Mechanical, antibacterial, biocompatible and microleakage evaluation of glass ionomer cement modified by nanohydroxyapatite/polyhexamethylene biguanide

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This study aims to look for the best concentration of nanohydroxyapatite (NHA) and polyhexamethylene biguanide (PHMB) incorporated into glass ionomer cement (GIC) in accordance with ISO:9917-1 and evaluate its mechanical, antibacterial, biocompatible and microleakages properties. NHA was incorporated into Fuji II GIC powder at 0–8.00 wt% concentration and specimens were prepared; the best concentration was sifted out according to ISO9917-1. Based on best NHA proportion, 0–0.80% PHMB was dispersed into powder and samples were respectively prepared. Mechanical properties include net setting time (ST), compressive strength (CS), microhardness (VNH), solubility and scanning electron microscopy (SEM) observation. Those met ISO standard were qualified to continue microleakage observation, antibacterial activity, and biocompatibility test. The results suggested that GIC/6%NHA/0.2% PHMB and GIC/6%NHA/0.4%PHMB showed great performances in mechanical, antibacterial, and microleakage improvements, and the cytotoxicity of modified GIC showed no statistical difference with pure GIC.

Keywords: Glass ionomer cement, PHMB, Nanohydroxyapatite, Modification

INTRODUCTION

Glass ionomer cement (GIC) has been widely utilized in tooth restoration due to its favorable aesthetics, bonding capability, fluorine release, and low stimulation to pulp\(^1,2\). However, failures have been reported after treatment for its mechanical and bacteriostatic insufficiencies\(^3\). Its vulnerability to dentition always leads to the fractures, failure of the bonding and microleakages\(^8\) between GIC and the tooth, where the cariogenic bacteria can easily enter. In addition, clinical surveys\(^4-6\) indicated that the fluoride released is inadequate for the inhibition from bacterial invasion, which usually results in secondary caries. These disadvantages have restricted further clinical application of GIC.

To overcome these drawbacks, scholars have tried to modify GIC with different materials. In antibacterial aspect, different newly-fabricated nano antibacterial compounds, including cross-linked quaternary ammonium polyethyleneimine nanoparticles\(^9\), chlorhexidine-encapsulated mesoporous silica nano particles\(^7\) and chitosan\(^8\) were incorporated. These additives enhanced the antibacterial activities of GIC but took a toll on GIC’s biocompatibility\(^6,9,10\). In addition, few experiments investigated the sustainability of agents release in a long term. Moreover, most of them seemed to neglect that microleakage is the direct causation of secondary caries. Therefore, a novel kind of agent with better performance is in demand.

In mechanical aspect, the conventional approach is to obtain a resin modified cement by adding resin monomer and initiator components. Though resin can significantly improve GIC’s mechanical properties, it goes with increased polymerization shrinkage, pulp stimulation and decreased fluoride release, and these aggravated the inherent advantages of GIC\(^11\). Recently, nano-metallics such as Ag, TiO\(_2\), ZrO\(_2\) and Cu were tried to optimize GIC. They showed conducive results in mechanical reinforcement\(^12-16\), but also triggered problems in cytotoxicity\(^13-16\), discoloration\(^12\), and poor interfacial bonding\(^14,15\). Simultaneously, most metallics failed the promotion of adhesion and the marginal adaption between GIC and teeth\(^17\). Likewise, some researchers tried to incorporate some other nanofillers such as forsterite, montmorillonite clay, bioactive glass, and niobium pentoxide, and then found that these materials only make slight mechanical enhancement but produce negative effects on fluoride release, radio-opacity, and biocompatibility\(^18-21\). Obviously, all these modifications above had inevitable drawbacks, restricting GIC’s clinical applications. Hence, a better scheme for optimization is in need.

Polyhexamethylene biguanide (PHMB) is a kind of efficient, safe, and broad-spectrum bactericidal disinfectant with no color, smell, volatilization, and corrosiveness\(^22\), which has been widely used in trauma treatment, ophthalmic disinfection, aquaculture, and many other fields. It can greatly eradicate bacterial by binding protonated groups to the anionic membrane of bacterial, leading to cytoplasmic leakage\(^22\). Compared to CHX and other halo-amines, phosphines, or quaternary ammonium (phosphine) based polymers, PHMB not only has superior antibacterial activity but also unprecedented biocompatibility\(^24,25\). Even with extensive use for the
past 40 years, PHMB-resistant mutants have not been reported26. All these features suggest it a potential choice for modification. Our previous study27 indicated that PHMB modified eugenol oxide (ZOE) root canal sealer had have its long-term antibacterial performance greatly improved and meanwhile setting time shortened. However, as organic polymers, PHMB will affect the integrity of the inorganic material when doped in, resulting in the slight decrease of mechanical properties. Hence, of significance is the selection of appropriate materials for the mechanical reinforcement.

Hydroxyapatite nanoparticle (NHA) is a promising bio-ceramic with superior osseointegration, biocompatibility and the promotion for enamel remineralization28, so it is widely utilized in dentistry. Numerous investigations indicated that NHA modified GIC could significantly improve the bonding strength with teeth29,30, reduce cytotoxicity31, and do not hinder the sustained release of fluoride31. Kheur et al.32 found that NHA modified GIC increased its bending strength by 166% and the bonding strength (BS) by 148%. Sharafeddin and Feizi33 also found that the penetration of NHA crystals into dentin and enamel can significantly improve the marginal adaption between teeth and GIC. These results suggested that NHA can either drastically boost the mechanical properties or reduce the microleakages between teeth and GICs, and this may be a new approach to effectively preventing secondary caries and improving the success rate of filling.

