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DOI:

[10.1016/j.msec.2020.111168](https://doi.org/10.1016/j.msec.2020.111168)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Zhu, J., Tang, D., Lu, Z., Xin, Z., Song, J., Meng, J., Lu, J. R., Li, Z., & Li, J. (2020). Ultra fast bone-like apatite formation on highly porous poly(l-lactic acid)-hydroxyapatite fibres. *Materials Science and Engineering C: Materials for Biological Applications*, [111168]. <https://doi.org/10.1016/j.msec.2020.111168>

Published in:

Materials Science and Engineering C: Materials for Biological Applications

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PII: S0928-4931(20)32009-9

DOI: <https://doi.org/10.1016/j.msec.2020.111168>

Reference: MSC 111168

To appear in: *Materials Science & Engineering C*

Received date: 4 May 2020

Revised date: 27 May 2020

Accepted date: 5 June 2020

Please cite this article as: J. Zhu, D. Tang, Z. Lu, et al., Ultrafast bone-like apatite formation on highly porous poly(l-lactic acid)-hydroxyapatite fibres, *Materials Science & Engineering C* (2018), <https://doi.org/10.1016/j.msec.2020.111168>

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Ultrafast bone-like apatite formation on highly porous poly(L lactic acid)-hydroxyapatite fibres

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Abstract

In order to provide a favourable environment for living bone formation, it is an essential condition to grow bone-like apatite layer at the interface between the tissue-implant and its surrounding tissues. Inspired by the chemical composition and the nano porous structure of natural bones, we developed an ultrafast and accessible route to accelerate effectively the formation of bone-like apatite on the surface of porous poly(L-lactic acid)-hydroxyapatite (PLLA-HA) composite fibres in 5 times simulated body fluid (5SBF). The key of the method lies in successful exposure of HA nanoparticles on the surface of PLLA fibres by acetone treatment of electrospun PLLA-HA nano/micro fibres. The recrystallization of PLLA chains uncovers more HA nanoparticles on the surface of every fibre which provide nucleation sites for calcium and phosphate ions. After only 2 hours of immersing in 5SBF, a full layer of apatite completely covered on the surface of porous PLLA-HA fibres. The results indicate that HA nanoparticles on porous fibre surface can accelerate the kinetic deposition of apatite on fibre

surface. Biological *in vitro* cell culture with human osteoblast-like cell for up to 7 days demonstrates that the incorporation of HA nanoparticles on the surface of porous PLLA fibrous membranes leads to significant enhance osteoblast adhesion and proliferation. The route can open avenues for development of fibrous PLLA biomaterials for hard tissue repair and substitution.

Keywords: hydroxyapatite; mineralization; poly(L lactic acid); electrospinning; porous fibre.

1. Introduction

In the recent decades, the leading technologies of development of biomaterials have evolved extensively to accommodate the requirements in osteogenesis field of meditational applications. With the introduction of tissue engineering, studies toward excavating the potentials of the performance of the polymeric biomaterials continue to carry out and the results are promising. It is yet too early to describe the time now mature because in the past century the development of such materials have raised massive revolutionary improvements in modern medicine and treatment for diseases and it is still growing [1-4].

The major idea of using tissue engineering approach for bone repair is to mimic the bone growth environment [5]. The key to success lays onto the imitation of the extracellular matrix (ECM) for the bone cells to attach to and reconstruct bony tissue [6]. Naturally developed bone structures by human body, such as trabecular and cortical, are constructed with mineralised collagen fibres which are the core element of the bone tissue [7]. Osteoblast mineralises base on the collagen fibres ECM framework and build up layers of calcium (Ca)-based mineral to form up the bone morphology [8]. It is a promising approach to construct an artificial biocompatible polymeric scaffold to imitate the fibrous ECM for osteoblasts attachment [9, 10].

Electrospinning technique, by breaking surface tension of polymeric solution under electric field and collecting woven scaffold formed from jets of polymer stands, can develop 2-D or 3-D scaffolds with nanofibers that have similar dimensions comparing to collagen ECM [11]. PLLA, as a biocompatible material with good mechanical integrity, has been electrospun to be nano/micro fibres and fibrous scaffolds to realise this task in *in-vitro* and *in-vivo* experiments [12]. The porous morphology of the scaffold provides enough spaces for cell attachment and later intrinsic vascularisation forming new skeletal system as well as nutrition and metabolites permeations [13]. Some studies suggested that the PLLA nanofiber scaffold promotes progenitor stem cells differentiation to osteoblasts, proliferation, mineralisation and whole endochondral ossification process [14, 15].

Many substances including proteins, polyamine, metal oxides, calcium salts, bioactive glass, or other polymers are proven to be beneficial for bone growth. More specifically, these substances enhance mesenchymal stem cells derived osteoblast attachment, proliferation or Ca deposition, or all of above [16]. As a result, preparing polymeric scaffold with such surface deposition are highly focused and debated. Ca contents that present in the tissue-scaffold interface are considered to be a good stimulus toward osteo-regeneration [17, 18]. Therefore, polymer scaffolds with hydroxyapatite (HA), CaCO_3 , amorphous calcium phosphate (ACP) and other Ca-rich substances have been widely studied up to the present [19-22].

