Can Tho University Journal of Science

website: sj.ctu.edu.vn

Incorporation of amine functional group on surface of hydroxyapatite prepared fromstriped catfish (*Pagasianodon hypophthalmus*) bone

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Article info.	ABSTRACT

This study was conducted to incorporate amine functional groups on the surface of hydroxyapatite to enhance its potential application in biomedical materials. Hydroxyapatite particles (HA) were synthesized from striped catfish (Pagasianodon hypophthalmus) bone and surface modified by grafting with (3-aminopropyl)triethoxysilane (APTES). Important factors affected grafting efficiency such as concentration of APTES, water content, reaction temperature and reaction time were studied. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM) were used to confirm the grafting reaction on HA surfaces. Grafting efficiency was evaluated based on intensity of characteristic absorption peaks of APTES. The experimental results showed that amine functional groups were successfully introduced on HA surface at optimal condition of 0.2 M APTES, 0.75 wt.% water content, at 60°C and 12-hour reaction time.

1. INTRODUCTION

Hydroxyapatite (HA), a calcium phosphate mineral with a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ presents in both human bone and teeth. HA crystals is a bioactive ceramic cover 65 to 70% by weight of the bone and 70 to 80 wt.% of dentin and enamel. Nano HA is attracting interest as biomaterial for prosthetic applications due to its high biological compatibility, non-toxic, biodegradable as well as its similarity in size, morphology and chemical composition with human hard tissue (Haixin Zhao et al., 2011; Kim, Lee, & Knowles, 2006; Rivera-Muñoz & M, 2011; Ylinen, 2006). HA particles of different shapes can be obtained from pure chemicals or natural sources such as catfish bone, fish bone, seashells and egg shells (Sadat-Shojai, Khorasani, Dinpanah-Khoshdargi, & Jamshidi, 2013). HA can be combined with polymers such as collagen, to create scaffold materials used in tissue engineering or bone regeneration. However, due to its inorganic occuring, HA is inert and has poor interaction with chemical compounds or bio-components to be introduced in. Therefore, surface modification is essential to overcome this disadvantage. For examples, in order to graft collagen on the surface, TiO_2, Fe_3O_4, SiO_2, and HA need to be modified by 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) or N-hydroxysuccinimide (NHS).

Besides EDC and NHS, (3-aminopropyl)triethoxysilane (APTES), 3-amino trimethoxypropyl silane (APTMS) and other agents such as IOTA 5603 and IOTA 5910 are typical agents for anchoring amine groups on the surface of SiO_2 through the silanolization of trialkoxysilane $(R'O)_3Si-R-X$ (R is the hydrocarbon (C_xH_y) and X is the functional group to be created on the surface of materials) were applied to effectively create amine groups on the surface of SiO_2 by using silanolization of trialkoxysilane $(R'O)_3Si-R-X$. Where R is the hydrocarbon (C_xH_y), and X is the functional group to be created on the surface of materials. R' is usually methyl or ethyl group and R'O can be easily hydrolyzed in the presence of water to form silanol that formed covalent bonds with OH group on inorganic materials (Howarter & Youngblood, 2006; Pasternack, Rivillon Amy, & Chabal, 2008).