The objective of this study is to obtain a novel GIC modified by PHMB/NHA in accordance with the recommendation outlined in ISO:9917-134 and investigate its mechanical, antibacterial, bonding, and biocompatible properties.

**MATERIALS AND METHODS**

*Preparation of NHA modified and NHA/PHMB modified glass ionomer powder and specimen*

Restorative GIC (Fuji II, GC, Tokyo, Japan) was used in this study. Nano-sized hydroxyapatite (20 nm, 99%) (HuaLan Chemical, Shanghai, China) was homogeneously mixed into powder dryly at the concentration of 0, 2, 4, 6, 8, 10% (w/w) respectively by a ball mill (ChaoYue, Jiangxi, China) with 7 mm zirconia balls at 80 rpm for 24 h. The best concentration of NHA was sifted out and then PHMB (powder, 99%) (YiKa Chemical, Shanghai, China) was manually mixed with NHA/GIC powder in agate mortar in the proportion of 0, 0.2, 0.4, 0.6, 0.8% (w/w of powder). Specimens were prepared strictly with the powder/liquid ratio at 2.7 g/1 g as recommended by the manufacturer, and the compositions of specimens are clearly illustrated in Table 1.

**Mechanical properties**

1. **Net setting time**

Following the ISO:9917-134, GIC was filled in ring molds (10 mm internal diameter, 5 mm height) on a glass plate at 37±1°C and a relative humidity of 90%. An intender of weight 400±5 g with a needle tip of which the diameter is 1.0±1 mm was pressed vertically on to the surface of specimen for 5 s at the interval of 30 s each time, 90 s after preparation. Recorded until the press indentation was no more integrated. Estimate the setting time upon finishing the first test and repeated all steps above at the interval at 10 s thirty seconds before estimation. The time from mixture finished to the last record was regarded as net setting time (ST).

2. **Compression strength**

Cylindrical specimens following ISO:9917-134 (6 mm height, 4 mm diameter) were prepared using acrylic-based resin mold. The specimens were polished with 400#, 800#, 1200# abrasive paper and then stored for 24 h. After that, the compressive test was conducted on a universal test machine (R Controller, Testresources, Shakopee, MN, USA) with crosshead speed of 0.5 mm/min. The compression strength (CS) was calculated with the formula:

$$CS = \frac{4P}{\pi d^2}$$ (MPa)

P is the load at fracture (N), and d is the diameter of specimen (mm).

3. **Surface microhardness test**

Three cylindrical samples (8 mm diameter, 5 mm height) of each group were prepared, each sample was polished by 1200#, 1500#, 2000# abrasive paper and then stored for 24 h. A microhardness tester (HV-1000IS, Shanghai Jujin, China) was used with the load of 200 N for 10 s. The microhardness (VHN) was calculated following the

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**Table 1** The composition of the samples

| Samples                                                        | Composition                                                                 |
|----------------------------------------------------------------|-----------------------------------------------------------------------------|
| Conventional glass ionomer cement (GIC)                        | Powder: fluoroaluminosilicate glass. Liquid: polyacrylic acid, itaconic acid, tartaric acid, maleic acid, water. |
| Nanohydroxyapatite modified glass ionomer cement (GIC/NHA)      | Powder: fluoroaluminosilicate glass, nanohydroxyapatite. Liquid: polyacrylic acid, itaconic acid, tartaric acid, maleic acid, water. |
| Nanohydroxyapatite and polyhexamethylene biguanide modified glass ionomer cement (GIC/NHA/PHMB) | Powder: fluoroaluminosilicate glass, nanohydroxyapatite, polyhexamethylene biguanide. Liquid: polyacrylic acid, itaconic acid, tartaric acid, maleic acid, water. |
Characterization of specimens by SEM
The specimens were collected after compression strength test and then gold sputter coated by a sprayer (108auto, Ted Pella, Redding, CA, USA). Scanning electron microscopy (SEM; S4800, Hitachi, Tokyo, Japan) was used to explore the features of the fractured surfaces at 1,000× magnifications.

Microleakage observation
1. Cavity preparation
Sixty freshly extracted third molars with similar size were collected (Approved by the university ethic committee by the code of LZUKQ-2020-025). All teeth were thoroughly cleaned and then immersed in 1% chloramine-T (Sigma-Aldrich, St. Louis, MO, USA) solution for disinfection. After saved for 24 h at 37°C, teeth were taken out for cavity preparation. A standard rectangular class V cavity was prepared on the buccal surface near cervical margin of each tooth by a high-speed handpiece with fissure bur. The cavity was 2 mm in depth, 4 mm in mesiodistal width, 3 mm in occlusogingival height, and no bevel angles at cavosurface margin (acute 90 degrees walls). All the dimensions were measured and guaranteed by using marked burs and periodontal probes.

2. Group division and restoration
The prepared teeth were divided into 6 even groups randomly (n=10): group 1 was restored with GIC (Fuji II, GC), group 2 was restored with GIC modified by NHA of best concentration, group 3 was restored with GIC modified by NHA of best concentration and 0.2%PHMB, group 4 was restored with GIC modified by NHA of best concentration and 0.4%PHMB, group 5 was restored with GIC modified by NHA of best concentration and 0.6%PHMB, group 6 was restored with GIC modified by NHA of best concentration and 0.8%PHMB. The cement/liquid ratio was strictly controlled at 2.7 g/1.0 g as recommended in instructions. At last, the teeth were polished and then stored in artificial saliva at 37°C for 14 days.

