Fabrication and characterization of HA-oyster shell based on biopolymer - propolis as an agent of dental enamel remineralization material

Aminatun Nisa, Mona Sari and Yusril Yusuf

Department of physics, Faculty of Mathematics and Natural Science, Universitas Gadjah Mada, Yogyakarta, Indonesia

Email: yusril@ugm.ac.id

Keywords: hydroxyapatite, oyster shell, gel, propolis, dental

Abstract
This study aims to make gel composites by synthesizing and characterizing hydroxyapatite (HA) from oyster shell (Crassostrea gigas) as an essential ingredient for remineralization gel in teeth. The method used to synthesize HA is the precipitation method. HA is synthesized with a variation of calcination for 8 h at 1000 °C and aging time for 24 h to get the best result. The size of the HA crystal obtained is 14 ± 4 nm, with a degree of crystallinity of 91.54%. The result of the HA-oyster shell is used to synthesize gel composites. The gels used as parameters are composition variations: basis gel (basis gel as a negative control), HA gel, propolis gel, and HA-propolis gel. The essential ingredients of oyster shell, HA, and composite gels are treated by physicochemical tests in SEM, XRD, and FTIR characterization. The gel composites are treated using antibacterial tests with Streptococcus mutants, Streptococcus sanguinis, and Lactobacillus acidophilus. The antibacterial test aims to determine the inhibition of bacteria that cause caries in teeth. The best antibacterial test results are found in HA-propolis gel with the inhibition zone diameter of S.Mutants 22 ± 0.2 mm, S. Sanguinis 22 ± 0.3 mm, and L.Acidophilus 21 ± 0.2 mm. In addition to the antibacterial test, the gel was treated with a feasibility test to determine the viability of viable cells (MC3T3-E1) when incubated for 48 h. The MTT test shows that the results of the HA gel sample gave significant cell growth, which was 92.80% at the low concentration. The physicochemical, antibacterial, and MTT (Viability) test results confirm that the HA-propolis gel composite could potentially improve dental enamel caries with the remineralization process.

1. Introduction
The maintenance of dental is one of the essential elements of health [1]. One example of dental health problems is dental caries [2–4]. Dental caries is a disease of dental tissue with tissue damage starting from the surface of the pit, fissure, or interproximal areas that spread to the pulp component. Caries is a gradually multifactorial disease [5]. Dental caries attacks the tooth, dentin, and cementum enamels that cause progressive demineralization of parts with the formation of a tooth cavity [5, 6]. Dental caries is an infection that can also damage the tooth structure. Caries can occur due to an erosive process that destroys tooth enamel slowly and invades the pulp. The main cause of the disease is the bacteria of leftovers in the teeth. However, many factors that cause dental caries are microorganisms, substrates, hosts (teeth and saliva), and time. Dental caries is generally caused by interactions between bacteria, especially Streptococcus mutans, Streptococcus sanguinis, and Lactobacillus acidophilus in tooth enamel [7–9].

Efforts to maintain and prevent dental caries can be made through several methods of remineralization: diet counseling (dietary habit), fluorine coating, and oral hygiene maintenance. Therefore, dental care can be done using biomaterial applications as an alternative to help dental remineralization. Remineralization of hard dental tissue is defined as the process of supplying calcium and phosphate ions from external sources of the tooth to depose ions into the crystal cavity in the tooth enamel demineralization and produce minerals in the enamel [6].
Visually, the tooth enamel demineralization appears as a white spot lesion. The lesion can develop into a cavity or become remineralized. If given a change of conditions, remineralization can be a dominant process, thus encouraging improvement of the lesions [10]. Remineralization is the process of replacing calcium and phosphate (minerals) that begin to erode on tooth enamel. [6].

Mouthwash, toothpaste, gum, and gel are commonly used as carriers in remineralization efforts. HA, often called calcium hydroxyapatite, is a type of apatite material with the chemical formula Ca$_{10}$(PO$_4$)$_6$OH$_2$ [11]. HA represents calcium phosphate compounds [12, 13] that are components of human bones and teeth [14, 15]. HA is considered promising in its application because it is often applied in medicine and dentistry. The method used in the synthesis process of HA is precipitation. Precipitation is a method that begins with the formation of solids (deposits) in a solution as a result of chemical reactions [16]. HA has been applied to the basic ingredients of natural resources and biogenic material [14, 17–19]. Oyster shell is the selected biogenic material in this study. The wastes of the oyster shell reach 0.12 tons year$^{-1}$ [20]. Therefore, The application of this study can help reducing the waste become useful materials. The oyster shell has thick tissues containing CaCO$_3$ that can be applied to a composite to overcome dental caries. The biogenic material is processed into HA. HA can be synthesized using hydrothermal, sol-gel, mechanochemical, precipitation, electrochemical deposition, and emulsion [21–23]. The HA synthesis method effects determine the morphology, crystallography, and purity of the phase of the HA particles produced and their mechanical properties. Of the several methods, precipitation is the most effective method [23].

