ORIGINAL ARTICLE

Stirring Time Effect of Beta-TCP Nanoencapsulation Synthesized from *Anadara granosa* Shells on Particle Size and Calcium Level

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ABSTRACT

Introduction: Anadara-granosa synthesization through hydrothermal-method produces beta-tricalcium(beta-TCP), a biomaterial that is able to provide a pathway for calcium-ions in dentin reparative formation thus qualifies TCP as pulp-capping material.Nanoencapsulation is needed as calcium-ions have shown its rapid solubility which is the main cause of high probability risk of tunnel defect.The present study aimed to understand the correlation between stirring time, particle size and level of calcium of beta-TCP nanoencapsulation synthesized from Anadara-granosa-shells. **Methods:** Anadara-granosa-shells powder was hydrothermically-processed for 18hours and sintered for 3hours. After homogenous beta-TCP powder mixed with aquadest in magnetic stirrer acquired, Na-alginate was added during the stirring process following CaCl2 drop by drop into the mixture.Sample divided into 6-test groups according to the stirring time; P1-one hours;P2-two hours;P3-three hours;P4-four hours;P5-five hours;P6-six hours.All samples centrifuged at 2500rpm for 6minutes and freeze-dried for 12hours.PSA-test and Calcium level-test were performed on the sample test groups, followed by ANOVA-test and post hoc with significance level of P-value=0,05. **Results:** Data showed average of particle-sizes P1=±336.44; P2=±325.7; P3=±340.94; P4=±452.6; P5=±556.6; P6=±593.93.ANOVA-test result indicated a significant difference and backed up by Gomes howell test result.Significant differences were found between group first-second-third and group four-five-six also between group five and group six. Calcium level test result was P1=±10.41; P2=±9.53; P3=±9.87; P4=±5.52; P5=±5.33; P6=±5.25.ANOVA-test showed a significant difference and supported by post-hoc LSD-test.Significant differences noted between group one and other groups also between group-two-three and group-four-five-six. **Conclusion:** In the process of Nanoencapsulation of Anadara-granosa-shells, particle size gradually increased and calcium level gradually decreased along with the longer stirring time was performed.

Keywords: Encapsulation, Calcium content, Particle size, *Anadara granosa*

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INTRODUCTION

Anadara granosa is bivalve species and easily found in Indonesia's sea (1). *Anadara granosa* is also a type of clam that is commonly served as Indonesian cuisine (2). The clams are usually served boiled, steamed and fried. Thus, the accumulated inedible clams shells end up as household waste and quite numerous amounts all over the country.

A study by Widyastuti (2018), has shown that *Anadara granosa* shells produced 90% of CaCO3 (3). Study by Sari (2018) shows that 12 hours of hydrothermal-proccessed and sintered *Anadara granosa* shells contained 21% of tricalcium phospate (TCP) (4).

TCP is an osteoconductive materials with a faster resorption rate than hydroxyapatite (5). TCP contains calcium and has a similar chemical composition with tooth structure (6,7). Calcium is the main active component in the formation of reparative dentin process. Theoratically, combination of its biocompatibility and calcium-release might allow TCP to stimulate the odontoblast cells in dentin bridge formation. TCP is also able to stimulate bone regeneration and the closure of the tooth apex better than calcium hydroxide (CaOH) (8).

TCP is resorbable and non-reactive component. TCP

also contains porusbioceramic component. Physical test of TCP's functional density showed beta-TCP is more stable than alpha-TCP. According to test result, beta-TCP might perform a better result in bone grafting than alpha-TCP. Beta-TCP is a promising material in dental procedures, for its osteoconductivity and bone replacement capabilities (9).

The most commonly used material for dentin reparative formation is CaOH. A long-term study of CaOH showed the shortcomings of CaOH's bond with dentin as it tended to get softened, disintegrated and soluble in dentin liquid. In the exposed pulp, reparative dentin will be deposited by forming a dentin bridge. An incomplete dentin bridge formation is called tunnel defect. Tunnel defect creates multiple perforations lead to microleakages between pulp tissue and pulp capping material, thus providing pathway of microorganism to penetrate into the pulp tissue as the inadequate dentin formed (8).

Studies by Gupta (2011) and Dangi (2013), showed particle size shrank in a higher stirring pace and longer stirring time (10,11). High speed stirring in magnetic stirrer increases the intensity of collision between solvent molecules and biomaterial (12,13). Nanoparticles formed as the intense collision between two materials continue and correspondingly in a longer stirring time (13).

Given the explanation above, beta-TCP in this study will be synthesized using hydrothermal method in room temperature but longer, 18 hours processing time. The sample of calcium will be nanoencapsulated to decrease the solubility speed and avoid tunnel defect. Nanoencapsulated materials will protect the active substance beta-TCP and maintain substance release that will be absorbed by the body under control so dentin reparative formation can be optimalized.