3. Artificial aging and microleakage assessment
After storage, all teeth underwent a 500-times thermal cycle at 5°C and 55°C. All teeth were smeared with nail vanish (Mufan, Zhejiang, China) and model wax (Yuwei, Shanghai, China) 1 mm away from cavity margin, then immersed in methyrosanilnium chloride dye for 24 h at 37°C. Subsequently, the teeth were removed from dye and rinsed by deionized water, then a hard tissue slicer (DTQ-5, Weiyijinxian, Guangzhou, China) was used to obtain an even 1 mm thick buccolingual section from each tooth. Sections were categorized and photographed in advance under a stereomicroscope (CX23, Olympus, Tokyo, Japan) at 40×. Photos were randomly sent to three blinded examiners. Then, a common evaluation method33,35) for assessing microleakage was applied. Each examiner scored the microleakage of every section at the positions of mesial margin, distal margin, and axial wall in each photo by a criterion measuring dye's penetration depth. Subsequently, the scores of sections in one group from one examiner were summarized, and the summarized scores of each group from 3 examiners were calculated the mean value. Then, mean values of all 6 groups were analyzed statistically. The scoring criterion is as follow:

0=no dye penetration;
1=dye penetration between the restoration and the tooth up to one-third of the distance between the tooth surface and the axial wall;
2=dye penetration extending beyond one-third of the distance up to two-thirds of the distance between the tooth surface and the axial wall;
3=dye penetration extending up to two-thirds of the distance between the tooth surface and the axial wall;
4=dye penetration reaching the axial wall;
5=dye penetration reaching the entire axial wall.

Solubility
Four specimens (8 mm diameter, 5 mm height) of each group were prepared as method in net setting time test. Each was weighed (m₀) before and immersed in artificial saliva in 37°C, the artificial saliva was daily updated. After 1, 7, 60 days, the specimen was taken out, dried at 37°C for 2 h each, then weighed (mₙ) and the solubility was analyzed following the formula:

VHN=\frac{1854.4P}{d^2} (MPa)

Where the P is the load and d is the length of diagonals. The mean value of 5 points on each sample was collected for further analysis.

Antibacterial activity
1. Direct contact test
The cylindrical specimens (8 mm diameter, 5 mm height) and polyethylene films (8*8 mm, Liangjie, Shanghai, China) were sterilized under ultraviolet light via a UV irradiator (HFsafe-1200LC, Likang, Shanghai, China) 1 h for both sides in advance. Each specimen was put in a well of 24-well plate, then 10 μL S. mutans (ATCC, Huayeyang Bio, Shenzhen, China) suspension (10⁶CFU/mL) was vertically dropped on the center of specimen’s surface. Subsequently, surfaces were carefully covered by films in a bid to form even and thin biofilms. The well plate was converted into an incubator with 37°C and over 90% relative humidity and cultured for 24 h. After that, the film and surface were washed by 1 mL LB culture medium (Haibo, Beijing, China), respectively. The LB washing solution was diluted 20 times and 100 μL of it was dropped on an LB ager plate, then incubated at 37°C for 24 h. After that, the colonies of each plate were photographed by a mobile phone and counted by ImageJ2x (Rawak Software, Stuttgart, Germany). All experiments were performed in triplicate.

2. WST-8 assay
WST-8 {2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-
Biocompatibility

1. MTT assay

MTT assay was used to evaluate the in vitro cytotoxicity of modified GIC by examining the absorbance of L-929 fibroblast at a wavelength of 490 nm. The L-929 cell line (Cell Bank at the Chinese Academy of Sciences, Shanghai, China) was cultured in H-DMEM (SH30022.01, HyClone, Beijing, China) in incubator with 5% CO₂ at 37°C. Cylinder specimens with the same dimensions of that in microhardness test were prepared and stored in artificial saliva for 24 h at 37°C. Specimens were thoroughly sterilized via 1 h ultraviolet light for both sides. Extraction solution of the material were prepared with DMEM serum free cell culture medium 5 mL each, and then incubated for 24 h. L-929 cells were incubated in 96-well plates with a 5×10³ cells/100 μL cell culture medium in each well, the supernatant was discarded after 24 h. Afterwards, the medium in each well was replaced with 100 μL of extraction solution of each group. Then, cells were cultured a incubator with 5% CO₂ at 37°C for 24, 48, and 72 h. Then, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) was added to the each well. The specimens were incubated with MTT for 4 h. Then MTT was discarded and 150 μL of dimethyl sulfoxide (DMSO) solution was added to each well. Afterwards the 96-well plate was put into a rotation shaker at 400 r/min for 5 min. The optical absorbance was measured at 490 nm wavelength by a microplate reader.

Statistical analysis

The results were analyzed by GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). LSD t-test and Dunnett’s t-test were used to test differences between control and experimental groups.

RESULTS

Mechanical properties

In Fig. 1, the mechanical properties of GIC and GIC modified by NHA are demonstrated. Overall, NHA can improve both compression strength and microhardness (p<0.05). The setting time was prolonged (p<0.05), but the incorporation of 2 wt% NHA showed no statistical difference.

In a bid to sift out the best addition proportion of NHA, the requirements for restorative cement...
materials in ISO:9917-1(33) were referred to. When the incorporation of NHA was 8 wt%, the setting time exceeded the maximum limit of ISO standard, which is 360 s. Also, when 8 wt% NHA was added, the compression strength took a plunge to about 80 MPa, unfulfilling the minimum requirement of 100 MPa. Based on these results, the proportion of 8 wt% was abandoned.

To summarize, only when the concentrations of NHA were from 2 wt% to 6 wt% did the materials meet ISO requirements(30), and when 6 wt% NHA was added, the CS and VHN of the material reached the highest (158.3 MPa and 126.4 MPa) while it was valid according to ISO:9917-1. Hence, the best concentration of NHA was selected as 6 wt%, and GIC/6%NHA was regarded as a new control group (C1) for the continuous tests.

