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Research Article

Biocompatibility of nanometre scale porous anodic aluminium oxide membranes towards the RK 13 epithelial cell line: A preliminary study

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ABSTRACT

Background: This study for the first time examines the biomedical potential of using anodic aluminium oxide (AAO) for culturing *Oryctolagus cuniculus* (European Rabbit) Kidney (RK-13) epithelial cells.
Methods: The cellular response of RK-13 cells towards in-house synthesised AAO membranes, a commercially available membrane and glass controls were investigated by examining cell adhesion, morphology and proliferation. The in-house membranes were anodized using a two-step procedure to produce a highly ordered hexagonal pore and channel structure.
Results: Cell proliferation over a 48 h period indicated that the AAO membranes were more than comparable with the glass control substrates. Subsequent microscopy observations revealed evidence of focal adhesion sites and cellular extensions interacting with the underlining porous membrane surface structure.
Conclusions: The study has shown that AAO membranes have the potential to culture RK-13 cells and indicate a possible tissue engineering technique for producing tissues.

Keywords: Nano-porous Anodic Aluminium Oxide, RK-13, Cell Adhesion, Cell Proliferation

INTRODUCTION

The development of porous aluminium oxide membranes via anodization has been extensively studied for a variety of applications for many years.[1,2] Using a two-step anodization process developed by Masuda *et al.*, it is possible to produce a highly ordered hexagonal pore and channel structure from a set of pre-arranged macroscopic parameters such as acid type, acid concentration, temperature and applied voltage.[3] The channels formed in the membrane makes it an attractive template for the manufacture of nanometre scale materials and devices.[4] These membranes have also been used for filtration[5], incorporated into chemical sensors[6,7] and even used as cell scaffolds for potential tissue regenerative procedures.[8,9]

From the medical perspective any material being considered as a cell scaffold or substrate needs to take into account biocompatibility and cell-substrate interactions. This is extremely important since these interactions will directly influence cell adhesion, morphology and proliferation.[10] Typically, cells are in the micrometre range and their component structures and associated environment are in the sub-micrometre to nanometre range. The significance of scale becomes

important when you consider molecules involved in cellular processes such as proteins, carbohydrates, nucleic acids and lipids are all nanometre scale structures. Consequently, interactions between cells and nanometre scale molecules can have significant influence on cellular processes such as migration, proliferation, and the synthesis of the extracellular matrix (ECM).[11] Because of this cellular interaction the surface chemistry and topography of a substrate can directly influence cell attachment and proliferation of anchorage-dependent cells. For example, Dalby *et al*., found that filopodial extensions from fibroblast cells could detect nanometre scale topographical features as small as 10 nm.[12] It is the relationship between cell and surface topography of an underlining substrate that has generated so much interest in recent years.[13]

The dominant surface feature sensed by cells on anodic aluminium oxide (AAO) membranes are the numerous nanometre scale pores that cover their surface.[14] A study by Karlsson *et al*., found that by varying membrane pore size one could influence protein secretion from osteoblast cells and in turn influence cellular processes such as attachment and differentiation.[15] A similar study by Nguyen *et al.*, also found smooth muscle cells sensed and responded to nanometre scale topographical features present on substrate surfaces.[16] Both studies, like many others have highlighted the influence of both micrometre and nanometre scale surface topographical features on cellular behaviour.[14] Therefore, to explore the possibility of using an engineered AAO membrane as a cell scaffold for potential tissue engineering applications is an essential step. The aim of this study was to investigate the viability of using a fixed pore size (100 nm) membrane for promoting cellular growth of *Oryctolagus cuniculus* (European Rabbit) Kidney (RK-13) epithelial cells. Cells were cultured on two different AAO membranes and a laboratory grade glass control. The first membrane was fabricated in-house and the second was supplied by Whatman® Anopore. The influence of substrate surface topography on cell adhesion and morphology was investigated using optical microscopy and field emission scanning electron microscopy (FESEM). Cell viability towards the substrates was evaluated over a 48 h period using the CellTiter 96® Aqueous One Solution cell proliferation assay procedure.