Due to the presence of hydroxyl groups, an amine functional group can be created on HA surface by using alkoxysilane amine agents such as APTES (Da Silva, Da Silva Filho, Da Fonseca, Arakaki, & Airoldi, 2006). The mechanism of grafting APTES on HA surface to create amine functional group was shown in Figure 1. In the presence of water, C_2H_5O- group of APTES was hydrolysed to NH_2-$(CH_2)_3Si(OH)_3$ with silanol groups. The silanol groups then reacted with OH^- of HA to form products such as SP1, SP2, or SP3 (Figure 1), and then amine groups were successfully produced on HA surface. These new introduced amine functional groups allowed the attachment of other bio-molecules to HA surface through nucleophilic reactions, which broadened the applications of HA in drug delivery, scaffold fabrication and adsorbent (Durrieu et al., 2004). The grafting APTES on HA surface is affected by major factors such as solvents, chemcial concentration, water content, reaction time, reaction temperature (Goonasekera, Jack, Cooper-White, & Grøndahl, 2013). Therefore, the study focused on the grafting of APTES on HA surface and parameters influencing the process. The HA was synthesized from catfish bone according to the method mentioned by previous study (Ho, Tao, Huynh, & Wang, 2020).
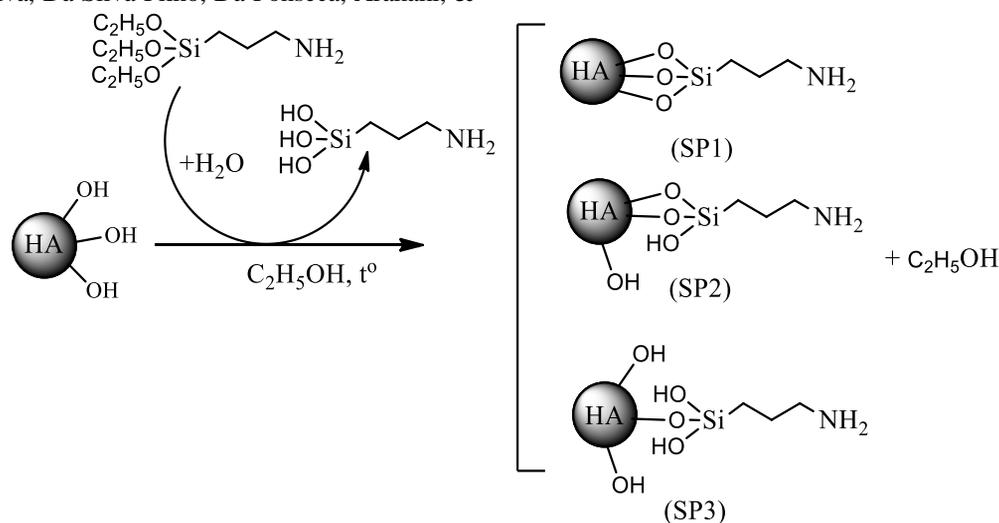
Figure 1. Grafting mechanism of APTES onto hydroxyapatite surface

2. THE EXPERIMENT

2.1. Chemicals

Catfish bone (*Pangasius hypophthalmus)* was provided from a local fish fillet processing factory. Chemicals such as NaOH, H_3PO_4 and NH_3, APTES were supplied by Merck (Vietnam).

2.2. Preparation of hydroxyapatite from catfish bones

Fish bone was boiled in water for 4 hours to remove adherent fish meat. Clean bone was dried, cut into small pieces and subjected to 0.1 M NaOH solution for 24 hours. The treated bone was washed with distilled water and ethanol to remove remaining fats and other impurities, dried and calcined at 900°C for 6 hours in a furnace. The calcined bone was ground into powder with 5-10 μm size range by high-speed ball mill. 10 grams of fish bone powder was then subjected to 60 mM H_3PO_4 aqueous solution with the ratio of 1/10 (g/mL). Reaction was carried out at 90°C, for 2 hours, at pH 11 (NH_3 was used to control solution pH). After the reaction, the

mixture was cooled to room temperature and aged for 24 hours before separating by vacuum filter. The sample was dried at 100°C and continuously calcined at 1000°C in 2 hours to stabilize the structure of HA product. X-ray diffraction (XRD) and scanning electron microscopy (SEM) were applied to characterize HA.

2.3. Hydroxyapatite surface grafted APTES

APTES was grafted onto the surface of HA surface to create amine functional groups based on the reaction mechanism described in Figure 1 (Da Silva et al., 2006). The incorporation of amine functional groups controlled the crystal dimensions and favours osteoblast proliferation, the activation of osteoblast metabolism and differentiation, which are all of high importance for potential biomedical applications. APTES was firstly hydrolysed in water to create silanol functional groups [NH_2-$(CH_2)_3Si(OH)_3$]. The silanol groups then reacted with the hydroxy groups (OH) of HA to form chemical bonding and finally APTES grafted on HA surface. Typically, experiments was conducted following the procedure: HA was first dispersed in pure ethanol with the concentration of 1/25 (g/mL) contained in bottle holder and applied in an ultrasonic bath for 5 minutes. The mixture was then stirred at 300 rpm in a water bath. APTES and water was added to the suspension with different concentration. After the reaction, the mixture was vacuum filtered and subsequently washed 3 times with distilled water and ethanol to obtain HA grafted APTES. The sample was then dried at 60°C for 24 hours to obtain final product. Important factors affecting grafting reaction such as APTES concentration, water content, reaction temperature and reaction time were investigated