In Fig. 2, the mechanical properties of group C1 and GIC modified by NHA/PHMB are shown. GIC’s setting time remained similar ($p>0.05$) until the addition of PHMB was over 0.4 wt%, when the setting time was statistically significant shortened ($p<0.05$). PHMB also decreased the compression strength to 122.4 MPa as the concentration started at 0.2 wt% ($p<0.0001$). Likewise, the microhardness went through a decline, nevertheless the similar value was obtained when PHMB was 0.2 wt% ($p>0.05$).

According to ISO:9917-1, all groups met the requirement for setting time. When PHMB was 0.6 wt%, the CS of GIC decreased to 102.2 MPa, approaching the bottom line. When PHMB was 0.8 wt%, it was no longer valid in aspect of CS referring to ISO:9917-1.

Characterization of the specimens by SEM

SEM were used to evaluate the microstructure of specimens’ surfaces. Figure 3 is the SEM images of microstructures of GIC, GIC/6%NHA, GIC/6%NHA/0.2%PHMB, GIC/6%NHA/0.4%PHMB, GIC/6%NHA/0.6%PHMB, GIC/6%NHA/0.8%PHMB after compression strength test. Each group was shot at 1,000× magnifications, respectively. In (a) and (b), NHA dispersed evenly in GIC matrix, and fewer cracks and pores can be observed compared with control group. Based on 6%NHA, cracks gradually increased to former extent as control group when more PHMB was added. In (c–f), it is clear to see the sphere impressions left by PHMB microparticles distributed evenly in the specimens.

Microleakage observation

Figure 4 illustrates the sectioned teeth and the criteria of evaluation. The mean value of scores and standard difference are listed in Table 2. GIC/6%NHA drastically decrease the microleakages compared with pure GIC ($p<0.001$). When PHMB was incorporated at 0.2, 0.4, and 0.6 wt%, the microleakage was significantly decreased ($p<0.05$) compared with control group, nevertheless there was no statistical significance within group 3, 4, and 5. When the PHMB was up to 0.8 wt%, the composites (GIC/6%NHA/0.8%PHMB) showed...
Fig. 3  The SEM images of microstructures of GIC with NHA concentration of (a) 0 wt%, (b) 6 wt%, and the GIC/6%NHA composites with PHMB concentration of (c) 0.2 wt%, (d) 0.4 wt%, (e) 0.6 wt%, (f) 0.8 wt%.

Fig. 4  The criteria of microleakage observation is as follow.
a: 0=no dye penetration; b: 1=dye penetration between the restoration and the tooth up to one-third of the distance between the tooth surface and the axial wall; c: 2=dye penetration extending beyond one-third of the distance up to two-thirds of the distance between the tooth surface and the axial wall; d: 3=dye penetration extending up to two-thirds of the distance between the tooth surface and the axial wall; e: 4=dye penetration reaching the axial wall; f: 5=dye penetration reaching the entire axial wall.

Table 2  Comparison of microleakage scores in different groups

| Group                   | Mean value       |
|-------------------------|------------------|
| GIC                     | 40.00±2.45a      |
| GIC/6%NHA               | 15.33±0.47b      |
| GIC/6%NHA/0.2%PHMB      | 23.33±3.40c      |
| GIC/6%NHA/0.4%PHMB      | 26.00±2.45c      |
| GIC/6%NHA/0.6%PHMB      | 27.33±6.46c      |
| GIC/6%NHA/0.8%PHMB      | 51.67±2.88d      |

Values followed by different letter right upside the corner (2nd column) are statistically significant ($p<0.05$).
significantly increased microleakages compare with other 5 groups ($p<0.01$).

**Solubility**

The mean solubility of GIC, GIC/6%NHA/0.2%PHMB, GIC/6%NHA/0.4%PHMB, GIC/6%NHA/0.6%PHMB and GIC/6%NHA/0.8%PHMB at day 1, 7, 60 are listed in Fig. 5. In day 1, all groups showed no statistical significance. In day 7, the solubility of GIC/6%NHA/0.4%PHMB group (3.04%) was significantly lower than others ($p<0.05$). In day 60, the solubility of GIC/6%NHA/0.4%PHMB group (6.77%) was statistically lower than control group and GIC/6%NHA/0.2%PHMB ($p<0.05$), but there was no statistical significance compared with GIC/6%NHA/0.6%PHMB and GIC/6%NHA/0.8%PHMB.

**Antibacterial activity**

1. Direct contact test

In Fig. 6, the *S. mutans* colonies on LB agar of control group (GIC), GIC/6%NHA/0.2%PHMB (1),

![Image](image1.png)

Fig. 5 The mean solubility of control group and experimental groups in day 1, day 7, and day 60. Groups with different letter labels are statistically significant ($p<0.05$).

![Image](image2.png)

Fig. 6 The *S. mutans* colonies on LB agar of control group (GIC), GIC/6%NHA/0.2%PHMB (1), GIC/6%NHA/0.4%PHMB (2), GIC/6%NHA/0.6%PHMB (3), and GIC/6%NHA/0.8%PHMB (4).

![Image](image3.png)

Fig. 7 The absorbance of WST-8 assay of different groups at 450 nm with the OD value of LB agar subtracted. Groups with different letter labels are statistically significant ($p<0.05$).
Table 3 The CFU of *S. mutans* and antibacterial rate of control group and experimental groups.

| Group                  | CFU     | AR (%) |
|------------------------|---------|--------|
| Control                | 278.0±24.1* | —      |
| GIC/6%NHA/0.2%PHMB     | 32.0±9.2b  | 88.5   |
| GIC/6%NHA/0.4%PHMB     | 9.7±1.9c  | 96.5   |
| GIC/6%NHA/0.6%PHMB     | 8.0±1.4c  | 97.1   |
| GIC/6%NHA/0.8%PHMB     | 3.3±0.5d  | 98.8   |

CFU: colony forming unit, AR: antibacterial rate. Values followed by different letter right upside the corner (2nd column) are statistically significant (p<0.05).

