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RESEARCH ARTICLE

POLYHYDROXYALKANOATE (PHA)/NANO-CALCIUM PHOSPHATE (nCAP)/CHITOSAN BIOCOMPOSITE FOR BONE REGENERATION: DEGRADATION, BIOACTIVITY AND CYTOTOXICITY EVALUATION

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Abstract. Due to an ageing culture, bone defects and fractures are becoming serious concerns to human health and quality of life. Natural polyhydroxyalkanoate (PHA) biopolymers are biodegradable plastics and are receiving great attention in medical applications. However, PHA has several weaknesses such as low mechanical properties and poor biological properties like less apatite growth on PHA. Bioceramic was combined with PHA to enhance its bone-related properties, which resulted in gains in compressive elastic modulus, maximum stress, and osteoblast responses, such as cell growth and alkaline phosphate activity. PHAs have been found in medical applications due to their compatibility with the body without any adverse effects. In this work, PHA was reinforced with nanocalcium phosphate (n-CaP) and chitosan (CH) to improve the ability and promote bone formation and can also act as natural carriers for growth factors and antimicrobial resistance. Simulated body fluid (SBF) and cytotoxicity of PHA and PHA/n-CaP/CH composites were investigated to determine the biocompatibility properties. SBF results show that an apatite layer with a Ca/P ratio of 1.51-1.64 was formed on the surface of the composites after 28 days of immersion via scanning electron microscopy (SEM-EDX) examination. The cytotoxicity test has revealed that all samples have no hazardous effects towards the osteoblast cell. Thus, cell proliferation appears to be doubled in composites with 3wt% of n-CaP, surpassing the established standard. Thus, the biological activity of PHA/n-CaP/CH composites appears to be better than pure PHA. PHA and PHA/n-CaP/CH composites can be potential bone graft substitutes for implant applications.

Keywords: Bioactive composite, polyhydroxyalkanoate (PHA), nano-calcium phosphate, biodegradable, bone regeneration.

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1. INTRODUCTION

Bones have a variety of functions, including serving as the primary supporting framework for the attachment of muscles, ligaments, and tendons, as well as providing mechanical support and protection for vital organs [1]. Due to an ageing culture, bone defects and fractures are becoming serious concerns to human health and quality of life. The number of bone fracture cases is approximately 178 million globally. The cases of bone fractures were 102 million in males compared with 76·4 million in females. Australasia, central Europe and Eastern Europe are the top three regions that have the highest rate of bone fractures. The combination of several biomaterials, such as biopolymer and bioceramic, known as biocomposites are designed and developed to improve the properties of single-phase material [2].

Polyhydroxyalkanoate (PHA) is a family of natural polyesters frequently employed in many applications, particularly in biomedicine. It is made by various microorganisms, offering unique qualities that other synthetic polyesters do not [3]. Due to the limited mechanical strengths, the PHAs have been combined with other materials to form a composite for biomedical applications. From the previous studies, the PHAs usually blended with other types of polymers such as biodegradable poly(dioxanone) (PPD). The mechanical and thermal properties of PHA/PPD were improved but not the cytotoxicity effects. Previous researchers were also reported for PHA blended with polyethylene glycol (PEG). The PEG affected blood compatibility which has positive effects in delaying clotting time and reducing platelet adhesion. However, there were no results on the mechanical properties reported from this study. In addition, PHAs also have been reinforced with zirconium dioxide (ZrO2). From the biological study, the PHA/ZrO2 composite has improved the tensile and strain properties but exhibited low degradation where there were no significant changes after being implanted in the femora of growing rats in 36 weeks [4-5].