2.4. Characterization methods

Fourier-transform infrared spectroscopy (FT-IR, Thermo Scientific Nicolet 6700 FT-IR Spectrometer, United States) was used to evaluate the grafting of APTES on HA surface. The sample was mixed with KBr powder. The mixture was subsequently ground for 3-5 minutes and pressed into a pellet. The spectra were recorded from 4000 to 400 cm^{-1} at a data acquisition rate of 1 cm^{-1} per poin. The resulting spectra were analysed by using OMINIC software.

3. RESULTS AND DISCUSSION

3.1. Synthesis of hydroxyapatite from catfish bone

After pretreatment with 0.1 M NaOH aqueous solution for 24 hours and calcination at 900°C for 6 hours, the catfish bone became whiter and lost around 45 wt.%. The treated catfish bone was then ground into small powder, reacted with H_3PO_4 60 mM aqueous solution at 90°C, pH 11 for 2 hours and calcined at 1000°C in 2 hours to obtain the final HA product (Figure 2a). The synthesized HA product was characterized by XRD diffraction. Typical peaks at 2θ (degree) at 10.8, 25.9, 28.9, 31.9, 32.2, 32.9, 34.1, 39.8, 46.7, 48.1, 49.5, 50.6, 51.3, 52.1 and 53.2 of HA sample was as same as those of the commercial one (Figure 3a). In addition, SEM analysis showed that HA particles have asymmetric spherical shape with size of about 1000 nm, (Figure 2b). FTIR spectra of the HA particles (Figure 3b) also showed the characteristic absorption bands at 1046 cm^{-1}, 962 cm^{-1}, 601 cm^{-1}, and 571 cm^{-1} which are assigned for PO_4^{3-} of hydroxyapatite while peak at 3571 cm^{-1} corresponds to the stretching vibration of hydroxyl groups of HA surface. It can be stated that HA was successfully prepared from catfish bone.
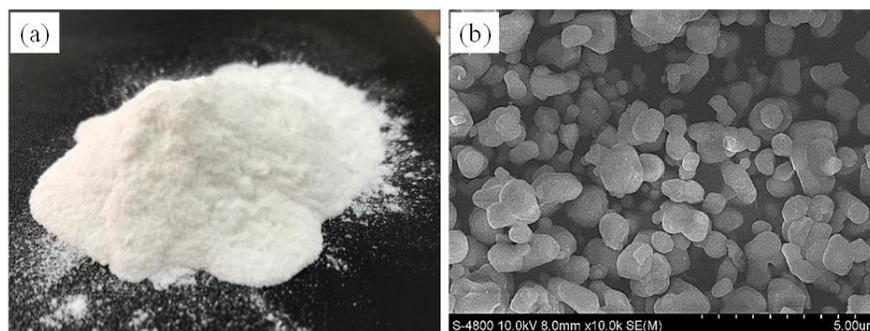
Figure 2. Hydroxyapatite powder prepared from catfish bone