The gel formulation chosen for this study can increase the contact time between the active material and tooth enamel. The preparation and concentration of materials influence the absorption of substances in the body. High ion concentrations increase the potential for remineralization compared to saliva [6]. In addition, in this study using glycerin, Na–CMC, and guar-gum in the composition of the base gel is an elemental composition that effortlessly flows and enters the surface of the tooth enamel. To maximize its function, HA gel can be added to propolis material that functions as an antibacterial agent [5, 6, 24]. Propolis collected by bees from tree buds, flowers, and pollen has a diversity of pharmaceutical properties including bactericidal, anti-inflammatory, antivirus, fungicidal, antioxidant, biostimulant, and anti-tumor. Propolis aids cytotoxic and healing activity in the bones, cartilage, and dental pulp which is biocompatible with human tissue [25]. Propolis has also been proven to be a natural ingredient that can repair demineralization in tooth enamel [24]. Propolis is a natural remedy with therapeutic and anti-cariogenic properties [25].

This study developed a biocomposite HA gel from the biogenic material of the oyster shell with propolis as a potent bacterial agent in preventing demineralization. The gel also contains remineralization components in CaCO$_3$, calcium phosphate, and nano calcium HA. The synthesis method of the HA-oyster shell used is the precipitation method. Fabrication of the gel composite is divided into four compositions, namely: basis gel, propolis gel, HA gel, and HA-propolis gel. Each of the results was applied to the physicochemical test; Scanning Electron Microscopy (SEM), x-ray Diffractometry (XRD), Fourier Transform Infrared Spectroscopy (FTIR), and in vitro tests (antibacterial tests and viability test). A physicochemical test of gels was carried out to determine the chemical structure and morphology of the gel composite. The antibacterial test is an inhibitory test on Streptococcus mutans, Streptococcus sanguinis, and Lactobacillus acidophilus, which aims to determine the inhibition of gels for caries accumulated in the mouth. The viability test was carried out to prove that the sample was safe and non-toxic to be applied to the teeth.

2. Materials and methods

Fabrication is divided into three stages: preparation of oyster shell’s calcium oxide (CaO), synthesis and HA characterization, and gel fabrication and characterization. This is carried out with variations of four compositions: base gel, propolis gel, HA gel, and HA-propolis gel, as shown in figure 1.

2.1. Materials

Oyster shell used as a source of CaCO$_3$ is taken from the breeding of shellfish waste in Tangerang, Indonesia. Precursor Diammonium hydrogen phosphate solution ([NH$_4$]$_2$HPO$_4$) 99.5%, and ammonium hydroxide (NH$_4$OH) 25% were purchased from Merck (NJ, USA).

HA-oyster shell gel was made in the Material Physics Laboratory, Faculty of Mathematics and Natural Sciences, Gadjah Mada University, Yogyakarta. The composition of HA gel is a material that is pro-analysis with high purity. The natural propolis material is an original product from Melia propolis brand purchased in Malang, East Java, Indonesia.

The essential basis gel compositions are guar-gum, Na CMC, and glycerin. The material is highly pure and safe for making gels.
2.2. Preparation CaO-oyster shell

The oyster shell was washed thoroughly with running water and rinsed with acetone. After washing, the oyster shell was dried with Sunlight for 24 h. Then the shell was soaked in acetone for 24 h and rinsed with distilled water. The cleansing aims to clean the shell from dirt and moss attached. Furthermore, the shell was milled using a ball mill to mash it into powder. When the process was completed, the powders were then filtered with a shaker to get a softer and more homogeneous texture. Oyster shell powder was heated at 1000 °C for 8 h, which aims to produce calcium oxide (CaO) served as a source of calcium (Ca) in the synthesis process of HA.

2.3. Fabrication and characterization of HA

Synthesis HA uses the precipitation method obtained from CaO and (NH₄)₂HPO₄, diluted with their respective compositions which the two samples become homogeneous solutions. The following are the steps of HA synthesis:

2.2.1 CaO powder (10 g) is dissolved into aquadest (H₂O) 70 ml. The dilution is carried out at temperatures reaching 60 °C for 1 h with setting on stirrer at 500 rpm.

