EFFECTS OF TEMPERATURE AND CONCENTRATION OF SIMULATED BODY FLUID ON BONE APATITE FORMATION USING ELECTROSPUN POLY(E-CAPROLACTONE) FIBRE SUBSTRATE

Nur Aqilah Ibrahim[1], Nor Dalila Nor Affandi[1*], Ahmad Mukifza Harun[2], Mohammad Khursheed Alam[3,4] and Noor Najmi Bonnia[1,5]

[1]Textile Research Group, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Malaysia.
[2]Engineering Faculty, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[3]College of Dentistry, Jouf University, 72721 Sakaka, Saudi Arabia.
[4]Department of Dental Research Cell, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, 600077 Chennai, India.
[5]Materials Science and Technology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.

*dalila@uitm.edu.my

Abstract. A versatile synthetic matrix material for bone regeneration using electrospun fibres was introduced in this study. In this work, the electrospinning parameters were controlled to produce favourable porous fibre substrate that can aid in forming calcium phosphate during an in-vitro biomineralisation process. The fibre substrate underwent two concentrations of simulated body fluid (SBF) to incubate the electrospun poly(ε-caprolactone) (PCL) fibre substrate at different temperatures (37 °C and 25 °C) for 7, 14, and 21 incubation days. The resultant substrate exhibited a large pore diameter with approximately 10 µm and uniform thick layer at about 0.36 ± 0.04 mm. From the SEM analysis, a hexagonal apatite structure was formed. An increase in bone apatite was observed when the SBF concentration increased from 1.0 x to 3.0 x. The EDX analysis showed that by increasing the incubation days to 21, the resultant crystal apatite decreased at body temperature of 37 °C. At room temperature of 25 °C, more crystal apatite was observed under the SEM as the incubation days increased. The current study suggested that the bioactivity of electrospun PCL substrate can be done at body temperatures as well as room temperature.

Keywords: electrospinning, poly(ε-caprolactone), simulated body fluid, bone apatite, temperature

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Introduction

Bone autografts and allografts are the most frequently employed methods for bone replacement in orthopaedic surgeries. However, the effectiveness of autografts is severely limited, attributed to the lack of transplantable bone, the complicated surgical procedure needed for the bone harvest, and donor site complications. Allografts may have introduced the additional risks of immune injection or disease transmission. Therefore, there is a critical demand for synthetic bone graft substitutes to overcome the shortcomings of bone grafting. A versatile, synthetic matrix material for bone regeneration is engineered using electrospun fibres. Electrospun fibres can be made using a technique known as electrospinning. Electrospinning is a powerful technique of producing synthetic polymer filaments by using electrostatic forces, which are capable of delivering the nano to micro range of fibres [1].

The electrospinning tool consists of a high voltage power supply, syringe pump, and a grounded conductive collector. There are several electrospinning parameters, such as solution concentration, the conductivity of the solution, collector composition, distance of the needle tip-to-collector, and needle tip design, which have significant influences on the formation of the polymer fibres during electrospinning process [2]. The electrospinning process uses a high voltage of electric field in a range of 5 kV to 20 kV to produce charges jets electrically from a polymer solution or melts. As the charged jets travel to the collector, solvent from the jets will evaporate, leaving the dried electrospun fibre in a range of 0.1 nm to 100000 nm [2]. Electrospun fibres, which exhibited several outstanding properties, such as a high surface area to volume ratio, good flexibility in surface functionalities and higher tensile strength, are suitable as a substrate for bone regeneration application [2].

One of the polymers that can be extruded using electrospinning is poly(ε-caprolactone) (PCL). PCL is one of the polymer materials suitable for surgery and medicine applications because of its biodegradability. In this work, the electrospinning parameters were controlled to produce a favourable porous PCL fibre substrate that can aid in forming a bone-like apatite structure from calcium phosphate during an in-vitro biomineralisation process. During the in-vitro bio mineralization process, the substrate is immersed into simulated body fluid (SBF), which act as a medium to promote the precipitation of the calcium phosphate. A review on the use of the SBF for assessing materials bio-activity has also been highlighted by Baino and Yamaguchi [3]. Previous studies suggested that the SBF ion concentrations can be increased by 1.5 and 3 times from the original SBF to accelerate the mineralization process [4-6]. In addition, the temperature used in the SBF plays an important parameter during the in-vitro biomineralisation process [4-8]. Several studies used 37 °C to form bone apatite on electrospun substrate [9-12]. However, the formation of the bone apatite on electrospun substrates at room temperature has yet extensively studied. Hence, the current study aimed to investigate the effects of room temperature on bone apatite formation at different SBF concentrations. By undertaking this research work, an in-vitro bioactivity for bone regeneration using electrospun PCL fibre substrate at different temperatures can be determined.

