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Research Report

Description of biofilm density on glass ionomer cement modified by 5% hydroxyapatite from the synthesis of chicken eggshells

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ABSTRACT

Background: Oral microbiome biofilms can appear on all surfaces of the oral cavity as well as restorative materials such as Glass Ionomer Cement (GIC). GIC is considered superior because it has the ability to produce fluoride, which has a cariostatic effect, but the fluoride produced is inadequate to inhibit the growth of biofilms, so modifications were made by adding hydroxyapatite from eggshell. **Purpose:** To evaluate the levels of hydroxyapatite contained in chicken eggshells and to assess the description of oral microbiome biofilm density on the surface of hydroxyapatite-modified GIC from chicken eggshell synthesis. **Methods:** The research group was divided into a control group, namely GIC without the addition of hydroxyapatite, and a treatment group, namely GIC with the addition of 5% hydroxyapatite. The oral microbiome biofilm obtained from the voluntary dental plaque swab was cultured on the specimen surface at a time span of 24 hours, 48 hours, and 72 hours. Biofilm density was examined using Scanning Electron Microscopy and analyzed using ImageJ software. **Results:** The average density of biofilm on GIC without the addition of hydroxyapatite at 24 hours, 48 hours, and 72 hours, respectively, was 2,640.02 p/µm, 1,130.08 p/µm, 16,797.33 p/µm. Meanwhile, the GIC with the addition of hydroxyapatite was 1,921.52 p/µm, 1.029 p/µm, and 5,764.50 p/m. **Conclusion:** Statistical analysis performed showed that time affected the density value of the oral microbiome formed, and the two groups of materials had different effects in reducing biofilm density descriptively but statistically did not have a significant difference.

Keywords: Biofilm density; Glass Ionomer Cement; Hydroxyapatite; Scanning Electron Miroscopy (SEM); Medicine

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INTRODUCTION

Caries are the most common problem in the oral cavity of children and adults.[1,2] Based on the data from Riskesdas in 2018, the prevalence of caries in Indonesia was 88.8%.[3] Caries are caused by the role of the bacterium Streptococcus mutans in biofilms on the surface of the teeth.[4] Biofilms are a combination of microorganisms consisting of microbial cells, surrounded by a matrix of polysaccharides and attached to the surface.[5,6] Biofilms in the oral cavity play a role in the formation of plaque and can be found on almost all surfaces of the oral cavity as hard tissues, soft tissues, dentures, and restoration materials.[7]

One of the commonly used restoration materials in dentistry is Glass Ionomer Cement (GIC). Because of the chemical, mechanical, and biological properties of GIC, it has been used in minimally preventive dentistry practices.[8] Since the mid-1970s, GIC has been considered superior because it has chemical bonds with enamel and dentin, a thermal expansion coefficient almost the same as dental tissue, and the ability to produce fluoride that could prevent caries.[9]

The ability of GIC to provide fluoride has been believed to have a cariostatic effect because it prevents demineralization and increases remineralization. On the other hand, Wang's research (2016) found that the release of fluoride has no advantages in inhibiting bacterial growth or reducing the density of biofilms formed in GIC.[10] This encourages the innovation of the combination of GIC with other materials to decrease the denseness of biofilms in an effort to prevent secondary caries.[11,12] Moshaverinia M. (2016) said that the addition of hydroxyapatite to GIC will cause fluoride produced by GIC to form fluorapatite bonds that more effectively act as bacteriostatics and bacteriocide to inhibit and stop the accumulation of bacteria on the substrate so that it is more resistant to acidic conditions and the biocompatibility of GIC increases.[13]A recent study by Yanti (2019) showed a significant reduction

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in biofilm thickness between the GIC group with the addition of 5% hydroxyapatite from the synthesis of blood clam shells and the GIC group without the addition of hydroxyapatite.[14] Moreover, there have been many studies on using natural waste materials as a source of hydroxyapatite, such as chicken egg shells.[15]

The high content of calcium carbonate ($CaCO_3$) in chicken egg shells can be utilized for hydroxyapatite synthesis.[16] The diverse use of eggs in food production makes egg shells a waste that costs globally.[17] According to data released by the Central Agency for Agricultural Data and Information Systems in 2018, chicken egg consumption reached 1,644,000 tons per year.[18] Based on this, the study aim to investigate the potential of incorporating eggshell hydroxyapatite into Glass Ionomer Cement (GIC) as a novel restoration material for caries prevention in Indonesia, by assessing its effect on GIC properties, specifically its ability to inhibit the accumulation of biofilms on dental surfaces.

