IN-VITRO APATITE GROWTH ON POROUS β-TRICALCIUM PHOSHPATE SCAFFOLDS COATED WITH PHVB

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ABSTRACT: The bioactive properties of polyhydroxybutyrate-co-valerate (PHBV) coated beta-tricalcium phosphate (β-TCP) have been studied in-vitro. Porous β-TCP scaffolds have been prepared using a template method and sintered at 1450 °C. The bio ceramics were then coated with PHBV solution before being immersed for 6 weeks in a simulated body fluid (SBF) at 37°C. At the end of the immersion time, insignificant changes in the SBF pH value was observed, suggesting good stability against hydrolytic degradation. X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analyses revealed the presence of apatite. Morphological analysis by SEM showed the formation of apatite crystals in the form of flakes and globular deposits on the scaffold surface. This bone-like apatite indicates good biological activity of the bio ceramics scaffold with PHVB coating suggesting that the composite has potential for bone tissue engineering applications.

KEYWORDS: *Beta-Tricalcium Phosphate; Polyhydroxybutyrate-Co-Valerate; Apatite; Simulated Body Fluid; Composite Scaffold*

1.0 INTRODUCTION

Bone tissue has a complex structure consisting of approximately 70% nano substituted hydroxyapatite (HA–$Ca_{10}(PO_4)_6(OH)_2$) and 30% collagen, mainly Type I, by weight, that contribute to its relatively high strength. The major functions of bones are to provide structural support, that is load bearing, protect of the internal organs, maintain the metabolic process, provide a calcium reserve and others [1-2]. Although bone has the ability to self-heal, this is only possible for small defects, while larger bone defects, above a critical size, need surgical treatment including the use of a scaffold. The application of bone tissue engineering, incorporating suitable biocompatible and bioactive scaffolds, is preferred in reconstruction of critical size bone defects.

Porous calcium phosphates such as HA have been extensively studied for the scaffold materials reconstruction of bone defects [3], but show limited bioresorbability properties to facilitate new bone formation. In contrast, tricalcium phosphate (TCP) is a biodegradable bio ceramic, which provide more desirable degradation characteristic and are better candidate bone replacement materials [4-5].

Bone scaffolds must provide sufficient mechanical strength and toughness, as well as ability to facilitate a good biological response to ensure successful application. Thus, extensive studies have tried to produce degradable scaffolds using ceramic-polymer composite scaffold systems to produce the required combination of high strength and toughness yet bioactive scaffolds by combination of the various polymers with the bio ceramic [6-9]. However, limited success has been reported as the polymeric scaffold can become fragile with increasing amounts of bioactive filler, thus limiting their content to 20 or 40 vol. % [10]. This in turn limits the osteointegration of the biocomposite. Alternatively, a high concentration of ceramic scaffold can be produced by combining two separate methods which are the fabrication of porous bio ceramics scaffold followed by a polymeric coating to improve the mechanical properties and bioactivity [5, 7-8, 11].

Biodegradable polymers, such as poly (l-lactic acid) (PLLA), and poly (glycolic acid) (PGA) are commonly used in bone tissue engineering. They are biocompatible and can support cell proliferation during cell culture and in vivo applications. However, it has been reported that they can generate acidic by-products during the degradation process that may cause a strong inflammatory response from the host tissue

[12-13]. Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a range of copolymers of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) and are degradable candidates for tissue engineering as well as in drug delivery applications [9, 12, 14-15]. These applications are largely due to their excellent biocompatibility as well as the ability for their degradation properties to be tailored to suit the intended application [16-17]. Doyle et al. [9] showed a slow loss of mechanical properties of PHB reinforced with HA during the in-vitro study as well as good in-vivo biocompatibility and bioactivity. Kose et al. [18] reported the stability of the PHBV porous scaffold up to 120 days when immersed saline solution. Afterwards, the degradation activity was observed through a reduction in pH levels. Reasonable cell culture activity was also observed during in vitro testing. The other advantage of PHBV is it does not produce acidic by-products during the degradation process unlike other degradable polymers such as PLA, PGA, or their co-polymers [12, 18].