(a) synthesized HA powder and (b) SEM image of synthesized HA powder. Reaction condition: Catfish powder/H₃PO4 = 1:10 g/mL, H₃PO₄ (60 mM), pH 11, reaction temperature 90°C and calcined temperature 1000°C for 2 hours
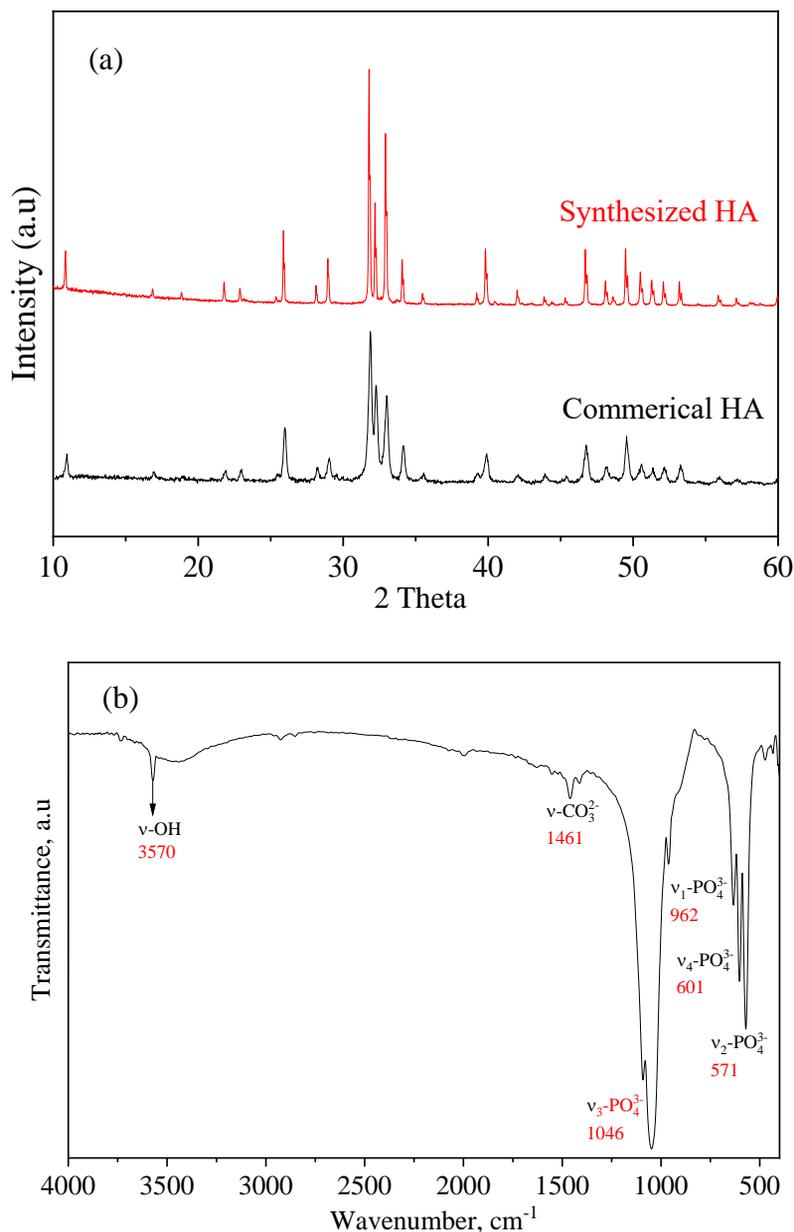
Figure 3. Characterization of hydroxyapatite product prepared from catfish bone

(a) XRD diffraction and (b) FT-IR spectrum. Reaction conditions: catfish powder/H_3PO_4 = 1:10 g/mL, H_3PO_4 (60 mM), pH 11, reaction temperature 90°C and calcine temperature 1000°C for 2 hours

3.2. Effect of APTES concentration on grafting efficiency

To investigate the effect of APTES concentration on grafting yield, different concentration of APTES was used. FT-IR spectra of HA sample showed characteristic absorption peaks of at 3570 cm^{-1} (v-H-O), 1046 cm^{-1} (v_3- PO_4^{3-}), 962 cm^{-1} (v_1-PO_4^{3-}), 601 cm^{-1} (v_4-PO_4^{3-}) and 571 cm^{-1} (v_2-PO_4^{3-}) which were correspondingly to HO$^-$ and PO_4^{3-} of HA (Balasundaram, Sato, & Webster, 2006; Liu et al., 2011; Motskin et al., 2009; Santos, Oliveira, Souza, Mansur, & Vasconcelos, 2004; Wang et al., 2011) (Figure 3b). APTES grafting led to the presence of a new peak at 2929 cm^{-1} (v-C-H), 1465 cm^{-1} (δ-C-H), 3400 cm^{-1} (v-N-H), 550 cm^{-1} (δ-N-H), and 1410 cm^{-1} (v-Si-CH$_2$). Furthermore,

there were also a peak at 471 cm^{-1} which was ascribed to the band of ν-Si-O-Si due to self-polymerization of APTES (Figure 5) (Barakat, Khil, Omran, Sheikh, & Kim, 2009; Howarter & Youngblood, 2006). The peak located at 1465 cm^{-1} is known as the overlap between (δ-C-H) and (ν-CO_3^{2-}) (Boerio, Armogan, & Cheng, 1980; Chang & Tanaka, 2002; Goonasekera et al., 2013) while absorption peak above 3700 cm^{-1} is assigned to stretching vibration of SiO-H of polymer (Culler, Ishida, & Koenig, 1985).