Calcium phosphate (Ca3(PO4)2), also known as CaP, is a modifiable bioactive material extensively employed for bone tissue augmentation and repair. It can promote osteoblast adhesion and proliferation (also known as osteoconduction) and encourage the production of new bone (also known as osteoinduction) [6]. Chitosan is a natural biopolymer derived from chitin which is available in the shells of crustaceans (crabs and shrimp). Researchers have explored using chitosan and other materials to create biopolymer composites to help bone regrow [7]. Studies have shown that chitosan in these composites can improve their mechanical properties, making them stronger and more durable. This is important because the composite must withstand the forces exerted on it during the healing process. Besides, chitosan has been found to exhibit antibacterial properties and support the attachment and proliferation of osteoblast cells that can help prevent infections and promote bone healing. It has been reported that chitosan-calcium carbonate with high mineral content was demonstrated to accelerate new bone formation substantially [8]. This work set out to combine bioactive ceramics with degradable polymers to create a biocomposite that offers a promising approach for applications in bone regeneration. The objective of this study is to identify the biological activities of PHA and PHA/n-CaP/CH composites in vitro, including their capability to generate the apatite layer, and their impact on cytotoxicity for cell division and growth. This work aims to provide suitable conditions for biomaterials to be used as bone scaffold material in bone tissue engineering.

2. MATERIALS AND METHODS

Polyhydroxyalkanoate (PHA) pellets in medical grade were purchased from Ecomann Biotechnology, Inc. (United States). The PHA pellets have a melt mass-flow rate (MFR) of 1 g/10 min (170°C/2.16 kg) (ASTM D570) and a true density or specific gravity of 1.27 g/cm3. The chitosan powders or poly (D-glucosamine) deacetylated chitin (C8H13NO5)n) were purchased from Ecomann Biotechnology, Inc (United States). The particle size of chitosan powder ranges from 30 - 40 μm with 99.9% purity and 0.25 g/mL density.

2.1 Preparation of n-CaP Powders

In the prior work, wet chemical precipitation was used to create the osteoconductive filler of n-CaP (nano calcium phosphate) powder [9]. It contains a 1.63 Ca/P ratio with a composition of 80% HAP + 20% β-TCP. The n-CaP powders have been recorded with 96% purity with less than 10% impurities of copper, nickel, zinc, iron, cobalt, cadmium, chlorine ion and sulfate ions.

2.2 Preparation of PHA/n-CaP/CH Composites

The n-CaP (nano calcium phosphate) and CH (chitosan) were blended with PHA in a Brabender Plastography Machine (D-47055 Duisburg, C. Melchers GMBH & Co, Germany) at a temperature of 130 °C and a rotational speed of 30 rpm for approximately 20 minutes. These parameters were set and used to prepare all PHA composites, as listed in Table 1. The CH composition was standardised at 10wt% to investigate the effects of n-CaP composition variation (0 - 15wt%) on the composites' biodegradability, bioactivity and cytotoxicity.

Table 1: Compositions of PHA, n-CaP, and CH (wt%) in preparing PHA/n-CaP/CH composites

Sample	PHA (wt%)	n-CaP (wt%)	CH (wt%)
PHA100	100	-	-
PHA87	87	3	10
PHA83	83	7	10
PHA79	76	11	10
PHA75	75	15	10

2.3 In Vitro Bioactivity Simulated Body Fluid (SBF) Test

Conventional SBF was prepared for the bioactivity test in this study, following the procedures proposed by Kokubo & Takadama (2006) [10]. The SBF solution was prepared by dissolving various chemicals (Table 2), such as NaCl, NaHCO3, KCl, K2HPO4.3H2O, MgCl, CaCl, and Na_2SO_4, in distilled water, and then buffering the solution with Tris (hydroxyl-methyl- aminomethane, NH2C(CH2OH)3) and hydrochloric acid (HCl) to a pH of 7.4 at 37 °C. Identical ions to those found in the human blood plasma are presented in the SBF solution.