METHODS

Materials

All high purity grade chemicals used in this study were supplied by Sigma-Aldrich (Castle Hill: NSW, Australia) and used without further purification. All aqueous solutions were made using Milli-Q® water (18.3 MΩ cm^{-1}) produced from a Barnstead Ultrapure Water System D11931 (Thermo Scientific, Dubuque, IA). High purity (99.99%) aluminium foil (100 mm square and 0.25 mm thick) was used in the synthesis of in-house membranes and was supplied by Alfa Aesar (USA). Anodisc membranes (diameter 25 mm, pore size 0.1 µm) were supplied by Whatman® Anopore (Anodisc 25, 0.1 µm, UK). While CellTiter 96® Aqueous One reagent solution supplied by Promega (USA) was used to determine viable cells numbers on the respective substrates.

Fabrication of in-house membranes

An extensive description of synthesizing nanometre scale porous AAO membranes by the authors is given elsewhere.[2,9] However, for completeness a brief procedure is discussed here. Fabrication begins by cutting up the aluminium sheet into 50 mm x 20 mm strips. The strips were annealed for 5 h in a nitrogen atmosphere to re-crystallise and release mechanical stresses in the strips. After annealing, strips were degreased and etched in 3.0 M sodium hydroxide for 5 min before being thoroughly washed in Milli-Q® water and then allowed to dry. A protective polymer layer was applied to one side of the strip before being placed into an electrochemical cell consisting of 0.3 M oxalic acid. The voltage was adjusted to 60 V and anodization period was set to 5 h. At the end of the first step, the resulting oxide layer formed on the expose surface was removed from the strip by immersion in a stirred acidic solution composed of phosphoric and chromic acid (70 mL/L and 20 g/L, respectively) at 60°C for 1 h. During the second anodization step the same operating parameters were followed except that the anodization period was reduced to 3 h. After anodization, the pores formed in the oxide layer were widened by chemical etching in a 5 % solution of phosphoric acid at 35°C for 15 min. Then a thin layer of Acrifix 192 was applied to the anodized side of the Al strip to provide physical support during the removal of the aluminium backing. The backing was removed using an acidic solution mixture composed of 0.1 M copper chloride and 7 % hydrochloric acid. During the next stage, the barrier layer oxide was removed by etching in phosphoric acid to produce a clear oxide membrane. The final stage involves sterilizing the membranes in a 30 % solution of hydrogen peroxide at 60 ºC for 15 min and then quickly rinsed in a solution of Milli-Q® water to remove any hydrogen peroxide. The membranes were then allowed to dry before being stored in airtight containers.

Membrane-cell characterisation

Optical microscopy was used to study cell-membrane interactions as evidenced by attachment and proliferation. An Olympus BX51 compound microscope (Olympus Optical Co. Ltd., Tokyo, Japan) was used for all optical studies and photographs were taken using the DP 70 camera attachment. Before microscopy, adherent cells were fixed on the membranes by using a 1:1 solution of acetone and methanol, and then dried. The surface covering of cells were stained using an aqueous solution containing 1 % Fuchsin acid. After 30 min the excess stain was rinsed off using Milli-Q® water and then allowed to air dry. After drying the membranes were ready for optical microscopy.

Field emission scanning electron microscopy (FESEM) was carried out using a Zeiss Neon EsB FIBSEM. The field emission electron gun provided both high brightness and high resolution (0.8 nm). Micrographs were taken at various magnifications ranging from 2 to 5 kV using the SE2 and In-Lens detectors. Prior to FESEM observation, sample preparation consisted of first soaking and washing in a 30 % solution of ethanol for 15 min. This was followed by sequential drying of the samples using progressively increasing concentrations of ethanol washes (2 washers of 50 %, 70 %, 80 %, 90 %, and 95 %), until being finally washed in 100 % ethanol for 30 min. Following ethanol washing, samples were immersed in a 50:50 solution of ethanol:amylacetate for 30 min. This was then followed by two sequential immersions in amylacetate over a period of 1 h before being placed into a critical point dryer. Finally, the dried samples were mounted on FESEM stubs using carbon adhesive tape before being sputter-coated with a 2 nm layer of platinum metal to prevent charge build up.