It is suggested that the amount of ionic Ca content among the surface of implant scaffold determines the mineralisation of the alkaline phosphatase (ALP) catalysed reaction of Ca deposition [23, 24]. How to increase the deposition of such materials upon the surface of the polymeric scaffold substrate becomes worth debating deeply. The aim of such coating is to

maximise the probability of calcium and phosphate (Ca-P) layer formation [25]. The layer provides connection between implants and living bone with cells and acts as an essential part of osteogenesis [26]. The enrichment of the Ca ions provides more nucleation sites for Ca-P structure generation. Substances such as CaCO_3 or HA effectively provide such opportunity for more Ca-P growth [27]. Osteoblasts are observed to be keen on such Ca-P structure rich environment and behave more actively indicated by increasing adhesion rate and Ca mineralisation by ALP [28]. Moreover, Ca-enriched areas among the scaffold surface help enhance the local hydrophilic property which is another improvement for cell adhesion [29].

High specific surface area is an inherent advantage of electrospun polymer fibrous scaffolds. Naturally, then, Ca materials have been introduced into these nano/micro fibres for fast mineralization and good bone regeneration. For ACP-PLLA and HA-PLLA composite nanofibers, the ACP nanospheres and HA nanorods played an important role in the formation of HA nanosheets on the surface of composite nanofibers [30]. Smooth gelatin nanofibers were successfully obtained at low concentrations of CaCO_3 which provides nucleation sites for the growth of Ca-P structures, leading to mineralization of the mats [26]. The deposition rate of Ca-P based material precipitates was higher on the surface of HA-poly(ϵ -caprolactone) (PCL) nanocomposite fibre mats than that of bioactive glass-containing PCL scaffolds [31]. While Ca nanoparticles on fibre surface play their roles in the mineralization, most of them are completely trapped inside the polymer matrix and have no chance to contact their external environment. Therefore, if the solid polymer-Ca composite fibres are changed to be some porous ones, it should be beneficial to provide more sites for Ca-P structures or bone cell attachment.

In this paper, PLLA nano/micro fibres were electrospun with HA nanoparticles. After PLLA/HA composite fibres were treated by acetone, the recrystallization of PLLA generated hierarchical porous structure throughout each individual fibre. While the porous fibres get ultra-high surface area, more HA nanoparticles were exposed on the fibre surface. The effects of porous PLLA-HA fibres on mineralization and their biocompatibility were examined.

2. Method

2.1 Materials

Sodium hydroxide (NaOH), hydrochloric acid (HCl), calcium chloride (CaCl_2), and sodium dihydrogen phosphate (NaH_2PO_4), materials for preparing SBF (NaCl , NaHCO_3 , KCl , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, CaCl_2 , Na_2SO_4 , tris(hydroxymethyl)aminomethane (Tris) and HCl), dimethylformamide (DMF), dichloromethane (DCM), ethanol and acetone were all analytical grade and bought from Sigma-Aldrich. Poly (L-lactic acid) (PLLA, PL65) was supplied by Corbion (Netherlands). Water used in this experiment was deionised (DI) water.

2.2 Synthesis of HA nanoparticles

HA nanoparticles were synthesized based on our previous studies [32, 33]. In short, 180 ml NaH_2PO_4 solution (0.1 M) was dropped into 300 ml CaCl_2 solution (0.1 M) with gentle stirring. Afterward, the pH value of the solution was adjusted to 7.0 by adding NaOH solution (0.1 M). After the reaction completed, the mixed solvent was continuously stirred for 1 hour and then kept for 24 hours. Finally, HA precipitation was collected and washed by DI water for 4 times to remove any unreacted residues. HA particles were washed by ethanol and then DMF for 3 times, respectively. The concentration of the final HA/DMF suspension was adjusted and

verified for the following process.

2.3 Electrospinning of PLLA-HA fibrous membranes

For preparation of the electrospinning solutions, PLLA and DCM were added into a sealed glass reagent bottle and stirred with an electromagnetic stirrer at 50 °C. After PLLA was completely dissolved, HA/DMF suspension were added into PLLA/DCM solution. The mixed solution was treated by the ultrasonic equipment for 30 min before electrospinning. The final composition of the electrospinning solution was: DCM : DMF = 9 : 1 in weight; PLLA : (DCM/DMF) = 2 : 98 in weight, HA : PLLA = 1 : 9 in weight.

The electrospinning was carried out using an electrospinning unit consisting of a high voltage power supply; a syringe pump for delivering the spinning solution at a constant rate; a syringe provided with a positively charged metal needle with inner diameter of 0.6 mm and a grounded rotating collector. The electrospinning was performed under following conditions: feeding rate: 3 mL/h, applied voltage: 25 kV, tip-to-collector distance: 17 cm, collector rotating speed: 200 rpm, humidity: 50% and temperature: 20 °C. The obtained fibrous membranes were dried in a fume cupboard at room temperature for 24 hours. Both PLLA and PLLA-HA fibrous membranes were all electrospun for comparison.