Fig. 8 The absorbance of L-929 at 490 nm cultivated for 24, 48, and 72 h by MTT assay.
* indicates statistical significance between groups (p<0.05).

GIC/6%NHA/0.4%PHMB (2), GIC/6%NHA/0.6%PHMB (3), and GIC/6%NHA/0.8%PHMB (4) are shown. The PHMB exhibited powerful enhancement of antimicrobial activity when only 0.2 wt% was added, as the modified GIC’s antibacterial rate vigorously raised to 88.5% compared with pure GIC. When the addition of PHMB was 0.4 wt%, its AR was up to 96.5%. Then the rate of increase of antibacterial rate (AR) turned to be mild after PHMB was over 0.4 wt%, as the AR against *S. mutans* of GIC/6%NHA/0.6%PHMB and GIC/6%NHA/0.8%PHMB were up to 97.1% and 98.8% respectively (Table 3).

2. WST-8 assay
The absorption of WST-8 assay of different groups at 450 nm is illustrated in Fig. 7. As LB agar was colored itself, which would affect the accuracy of antibacterial rate, the optical density (OD) value of LB agar has been subtracted for each group already. The mean OD value of GIC/6%NHA/0.2%PHMB and GIC/6%NHA/0.4%PHMB showed significant decrease compared with control group (p<0.001), but no statistical significance was found between these two groups.

Similarly, the mean OD value of GIC/6%NHA/0.6%PHMB and GIC/6%NHA/0.8%PHMB was significantly lower than control group (p<0.0001) and two former experimental groups (p<0.001), and no statistical significance was obtained within these two groups.

Cytotoxicity
1. MTT assay
The OD values of L929 by MTT assay in different groups were shown in Fig. 8. There was no statistical significance found between control groups and experimental groups in 24, 48, and 72 h. With the time going through, the OD values of all groups in different time point were statistically increased, which indicated that the cell proliferation was correspondingly increased.

DISCUSSION
The high brittleness, solubility, poor toughness, and insufficient fluoride release have hampered GIC’s application for a longevous restorative material. Many trials to optimize GIC have been made, but bare attempt has improved both mechanical and antibacterial activities. This investigation was to use NHA to enhance the physiochemical properties of GIC, and then incorporate a novel antimicrobial agent PHMB, thus obtaining both the antibacterial and mechanical optimization of GIC.

According to the mechanical test, GIC/NHA
composites exhibited a good performance. The GIC/6%NHA obtained the greatest improvement of 53.83% and 70.30% increase in CS and VHN. This can be attributed to the mechanism that homogeneously distributed NHA particles fully occupy the space between fluoaluminosilicate particles. This provides polyacrylic acid, the main component of GIC liquid agent, with more bonding sites to form salt bridging and crosslinking, enhancing the interaction between GIC matrix. Also, the chemical reactivity between NHA and polyacrylic acid improves the intrinsic integrity of GIC matrix. Evidence of the intrinsic reinforcement can be also seen in SEM images, as GIC/6%NHA vigorously decrease the voids and cracks in GIC phase. However, the GIC/8%NHA indicated a sharp decline in CS, unfulfilling the minimum requirement of CS (100 MPa) in ISO:9917-1. And during our manipulation, the GIC/8%NHA powder was found hard to mix with the liquids. This may be explained that the too much NHA overwhelmed the interaction with polyacrylic acid. Secondly, a mass of NHA could easily agglomerate and lead to inhomogeneous dispersal in matrix. Hence, the stress concentration occurred and lead to clumps of GIC when it is withstanding forces. The ST was prolonged with increasing NHA added, and it was remained in the rational range of 90–360 s regulated in ISO:9917-1 until 8% addition of the NHA, which exceeded the maximum limit. Therefore, the best concentration of NHA was sifted out at 6 wt%, as it may provide the dentists with more adequate time to mix the liquid and powder during clinical manipulations, without taking too much time for its solidification and procrastinating the treatment. This finding corresponds with relevant previous literature, while the mechanism was poorly defined. A possible explanation is that increased calcium ions from NHA preempt to seize the acid ions, postponing the interaction of polyacrylic acid with aluminum ions. Then, the GIC/6%NHA was regarded as the second control group (C1) for continuous tests.

In second mechanical test, CS, VHN, and ST became lower with PHMB incorporated compared to GIC/6%NHA. The decline of mechanical properties corresponds with our previous study, this may lie in that PHMB, as organic polymers, affected the integrity of the inorganic material, resulting in weak zones in the matrix. In SEM images, more voids and cracks could be seen when more PHMB was added. Nevertheless, compared to conventional GIC of which the CS and VHN are 102.9 and 74.2 MPa, GIC/6%NHA/0.2%PHMB composite’s CS and VHN increased to 122.4 and 118.5 MPa. Meanwhile, those of GIC/6%NHA/0.4%PHMB were 116.0 and 105.5 MPa respectively, the enhancement of these two groups remained considerable. We firstly doped PHMB into GIC liquid to mix with powder. In this humid environment, PHMB gradually dissolved and exhibited acidity, which may accelerate the displacement of aluminum ions from fluoaluminosilicate particles, thus decreasing the solidification time.

