BIOMIMETIC FIBROUS COMPOSITE MEMBRANES FOR BONE TISSUE ENGINEERING

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Abstract

One of the major challenges in biomaterials and tissue engineering is to guide the cell differentiation to the specific phenotype, therefore allow the formation of the tissue of certain type. This can be achieved by manipulating the structural, geometrical and chemical characteristics of the tissue engineering constructs. In our studies we concentrated on the chemical modifications of the polymer based materials for tissue engineering. The primary aim of our study was to incorporate nano size hydroxyapatite (n-HAp) crystals into the polymer fibres and form membranes, which are a core to the construction of novel scaffolds for tissue regeneration. We hypothesised that n-HAp will significantly improve the bioactivity of the polymer based membranes due to the presence of chemical cues. We developed a simple method to fabricate PLDL/n-HAp composite membranes using electrospinning process. The investigation showed that the incorporation of the n-HAp particles in the polymer spinning solution induced changes in the material surface morphology. FTIR analysis confirmed the presence of apatite on the surface of the membrane' fibers. The bioactivity analysis, which was based on SEM observation of the membranes surface, showed that after only 7 days immersion in SBF, the PLDL/n-HAp –membranes were completely covered by the apatite layer. This was not observed for pure PLDL membranes.
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Introduction

A biomimetic material for bone tissue engineering can be any scaffolding material that mimics one or multiple characteristics of the natural bone. Many extracellular proteins have a fibrous structure with diameters on the nanometer or micrometer scales. For example, collagen, which is the most abundant extracellular matrix (ECM) protein in the human body, possesses a fibrous structure with fibre bundle diameter varying from 50 to 500 nm [1]. It is well established that natural bone consists the nano-size crystals of hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) grown in intimate contact with the ECM rich in collagen fibres. When we consider materials that support tissue regeneration, scaffolds are the most popular and under continues development in the major research laboratories worldwide. Ideally scaffolds should: mimic natural ECM, provide structural support to the repair region, allow for the cell attachment, proliferation and migration, induce the tissue specific differentiation of stem and progenitor cells, stimulate tissue formation and be designed to biodegrade over time ultimately leaving tissues with native structure and function [2]. The incorporation of nanofillers such as HAp into the polymer matrix can improve mechanical properties, and can provide favourable environment for osteoconduction, the localized adsorption of specific proteins, and cell proliferation [3-5]. Various studies have assessed the effect of incorporation of an inorganic phase into biocompatible polymers on their properties. Deng X et al. have reported the production of 3D scaffold of PLA/d-HAP nanocomposites by solvent-cast technique [6]. In this study the dispersion of nanocrystals in the polymer matrix was homogeneous at a microscopic level. The tensile modulus for the nanocomposites increased with d-HAP loading. More recently a new method based on electrospinning was presented [7]. Uniform PLA-g-HAP/PLA composite nanofiber mats were successfully prepared and they exhibited improved mechanical properties. The authors studied the degradation of scaffold containing PLA-g-HAp and concluded that degradation of fibrous mat could be accelerated depending on the PLA-g-HAp content. When PLA-g-HAp content was high, degradation rate increased because of the enhanced wettability of the composite fibers and the escape of the nanoparticles from fiber surfaces during incubation [7].

The electrospinning technology (ESP) is an economical fabrication method that is easily set-up for the production of membranes [8]. Many different polymers (e.g. collagen, silk, fibroin, fibrinogen, PGA, PLLA, PLGA, and PCL) have been successfully electrospun for a number of medical applications [9]. However, until now it was not reported that nano-size HAp was successfully incorporated into the polymer fibres. This raise the tantalising possibility to develop technology to fabricate such materials, which are intended to support tissue regeneration. To initiate the electrospinning process, a selected polymer material is dissolved in the appropriate solvent and this solution is loaded into a syringe. The jet coming from needle is drawn towards a collector due to an electric field ranging from 10 to 30 kV [10]. With increased voltage, the polymer droplet elongates to form a conical shape known as the Taylor cone and the surface charge on the polymer droplet increases with time. Once the surface charge overcomes the surface tension of the polymer droplet, a polymer jet is initiated [8]. Evaporation of the solvent from the jet after leaving the needle results in fibre deposition on the collector. By rotating the collector, a non-woven membrane with a preferential orientation of the fibre is created [11-12]. This process results in the production of a nonwoven fibrous mat. These membranes can have fibre diameter in the order of nanometers to microns. A number of processing parameters such as: applied voltage, polymer flow rate, and capillary-collector distance can greatly influence the properties of the generated fibres [10]. By combining both polymer and mineral phase during the fabrication process, it is possible to tailor the microstructure and chemistry of the membrane, therefore the biological performance of the material.