To generate porous structure on every single fibre, the fibrous membrane was immersed into acetone for 5 min at room temperature and then dried for 24 hours. Finally, four samples were prepared: PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes, as shown in Figure S1.

2.4 Mineralization in 5SBF

The preparation of 5SBF solution was based on our previous study [34]. To prepare 1000

ml of 5SBF, 700 ml DI water was added into a big plastic beaker with gentle stirring. The first group of chemicals, including NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O and MgCl₂·6H₂O, were dissolved in DI water in the order shown in Table S1. During this process, the temperature was kept between 35 °C and 38 °C. Then the pH value was adjusted to 6.4 by 1.0 M HCl. After the first group of chemicals was dissolved completely, CaCl₂ was added slowly in the solution to avoid precipitation. Then Na₂SO₄ was added into the solution after CaCl₂ was dissolved completely. The pH value of the solution was adjusted to 6.4 by Tris and 1.0 M HCl. Finally, the volume of the solution reached 1000 ml by adding DI water. In the process of adjusting pH, the temperature should be kept preferably between 36 °C and 37 °C, and the pH changes should be less than 0.02 after pH value arriving at 6.2.

A piece of fibrous membrane with dimensions of 20 × 20 mm² (thickness about 80 μm) was firstly wetted by 50% alcohol and washed by DI water and 5SBF for 5 times, respectively. This was performed to force the solution into the pores of the fibrous membranes. The samples were immersed in 50 ml 5SBF in a plastic bottle with the temperature maintained at 37 °C in a water bath. After being maintained at such conditions for 1, 2, 4 and 6 hours, the specimens were removed from 5SBF, washed carefully with DI water to remove the soluble inorganic ions, and dried in air. The mass changes of the samples after incubation in 5SBF were measured by using an analytical balance (accuracy: 0.1 mg). Six specimens with identical dimensions were used for each sample.

2.5 Characterizations

SEM: The morphologies of HA nanoparticles and the fibrous membranes were examined using scanning electron microscopy (SEM, Carl Zeiss AG-UL TRA 55) at 1.5 kV. All samples were

pasted onto a carbon tab and coated with gold.

TEM: HA nanoparticles were dropped onto the carbon-coated copper grids for the TEM test by a transmission electron microscope (TEM, Tecnai 20).

FTIR: HA particles were dried in an oven at 60 °C for 48 hours to avoid errors of measurements. They were mixed with potassium bromide (KBr) with 1 : 20 mass ratio and grounded in an agate mortar. Each of the membrane samples was teared out a small piece for the FTIR analysis. FTIR data were recorded by a Fourier transform infrared spectrometer (Nicolet 5700).

XRD: Crystallinity and structure of HA powders as well as membrane samples were analysed by X-ray diffractometer (XRD5, PANalytical X'Pert Pro). Data collections from 10 to 60° coupled 2theta/theta at 0.033° step 100 s/step was undertaken.

DSC: Differential scanning calorimetry Q1000 (TA instrument Ltd. USA) was used for data acquisition. The temperature range was set from 20 °C to 300 °C with a heating rate at 10 °C/min. Each of the samples was measured three times, and the results were analysed based on the average data.

Contact angle: Contact angle equipment KRUSS DSA 100 was used for testing membrane samples. All samples were put on a glass plate. For the uniform surface of membranes, another glass cover plate was used to compact the sample. The droplet used in the test was DI water.

BET: Surface area membrane samples were analysed with Gemini 2360 BET Surface Area Analyser. All samples were run three times each for collecting data.

Mechanical test: The mechanical test was conducted by Instron 3344 with a tensile speed of 5 mm/min. The samples were cut into sample stripes fitting the size of an assisted paper window, as shown in Figure S2.

2.6 Cell culturing

PLLA, porous PLLA, PLLA-HA and porous PLLA-HA membranes ($8 \times 8 \text{ mm}^2$, 30-50 μm in thickness, six pieces for each group) were cut for cell culture, respectively. The fibrous membranes were soaked in absolute ethanol for 24 h, then were exposed to UV light for 3 h for every sides, respectively. Before cell seeding, these membranes were sterilized with ethanol (70%, 30 min) then washed with phosphate buffered saline (PBS, Sigma, 15 min) 3 times and finally soaked in complete medium overnight.

Human osteoblast-like cell, Saos-2, was obtained from ATCC and cultured in T 25 culture flasks (SPL, Korea) in an incubator (37 °C, CO₂ (5%)). Dulbecco's modified medium (DMEM, Invitrogen, USA), was supplemented with foetal bovine serum (10%, FBS, Invitrogen, USA), penicillin (1%, Invitrogen, USA), streptomycin (1%, Invitrogen, USA). All cells used in this study were at passage 10 or less. Cells were harvested by adding trypsin with EDTA (0.25%, Invitrogen, USA). The trypsin was neutralized by adding DMEM media with FBS (10%). Then the cell suspension was centrifuged and 60,000 cells/cm² were seeded on each sample on 24 well plates, and incubated (37 °C, CO₂ (5%)) until the evaluation.