In this study, the stirring time is used as an indicator to figure out the correlation between particle size and level of calcium of beta-TCP nanoencapsulation synthesized from *Anadara granosa* shells with different time taken for each test groups.

MATERIALS AND METHODS

Synthesization of beta-TCP

The powder making of beta-TCP started with thoroughly cleaning *Anadara granosa* shells without soap or whitening product and grinded the shells using mortar and pastle. The powder sieved using 200 mesh sieve. 10 grams of sieved *Anadara granosa* was mixed with 100 ml of aquadest and 6.9 grams $NH_4H_2PO_4$ and stirred in magnetic stirrer for 30 minutes. The homogenous mixture kept in a reactor inside electric oven with 200°C temperature for 18 hours. Reactor then cooled down in room temperature before repeatedly washed using aquadest in magnetic stirrer until sample got separated from aquadest and reached pH 7. Last wash done with methanol to limit the agglomeration of beta TCP particle during sample drying. Sample was dried in electric oven in 50°C temperature for 3 hours and sintered in 900°C temperature for 3 hours to remove the impurities and enhance the crystallinity of the sample to produce active substance beta-TCP (8). After beta-TCP acquired, encapsulation from polymer of natrium alginate and $CaCl_2$ crosslink was proceed.

Nanoencapsulation of beta-TCP

A 0,5 grams of beta-TCP powder mixed with 50 ml of aquadest in magnetic stirrer until sample homogenous then 0,5 grams of natrium alginate added into beaker glass during the stirring in magnetic stirrer until completely mixed. Drop by drop of 0.5 grams of $CaCl_2$ liquid dissolved in 25 ml of aquadest added slowly into the previously processed homogenous beta-TCP and natrium alginate liquid in the magnetic stirrer until all the liquid mixed in. During the stirring, sample divided into 6 test groups according to the stirring time, P1 group for 1 hour, P2 group for 2 hours, P3 group for 3 hours, P4 group for 4 hours, P5 for 5 hours and P6 for 6 hours. All samples centrifuged at 2500 rpm speed for 6 minutes. Filtrates were thrown away and the sendiments were kept. The acquired sendiments saved inside freezer overnight and freeze-dried for 12 hours.

Particle size analysis

PSA characterization of encapsulated beta-TCP aims to determine the particle size using VASCO-*Particle Size Analyzer* Tool. 1 gram of encapsulated beta-TCP dissolved into 8 ml of aquadest and sonicated for 10 minutes to get a homogenous sample. Samples was placed in disposable plastic cuvets accordingly. Sample was measured with beta nanoparticle analyzer for 5 times per group. Optimum attenuator gap width is 6 to 8. Cloudy sample will be showing 6 in the attenuator, which means sample needs to be dissolved. Too transparent sample will show 8, means sample substance is needed to be added more.

Calcium level test with complexiometry

0.5 gram of sample dissolved in aquadest in volumetric flask until meniscus reached 100 ml. The next sample added with 2 ml of NaOH 8N and also Calcon indicator. Titration with 0.1N EDTA reagent carried on until the color changed from orange to blue. 0.1N EDTA titrant volume then noted down.

Statistical analysis

Data were analyzed using SPSS 23 produced by IBM®, US. ANOVA and post hoc test with significance level of P value= 0.05 were used in this study.

RESULTS

PSA test result showed various particle sizes. Decreased

particle size happened in 2 hours stirring group compared to 1 hour stirring group's particle size. However, particle size increased after 3 hours of stirring and is still increasing as a longer stirring time given to the other groups (Fig. 1).

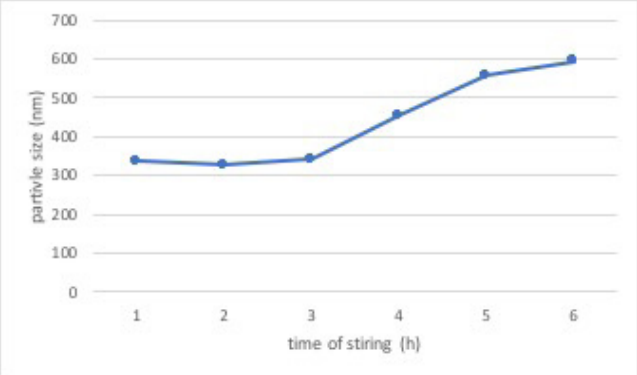

Figure 1: Particle sizes graphic of test groups according to stirring time

ANOVA test result was a significant difference (p= 0,000), of which Post Hoc Gomes Howell test result showed the significant differences between group P1, P2, P3 and group P4, P5, P6, also between group P5 and group P6 (Table I).