The aim of this study was to produce a bioactive biopolymer – ceramic membranes, which will be used in the future as scaffold for bone tissue engineering.

Materials

Nano-hydroxyapatite (n-HAp) was synthesised at the Department of Technology of Ceramics and Refractories, AGH-UST (Cracow, Poland). An average size of the n-HAp particles was 23 nm. The specific surface area of the n-HAp was 79.9 sq. m/g. L-lactide/DL-lactide (PLDL) copolymer was obtained from PURAC, The Netherlands. Acetone (POCH, Poland) was used as a solvent.

Fabrication of PLDL membranes

Fibrous membranes were prepared by electrospinning from PLDL and composite PLDL/n-HAp solutions. 1g of PLDL copolymer was dissolved at room temperature under magnetic stirring in 50 ml of acetone. For the composite membranes the PLDL/acetone solution was mixed with 20wt.% of n-HAp and the suspension was ultrasonicated. Both solutions were stirred overnight until the solution became homogeneous. Electrospinning was carried out using custom-made apparatus consisting of a power supply, syringe and electrodes. The solutions were fed through a capillary tip (diameter 0.7 mm) using a manual syringe (25 ml) and spun at a working distance of 20 cm with a driving force of 30 kV. The solution flow rate was 15 ml/h. The fibres were dried in flight and collected on the aluminium foil wrapped on a rotating metal drum. Composites PLDL/n-HAp membranes and PLDL membranes (as control), were obtained using this method.

Methods

Membranes morphology

The microstructure of polymer fibrous membranes was investigated using scanning electron microscopy (SEM, Jeol, JSM 5500). Before SEM observation, all of the samples were cut from the electrospun membrane (5 x 5 mm) and gold coated (Jeol JFC 1200 sputter). The average fibre diameter of the fibres was measured from the SEM images.

Membranes surface chemistry

The infrared spectra (IR) of the membranes were study using Fourier transformed infrared spectroscopy (FTIR) using spectrophotometer Nicolet 6700. The IR spectra were recorded using fotoacustic reflectance device (MTEC Photoacoustics 300 THERMO NICOLET) at the range of 400-4000 cm^{-1} using at least 64 scans and 4 cm^{-1} resolution.

Pore size distribution

Pore size distribution was determined using the PMI capillary flow porometer. In this study pores were considered as capillaries. In this technique the membrane samples were soaked in a “wetting liquid” (Isopropyl alcohol), which fully and spontaneously fills all the pores in the sample. The gas pressure on one side of the wet sample was then gradually increased. When the pressure was sufficient to empty the largest pore in the sample, the gas begun to flow through the membrane. The measurement of the pore size of the membrane was based on the physical phenomenon of surface tension of the liquids [13]. With increasing pressure, the gas removed liquid from smaller pores and the gas flow rate increased. The minimum force required to empty the fluid from the pore of the membrane was calculated using the fundamental equation of porometry [14]:

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

where:

D – pore diameter,

γ – surface tension of wetting liquid,

θ – contact angle of the wetting liquid with the sample,

p – differential pressure.

Bioactivity of the membranes

Bioactivity was investigated by analysing the formation of crystalline apatite on the surface of membranes upon immersion in Simulated Body Fluid (SBF). SBF was prepared according to Kokubo et al. [15]. PLDL and PLDL/n-HAp membranes were incubated up to 14 days in 1.5xSBF fluid of pH 7.4, at the temperature of 37°C, in closed polyethylene containers. SBF solution was replaced every 2.5 days. After 1, 3, 7 and 14 days of immersion, the samples were washed with distilled water and dried at room temperature. After each time point, the surface morphology of the samples was examined using SEM.

Results and Discussion

The macroscopic images of membranes are shown in FIG. 1. The morphology of electrospun membranes was influenced primarily by the properties of the polymer solution: polymer concentration, surface tension and hydroxyapatite agglomeration. Furthermore, the following parameters influenced the fibre formation: voltage, distance between capillary and collector, and solution flow rate. SEM images and the diameter distribution are shown in FIGS. 2, 3 and 4.

