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Patterned substrates fabricated by a controlled freezing approach and biocompatibility evaluation by stem cells

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ABSTRACT

Patterned substrates have been widely used in the studies investigating how to regulate cell growth and alignment. Such substrates may be fabricated by various techniques such as photolithography, soft lithography and microcontact printing. We report here a facile approach to fabricate aligned and grid surface patterns by a controlled freezing approach and further investigate their biocompatibility. The fabrication has been demonstrated with polymers (hydrophilic & hydrophobic), nanoparticles (organic & inorganic), or mixtures of these components. For the aligned surface patterns, the spacings between the patterned ridges can be tuned by varying the freezing rates. The biocompatibility of the substrates is evaluated by WST-8 viability tests with cell counting kit-8 (CCK-8) and by culturing with mouse mesenchymal stem cells (mMSCs). Three surface-patterned substrates (PLGA, PLGA nanospheres with chitosan, and silica colloids) are evaluated in more details to show that the mMSCs can grow alongside the aligned ridges while the cells grow randomly when plain glass slides are used as control. Further observations show that PLGA substrates undergo degradation, and are thus unsuitable for cell culture over the longer term. On the other hand, the PLGA-chitosan substrate and silica substrate were stable and could maintain mMSC alignment throughout the culture period.

1. Introduction

Stem cells are able to self-renew and differentiate to produce specialized progeny, and thus have enormous potential for tissue engineering-based therapies [1]. For instance, mesenchymal stem cells (MSCs) have been used to generate cartilage for airway replacement [2], and bone tissue for the repair of long-bone defects [3]. However, in order to differentiate and function appropriately, it is very important for the cells to be correctly aligned under certain conditions [4,5]. Surface patterned substrates have been used to investigate the effect of surface topography on stem cell growth, alignment and differentiation. This is because the 2D culture systems can simplify the analysis when deconstructing the stem cell niche and assessing the effects of individual niche components on stem cell fate [6]. For example, a micropatterned bioresorbable poly(lactide-*co*-glycolide) (PLGA) substrate fabricated by soft lithography was found to control human MSC (hMSC) differentiation along different lineages [7]. It was also shown that scaffold alignment and optimized mechanical simulation were sufficient to drive MSC differentiation without the need for additional chemical stimuli [4]. A number of methods for directing cell alignment using patterned substrates have been described; for instance, a mould patterning method with solvent casting to form CeO_2 nanoparticle lines within a PLGA film could induce the CeO_2-dependent alignment of cardiac stem cells and MSCs [8]. Drug-laden PLGA microspheres patterned into grooves by Teflon chips could direct cellular alignment and osteogenic commitment of adipose-derived stem cells for bone regeneration [9].

There are different ways to fabricate surface patterns [10–12], including (i) self-assembly; (ii) breath figures as templates; (iii) laser or light irradiation; (iv) pre-formed patterns as templates; and (v) direct writing [13]. Self-assembly of block copolymers can produce patterns with lamellar, cylindrical or spherical structures based on microphase separation of dissimilar polymer chains into ordered domains [14,15]. Based on arrays of condensed water droplets, breath figure templating generates ordered porous films on substrates or 3D objects [16,17]. For these self-assembly approaches, the structures are formed as a result of self-assembly via selection of polymers & substrates and control of preparation conditions. It may be difficult to create desired complex patterns. Inkjet printing and dip-pen nanolithography are widely used to fabricate patterns with computer-controlled programs [13,18,19]. These direct writing techniques would require the use of dedicated facilities.

The most commonly used approaches to fabricating surface patterned structures may be laser lithography [20] or photolithography [21–23]. The potential problems with photolithography are polymer degradation caused by photo-oxidation and lack of ability to form

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large-area low-cost high-resolution patterns [24]. Soft lithography employs the pre-patterned structures and the fabrication process relies on replication and pattern transfer [25]. This technique generates well-defined patterns with controllable surface properties, providing a promising platform for patterning cells and bioactive molecules [26,27]. Microcontact printing is also very useful for patterning chemicals and biological materials onto surfaces [28]. Both soft lithography and microcontact printing require pre-formed stamps or substrates which again are usually prepared by photolithography or microfabrication.

We describe here an alternative approach to preparing surface patterns by controlled freezing and subsequent freeze-drying. Freeze-drying is a versatile technique to fabricate 3D porous materials which are widely used as scaffolds for tissue engineering [29]. Particularly, directional freezing can be used to produce 3D aligned porous materials [30,31]. In this method, a temperature gradient is applied to a solution or suspension, which orientates the growth of ice crystals. Sublimation of ice by freeze-drying results in the formation of aligned porous structures. 3D porous scaffolds with anisotropic or aligned structures have been prepared by directional freezing to guide cell growth and alignment [32–34]. Although a previous report briefly mentioned that it was possible to produce 2D surface patterns with gold nanoparticles [35], there has been no study on fabrication of 2D surface patterns with various components and the use of such prepared patterns. Due to the wide use of patterned substrates in biological studies, in this study, we aim to present a versatile approach for preparation of surface patterned substrates (aligned and grid patterns) from different materials by directional freezing. The cell viability and the aligned growth of mMSCs with the substrates are evaluated.

