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Bioactivity Evaluation of Bio-Hydroxyapatite Derived from Spanish Mackerel in Composite Scaffolds for Bone Tissue Engineering

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Abstract. This research focuses on the interaction between fish bone extracted hydroxyapatite and polyvinyl alcohol as the composite material of a scaffold with the ions presented in a simulated body condition to study on the composite's bioactivity and biodegradability. This was achieved by synthesizing scaffold with different amount of hydroxyapatite particles using the solvent casting method and with that done, the scaffolds were soaked in simulated body fluid for 7 and 14 days. Studies and investigations were done based on the weight difference before and after immersion of samples in simulated body fluid and the FTIR characterization of the samples before and after immersion. The weight analysis of the samples showed that along with degradation of the scaffolds as time passes, degradation of scaffold material and formation of deposits were present. The degradation of materials is due to the bioactivity reaction between the simulated body fluid and the scaffold where PVA is resorbed by the water contents and ions in relation to apatite in the fluid bonded onto the HAp contents in the scaffold. The most optimum composition for a long period of degradation and regeneration of apatite amongst the compositions studied was determined to be that with 100 phr PVA and 10 phr HAp. The FTIR results obtained also showed that the functional groups of both PVA and HAp exist in the samples before and after immersion as the absorption band for both the materials were present. The bands shifted had clearly shown that the amount of HAp related bonds had increased in amount. All in all, the results showed that fish bone extracted hydroxyapatite formed composite with PVA is a low cost biocomposite which could be a potential substitute of metal in bone grafting and a potential development in bone tissue engineering.

INTRODUCTION

Bone grafting is used when bone repairing, or bone rebuilding is required due to fractures or diseases [1]. The current technology uses metal steel such as stainless steel and titanium to conduct such implantation. To ease the process of bone implantation, the aim is that bone growths can be induced by planting a self-degradable bone scaffold into human bodies without having to remove the undegradable metal afterwards. However, the degradation period of bone scaffold is also a very important factor to consider. The bioresorption rate of the scaffold should be controllable as different bones requires different recovery period, such that spine bones should take a considerable 9 months for recovery while craniomaxillofacial osteosynthesis might take up to 6 months of recovery [2]. Hence, the degradation of bone scaffolds should be controlled within the desired period to ensure sufficient space and time required are provided for complete bone regeneration and recovery. The degradation period of the scaffold is expected to be linear to the desired timeframe. The said bone scaffold is also expected to be biocompatible with natural bones in human bodies for the formation of blood vessels so that nutrients supply around the area is provided and hence, the scaffold could be absorbed and regenerated by the body [3]. Meanwhile, the criteria that is expected of a bone scaffold should also be flexible and strong, imitating the extracellular matrix network in a human body which is made up of connective tissues. The expectations are such that scaffold should at least have a pore diameter of 100 μm [4] and the optimum range at 200 to 350 μm [5] for diffusion of oxygen and nutrients to encourage the regeneration of bone tissues [4]. In order to achieve this, the choice of scaffold material is very critical.

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The nature of bone is hydroxyapatite (HAp), which is made up of calcium phosphate with the molar ratio of calcium to phosphate as 1.67. HAp could either be synthetic or natural. Even though synthetic HAp is more commonly used in the field, studies had proven that natural HAp is better as it has a higher metabolic activity and gives better dynamic responses to its surrounding environment than synthetic HAp. Also, synthetic HAp requires many chemicals in addition to stabilize its structure and stay fixed in a human body. There are many sources for the extraction of natural HAp, such as egg or sea shells and animal bones. Fish bone which is low in cost with high abundance in market and could be easily fabricated into the desired powder form of HAp through calcination is one of the potential animal bones used to extract natural HAp. The abundance of fish bones comes from the food industry where there are more than 91 million tons of fishes consumed every year, producing a corresponding amount of fish bones as by-products of food processes annually [6-7]. Fish bones take up about 40 to 50 % of the total weight of fishes, making it a great amount of threat to the environment as pollution if not dealt with wisely. Recent studies on fish bones showing its potential to produce high quality bioengineering material with the calcium phosphate ceramic contents that are present in the bones [8]. Besides showing similar chemical properties with human bones, reports also show that fish bones are safe with lower disease transmission risk, making it desirable. However, scaffolds consisting of solely HAp do not possess compatible mechanical properties as they are very porous, dense and brittle despite having compatible bio-integration and cell attachment properties. Hence, it is usually necessary to further reinforce the scaffold's flexibility by combining the HAp with polymers.

