scientific, USA). For negative cell control, HOK cells were incubated in serum-free DMEM with no GIC extract. For background control, there were no cells in the wells and DMEM with 10% CCK-8 solution were used for measurement.

\textit{Shear bond strength}

The collected teeth were randomly divided into the conventional group and three experimental groups as mentioned earlier, of 6 each. Thirty-five percent of phosphoric acid was used to etch each specimen for the 60 s. Different groups of GICs were used to stick orthodontic brackets (the adhesive surface area is 8 mm\textsuperscript{2}) to the teeth, and then photo-cured with the curing light for 15 s per side. The specimens were subjected to the shear bond strength test with a computer-controlled Universal testing machine (5565, Instron, USA), at a cross-head speed of 1.0 mm/min on the upper part of the bracket until the bond failed. The debonding force that applied before the bracket falls off was referred to as the maximum shear bond force and was then recorded. The failure mode was assessed by examination with a stereomicroscope (SZ61, Olympus, Tokyo, Japan), and the amount of bonding resin remaining on the tooth crown surface was scored using the modified adhesive remnant index (ARI)\textsuperscript{26}. 1, All composite remained on tooth; 2, More than 90% of the composite remained on tooth; 3, More than 10% but less than 90% of the composite remained on tooth. 4, Less than 10% of the composite remained on tooth; 5, No composite remained on tooth.

\textit{Durability of shear bond strength}

A common method to perform the durability test is using artificial saliva at 37°C, which can imitate the
characteristics of the oral cavity. After photo-curing, 24 teeth were soaked in the artificial saliva prepared according to Hahnel’s method\(^2\) (4.1 mmol/L KH\(_2\)PO\(_4\), 4.0 mmol/L NaHPO\(_4\), 24.8 mmol/L KHCO\(_3\), 16.5 mmol/L NaCl, 0.25 mM MgCl\(_2\), 4.1 mmol/L citric acid and 2.5 mmol/L CaCl\(_2\), pH=6.7) for 30 days\(^2\). Before using, the artificial saliva were sterilized with a 0.22 μm filter, and were changed every week during the aging period. Subsequently, they were tested for the shear bone strength to determine the durability of the modified GICs and the conventional GIC.

**Morphological observation**

To visualize the microscopic morphology, GIC disks were sputter-coated with gold and observed with the Scanning electron microscopy (SEM; Inspect F, FEI, Holland) at magnifications of 1,000× and 5,000× under 20 kV, with a working distance ranging from 10.4 to 11.4 mm, and in the secondary electron (SE) mode to obtain the SEM images. This examination was essential to analyze rough surfaces, which may be beneficial to the bacterial adhesion and accumulation\(^9\).

**Ion release measurement**

Different GIC disks (manufactured according to 2.1) were placed into a 24-well plate (Costar, Corning, USA) and 2.0 mL of deionized water was added to each well and kept at 37°C. The solution in the wells was replaced every day. The release of calcium ion and phosphate anion from modified GIC and conventional GIC group was measured on day 1, 3, 5, 7, 14, 21 and 30 by Standard kit assay to react with the extracts, and the absorbance of the extract was measured with a spectrophotometer (Multiskan GO, Thermo Scientific). The content of free fluorine was measured with Ion Chromatograph (ICS-90, Dionex, USA).

**Remineralization-de-mineralization cycle test**

Human teeth were longitudinally cut into two parts including the buccal sides and the lingual sides, and both sides were embedded in resin with the crown exposed (Fig. 1). The micro-hardness of all the lingual sides were tested (MMT-X7A, Matsuzawa, Japan). The 12 buccal sides were randomly divided into 4 groups as mentioned above. Then, crown sections of the 4 groups were embedded with different modified GICs and were polished with the same method as described previously to ensure a 2×2 mm enamel exposure.

After the treatment, demineralization, and remineralization procedures were performed continually every day. The specimens were immersed in 5.0 mL of demineralization solution (2.2 mmol/L KH\(_2\)PO\(_4\), 2.2 mmol/L Ca(NO\(_3\))\(_2\), 50.0 mmol/L CH\(_3\)COOH, 1.0 mmol/L NaN\(_3\), pH=4.5) at 37°C for 6 h per day. Later, specimens were rinsed three times with distilled water. Subsequently, the specimens were immersed in 5.0 mL of remineralized liquid (1.5 mmol/L CaCl\(_2\), 0.9 mmol/L KH\(_2\)PO\(_4\), 130.0 mmol/L KCl, 1.0 mmol/L NaN\(_3\), 20.0 mmol/L HEPES, pH=7.0) at 37°C for 17 h. Measurements were performed continuously for 14 days. A Vickers microhardness indenter was used to evaluate the baseline microhardness under 100 gm load applied for 15 s.