As expected, intensity of characteristic peaks between 1550 and 1410 cm^{-1} increased with an increasing APTES concentration. This suggested that the increase of APTES concentration provided better interaction between molecules, and therefore higher grafting efficiency on HA surface. This result had similar trends as grafting APTES on TiO_2 surface (Song, Hildebrand, & Schmuki, 2010; Zhang & Srinivasan, 2004). Figure 4 also showed that as APTES concentration increased, the intensity of Si-O-Si group (471 cm^{-1}) increased. The presence of new peaks compared to HA spectra confirmed that APTES was successfully grafted on HA surface. Highest absorption intensity of characteristic peaks was obtained at APTES 0.2 M.
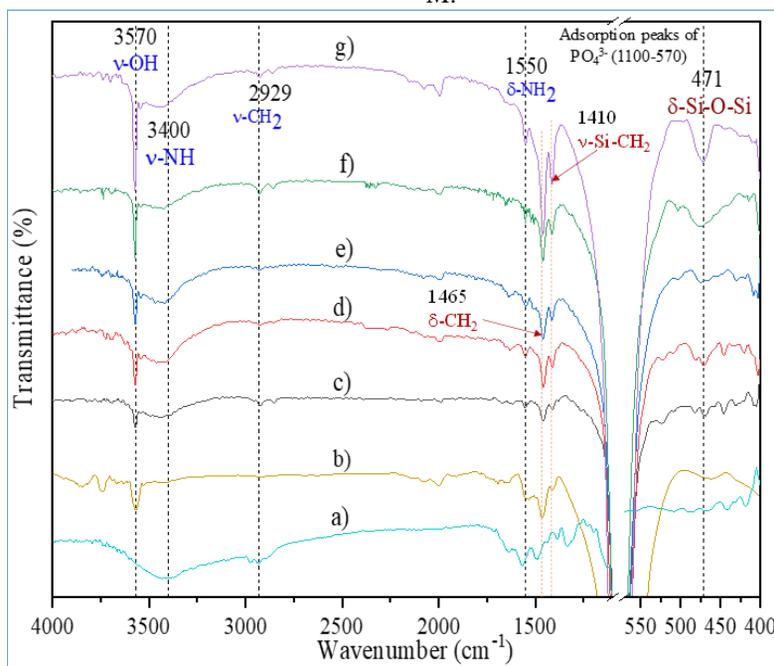
Figure 4. FT-IR spectra of (a) APTES, (b) HA from catfish bone, (c-g) has grafted APTES with different APTES concentration

(c) 0.01 M, (d) 0.05 M, (e) 0.1 M, (f) 0.15 M and (g) 0.2 M.

All experiments were fixed at HA/ethanol ratio 1:25 (w/v), 40 °C, 0.75 wt.% water content for 4 hours.
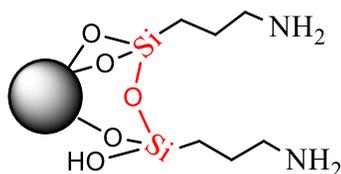
Figure 5. The formation of Si-O-Si bond

3.3. Effect of water content on grafting efficiency

Water took an important role in the hydrolysis of ethoxy groups of APTES molecules to form silanol groups to react with the OH group on HA surface (Pasternack et al., 2008). To investigate the effect of water content, experiments were conducted in different water content varied from 0 wt.% to 1.5 wt.%. Experiment condition was controlled at HA/ethanol ratio 1:25 (w/v), 40°C, 0.2 M APTES for 4 hours. Typical peaks characterized for HA and APTES were also obtained in FTIR spectra (Figure 6c-g). It was also notified that intensities of these peaks changed remarkably when the water content was changed. The intensity of NH_2 increased as a function of water content and reach a

maximum at 0.75 wt.% water content. The results agreed with the fact that more water would accelerate the hydrolysis of APTES ethoxy group, *allowing* the resulting *hydroxysilane* to *attach* on the HA surface (Engelhardt & Orth, 1987; Howarter & Youngblood, 2006). However, when the water content was higher than 0.75 wt.%, self polymerization of APTES (Figure 7a) was preferred leading