Materials and Methods

Preparation of PCL Solution. PCL (Sigma Aldrich, Mw 80,000 g/mol) in a granular form was dissolved in dichloromethane (Sigma Aldrich) (anhydrous, ⩾ 99.8 %) in a 50 ml Schott Duran bottle. The polymer solution was prepared at 10 wt % concentration due to its ability to form cylindrical fibres, as in the study [13-14]. The mixture was shaken at room temperature with a speed of 150 rpm for 24 hours. The mixture solution was then left at room temperature for another 24 hours before the solution was used to fabricate fibres by using electrospinning.

Electrospinning process. The formation of PCL fibre substrate using electrospinning was reported elsewhere [13-14]. The electrospinning setup consisted of a spinneret with a single nozzle needle gauge (23 G), 0.10 ml/min of flow rate, 15 cm of needle tip -to -the collector and 15 kV of applied voltage. The condition of the electrospinning process was carried out at room temperature. The fibres were fabricated onto a grounded collector with a flat vertical placed on an aluminum foil. The PCL fibre substrate was fabricated onto the collector for three hours and was then dried in a desiccator with 10 ºC. The resultants of PCL substrates were characterised for their morphological structures, fibre diameter, substrate pore diameter, substrate thickness before underwent in-vitro bio mineralisation process.

Morphological structures of PCL fibre substrate and CaP formation. The morphological structures of PCL fibre substrates were characterized by using a Scanning Electron Microscopy (SEM). The PCL fibre samples were cut into 1 cm x 1 cm size dimensions followed by gold coating for 60 seconds before observation to increase the conductivity of the samples. Each sample was then attached to a SEM stub by using carbon tape. The magnification used was 500 x and 10000 x with a constant acceleration voltage of 5 kV. The formation of bone apatite onto the surface of the electrospun PCL substrate after the in-vitro biomineralisation was also characterized by using the SEM. Samples at days 7, 14, and 21 were taken out from a simulated body fluid (SBF) media and gently rinsed off with distilled water. The SEM analysis was conducted to observe the formation of calcium phosphorus (CaP) onto the PCL fibre substrate. Each sample was coated with gold for 60 seconds and attached to the SEM stub by using carbon tape.

PCL fibre diameter and substrate thickness. The ImageJ software was used to measure the mean diameter of the fibres and apatite size formed on the fibre substrate. From Field Emission Scanning Electron Microscopy (FESEM) and SEM images of electrospun PCL fibres substrate, at least 50 of the fibre diameter measurements were randomly selected. 50 readings of apatite size were also analysed for each sample. Substrate fibre thickness was measured by using a digital micrometre gauge (digimatic micrometer gauge). At least 10 readings were taken for each sample in random are-as and then averaged.

Pore size distribution of PCL fibre substrate. A mercury porosimeter (AutoPore V-Micromeritics) was used to analyse the pore size distribution of electrospun PCL fibres substrate. The mercury porosimeter employed a pressurized chamber (up to 60,000 psi) to force mercury to intrude into the voids of the electrospun PCL fibre substrate. As pressure was applied, mercury filled the larger pores, followed by the smaller pores. The pore size distribution of the substrate was determined automatically by the porosimeter using the Washburn equation [15].

$$D = (-4\gamma\cos\theta)/P \quad (1)$$

where D is the pore diameter (μm), γ the surface tension of mercury (485 mN/m) or 485 dyne/cm), θ the contact angle of mercury (approximately 130º), and P the applied pressure (psi).

Energy dispersive X-ray (EDX) analysis. The substrates of electrospun PCL fibre after the in-vitro biomineralisation process were tested to determine the detailed element on the substrate by using the EDX. After in-vitro biomineralisation process, all samples were analysed for calcium (Ca) and phosphate (P) elements in electrospun PCL fibre substrates.

