Colloids and Surfaces B: Biointerfaces 116 (2014) 576–581

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Galactosylated electrospun membranes for hepatocyte sandwich culture

Hsiu-Wen Chien [a], Juin-Yih Lai [b], Wei-Bor Tsai [a,*]

[a] Department of Chemical Engineering, National Taiwan University, No. 1, Roosevelt Rd., Sec. 4, Taipei 106, Taiwan
[b] R&D Center for Membrane Technology and Department of Chemical Engineering, Chung Yuan Christian University, Chungli, Taoyuan, Taiwan

ARTICLE INFO

Article history:
Received 11 October 2013
Received in revised form 7 January 2014
Accepted 24 January 2014
Available online 4 February 2014

Keywords:
Electrospun membrane
Polyelectrolyte multilayer deposition
Sandwich culture
Hepatocytes

ABSTRACT

In this work, we developed a galactocylated electrospun polyurethane membrane for sandwich culture of hepatocyte sandwich culture. The electrospun fibrous membranes were bio-functionalized with galactose molecules by a UV-crosslinked layer-by-layer polyelectrolyte multilayer deposition technique. The galactosylated electrospun membranes were employed as a top support membrane for the sandwich culture of HepG2/C3A cells on a collagen substrate. Our results demonstrate that HepG2/C3A cells covered by the galactosylated PU membranes form multi-cellular aggregates and lead to improved albumin secretion ability compared to the control membranes (unmodified PU or poly(ethylene imine)-modified PU). Our study reveals the potential of galactosylated electrospun membranes in the application of liver tissue engineering and the regeneration of liver-tissue substitutes.

1. Introduction

Hepatocytes, which are responsible for the major functions of the liver, are commonly isolated and cultured *in vitro*, *e.g.* for hepatic tissue engineering studies and drug screening for hepatotoxicology [1]. However, hepatocytes in two-dimensional (2D) culture tend to lose their differentiated functions. Therefore, much effort has been devoted to the development of three-dimensional (3D) microenvironments that simulate the *in vivo* extracellular matrix (ECM) in order to retain the essential hepatic functions. For example, sandwich culture of hepatocytes between two collagen layers is a well-established *in vitro* model for re-establishing hepatic polarity and maintaining differentiated functions of hepatocytes [2,3]. Different from the traditional 2D culture, the top support for the sandwich culture serves as a matrix for cell attachment beside the base substrate, creating 3D cellular structure and polarities. Therefore, sandwich cultures provide a microenvironment similar to that found in the liver and promote hepatocyte cell–cell and cell–ECM interactions [2]. Thus, sandwich cultures serve as a tool for investigation of liver physiology, drug metabolism/toxicity testing [3], and hepatocyte-based bioreactors [4]. However, applications of the ECM-based sandwich culture may be limited by low mass transfer owing to the top gelled ECM layer, batch-to-batch variation in the ECM compositions and uncontrollable ECM coatings [5].

To address these issues, synthetic porous membranes have been proposed as an alternative for collagen gels in hepatocyte sandwich culture. Du et al. established a synthetic sandwich culture by overlaying hepatocytes that are cultured on a galactosylated polyethylene terephthalate (PET) film with a porous PET track-etched membrane [5]. They demonstrated that the synthetic sandwich culture could achieve better mass transfer through the top porous PET support in comparison to the collagen gel. The hepatocytes in the sandwich culture with synthetic membranes exhibited a similar process of hepatic polarity formation, better cell–cell interaction and improved differentiated functions compared to hepatocytes in the collagen sandwich culture. These results suggest that better mass transfer through the top support benefits hepatocyte sandwich cultures.