**Three species biofilm culture**

*Streptococcus mutans* (S. mutans; UA 159), *Streptococcus sanguinis* (S. sanguinis; ATCC 10556) and *Streptococcus gordonii* (S. gordonii; ATCC 10558) strains were procured. *S. mutans*, *S. sanguinis*, and *S. gordonii* were routinely cultured for 12 h. For three-strain biofilm formation, cultured *S. mutans*, *S. sanguinis*, and *S. gordonii* in the ratio of 1:1:1 were vortex blended to acquire an inoculum and cultured with 4% Brain Heart Infusion broth (BHI; Oxoid, Thermo Fisher Scientific) and 1% sucrose. While the medium used for biofilm

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**Fig. 1** Remineralization-de-mineralization cycle test procedure.
Premolars were longitudinally cut into buccal sides and the lingual sides, and were embedded in resin with the crown exposed. The micro-hardness of all the lingual sides were tested. Buccal sections were embedded with different modified GICs with a 2×2 mm enamel exposure. After the 14-day remineralization-de-mineralization cycle, micro-hardness of all the buccal sides were tested.
development was replaced every 12 h. After 48 h of biofilm formation, all the specimen disks with the three-species biofilm were washed three times with phosphate buffer saline (PBS) to remove the planktonic bacterial cells, then they were transferred to new 24-well plates for further experiments.

**MTT assay**
The 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) assay was used to evaluate the metabolic activity of the three-species biofilm. After rinsing specimen disks for 48 h, each disk was incubated in 1 mL of 0.5 mg/mL MTT solution (in PBS) at 37°C for 3 h in dark. Later, all the disks were transferred to new 24-well plates, and 1 mL of dimethyl sulfoxide (DMSO; MP Biomedicals, USA) was added for solubilizing the formazan crystals. After incubation of 30 min, 100 μL of the DMSO solution from each well was transferred to a new 96-well plate, and the absorbance of the solution was measured with the spectrophotometer at 560 nm. Each group has six replicates.

**Quantitative real-time polymerase chain reaction (Q-PCR)**
The biofilms from the four groups were collected, and total DNA was isolated using the TIANamp Bacteria DNA kit (TIANGEN, Beijing, China). The enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM sodium EDTA, and 1.2% Triton X-100) containing 25 mg/mL lysozyme was used to lysing bacteria and releasing bacterial contents at 37°C for 1.5 h.

The quantitative detection of the DNA from *S. mutans*, *S. gordonii*, and *S. sanguinis* was performed using TaqMan real-time polymerase chain reaction (Q-PCR), the results denoted the gene expression of *S. mutans*, *S. gordonii*, and *S. sanguinis* in the biofilms. Q-PCR reaction in each well contained 1.5 μL of template, 10 μL of TaqMan Universal PCR Premix Ex Taq, 250 nmol/L of sense and antisense primers, and 250 nmol/L of TaqMan probe. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, then 40 cycles of denaturation at 95°C for 10 s, and annealing at 56°C for 30 s, fluorescence was detected after each cycle. The quantification of the three strains was based on the standard curves generated using standard strains.

**Statistical analysis**
All data were expressed as mean±S.D. of three independent experiments, each performed in triplicate. Data obtained from the cytotoxicity test, shear bond strength, durability, remineralization-decalcification cycle test, ion release, and MTT test were analyzed using one-way ANOVA and SNK q-test was performed for multiple comparisons. Statistical analyses were performed using IBM SPSS Version 20.0 software (IBM, Armonk, NY, USA).

## RESULTS

**Analysis of mechanical and physical performance**
As illustrated in Fig. 2, the shear bond strengths of group A to D were 9.80±2.43, 9.31±1.62, 5.23±0.83 and 4.04±1.61 MPa, respectively. The conventional GIC exhibited the highest strength, and group B exhibited no statistically difference from group A (p>0.05). However, significant differences were observed in group C and D compared with conventional GIC (p<0.05). The shear bond strengths of the specimens after durability treatment for group A to D were 7.75±2.04, 6.85±1.80, 4.03±1.20, and 2.77±1.64 MPa, respectively. Group C and D still remained at a significantly lower level of the strength compared with group A and B (p<0.05). It is worth to note that within each group, no significant difference in the shear bond stress was recorded before and after the durability test. ARI result (Table 1) shown failure modes of each groups before and after durability treatment. All groups were associated with mixed failure modes, while group D after durability treatment displayed tendency to adhesive failure modes.