Polymers that could be used for the composite mixing are usually from natural sources, such as chitosan [9], cellulose [10], collagen [11] and chitin [12] or from synthetic sources, such as polyvinyl alcohol (PVA), polyhydroxybutyrate [13], polyethyene glycol [14]. PVA being a synthetic polymer with high biocompatibility with human bodies at low toxicity and most importantly, low cost, has been commonly used as cartilage and for skin grafting. With the PVA's ability to produce covalent bonding with HAp particles mechanically to combine the two materials, more research has been focusing on the use of PVA in tissue engineering. Another reason to which PVA is being researched on is that tissue engineering is highly related to the regeneration of cells and PVA shows excellence in the few aspects below, pH stability, semi permeability and hydrophilicity, which are some of the very critical factors for the sustainment of cells [15].

The bioactivity of a scaffold is shown through the precipitation of hydroxyapatite after immersion in simulated body fluid (SBF) prepared using the Kokubo method [16]. SBF is a model solution containing the components present in blood plasma. However, this method can only show whether or not precipitation occurs on the scaffold and hence, the variable to be controlled while using this method would be the soaking duration. The state of the scaffold at different duration should be studied in order to analyse the trend of the bioactivity. Hence, the objective of this study is to analyse the properties of different compositions of HAp-PVA-made scaffolds in terms of biodegradability and bioactivity. In this study, samples of HAp-PVA scaffold with different HAp compositions were synthesised and prepared. The scaffold samples were prepared in the form of solvent casting. To characterise the scaffold samples, the scaffolds casted were first weighed and analysed using FTIR. The scaffolds would then undergo immersion in the SBF for 7 and 14 days before it underwent weighing and FTIR analysis again.

METHODOLOGY

Materials

Scomberomorus commerson, English named as Spanish mackerel or "ikan tenggiri" by the local in Malaysia, were acquired from local morning market in Kuala Lumpur, Malaysia. The spine part of the fish bones were selected and stored in freezer for future used. 88.3 % partially hydrolysed polyvinyl alcohol was obtained from R&M Chemical, UK. Glycerol at 99.5% purity was purchased from Merck Sdn. Bhd., Malaysia. The required chemicals for SBF solution were obtained from several sources as listed: sodium chloride (A.R/ACS, R&M), sodium bicarbonate (C.P., Bendosen), potassium chloride (Friendemann Schmidt), di-potassium hydrogen phosphate (Friendemann Schmidt), magnesium chloride hexahydrate (Chemsoln), hydrochloric acid of 1 mol/L or 1N (R&M), calcium chloride anhydrous, granular (C.P., R&M), sodium sulphate anhydrous (A.R, R&M), and tris(hydroxymethyl)aminomethane with purity >99% (MB grade, Chemsoln). All these materials were used as received.

Composite scaffold material preparation

The procedure for the bone calcination preparation and HAp extraction was discussed earlier [17]. In brief, the bones were boiled in water for an hour to remove the traces of skin and meat, followed by drying at 60°C for 6 hours in oven. The cleaned bones were calcinated at 800 ºC at the heating rate of 10 ℃/min for 4 hours, followed by the cooling and grinding into fine powder. Finally, the powder were sieved via mesh size of 200.

Samples containing different amount of HAp (0, 5, 10, 20 and 30 phr HAp) were prepared as shown in **Table 1** below. The samples were obtained by casting method. 10 g PVA were dissolved in 200 ml distilled water and 2 g glycerol followed by adding the measured HAp powder. The solution were heated in a water bath at 97 ± 2℃ and stirred constantly at 600 RPM with a mechanical stirrer for one hour to ensure all PVA were dissolved and a homogenized dispersion of HAp in the mixture. The mixtures were cast at similar weight on petri dish, and dried in an oven at 60 ℃ for 24 hours.