2.2.2 In the same way, (NH₄)₂HPO₄ (0.0648 mol) as a source of phosphate dissolved with distilled water (H₂O) 70 ml of dilution is carried out at temperatures reaching 60 °C for 1 h with setting at 500 rpm.

2.2.3 (NH₄)₂HPO₄ solution is titrated into the CaO solution at flow rate of 1 ml min⁻¹. Titration is carried out in a state of solution being stirred on a hot plate magnetic stirrer.

2.2.4 Control the pH values of the mixture CaO and (NH₄)₂HPO₄ solutions were carried out by adding 15 ml of ammonia solution (NH₄OH) to the solution (NH₄)₂HPO₄. The aim is to form an alkaline (NH₄)₂HPO₄ solution (pH > 9).

2.2.5 Mixing is carried out by stirring for 1 h at 500 rpm at 60 °C to make the solution homogeneous.

2.2.6 The solution is precipitated (aged) for 24 h and filtered for 24 h. Next, the result of filtering the solution is heated (oven) at 100 °C for 8 h. Finally, the filtering result was heated and calcined (furnace) at a temperature of 1000 °C for 8 h. The result of filtering the calcined solution is called HA.

2.2.7 The HA-oyster shell was treated with physical testing (SEM, XRD, and FTIR) to determine morphology, crystal properties, and chemical structures.
2.4. Fabrication and characterization of gel

Gel fabrication is made by optimizing the gel composition. The elemental composition of the gel consists of Na-CMC, guar gum, and glycerin. Na CMC and guar gum function as thickening agents, and glycerin serves as gel structure binders, humectants, and moisturizers. Therefore, the following is the process of making the gel:

2.4.1. Na-CMC dilution

The dilution was carried out with 2% Na-CMC dissolved in aquadest. The solution was stirred at 500 rpm for 1 h until a homogeneous solution formed.

2.4.2. Guar-gum dilution

The dilution was carried out with a 2% guar gum concentration dissolved in aquadest. The solution was stirred at 500 rpm for 1 h until a homogeneous solution formed.

2.4.3. Glycerin dilution

Glycerin dilution was carried out using a ratio of 1:1.

2.4.4. Homogenization of composition

Na-CMC solution, homogenized with aquadest, is slowly poured into the guar gum solution while the solution is stirred. Both solutions became homogeneous within 1 h. Next, the mixtures of Na-CMC and guar gum were added with diluted glycerin. The three composition mixtures were stirred at 500 rpm for 1 h until the mixture was homogeneous. These compositions are called gel base.

2.4.5. Gel composition

The synthesized base gel was applied to 4 variations of gel composition: base gel, propolis gel, HA gel, and HA-propolis gel. Each composition variation was analyzed using SEM, XRD, and FTIR characterization. In addition to the characterization, the sample was also be tested in vitro by conducting an antibacterial test using three bacteria: Streptococcus Sanguinis, Streptococcus Mutants, and Lactobacillus Acidophilus. The viability test was also treated on these cells.

2.5. Physicochemical characterizations

The fabrications of HA-oyster shell and gel were given test treatment in a physicochemical test.

2.5.1. Scanning electron microscopy (SEM)

SEM characterization test was used to determine the morphological structure of the sample. From the SEM results, the sizes of the HA crystal grains were measured using the ImageJ software. The measurement results were displayed as a graph of the frequency Gauss distribution to the size of the sample diameter. In addition to process sample sizes, this test calculates the Ca/P value of HA using scanning electron microscopy energy-dispersive x-ray spectroscopy (SEM-EDS).

2.5.2. Fourier transform infrared (FTIR)

FTIR is a characterization method to identify the functional group, bond type, chemical composition, and vibration of molecular bonds in compounds. The compound analysis was carried out from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\). In this case, FTIR was used to identify the functional groups in the CaO, CaCO\(_3\), HA, and gel samples.

2.5.3. X-Ray diffractometer (XRD)

X-Ray diffractometer (XRD) is used to characterize and identify the crystalline phase in a sample by determining the lattice structure and particle size. The data were resulted from the intensity value of the 2\(\theta\) angle formed. The results of the XRD data are processed into a software origin pro9 to be presented as a curve that displays the peaks of the diffraction results. The data presented in the form of a curve is done by Gaussian fitting at the selected peaks to get a value of 2\(\theta\) and the value of full width at half maximum (FWHM). The fitting diffraction peaks were matched with International Center for Diffraction Data (ICDD) data. The data obtained could be used to obtain lattice parameters, crystal size, microstrain, and degree of crystallinity in HA samples.