Cell culturing, adhesion and proliferation

The cell line used in this *in vitro* study was the *Oryctolagus cuniculus* (European Rabbit) Kidney (RK 13) epithelial, supplied by the Animal Health Laboratories, Animal Virology, Department of Agriculture and Food, 3 Baron Hay Court, Kensington, Western Australia 6151, Australia. The cell culturing protocol was carried out in accordance with the Animal Health Laboratories procedure VIW-17 using a Cell Growth Medium 199 (Sigma-Aldrich) and 10 % fetal calf serum (FCS).[17] An extensive description of the standard cell culturing procedure used by the authors is presented and discussed in reference.[8]

Cell adhesion studies consisted of preparing 3 sets of sample substrates consisting of in-house AAO membranes, Anodisc membranes and glass controls. Each set consisted a substrate designated for each of the 4, 24 and 48 time intervals. A total of 9 substrates were individually placed into their respective well in a cell culture plate (Cellstar® Greiner Bio-One, Germany). Then a 1 mL solution of RK-13 cells ($1x10^5$ cells/mL) suspended in DMEM culture medium and 10 % FCS was transferred to each well via a pipette. Following this a further 1 mL of DMEM medium was added to each well using a pipette. The cells were then incubated at 37°C with a 5 % CO_2 atmosphere for 4 h. After 4 h, the first 3 substrates were removed from their wells and washed several times using PBS to remove unattached cells and DMEM medium. The remaining 6 substrates were transferred to fresh culture medium loaded wells on another cell culture plate. The transfer procedure ensured all unattached cells were removed and the original depleted culture medium was replenished before continuing incubation. The removed substrates were then prepared for microscopy and cell adhesion studies. This procedure was repeated for the 24 h and 48 h time periods. The cell adhesion procedure was carried out in triplicate to ensure consistency.

A cell proliferation assay was carried out to determine the number of viable cells proliferating over the surface of each respective substrate over a 48 h period using the CellTiter 96® Aqueous One Solution assay procedure. Substrate sets were individually placed into separate wells on cell culture plates, with individual substrates being used for each time interval (4, 24, and 48). Sufficient substrates were used to make up triplicate sets. The wells were then filled by adding a 1 mL solution of RK-13 cells ($1x10^5$ cells/mL) suspended in DMEM culture medium with 10 % FCS and 1 % PBS using a pipette. The cells were then incubated at 37°C in a humidified atmosphere with a 5 % CO_2 atmosphere for 4 hours. After 4 hours, the first set of substrates samples were transferred to pre-filled wells containing fresh culture medium (500 µL) located on a new cell culture plate. Then a 60 µL solution of CellTiter 96® Aqueous One Solution reagent was added to the wells using a pipette as per supplier's recommendations. This was followed by another hour of incubation before 120 µL aliquots were removed from the wells and placed into fresh wells located on a 96 Well Tissue Culture Plate (83.1835, Sarstedt Inc. Newton, USA). Then the absorbance at 490 nm was recorded using an ELISA 96 well automatic plate reader fitted with a Microplate Spectrophotometer equipped with Microplate Manager 5.2.1 software for data analysis (Bio-Rad, Australia). The procedure was then repeated for the 24 and 48 proliferation periods. The results of the CellTiter 96® Aqueous One Solution cell proliferation assay (including triplicates) were analysed using Microplate Manager 5.2.1 software for data analysis (Bio-Rad, Australia).

RESULTS

Membrane surface topography

Membrane surface structure and topography was studied using FESEM. The surface terrain of in-house membranes revealed a smooth undulating surface landscape with arrays of uniformly sized pores. Analysis of several randomly selected 1 μm^2 survey locations on each membrane type were selected and the pore arrays were examined. The in-house membranes were found to have pores with a mean diameter of 102 ± 12 nm and a mean inter-pore distance of 148 ± 12 nm. Overall the membranes were 40 µm in thickness and had a mean pore density of 54 ± 3 pores/μm^2. The comparative membrane supplied by Whatman® was found to have a nominal thickness of around 60 µm, a mean pore diameter of 120 ± 45 nm and a mean inter-pore distance of around 0.32 µm.[9,18] The surface topography was also found to be much rougher than the in-house membranes, with numerous jagged edges protruding up from the inter-wall structures between the pores. Detailed surface studies of both membrane types have been carried out by the

authors and the reader is referred to these studies for further information.[9,19,20]

Cell adhesion studies

Optical microscopy and FESEM investigations of RK-13 cells after 24 h of cultivation on all three substrates reveal good cell adhesion. The cells also exhibited a flattened polygonal morphology and wide spread coverage over all three substrate surfaces as seen in (Figure 1). Examination of (Figure 1(c)) Whatman® Anopore and (Figure 1(e)) In-house AAO membranes reveals the attached cells are comparable to those attaching to the glass control in (Figure 1(a)). It is also evident at the 24 h period that the number of viable cell present on all three substrates is similar.