Since ECM proteins provide essential biological signals for maintaining cell physiology, we evaluated retaining a collagen substrate layer, while replacing the top-layer collagen gel by a synthetic membrane for hepatocyte sandwich culture. Compared to commercial membrane products, electrospun fibrous membranes appear to be a better choice as the top support of the sandwich culture. Electrospun fibers, fabricated from electrically driven polymer jets, have been employed in various fields such as biomedical engineering and environmental engineering [6,7]. The morphological similarity between the ECM and electrospun fibers suggests an outstanding potential for cell/tissue engineering applications [8,9]. The advantages of electrospinning also include easy fabrication, modulation of fibrous structures by adjusting electrospinning

* Corresponding author. Tel.: +886 2 3366 3996; fax: +886 2 2362 3040.
E-mail address: weibortsai@ntu.edu.tw (W.-B. Tsai).

0927-7765/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.colsurfb.2014.01.040

conditions, and a wide variety of materials that can be used. Furthermore, by controlling the collection time, the fiber coverage density of the electrospun fibrous membrane can be controlled, which in turn modulates the mass transfer through the membrane. Therefore, we have evaluated this type of membranes as the top support of the sandwich culture of hepatocytes.

In this study, polyurethane (PU) fibrous membranes were fabricated by electrospinning and then biofunctionalized with galactose molecules *via* a layer-by-layer (LBL) polyelectrolyte deposition technique (Fig. 1). This technique is based on alternating adsorption of positively and negatively charged polyelectrolytes, resulting in a thin coating on a substrate material [10]. One of the advantages of this surface modification technique is that it is not restricted to specific types, sizes and shapes of substrate materials. We previously deposited a tri-layer polyelectrolyte film of poly(ethylene imine) (PEI), poly(acrylic acid)-*g*-azide (PAA-*g*-AZ) and PEI on polymeric substrates. After exposure to UV irradiation, the three-layer polyelectrolyte film was crosslinked *via* a phenylazide-based reaction and covalently conjugated on the substrate material [11]. In this study, galactose molecules were first conjugated onto PEI, and then adsorbed as the outmost layer of a tri-layer polyelectrolyte film on the outer layer of PU electrospun fibers. The galactosylated PU membranes were then evaluated for hepatocyte sandwich culture.

2. Experimental

2.1. Materials

Polyurethane (PU, Pellethane™, 2103-80AE) was obtained from Dow Chemical Company, USA. Poly(ethylene imine) (PEI, $M_w \sim 750$ kDa, cat. no. P3143) was received from Sigma-Aldrich. Collagen was purified from bovine skin according to a previously described procedure [12]. All other chemicals were purchased from Sigma-Aldrich unless specified otherwise.

Poly(acrylic acid-*g*-azidoaniline), abbreviated as PAA-*g*-AZ, was synthesized using a previously published procedure [13]. The content of AZ in PAA-*g*-AZ, estimated from the ratio of the peaks of the azidophenyl protons at 6.5–7.5 ppm and the methylene protons of the polymer main chain at 1.3–2.5 ppm in the ^{1}H-nuclear magnetic resonance (^{1}H-NMR, Avance-500 Hz, Bruker) spectrum was 6.0 mol%. Poly(ethylene imine)-*g*-galactose (PEI-*g*-Gal) was synthesized according to a previous protocol [14]. The coupling percentage of lactobionic acids to PEI, estimated from the ratio of the peaks of the protons in the methylene groups and the methane groups of the β-galactose at 3.5 to 4.0 ppm and the methylene protons of the PEI main chain at 2.5 to 3.0 ppm in the ^{1}H-NMR spectrum, was 37.7 mol%.

