SHORT COMMUNICATION

Morphological Characterization of Calcium Carbonate Nanoparticles Derived from Blood Cockle Shells (*Anadara granosa*)

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ABSTRACT

Blood cockle shells are a type of shellfish with distinctive bright red color that is commonly found in coastal areas of Asia, a rich source of protein, vitamins, and minerals, including calcium carbonate. Calcium carbonate ($CaCO_3$) is a common mineral supplement that slowly paved its way into nano research to support bone health that also used in the treatment of bone infections due to its inherent properties; reduces inflammation, promotes bone healing, crucial role in the immune system, and slow degradability. The latest invention on $CaCO_3$ from cockle shells, is an alternative mode of drug delivery to circumvent existing antibiotic resistance mechanisms and allow direct drug delivery to the target site, using locally sourced ingredients to reduce long-term high production costs. A top-down approach $CaCO_3$ nanoparticles from blood cockle (*Anadara granosa*) shells were developed, known as calcium carbonate nanoparticles (CNPs). This research aims to characterize the morphology of CNPs from blood cockle shells via Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Atomic Force Microscopy (AFM). A homogeneously spherical CNPs. with a mean diameter of 36.5 ± 2.3 nm was observed on TEM, so as FESEM. From AFM, the mean diameter for CNPs was 38.6 ± 3.6 nm The CNPs were spherical in shape, homogenously scattered, less agglomerate, porous with rough surfaces. The size and morphology of the CNPs from blood cockle shells were suitable for transport carrier in situ bone infection, according to the results.
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Keywords: Blood cockle shells; Calcium carbonate; Nanoparticles; Morphology; TEM; SEM; AFM

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INTRODUCTION

Piper sarmentosum Cockle shell or locally known as 'kerang' is one of the marine organisms with high calcium carbonate contents on their shells that are easily found in Malaysia. Blood cockle or its scientific name, *Anadara granosa*, can be effectively found in nearly all coastal zones of Malaysia. According to statistics by the Department of Fisheries Malaysia in 2011, about 57,544.40 tons of cockle were harvested along the west coast of peninsular Malaysia. Based on this value, can concluded how massive shells waste is generated (1). The shells were considered as waste once the flesh is eaten. This cockle industry has resulted in a significant amount of waste, in addition, if the shells waste were dumped irresponsibly, it will produce unpleasant smells and sites (2,3).

Regarding these matters, the recycling of waste products is necessary as the quantity of cockle shell waste has risen significantly over the years. Recycling reduces environmental pollution and protects natural resources, which are its key advantages. Pertinent to this, research is designed to maximize the potential usage of cockle shells in various sectors including medical.

Recent developments in advanced targeted drug delivery, tissue engineering, molecular imaging, and diagnosis have all seen significant increases in

the usage of nanoparticles in the biomedical field in recent years (4). Conventional ways of drug delivery were through subcutaneous, oral, intramuscular, or intravenous routes, which have resulted in medication distribution across blood capillaries, accumulating in specific concentrations at the target spot to perform therapeutic effects. However, it has been noted that poor bioavailability factors due to insolubility, hydrophobic nature, poor absorption, and quick metabolism of certain diseases, there is a paucity of the desirable of these drugs (5). In the quest to overcome these limitations, the fast-growing field of nanotechnology focuses on diverse exploratory ways of using natural products as a carrier for researchers in the field of biomedical and pharmaceutical sciences.

Calcium carbonate derived from cockle shells are common sources in aragonite polymorphic form, it is biodegradable and has slow decomposition (6). It also possesses a desirable large surface area, resulting in high loading capacity and encapsulation for carriers in drug-controlled release properties, compared to other organic nanocarriers such as dendrimers, micelles, and liposomes (7). Additionally, it has exceptional qualities for a drug delivery system, due to its unique characteristics in the dual application as nanocarrier and bone substitution in bone-related diseases (8).

This research is motivated to study distinctive morphological surface structure, sizes, and shape of CNPs derived from blood cockle shells under an electron microscope via Transmission Electron Microscopy (TEM), Field Emission Scanning Electron Microscopy (FESEM) and Atomic Force Microscopy (AFM). The experimental work presented here provides knowledge into how these natural biomaterials with simple processing methods and low-cost production can become a promising biomaterial as a carrier in drug delivery systems.