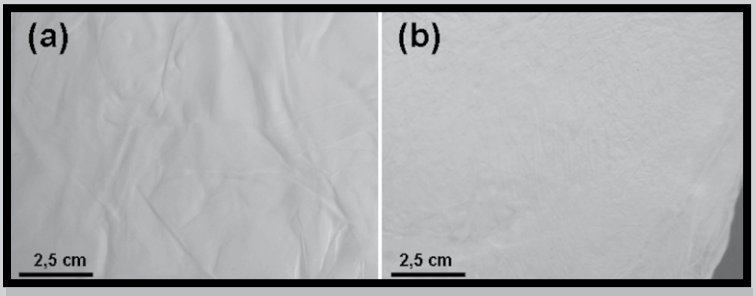

FIG. 1. Images of electrospun membranes: (a) PLDL, (b) PLDL/n-HAp.

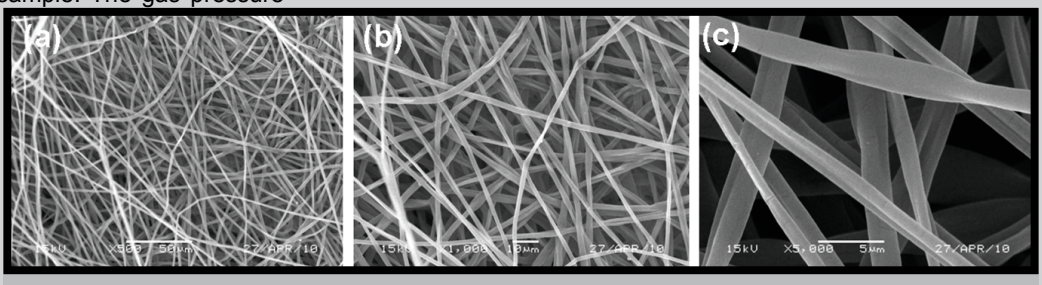

FIG. 2. SEM images of electrospun PLDL membranes.

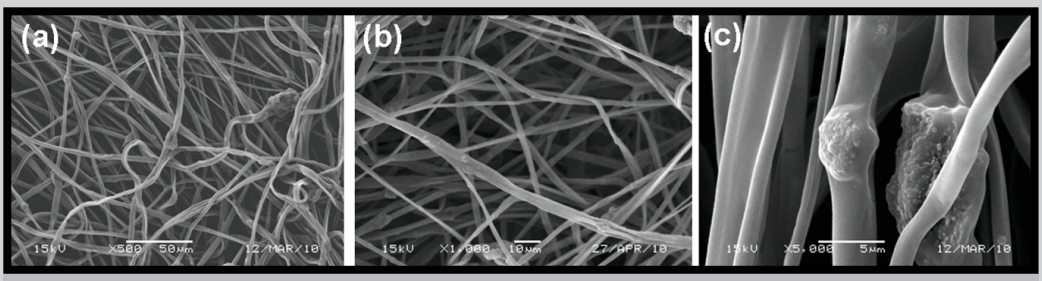

FIG. 3. SEM images of electrospun composites (PLDL/n-HAp).

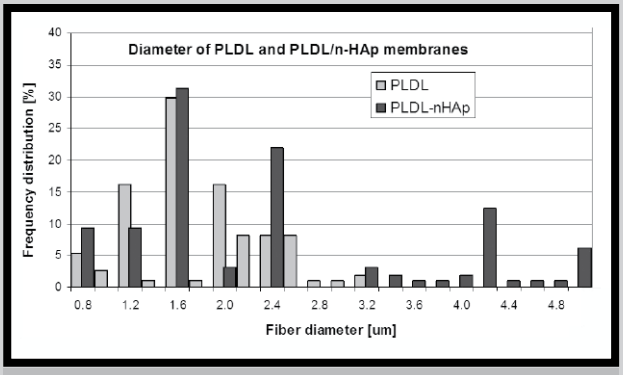

FIG. 4. Fiber's diameter distribution for modified and unmodified membranes.