TABLE 1. Biocomposite sample composition

Composition No.	PVA, phr	Glycerol, phr	HAp, phr
1	100	20	0
2	100	20	5
3	100	20	10
4	100	20	20
5	100	20	30

In-vitro bioactivity of composite scaffold material

The apatite formation of the composites was studied by soaking the prepared samples in SBF solution, which was prepared according to Kokubo method [16]. To prepare the SBF solution, reagent-grade sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$), calcium chloride ($CaCl_2$) and sodium sulphate (Na_2SO_4), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) were dissolved in deionized water in a plastic beaker and buffered to pH 7.40 with tris(Hydroxymethyl)aminomethane (Tris) and 1M of HCl solution 1 mol L−1) at nearly 37°C.

30 × 30 mm casted samples were weighed prior to soaking in the SBF solution. The amount of SBF required is calculated according to equation (1).

$$V_s = \frac{S_a}{10} \tag{1}$$

Where V_s is the volume of SBF required in ml and S_a is the apparent surface area of the specimen in mm^2.

The 30 × 30 mm samples were immersed in the SBF solution at 37°C for 7 and 14 days. After the immersed time, the samples were cleaned with distilled water and dried for further analysis.

The samples' functional groups and chemical structure were studied through Perkin Elmer Spectrum 100, a Fourier-transform infrared spectrometer (FTIR), at 4 cm^{-1} resolution with frequency range set from 525 to 1400 cm^{-1}.

RESULTS AND DISCUSSION

Sample Weight Before and After Immersion in Simulated Body Fluid

The weight of the samples before and after immersion in the SBF for both 7 and 14 days are tabulated and shown below where the compositions are as listed in **Table 1** according to the composition number respectively.

TABLE 2. Sample weights before and after immersion in SBF (7 days)

Composition No.	Before Immersion, g	After Immersion, g	Weight Difference, g
1	0.164	0.160	-0.004
2	0.160	0.140	-0.020
3	0.234	0.163	-0.071
4	0.172	0.150	-0.022
5	0.244	0.260	+0.016

TABLE 3. Sample weights before and after immersion in SBF (14 days)

Composition No.	Before Immersion, g	After Immersion, g	Weight Difference, g
1	0.128	0.112	-0.016
2	0.207	0.155	-0.052
3	0.181	0.202	+0.021
4	0.137	0.148	+0.011
5	0.318	0.209	-0.109

It can be observed that after 7 days immersion, the sample weight for composition 1, 2, 3 and 4 decreased upon soaking in the SBF whilst composition 5 had an increment in weight. As for 14 days immersion, the samples of composition 1, 2 and 5 decreased after soaking in the SBF whilst the samples for composition 3 and 4 increased.

For composition 1, it is the composition without any HAp at all, consisting of only water, PVA and glycerol. It is expected that there will not be any apatite precipitation at all for composition 1 and hence, the decrease in weight is valid. Also, this shows that PVA is degrading as it is immersed in SBF. It can also be seen that more of the scaffold had been degraded as the period of immersion is longer. The difference in weight for the composition 1 sample after 7 days immersion is 0.004 g and 0.016 g after 14 days. The weight loss increased by 4 times of it after a week's times.

For composition 2, the composition consists of the least amount of HAp as compared to composition 3, 4 and 5. The weight loss is due to the scaffold being resorbed and degraded after it was immersed in the fluid. However, even though the samples after 14 days immersion has slightly more weight loss, the difference is only of 2.6 times more, which is lower than that for composition 1. This could be explained as precipitation of apatite is formed on the surface of the specimen during the immersion. But the precipitation rate is lower than the degradation rate and hence, weight loss in the specimen did exist, but at a lower rate. This also shows that 7 days of scaffold implantation is definitely not sufficient for the regeneration to take place completely as the degradation and absorption of the components are still taking place at a higher rate than precipitation.

The verdict above is further supported by observing the weight differences between the 2 different durations for compositions 3 and 4. The weight loss for composition 3 after 7 days of immersion is 0.071 g, but after 14 days of immersion, the weight of the sample increased by 0.021 g. As for composition 4, there is a weight loss of 0.022 g after 7 days and gain of 0.011 g after 14 days immersion. The weight loss is due to the degradation of the scaffold for absorption. At a longer period of immersion, precipitation and regeneration of apatite started to form, and hence weight gain is present in the samples. This shows that by 14 days of scaffold implantation, regeneration of HAp has already begun. Besides that, after 7 days of immersion the degradation rate of scaffold is the highest in composition 3 as compared to the rest. This shows that at 7 days of immersion, the resorption rate is at peak with the composition of 100 phr PVA/10 phr HAp. When the composition is higher than that, precipitation would have begun on the scaffold surface. This can also be observed in lower weight loss in composition 4 after 7 days immersion. The weight increment of composition 3 is the highest. This could be explained as the precipitation rate of composition 3 is the highest.