2.6. In vitro characterizations (antibacterial test and viability test)

The in vitro test in this study was in the form of an antibacterial test against bacteria that cause dental caries accumulated in the mouth, namely streptococcus mutants, streptococcus sanguinis, and lactobacillus acidophilus. This test was conducted to determine the inhibitory power of the gel against bacteria that cause dental caries.
bacterial inhibition test was carried out by measuring the diameter of the clear zone of the sample. Four samples were tested in this test: base gel, propolis gel, HA gel, and HA-propolis gel.

A viability test using the MTT test is used to assess the metabolic activity of cells using culture for a particular time. NAD(P) enzyme is a cellular oxidoreductance enzyme that changes MTT colour to purple. In addition, the MTT test determines the cytotoxicity that is toxic to living cells. The absorption of this solution was then measured using a spectrophotometer at a wavelength between 500–600 nm [26].

3. Results and discussion

3.1. Results of HA-Oyster shell fabrication

The physicochemical properties of nano-HA from the oyster shell were determined using SEM-EDS, XRD, and FTIR, as shown in figure 2. As shown in figure 2(a), the synthetized HA had the small agglomerate shape and solid structure (shown by the yellow arrow). Figure 2(b) showed the constituent elements of HA by measuring the ratio of Ca/P. These analyses had been studied in previous research [6], where HA based on oyster shell had a Ca/P molar ratio of 1.63. The XRD analysis determines the crystal structure and atomic-scale parameters by identifying Bragg peaks concerning material JCPDS data. The JCPDS identified the diffraction peaks of HA. The crystallite size, microstrain, and degree of crystallinity of the synthesized HA were 14.15 ± 3.73 nm, 0.04 ± 0.016, and 91.54%, respectively. The distance between the crystal planes of the HA was determined using the Scherrer equation and calculated to be 2.88 Å (shown in figure 2(d)). This result is close to the crystal plane of the HA at 2.88 Å, making it appropriate by international standards (ISO 13779–3, ISO 13175–3) for HA implants. As shown in figure 2(c), the synthesized HA exhibited the functional group of HA. The HA exhibited the stretching mode of OH⁻ at 3572.56 cm⁻¹ and the bending modes of stretching (P–O) way of PO₄³⁻ at 1142.64; 1043.92; 975.14, and 561.66 cm⁻¹. In addition, HA exhibited the functional group of CO₃²⁻ only at 886.96 and 1476.66 cm⁻¹.

3.2. Physicochemical characterization of synthesized

3.2.1. Biocomposite gel

SEM analysis was conducted to observe the morphological structure of 4 gel samples: base gel, propolis gel, HA gel, and HA-propolis gel. The results of the SEM analysis are shown in figure 3. There are no spherical particles in the gel base morphology. The propolis gel is shown in figure 3(b) and has almost the same morphological structure as the base gel. However, there are few particles in some gaps in the gel structure from propolis gel. Figures 3(c) and (d) show round and fine-sized particles, with the gel adding HA. In the HA gel, several particles
are seen grouped (agglomeration shown by the yellow arrow) in an area. Particle size analysis on HA gel was carried out by taking 77 particle samples. The particle size produced by the measurement is $0.57 \pm 0.09 \mu m$. The mean particle size of the HA gel is presented in the normal distribution diagram shown in figure 4(a). In figure 4(b), the HA-propolis gel showed smaller particle sizes in each gel area. The particles are dispersed unevenly and appear over long distances in the gap area. The number of particles in the HA-propolis gel is more than the HA gel. Particle measurement in the HA-propolis gel used 88 samples with a particle size of $0.26 \pm 0.06 \mu m$ (figure 4(b)). In this case, the detected spherical particles in a gel indicated that there was a detectable content of HA in the extract, even though it was small. And not only on HA but also on propolis that has been dissolved. The presence of spherical particles indicates that the HA or propolis has been well homogenized in the gel extract, which can be seen in SEM analysis. The textures formed on the four gel samples were included in several requirements of SNI 12–3524–1995, including; a pH value of 4.5–10; measuring the pH value using a digital...
factor affects the gel synthesis result. The composition of the solvent dominates samples detected by spectrum, nevertheless, the base gel and propolis have sharp transmittance band shapes. The solvent composition described in the O-bubbles, no visible foreign objects, and a weak elemental content of tin and Mercury.

