Antibacterial Ability of Mesoporous Carbonated Hydroxyapatite

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Abstract. Mesoporous carbonated hydroxyapatite (Meso-CHA) is a bioceramic materials that offer good biocompatibility and bioactivity properties that suitable for bone defects or diseases treatment and therapy. Antibacterial study is very important to ensure that materials is biocompatible enough to be used on or inside the human body. The aim of this study is to investigate the antibacterial ability of the Meso-CHA (without addition of antibacterial agents) towards Escherichia coli (E. coli) bacteria. Meso-CHA samples were synthesized using chemical precipitation method where surfactant P-123 was used to introduce pores within nanoparticles structure. X-ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) analysis proved that pure phase of Meso-CHA was obtained. Synthesized Meso-CHA demonstrated higher pore properties (surface area = 146.92 m²g⁻¹, pore size = 2.35 nm, and pore volume = 0.2437 cm³g⁻¹) compared to commercial HA (surface area = 7.84 m²g⁻¹, pore size = 2.05 nm, and pore volume = 0.0085 cm³g⁻¹). The antibacterial study demonstrated that Meso-CHA has low antibacterial properties with Minimum Inhibitory Concentration (MIC) of 200 mg.ml⁻¹ compared to HA. Carbonate ion addition into the Meso-CHA structure does not improve the antibacterial ability of the materials.

1. Introduction
Bioceramic materials are one of the biomaterials that are widely used in various type of biomedical applications. Some of the examples are calcium phosphates, alumina, zirconia, and bioactive glasses [1]. Hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂, HA) is one of the most studied materials from the calcium phosphates family for application in biomedical field because of its good biocompatibility property. Moreover, HA possesses chemical similarity to bones and hard tissues components where it can form bonds with surrounding tissues which is suitable for bone implantation, bone coating, dental application, and drug delivery application [2-6].
Previously, several studies had demonstrated that porous bioceramics like porous HA has better potential in bone implants application rather than using dense HA. Materials with porous structure allows better bone ingrowth due to its bioactive property [2] and porous structure. Porous materials also offer better efficiency in drug delivery application with suitable pore characteristics [7, 8]. Porous material is an amorphous solids or crystalline that consists of empty pores, cavities, or channels within the particles where according to the International Union of Physical and Applied Chemistry (IUPAC), pore sizes can be divided into three categories which are micropore (<2 nm), mesopore (2-5 nm), and macropore (>50 nm) [9]. Materials with mesopore structure is said to has a good potential for drug delivery application where it offers smaller pore size, larger surface area, and higher porosity compared to macroporous material which has lower porosity and exhibit burst release profile [10].

The main target of the mesoporous bioceramic is to be used in the bone defects treatment and therapy. Thus, it is crucial to choose the materials that mimic closely with the bone tissue mineral component. The major inorganic composite of our natural bones and teeth is a biological apatite, the impure and non-stoichiometric version of HA [11] that contains several other ions such as CO$_3^{2-}$, Mg$^{2+}$, Na$^+$, Fe$^{2+}$, HPO$_4^{2-}$, F$^-$, and Cl$^-$. Therefore, carbonated hydroxyapatite that is nonstoichiometric, nanosized with low crystallinity and contain carbonate ion is a good alternative for any application, treatment and therapy related to bones.

Biocompatibility is the important aspect for biomaterials; this includes the cytotoxicity and antibacterial properties of the materials. It is important that the materials did not facilitate any bacterial infection and could also act as an antibacterial agent to prevent bacterial colonization on prostheses and implants [12]. Thus, this study aims to investigate the antibacterial properties of the in house synthesised mesoporous carbonated hydroxyapatite using Escherichia coli (E. coli) bacteria.

2. Methodology

2.1. Materials
Calcium nitrate tetrahydrate (Ca (NO$_3$)$_2$.4H$_2$O) and diammonium hydrogen phosphate ((NH$_4$)$_2$.HPO$_4$) were used as the calcium and phosphate precursor respectively. Carbonate ion was introduced into the structure with the addition of ammonium hydrogen carbonate (NH$_4$HCO$_3$). Non-ionic triblock copolymer Pluronics® P123, (PEO$_{34}$PPO$_{69}$ PEO$_{34}$) donated by BASF, USA was used as the surfactant for pores template. Sodium hydroxide (NaOH) was used to set pH of the mixture at pH 11 throughout the mixing process. Commercial HA, (HAP-200) obtained from Taihei, Japan was used as a control sample.