While PHBV possess good biocompatibility and is a non-toxic substrate for cell culture [19], its bioactive properties are still insufficient, and this limits its application as a bone replacement material. Thus, incorporation of bioactive inorganic phase is often used. Duan et al. [20] successfully fabricated porous PHBV scaffolds by incorporating Ca-P using selective laser sintering which reported a promising initial compressive strength and dimensional accuracy. Wang et al. [21] attempted to integrate β-Ca_2SiO_4 nanoparticles into PHBV via solvent casting and particulate incorporation. They reported that, while the process was able to produce high porosity scaffolds which are important for cell seeding, the bioactive phase is only in the range of 2.5-5%, which may limit the bioactivity. However, these studies focussed on the incorporation of the bioactive phase calcium phosphate such as HA and TCP in the PHVB polymeric scaffold system to improve their bioactive properties. Various studies show that bone-like apatite will precipitate on bioactive materials, such as calcium phosphate and bio-glasses [22-23]. In contrast, although a polymeric coating is favourable in order to improve the compressive strength of the TCP scaffold as used in this study, the hydrophobic characteristic may hinder the bio ceramics exposure and subsequently reduce the bioactivity. Therefore, the aim of this study is to evaluate the preliminary biological properties of the PHVB coated β-TCP scaffold in-vitro.

This paper presents the preparation of a β-TCP scaffold using a replication method and the used of high temperature sintering to transform it from the original α-TCP form. The porous scaffold is then

coated with PHVB before being subjected to immersion in stimulated body fluid (SBF) for up to 6 weeks to evaluate their biological properties.

2.0 METHODOLOGY

2.1 Scaffold fabrication

Porous β-TCP/PHBV composite scaffolds were prepared via our previously described polymer replication method [24]. Calcium phosphate ($Ca_3(PO4)_2$) powder (Sigma Aldrich, United Kingdom) was dispersed in 2 wt.% polyvinyl alcohols aqueous solution at a powder to liquid ratio of 5/5 (wt./vol.), this approach produces a well-dispersed slurry. Polyurethane foam templates (Wansern Technology Sdn. Bhd., Malaysia) with dimension of 1 cm × 1 cm × 1 cm were immersed into the slurry and gently squeezed several times. This allows the slurry to penetrate the foams before the excess slurry was squeezed out. In order to avoid the blockage of pores, compressed air was used to blow out any residual solution. The ceramic-slurry coated PU foams were then left to dry in an oven (Mermet, Germany) at 60°C for 24 hours.

Following the drying, the ceramic-slurry coated PU foams were sintered in an electric furnace (Carbolite Furnace Manifesto, UK) using a four-stage firing schedule. First, the coated foams were heated from room temperature to 400°C at a heating rate of 1°C min^{-1} to burn-out the polyurethane foam and to prevent thermal shock. The foam was then heated to 1450°C at a faster rate of 5°C min^{-1} to allow a sintering reaction of the calcium phosphate slurry followed by being held at the maximum temperature for 4 hours to complete sintering process before it was cooled down to room temperature at a rate of 5°C min^{-1}. Subsequently, 5g of PHVB (Sigma Aldrich, United Kingdom) were dissolved in 50 ml chloroform and refluxed at 60°C for 30 minutes. The ceramic scaffold was then immersed into the solution and dried at room temperature in a fume cupboard overnight. The obtained polymeric coated scaffolds were further dried in a vacuum oven (Mermert, Germany) for 24 hours at 37°C.

2.2 Scaffold Characterization

The scaffolds were immersed in stimulated body fluid (SBF) (pH 7.4-7.6) at 37°C for a duration of up to 42 days (6 weeks) using an IN30 incubator (Mermert, Germany). Five samples were used at each immersion period. Prior to the immersion test, the SBF solution was

prepared as detailed in [25]. The SBF was replaced weekly and the pH values were measured using a pH meter model HI 2211-01 (Hanna Instruments, USA). The samples were taken out at interval of 7, 14, 21, 28, 35 and 42 days. The change of mass (wt. %) was calculated by using Equation (1).

$$\text{Mass}\,(\text{wt.}\%) = \frac{\text{Wo - Wt}}{\text{Wo}} \text{x}\,100\% \quad (1)$$

where Wo is the initial weight and Wt is the final weight. The phase and crystallographic structures of the synthesized samples were identified by X-Ray Diffraction (X'Pert Pro MPD PW 3060/60; PanAnalytical, Netherland) operating at room temperature using Cu Kα radiation (α=1.54178 Å), over the range of 10° - 90°. Following this the variations of the structural characteristic groups and bonds obtained from these bioceramic scaffolds were captured using a FT/IR 6100 (JASCO, Germany) Fourier Transform Infrared Spectroscopy (FTIR) system. The surface morphologies of samples after experiments were examined using an EVO 50 SEM (Carl Zeiss SMT, UK). All samples were gold coating prior to the examination.