GIC is sensitive to water and has high solubility at early stage of setting, it may cause structure deformation, failure of setting and decrease of mechanical properties. This is reasoned that initially formed polyacrylic acid calcium is weak and adsorbable to water, so it is easily corroded in early 24 h. According to SEM images, we can see the most cracks and pores on the rough surface of GIC, the porous structure may lead to disintegration of GIC by erosion in a long run. The solubility results were shown in Fig. 5, the solubilities of all groups showed no statistical differences in the first day. This is because the setting was uncompleted and the ammonium polyacrylate had not well produced. In day 7 and day 60, pure GIC holds the highest and groups with NHA/PHMB showed statistical decrease of solubility. This may be attributed to the improvement of microstructure of GIC as seen in SEM images, the addition of NHA had GIC tightened and compacted, and the PHMB decreased this impact, so the solubility relatively rose high with more PHMB.

An assessment of microleakage is an important approach to evaluate the comprehensive behavior of GIC in complex oral environment, as the depth of dyes can quantify and visualize the changes of bonding properties and polymerization shrinkage of the material after going through thermodynamic cycle, fluid scour and bacterial corrosion. Studies indicated that the penetration of NHA crystals into dentin and enamel can significantly improve the marginal adaption and adhesion between teeth and GIC. In this investigation, compared with conventional GIC, GIC/6%NHA/0.2%PHMB composite showed a 41.7% decrease of microleakage, and that of GIC/6%NHA/0.4%PHMB composite was 35.0%, which were great improvements to impede the S. mutans invasion and may eventually prevent secondary caries.

Secondary caries is another main reason for the failure of dental restoration. Low PH value in setting stage and fluoride release are the main mechanisms for GIC’s antibacterial property. However, traditional GIC has insufficient fluoride release, and cariogenic bacteria can invade the material interface, form biofilms, and survive for a long time. So, of significance is to enhance the antimicrobial activity of GIC. In relevant previous studies, NHA has been found a promising bio-ceramic and there were mechanical improvements but no antibacterial enhancement when applied in GIC. While in our previous study, PHMB exhibited unprecedented strong antibacterial property in ZOE. The main mechanism of PHMB is that its protonated groups bind to the anionic membrane of bacterial, leading to cytoplasmic leakage. In addition, PHMB was proven strong adsorption that it can permeate through dentin tubules and kill the bacterial in deeper layers. In our study, DCT and WST-8 assay were used to investigate the antibacterial effect of modified GIC. In DCT test, 88.5% S. mutans were eliminated with GIC/6%NHA/0.2%PHMB composite compared to pure GIC after 24 h, and that of other experimental groups are higher. The antibacterial activity was shown very strong at a low concentration of PHMB, this was attributed to that, guanidine in PHMB can be bound to cell membrane in the form of double hydrogen bond, which makes it more firmly bound to...
cell membrane and has stronger antibacterial activity compared with halo-amines, phosphines, or quaternary ammonium (phosphine) based polymers. WST-8 assay exhibited the similar trend of antibacterial rate as the absorbance got lower when there was more PHMB, but the activities of 0.2%–0.4% PHMB were relatively low and did not correlate with the colony count data. This may be due to the colored LB medium itself, which interfered the observation and led to the inaccurate data. Though the OD value of culture medium has been subtracted, it still could trigger mass disperse to the true situation as the OD value is not calculated in a linear model. The persistent downward of antibacterial effect of many dental materials is a common problem, while several studies have indicated PHMB's potential for the long-term disinfectant activity when applied in dental materials, yet it has not been systematically investigated. In our previous study, the AR of ZOE modified by 0.2% PHMB remained 85.2% after 14 days. This long-lasting effect could be attributed to PHMB's strong adsorption into dentinal tubules, which helps keep a sterile environment and avoid reinfection. However, how long could the disinfection of modified GIC maintain is still unclear. So, in a bid to figure out its sustainable effectiveness, we intend to carry out further study in the future, as well as in-vivo experiments to uncover whether the antimicrobial activity of modified cement is effective in oral environment.

Cytotoxicity evaluation was crucial for the feasibility of the application of modified GIC, however, previous studies have been prone to neglect this assessment hitherto. In this investigation, L-929 with pure GIC and modified GIC was used for MTT assay. It can be seen there was no statistical difference among control groups and experimental groups at 24, 48, and 72 h, which indicated that the low cytotoxicity of modified GIC. This is due to the excellent biocompatibility of NHA and PHMB. NHA was like the hydroxyapatite in human bones and was widely recognized as a safe bio-ceramic. PHMB eliminates bacterial via electrostatic effect. Because the outer layer of mammalian cell membrane is electrically neutral, and the outer layer of bacteria/fungi cell membrane is negatively charged, the positively charged guanidine polymers have strong selectivity to bacteria or fungi cell membrane, thus reducing the toxicity of guanidine polymers to mammalian cells. In this study, the OD value of all groups statically increased as time went by, which represented that the cell proliferation was correspondingly increased, this may because the setting was in process and the PH value of material became higher from a low level.

The leach property of sterilizing agents from materials is an important issue, as it can affect the antimicrobial sustainability and short-term cytotoxicity in vivo. Compared with the small molecular antibacterial agent, that with high molecules, such as PHMB, is not easy to be leached and have more durable antibacterial properties as well as no pollution. Moreover, PHMB is a kind of biocidal polymers, eliminating microbes with nonspecific mechanism bodily rather than biocide-releasing polymers and polymeric biocides, in which the polymers themselves are in essence only used as carriers connecting small molecular antibacterial agents. However, it is needed to investigate the elution of PHMB from composites and relevant evaluations are supposed to be carried out in the future.