MATERIALS AND METHODS

Calcium carbonate nanoparticles (CNPs) were prepared according to our previous method of Fu et al. (9). However, some modifications were made to obtain the best results of nanoparticles. The blood cockle shells (1 kg) obtained from the local market were boiled at 100 °C for 15 minutes, then thoroughly cleaned with a brush and washed with clean water, and later dried in an oven (Memmert UM500, GmbH Co, Germany) at 50 °C for 5 days. In this study, the production of CNPs was improved by using a laboratory mixer (RT -80rpm 2500, Taiwan) and a planetary ball mill (2-Places Programmable Ball Mil, Dai Han Brand, Korea), in addition to the method used previously.

The micron-sized powder was then sieved through an automatic steel test sieve machine (Endecott Ltd., London, England), which includes test sieves with different apertures, starting with 90 µm, followed by 75 µm and 50 µm. The uniform micron size of the cockle powder is then converted into nanoparticles using Dodecyl dimethyl betaine (BS-12) (Sigma-Aldrich (St. Louis, MO USA), via mechanochemical technique according to our previously described method (10). Mechanically 2 g of micron powder with 50 ml deionized water (HPLC-grade of resistance >18 MΩ obtained from a MilliRO6 plus Milli-Q-Water System (Organex) and 0.5 ml of surfactant BS-12 at 1000 rpm at room temperature for 2 hr using heating homogenize stirrer machine (Systematic Multi Hotplate stirrer (Witeg, Wise Stir SMHS). Then filtered using filter paper size 12.5 cm (Filtres Fioroni, China) and dried in an oven at 50 °C in a sterile container before use. The dried nanosized powder was mechanically rolled again using roll mill machine (Wisd® Ball Mill, Korea) at 200 RPM for 120 hr. The final powder fraction was packed in polyethylene plastic bags for further use. The nanoparticle powder was later physically characterized by FESEM, TEM, and AFM.

Transmission Electron Microscopy (TEM)

Characterization of the synthesized CNPs was done using Transmission Electron Microscopy (TEM) (HitachiH-7100, Japan). The nanoparticles powder was first put into micro vials containing absolute alcohol, then sonicated using (Power Sonic 505, S Korea for 30 minutes. Later, 5µL of the sonicated solution would be pipetted out onto an Ultrathin Carbon Film/Holey Carbon 400 mesh copper grid (Ted Pella, Inc.) and dried at an ambient temperature of 37 °C overnight. The magnification measurement was operated at a voltage of 150 kV (10).

Field Emission Scanning Electron Microscopy (FESEM)

The surface morphology, agglomeration, and size of the aragonite CNPs nanostructure was then examined using a Field Emission Scanning Electron Microscopy FESEM, (Hitachi 4700 SEM/EDX) operated at a voltage of 5 kV. All samples were dispersed onto carbon conductive adhesive, then coated with gold using a magnetron sputter coater from Emitech Inc for 1 min before being examined under the electron microscope and detected automatically using IQ Materials Image Analysis software (17).

Atomic Force microscopy (AFM)

AFM analysis was used to check the particle size, shape, and surface of the nanoparticles. For AFM analysis, each sample was sonicated for 10 mins, followed by centrifugation at 15,000 rpm for 10 mins. Then, a small thin film of the pellet on a glass slide was prepared and examined under an electron microscope.

RESULTS

Transmission Electron Microscopy (TEM)

The shapes of the CNPs nanoparticles appear to be polydisperse, with some having well-defined edges

and others being more irregular in nature as seen in Figure 1. In some instances, TEM images may also reveal the presence of nanoparticle aggregates or clusters. Photomicrograph of Figure 2 shows the of CNPs after vigorous mixing in BS-12 and roller mill, showing spherical nanoparticles with an average size of 36.5 ± 2.3 nm with minimal clamping. The TEM images show that the surface of the CNPs nanoparticles possesses a textured appearance, such as ridges, grooves, or irregularities as an additional roughness and contribute to the overall morphology of the nanoparticles as shown by the arrow.