Table 2: Amounts, purities and formula weights of chemical reagents used to prepare 1000 ml of SBF solution following the Kokubo method [10]

Chemical reagent	Amount	Purity (%)	Formula weight
NaCl	8.035 g	99.5	58.4430
$NaHCO_3$	0.355 g	99.5	84.0068
KCl	0.225 g	99.5	74.5515
$K_2HPO_4.3H_2O$	0.231 g	99.0	228.2220
$MgCl_2.6H_2O$	0.311 g	98.0	203.3034
1.0M HCl	39 mL	39 mL	-
$CaCl_2$	0.292 g	95.0	110.9848
Na_2SO_4	0.072 g	99.0	142.0428
Tris	6.118 g	99.0	121.1356
1.0 M HCl	0.5 mL	0.5 mL	-

The biological activity test was performed in an incubator at a controlled temperature of 36.5 °C. All samples were immersed in the SBF solution for different time intervals: 3, 7, 14 and 28 days, while the medium refreshes every two days. After this period, all samples were removed from SBF and dried at 70 °C. At the same time, the pH of the SBF solution was measured from Day 3 to Day 28.

2.4 In Vitro Cytotoxicity Test

The in vitro cytotoxicity test was performed on the samples to verify their safe use in the human physiological environment. Human foetal osteoblast cells (hFOB 1.19, ATCC, USA) were cultured in Ham's F12 Dulbecco's Modified Eagle's Medium (Sigma, USA) at 37 °C in a humidified 5% CO2 incubator. The medium was supplied with 0.4 mg/mL Geneticin (G418, Gibco, USA) and 10% foetal bovine serum (FBS, Sigma, USA), henceforth, considered as a complete medium. The cells that were dissociated by Tryple E Express (Gibco, USA) were cultured in a 75 cm2 flask [11]. The 80-90% confluence cells were then used for the in vitro cytotoxicity test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Before cell seeding, the samples were sterilised under ultraviolet (UV) light for 30 minutes on each surface. The sterilised samples (n=3) were seeded with the osteoblast cells in a 24-well plate. The non-treated cells were set as the Positive Control. Both treated and non-treated cells were incubated for 24 hours in a standard culturing environment: 37 °C, 95% humidification, and 5% CO2. After the prescribed day, the samples were removed, placed in a new well plate, and washed with PBS to remove non-attached cells. A total of 50 µL MTT (5 mg/mL) solution and 250 µL medium were added to each well and incubated for 4 hours for a complete reaction. After 4 hours, 62.5 µL solution from each well was discarded and 125 µL DMSO (filtered before use) was pipetted into the remaining solution to dissolve the formazan crystals. Finally, the formazan products were measured at 570 nm wavelength using a microplate reader (Thermo, USA). The data were presented as a percentage of cell viability based on Equation 1, which is the absorbance value for the specified sample and Ac is the absorbance value for the Positive Control.

$$\text{Cell viability (\%)} = [(As-Ac)/Ac] \times 100 \quad (1)$$

3. RESULTS AND DISCUSSION

3.1 Formation of Apatite Layer Following SBF Bioactivity Analysis

The PHA and PHA/n-CaP/CH composites were sequentially submerged in the SBF solution for 3, 7, 14, and 28 days without pre-washing, followed by the SEM and EDX analyses. The obtained results are shown in Figure 1. The importance of surface morphology after the SBF test is to understand the material's surface structure. The apatite growth can be detected under SEM after 3 to 28 days [10]. Specifically, the PHA100 contained small and irregular particles that seemed to be in masses with fine-grained surfaces. After 3 days of immersion, PHA in Figure 1(a) exhibit small apatite in granular form in white colour. In this stage, since the PHA is not a bioactive material, the apatite grows more slowly compared to PHA/nCaP/Chitosan. The apatite starts to grow after 7 days of immersion as shown in Figure 1 (b). PHA100 was constantly immersed in the SBF solution, apatites were formed on the surfaces as shown by the spherical, cauliflower-like cluster of spheroids [12] in Figure 1(c) at day 14 and Figure 1(d) at day 28, which mostly the deposition of ions from the SBF solution. The sample surfaces undergo obvious changes for the PHA/n-CaP/CH composites, starting from Day 3 of immersion. The presence of numerous thin and flaky-like apatite layers was observed entirely on the surfaces of all PHA/n-CaP/CH composites, specifically on the PHA87, where the structure became denser as the n-CaP content increased, as shown in Figure 1(e), (i), (m) and (q). This demonstrates a morphology resembling bone-like apatite generated on bioactive materials in the traditional biomimetic method using SBF.