After 7 days of immersion, composition 5 had shown a slight increment in weight. This is because precipitation has already occurred due to the large amount of HAp on the surface of the scaffold. However, after 14 days of immersion in SBF, the weight of composition 5 had dropped immensely. The decrease of weight is believed to have happened as the precipitation of apatite on the scaffold surface had reached its maximum, but degradation of the component continued to take place, resulting in a higher degradation rate than deposition rate.

The formation of apatite as a precipitate on the surface of the scaffolds could be explained as follows. HAp commonly consists of negative ions, OH^- and PO_4^{3-}, and positive ions, Ca^{2+}. The negative ions of HAp that are present on the surface of the scaffold is the main cause of apatite formation, leading to precipitation. These negatively charged ions of HAp would usually attract the calcium ions that are present in the SBF, resulting in an extra positive charge on the initially neutral surface. This positive charge will then further attract the OH^- and PO_4^{3-} ions that are present in the SBF [18]. The above scenario then results in the formation of a rough and entangled layer of apatite, which will further induce apatite heterogeneous nucleation to form apatite nuclei [10]. Upon soaking, the precipitation will continue until the whole sample surface is covered by consuming the relevant ions present in the surrounding.

As a summary, it could be understood that with a higher amount of HAp, the precipitation rate would be higher as well. However, the results also show that composition 3 is by far the most optimum choice for a longer duration of implantation.

Fourier-Transform Infrared Spectrometer (FTIR)

FTIR analysis was conducted for pure HAp and PVA, sample scaffolds before immersion in SBF and after immersion in SBF in which the results are shown in FIGURE 1., FIGURE 2. and FIGURE 3. respectively.

FIGURE 1. shows the spectra of pure PVA and HAp. The distinct difference between these two components could be observed clearly from the spectra. For PVA, he broad band at 3251 cm^{-1} show that O-H stretching alcohol intermolecular bond exists. where the two narrow peaks of 2936 cm^{-1} and 2908 cm^{-1} show that C-H stretching vibrations are present. The short peak of 1590 cm^{-1} indicates the presence of carbonyl group. The band of 1419 cm^{-1} also indicates the symmetric bending mode of CH_2, C-H and O-H groups whilst the stretch at 1327 cm^{-1} shows a wagging vibration of CH_2. The bands at 1139 cm^{-1} and 1088 cm^{-1} signify that there is a C-O-C bridge stretching and C-O stretching as secondary alcohol respectively. The weak band of 843 cm^{-1} shows that there is a stretching vibration for C-C bond. The bands above had shown various functional groups of PVA distinctively. For HAp, the spectrum showed bands that are of the range 500 cm^{-1} to 600 cm^{-1}, 970 cm^{-1} to 1100 cm^{-1} are correspondent to the tetrahedral phosphite ion group's P-O vibration attributes, v_4 and v_3 respectively. The peak at 547 cm^{-1} is related to the bending mode of O-P-O where symmetric P stretching is involved. The peak at 979 cm^{-1} shows the symmetric stretching of the phosphate group that is present in the HAp.

As it could be observed in FIGURE 2. the spectra showed the increasing amount of HAp functional groups with the increase of HAp amount in the composition. Also, the difference between composition 1 with the rest of the compositions is very distinctive as well. This is due to the lack of HAp particles in composition 1, where the bands representing the function group PO_4^{3-} do not exist. The main difference between pure PVA with the composite would be that there is a shift of absorption band for the hydroxyl group, where the O-H stretch had shifted to 3271 cm^{-1}, which is of a higher value. This is due to the addition of glycerin into the mixture in which glycerin would plasticise PVA by forming strong hydrogen bonding with the hydroxyl groups in PVA. With this effect, the hydrogen bonding in PVA is reduced, thus the shift in value, signifying the weakening of the band [19]. Another significant change is that the carbonyl group does not exist in the composites as well. This is because of the presence of water in the composite mixture. The oxygen particle of the carbonyl groups in PVA tends to provide a site for the hydrogen bonding to take place. Besides that, there is also a shift in the C-O-C bridge stretching which was previously 1139 cm^{-1}. The C-O-C bridge had shifted to a higher value of 1145 for the composites, signifying a slight weakening effect in the C-O-C bridge.