CONCLUSIONS

NHA was uniformly dispersed in GIC by 24 h dry ball-milling, and the best concentration of NHA was sifted out at 6% according to ISO:9917-1. Significant enhancement of mechanical properties was observed with the addition of 6%NHA and different concentration of PHMB. When 6%NHA and 0.2% PHMB were added, the CS and VHN increased 18.9% and 59.7% compared to pure GIC, and that of GIC/6%NHA/0.4%PHMB were 12.7% and 42.1%. The solubility of experimental groups in day 7 and day 60 was statistically lower than pure GIC. Microleakage was greatly decreased at 41.7% and 35.0% respectively in the composites of GIC/6%NHA/0.2% PHMB and GIC/6%NHA/0.4%PHMB. Additionally, modified GIC exhibited unprecedented antimicrobial activity, as the AR of S. mutans reached 88.5% in GIC/6%NHA/0.2%PHMB, and AR was even higher when more PHMB was added. The cytotoxicity evaluation showed that the addition of NHA and PHMB had no effect on the cytotoxicity of GIC, which indicated it a safe and biocompatible modification.

In conclusion, the present work shows that NHA and PHMB were promising to improve the mechanical properties, solubility, antibacterial properties and microleakage of GIC, and have no adverse effect on its cytotoxicity. We believe that the incorporation of NHA/PHMB can be a feasible way for GIC’s optimization, however more tests such as long-term antibacterial study, water aging evaluation, wear resistance and in vivo research are still needed for the clinical application.

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REFERENCES

1) Wiegand A, Buchalla W, Attin T. Review on fluoride-releasing restorative materials—Fluoride release and uptake characteristics, antibacterial activity and influence on caries formation. Dent Mater 2007; 23: 343-362.
2) Hume WR, Mount GJ. In vitro studies on the potential for pulpal cytotoxicity of glass-ionomer cements. J Dent Res 1988; 67: 915-918.
3) Francois P, Fouquet V, Attal JP, Dursun E. Commercially available fluoride-releasing restorative materials: A review and a proposal for classification. Materials 2020; 13: 2313.
4) Farrugia C, Camilleri J. Antimicrobial properties of conventional restorative filling materials and advances in antimicrobial properties of composite resins and glass ionomer cements —A literature review. Dent Mater 2015; 31:
5) Xie D, Weng Y, Guo X, Zhao J, Gregory RL, Zheng C. Preparation and evaluation of a novel glass-ionomer cement with antibacterial functions. Dent Mater 2011; 27: 487-496.

6) Beyth N, Houri-Haddad Y, Baraness-Hadar L, Yudovich-Farber I, Domb AJ, Weiss EI. Surface antimicrobial activity and biocompatibility of incorporated poly-ethylennime nanoparticles. Biomaterials 2008; 29: 4157-4163.

7) Huivy Y, Hongye Y, Kang L, Jian Y, Cui H. Effects of chlorhexidine-encapsulated mesoporous silica nano particles on the anti-biofilm and mechanical properties of glass ionomer cement. Molecules 2017; 22: 1225.

8) Kumar RS, Ravikumar N, Kavitha S, Mahalaxmi S, Jayasree et al. Characteristic of chitosan-modified glass ionomer cement and their effects and the adhesion and proliferation of human gingival fibroblasts: An in vitro study. J Mater Sci Mater Med 2010; 30: 39.

9) Yuan Z, Ming W, McGrath CPJ, Li L. In vitro and in vivo evaluation of electrophoresis-aidsed casein phosphopeptide-amorphous calcium phosphate remineralisation system on pH-cycling and acid-etching deminerised enamel. Sci Rep 2018; 8: 8904.

10) Zhov J, Xu Q, Fan C, Ren H, Xu S, Hu F, et al. Characteristics of chitosan-modified glass ionomer cement and their effects and the adhesion and proliferation of human gingival fibroblasts: An in vitro study. J Mater Sci Mater Med 2010; 30: 39.

11) Dias A, Magn M, Delbem A, Cunha L, Pessan J. Clinical performance of glass ionomer cement and composite resin in Class II restorations in primary teeth: A systematic review and meta-analysis. J Dent 2018; 73: 1-13.

12) Paiva L, Fidalgo TKS, Da Costa LP, Maia LC, Balan L, Anselme K, et al. Antibacterial properties and compressive strength of new one-step preparation silver nanoparticles in glass ionomer cements (NanoAg-GIC). J Dent 2018; 69: 102-109.

13) Kantovez K, Fernandes F, Feitosa I, Lazzarini M, Denucci G, Gomes O, et al. TiO2 nanotubes improve physico-mechanical properties of glass ionomer cement. Dent Mater 2020; 36: e85-e92.

14) Anusha Thampi V, Prabhu M, Kavitha K, Manivasan P, Prabu P, Rajendran V, et al. Hydroxyapatite, alumina/zirconia, and nano-bioactive glass cement for tooth-restoring applications. Cera Int 2014; 40: 14355-14365.

15) Rahman IA, Ghazali NAM, Bakar WZW, Masudi SM. Modifications of glass ionomer cement powder by addition of recently fabricated nano-fillers and their effect on the properties: A review. Eur J Dent 2019; 13: 470-477.

16) Suyedan FS, Fathi MH, Edris H, Doostmohammadi A, Mortazavi V, Hanifi A. Effect of forsterite nanoparticles on the properties of glass ionomer cements. Acta Biomater Odontol Scand 2016; 2: 138-143.

17) Oulé MK, Azizwi R, Bernier AM, Kablan T, Maupertuis AM, Mauler S, et al. Polyhexamethylene guanidine hydrochloride-based disinfectant: a novel tool to fight meticillin-resistant Staphylococcus aureus and nosocomial infections. J Med Microbiol 200; 57: 1523-1528.