MATERIALS AND METHODS

This study employed an experimental laboratory design known as the post-test control-only method from August 2021 to October 2021, involving multiple laboratories. The synthesis of hydroxyapatite was conducted in the Chemical Laboratory of the Chemical Study Program at the Faculty of Teacher Training and Education at Universitas Syiah Kuala and the Titrimetry and Gravimetry Laboratory of SMK SMTI Aceh. The Chemical Engineering Testing Laboratory of the Lhokseumawe State Polytechnic performed the X-ray diffraction (XRD) analysis of the specimens. Furthermore, the Microbiological Laboratory of the Faculty of Dentistry at Universitas Syiah Kuala carried out the biofilm culture, while the Scanning Electron Microscopy (SEM) analysis took place in the Physical Materials Laboratory of the Faculty of Math and Science at Universitas Syiah Kuala. These laboratories played crucial roles in different stages of the study, contributing to the overall experimental design and data analysis.

In this study, a total of six specimens were utilized, divided into two groups: the control group, consisting of GIC without the addition of hydroxyapatite, and the treatment group, consisting of GIC with hydroxyapatite synthesized from chicken eggshells. For the control group, the specimens were immersed in media containing biofilm bacteria, with one specimen allocated for each time interval. Similarly, for the treatment group, GIC was modified with the addition of 5% hydroxyapatite synthesized from chicken eggshells, and the specimens were also soaked in media containing biofilm bacteria, with one specimen assigned for each time interval. The time intervals for both groups were set at 24 hours, 48 hours, and 72 hours. Each specimen had a diameter of approximately 5 mm and a height of 2 mm, with the requirement of being flat, smooth, and devoid of pores.

The 200-gram chicken eggshell is washed and cleaned with aquadest to remove mucous membranes and dirt, then dried in an oven at 110°C for 2 hours. Next, the eggshell is mashed with ball milling, then sifted using a 200-mesh sieve. Eggshell powder is burned using a furnace at a temperature of 1000°C for 5 hours, which aims to eliminate organic components and other metals other than calcium (Ca) and convert calcium carbonate (CaCO3) contained in chicken eggshells into Calcium Oxide (CaO), which is used as a material for making Ca precursors. Next, is the manufacture of a 10 M solution of Calcium Nitrate ($Ca(NO_3)_2$) as a precursor to Ca. CaO powder from the combustion of chicken eggshells was weighed with digital scales at 56 g according to stoichiometric calculations, then dissolved in a 68% nitric acid solution (HNO_3) of 81 ml. Aqueous is added by 19 ml until the total volume is 100 ml and stirred with a magnetic stirrer to make the suspension homogeneous. Furthermore, a 6 M solution of Phosphoric Acid (H_3PO_4) of 100 mL is made, which is a precursor to phosphate. H_3PO_4 solution with a concentration of 85% measured in as much as 28.5 ml is put into a measuring cup according to stoichiometric calculations and then dissolved with aquadest in as much as 71.5 ml until the total volume becomes 100 ml and stirred until the solution is homogeneous. Hydroxyapatite synthesis is carried out by dripping a 100 ml H3PO4 solution using a burette into an Erlenmeyer tube containing a solution of $Ca(NO_3)_2$ 100 ml and then heating at a temperature of 40°C (kept constant) with a stirring speed of 300 rpm. Stirring is continued without heating for 30 minutes after the phosphate solution is finished reacting. The pH of the solution is kept at pH 10 by adding Ammonium Hydroxide (NH_4OH). Then aging is carried out for 24 hours by putting it in an incubator. The formed precipitate is then filtered using Whatman 42 filter paper and washed with aquadest to remove the by-product, namely ammonium nitrate (NH_4NO_3). The filtered precipitate is then oven-dried at 110°C for 5 hours. Then sintering is carried out on the dry precipitate by putting it in a furnace at a temperature of 900 ° C for 5 hours.[15]

The product characteristics of the resulting hydroxyapatite particles are determined using the XRD (X-Ray Diffraction) test tool to determine the composition of the compounds formed. The acquired diffraction peaks are then matched to X-ray diffraction standards in the Joint Committee on Powder Diffraction Standards (JCPDS).