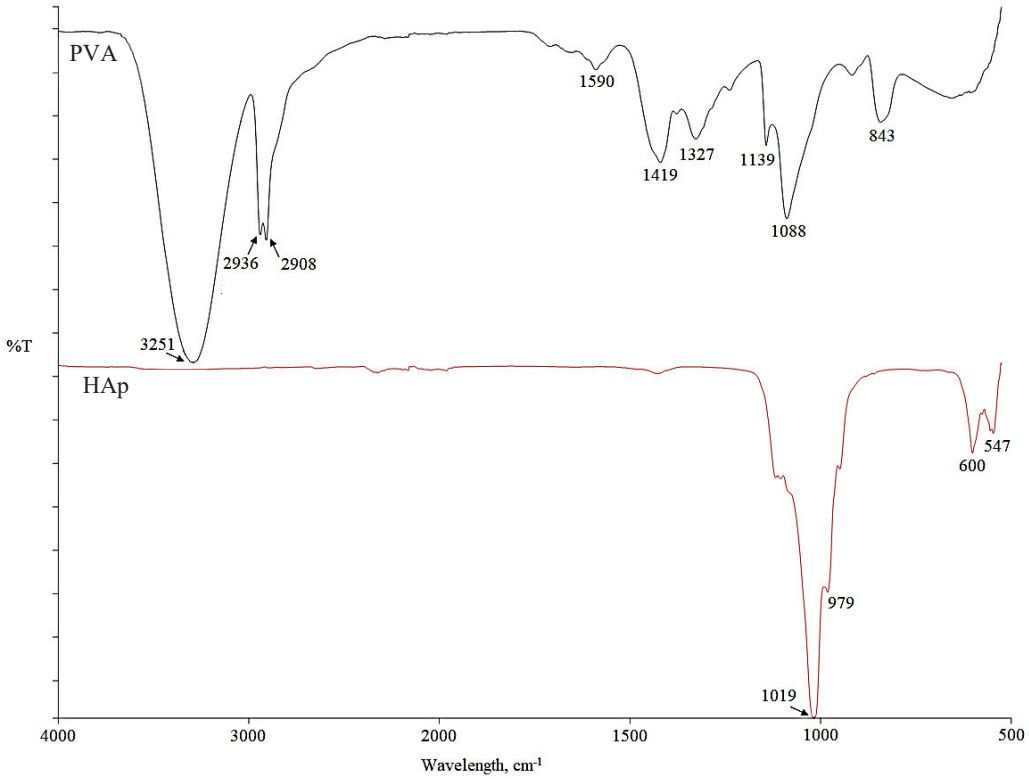

FIGURE 1. Spectra of pure PVA and HAp under FTIR

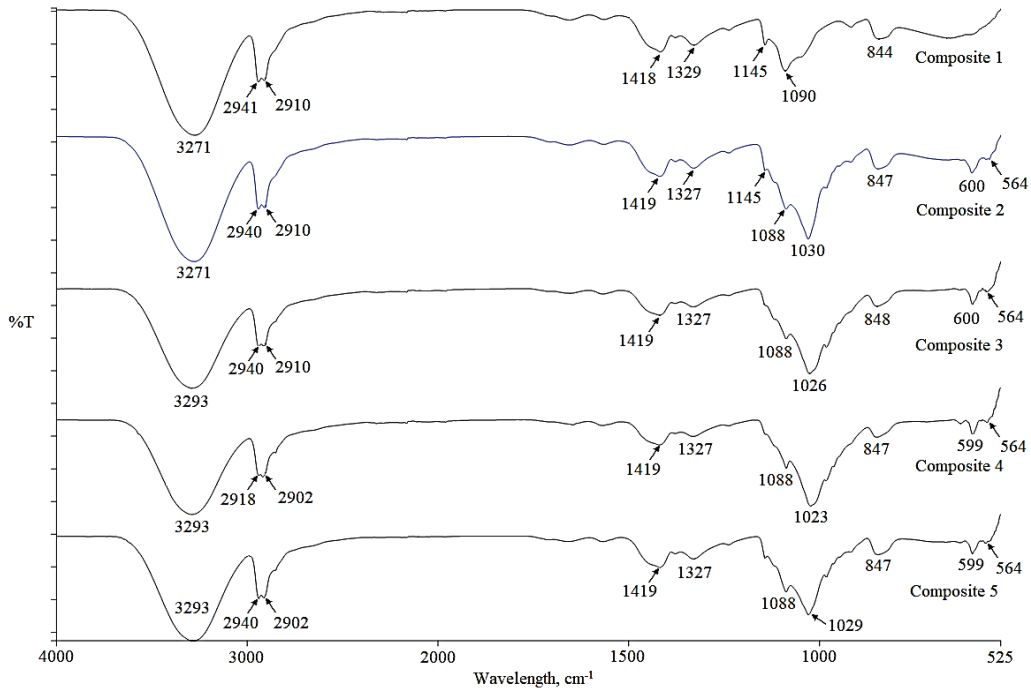

FIGURE 2. Spectra of samples under FTIR before immersion in SBF

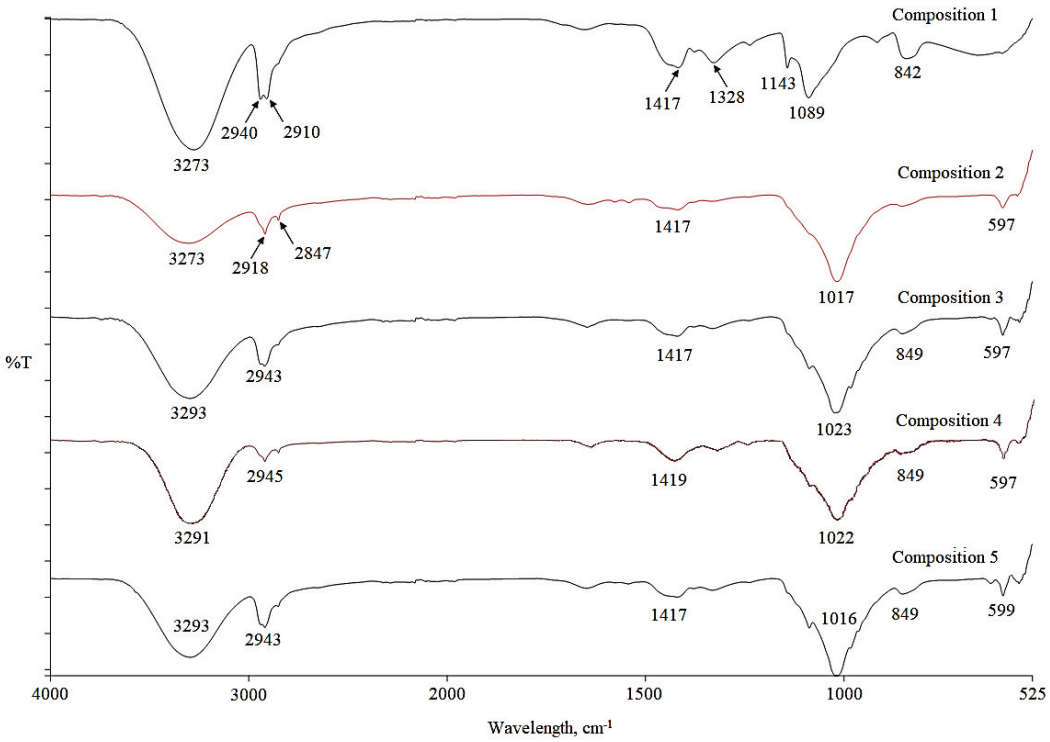

FIGURE 3. Spectra of samples under FTIR after immersion in SBF for 7 days

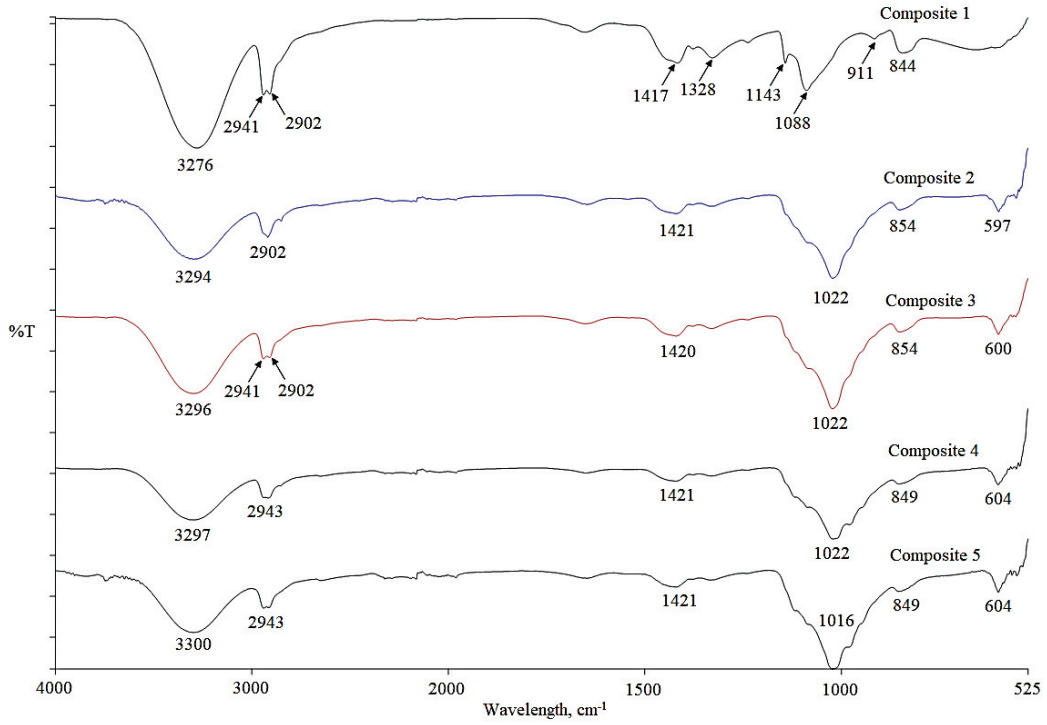

FIGURE 4. Spectra of samples under FTIR after immersion in SBF for 14 days

FIGURE 3. and 4. show the spectra of samples after immersion in SBF for 7 days and 14 days respectively. From the spectra, it could be seen that the fraction percentage of the bands have decreased as compared to the samples before immersion. The shift of hydroxyl group continues after immersion. The absorption band of O-H group had further shifted to slightly higher values ranging from 3272 cm^{-1} to 3300 cm^{-1} with the increasing duration of immersion and increasing amount of HAp in the composite while it was initially ranged between 3271 