3.0 RESULTS AND DISCUSSION

The series of changes, such as the material dissolution and deposit formation on the surfaces of the samples, occurred when composite materials were soaked in SBF for 6 weeks. The pH during the in-vitro degradation is shown in Figure 1. The value remained virtually unchanged up to 6 weeks from its initial value (pH 7) indicating the stability of the scaffold against the hydrolytic degradation as reported in the literature [18]. The weight of the scaffold remains relatively constant up to 4 weeks, before a small weight gain (4.2%) was observed in final immersion of week 6 (42 days) (Figure 2). This suggests apatite mineral has started to precipitate on to the scaffold surface.

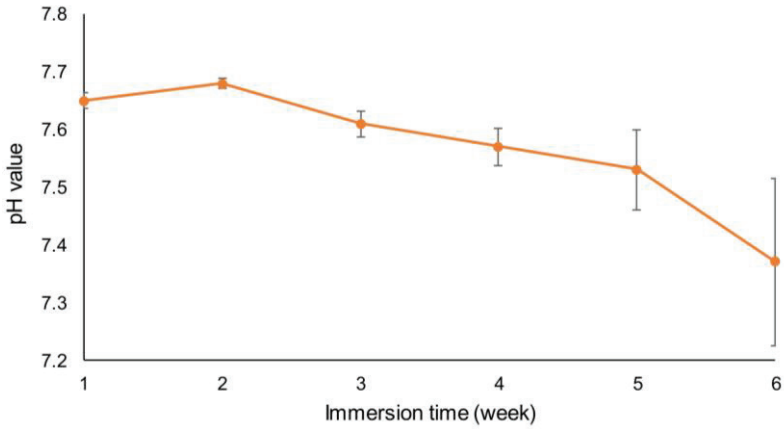


Figure 1: pH value change for SBF during the immersion process

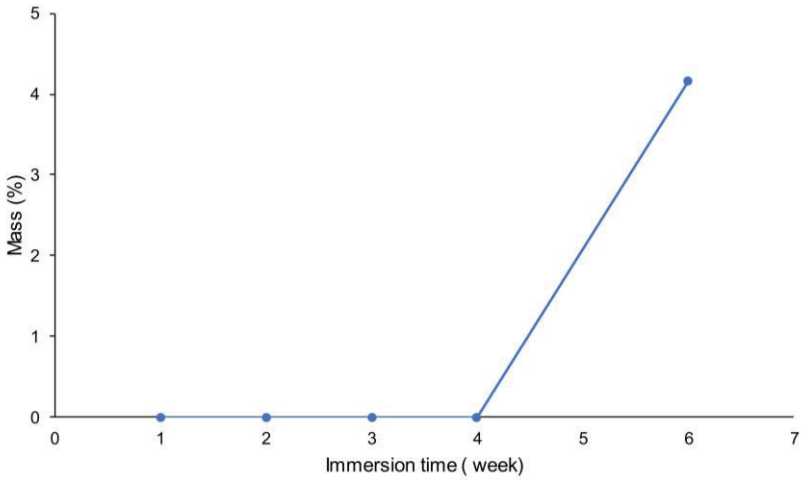


Figure 2: Change of mass during the immersion process

The β-TCP/PHBV scaffolds incubated in SBF at week 6 were also characterized by X-ray diffraction and their characteristics were compared prior to immersion (Figure 3). Broad and diffuse peaks at $2\theta = 25.9°$ and 31.8–32.9° clearly appear in the resulting XRD patterns, which may be ascribed to the characteristic peaks of apatite [26-27].