to the formation of coating layer on HA rather than grafting (Damia, Sarda, Deydier, & Sharrock, 2006; Song et al., 2010; Zhang & Srinivasan, 2004). In the absence of water, grafting of APTES would be occurred between hydroxyl group of HA and amine group of APTES, therefore, no amine groups could be formed on HA surface (Figure 6b) (Song et al., 2010; Zhang & Srinivasan, 2004).
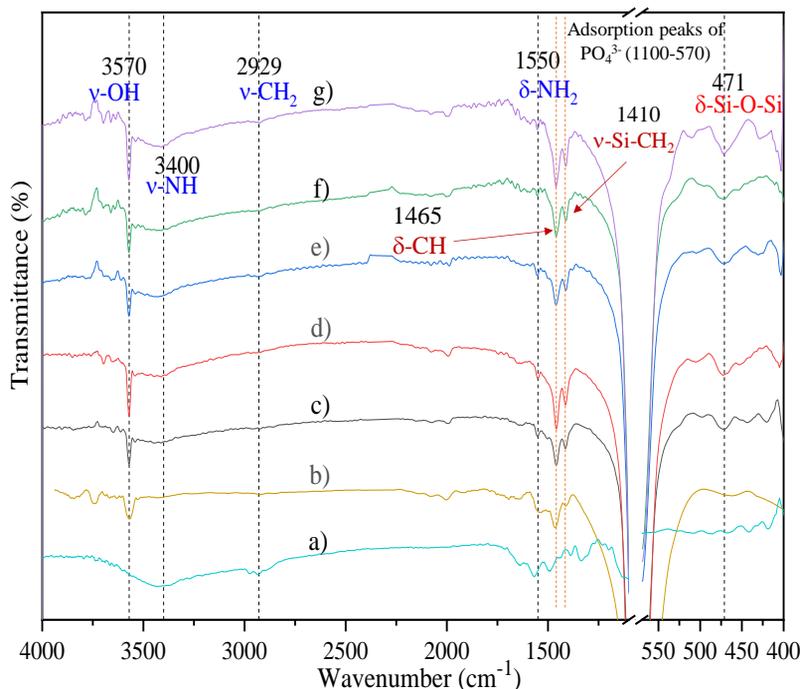
Figure 6. FT-IR spectra of (a) APTES standard, (b) HA from catfish bone, HA-APTES samples, prepared with different water content: (c) 0.5%, (d) 0.75%, (e) 0.1%, (f) 1.25% and (g) 1.5%

Experiment conditions: HA/ethanol ratio 1:25 (w/v), 40 °C, APTES 0.2 M, 4 hours.
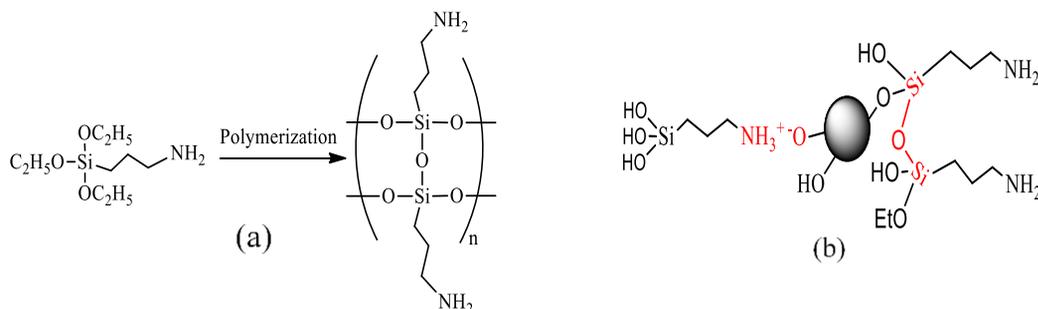
Figure 7. Formation of ionic bonds between amine group of APTES and hydroxyl group of HA (a) and polymerization of APTES to form a coating layer on HA surface (b)