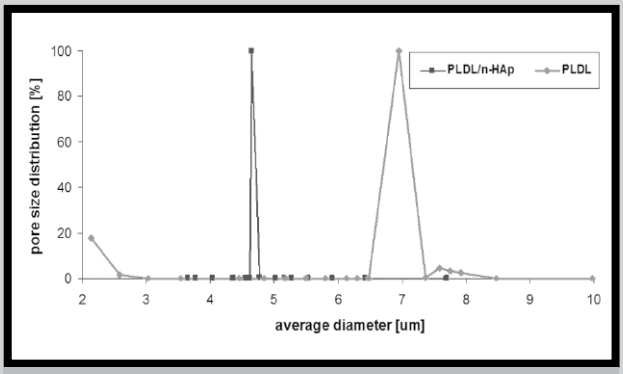

FIG. 5. Pore size distribution for modified and unmodified membranes.

The analysis of the SEM images revealed that the electrospun membranes were globally homologous, and were formed from randomly oriented fibers. It was found that the average diameter of the fibres was greater for the n-HAp/PLDL composite membranes. It can be speculated that this was associated with the viscosity of the solution, which alter fibres formation. It was evidenced that n-HAp particles were build into the fibres structure (FIG. 3). However, the particles size was greater than the diameter of the fibres. It is likely that the large number of nanosize paticles were also incorporated into the polymer structure, which was not evidenced by SEM studies due to the resolution limitations. The average fiber diameter was respectively 1.7 ± 0.5 and 2.8 ± 1.4 μm for unmodified and composite membrane (FIG. 4). Interconnected voids were present within the fibers, resulting in a porous network. The addition of hydroxyapatite into the spinning solution influences the porosity of obtained membrane; the sizes of the main pore fraction decreased of 30% (FIG. 5). PLDL/n-HAp membrane showed a very narrow distribution of pore size centered about 4.8 μm, whereas for unmodified PLDL the main pore fraction is in the range of 6.5-7.5 μm.

The FTIR spectra of HAp, PLDL and PLDL/n-HAp composite membranes were presented in FIG. 6. This study confirmed that n-HAp was successfully incorporated into PLDL membrane produced by electrospinning, which was evidenced by the presence of the phosphate groups' peaks, which are associated with n-HAp. Analyzing the FTIR spectrum of hydroxyapatite powder one can observe clearly visible bands dedicated to PO_4^{3-} in the 900-1200 cm^{-1} region, the most intensive are attributed to stretching vibrations. Band at 963 cm^{-1} correspond with non-degenerated symmetric vibrations v1 P-O. Absorption maxima at 1033 cm^{-1} and 1107 cm^{-1} are attributed to triple-degenerated asymmetric stretching vibrations v3 P–O, while bands at 566 cm^{-1} and 605 cm^{-1} are attributed to triple-degenerated bending vibrations v4 O–P–O. In addition to bands assigned to phosphate groups, there are also visible bands near 1350-1550 cm^{-1} and 1400-1580 cm^{-1} attributed to carbonate groups v3 CO_3 and near 850-890 cm^{-1} to v2 CO_3 groups.

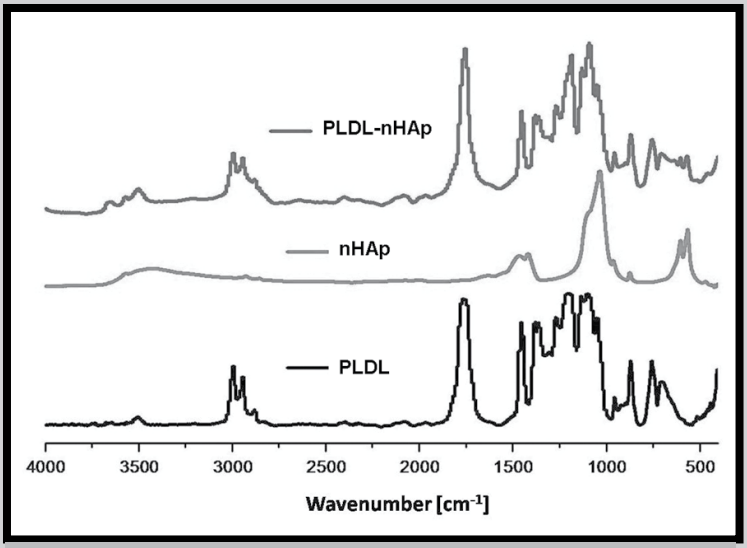

FIG. 6. FTIR spectrum for a control sample (PLDL), pure HAp and composite membrane (PLDL/n-HAp).