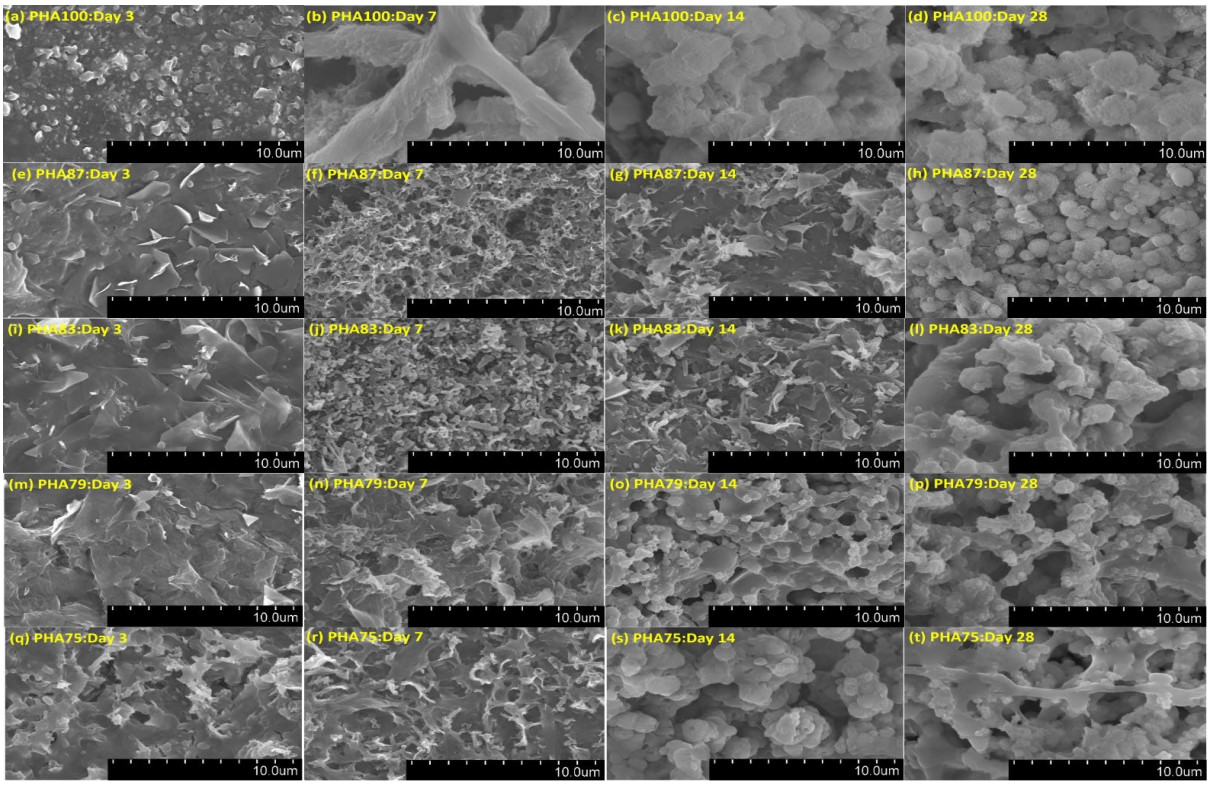

Figure 1: SEM of PHA and PHA/n-CaP/CH composites after (i) 3, (ii) 7, (iii) 14, and (iv) 28 days of SBF immersion