cm^{-1} and 3293 cm^{-1}. Besides the shift in value of the hydroxyl group, the rest of the absorption bands remain almost the same with difference of less than 5 cm^{-1}. However, it could be observed that the intensity of phosphate ion group increased at 1016 cm^{-1} to 1023 cm^{-1}, comparing between before, 7 days after and 14 days after immersion. This could be explained as there are more HAp that have been formed and precipitated on the surface of the scaffold. The precipitation shows that there is a bioactivity reaction between the fluid and the scaffold. As it was explained before, the precipitation is due to the attraction of positive ions in relation to apatite from the fluid, which are the Ca^{2+} ions onto the negative ions present on the scaffold surface, which are the OH^- and PO_4^{3-} ions. Given that OH bonds do exist in HAp, it is normal to expect an increment in the O-H group since there is also an increment in the other bonds that exist in HAp, such as the phosphate groups. However, the range of values did not increase by much. This could be explained as degradation of PVA had happened, leading to a reduction in O-H bonds within the scaffold. The growth of HAp that had precipitated on the surface had increased the O-H bonds instead, resulting in only a very slight difference in amount. This has then shown the biodegradation property of the scaffold. At this instance where the study uses SBF as an agent of bioactivity analysis, the only interpretation that could be done is on the precipitation of apatite on the surface of the scaffold as it is not a real body fluid with microorganism growths [16]. Such result is similar to most of the studies that had been done using the same agent where degradation and precipitation could be observed after a period of scaffold immersion [11][15-17].

The summary of wave number from the spectra collected with the absorption band assignments or representations as discussed is tabulated in TABLE 4.