2.7 MTS

After the cells were incubated on the above-mentioned membranes for 1, 3 and 7 days, MTS assay was added to the culture medium in each well to monitor the cell proliferation using Cell Titer 96 Aqueous Assay. Prior to this assay, the cellular constructs were rinsed with PBS followed by incubation with MTS (20%) reagent in serum free medium for 3 h. The absorbency of the solution from each well via 490 nm wavelength was read by Micro-plate reader infinite F200 (TECAN, Switzerland).

2.8 Alkaline phosphatase assay

The alkaline phosphatase (ALP) activity was measured using an ALP assay kit (Zhongsheng Beikong, China). Saos-2 cells were incubated on the samples in a 24-wells plate for 7 days. After removing the culture medium, the cell layers were washed with PBS and then detached using trypsin/EDTA. After centrifugation, the cell pellets were washed with PBS and resuspended by vortexing them in 0.1 mL of DI water with 0.1% Triton X-100. The cell pellets were disrupted via a cyclic freezing/thawing process. The prepared cell lysates were used to determine the ALP activity according the ALP assay manufacturer's instructions.

2.9 Live/dead cells assay (FDA/PI Stain)

The cell permeable esterase-substrate fluorescein diacetate (FDA) and the cell impermeant nucleic acid stain propidium iodide (PI) were combined to assess the viability of Saos-2 grown on the above-mentioned membranes. Saos-2 cells were seeded on the membrane surface in 24-well plates and cultured for 1, 3 and 7 days. Then membranes with cells were rinsed three times in PBS and the cells were stained by rinsing PBS with FDA (1.0 $\mu\text{g/mL}$) and PI (1.0 $\mu\text{g/mL}$) for 5 min at dark and room temperature. Cell images were taken by a fluorescent microscope. Cell viability in each section (11,828 μm^2) was assessed by counting the number of viable cells (green) and dead cells (red).

2.10 Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. Statistically significant differences were determined by one- or two-way analysis of variance (ANOVA) and Bonferroni post-test. Statistical significance was accepted at $p < 0.05$ and indicated in the figures as *.

3. Results and discussion

3.1 HA nanoparticles

HA nanoparticles have been widely used to improve the biocompatibility of polymer composite scaffolds for better mineralization, proliferation and differentiation of osteoblasts for a long culturing period. However, like other nanoparticles, it is a challenge to uniformly disperse dry HA powders into polymer matrix. The aggregation of nanoparticles not only seriously limits the function of each particle, but also reduce the processability of polymer composites. Specifically, for electrospinning process, big particles have negative effect on the morphology and uniformity of nano/micro fibres.

To address these issues, HA nanoparticles were fabricated by an *in situ* precipitation synthesis approach in this study [32, 33, 35]. After the HA was synthesized from an aqueous system, HA precipitation was washed by absolute ethanol for several times to replace water. Then ethanol was replaced by DMF. HA was finally collected as a HA/DMF slurry for the following electrospinning solution. From the SEM and TEM images of HA nanoparticles, we can see some spindle-like crystals with a length of about 80-120 nm and a diameter of about 10-20 nm, as shown in Fig. 1(a & b).

HA has two primary characteristic peaks indicating the presence of PO_4^{3-} and CO_3^{2-} [32, 36]. In the HA spectrum (Figure 1(c)), the bands at 602 and 562 cm^{-1} represent the O-P-O bending mode and the bands at 1091, 1034 and 962 cm^{-1} correspond to P-O stretching vibration [32, 37]. The existence of CO_3^{2-} is shown by bands of 1422 and 874 cm^{-1} which are C-O stretching mode [37]. The reason for the existence of CO_3^{2-} is the CO_2 in the air replace some parts of HA to form the carbonate ions. The band at 1,635 cm^{-1} is attributed to H_2O group in HA.

In Figure 1(d), XRD pattern shows crystallographic structures of HA particles. The characteristic peaks appear at 25.8° , 31.8° , 32.9° , 34.1° , 40° , 46.8° , 49.6° and 53.5° , corresponding to diffraction planes including (002), (211), (300), (202), (310), (222), (213) and (004) [37, 38]. Three planes (211), (300) and (202) are significant peaks of commercial HA [37]. The synthesised HA nanoparticles own these major peaks, implying the successful preparation of HA.

Figure 1. (a) SEM, (b) TEM, (c) FTIR and (d) XRD of HA nanoparticles.

3.2 Electrospun PLLA and PLLA-HA fibres

As a biodegradable PLLA, it has been extensively studied and applied in tissue engineering field. Electrospun PLLA nano/micro fibres and fibrous membranes have been used

as porous substrates or scaffolds for various cells. Like most of other studies, PLLA fibres were collected as non-woven fibrous membranes based on optimized electrospinning parameters in our primary works [33], as shown in Figure 2(a). The electro-spinnability of PLLA is quite good, and PLLA fibres are uniform and exhibit a circular cross section.