Specimens are made by mixing powder and liquid. The GIC group with the addition of hydroxyapatite is made by adding 0.25 grams of hydroxyapatite powder and 4.75 grams of GIC powder. The mixture will form a modified GIC powder with the addition of 5% hydroxyapatite. The manipulation process is carried out by mixing each GIC and GIC powder with the addition of hydroxyapatite to the GIC solution in a ratio of 1:1. Then polishing is done until the surface is smooth and flat.

Microbiome biofilm samples were taken from the subjects by doing a swab on dental plaque using a sterile cotton swab. The swab is performed on the teeth of the buccal mandibular first molar. The swabs were then placed in test tubes containing Brain Heart Infusion Broth (BHIB) and incubated aerobically at 37°C for 24 hours. Once

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bacterial colonies formed in the BHIB, saliva was applied to the specimen surface as a coating. The microbiome biofilm culture was established by applying the cultured biofilm onto the surface of the GIC and GIC with hydroxyapatite specimens using micropipettes. The specimens were then incubated at 37°C for 24 hours, 48 hours, and 72 hours.

The density of the biofilm was examined using Scanning Electron Microscopy (SEM) to observe and photograph the transverse section of the biofilm-covered specimens. Biofilm density measurements were conducted using SEM images and analyzed with ImageJ software and reported in units of p/µm.

The picture of the density of biofilms that appear in the GIC group without the addition of hydroxyapatite and with the addition of hydroxyapatite is characterized by the presence of a deeper black color compared to the area that is around it. The results of the images that appear are then analyzed using ImageJ software.

The resulting research data was described and presented through tables and figures. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software. The Shapiro-Wilk test was employed to assess the data distribution. If the data exhibited normality and homogeneity with $p > 0.050$, it was analyzed using one-way ANOVA. If the data deviated from normality with $p < 0.050$, the Kruskal-Wallis analysis was employed.

RESULTS

The XRD results show peaks on the diffractogram formed at angles of $2\theta = 29.05°$, 32.24°, 33.65°, 37.38°, and 49.34° without the formation of peaks from foreign phases. The absence of foreign peaks indicates pure hydroxyapatite has been formed and can be used as a material for GIC modifications (Figure 1).

The picture of the density of biofilms that appear in the GIC group without the addition of hydroxyapatite and with the addition of hydroxyapatite is characterized by the presence of a deeper black color compared to the area that is around it (Figure 2).

The biofilm density data obtained from this study were then analyzed statistically using SPSS statistics. The results of the Kruskal-Wallis test on the effect of biofilm density based on incubation time showed that there was a significant difference with a p-value of <0.05 meaning that time affects

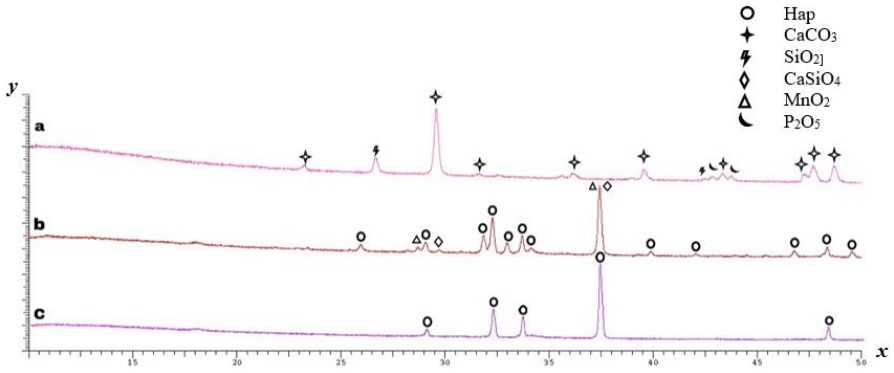

Figure 1. Comparison of XRD diffractogram patterns of a. chicken egg shell samples; b. chicken egg shell after calcination; and c. after the synthesis of hydroxyapatite.