In the case of PLDL/n-HAp FTIR spectrum the small peaks at 566 cm^{-1}, 605 cm^{-1} and 1033 cm^{-1} assigned to phosphate groups are present, confirming the presence of HAp in the composite membrane.

Bioactivity study showed that the formation of apatite from SBF solution significantly increased for the composite membranes. During the incubation of the composite membranes in SBF the deposition of the apatite layer was observed. Bone-like crystalline apatite layers covered the surface of the composite membranes, as shown in FIG. 8. Spherical calcium phosphate precipitated on the membrane surface, indicating rise to the bioactivity. This uniform and dense apatite film was formed on the samples after 7 days of incubation in 1.5×SBF, while no apatite formation was observed for control PLDL membranes (FIG. 7). The fibers of the later membranes had relatively smooth surface and more compact structure.

Conclusions

The electrospinning was successfully used to fabricate the fibre composite membranes composed of L-lactide/DL-lactide copolymer and nano-size hydroxyapatite. The n-HAp particles were incorporated into the PLDL fibres structure and present on the fibres surface. Immersion test performed in SBF indicated increase in the bioactivity of composite membrane. However, the sizes of pores are too small for bone ingrowth. It is well known that pore size distribution, porosity and pore interconnectivity are critical factors for materials used to regenerate tissues; they provide the optimal spatial and nutritional conditions for the cells and determine the successful tissue ingrowth. The optimal scaffolds for bone tissue regeneration should be porous and contain two types of pores: (i) larger than 200 μm (pores in which cells could grow) and (ii) smaller pores dedicated to the diffusion of the nutrients and metabolites of bone forming cells. For these reason our future study will concentrate on the optimisation of the fabrication process to allow the formation of scaffolds with bimodal pore size distribution.

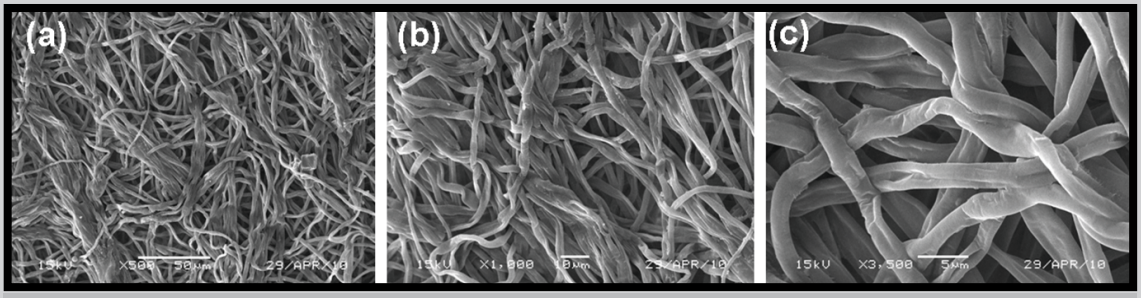

FIG. 7. SEM micrographs of PLDL membranes after 7 days immersion in SBF.

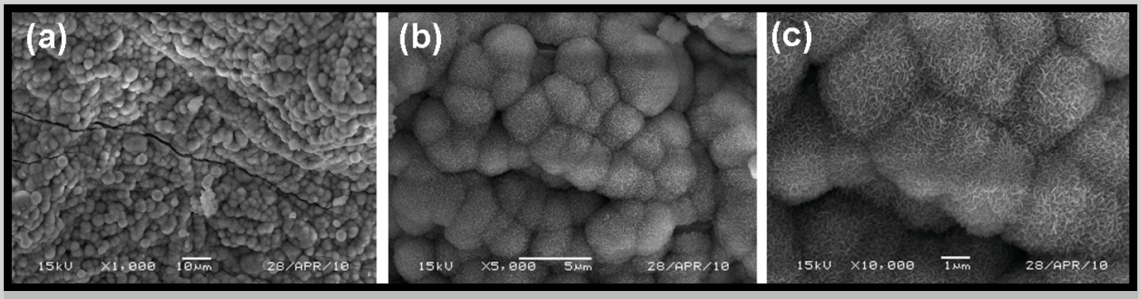

FIG. 8. SEM micrographs of composite PLDL/n-HAp membranes after 7 days immersion in SBF.

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