Supporting Information

Experimental Section

Materials

Poly(L-lactic acid) (PLLA) with an inherent viscosity of 7.0 dl/g was purchased from PURAC (Netherlands) and used as received. Sodium hydroxide (NaOH), hydrochloric acid (HCl), calcium chloride ($CaCl_2$) and sodium dihydrogen phosphate (NaH_2PO_4), Chloroform, N, N-dimethylformamide (DMF) were bought from Acros (Belgium). Wool keratin solution (0.25 g/l) was prepared in our lab.

Wool keratin solution

100 g wool fibers were immersed in 1000 ml sodium hydroxide (NaOH) solution (5% wt in water) with stirring until the wool fibers were dissolved completely.

Synthesis of HA/keratin Nanocomposite

100 ml $CaCl_2$ solution (0.1 M) was mixed with 200 ml wool keratin solution and maintained for 20 min. NaH_2PO_4 (60 ml, 0.1 M) was then added slowly in drops with gentle stirring. The molar ratio of Ca/P in the solution was set as 1.67, equal to stoichiometric hydroxyapatite. The pH value of the solution was adjusted to 7.0 by NaOH solution (0.1 M) slowly. After the solution was maintained for 24 h, the HA/keratin precipitation deposited on the bottom of the bottle. The precipitate was harvested by centrifugation at 10,000 rpm for 4 min. Then the HA/keratin was re-suspended in deionized water and centrifugated again. The washing procedure was repeated five times to remove all un-reacted ions. Then the composite was dispersed into absolute ethanol (20 ml) and centrifugated. This process was repeated five times to replace water totally by ethanol. The ratio of the final HA/keratin was 78:22 (in weight, characterized by TGA). By the same method, pure HA without keratin was also fabricated as a control. Part of the final products were dried and ground into fine powder for later characterizations.

Extraction of keratin

Hydrochloric acid (10%) was added slowly into 200 ml wool keratin solution. When the pH value of the solution reached 5.55, the keratin precipitation was collected and named as Keratin 5.55. Keratin 5.55 was washed by DI water for several times to remove any ions. And then, it was washed with ethanol for several times to replace water completely.

Electrospinning of PLLA/HA/keratin Membrane

PLLA was dissolved in chloroform and N,N-dimethlformamide (DMF) (10:1, w/w) at a concentration of 1.0 % (w/w). The prepared HA/keratin with ethanol was dispersed into PLLA solution by ultrasonic (PLLA:(HA/keratin)=9:1, w/w) at room temperature for 24 h. For electrospinning of PLLA/HA/keratin composite fibers, PLLA/HA/keratin spinning solution (10 ml) were placed in a syringe with metal capillary, and fed by a syringe pump at a volume flow rate of 0.1 ml/min. A grounded rotating drum wrapped with aluminum foil was located at a fixed distance of 20 cm away from the capillary tip and used as the collector. The capillary needle spinneret was connected with a high voltage power supply applying 20 kV. Ambient conditions for current electrospinning process are 52 % humidity and 22 ℃ room temperature. For comparison, pure PLLA fibrous membrane was also electrospun as controls. All electrospun nanofibrous membranes were vacuumed for over 7 days in a vacuum to remove any potential residual solvents.

Using the same procedure, PLLA/keratin fibrous membranes were also electrospun (PLLA:keratin=9:1, w/w).

Characterizations

The morphologies of the washed HA/keratin and electrospun fibrous membranes were observed with a scanning electron microscopy (SEM, JEOL, JSM-6490). After a small drop of the HA/keratin/ethanol suspension was dried on the surface of a piece of silicon slice, the morphology of HA/keratin composite was also observed. A small piece of PLLA, PLLA/keratin and PLLA/HA/keratin fibrous membrane and HA/keratin composite were dispersed in absolute ethanol under an ultrasonic treatment, respectively. The solution was then dropped onto the carbon-coated copper grids for the TEM observation using a transmission electron microscope (TEM, JEOL, JEM-1230). To investigate the structure and crystallinity of the samples, HA/keratin and PLLA/HA/keratin were examined with a X-ray powder diffractometer (XRD, Bruker D8 Advance) in the range of 2θ=10-40 ° with a step of 0.05 ° and a scanning rate of 2.0 s/step. FTIR spectra of HA, keratin, HA/keratin, PLLA and PLLA/HA/keratin membrane were recorded on Fourier transform infrared (Nicolet 5700, Thermo Co. USA) in the range of 450-4000 cm-1 with a resolution of 4 cm^{-1}.

The tensile properties of electrospun fibrous membranes were evaluated using a tabletop MicroTester (Instron 4411, USA) using a load cell of 10 N capacity at ambient conditions of 25 °C and 74% humidity. Rectangular specimens of dimensions 10 × 20 mm were cut (using a specimen frame) and peeled off from the nanofiber sheet spun on aluminum foil and tested at a cross-head speed of 10 mm/min. Ten samples were tested for each type of electrospun membrane during this study. Tensile strength and elongation at break were calculated based on the generated stress-strain curves of each membrane.

Cell Expansion and Seeding

PLLA, PLLA/keratin and PLLA/HA/keratin membranes (8×8 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) three 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 ℃, CO_2 (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 were centrifuged and 60,000 cells/cm^2 were seeded on each sample on 24 well plates, and incubated (37 ℃, CO_2 (5 %)) until the evaluation.

MTS

After the cells were incubated on the above-mentioned membranes for 1, 3 and 7 days, MTS assay (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium, inner salt) was added to the culture medium in each well to monitor the cell proliferation using CellTiter 96 Aqueous Assay. The mechanism behind this assay is that, metabolically active cells react with tetrazolium salt in MTS reagent to produce a soluble formosan dye that can be observed at 490 nm. 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).

Alkaline phosphatase activity (ALP)

Samples were removed of medium and washed twice with a buffer solution on 1, 3 and 7 days. The fibrous membranes were then submerged into 1 ml of 1% Triton 100× solution for cell lysis. They were then centrifuged and the supernatant was used to calculate alkaline phosphatase (ALP) activity by p-nitro phenyl phosphate (p-NPP) method. ALP catalyzes the cleavage of p-NPP to give p-nitrophenol and orthophosphate, which develops a yellow color in the presence of a base. A 0.5 ml of supernatant and 0.5 ml of diluted p-NPP (100 µl of p-NPP concentrate per 2 ml of 100 mm sodium bicarbonate/carbonate buffer, pH 10) were mixed and incubated for 45 min. The absorbance of this mixture was read at 405 nm. The absorbance was converted to the units of ALP per liter.

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 µg/mL) and PI (1.0 µg/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).

DAPI/Phalloidin Stain

After Saos-2 cells were cultured on the above-mentioned membranes for 1, 3 and 7 days. The samples were gently washed with 37 ℃ PBS, fixed with paraformaldehyde (4 % in PBS) at room temperature for 15 min, and permeabilized with Triton X-100 (Sigma, 0.1 % in PBS) for 5 min at room temperature sequentially. F-actin staining was performed by incubating cells with TRICE-phalloidin (10 µg/ml, Sigma) in dark for 30 min to visualize the cytoskeleton, and the nucleuses of cells were additionally counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, 1.0 µg/ml in PBS). The samples were visualized using a Leica fluorescence microscope. In addition, the cell areas were quantitatively measured by selecting the full area of the cells based on the color differences and the number of pixels covered by the cell. The data were reported as a boxplot.

Statistical Analysis

Data are expressed as mean ± 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 $p < 0.05$.