Preparation of simulated body fluid. The preparation of a metastable solution of SBF was based on ISO 23317 standard method ("International Standard ISO 23317 Third Edition") and a previous study [4] and marked as SBF 1.0 time (SBF 1.0 x). To accelerate the mineralisation process, the SBF concentration was increased by 3 times and marked as SBF 3.0 x. The chemical compositions of SBF 1.0 time (SBF 1.0 x) and SBF 3.0 times (SBF 3.0 x) are given in Table 1 and 2. All the chemicals were weighted and were then added to the solution in the same order as given in Table 1 and Table 2, respectively. The total SBF solution for each concentration was 1000 mL. After adding $(HOCH_2)3CNH_2$ Tris, the temperature of the SBF solution was maintained at 36.5 ºC, and the pH was adjusted to 7.4 by using a 1m-HCL solution. Before using the SBF solution, the stability was examined. A stable SBF did not produce any precipitation. If the precipitation occurred, the SBF solution was discarded, and a new solution was prepared.

Table 1. Order and amount of reagents for preparing 1000mL of SBF 1.0 x.

Order	Reagent	Amount
1	NaCl	8.0368 g
2	$NaHCO_3$	0.3570 g
3	KCl	0.2272 g
4	$K2HPO_4.3H_2O$	0.2328 g
5	$MgCl_2.6H_2O$	0.3225 g
6	1m-HCl	39 ml
7	$CaCl_2$	0.2938 g
8	Na_2SO_4	0.0716 g
9	$(HOCH_2)3CNH_2$	6.1190 g
10	1m-HCl	5 ml

Table 2. Order and amount of reagents for preparing 1000mL of SBF 3.0 x.

Order	Reagent	Amount
1	NaCl	24.1069 g
2	$NaHCO_3$	1.0660 g
3	KCl	0.6748 g
4	$K2HPO_4.3H_2O$	0.6930 g
5	$MgCl_2.6H_2O$	0.9377 g
6	1m-HCl	117 ml
7	$CaCl_2$	0.8698 g
8	Na_2SO_4	0.2208 g
9	$(HOCH_2)3CNH_2$	18.3570 g
10	1m-HCl	15 ml

In-vitro biomineralisation process. The in-vitro biomineralisation was based on the previous study [16]. The purpose of this process to observe the formation of bone-like apatite from calcium phosphate onto substrate. The substrate of electrospun PCL fibres was immersed into the SBF at two different temperatures which were 37 °C (body temperature) and 25 °C (room temperature), respectively. Each sample was cut into 0.1 cm x 0.1 cm in a square and then incubated in 1 ml of the SBF. All the samples were left and observed for over 21 days, which the substrate was taken out on days 7, 14, and 21 of the incubation periods. The formation of bone apatite on the electrospun PCL substrate was characterized for the morphological structures and EDX.

Results and Discussion

Morphological structures of PCL fibre. Figure 1 depicts the morphological structures of PCL fibre substrate produced from electrospinning process. The resultant electrospun PCL substrate had a diameter of 6.037 ± 1.36 µm, pore diameter of 10 µm and substrate thickness of 0.36 ± 0.04 mm. The substrate was further tested for bio-mineralization process

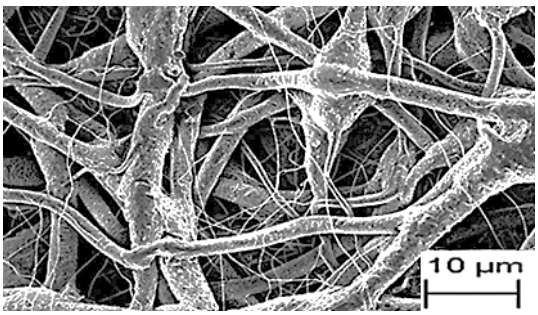

Figure 1. FESEM image of an electrospun PCL fibre substrate produced at the 0.10 ml/min flow rate with a 15 cm distance of needle tip -to -the collector.