TABLE 4. Summarised FTIR spectra peaks and their representations

Samples	**Wave Number, cm^{-1}**	**Representations**
Pure PVA	3251	O-H stretching alcohol intermolecular bond
	2936, 2908	C-H stretching vibrations
	1590	Carbonyl group
	1419	Symmetric bending mode $\nu_\delta(CH_2)$
	1327	Wagging vibration of CH_2
	1088	C-O stretching as secondary alcohol
	843	ν (C-C) stretching vibration
Pure HAp	1019, 979, 600, 547	PO_4^{3-} groups
		Before Immersion
100 PVA / 0 HAP	3271	O-H stretching alcohol intermolecular bond
	2941, 2910	C-H stretching vibrations
	1418	Symmetric bending mode $\nu_\delta(CH_2)$
	1329	Wagging vibration of CH_2
	1145	Asymmetric C–O–C bridge stretching
	1090	C-O stretching as secondary alcohol
	844	ν (C-C) stretching vibration
PVA / HAP	3271-3293	O-H stretching alcohol intermolecular bond
	2902-2918	C-H stretching and vibration
	1419	Symmetric bending mode $\nu_\delta(CH_2)$
	1327	Wagging vibration of CH_2
	1088, 1020-1030, 600, 564	PO_4^{3-} groups
	847-853	ν (C-C) stretching vibration

TABLE 4. Summarised FTIR spectra peaks and their representations (cont.)

After Immersion (7 days)		
100 PVA / 0 HAP	3272	Symmetrical stretching ($\upsilon 1$) of OH
	2940, 2910	(-CH2-) symmetric and asymmetric stretching
	1417	Symmetric bending mode $\nu_\delta (CH_2)$
	1328	C-N stretching
	1143	C-O-C bridge stretching
	1089	C-O stretching as secondary alcohol
	842	CO_3^{2-}
PVA / HAP	3272-3293	O-H stretching alcohol intermolecular bond
	3300	C-H bond stretching - Sp hybridized, one half s character
	2910-2945	(-CH2-) symmetric and asymmetric stretching
	1417-1419	CO_3^{2-}
	1016-1023, 597-599	PO_4^{3-} groups, O–P–O bonds
	849	CO_3^{2-} and ν (C-C) stretching vibration
After Immersion (14 days)		
100 PVA / 0 HAP	3276	O-H stretching alcohol intermolecular bond
	2902-2941	(-CH2-) symmetric and asymmetric stretching
	1417	CO_3^{2-}
	1328	C-N stretching
	1143	C-O-C bridge stretching
	1088	C-O stretching as secondary alcohol
	911	Angular deformation outside O-H bond
	844	CO_3^{2-} and ν (C-C) stretching vibration
PVA / HAP	3294-3300	O-H stretching alcohol intermolecular bond
	2902-2943	(-CH2-) symmetric and asymmetric stretching
	1420-1421	CO_3^{2-}
	1016-1023, 597-604	PO_4^{3-} groups, O–P–O bonds
	849-854	CO_3^{2-} and ν (C-C) stretching vibration

CONCLUSION

The aim of this study was to investigate the feasibility of the bone scaffold produced using the biocomposite made up of PVA and HAp focusing on the bioactivity and bioresorption rate of the scaffolds. The results showed that precipitation and formation of apatite was present on the specimen after soaking. It also showed positive amount of degradation upon soaking, which is a desired outcome. It was also found that the most optimum composition for a longer period of resorption would be composition 3 which contains of 100 phr of PVA with 10 phr of HAp. From the results, it could be seen that with increasing amount of HAp particles in the composite, deposition of apatite happened faster. Functional groups present in each pure material as well as the samples were also identified. The spectra obtained from FTIR showed that upon the formation of the composite after immense stirring under the temperature of 97 ℃, bonding between the PVA and HAp do exist. Also, after being immersed in the SBF solution, the functional groups in the composite did not have much changes since the deposition of apatite take place without changing the composite chemically. However, the FTIR did show positive outcome where there is an increase in the amount of HAp due to precipitation and thus, the evidence of bioactivity reaction between the scaffold and the fluid. Hence, it could be concluded that this biocomposite-made bone scaffold is feasible in terms of its bioactivity and resorption rate can be used as an agent for artificially induced bone regeneration in a human's body. In the near future where the further analysis is carried out and technology is more matured, the use of PVA-HAp composite as bond scaffold could be used to substitute metal-aided bone growth to ease the whole process of bone repairment.

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