Then, the immersion of samples in the SBF solution for 7 days caused the surfaces of PHA/n-CaP/CH to be uniformly covered with the torn-like structure of the CaP apatite layer as shown in Figure 1, column (ii). After seven days, the torn-like structure of apatite grew increasingly with n-CaP composition. When the SBF immersion reached 14 days, the apatite structure began to shift and appeared to be predominantly formed in the shape of a cauliflower-like cluster of spheroids, as shown in Figure 1, column (iii). The cauliflower-like structure appears in PHA 79 and PHA 75 compared to PHA 87 and PHA 83 which exhibited the torn-like structure. After 28 days of SBF immersion, the dense coral-like structure made up of a cluster of spheroids continues to be interconnected, as depicted in Figure 1, column (iv) (h), (l), (p) and (t). This observable bulk formation of the apatite layer was mostly the result of continuous apatite formation in the SBF, where ion exchange occurred between the SBF solution and the PHA/n-CaP/CH composites. The interconnected nanopores, especially the meso/nanopores, can efficiently assure the absorption of required ions for bone formation, especially the Ca2+ and PO43- ions [13]. In the mineralisation process of bone healing, calcium and phosphate ions were released from the PHA/nCaP/CH composites and exchanged with ions in the surrounding tissue fluids. This exchange creates a concentration gradient that drives calcium and phosphate ions deposition onto the surface of the composite. The deposited ions then react to form hydroxyapatite crystals, which are the mineral component of natural bone. These crystals continue to grow and align to form a mineralised matrix that provides structural support for the growth of new bone.

All deposits contained calcium and phosphorus, according to EDX measurements taken with SEM alongside the presence of oxygen (O), carbon (C), magnesium (Mg), sodium (Na) and chlorine (Cl) peaks, which are also considered to be significant in the formation of apatite. Table 3 shows the calcium-to-phosphate ratio from the percentage of atomic calcium and phosphorous elements that were scanned on the growing apatite layer on the sample surface. The adsorption of Ca2+ ions from the SBF solution on the sample surfaces is one of the processes that led to the formation of apatite, followed by the inclusion of PO43- groups to produce a surface apatite layer. It has been shown that an increase in

the hydrophilicity (polar component) of the surfaces of polymer substrates stimulates the precipitation of apatite [14]. The ratio of calcium to phosphorous in calcium phosphate-based materials is important because it mimics the natural composition of human bone. The human bone has a calcium-to-phosphorous ratio of 1.5 to 1.676, and therefore, materials with a similar ratio are the most effective in promoting bone regeneration. This ratio promotes the formation of hydroxyapatite, which is the mineral component of natural bone. Hydroxyapatite provides an excellent substrate for cells to adhere and proliferate, allowing for new bone formation [15]. The calcium-to-phosphorus ratio is important in bone development because calcium and phosphorus are essential minerals for bone formation. Calcium serves as the primary building block for bones, whereas phosphorus plays a critical role in mineralizing and strengthening the structure of bones. An imbalance in the calcium-to-phosphorus ratio can lead to decrease bone density, mineralization, and impaired bone growth.

Table 3: Calcium-to-phosphate ratios of PHA/n-CaP/CH composites taken from EDX.one growth

Sample	Ca/P ratio
PHA100	1.511
PHA87	1.539
PHA83	1.631
PHA79	1.641
PHA75	1.643

When an artificial material is implanted in a living body, bone-like apatite should be formed on the surface to initiate the interface integration with the physiological bone. The changes in the pH of the SBF solution were also recorded from Day 3 to Day 28. The records indicate that the pH values gradually increased from Day 3 (7.2) for all composites to Day 28 (7.5), as shown in Figure 2. The ionic exchange of H^+ or H_3O^+ ions with Ca^{2+} and Mg^{2+} ions from the SBF solution has caused the pH to rise. Besides, the pH of the human body is 7.35 to 7.45, where there should be no pathological conditions, with 7.40 being the average [16].