18) Locock KE, Michl TD, Valentin JD, Vasilyev K, Hayball JD, Qu Y, et al. Guanylated polyacrylates: A class of potent antimicrobial polymers with low hemolytic activity. Biomacromolecules 2013; 14: 4021-4031.

19) User Celik E, Tunac AT, Ates M, Sen BH. Antimicrobial activity of different disinfectants against cariogenic microorganisms. Braz Oral Res 2016; 30: e125.

20) Mattheis C, Wang H, Meister C, Agarwal S. Effect of guanidination on the properties of poly(2-aminoethylmethacrylate)-based antibacterial materials. Macromol Biosci 2013; 13: 242-255.

21) Muthamali B, Muller R, Vaidyanathan T, Srinivasan K, et al. Antibacterial properties and compressive strength of new one-step preparation silver nanoparticles in glass ionomer cements (NanoAg-GIC). J Dent 2018; 69: 102-109.

22) Hii SC, Luddin N, Kannan TP, Ab Rahman I, Nik Abdul Ghani NR. The biological evaluation of conventional and nano-hydroxyapatite-silica glass ionomer cement on dental pulp stem cells: A Comparative study. Contemp Clin Dent 2019; 10: 324-332.

23) Sarker A, Hossain M, Rahman M, et al. Comparative evaluation of shear bond strength of nano-hydroxyapatite incorporated glass ionomer cement and conventional glass ionomer cement on dense synthetic hydroxyapatite disk: An in vitro study. Indian J Dent Res 2015; 26: 170-175.

24) Malik S, Ahmed MA, Choudhry Z, Mughal N, Amin M, Lone MA. Fluoride release from glass ionomer cement containing fluoroapatite and hydroxyapatite. J Ayub Med Coll Abbottabad 2018; 30: 198-202.

25) Naveed S, Ahmad MA, Choudhry Z, Mughal N, Amin M, Lone MA. Fluoride release from glass ionomer cement containing fluoroapatite and hydroxyapatite. J Ayub Med Coll Abbottabad 2018; 30: 198-202.

26) Regan J, Moore KE, Cationic antimicrobials: Diversity of action under a common epithel. J Appl Microbiol 2005; 99: 703-715.

27) Dominguez R, Chen R, Liu JT, Huang ZX, Bao GJ, He XX. A novel zinc oxide eugenol modified by polyhexamethylene biguanide. J Mater Sci Mater Med 2019; 30: 198-202.

28) Farooq I, Moheet I, Alshwaimi E. In vitro dentin tubule occlusion and remineralization competence of various toothpastes. Arch Oral Biol 2015; 60: 1246-1253.
and mechanical properties of graphene oxide: cement nanocomposites. Sci World J 2014; 2014: 276323.

40) Lee JJ, Lee YK, Choi BJ, Lee JH, Choi HJ, Son HK, et al. Physical properties of resin-reinforced glass ionomer cement modified with micro and nano-hydroxyapatite. J Nanosci Nanotechnol 2010; 10: 5270-5276

41) Silva FWGdP, Queiroz AMD, Freitas ACd, Assed S. Utilização do ionômero de vidro em odontopediatria. Odontol Clinico-Científica (Online) 2011; 10: 13-17.

42) Brito CR, Velasco LG, Bonini GA, Imparato JC, Raggio DP. Glass ionomer cement hardness after different materials for surface protection. J Biomed Mater Res A 2010; 93: 243-246.

43) DeSchepper EJ, White RR, von der Lehr W. Antimicrobial effects of glass ionomers. Am J Dent 1989; 2: 51-56.

44) Van Dijken JW, Kalfas S, Litra V, Oliveby A. Fluoride and mutants streptococci levels in plaque on aged restorations of resin-modified glass ionomer cement, compomer and resin composite. Caries Res 1997; 31: 379-383

45) Li P, Poon YF, Li W, Zhu HY, Yeap SH, Cao Y, et al. A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. Nat Mater 2011; 10: 149-156.

46) Zaugg LK, Zitzmann NU, Hauser-Gerspach I, Waltimo T, Weiger R, Krstač G. Antimicrobial activity of short- and medium-term applications of polyhexamethylene biguanide, chlorhexidine digluconate and calcium hydroxide in infected immature bovine teeth in vitro. Dent Traumatol 2014; 30: 326-331.

47) Basrani B, Santos JM, Tjäderhane L, Grad H, Gordus O, Huang J, et al. Substantive antimicrobial activity in chlorhexidine-treated human root dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002; 94: 240-245.

48) Gabriel GJ, Som A, Madkour AE, Eren T, Tew GN. Infectious disease: Connecting innate immunity to biocidal polymers. Mater Sci Eng R Rep 2007; 57: 28-64.

49) Vermeersch G, Leloup G, Delmee M, Vreven J. Antibacterial activity of glass-ionomer cements, compomers and resin composites: Relationship between acidity and material setting phase. J Oral Rehab 2005; 32: 368-374

50) Kenawy ER, Worley S, Broughton R. The chemistry and applications of antimicrobial polymers: A state-of-the-art review. Biomacromolecules 2007; 8: 1359-1384.

51) Ding W, Peng K, Zou T, Wang R, Guo J. Development of non-leaching and eco-friendly polyhexamethylene guanidine hydrochloride based antimicrobial waterborne polyacrylates. Pigment Resin Technol 2017; 46: 458-468

52) Siedenbiedel F, Tiller J C. Antimicrobial polymers in solution and on surfaces: Overview and functional principles. Polymers 2012; 4: 46-71.