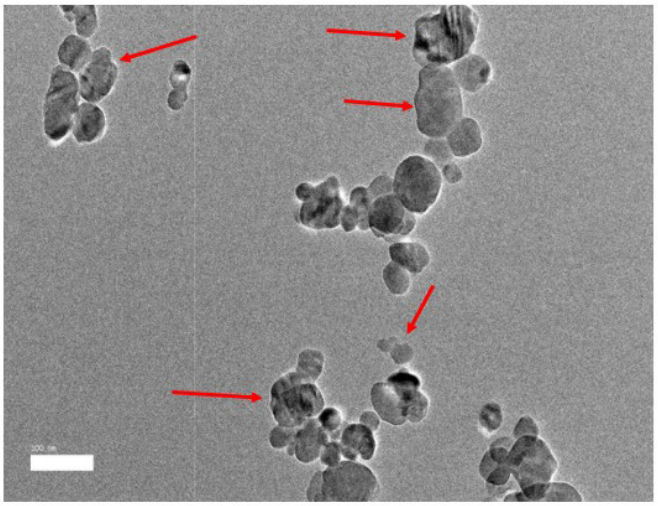

Figure 1 : The photomicrograph of CNPs via TEM with minimal aggregation, irregular surface and pores.

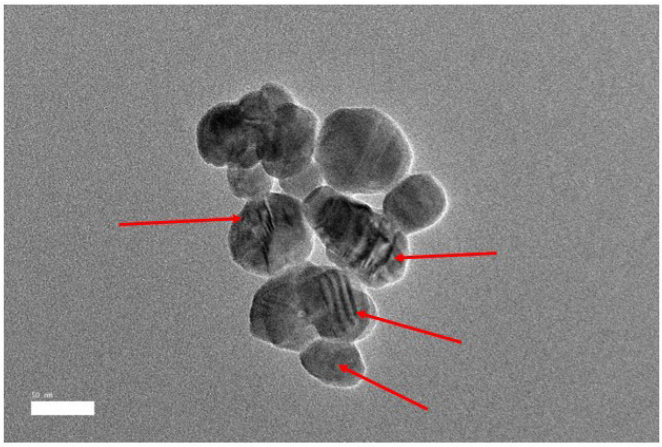

Figure 2 : The photomicrograph of homogenous cockle-shell derived calcium carbonate nanoparticle CNPs on transmission electron microscope (TEM) demonstrating it nanoparticle size with an average diameter size of 36.5± 2.3 nm size with irregular surface and pores after vigorous mixing in BS-12 and roller mill.

Field Emission Scanning Electron Microscopy (FESEM)

The morphology of CNPs before and after adding BS-12 with a vigorous roller mill is shown in Figure 3. Micron size CaCO3 with diameters ranging from 50 -500 nm were produced in the absence of BS-12. Structurally, the individual micron particles were cubic in shape, blunt vertically, and had smooth edges. They were also partially or completely overlapping (clumping features).

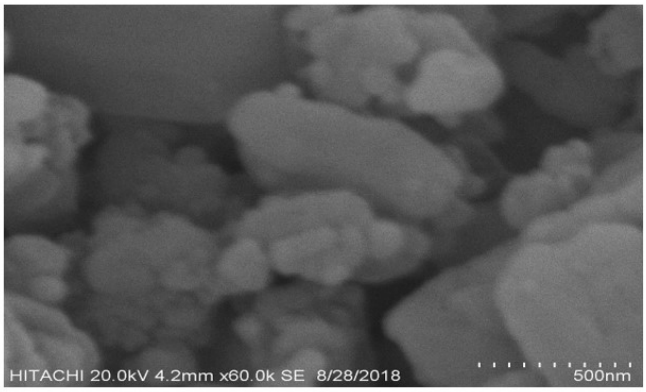

Figure 3 : SEM photomicrograph of micron size and cubic shape $CaCO_3$ in the absence of BS-12.

A sample of CNPs exhibited spherical, well-dispersed crystals with even size as shown in Figure 4 after adding BS-12 and vigorous roller mill. The CNPs prepared had an average size of 39 ± 3 nm showing homogenous spherical shape nanoparticles similarly seen on TEM. The nanoparticles exhibit a small range of sizes, ranging from approximately 30 to 50 nm. The FESEM images show that the surface of the CNPs nanoparticles is covered with intricate surface features including ridges, grooves, and spiky structures, giving the particles a rough appearance. Minimal aggregation and clustering of CNPs nanoparticles are also observed. There were also presence of small voids or openings on the surface of the nanoparticles called pores, contributing to the overall porous nature of the nanoparticles seen in TEM.