The pH meter. The pH value of the gel ≥ 5 which showed homogeneous phase, lack of agglomeration, lack of bubbles, no visible foreign objects, and a weak elemental content of tin and Mercury.

The FTIR functional group (figure 5(a)), phosphate group in HA gel, and HA-propolis show a very narrow peak spectrum, nevertheless, the base gel and propolis have sharp transmittance band shapes. The solvent composition factor affects the gel synthesis result. The composition of the solvent dominates samples detected by FTIR.

The gel without adding HA uses water solvents, and the gel with the addition of HA uses acetic acid solvents. The acetic acid used in the gel makes HA quickly dissolve and evaporate, thereby reducing the phosphate content of the composition. Therefore, the gel with the addition of HA has a thin transmittance band in the phosphate group.

The structure of the functional group in the sample is a functional group that can be applied in the biomedical field, namely biocomposite gel because the functional group does not contain toxic elements and has low crystallinity in the FTIR characterization results. In the FTIR results, the bands in the spectrum were observed at 1635.45 cm⁻¹, which are C=O, C=C, and N–H groups which are skeletal aromatic rings of flavonoids and amino acids that confirm the presence of long-chain alkyl compounds in the gel extract. Stretching and bending bands were observed at 1450 cm⁻¹, corresponding to the aromatic ring of phenolic compounds specific to propolis and glycerin. In addition, the broad band with a maximum at 3367.76 cm⁻¹ described in the O–H band strain vibration was also complemented by the presence of phenolic compounds in the gel extract. This follows the reference by Woźniak et al, 2020 [27].

From the results of the FTIR characterization, it was confirmed that this gel was safe to be applied to the mouth. This gel contains safe compounds and does not contain Mercury (The gel fabrication does not use a mixture of Mercury/heavy metals. This can be seen in the FTIR functional group of the gel. In general, various functional groups such as amines (–NH), carboxylate anions (COO–), hydroxyl (–OH), and others: (N=O) (–C=N), (–C=O), (–C–N), and (–C–H) have been proposed to be responsible for the heavy adsorption metal ions on the surface of the adsorbent cell. However, these groups were not formed in the IR spectra of this gel) which the Mercury functional group does not appear in the FTIR results of this study [28].

Figure 5(b) shows the results of the four samples XRD characterization. The analysis results of gels have a peak and amorphous shape. The amorphous structure in the XRD gel pattern is caused by the dominance of the solvent in the sample, namely H₂O. In addition, the materials used are Na-CMC, guar gum, and glycerin, which have hygroscopic properties and are readily soluble when reacted with solvents. Therefore, the sample is not crystalline [29, 30].

HA has a sharp peak of diffraction and good crystallinity. However, in this sample, HA is synthesized with basis and propolis gel materials that make the structure of the compound forming HA damaged. In addition, the XRD spectra of the gel (figure 5(b)) have broad peaks and an amorphous shape with the addition of gel base composition. XRD spectra of the gel showed that Na-CMC substitution, glycerin, and guar gum could change the crystal structure of HA. Therefore, the XRD spectra could not detect the crystal peaks in the HA Gel and HA-Propolis Gel. The structure of HA is easily damaged when it binds to other polymers such as propolis and some compositional materials in the gel base.

The gel biocomposite substituted with HA reduces the crystal size and x-ray density due to the addition of propolis and other materials called distortion. Distortion causes crystal defects at the distance between the crystal planes (dislocations), which causes damage or loss of crystal structure. Dislocations in the material are observed with prominent XRD peaks influenced by increasing temperature and decreasing crystal size. The
success of the antibacterial gel is seen from the characterization of XRD with an amorphous peak, following the references made by previous studies [29, 31]. The amorphous phase produced broad XRD peaks at low 2 theta angles of 10–30 degrees because the sample does not have crystalline properties. This occurs because of the dominance of polymers that damage the crystal structure thus the peak is amorphous. In the fabrication of the gels that are applied to the teeth have to be homogeneous structures, and lack of agglomerate. This is necessary because the gel is easily absorbed by the teeth and easily blends with the mucosa around the teeth. Therefore, an amorphous structure is required in dental applications.