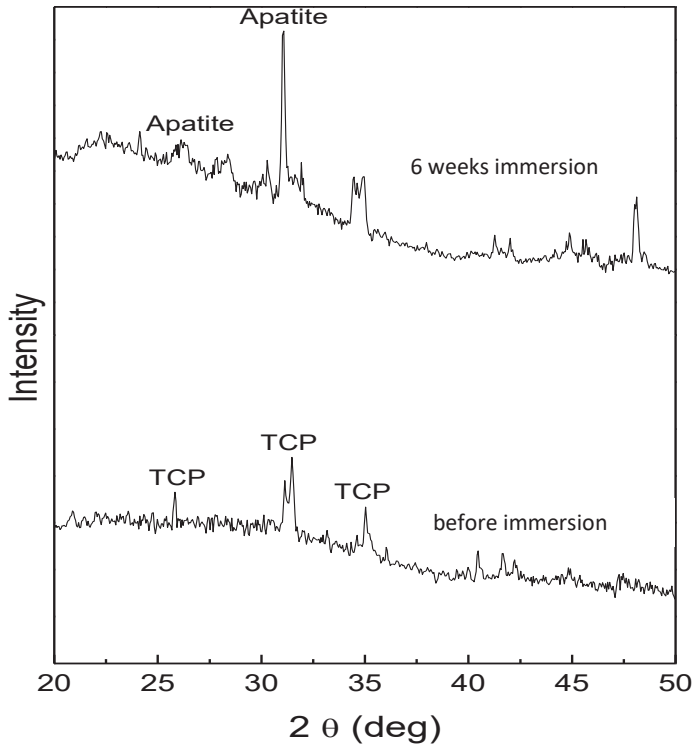


Figure 3: XRD patterns of β-TCP/PHBV before and after 6 weeks immersion in SBF showing apatite deposition

FT-IR spectra of the scaffold prior to SBF immersion and after 6 weeks in-vitro immersion are shown in Figure 4. The appearance of new peaks after 6 weeks immersion was presented at 550–650 cm^{-1} and 950–1100 cm^{-1}, which were attributed to $PO4^{3-}$ vibration. Furthermore, the formation of bands at 1400-1500 cm^{-1} is due the carbonate vibrations and is characteristic of carbonated apatite [23, 28]. The hydroxyl group vibration mode was also observed at 2500-3700 cm^{-1}.