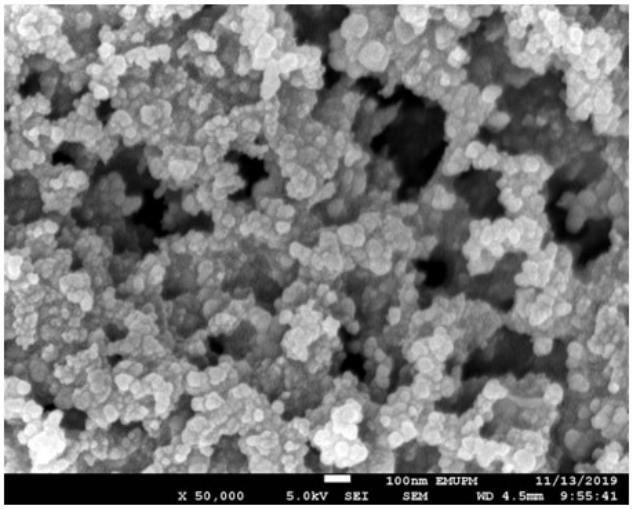

Figure 4 : FESEM photomicrograph displayed the homogenous spherical shape of CNPs.

Atomic Force microscopy (AFM)

Numerous uniformly sized and spherical shaped nanoparticles with rough surfaces, with a multitude of small protrusions and depressions, indicating a complex

and textured morphology were visible in the cross-sectional view of the surface morphology of CNPs as shown in Figures 5 and 6. The topography image also showing regularly shaped particles with varying heights. A size distribution analysis indicates a majority of CNPs particles were around 40 to 50 nm, with a relatively narrow size range. The nanoparticle distribution appears to be homogeneous across the scanned area, with only a few agglomerations observed. The AFM image reveals the presence of nanoscale pores or cavities on the surface of CNPs nanoparticles that irregularly distributed and have varying sizes and depths, which contribute to the nanoparticles' overall roughness. Occasionally, CNPs nanoparticles agglomerations are observed, but very minimal. While most of the nanoparticles appear to have relatively rough surfaces, a few instances of surface defects are observed including small cracks or scratches on the surface, which could have happened during the preparation process.

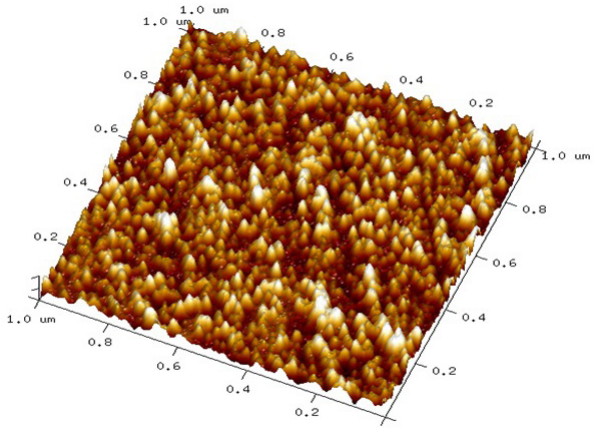

Figure 5 : AFM photomicrograph displayed rough surfaces homogenous spherical shape of CNPs.

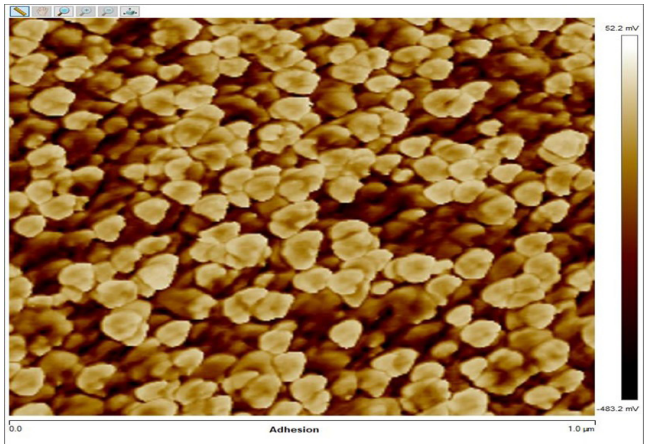

Figure 6 : AFM photomicrograph displayed the homogenous spherical shape of CNPs.

DISCUSSION

This study was done to create homogenous Calcium carbonate (CaCO3) nanoparticles CNPs derived from blood cockle shell (*Anadara granosa*) for possible use as a carrier in the medical industry drug delivery system. Previous research showed, the ideal physiochemical characterization and purity of the nanoparticles as a nanocarrier is a brilliant step in understanding therapeutic efficiency. The properties of the nanomaterial chosen play an essential role in controlling the drug release, resulting in efficient biodistribution and safety (10,11).