3.3. In vitro characterization (antibacterial test and viability test) of synthesized biocomposite gel
Figure 6(a) showed the test of S. mutans bacteria on the four gel samples; base gel, propolis gel, HA gel, and HA-propolis gel. The test of S. mutans bacteria was repeated three times on the sample. The result obtained is the addition of diameter periodically to the gel added with HA and propolis. The addition of diameter is indicated by the size of the circle given by the bacteria. The same treatment was also applied to figures 6(b) and (c) with the bacterial tests of S. sanguinis and L. Acidophilus.

The antibacterial test was carried out on four gel samples: basis gel, propolis gel, HA gel, and HA-propolis gel. The antibacterial test was carried out to determine the inhibition/resistance of the gel sample to the accumulation or the presence of bacteria that cause dental caries (as shown in figure 6). There are many species of bacteria in the oral cavity, but few cariogenic bacteria that cause caries, such as Streptococcus mutans and Lactobacillus acidophilus.

As shown in figure 6, the test aims to compare the effectiveness of biocomposites gel as an antibacterial by measuring the clear zone and the bacterial zone. The bacterial growth zone, S. mutans bacterial [figure 6(a)] testing in HA gel showed a diameter value of $15.2 \pm 0.40$ mm, propolis gel of $19 \pm 0.20$ mm, and HA-propolis gel of $21.8 \pm 0.18$ mm. HA-propolis gel occupies the most significant position at its diameter value, so it has the most potent inhibition and inhibits S. mutans among other gel samples. S. Sanguinis bacterial [figure 6(b)] testing
in HA gel showed a diameter value of 13.08 ± 0.10 mm, propolis gel of 18.7 ± 0.22 mm, and HA-propolis gel of 22.1 ± 0.31 mm. In the bacterial testing of L. Acidophilus [figure 6(c)], the HA gel showed a diameter value of 12.08 ± 0.10 mm, the propolis gel of 18.8 ± 0.18 mm, and the HA-propolis gel of 22.35 ± 0.22 mm. The inhibition test results are also plotted in a stem diagram using the ANOVA test to determine the significance value (figure 7). The significance value of the ANOVA p test is < 0.05 meaning a significant effect between one diameter variable on the bacterial variable. HA-propolis gel had the largest diameter value in each different bacterial type. The combination gel of HA and propolis strongly inhibited bacteria accumulating in the teeth/mouth. The most significant diameter value proves that HA-propolis gel has the most potential among other samples. Figure 6 shows that the results of the bacterial test (S. mutans, S. Sanguinis, L. Acidophilus) gave the most increased result in the diameter of the HA-propolis gel. The repetition was carried out three times and resulted in almost the same data as the first experiment.

In addition to propolis, HA also has properties as a potent antibacterial agent. Therefore, HA has potential in the process of tooth remineralization; this has been proven in a previous study [6]. Hydroxyapatite powder plays a vital role in dentistry and is associated with the exposure of dentinal tubules. HA in toothpaste and dental gels reduces the deposition of accretions on teeth. HA can also be used as a component of dental cement and fillings. HA is worth mentioning that microporous structures of hydroxyapatite can serve as carriers of medicinal substances directly to the destination. The studies on HA systems for the controlled release of anticancer drugs, antibiotics, and growth factors were reported in several papers [32]. It was found that nanoparticle composites could inhibit bacterial and fungal growth. The initial powders of the nanocomposites used in this study were prepared by precipitation. This method is simple and produces particles of tiny size on the nanoscale. Particle size affects their antibacterial properties. Decreasing the particle size can increase the surface area and contact with the surrounding environment increase, resulting in increased solubility. Therefore, a nanosized amorphous structure could have an optimal antibacterial effect due to faster ion release than structures with typical grain size. In addition, factors such as the production of active oxygen species due to the presence of HA, electrostatic interaction between HA nanoparticles and the cell wall, so the penetration of the HA nanoparticles into the cell and the reformation of HA can be prevented by cell wall formation and growth of bacteria. As a result, the antibacterial potency of biomaterials can be different according to the characteristics of other species of bacteria, especially their structural properties [33].

The viability test through MTT Assay showed the interaction of MC3T3E1 cells on HA/propolis and its cytocompatibility at 48 h [figure 9]. HA affects the supply of nutrients for cell growth at low concentrations. The MTT test showed that the results of the HA Gel sample gave significant cell growth, which was 92.03% at a low concentration, 31.25 μg ml^{-1}, which decreased to 74.60% at a dose of 125 μg ml^{-1} [figure 8]. The sample concentration did not affect cytotoxicity cell but increased viability close to the control value (100%). At 250 μg ml^{-1} and 500 μg ml^{-1}, the distribution of cells was not concentrated in the nucleus. This indicates a decrease in the percentage of cell viability. Cell
death can occur due to a lack of nutrients in the medium or high cell density. The morphology of MC3T3E1 cells proved that HA-propolis gel is cytocompatible to protect cell lines [figure 9].