After the HA was synthesized from an aqueous system, the HA-water precipitation was washed by absolute ethanol for several times to replace water. Then HA was washed by DMF to replace ethanol and finally collected as a HA/DMF slurry. Since the HA nanoparticles were kept under wet conditions during the whole procedure, the HA/DMF slurry can be dispersed easily and homogeneously in the polymer solution without big aggregations. For PLLA-HA composite fibres, no HA bumps, beads or aggregation can be observed along the fibres, showing that the nanoparticles are dispersed uniformly into the polymer matrix well, as shown in Fig. 2(c & d). Moreover, it is worth noticing that the surface of PLLA-HA fibre is rougher than the smooth PLLA fibres, which should be caused by the HA particles inside.

The diameter distribution of PLLA fibre is quite narrow with an average diameter about $1.17\ \mu\text{m}$. The addition of HA particles increases the diameter of the composite fibres to about $1.38\ \mu\text{m}$. And the diameter distribution is a little bit wider, as shown in Figure 2(e & f).

Figure 2. SEM of electrospun PLLA (a & b) and PLLA-HA (c & d) fibres with different magnifications, and their diameter distributions (e & f).

3.3 Porous PLLA and PLLA-HA fibres

Some papers studied the porous surface of electrospun PLLA fibres by using different evaporation speeds of solvent and nonsolvent [39, 40]. Different phase separated structures were generated during electrospinning process and led to porous fibres with rough surfaces. Inspired by this mechanism, we developed a two-step strategy, including electrospinning and post treatment, to fabricate high porously PLLA fibres [41, 42].

During electrospinning step, a binary solvent system is used to prepare PLLA solution, where DCM and DMF are solvent and nonsolvent of PLLA, respectively. Since the vapour pressure of DCM (58.1 kPa, 25 °C) is much higher than that of DMF (0.49 kPa, 25 °C), it is predicted to evaporate quickly during the electrospinning process [40]. When the liquid jet is forced to push out from the needle for creating fibres on the collector, the higher volatile solvent

and lower volatile nonsolvent (DCM & DMF) trigger the phase separation. The fast evaporation of DCM makes the homogenous polymer solution into two phases, the polymer-rich phase and the polymer-poor phase. While the polymer-rich phase establishes the fibre matrix, most of DMF in polymer-poor phase is trapped among polymer phases. After PLLA fibres are completely dried, the space occupied by DMF leaves physical pores or only phase interfaces inside fibres which depends on the percentage of nonsolvent in the solvent system. Meanwhile, polymer phases are elongated to be fibrils along with the fibre axial direction.

Morphology studies of the electrospun fibres obtained by a binary solvent system have shown that the nonsolvent in the spinning solution has a significant influence on the final fibre structure [39]. Only high ratio of nonsolvent (>30%) could result porous structure. However, we don't intend to directly form pore structures by nonsolvent, so only 10% nonsolvent was added to the electrospinning solution. Although no physical pores can be observed on electrospun PLLA and PLLA-HA fibres, there should be some phase interfaces inside the fibres which play a key role during the post treatment process by acetone.

Polymer fibres are immersed into acetone for 5 minutes at room temperature. In this case, as explained in literature reviews, the acetone has similar solubility parameter as PLLA, so it penetrates the amorphous PLLA phase resulting in swelling [43, 44]. Subsequently, the glass transition temperature (T_g) and crystallisation temperature of PLLA are both reduced, increasing the mobility of polymer chains at the same time. When T_g is decreased below the exposure temperature, the crystallisation is kinetically favourable, and then the solvent-induced crystallisation starts [43]. The acetone comes across the glassy PLLA, and makes

intermolecular cohesive forces between parts of PLLA chains into disruption, thus increases the mobility of polymer chains. This process reaches equilibrium in a short time. Additionally, the phase interfaces inside the fibre promote acetone to permeate into the whole fibre more efficiently. Finally, shish-kebab-like structures and porous structure were formed on the fibres (Figure 3).

Acetone treatment leads to the sufficient crystallization of PLLA fibres which still keep their integrated shapes (Figure 3(a)). Accordingly, for each individual fibre, the crystal grows to be vertical lamellar scales on fibre surface (Figure 3(b)). For PLLA-HA fibre after treatment, its porous structure is much smaller than that of PLLA. It is believed that HA nanoparticles hinder the moving range of polymer chains and result small pores. Even so, some HA nanoparticles covered by PLLA are exposed. More Ca and P are detected on porous PLLA-HA membranes by element mapping (Figure S3). As for fibre diameter, the porous PLLA fibres reach about $3.4\ \mu\text{m}$ which is about 3 times of the original ones. The diameter of porous PLLA-HA is about $1.80\ \mu\text{m}$ which increases about 30% after acetone treatment.

Figure 3. SEM of porous PLLA (a & b) and porous PLLA-HA (c & d) fibres with different magnifications, and their diameter distributions (e & f).