**Analysis of ion release**
Daily release of fluoride, calcium and inorganic phosphate from the modified GIC in deionized water is shown in Fig. 3. As Fig. 3a suggested, mean daily release of fluoride of the four groups were significantly different among each group on the 1st day (p<0.05). Higher fluoride release was also found on day 3 and 5 with modified GIC compared with the control group (p<0.05). Moreover, higher changes of calcium release occurred when the GIC samples were modified with CPP-ACP compared with the conventional material (p<0.05), which shown nearly no released calcium detectable (Fig. 3b). Similar result was found with inorganic phosphate (Fig. 3c), which revealed significant higher release of inorganic phosphate in all modified GIC groups in the first 7 days (p<0.05) and an even higher release with the 5% CPP-ACP group in the first 30 days (p<0.05). As obviously indicated in the figure, it was evident that the
Table 1  ARI score result

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<th>Modified ARI scores</th>
<th>Before durability treatment</th>
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ARI, Adhesive remnant index score; Group A, conventional GIC group; Group B, GIC modified with 1% CPP-ACP; Group C, GIC modified with 3% CPP-ACP; Group D, GIC modified with 5% CPP-ACP; SD, standard deviation

Fig. 3  Daily release of fluoride, calcium and inorganic phosphate.
(a) Mean daily release of fluoride of the four groups were significantly different among each group on the 1st day ($p<0.05$). Significant higher fluoride release was also found on day 3 and 5 with modified GIC compared with the control group ($p<0.05$). (b) Significantly higher calcium release occurred when the GIC samples were modified with CPP-ACP compared with the conventional material ($p<0.05$). (c) Significant higher release of inorganic phosphate was found in all modified GIC groups in the first 7 days ($p<0.05$) and an even higher release with the 5% CPP-ACP group in the first 30 days ($p<0.05$).

release of these three ions was directly proportional to the proportion of CPP-ACP of the modified GIC.

Microhardness of the enamel following the remineralization-demineralization cycle
Figure 4a represented the microhardness of enamel of each group before and after the remineralization-demineralization cycle. As shown in Fig. 4b, the microhardness reduction of the enamel of each group were 165.25±7.84 VHN (conventional GIC), 92.51±11.97 VHN (1% group), 56.56±12.99 VHN (3% group), and 30.13±52.78 VHN (5% group), respectively. There were significant differences between conventional GIC and modified GICs ($p<0.05$). The microhardness reduction of enamel after the remineralization-demineralization cycle significantly decreased with the increased proportion of CPP-ACP added to the GIC, indicating that CPP-ACP considerably prevented the demineralization process of the enamel.

Micromorphological observation of the modified GICs disks
As depicted in Fig. 5, the microstructures of conventional GIC group and 1% group examined by SEM at 5,000×
Fig. 4 (a) The microhardness of enamel of each group before and after the remineralization-demineralization cycle. (b) The microhardness reduction of enamel after the remineralization-demineralization cycle (p<0.05). There were significant differences between conventional GIC and modified GICs (p<0.05). The microhardness reduction of enamel after the remineralization-demineralization cycle significantly decreased with the increased proportion of CPP-ACP added to the GIC, indicating that CPP-ACP considerably prevented the demineralization process of the enamel.

Fig. 5 The microstructures of different modified GIC disks at magnification of 1,000× and 5,000× observed by SEM. The microstructures of conventional GIC group and 1% group examined by SEM at 5,000× magnification exhibited similar structure, although a more roughened surface was observed in 1% group at 1,000× magnification with larger particles. Significant differences were observed for 3 and 5% groups compared with conventional GIC group and 1% group. Both the two groups, 3 and 5% groups exhibited visible pores on the surface, while 5% group exhibited particles nearly detached from the material. These results indicated that a more porous and roughened surface will be produced with the incorporation of more CPP-ACP to the conventional GIC.

Regulation of microbial composition
MTT assay was used to investigate the effects of CPP-ACP on the growth of biofilm, as shown in Fig. 6a, the absorbance values (A540/cm²) of the experimental groups,
Fig. 6 (a) effects of CPP-ACP on the growth of three-species biofilms investigated by MTT assay was used to investigate the effects of CPP-ACP on the growth of biofilm, as shown in (a). Noticeably, the absorbance of 5% group exhibited an average decrease of 39% in biofilm adhesive to the specimen disks compared to the conventional GIC group. (b) the variation in the ratio of three bacteria in the multi-species biofilms.