3.4. Effect of reaction temperature on grafting efficiency

To investigate the effect of temperature on grafting efficiency, experiments were conducted at different temperature ranged from 30°C to 70°C with HA/ethanol ratio 1:25 (w/v), water content 0.75%, APTES 0.2 M for 4 hours. FTIR analysis confirmed the grafting of APTES on HA surface with characteristic peaks of HA at 3570 cm^{-1}, 1046 cm^{-1}, 962 cm^{-1}, 601 cm^{-1} and 571 cm^{-1} as well as for APTES at 3400 cm^{-1}, 2929 cm^{-1}, 1465 cm$^-$, 550 cm^{-1} and 1410 cm^{-1} (Figure 8c-g). The intensity of absorption peaks at 1550 cm^{-1}(NH$_2$), 2929 cm^{-1}(CH$_2$) and 3570 cm^{-1} (ν-SiO-H) increased with increasing temperature. This suggested that grafting efficiency increased with the increase of temperature. The highest intensity was obtained at reaction temperature of 70°C. However, higher temperature led to a reduction in peak intensity (ν-SiO-H) at 471cm^{-1} (Figure 8g). This phenomenon can be explained by collision theory that raising the reaction temperature would bring more collisions, hence increase reaction rate i.e. grafting efficiency of APTES on HA. This result is entirely consistent with the study of Pasternack *et al.* (Pasternack et al., 2008). In addition, it was recommended that grafting temperature should not exceed 70°C since it is close to the boiling point of ethanol (78°C).
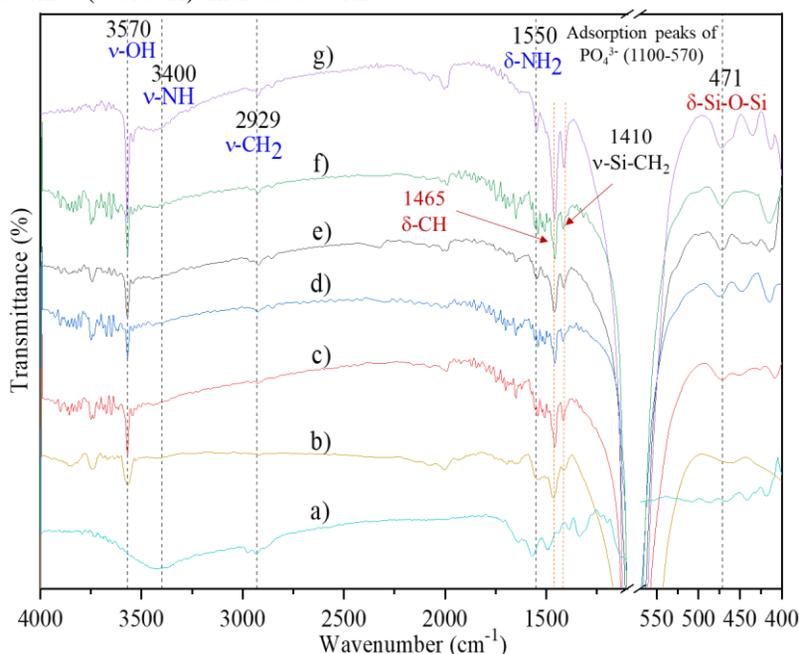
Figure 8. FT-IR spectra of (a) APTES standard, (b) HA from catfish bone, HA - APTES samples, prepared at different temperature: (c) 30°C, (d) 40°C, (e) 50°C, (f) 60°C and (g) 70°C

Experiment conditions: HA/ethanol ratio 1:25 (w/v), APTES 0.2 M, 0.75 wt.% water content for 4 hours.

3.5. Effect of reaction time on grafting efficiency

To understand the effect of reaction time on grafting efficiency, the experiments were conducted at different intervals of time from 2 to 24 hours. All experiments were performed at HA/ethanol ratio 1:25 (w/v), water content 0.75%, APTES 0.2 M, and reaction temperature 70°C. Typical absorption peaks of HA and APTES were identified in FTIR spectra (Figure 9). Data obtained also showed that the intensity of absorption peaks increased with increasing reaction time. Especially, there was a sharp increment in the intensity of -NH$_2$ peak at 12 hours. However, intensity of characteristic absorption peaks is no different when reaction time is raised to 24 hours. It can be clearly understood that prolong reaction time would increase NH$_2$-(CH$_2$)$_3$Si(OH)$_3$ concentration, increase the formation of HA-O-Si bond, therefore increasing grafting efficiency. However, longer reaction time could also cause self-reaction of silanol groups (Si-OH) to form Si-O-Si bonding or self-polymerization of APTES, resulting in a covered film on HA surface. These observations were confirmed by the increase in absorption intensity at 471 cm^{-1} (Song et al., 2010)