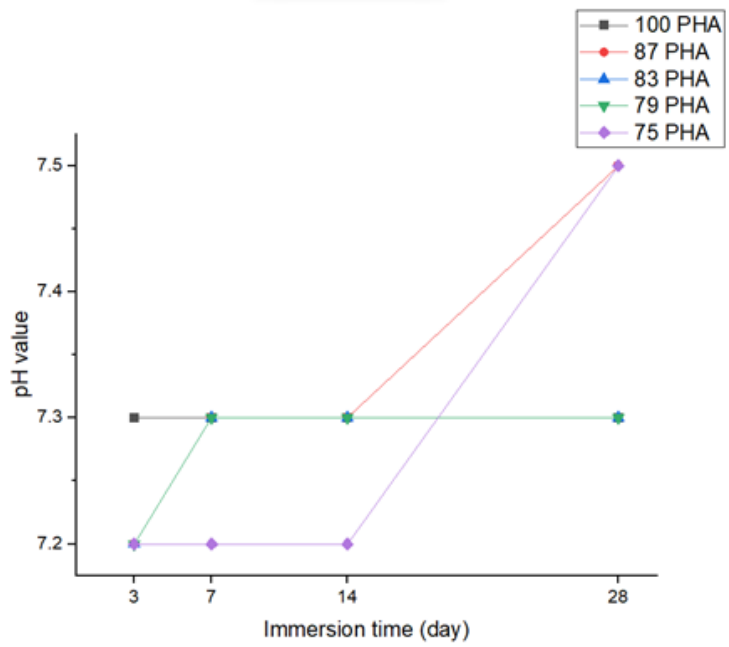

Figure 2: Changes in pH value during a total of 28 days of immersion time in SBF solution under 37 °C

3.2 Cells Compatibility Evaluation

The biocompatibility of the PHA and PHA/n-CaP/CH composites was evaluated through an in vitro cell culture experiment of cytotoxicity test further to investigate the composites' potential in bone regeneration applications. To determine the viability of the cells at the lowest and highest concentrations of n-CaP, human fetal osteoblast (hFOB) cells were seeded on the samples of pure PHA (100PHA) as well as PHA/n-CaP/CH composite with the highest n-CaP content of 15wt% (PHA75) and the lowest n-CaP content of 3wt% (PHA87). From the MTT absorbance values and cell viability percentages, all samples exhibit biocompatibility with osteoblast cells and show higher proliferation compared to the Positive Control (cells without sample) as shown in Figure 3. The PHA87 sample produced double the cell viability percentage compared to the Positive Control within 24 hours of incubation. In addition, the PHA75 sample with 15wt% of n-CaP content was comparable since it met the inclusion criteria for cytotoxicity examination, supported by considerable research on eggshell-based hydroxyapatite [17]. Figure 3 shows that the PHA and PHA/n-CaP/CH composites that were cultured with osteoblastic cells for 24 hours did not cause cytotoxicity. As mentioned earlier, a calcium-to-phosphate ratio of 1.5 to 1.67 promotes bone regeneration because it promotes hydroxyapatite formation. Besides, waste materials from natural resources with a calcium-to-phosphate ratio of 1.5 to 1.67 reveal a more crystalline structure [18], providing an excellent substrate for cells to adhere and proliferate, thus supporting cell growth. The adhesion and growth of cells associated with osteoblasts have also been stimulated by chitosan, making it an ideal carrier for substances that support bone growth [19] Moreover, owing to its antibacterial properties, any aggressive immune responses in the implantation area could be avoided.