Fig. 7 DMEM was selected as blank control to evaluate the cell toxicity in the Figure, no significant toxicity was observed with different concentrations of CPP-ACP (1%, 3%, or 5% group) at the dilution rate on $2^0$ and $2^{10}$ as compared to the conventional GIC group or the DMEM group after 24 h incubation ($p>0.05$). This result suggested that modified GICs at indicated proportions of CPP-ACP did not exhibit any significant cytotoxicity and they were safe to the HOKCs.

DISCUSSION

Dental caries is initiated by early demineralization of tooth hard tissue by organic acids. However, fluoride ions, in the presence of calcium and phosphate ions, can possibly reverse this demineralization of early caries lesions through remineralization. GIC, a fluoride-releasing material that has been increasingly used in dental clinics, has been recognized to exhibit a promising remineralization and biofilm regulation ability when modified with other materials such as CPP-ACP. Therefore, a novel remineralization system of modified GIC using three different concentrations of CPP-ACP (1, 3, and 5% group) on the three-species biofilm has been developed in the present study and its physical performance, remineralization efficacy, biocompatibility, and prebiotic effect were investigated.

Physical properties should be the first issue to be taken into consideration while investigating a novel bracket bonding material. Though result revealed that mixture of CPP-ACP reduced the shear bond strength of GIC and the added concentration exhibited a positive

3.16±0.12, 3.04±0.12, and 2.10±0.39, respectively, showed a significant decrease compared with the conventional GIC group (3.43±0.13; $p<0.05$). Noticeably, the absorbance of 5% group exhibited an average decrease of 39% in biofilm adhesive to the specimen disks compared to the conventional GIC group. Figure 6b presented the variation in the ratio of three bacteria in the multi-species biofilms. Significant decrease in proportions of $S$. mutans biofilms in the experimental groups was noticed compared with the conventional GIC group, with a ratio of 21.0, 2.0, 2.0, and 0.0% respectively. Moreover, the proportions of $S$. gordonii were 14.0% (Conventional GIC group), 7.0% (1% group), 37.0% (3% group), and 38.0% (5% group) for each group. Taken together, these findings suggested that with a higher proportion of CPP-ACP (3 or 5%) incorporated to the GICs, the $S$. gordonii of the biofilm exhibited a profound growth.

Cell cytotoxicity

DMEM was selected as blank control to evaluate the cell toxicity. As presented in Fig. 7, no significant toxicity was observed with different concentrations of CPP-ACP (1, 3, or 5% group) at the dilution rate on $2^0$ and $2^{10}$ as compared to the conventional GIC group or the DMEM group after 24 h incubation ($p>0.05$). This result suggested that modified GICs at indicated proportions of CPP-ACP did not exhibit any significant cytotoxicity and they were safe to the HOKCs.
correlation with a reduction in the degree of mechanical properties, which were in accordance with previous study\(^{10}\), all groups before and after the durability treatment shown acceptable strength considered with clinical bracket bonding requirement\(^{27,28}\). Laboratory tests also suggested that an adhesion of 5.8–7.8 MPa is enough to ensure proper fixture of a fixed appliance\(^{29}\). Moreover, the ARI scores shown even though group D after durability treatment displayed tendency to adhesive failure modes, which reflected the relatively low bonding strength\(^{30}\), all groups were associated with mixed failure modes.

Possible reason for the decrease of shear bond strength with experimental group may due to the change of chemical structure of the material. As GIC consists of calcium aluminosilicate glass particles mixed with polyacrylic acid, therefore, these phenomena may be attributed to the acid-base reaction with calcium aluminosilicate in GIC. Briefly, at the initial reaction stage calcium poly salts are formed, followed by the formation of aluminum poly salts, which then strongly crosslinks with the polyanion chain. However, at a concentration of CPP-ACP in modified GIC, the sites of formation of aluminum poly salt may be occupied by the calcium poly salts, consequently preventing the maturation of the crosslinking to the GIC\(^{10}\), which leads to decrease of physical properties of the adhesives. Despite shear bond strength, other relevant physical parameters of this novel material have been investigated following the International Organization for Standardization (ISO) Standard 9917:2003 for dental water-based cements by previous study\(^ {31} \). Hanan et al. revealed in their study that though the incorporation of up to 5% CPP–ACP into GIC decreased the cements’ strength and prolonged setting time, and compressive and diametral tensile strength significantly decreased when 3 or 5% CPP–ACP was incorporated, all values remained within ISO limits\(^{10}\), which pointed out CPP-ACP modified GIC to be potential alternative adhesive agent for bracket bonding.