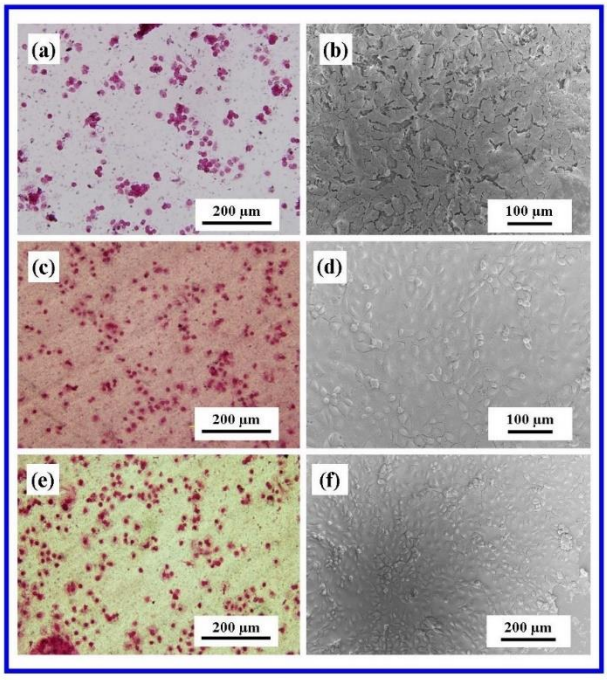

Figure 1: Optical microscopy of RK-13 cells on (a) glass, (c) Whatman® Anopore and (e) in-house AAO membrane. FESEM micrographs of cells on (b) glass, (d) Whatman® Anopore and (f) in-house AAO membrane.

An enlargement of a representative RK-13 cell attached to an in-house AAO membrane is presented in (Figure 2). The cell has been colourised to highlight its presence. Examination of the cell reveals the presence of numerous microvilli extending from the cell wall. The entire upper cell surface is covered by microvilli (yellow arrows) and confirms the cells are actively involved in adsorption, adhesion, and secretion. It also confirms the cells are interacting with the surrounding extra cellular matrix. (Figure 2) also reveals the presence of numerous filopodia at the boundary of the cell. The position of several filopodia are highlighted by red arrows. Filopodia can be seen spreading out from the cell to form anchoring points on the underlining porous membrane surface. The filopodia are also important in adhesion during cell migration and their presence clearly demonstrates the cell is actively interacting with the underling membrane surface and surrounding extra cellular matrix.[21]

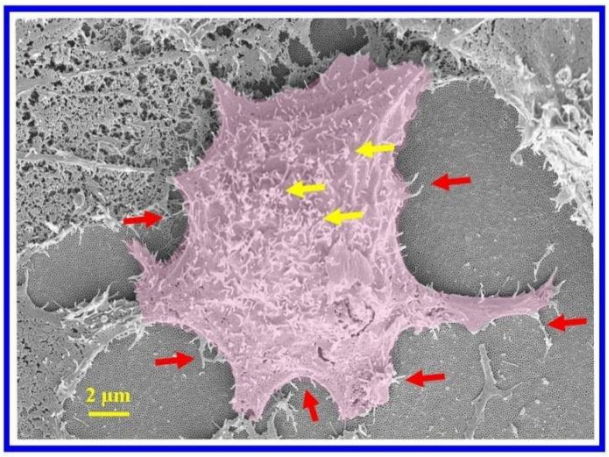

Figure 2: An enlarged and colourised FESEM micrograph of a RK-13 cell membrane showing the presence of numerous microvilli (yellow arrows) and the presence of filopodia (red arrows).

Cell proliferation study

The results of adhesion studies have clearly shown that RK-13 cells can easily attach to the glass control and both porous membranes. However, to determine the long-term viability of cells, a cell proliferation assay was carried out since proliferation is a good indicator of long-term survivability. Importantly, during the proliferation study no evidence was found to indicate the presence of infection or toxicity effects. (Figure 3) presents the results of the cell proliferation assay carried out over a 48 h period. (Figure 3(a)) presents the results of the proliferation study and (Figure 3(b)) presents a line graph (trend) of the study. Both graphs clearly show a positive increase in the number of viable cells surviving on all substrates.