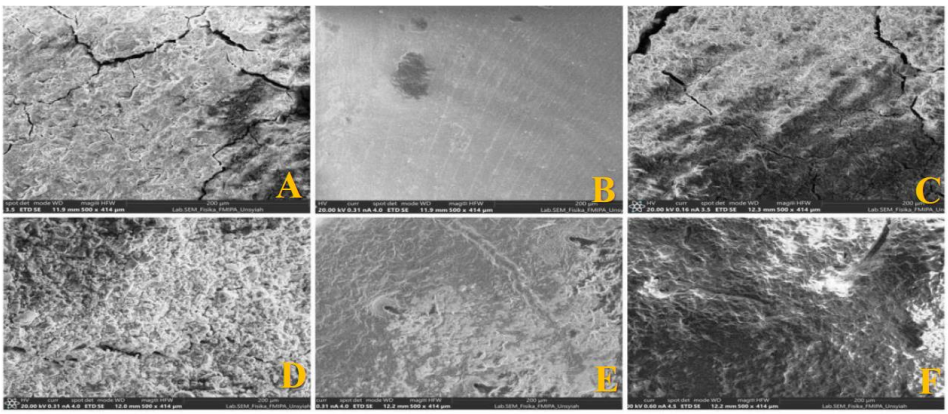

Figure 2. Density images of oral microbiome biofilms formed on the specimens: A. GIC without addition of hydroxyapatite after 24 hours, B. GIC without addition of hydroxyapatite after 48 hours, C. GIC without addition of hydroxyapatite after 72 hours, D. GIC with addition of hydroxyapatite after 24 hours, E. GIC with the addition of hydroxyapatite after 48 hours, and F. GIC with the addition of hydroxyapatite after 72 hours.

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Table 1. The results of the measurement of the average oral biofilm density of the microbiome based on time in the GIC group without the addition of hydroxyapatite and GIC with the addition of hydroxyapatite

Microbiome Oral Biofilm Density (Mean)	Group	Number of Samples	Incubation Period		
			24 hours (p/µm)	48 hours (p/µm)	72 hours (p/µm)
	GIC	N = 3	2640.02	1130.79	16797.33
	GIC + HA	N = 3	1921.52	1029.99	5764.50

the density of microbiome biofilms, meaning that each group has significant differences between each other based on the incubation time, while the results of the Kruskal Wallis test regarding the influence of biofilm density based on the material group showed a p-value of >0.05 meaning descriptively groups of materials have different effects in reducing the density of biofilms but statistically do not differ significantly, meaning that both groups of materials have the same effect on the density of biofilms (Table 1).

DISCUSSION

Hydroxyapatite is a bio ceramic of the calcium phosphate group, which is the main component of tooth enamel.[16] Hydroxyapatite can be synthesized from natural materials by various methods, one of which is the precipitation method of H_3PO_4. The resulting hydroxyapatite powder was analyzed by an XRD test, which was carried out to identify the crystal structure, phase, degree of crystallinity, lattice parameters, as well as the types of elements and compounds contained in the material. In this study, an XRD test was performed to identify the crystal structure, phase, elements, and compounds contained in hydroxyapatite synthesized from chicken eggshells. Seen in Figure 1. There are very significant differences in the phases formed in diffractograms a, b, and c, where in figure a, the majority of peaks formed are calcium carbonate compounds, while in diffractograms b and c the majority formed is hydroxyapatite. But the fundamental difference in diffractogram b is that there are still some impurity compounds, such as MnO_2 and $CaSiO_4$, while in diffractogram c only hydroxyapatite is formed. The results of the c diffractogram test match the JCPDS standard data (09-0432), so it can be ascertained that the powder formed after the synthesis process is hydroxyapatite.

The hydroxyapatite powder that has been obtained is then used to mix with GIC to see the comparison between the group with the addition of hydroxyapatite and the group without the addition of hydroxyapatite. Each group was incubated for 24 hours, 48 hours, and 72 hours and viewed using SEM with 500x magnification. The description of biofilm density that appears in the GIC group without the addition of hydroxyapatite and with the addition of hydroxyapatite is characterized by a darker color compared to the surrounding area; this is due to the ability of the biofilm to bind to the color protein given during the staining process using crystal violet. The images obtained from the SEM tool were then analyzed using ImageJ software, which can calculate the density of biofilms formed in both groups. Figures 2 A and D, as well as Table 1, show the description of biofilm density after 24 hours of incubation. Figures 2A and D and the measurement results in Table 1 show that biofilms were formed in all groups of specimens. The density of the biofilm formed after 24 hours was still quite low, namely 2640.02 p/µm in the GIC group without the addition of hydroxyapatite and 1130.79 p/µm in the GIC group with the addition of hydroxyapatite. This is presumably because the fluoride produced by GIC can inhibit the attachment of the biofilm.