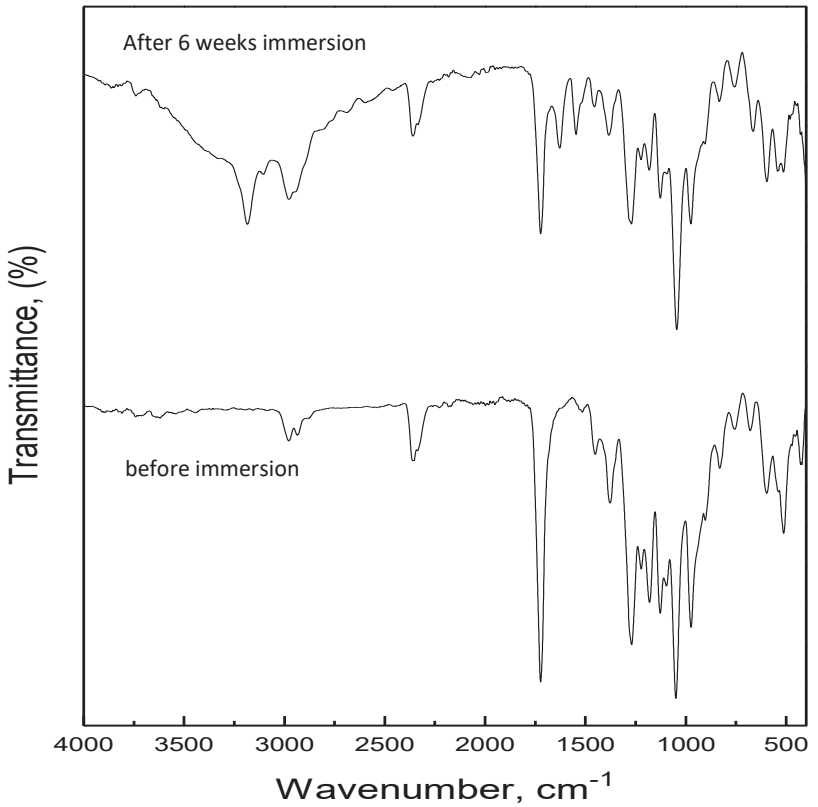
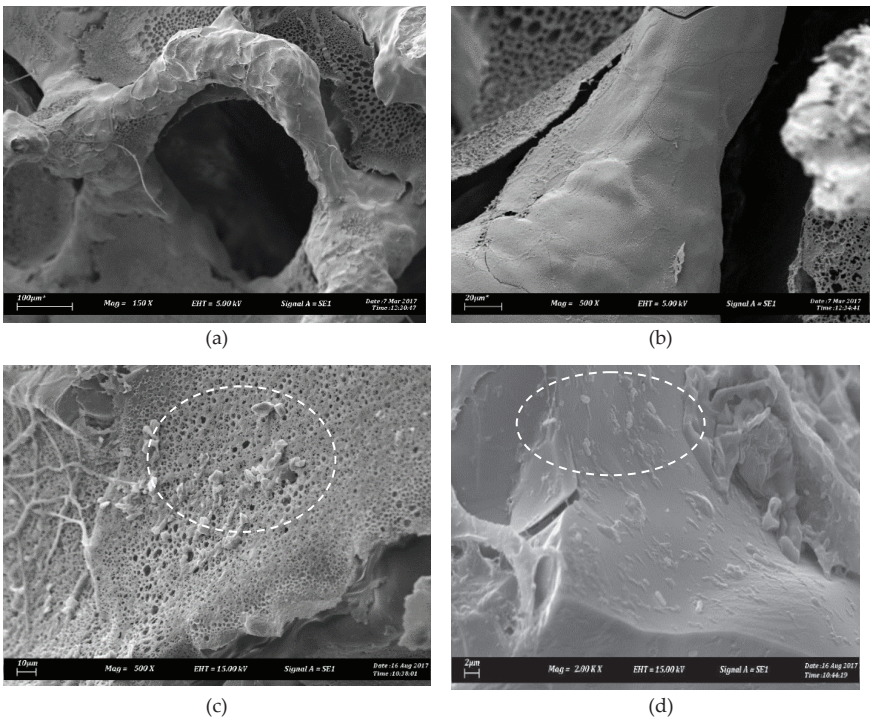


Figure 4: FTIR analysis of surfaces of β-TCP/PHBV before and after 6 weeks immersion in SBF solutions showing apatite deposition

SEM micrographs of the scaffold before and after 6 weeks immersion in SBF are shown in Figure 5. The scaffold contains interconnected pores of different shapes in the size range 200-400 μm after the PHVB coating (Figure 5a). The architecture and structure are not significantly different from the uncoated β-TCP scaffold reported by our earlier study [29]. This is desirable to achieve osteogenic cell ingrowth. Prior to the immersion, the β-TCP/PHBV scaffold revealed a smooth surface for the β-TCP ceramic strut coated with a thin layer of PHBV (Figure 5b). The incubation in the SBF subsequently led to the nucleation and growth of the apatite layer. Figure 5c shows the mineral starting to precipitate on the smooth surface of the scaffold, assembling as small flake-like pieces. At higher magnification, a few mineral crystals with globular structure are also observed (Figure 5d). Previous studies have reported the apatite mineral in form of flakes [30-33]. The precipitated apatite mineral in this study is in the form of flaky and globular structures similar to that shown in previous study [22]. Apatite crystal morphology may be influenced by the underlying

characteristic of the substrate used that differs in topography and charge [33].



(a) (b)

(c) (d)

Figure 5: SEM micrograph of scaffolds after immersion in SBF (a)-(b) 0 week (c)-(d) 6 weeks and marker bars (a) 100µm, (b) 20µm, (c) 10µm and (d) 2µm

4.0 CONCLUSION

In conclusion, the scaffold possesses an open architecture with interconnected pores after the application of PHVB coating, as well as hydrolytic degradation stability. After being immersed for 6 weeks in SBF at 37°C, a bone-like apatite was formed on the β-TCP/PHBV surface, which was both confirmed by FTIR and XRD analyses, thus indicating a good bioactivity of the composite. Hence, the composite showed a promising application prospect as an artificial bone replacement material.

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