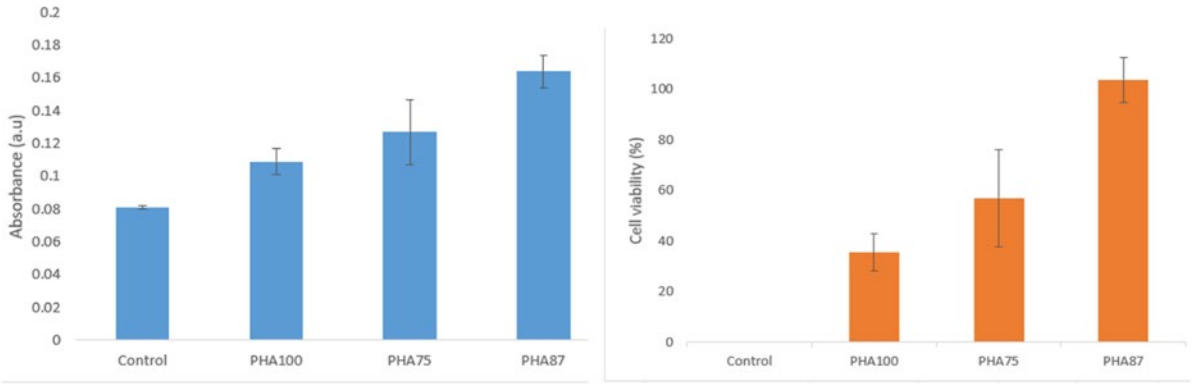

Figure 3: Absorbance values (left) and percentages of cell viability (right) on PHA100, PHA75 and PHA87 after 24 hours of incubation with hFOB cells

PHA/n-CaP/CH composite shows good biological activity rather than any previous biocomposites [11]. After a day, water may penetrate polymeric chains in the PHA/n-CaP/CH biocomposite. Compared to other biocomposites, SBF solution for PHA/n-CaP/CH biocomposite remains in the range of 7.3 to 7.5 which is near to the pH of the human body 7.35 to 7.45 whereas other biocomposites show lower pH. The Ca/P ratio of other biocomposites also shows a different ratio than Ca/P standard stochiometry for bone (1.67), where if there is an imbalance in the Ca/P ratio, it can lead to decreased bone density, mineralization, and impaired bone growth. Lastly, other biocomposite shows a lower percentage of cell viability than PHA/n-CaP/CH biocomposite, which shows this biocomposite has better non-toxic properties and can promote cell proliferation.

4. CONCLUSIONS

From the SBF analysis, the micrographs have shown that the growth of an apatite layer that covered every square inch of the samples illustrates how a particular implanted material can interact with tissues to form interfacial connections when they encounter physiological fluid. Besides, the

average pH of 7.40 during 28 days of SBF immersion shows that PHA and PHA/n-CaP/CH composites can be potential bone graft substitutes. The cytotoxicity test revealed that all samples show no hazardous effects towards the osteoblast cell. Thus, the amount of cell proliferation appears to be doubled in composites with 3wt% of n-CaP thus, surpassing the established standard. The objective of the study was successfully achieved where the biological activity of PHA and PHA/n-CaP/CH composites provide significant findings and valuable information for its application in bone tissue engineering application.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards

References

[1] Li, J., Zhang, Z.., Udduttula, A., Fan, Z., S., Chen, J. H., Sun, A. R. J. & Zhang P. (2021). Microbial-derived polyhydroxyalkanoate-based scaffolds for bone tissue engineering: biosynthesis, properties, and perspectives. *Frontiers in Bioengineering and Biotechnology*, 9, 1-17.

[2] GBD 2019 Fracture Collaborators. Global, regional, and national burden of bone fractures in 204 countries and territories, 1990-2019: a systematic analysis from the Global Burden of Disease Study 2019. Lancet Healthy Longev. (2021). [Online]. [Accessed 18th November 2024]. Available from the World Wide Web: https://pubmed.ncbi.nlm.nih.gov/34723233/.

[3] Aisyah, R., Najah, M. I. & Adzila, S. (2023) Study of hardness and density of polyhydroxyalkanoate (PHA) reinforced with biphasic calcium phosphate (BCP) from eggshell waste. *The Engineer Story*, 1, 9-11.

[4] Najah, M. I., Aisyah, R., Adzila, S. & Haq, R. H. A. (2022) Mechanical properties of calcium phosphate reinforced polyhydroxyalkanoate (PHA) biocomposite. *Journal of Thermoplastic Composite Materials*, 36, 3294–3311.