As the mineral content in enamel is associated with hardness, GIC incorporated with CPP-ACP can also considerably affect the mechanical strength of enamel, leading to an increase in microhardness. This finding is consistent with the results obtained from the Vickers microhardness test in which we measured the enamels after they had undergone a remineralization-deminerelization cycle, which revealed that the microhardness of enamel was noticeably reduced and the decrease in the rate of hardness was negatively correlated with the concentration of CPP-ACP in GIC.

With evidence that the release of fluoride, calcium and inorganic phosphate was directly proportional to the proportion of CPP-ACP of the modified GIC, the results of ions release experiment were consistent with the result of the microhardness test. These findings proved that adding CPP-ACP into GIC can not only enhance the existed capability of fluoride and inorganic phosphate release, but also empower GIC with calcium release capability\(^{10}\). As for fluoride, it has been indicated that an initial high level of fluoride release will reduce the viability of bacteria thus inhibit dental caries by inducing remineralization of enamel or dentin\(^{32}\), which suggested a significant higher release of fluoride in the first 5 days of CPP-ACP modified GIC provides potential benefit for caries prevention. It is also notable that the release of fluoride and calcium on the 1st day was obviously much higher than other days. It might due to the initial fluoride burst effect and the superficial rinsing effect\(^{33}\), and during the subsequent days release is attributed to its ability to diffuse through cement pores and fractures\(^{34}\).

Although the development of carious lesions has multifactorial etiology, the role of microorganisms has been well documented. Also, S. mutans, an acid-producing, aciduric, acidophilic microorganism, is considered as the major cariogenic bacteria\(^{35}\). Most of the previous studies employed S. mutans to test the antibacterial efficacy of CPP-ACP. Consistently, in the present study, CPP-ACP exhibited efficient antibacterial potential in situ\(^ {12,36,37} \). However, as it is well recognized that the poly-microbial biofilm should be included to simulate the oral cavity instead of using just single species of bacteria\(^{38,39}\), thus the three-species biofilm comprising S. mutans, S. sanguinis, and S. gordonii, was used in our study to provide a more accurate clinical representation of the impact that CPP-ACP modified GIC exerts on the biofilm\(^{22}\).

A significant reduction of nearly 39% in biofilm metabolism in the 5% CPP-ACP group was observed compared with the conventional GIC, suggesting that GIC with CPP-ACP has the capability of effectively inhibiting the metabolic activity of biofilms. As a higher surface roughness may contribute to increased biofilm adhesion, an even roughness of all specimens can prevent bias. The result of the Q-PCR assay indicated that the modified GICs exhibited a prebiotic effect that favors commensal species in biofilms. Compared with the conventional GIC group, S. mutans was markedly inhibited among the three-species in the biofilm grown on the modified GICs disks, which is consistent with the previous findings that both the pure CPP-ACP and CPP-ACP modified GIC can significantly inhibit the adhesion and growth of S. mutans\(^ {12,40} \). However, considering the results of MTT and Q-PCR, our study for the first time has demonstrated that the proportion of S. gordonii exhibited an increased predisposition to CPP-ACP modified GICs and that CPP-ACP modified GICs also exhibit noticeable prebiotic effect\(^ {21} \). Moreover, a significant effect of CPP-ACP modified GICs on the proportion of S. mutans and S. gordonii indicated that the modified adhesive significantly impacts biofilm metabolism, thus CPP-ACP modified GICs substantially promotes the inhibition and reduction of development of early caries around orthodontic brackets. There are two mechanisms that can be attributed to the prebiotic effect of CPP-ACP modified GICs. Firstly, CPP-ACP exhibits anti-adhesion capabilities to the bacteria: the binding of the CPP-ACP nano-complexes to the surface macromolecules of S. mutans is mediated by calcium...
cross-linking with cell surface phosphate moieties through hydrophobic and hydrogen-bond mediated interactions. Furthermore, this binding can compete with free calcium for plaque calcium-binding sites, reducing the degree of calcium bridging between the adhering cells, which affects the bacterial adhesion and biofilm development. Alternatively, mechanism of buffering has also been hypothesized in which CPP-ACP may act as a reservoir of peptides, calcium, and phosphate ions which counterbalances any alteration in pH, this may influence the ecological equilibrium of the bacterial biofilm inside the plaque.

In conclusion, the modified GICs with CPP-ACP exhibit anti-demineralization effects. Furthermore, the modified material demonstrated to have a significant potential leading to attenuation of the plaque’s cariogenicity, thus, regulating the biofilm composition into a healthier system. However, considering the rough micromorphology of the proposed material surface, further improvement in mechanical and physical performances of the modified GICs is warranted prior to its application in clinical settings.

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CONFLICT OF INTEREST

The authors declare that the research has no conflict of interest.

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