4. Conclusion

Synthesis of HA Oyster shell and fabrication of composite gel was successfully carried out. Indicators of success seen from;
4.1. Antibacterial test
The larger the zone of inhibition bacteria, the greater the inhibition of the gel to inhibit caries-causing bacteria on the teeth. HA - Propolis gel has the most prominent inhibition, with the test results of S. mutans bacteria showing a diameter of 21.8 ± 0.18 mm. The diameter indicated the ability of the gel sample to inhibit bacteria. Therefore, the HA-propolis biocomposite gel has the potential to inhibit the bacteria that cause dental caries. Alternative HA-propolis gel fabrication can be used to help treating caries problems in teeth, such as demineralized teeth and cavities. This study proves that HA-propolis gel can be a potent antibacterial agent against bacteria that cause dental caries.

4.2. Viability test
The greater the percentage of viability, the safer the gel to be applied to the bone cells of the teeth. The viability test proved that the HA-propolis gel is cytocompatible to protect the osteoblast cell line seen from the morphology of the MC3T3E1 cell, where the HA-propolis gel is safe and non-toxic.

4.3. Toothpaste quality requirements
Requirements according to standard SNI 12–3524–1995 (pH 4.5–10.5, soft gel texture, homogeneous, no visible air bubbles/clumps, separated particles, and no visible foreign matter).

Acknowledgments
This research was funded by the Ministry of Education, Culture, Research, and Technology (1724/UN1/DITLIT/Dit-Lit/PT.01.03/2022), and Indonesian Endowment Fund for Education (LPDP KET-4099/LPDP.4/2020).

Data availability statement
The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

ORCID iDs
Yusril Yusuf https://orcid.org/0000-0001-9104-9333

References
[1] Dorozhkin S V 2019 Dental applications of calcium orthophosphates (CaPO4) Journal of Dentistry Research 1 024–54
[2] Elkassas D and Arafa A 2014 Remineralizing efficacy of different calcium phosphate and fluoride-based delivery vehicles on artificial caries like enamel lesions Journal of Dentistry 42 666–74
[3] Amalina R, Soekanto S A, Gunawan H A and Sahlan M 2017 Analysis of CPP-ACP complex in combination with propolis to remineralize enamel J Int Dent Med Res 10 814–9
[4] Rugg-gunn A 2013 Dental caries: strategies to control this preventable disease Acta Med Academia 42 117–30
[5] Cochrane N J, Cai F, Huq N L, Burrow M F and Reynolds E C 2010 Critical review in oral biology & medicine: new approaches to enhanced remineralization of tooth enamel J. Dent. Res. 89 1187–97
[6] Sari M, Ramadhanti D M, Amalina R, Chotimah, Ana I D and Yusuf Y 2022 Development of a hydroxyapatite nanoparticle-based gel for enamel remineralization—A physicochemical properties and cell viability assay analysis Dental Mater. J. 41 68–77
[7] Goyal D, Kaur P and Majeed A 2019 Effect of plant chewing stick extracts on certain cariogenic activities of s. mutans International Journal of Science and Research (IJSR) 8 1116–20
[8] Yamaguchi M, Terao Y, Ogawa T, Takahashi T, Hamada S and Kawabata S 2006 Role of Streptococcus sanguinis sortase A in bacterial colonization Microbes and Infection 8 2791–6
[9] Hamada S and Slade H D 1980 Biology, immunology, and cariogenicity of Streptococcus mutants Microbiological Reviews 44 331–84
[10] Tschope P, Zandim D L, Martus P and Kielbassa A M 2011 Enamel and dentine remineralization by nano-hydroxyapatite toothpaste Journal of Dentistry 39 430–7
[11] Sari M, Hening P, Chotimah, Ana I D and Yusuf Y 2021 Bioceramic hydroxyapatite-based scaffold with a porous structure using honeycomb as a natural polymeric porogen for bone tissue engineering Biomater. Res. 25 1–13
[12] Ana I D, Satria G A P, Dewi A H and Ardhani R 2018 Bioceramics for clinical application in regenerative dentistry Adv Exp Med Biol 1077 309–16
[13] Ana I D 2019 Bone substituting materials in dental implantology (Switzerland: Springer Nature Switzerland AG)
[14] Sari M and Yusuf Y 2018 Synthesis and characterization of hydroxyapatite based on green mussel shells (perna viridis) with the variation of stirring time using the precipitation method IOP Conf. Ser.: Mater. Sci. Eng. 342 357–70
[15] Kumar G S, Girija E K, Venkatesh M, Karunakaran G, Kolesnikov E and Kuznetsov D 2017 One step method to synthesize flower-like hydroxyapatite architecture using mussel shell bio-waste as a calcium source Ceram. Int. 43 3457–61
[16] Fadli A, Yenti S R, Rasyidin R and Sari M 2019 The effect of time and number of balls on shaker milling process in hydroxyapatite powder synthesis IOP Conf. Ser.: Mater. Sci. Eng. 532 012012
Rujitanapanich S, Kumpapan P and Wanjanoi P 2014 Synthesis of hydroxyapatite from oyster shell via precipitation Energy Procedia 56 112–7