[5] Janice, L., Mingliang, Y., Jian, L. & Zibiao, L. (2017) Emerging bone tissue engineering via Polyhydroxyalkanoate (PHA)-based scaffolds. *Materials Science and Engineering C.,* 79, 917–929.

[6] Pierfelice, T. V., D'Amico, E., Iezzi, G., Piatteli, A., Pietro, N. D., D'Arcangelo, C., Comuzzi, L. & Petrini, M. (2022) Nanoporous titanium enriched with calcium and phosphorus promotes human oral osteoblast bioactivity. *International Journal of Environmental Research and Public Health*, 19, 6212.

[7] Aminatun, Suciati, T., Sari, Y. W., Sari, M., Alamsyah, K. A., Purnamasari, W. & Yusuf, Y. (2023) Biopolymer-based polycaprolactone-hydroxyapatite scaffolds for bone tissue engineering. *International Journal of Polymeric Materials and Polymeric Biomaterials*, 72, 376-385.

[8] Ressler, A. (2022) Chitosan-based biomaterials for bone tissue engineering applications: a short review. *Polymers*,14, 3430.

[9] Razak, A, Isa, N. M. & Adzila S. (2021) Synthesis of calcium phosphate extracted from eggshell waste through precipitation method. *Biointerface Research Applied Chemistry*, 11, 15058-15067.

[10] Kokubo, T. & Takadama, H. (2006) How useful is SBF in predicting in vivo bone bioactivity?. Biomaterials, 27, 2907-2915.

[11] Huang, J., Xiong, J., Liu, J., Zhu, W. & Wang, D. (2013) Investigation of the in vitro degradation of a novel polylactide/nanohydroxyapatite composite for artificial bone. *Journal of Nanomaterials*, 2013, 3-3.

[12] Hadi, Z., Hekmat, N. & Soltanolkottabi, F. (2022) Effect of hydroxyapatite on physical, mechanical, and morphological properties of starch-based bio-nanocomposite films. *Composites and Advanced Materials*, 31, 26349833221087755.

[13] Cao, J., Lian, R., Jiang, X. & Liu, X. (2020) Formation of porous apatite layer after immersion in SBF of fluorine-hydroxyapatite coatings by pulsed laser deposition improved in vitro cell proliferation. *ACS Applied Bio Materials*, 3, 3698-3706.

[14] Duarte, C. M. F., Santos, S. C., Cruz, L. B. D., Acchar, W. & dos Santos, E. A. (2022) Surface energy changes involved in apatite formation in copper-containing bioactive glasses. *Materials Research*, 2021, 0436.

[15] Islam, M. S., Abdulla-Al-Mamun, M., Khan, A. & Todo, M. (2020) Excellency of hydroxyapatite composite scaffolds for bone tissue engineering. *Biomaterials*, 10, 1-22.

[16] Zerankeshi, M. M., Bakhshi, R. & Alizadeh, R. (2022) Polymer/metal composite 3D porous bone tissue engineering scaffolds fabricated by additive manufacturing techniques: A review. *Bioprinting*, 25, e00191.

[17] Palakurthy, S., Azeem, P. A., Reddy, K. V., Penugurti, V. & Manavathi, B. (2019) A comparative study on in vitro behavior of calcium silicate ceramics synthesized from biowaste resources. *Journal of American Ceramic Society*, 103, 933–943.

[18] Mohd Pu'ad, N. A. S., Koshy, P., Abdullah, H. Z., Idris, M. I. & Lee, T. C. (2019) Syntheses of hydroxyapatite from natural resources: Review article. *Heliyon*, 5, 1-14.

[19] Tian, Y., Wu, D., Wu, D., Cui, Y., Ren, G., Wang, Y., Wang, J. & Peng, C. (2022) Chitosan-based biomaterial scaffolds for the repair of infected bone defects. *Frontiers in Bioengineering and Biotechnology*, 10, 899760.