3.4 FTIR

Figure 4(a) shows FTIR absorption bands of PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes. The band at 1756 cm^{-1} represents the -C=O stretching mode of PLLA [32]. This peak shifts to 1750 cm^{-1} in PLLA-HA composite nanofibers. This might be due to the hydrogen bond formation between the -OH^- group of HA and the -C=O group of PLLA in PLLA-HA composite fibres [45]. For composite membranes, the peaks at 602 and 562 cm^{-1} corresponding to PO_4^{3-} groups of HA confirm that these nanoparticles are incorporated in the polymer matrix. And there are no differences in types of characteristic bands for PLLA and PLLA-HA before and after acetone treatment.

3.5 BET surface area

The specific surface area of PLLA fibres is about $4.2\text{ m}^2/\text{g}$. After acetone treatment, the

solid fibres are changed to be porous structure which dramatically increases their surface area ($>90 \text{ m}^2/\text{g}$). The addition of particles slightly increases the surface area of PLLA-HA fibres mainly because the HA particles change the smooth PLLA fibres to be rougher ones. Meanwhile, HA particles also restrict the recrystallization of PLLA chains and the pore structure. Therefore, the specific surface area of porous PLLA-HA fibres is smaller than that of porous PLLA fibres, as shown in Figure 4(b).

3.6 Contact angle

As a hydrophobic polymer, PLLA fibrous membrane has a quite high contact angle. For the porous PLLA fibres, the nano vertical scale structure increases the surface roughness which cannot be easily penetrated by water drops. The water drops can be considered as staying on a semi-solid and semi-air surface, avoiding water penetration [46]. With the addition of hydrophilic nanoparticles, this situation of PLLA-HA is only slightly modified because most of particles are covered by polymer materials, as indicated by contact angle in Figure 4(b). For porous PLLA-HA fibres, more HA particles on the fibre surface improves the fibre hydrophobicity.

Figure 4. (a) FTIR spectra and (b) BET surface area and contact angle of PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes.

3.7 XRD

Generally, electrospun PLLA fibres has lower degree of crystallinity than fibres from melt or films from dilute solutions, which is attributed to the rapid solidification of the stretched polymer chains during the electrospinning process [47, 48]. Although the crystallinity of some PLLA fibres can be improved by adjusting electrospinning parameters, most of electrospun PLLA fibres are nearly amorphous because the short electrospinning process allows very little time for crystallization to occur [49, 50].

PLLA and PLLA-HA fibrous membranes have a weak peak at 16.5° , implying that these fibres are in a low crystal status, as shown in Figure 5(a). After acetone treatment of PLLA and PLLA-HA fibres, the intensity of their peaks is much enhanced which means higher degree of crystallinity. Moreover, the peak of porous PLLA-HA is weaker than that of porous PLLA which is caused by HA particles hindering crystallization. In addition to the remarkable peak of PLLA at 16.5° which is ascribed to (200)/(110) [44], three new diffraction peaks can be observed in porous membranes. They are at 14.7° , 18.9° and 22.3° , corresponding to (010), (203) and (015) diffraction planes of α form, implying that crystallisation of PLLA induced by acetone tends to create the α form [44]. The XRD patterns can confirm the recrystallisation of PLLA after immersing into acetone. Furthermore, there are week peaks of HA crystals corresponding to diffraction planes (211) and (300) in PLLA-HA composite membranes.

Figure 5. (a) XRD patterns and (b) DSC curves of PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes.

3.8 DSC

DSC curves of fibrous membranes have been displaced in Figure 5(b) for comparing fibres before and after acetone treatment. For PLLA membrane, peaks attributed by glass transition (T_g), cold crystallisation (T_{cc}), melting of crystalline phase can be observed at 64 °C, 73 °C and 184 °C, respectively. The amorphous state is confirmed from the distinct T_g and T_{cc} , and the area under T_{cc} , which has a proportional relationship to crystallinity. The addition of HA nanoparticles slightly changes these three temperatures to 62 °C, 70 °C and 184 °C, respectively. It implies that nanoparticles influence the PLLA crystallisation to some extent.

After samples are treated by acetone, their peak of T_{cc} disappears, and two glass transition peaks (T_{gl} and T_{gh}) appear. While acetone permeates into the PLLA, it works as a plasticiser to decrease the T_g of PLLA. Thus, there is another T_g appearing known as T_{gl} derived from amorphous polymer phases which is ranging from 40 °C to 50 °C, lower than the original T_g of untreated PLLA. Compared with untreated samples, porous PLLA and porous PLLA-HA have a very weak peak of T_{cc} , indicating their crystallinities are much higher than that of PLLA and PLLA-HA.