The effects of body temperature (37 ºC) and room temperature (25 ºC) on the formation of bone-like apatite on electrospun PCL substrate. Figures 2 (a-c) illustrate the electrospun PCL fibre substrate in SBF 1.0 x at 37°C for days 7, 14, and 21 of incubation. From observation, little deposition of crystal apatite was present on day 7. The crystal apatite with hexagonal phase started to deposit within the electrospun PCL substrate as well as the PCL surfaces when the incubation day increased from day 14 (Figure 2 (b)) to day 21 (Figure 2 (c)). Similar results were also discovered at SBF 3.0 x, where a layer of crystal apatite was clearly seen on the electrospun PCL substrate (Figures 2 (d-f)). As the SBF concentration increased from 1.0 x to 3.0 x, more apatite particles were deposited into the electrospun PCL substrate. This indicated that an increase in SBF concentration has accelerated the bioactivity process, resulting in more crystal apatite depositions on the substrate. The size of the apatite was approximately 1.8 µm. The morphological structure of electrospun PCL fibre substrate after the in-vitro biomineralisation process at 37 ºC body temperature using the SBF as a medium resulted in a successful deposition of crystal apatite. Although the characteristic nature of PCL is hydrophobic, the increase of SBF concentration resulted in the electrospun PCL fibres being more effective for the in-vitro bio mineralization. To confirm the analysis, all the samples were tested with EDX.

The bioactivity process through the in-vitro biomineralisation of electrospun PCL substrate was also handled at 25 ºC room temperature in an incubation oven to investigate the efficiency of the room temperature to nucleate the apatite from the SBF. Most of the studies [16-18] incubated electrospun substrate in SBF at a body temperature. Figures 3 (a-f) depict the typical SEM images of the electrospun PCL fibre substrate, in which the deposition of bone-like apatite can be seen within the fibres. The deposition of the apatite increased when the SBF concentration increased from 1.0 x to 3.0 x. At SBF 1.0 x, more apatite was formed when the incubation day increased from day 7 to day 21 (Figures 3 (a-c)). The size of the crystal apatite was approximately 1.8 µm. The crystal apatite in the current study was larger than the previous study [8, 19]. Previous studies reported that the size of crystal apatite was in a range of 13 nm to 50 nm [8, 19]. The current study observed that several apatite depositions on the PCL substrate form a large apatite. When the electrospun PCL substrate was incubated in the SBF 3.0 x from day 7 to day 21 of incubation days, more crystal apatite was deposited into the substrate. The SEM micrographs shown in Figures 3 (d-f) signify the presence of crystal apatite deposition within the electrospun PCL fibres. The amount of the apatite was also high as compared to SBF 1.0 x. To identify the amount of apatite deposited on the substrates, an EDX test was conducted on the samples.

As reported by several studies, an addition of other materials is required to increase the ion precipitation for calcium and phosphate [17, 20]. However, findings on the morphological structure as shown in Figures 3 (a-f) prove that room temperature (25 ºC) has the potential to nucleate crystal apatite from the SBF into electrospun PCL fibre substrate even without adding other materials.