Table I: Result of Post Hoc (Gomes Howell) test of particle sizes data

GROUP	1	2	3	4	5	6
1		0.186	0.935	0.000*	0.002*	0.001*
2			0.141	0.000*	0.002*	0.001*
3				0.000*	0.002*	0.001*
4					0.036*	0.009*
5						0.769
6						

Calcium level test showed group P1, P2, P3 have higher calcium level than group P4, P5, P6 (Fig. 2).

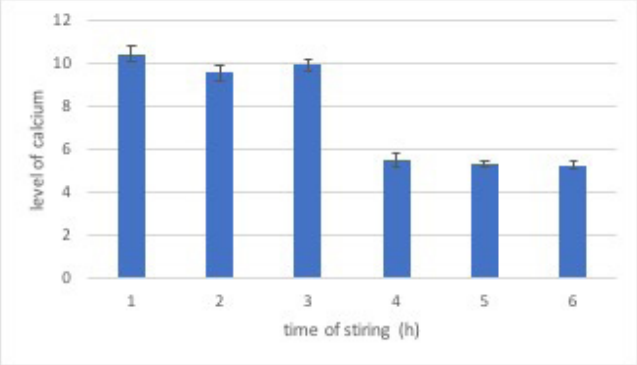

Figure 2: Calcium level of test groups according to stirring time

ANOVA test result showed a significant difference (p= 0.000), of which Post Hoc LSD test result showed the significant differences between group P1 and other groups, also between group P2, P3 and P4, P5, P6 (Table II).

Table II: Result of Post hoc (Gomes Howell) Calsium level

GROUP	1	2	3	4	5	6
1		0.002*	0.038*	0.000*	0.000*	0.000*
2			0.165	0.000*	0.000*	0.000*
3				0.000*	0.000*	0.000*
4					0.417	0.252
5						0.723
6						

DISCUSSION

Previous study by Sari (2018) shown that *Anadara granosa* shells hydrothermically processed for 12 hours and 3 hours of sintering produced 21% of beta-TCP content (4). Another previous study related to this study is by Istirafah (2012), which stated the amount of beta-TCP produced is increasing along with the longer stirring time is taken. Acquired beta-TCP content increased simultaneously the longer hydrothermic process time is given (14). A study by Suyadi (2010) showed high sintering temperature eliminated carbonate compound to attain beta-TCP compound (15).

The purpose of this study is to obtain a higher level of beta-TCP by modifying the method of previous study from 12 hours to 18 hours of hydrothermal process and 3 hours of sintering, thus a 79% composition of TCP is achieved.

Nano size particles have a higher ratio of surface area and volume compared to same material in bigger size, resulting of a more reactive particles. Reactivity of a material depends on the amount of atoms reacting to other materials on the surface area (16).

Homogenization of beta-TCP with polymers and crosslinker using magnetic stirrer keeps overall process under control in high speed, producing stable and homogenous particles without agglomeration to acquire stable nano particles after drying process (17,18).

In this study, encapsulation was done to help forming smaller particle sizes. Gardin etc. (2012) shared the ideal particle size of beta-TCP is about 50-500 nm. Beta-TCP in nano sizes able to increase cell adhesion, proliferation and differentiation for tissue fuctional system (19).

P1, P2 and P3 groups with one, two and three hours stirring time have the average smallest particle size, showing the approximate optimum mixing time with mean time of two hours. This result shows a similarity with study of Balavady (2014) which showed 2 hours of stirring time optimally disintegrated particle into the ideal size of nanoparticle without agglomeration (20). Similar to study by Dangi (2013) shows how the lack of stirring time creates an increased and non-ideal p agglomeration process of the particle (11). Encapsulated

beta-TCP particle sizes from synthesized *Anadara granosa* shells mixed with natrium alginate as pulp capping material of each test groups according to the stirring time had shown a significant difference. P2 group of beta-TCP *Anadara granosa* shells stirred together with natrium alginate for two hours acquired particle size most close to the pre-determined ideal particle size for pulp capping material.

Calcium level test is performed as the major component needed in dentin reparative formation for pulp capping is calcium (21). Calcium's primary function in the formation of bone and teeth (19). The result of this study showed P1 group had the highest calcium level.

Based on the result of the study, nanoencapsulation is a method to protect the main component in a relatively thin capsule. Nanoencapsulated component is easier to test and also works as controller of the component release as it controls the solubility of the product (22,23). However, nanoencapsulation might decrease calcium level of the active component as the capsule composition or the given polymer might affect the component unintentionally (24) as well as supposedly an effect of the crosslinker agent. Polymers and crosslinkers create a bond with the active component, thus might affect and decrease the amount of the components (25).

CONCLUSION

Anadara granosa shells hydrothermically processed for 18 hours and sintered for three hours produced 79% of beta-TCP. Encapsulated beta-TCP with natrium alginate stirred for one to three hours acquired average particle sizes most close to the ideal particle size for pulp capping material. Encapsulation of beta-TCP and natrium alginate decreased the level of calcium caused by the capsule composition or the given polymers.

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