as well as SEM images of HA-APTES samples (Figure 9). No significant difference between the surface of pristine HA and HA-APTES grafting in 2 hours was found (Figure 10a and 10b) while there was a covered layer of APTES on HA surface accompanied by an increment in HA particle size after 24 hours grafting (Figure 10c).
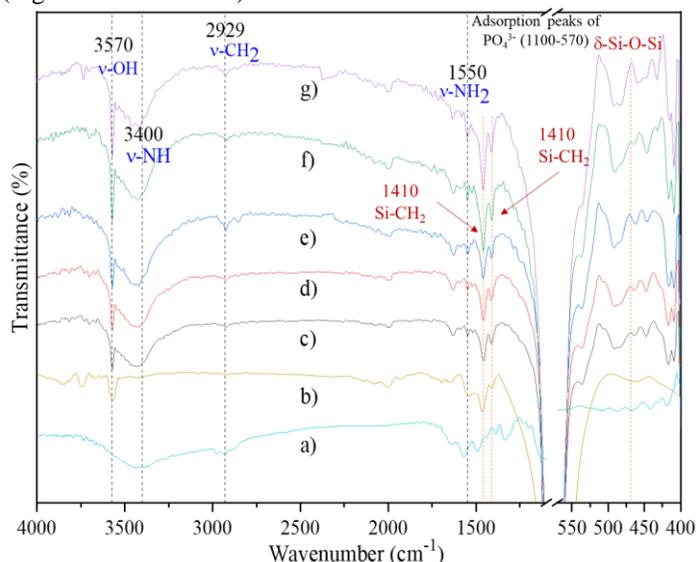
Figure 9. FT-IR spectra of (a) APTES standard, (b) HA from catfish bone

HA - APTES samples were prepared with different time: (c) 2 h, (d) 4 h, (e) 8 h, (f) 12 h and (g) 24 h. Experiment conditions: HA/ethanol ratio 1:25 (w/v), APTES 0.2 M, 0.75 wt.% water content.
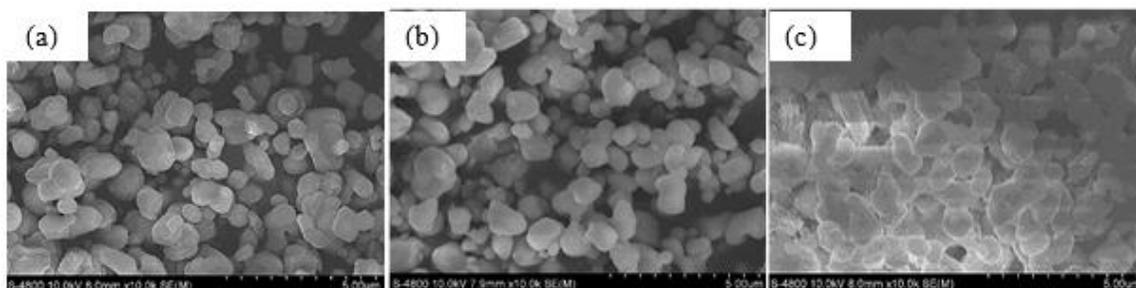
Figure 10. SEM images of (a) HA from catfish bone; (b) HA-APTES samples were prepared at 2 hours and (c) 24 hours

Experiment conditions: HA/ethanol ratio 1:25 (w/v), water content 0.75%, APTES 0.2 M, reaction temperature 70 °C.

4. CONCLUSION

Hydroxyapatite particles with size around 1000 nm were succesfully prepared from catfish bones treated with 60 mM H_3PO_4 solution, at a catfish powder/H3PO4 of 1:10 g/mL, pH 11 and 90°C followed by calcination at 1000°C for 2 hours. Incorporation of amine functional groups into HA particles was determined by grafting efficiency of APTES to HA surface. Experimental results showed that APTES was successfully grafted on HA under reaction condition of 0.2 M APTES solution, 0.75 wt.% water content, at 70 °C for 12 hours.

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