After the first 4 h, analysis revealed cell numbers for all three substrate types were similar. With both membrane types having a slightly larger number of cells than the glass control. At the end of 24 h period, the glass control had around 5 % more viable cells than the Whatman® Anopore membrane and 11 % more than the in-house AAO membrane. However, by the end of the 48 h period there was a significant difference seen between all three substrates. Analysis of viable cell numbers revealed the glass control had 1550 cells/mm^2, while the Whatman® Anopore membrane had 34 % more cells and the in-house AAO membrane had 53 % more cells. During the first 24 hours of the proliferation study cells cultured on glass tended to be similar to both membrane types. However, after 48 h the number of viable cells were significantly greater on both membrane types.

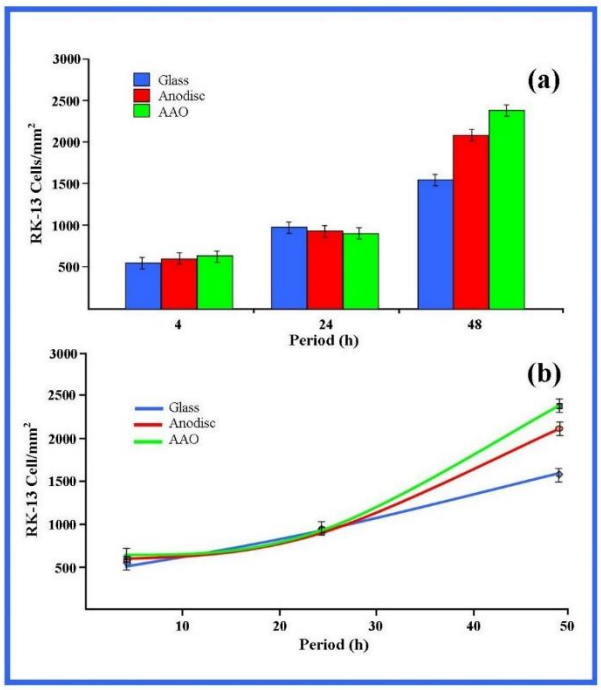

Figure 3 (a) Number of viable attached RK-13 cells on the glass control, Whatman® Anopore and in-house AAO membranes. (b) Trends in viable RK-13 cell numbers on the three substrate types.

DISCUSSION

FESEM studies of membrane surface structures and topography revealed the terrain of in-house membranes were smooth and undulating. The surface texture was produced by large numbers of uniformly distributed pores typically around 102 ± 12 nm in diameter. The Whatman® membranes had pore diameters around 120 ± 45 nm and had a much rougher surface topography with numerous jagged edges protruding up from the inter-wall structures between the pores. Both membranes were found to be comparable with the glass control in terms of cell proliferation. The study has revealed that both membrane types could be used to successfully cultivate the RK-13 cell line. The assay also suggests nanometre scale texturing of the surface via pore structuring in the membrane has influenced both cell attachment and cell proliferation, as indicated by the larger numbers of viable cells found on the membranes. The results of this study have shown that it is possible to culture RK-13 cells that retain histological characteristics and enhanced proliferation rates on AAO membranes. However, further studies are needed to translate this preliminary research into a viable tissue regeneration technology for the repair of damaged epithelia linings of organs.

CONCLUSION

In this study, the biomedical potential of culturing the RK-13 cell line on AAO membranes with nanometre scale surface texturing was investigated. Both the in-house membrane and the commercially available Whatman® Anopore membranes were used without any further surface modification. Importantly, over the 48 hour cell proliferation study there was no cytotoxic effects detected and all substrates promoted cellular growth. Microscopy studies revealed the presence of both microvilli and filopodia that clearly indicated the cells were actively involved in adsorption, adhesion, and secretion. Filopodia could be seen anchoring cells to the underlining surface and surrounding extra cellular matrix. The number of viable cells at the end of the proliferation assay revealed that both membrane types were capable of promoting cell attachment and cell proliferation. The study also found the in-house membrane tended to produced greater numbers of viable cells. However, further studies are needed to investigate the effect of surface texturing and the influence of surface texturing on cell processes. Future studies are expected to provide additional data that can be used to translate this technique into potential tissue regeneration therapies.

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