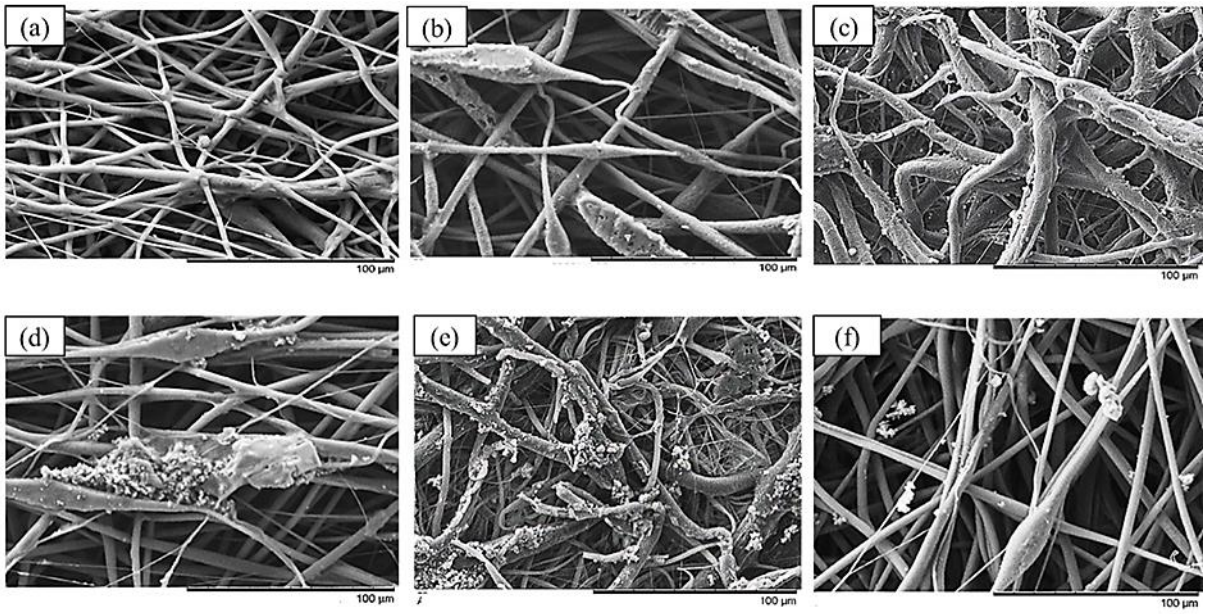

Figure 2. Typical SEM images of the electrospun PCL fibre after the in-vitro biomineralised process in different concentrations of SBF at 37 ºC. (a) SBF 1.0 x at day 7 (b) SBF 1.0 x at day 14 (c) SBF 1.0 x at day 21 (d) SBF 3.0 x at day 7 (e) SBF 3.0 x at day 14 and (f) SBF 3.0 x at day 21.

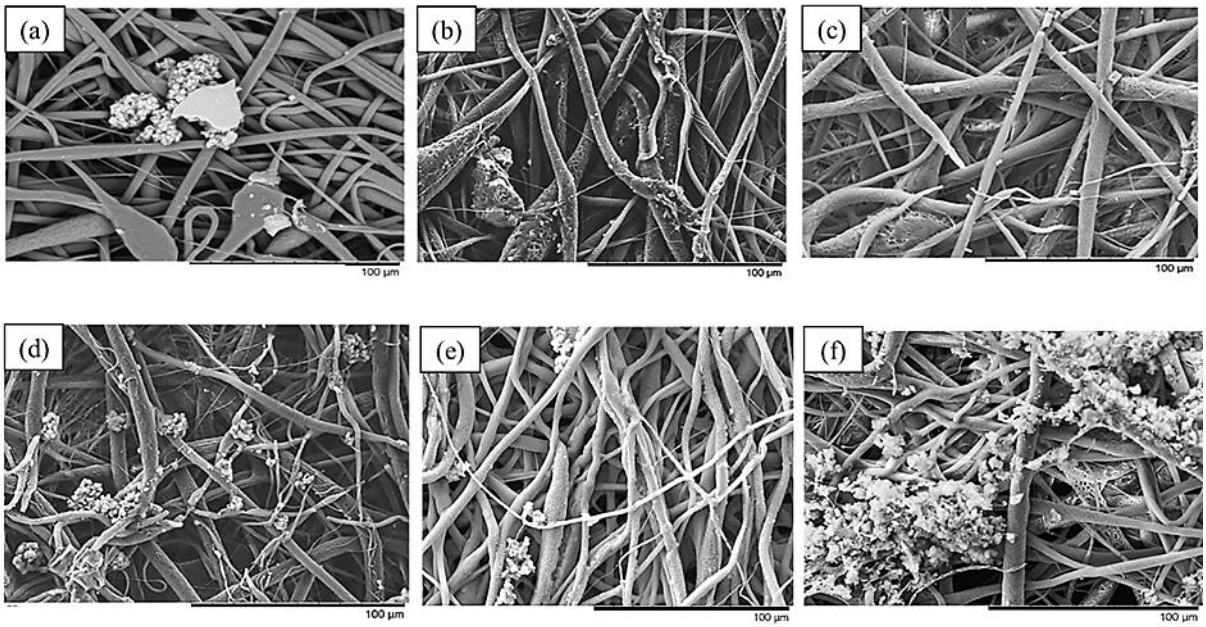

Figure 3. Typical SEM images of the electrospun PCL fibre after the in-vitro biomineralised process in different concentrations of SBF at 25 ºC. (a) SBF 1.0 x at day 7, (b) SBF 1.0 x at day 14 (c) SBF 1.0 x at day 21 (d) SBF 3.0 x at day 7 (e) SBF 3.0 x at day 14 and (f) SBF 3.0 x at day 21.