3.9 Mechanical property

The representative stress-strain curves of PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes are presented in Figure 6(a). The average tensile strength of PLLA-HA fibrous membranes are presented in Figure 6(a). The average tensile strength of PLLA fibrous membrane is 4.9 ± 0.2 MPa, with an elongation at break of 41%. PLLA/HA showed a tensile strength of 3.16 ± 0.12 MPa. The tensile strength of PLLA/HA scaffolds fabricated during our study is very similar to those prepared by other researchers [45, 51]. Porous PLLA and porous PLLA-HA samples have lower tensile stress than that of PLLA and PLLA-HA because the crystallisation induced by acetone results in brittleness of membranes. Consequently, elongation at break of the porous membranes is also reduced remarkably due to increased crystallisation in Figure 6(b).

Figure 6. Results of tensile mechanical testing of PLLA, porous PLLA, PLLA-HA and porous PLLA-HA fibrous membranes, (a) representative stress-strain curves; (b) peak stress and elongation at break.

3.10 Mineralisation

SEM images of electrospun PLLA and porous PLLA fibrous membranes after mineralization are shown in Figure 7 and 8, respectively. After 1 and 2 hours immersing into 5SBF, there almost no apatite are observed on these fibres. After 4 hours, some small apatite

clusters appear on the fibre surface. However, the distribution of apatite clusters is not good because there are no guided nucleation sites for Ca and P ions. More inorganic matter with nanosheet structure is observed on the surface of these fibres after extending the mineralization time to 6 hours. Without HA particles, although some big apatite spheres were also observed on porous PLLA fibres, they have no too much more advantage than PLLA fibres for apatite growing. It is also notable that apatite growth only happens on the surface fibres. It should be caused by the high hydrophobicity of PLLA fibres which hinders the ion-exchange between 5SBF and the interior spaces among fibres.

Figure 7. SEM images of PLLA membrane immersing in 5SBF for (a) 1 hour, (b) 2 hours, (c) 4 hours and (d) 6 hours.

Figure 8. SEM images of porous PLLA membrane immersing in 5SBF for (a) 1 hour, (b) 2 hours, (c) 4 hours and (d) 6 hours.

No obvious apatite is observed on PLLA-HA fibres after one-hour mineralisation. Some apatite particles begin to appear on fibre surface after 2 hours. These particles continue to grow larger after 4-hour and 6-hour mineralisation. Compared to PLLA fibres without HA, some HA particles on PLLA-HA fibre surface provide nucleation sites for Ca and P ions. More importantly, apatite can grow on the fibres inside the membrane because of their improved hydrophilicity (Figure 9).

For porous PLLA-HA, lots of small apatite particles are uniformly attached on the fibre surface when they are immersed into 5SBF for only one hour (Figure 10(a)). While PLLA-HA fibres are recrystallized by acetone treatment, the resulted porous structure exposes more HA particles to the external environment. The fast nucleation of apatite is mostly attributed to these HA particles on the porous fibre surface. The amount of mineral phase

increases with increasing incubation time, whereby a continuous mineral layer is formed on the porous PLLA-HA fibres after 2 hours incubation (Figure 10(b)). While the fibres are completely covered by apatite, the apparent fibre diameter increases mainly due to the deposition of the mineral phase on the surface of porous PLLA-HA fibres. After 4 and 6 hours of immersion, the mineral layer keeps growing which occupies most of the interspace among fibres (Figure 10(c & d)). The morphology of the mineral layer is quite similar to apatite coating on various fibrous substrates by SBF immersing method [52, 53]. This finding clearly indicates that the porous surface of the electrospun PLLA-HA fibres by acetone treatment significantly enhances the binding ability with Ca and P ions and promotes the heterogeneous apatite mineral growth.

Figure 9. SEM images of PLLA-HA nonporous and porous membrane immersing in 5SBF for (a) 1 hour, (b) 2 hours, (c) 4 hours and (d) 6 hours.

Figure 10. SEM images of porous PLLA-HA membrane immersing in 5SBF for (a) 1 hour, (b) 2 hours, (c) 4 hours and (d) 6 hours.

Figure 11(a) shows the overall mass percentage changes of electrospun PLLA and PLLA-HA fibres mineralized under different incubation periods. Compared to solid PLLA fibres, the porous structure of PLLA fibres does not significantly improve the mineralization speed. Some HA particles on PLLA-HA fibre surface help them to get more apatite bulks. Clearly, more HA exposed on porous PLLA-HA fibres greatly improve the rate of apatite mineral growth.

The nature of the mineral phases is studied by EDS, where their spectra show that the major elements of mineralized scaffold consist of carbon (C), oxygen (O), phosphorus (P), and calcium (Ca) as shown in Figure S4. The elements of C and O can be from both PLLA fibres and deposited mineral, but Ca and P of porous PLLA-HA fibres could only be from the mineral phases because they completely cover the fibres after 6 hours mineralization. It is

interesting to notice that the Ca/P ratio of apatite on PLLA is very close to HA (1.67). However, although much more Ca-P crystals are deposited on porous PLLA-HA fibres, their ratio decreases to 1.36 which is closer to that of HA with the transformation of dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) to Ca-deficient HA (Figure 11(b)) [54].