A human hepatoblastoma cell line, HepG2/C3A, was received from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cell culture medium contained αMEM (HyClone, USA) supplemented with 10% fetal bovine serum (JRH, Australia), 1% non-essential amino acids (GIBCO, USA), 1.0 mM sodium pyruvate, 2 mg/mL $NaHCO_3$, 0.5% of fungizone (GIBCO, USA), 0.25% gentamycin (GIBCO, USA) and 0.679% β-mercaptoethanol. Phosphate-buffered saline (PBS) was prepared as 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

2.2. Fabrication and surface galactosylation of electrospun PU membranes

PU fibrous membranes were fabricated by a conventional electrospinning technique. Briefly, 10% (w/v) PU solution in DMF/THF (1/1, v/v) was electrospun at 11 kV and 0.5 mL/h through a 16G blunt-end needle toward an aluminum collector at a distance of 10 cm. The samples used in sandwich culture as top supports were collected for 1, 3 or 5 min, while the PU membranes for characterization and 2D cell culture were collected for 1 h.

Galactose molecules were conjugated on electrospun PU membranes *via* LBL polyelectrolyte multilayer deposition (Fig. 1). Prior to LBL deposition, PU membranes were treated with radio-frequency glow discharge oxygen plasma (50 W, 0.07 torr, 2 min, 23.5 sccm) to introduce negatively charged functional groups on the surface. The membranes were then immersed in a PEI solution (1 mg/mL) for 30 min, followed by rinses with deionized water. Next, the membranes were immersed in PAA-*g*-AZ solution (1 mg/mL) in the dark for 30 min. Finally, after rinsing again with deionized water, the PU membranes were immersed in PEI or PEI-*g*-Gal solution (1 mg/mL) for 30 min, followed by rinsing with deionized water. After drying in air, the membranes were exposed to UV for 1 min to achieve crosslinking. The untreated PU membranes and those modified by PEI/PAA-*g*-AZ/PEI and PEI/PAA-*g*-AZ/PEI-*g*-Gal were abbreviated as PU, PEI and Gal, respectively.

2.3. Characterization of electrospun PU membranes

The morphology of electrospun PU fibers was investigated using scanning electron spectroscopy (SEM, JSM-5310, JEOL, Japan). The average diameters of fibers were determined from more than 100 PU electrospun fibers that were randomly selected from the SEM images.

The fiber coverage densities of the membranes collected for 1, 3 and 5 min were determined from optical microscopic images of the samples using NIH ImageJ software. The software determined the fiber-covered area and the void area. Fiber coverage density (%) was defined as the ratio of fiber-covered area/total surface area × 100%.

For water contact angle measurements, electrospun PU membranes were collected for 1 h. The wettability of the membranes was determined using a goniometer (FTA-125, First Ten Angstroms) with deionized water by the static sessile drop method. Ten droplets (10 μL) were measured for each sample.

2.4. Cell culture on electrospun membranes

PU, PEI and Gal membranes were sterilized by UV exposure for 1 h prior to cell seeding. HepG2/C3A cells were then seeded on the membranes at a density of 5×10^4 cells/cm^2 and cultured for 3 days. For SEM analysis, the samples were fixed by 0.25% glutaraldehyde in PBS for 10 min and 2.5% glutaraldehyde for another hour. The samples were then dehydrated in a graded series of ethanol: 30%, 50%, 70%, 80%, 90%, 95% and 100%, followed by CO_2 critical point drying. After gold sputtering, cells were imaged by SEM.

2.5. Sandwich culture with electrospun membranes

For 3D culture, the cells were sandwiched between a membrane and collagen substrate (Fig. 1B). Collagen substrates were prepared by adding 35 μL ice-cold, neutralized 1 mg/mL collagen solution onto a 10-mm glass coverslip kept at 37 °C for overnight gelation. HepG2/C3A cells were then seeded at 5×10^4 cells/cm^2 on the collagen substrate. After 9 h of incubation, the cells were covered by an electrospun fiber membrane for further culture in a 37 °C humidified incubator. The culture media were changed every day.

Cell viability was examined by live/dead staining. Briefly, after 2 days of culture, cells were stained with 1 mg/mL fluorescin diacetate (green for live cells) and 1 mg/mL propidium iodide (red for dead cells) for 15 min at 37 °C in the dark. After rinses with PBS, the samples were observed under a confocal microscope (Nikon TE2000-U, Japan).