2. Materials and methods

2.1. Solutions and nanoparticle suspensions

All polymers were used as received and the relevant aqueous solutions with the desired concentrations were prepared in distilled water. In_2O_3, TiO_2, and ZnO particles (all purchased from NanoTek®) were suspended in 0.1 wt.% aqueous poly(sodium acrylate) (PSA, Mw 2100, Sigma Aldrich) solution. Silica colloidal suspensions were diluted to the desired concentrations from the as-purchased Ludox silica colloidal suspensions (Sigma Aldrich).

Polystyrene microspheres were prepared by surfactant-free suspension polymerization with average diameters around 450 nm and a solid content of ~10% [36]. They were then washed and dispersed in 5 wt.% poly(vinyl alcohol) (PVA, Mw 10K, Sigma Aldrich) solution at the concentration of 2.0 wt.%. Silica microspheres were prepared by a modified Stöber method [37] while PLGA (Resomer® RG 503H, Boehringer Ingelheim) nanospheres were prepared by a common oil-in-water emulsion evaporation method (see supporting information).

2.2. Fabrication of surface patterns

A freezing stage controlled by a computer with software (FDCS freeze-drying system, Linkam Scientific Instrument Ltd.) was used to perform directional freezing on glass slides. 2 μl of the stock solutions or suspensions was deposited on glass slide with cover. The glass slide was placed on two temperature-controlled metal plates separated by a distance of 2 mm in the horizontal axis. Under the standard condition, one metal plate was set up at a temperature of −15 °C (−6 °C for PLGA-dioxane solutions) and the other plate at 20 °C. The glass slides were moved between the two plates at an accurate rate as controlled by the computer, in the range of 20–1000 μm/s. It should be noted that the slide moving rates were used to represent the freezing rate. The slide moving rate is proportional to the freezing rate and can be finely controlled to vary the freezing rate. After freezing, the frozen sample on glass slide was placed into liquid nitrogen and then transferred into a Virtis AdVantage freeze dryer (Biopharma Process Systems) with shelf temperature at −10 °C for 48 h.

To make the surface hydrophobic, the as-purchased glass slides were treated with chloro(dimethyl)octylsilane [38] and then used for PLGA-dioxane solution processing.

The surface patterns of silica particles were sintered in a furnace (Carbolite, CWF1500) at 400 °C for 6 h so that the patterns could be firmly stuck to the glass slide. The substrates with patterned silica colloids were thus treated before employed for mMSC growth.

2.3. mMSC cell culture

The D1 clonal MSC line, derived from bone marrow of BALB/c mice, was purchased from ATCC (CRL-12424). For routine culture, the cells were maintained on tissue culture plastic in standard culture medium (high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine) at 37 °C and 5% CO_2. The patterned substrates for cell culture were fabricated on circle glass slides with a diameter of 15 mm. The substrates were sterilized by UV light for 15 min on each side before culturing.

For cell viability WST-8 test, the patterned substrates fabricated with silica colloids (Ludox HS-30), aqueous chitosan solution, and PLGA-dioxane solution were placed in a Corning Costar 24-well plate. A tissue culture cover slip (Sarstedt) was used as a positive control while the background control was only the standard medium with no cells. All the samples were tested in triplet and the average values were used. 1 cm^3 D1 mMSC cells suspension at the concentration of 10,000/cm^3 was seeded in each well and incubated for 24 h. The medium was then aspirated and cell counting kit −8 (CCK-8, Sigma Aldrich) at a concentration of 20 μl in 200 μl medium was added into each well. After incubating for 3 h, 100 μl supernatant from each well was transferred into a 96-well plate and analysed by an Anthos Labtec LP 400 microplate reader at the wavelength of 450 nm.

For the culture study, the mMSCs were seeded onto 15 mm diameter circle substrates in 24-well plates (Nunc) in 250 μl droplets. Medium was topped up to 0.5 cm^3 after 1 day. The mMSCs were seeded at a density of 6000 cells per cm^2 for 3 to 5 days in standard medium. At the end of the culture period, cells were fixed with 4% (w/v) paraformaldehyde and stained with phalloidin labelled with Alexa Fluor 488 (Invitrogen) to visualize the F-actin cytoskeleton. Images were captured under a Leica DM2500 fluorescence microscope (Leica, Heidelberg, Germany) using a DFC350FX camera. For confocal microscopy, a Zeiss LSM 510 confocal laser scanning system mounted on a Zeiss Axiovert 200M (Carl Zeiss, Germany) was used.

2.4. Characterization

Surface pattern morphology was investigated using scanning electron microscopy (SEM, S-4800 Hitachi). The samples were coated with gold using a sputter-coater (EMITECH K550X) for 2 min at 25 mA. The spacing were measured based on the SEM images across a width ~0.5 mm (500 μm). The average values were used to plot the graph of spacings vs freezing rates. The height of the aligned ridges on the surface patterned substrates was measured from the SEM images. During the SEM imaging, the samples could be tilted to a certain angle, which helped to measure the ridge height. The SEM technique was also used to observe mMSC growth on the patterned substrates. After four days of culture, mMSCs on the PLGA-chitosan substrate were fixed, dried, and coated with Au before imaging. The in-situ directional freezing was investigated using Olympus CX41 microscope equipped with a digital camera and a computer-controlled freeze stage. The Fourier Transform Infra Red (FTIR) imaging was performed using a focal plane array (FPA) detector. Each spectrum corresponded to a specific position on the surface of the ATR crystal (diamond). By plotting the integrated absorbance of a specific spectral band in the mid-IR spectrum, it was possible to obtain the distribution and amount of a particular compound in