It is well known that precipitated Ca-P phases in aqueous solutions mainly include hydroxyapatite [$\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$, HA], octacalcium phosphate [$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$, OCP], and dicalcium phosphate [$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD]. Their Ca/P ratios are 1.67, 1.33, and 1.00, respectively. Although HA is considered the most thermodynamically stable in physiological environment, OCP formation in similar environments has also been observed in certain cases even though the driving force of HA formation was larger than that of OCP [55, 56]. Many factors, such as temperature and ion concentration, could influence the nucleation and growth rate of the apatite layer. Moreover, the decreasing of concentration of free Ca^{2+} ions in SBF reduces the Ca/P ratio thereof [54]. In this study, much more apatite was formed on porous PLLA/HA composite fibres than PLLA fibres. Accordingly, the Ca^{2+} concentration in 5SBF for PLLA/HA decreased and calcium-deficient hydroxyapatite with lower Ca/P was formed, especially on the out-layer of apatite coating of PLLA/HA fibres.

Figure 11. (a) Percentage mass increase versus mineralization time, and (b) EDX atomic

analysis after 6 hours mineralization of electrospun PLLA, PLLA-HA, porous PLLA and porous PLLA-HA.

3.11 Cell proliferation ability assessment

Figure 12 shows the distributions of live and dead human osteoblast-like cells, Saos-2, on PLLA, porous PLLA, PLLA-HA and porous PLLA-HA membranes after 1, 3 and 7 culturing days. Thanks to the cell affinity of HA nanoparticles, there appear to be substantially more live cells (stained green) on PLLA-HA composite membranes than on PLLA membranes after one day of culturing. After 3 and 7 days, the presence of HA further accelerated cell proliferation on PLLA-HA composite membranes compared to PLLA membranes. Fewer live cells and more dead cells (stained red) are observed on porous PLLA fibres because of their high hydrophobicity. After 3 and 7 days of culturing, the number of live cells continues to increase on PLLA, PLLA-HA and porous PLLA-HA membranes, while the number of dead cells (stained red) decreases (Figure 13(a)). Porous PLLA-HA membrane has most live cells and fewest dead cells because of its good biocompatibility. It also support uniform spreading and higher proliferation than other three membranes.

This observation is further supported by quantitative proliferation results with an MTS assay which directly correlate to the cell density (Fig. 13(b) left). Cell density on porous PLLA-HA membranes is significantly higher than that on other membranes after 3 and 7 days. The functional activity of the cells on the fibres was assessed by measuring the ALP expressed by the cells after culturing for 1, 3 and 7 days, as shown in Figure 13(b) (right). The cells on porous PLLA-HA membrane exhibit significantly higher ALP levels than those on other membranes. These results indicate that cell attachment and proliferation are

significantly enhanced by the exposure of HA nano composites on the surface of porous PLLA-HA fibres.

Figure 12. Live (green)/dead (red) assay of Saos-2 after 1, 3 and 7 days of *in vitro* culture on PLLA, porous PLLA, PLLA-HA and porous PLLA-HA membranes. Scale bar 200 μm .

Figure 13. (a) number of live and dead cells, (b) cell proliferation as assessed by MTS absorption at 490 nm (left) and ALP release from Saos-2 on PLLA, porous PLLA, PLLA-HA and porous PLLA-HA membranes (right). (Data are presented as mean $\pm$ standard deviation with $n = 6$. * $p < 0.05$.)

4. Conclusion

Keeping HA nanoparticles in a wet status is an effective way to enable their uniform distribution in the electrospinning polymer solution. PLLA-HA composite fibres were electrospun with no beads and bumps. Meanwhile, the fast electrospinning process resulted low crystallinity of PLLA fibres which left much room for recrystallization of PLLA phases. While electrospun PLLA fibres were immersed into acetone, the rearrangement of PLLA chains transformed the solid fibres to porous structures. As a result, the specific surface area of porous fibres was dramatically increased. Moreover, more HA nanoparticles were exposed on the fibre surface which provided nucleation sites for Ca and P ions in 5SBF. The process of apatite mineralization was greatly accelerated. A full layer of apatite was generated on porous PLLA-HA fibres in two hours. Biological *in vitro* cell culture with human osteoblast-like cell for up to 7 days demonstrated that the incorporation of HA nanoparticles into porous PLLA fibrous membranes led to significant enhance osteoblast adhesion and proliferation. The

results of this study suggest that porous PLLA-HA fibrous membranes can be potential scaffolds for bone regeneration.

Acknowledgement

We acknowledge the support of the Electron Microscopy Center and XRD suite in The University of Manchester. Finally, the authors thank to Corbion (The Netherlands) company for their supply of PLLA material.

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Graphical abstract

Highlights

- Uniform poly(L lactic acid)-hydroxyapatite (PLLA-HA) composite fibres were electrospun without aggregation.
- Recrystallization of PLLA in acetone generated highly porous structure for all fibres.
- More HA nanoparticles were exposed on fibres surface by the rearrangement of PLLA phases.
- Bone-like apatite formed on porous PLLA-HA fibrous membranes in 2 hours.