As reported by Lin et al (12), no specific guidelines were stated for characterization of nanomaterials, but the standard test used mainly to measure the size, shape, surface potentials, crystallity and chemical composition before therapeutic applications. TEM is extensively used for the particle size measurements of a wide range of nanomaterials. The main difference between FESEM and TEM is that SEM creates an image by detecting reflected or knocked-off electrons, while TEM uses transmitted electrons (electrons that are passing through the sample) to create an image. Therefore, TEM had information on the inner structure of the sample, such as crystal structure and morphology while SEM provides information on the sample's surface and its composition (13).

AFM is another imaging method to obtain nanoparticle measurements. While TEM imaging is used for length and width measurements, AFM is used to measure length and height of particles via a probe with a very sharp tip. AFM provides high contrast images compared to TEM and less detailed sample preparation process (14). Both methods have different advantages and disadvantages in terms of required expertise and time for sample preparation and imaging. In conclusion, there is no single test to characterize the nanoparticles that fit all the issues.

Based on my experiment, the size and shape of the synthesized CNPs nanocarrier concur with the finding of others (10,15,16). The difference between the present experiment with those researchers was the agglomeration rate. The previous study reported a high degree of agglomeration, compared to the current study. This might be due to the mechanical process via roller mill and carbonation processes in the presence of surfactant (BS-12) resulting in more homogeneous yet less agglomerate nanoparticles. Some authors (17,18) have also suggested that the presence of agglomeration was normal for (CaCO3) particles because of its hygroscopic nature.

The cubic shape with smooth edge, and micron size (CaCO3) particles as seen in TEM were due to the calcination process (thermal process) that change its shape into spherical after adding the surfactant of BS-12. This has also been explored in prior studies (19,20). In other words, the surfactant (BS-12), serves as an organic template that controlled the size, morphology, and crystal structure of synthesized (CaCO3) particles by expressing the catalytic function in facilitating the size reduction from micron to nanoparticles.

In this study, the CNPs showed a spherical shape, which in contrast with (21), reported a cubic shape, and (22) reported rod shape CNPs. The difference in shape was due to the type of polymorph that is present in limestone and the variety of synthesis procedures employed. It was reported in literature that, rhombic-shaped calcite has less of an impact on the quality of drug delivery materials. Concerning these issues, the spherical shape CNPs possesses a larger surface area for biological interaction in drug delivery (15), they can also flow freely in the blood vessel by avoiding the biological barrier (23) and suitable as a carrier. This has also been explored in prior studies (24), mentioning that spherical shape nanoparticles were 500% more efficiently taken by cell compared to rod shape, in view of more time needed for membrane engulfment in lengthy particles.

Other criteria for a good nanocarrier were the size of the nanoparticles. The size of CNPs produced in this study concurs with Kamba et al., (22) Isa et al., (16), and Jaji et al., (11). A longer time of body circulation and less hepatic filtration were demonstrated by nanoparticles smaller than 200 nm (25). In addition to that, small sizes particles will increase drug efficacy by promoting efficient interaction with cell membranes via the endocytosis system compared to larger particles (26). On the contrary, particles size less than 5nm most likely will be eliminated by the kidney if administered orally before reaching their target site, while particles > 200 nm get sequestered by both liver and spleen through the reticuloendothelial system. (27). The nanoparticles size obtained in this study falls within the acceptable range for an effective drug carrier in a release study.

A series of studies have indicated that porosity features in the nanocarrier are likely a credit for high loading and encapsulation percentage for a good potential carrier in drug delivery system, whereby it is an additional advantage for adsorption, loading ability as carriers and delivering the therapeutic agent to the target site (28-30). The porous structure and rough surfaces of calcium carbonate nanoparticles (CNPs) as seen in AFM were similar to the finding report of Lee et al., and Bharatham et al., (29,30). But CNPs in a recent study has less aggregation, due to differences in mechanical synthesis, attributed by mechanical grinding in a roller mill aided a better dispersion of the nanoparticles. Additionally, the porous and hollow surfaces may have enhanced interactions with biological body systems (22).

In summary, the novelty of these morphological research on calcium carbonate from cockle shell nanoparticles or CNPs involves the uniqueness of this natural abundant source as ground-breaking aspects of the study. Morphological features of CNPs in this experiment reveal less agglomeration rate, more homogenous spherical shape nanoparticles, sizes less than 200 nm that suitable for drug carrier and porous in nature for high encapsulation percentage.

CONCLUSION

The finding of this study conferred calcium carbonate nanoparticles made from blood cockle shells (CNPs) an easy-to-prepare nano biomaterial with promising morphological features that are efficient as a carrier in drug delivery systems.

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