The results of biofilm density after 48 hours in both groups can be seen in Figures 2B and E and Table 1. This figure shows that the lowest biofilm density results occurred after 48 hours in both groups. This is presumably because the fluoride effect possessed by the two materials was highest at 48 hours, and this effect contributed to the inhibition of extracellular polysaccharide production in the biofilm. This is in accordance with research by Ionescu (2019), which shows that the most prominent and relevant effect of fluoride is in the early phase of biofilm formation, but this effect will reach a threshold and decrease after the biofilm reaches the maturation phase so that bacterial growth can occur.[19] Thus, in Table 1 and Figures 2 C and F it can be seen that the biofilm density in GIC with the addition of hydroxyapatite had a significant difference with the GIC group without the addition of hydroxyapatite after 72 hours, whereas the growth of biofilm in GIC without the addition of hydroxyapatite experienced a very high increase. compared the growth of biofilms in the GIC group with the addition of hydroxyapatite.

The high density of biofilms in the GIC group without the addition of hydroxyapatite is also due to the biofilm's ability to affect the mechanical properties of the GIC. According to Chau (2014), the biofilm present in the GIC can reduce the mechanical properties of the GIC and make the surface of the GIC rough so as to encourage further biofilm formation. Meanwhile, according to Nurshamimi (2021), the addition of hydroxyapatite plays an important role in improving the mechanical properties of GIC. Hydroxyapatite has the ability to dissolve in acidic solutions, which can increase its solubility rapidly when the pH is below 2.05 when in contact with polyacrylic acid used as a GIC solution. Under these conditions, Ca^{2+} ions can be separated from the hydroxyapatite surface and form an intermediate layer that is very difficult to destroy. This can increase the acid-base reaction in the GIC structure and make the GIC stronger. The addition of hydroxyapatite will also increase the density of the GIC because hydroxyapatite can fill the distance between the glass particles in the empty GIC.[20] The results of this study are in line with previous studies, which were able to improve the physical and mechanical properties of GIC with the addition of hydroxyapatite at a certain concentration. However, there have been no previous

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studies examining the effect of adding hydroxyapatite from the synthesis of chicken eggshells on the formation of biofilms. Based on the results of this study, it can be seen that the addition of GIC can affect the density of the biofilm formed descriptively, but statistically, the results show that both materials have the same ability to reduce the density of the biofilm.

The present study employed Scanning Electron Microscopy (SEM) for sample imaging. However, it is recommended to further investigate using a more advanced instrument such as Confocal Laser Scanning Microscopy (CLSM). Additionally, it is advisable to conduct further research concerning the surface roughness and hardness of Glass Ionomer Cement (GIC) with the addition of hydroxyapatite from the synthesis of chicken eggshells. Appropriate instruments, such as surface profilometers and surface hardness tests, can be employed to analyze these parameters. The aforementioned additional research will contribute to a deeper understanding of the characteristics and performance of GIC materials enhanced with synthetic hydroxyapatite derived from chicken eggshells.

CONCLUSION

In conclusion, this study aimed to investigate the potential of incorporating hydroxyapatite synthesized from chicken eggshells into Glass Ionomer Cement (GIC) as a novel restoration material for caries prevention. The XRD analysis confirmed the successful synthesis of pure hydroxyapatite from eggshells. The addition of hydroxyapatite to GIC showed promising results in inhibiting the accumulation of biofilms on dental surfaces, as observed in SEM images. The biofilm density was significantly reduced in the group with the addition of hydroxyapatite compared to the control group without it. These findings suggest that the modified GIC with hydroxyapatite has the potential to enhance the prevention of secondary caries. Further studies and clinical trials are needed to validate these results and explore the effectiveness of this novel restoration material.

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