EDX analysis on the bone-like apatite from electrospun PCL substrate at body temperature (37 ºc) and room temperature (25 ºC). The amount of bone-like apatite containing calcium (Ca) and phosphorus (P) on the electrospun PCL substrate was investigated using the EDX. From the analysis, the samples contained carbon (C), oxygen (O), calcium (Ca), and phosphorus (P) elements. The current study focused on calcium (Ca) and phosphorus (P) because both common elements exist in the bone-like apatite. At a body temperature of 37 ºC, the Ca/P ratio increases with an increase in the SBF concentration (Table 3). Apart from the SBF concentration, the incubation day also affected the Ca/P ratio of the apatite. At SBF 1.0 x, the amount of Ca/P ratio increased from 0.12 % to 0.38 % when the incubation day increased from day 7 to day 14. When the incubation day was further increased to day 21, the Ca/P ratio was observed to reduce to 0.12%. Aside from SBF 1.0 x, a reduction in Ca and P elements was also found at 3.0 x when the incubation days increased from day 7 to 21. A possible reason for the reduction was due to the detachment of crystal apatite from the electrospun PCL fibre substrate at a temperature of 37 ºC. As a result, a small amount of Ca and P elements were left in the substrates. As reported by Qu and Wei [22], ions in SBF tend to decompose, causing the amount of apatite to decrease at high temperatures usually above room temperature. A small amount of apatite on the electrospun PCL fibre substrates was also reported by other studies [22-23].

Table 4 tabulates the Ca/P ratio of the crystal apatite on electrospun PCL substrate after in-vitro biomineralisation at room temperature of 25 ºC. As mentioned earlier, the increase of SBF concentration was found to increase the apatite amount. An increase in SBF concentration has accelerated the bioactivity process, resulting in more crystal apatite depositions on the substrate. The acceleration of the bioactivity process at higher SBF on the electrospun substrate was also reported in another study [22]. The results were confirmed by the EDX analysis, where the deposition of calcium (Ca) and phosphate (P) increased as the SBF concentrations increased from 1.0 x to 3.0 x. At SBF 3.0 x, an increase in Ca/P ratio was observed at a longer period of incubation. During a long incubation period, it allowed more apatite to precipitate into the

electrospun PCL substrates. The results were contradicted to several past studies [17, 22-23], where they reported that the PCL formed a low deposition of bone-like apatite that contained calcium and phosphate ions. This was due to the strong hydrophobicity of PCL which then resulted in an inactive capillary force. However, the current study has found that the hydrophobicity of PCL may not be the only factor for the low deposition of the apatite. The temperature may also affect the deposition of the apatite in the substrate. From the results, it showed that 25 °C has more apatite than 37 °C probably due to the apatite being more stable at 25 °C. At 37 ºC, ions in SBF tend to decompose, causing the amount of apatite to decrease at high temperatures usually above room temperature. Similar findings were also reported by Qu and Wei [22].

Table 3. Ca/P ratio of bone apatite in the electrospun PCL substrate incubated at 37 ºC.

	SBF concentration	1.0 x	3.0 x
		Ca/P ratio	Ca/P ratio
	*Control	0	0
	7	0.12	1.09
Day	14	0.38	0.86
	21	0.12	0.18

*Electrospun PCL before immersion in SBF

Table 4. Ca/P ratio of bone apatite in the electrospun PCL substrate incubated at 25 ºC.

	SBF concentration	1.0 x	3.0 x
		Ca/P ratio	Ca/P ratio
	*Control	0	0
	7	0.27	0.74
Day	14	0.26	0.46
	21	0.49	1.55

*Electrospun PCL before immersion in SBF

Conclusion

This study found that temperature may influence the deposition of apatite. Overall, the electrospinning process can be controlled to have the desired fibre that is optimal for various applications. For bone regeneration application, as in this study, PCL is excellent to be used either at body temperature or room temperature to predict the in-vitro biomineralisation.

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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 does not require any ethical procedures.

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