Hamester M R R, Balzer P S and dan Becker D 2012 Characterization of calcium carbonate obtained from oyster and mussel shells and incorporation in polypropylene Mater. Res. 15 204–8

Zhu B, Macleod L C, Kitten T and Xu P 2018 Streptococcus sanguinis biofilm formation & interaction with oral pathogens Future Microbiology 13 915–32

Wu S C, Hsu H C, Hsu S K, Tseng C P and Ho W F 2019 Effects of calcination on a synthesis of hydroxyapatite derived from oyster shell powders J. Aust. Ceram. Soc. 55 1031–8

Pang Y X and Bao X 2003 Influence of temperature, ripening time and calcination on the morphology and crystallinity of hydroxyapatite nanoparticles J. Eur. Ceram. Soc. 23 1697–704

Wu S C, Hsu H C, Hsu S K, Chang Y C and Ho W F 2016 Synthesis of hydroxyapatite from eggshell powders through ball milling and heat treatment Journal of Asian Ceramic Societies 4 85–90

Laonapakul T 2015 Synthesis of hydroxyapatite from biogenic wastes KKU Eng J 42 269–75

Wu S C, Hsu H C, Hsu S K, Chang Y C and Ho W F 2016 Synthesis of hydroxyapatite from eggshell powders through ball milling and heat treatment Journal of Asian Ceramic Societies 4 85–90

Machado B, Pulcino T, Silva A, Melo D, Silva R and Mendonca I 2016 Propolis as an alternative in prevention and control of dental cavity Journal of Apitherapy 1 47

Ragunathan S, Govindasamy G, Raghul D R, Karuppaasamy M and Vijayachandra Togo R K 2020 Hydroxyapatite reinforced natural polymer scaffold for bone tissue regeneration Mater. Today Proc. 23 111–8

Woźniak M, Kwaśniewska-Sip P, Krueger M, Roszyk E and Ratajczak I 2020 Chemical, biological and mechanical characterization of wood treated with propolis extract and silicon compounds Forests 11 907

Singh S K, Chaudhary A, Rai D K and Rai S B 2009 Preparation and characterization of a mercury based indian traditional drug- ras-sindoor Indian Journal of Traditional Knowledge 8 346–51

Asawahame C, Satirittangtham K, Eitsayeam S, Tragoolpua Y, Sirithunyalug B and Sirithunyalug I 2015 Formation of orally fast-dissolving fibers containing propolis by electrospinning technique Chiang Mai Journal of Science 42 469–80

Esposti I D, Ionescu A C, Brambilla E, Tampieri A and Iafisco dan M 2020 'Characterization of a toothpaste containing bioactive hydroxyapatites and in vitro evaluation of its efficacy to remineralize enamel and to occlude dentinal tubules,' Materials (Basel). 13 1–13

Patty D J, Yusril Y, Ari D N and Ika D N 2021 In vitro bioactivity of 3D microstructure hydroxyapatite/collagen based-egg white as an antibacterial agent Wiley Journal of Biomedical Material Research 110 1412–24

Lamkhao S, Phaya M, Jansukam C and Chandet N 2019 Synthesis of hydroxyapatite with antibacterial properties using a microwave-assisted combustion method Scientific Reports, May 2018 1–9

Seyedmajidi S, Rajabnia R and Seyedmajidi M 2020 Evaluation of antibacterial properties of hydroxyapatite/bioactive glass and fluorapatite/bioactive glass nanocomposite foams as a cellular scaffold of bone tissue Journal of Laboratory Physicians 10 265–70