2.2. Preparation of Mesoporous Carbonated Hydroxyapatite (Meso-CHA)

Meso-CHA was synthesized using precipitation method. The Ca/P ratio was set to be 1.67. Surfactant-calcium ion mixed solution was prepared by dissolving 3 g of P123 in 100 ml of distilled water followed by the addition of 9.45 g (0.04 mol) of Ca (NO$_3$)$_2$.4H$_2$O. The solution was then stirred for 30 minutes until all solids were dissolved. Then, 3.17 g (0.024 mol) of (NH$_4$)$_2$.HPO$_4$ was dissolved in 60 ml of distilled water followed by the addition of 3.795 g of ammonium hydrogen carbonate (NH$_4$HCO$_3$); the mixture was called as phosphate-carbonate mixed solution. Subsequently, phosphate-carbonate mixed solution was dripped slowly into the surfactant-calcium mixed solution under continuous stirring, yielded a solution contain white precipitate. The alkalinity of the white solution was maintained at pH 11 by NaOH drops. The white solution then was aged in room temperature for 24 hours. Then, the white solution was centrifuged at 3000 rpm for 20 minutes to obtain the white precipitate. The precipitate washed and centrifuged repeatedly for five times with distilled water. Next, the white precipitate was dried in an oven at 100°C for 24 hours. The dried white precipitate was then ground into fine powder using a mortar and pestle and further calcined in a furnace at 550°C for 6 hours which hence completes the synthesis process of mesoporous carbonated hydroxyapatite powder.
2.3. Characterization
The phase and crystallographic structures of synthesized sample were identified by Rikagu Ultima IV, X-ray Diffraction (XRD) diffractometer using CuKα radiation (λ = 0.15406) over the range of 10° ≤ 2θ ≤ 90. The Fourier Transform Infrared (FTIR) analysis was performed using PIKE Technologies spectrophotometer in the range of wave numbers of 4000-400 cm⁻¹ for the identification of functional group that represent carbonated hydroxyapatite. Morphology of the Meso-CHA nanoparticles were observed using JSM-6490LV, JEOL Scanning Electron Microscope (SEM). Nitrogen adsorption-desorption analysis was conducted using Micromeritics TriStar to evaluate the surface area and pore characteristics of the samples. Specific surface area of the samples was calculated using Brunauer-Emmett-Teller (BET) while pore size distribution (PSD) of the samples was measured from the desorption data of the isotherms using Barrett-Joyner-Halenda (BJH) model.

2.4. Antibacterial Activity of Mesoporous Carbonated Hydroxyapatite
The antibacterial activity of mesoporous carbonated hydroxyapatite was conducted using the broth dilution method. Fresh overnight grown Escherichia coli (E. coli) bacteria was prepared and were transferred aseptically into distilled water before adjusting its turbidity to 0.5 McFarland consisting 1-2 × 10⁸ CFU/ml bacteria. Next, Meso-CHA powder were extracted in Tryptic Soy Broth (TSB) before continuing with two-fold serial dilution consisting of 10 different concentrations of sample solutions where TSB were also used as the broth medium for the test. After sample dilution, bacterial inoculum was diluted into TSB until it gives the concentration of 5 × 10⁵ CFU/ml bacteria before adding into the diluted sample solutions. Then, the solutions were incubated for 18 hours at 35°C. Minimum inhibitory concentration (MIC) of the solutions were measured using Genesys 20, UV-VIS spectrophotometer.

3. Results and discussions

3.1. Characterization of Meso-CHA Nanoparticles
The XRD pattern of all sample in Figure 1 are attributed to CHA reflections and no characteristic peaks of impurities are observed which are well consistent with PDF Number 01-089-7834 with lattice constant of a = b = 9.3892 Å and c = 6.9019 Å with space group of hexagonal P6₃/m. The three main characteristic peaks of CHA at 2θ = 25.8°, 31.8°, and 33.0° that corresponding to diffraction plane of (002), (211), and (300) are clearly observed in the XRD pattern of both samples. The XRD peaks of Meso-CHA is broader compared to HA that is narrower and sharper especially at diffraction peak of plane (211), (112), (300), and (202). This result indicate that Meso-CHA is less crystalline compared to HA sample. Material with lower crystallinity will degrade faster than high crystalline materials. This characteristic is appropriate for Meso-CHA since it is targeted to be applied as bone scaffold or drug delivery carrier which need to be degrade at certain period of time.

![Figure 1. XRD patterns of Meso-CHA and commercial HA](image-url)
Figure 2 shows the FTIR spectrums of Meso-CHA and HA. The absorption peaks at 1041.74 cm\(^{-1}\) and 1044.71 cm\(^{-1}\) attributed to the stretching vibration (v\(_3\)) of the phosphate (PO\(_4^{3-}\)) groups for Meso-CHA and HA, respectively. While, the absorption peaks at 572.23 cm\(^{-1}\) and 573.90 cm\(^{-1}\) are corresponding to bending vibration (v\(_4\)) of the phosphate (PO\(_4^{3-}\)) groups Meso-CHA and HA, respectively. The characteristic bands of B-type CO\(_3^{2-}\) substitution are observed at 880.6 cm\(^{-1}\) and 1474.66 cm\(^{-1}\) for Meso-CHA while commercial HA at 882.2 cm\(^{-1}\) and 1449.10 cm\(^{-1}\) [13]. Even though CO\(_3^{2-}\) ions are present in both samples, Meso-CHA has more intense carbonate bend compare to HA. This result is due to the addition of carbonate ion during the synthesis process. The broad bend at 3773.38 cm\(^{-1}\) (Meso-CHA) and 3465.18 cm\(^{-1}\) (HA) corresponding to OH group [14, 15].

![Figure 2. FTIR spectra of meso-CHA and commercial HA](image)

3.2. Morphology of Meso-CHA Nanoparticles

Figure 3 (a) and (b) shows the SEM images of Meso-CHA and HA samples at 20,000× magnification. SEM images revealed that Meso-CHA consists of fine spherical nanoparticle and highly agglomerated (Figure 3(a)). Unfortunately, due to highly agglomerated nanoparticles, the spherical-shaped of Meso-CHA was not able to be seen clearly in the SEM image. Similarly, HA nanoparticles are highly agglomerated, however HA has rod-like structure that look like rice grains (Figure 3(b)). The agglomeration in both sample is mostly caused by the Van der Waals force between the nanoparticles [28].

![Figure 3. SEM images of (a) Meso-CHA and (b) HA](image)
3.3. Pore Characterisation

Figure 4 (a) shows the nitrogen adsorption-desorption isotherms of the samples. Meso-CHA exhibit Type IV curve with H1 hysteresis loop after the calcination, and a well-defined step at approximately P/P0 0.89 to 0.99, implying a small pore size distribution which is often observed at agglomerates or compacts of approximately uniform spheres in a fairly regular array [16]. Meso-CHA surface area ($S_{\text{BET}}$) is 146.92 m$^2$g$^{-1}$. Contrarywise, CHA sample showed a Type II character indicates that non-porous particles were produced after the calcination with a low specific surface areas ($S_{\text{BET}}$), of 7.84 m$^2$g$^{-1}$.

Table 1 shows that surface areas ($S_{\text{BET}}$), pore size and pore volume of Meso-CHA is higher than HA. Pore size distributions of the samples are shown in Figure 4 (b). The pore size of Meso-CHA is mainly distributed at 2.32 nm which is falls within the mesopore range (2-50 nm). Meanwhile, HA exhibited pore size of 2.05 nm with a very low pore volume which is 0.0085 cm$^3$g$^{-1}$; that most probably due to inter-particulate pores (pores between the particles). This result strongly supported the isotherm result earlier that commercial HA is consisting of non-porous nanoparticles, while Meso-CHA consists of mesoporous nanoparticles.

3.4. Antibacterial Study

Figure 5 shows the antibacterial ability of Meso-CHA and HA. Based on the graph, Meso-CHA shows higher absorbance percentage value than the control (E. coli in broth) at most solution concentrations compared to HA. However, the highest Meso-CHA concentration which is 200 mg.ml$^{-1}$, the absorbance percentage of Meso-CHA sample solution was at its lowest which is at 77% with slight difference to HA which is 76%. Then, at sample concentration of 100 mg.ml$^{-1}$, the percentage of Meso-CHA sample solution increases abruptly to 101% compared to commercial HA which is 80%.
Figure 5. Result of antibacterial ability of meso-CHA and commercial HA

A higher absorbance percentage value indicates that the solutions have higher bacteria growth which will also cause the solution to be cloudy. So, the antibacterial ability of the sample will be low due to a larger number of bacteria grown in the solution and the Meso-CHA has low ability to inhibit the bacteria growth in the solution. On the other hand, a clearer solution will produce lower absorbance percentage value indicate that a smaller number of bacteria is present and it shows that the sample is able to inhibit bacteria growth.

In this study, the antibacterial ability of Meso-CHA was found to be low compared to commercial HA. This may be due antibacterial agent such as silver or copper ion was not introduced into the Meso-CHA structure despite of knowing that HA is a weak antibacterial material [17]. The main aim of this study is to investigate the antibacterial ability of Meso-CHA without the additional of metal ions to see whether it has a better antibacterial ability compared to commercial HA or not. Therefore, it can be concluded that Meso-CHA apparently has low antibacterial ability than commercial HA with the minimum inhibitory concentration (MIC) of 200 mg.ml$^{-1}$.

4. Conclusion

In conclusion, Meso-CHA with high surface area (146.92 m$^2$g$^{-1}$) was successfully synthesized through precipitation method using P-123 surfactant as pore template. The pore size of Meso-CHA is 2.32 nm which is within the range of mesopore sizes. Meso-CHA exhibit low antibacterial ability compared to HA. For future work, antibacterial agents such as silver or copper ion should be doped into the Meso-CHA to enhance antibacterial properties of the material.

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