
Can anodic aluminium oxide nanomembranes treated with nanometre scale hydroxyapatite be used as a cell culture substrate

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Abstract: In this study we investigate the biomedical potential of composite membranes composed of anodic aluminium oxide (AAO) and nanometre scale hydroxyapatite (HAP). The nano-porous AAO membranes were produced using a temperature controlled two-step anodization technique. The AAO/HAP composite membranes were formed using the solution template wetting technique. The *Cercopithecus aethiops* (African green monkey) Kidney (Vero) epithelial cell line was used to demonstrate the biocompatibility of the synthesised membranes and composites. Investigating cell adhesion, morphology and proliferation over a 72 h period assessed cellular interactions and responses of the cell line to the various membranes types.

Keywords: Biomaterials, Anodic Aluminium Oxide, Bioceramics, Regenerative Medicine

1. Introduction

Anodic aluminium oxide (AAO) membranes produced by the electrochemical process of anodization in a polyprotic acid (e.g., oxalic, phosphoric or sulphuric acid) have a nanometre scale porous structure. This novel structure has the potential to be used as a cell scaffold for tissue engineering applications [1]. During short, single anodization periods, the resultant oxide layer formed a disordered array of pores, which were result of random nucleation sites produced on the metal surface at the start of oxide formation [2, 3]. Studies by Masuda and Fukuda found that it was only possible to produce a highly ordered, self-organized pore growth mechanism under specific anodizing parameters [4]. For example, long anodization periods (up to a maximum of 160 hours) allowed the pores to self-adjust from their random initiation sites. However, these ordered pore positions could only be seen at the metal/oxide interface after the barrier layer was removed. Further refinements to improve pore ordering during the anodization process resulted in the development of a two-step process. During the first step a long anodization period is used to form a highly ordered pore array at the metal/oxide interface. At the end of anodization, the oxide layer is

removed from the Al substrate to reveal a highly periodic and indented surface landscape. The indented landscape forms the initiation sites for future pore formation in the second anodization step [5]. It is during the second step that a densely packed, highly ordered pore array is formed in the developing oxide layer [6, 7]. Optimization of the two-step technique results in the production of straight, parallel and densely packed hexagonally arrayed pore channels with high aspect ratios that traverse the thickness of the oxide layer [8]. The advantage of using the two-step anodization technique is that it is possible to dictate the type of nanometre scale porous structure formed by adjusting controllable macroscopic parameters such as electrolyte type and concentration, temperature and applied voltage [2, 8]. This refined anodization technique has made it possible to use AAO membranes as templates for the manufacture of nanometre scale materials [9, 10], biological/chemical sensors [11, 12], filter membranes [13] and medical scaffolds for tissue engineering [14-16].

The potential biomedical application of AAO membranes as cell scaffolds for tissue engineering applications presents a

number of attractive possibilities. For example, the surface chemistry and topography of a potential material for use with cells must be biologically compatible for this substrate to induce productive cell-material interactions. In the case of AAO membranes, the unique surface properties have been investigated by a number of researchers for a number of cells such as keratinocytes and fibroblasts [17], hepatocytes [18], endothelial and vascular smooth muscle cell [19], and osteoblasts [20]. The results of these studies all confirm the importance of addressing the behaviour of a specific cell line to the scaffold environment. Therefore, cell-material interactions and the surface environment of the scaffold are of fundamental importance since they can have a significant influence on cell activity, adhesion, morphology and proliferation [21]. Therefore, any material that is considered for potential use as a cell scaffold must be highly biocompatible and promote normal cell activity. Furthermore, the material must be able to withstand normal sterilization procedures commonly used in the biomedical field.

In this study a solution template wetting technique is used to infiltrate nanometre scale hydroxyapatite (HAP) into the porous structure of AAO membranes to form a unique composite. Hydroxyapatite (HAP) $[\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6]$ is a hexagonal structured ceramic and is a member of the calcium phosphate family. Its structure and close chemical similarity to the mineral component found in natural bone tissue has attracted considerable interest in using synthetically produced HAP in a variety of biomedical procedures for the replacement of damaged or diseased bone tissues [22, 23]. Its success in these clinical procedures is due in part to its very attractive material properties such as: 1) its good biocompatibility with body tissues; 2) its biodegradability *in situ* is slow; 3) it provides good osteoconductivity; and 4) it has good osteoinductivity capabilities [24-26]. And because of its favourable biological response with a wide range of cells types found in skin, muscle and gums it is an ideal candidate for orthopaedic and dental implants [27].

The aim of this study was to investigate the viability of using an engineered AAO/HAP composite membrane as cell scaffold for promoting cellular growth of the *Cercopithecus aethiops* (African green monkey) Kidney (Vero) epithelial cell line. The cells were cultured on two different kinds of AAO membranes, each with a nanometre scale porous structure, two AAO/HAP composites based on each type of membrane and a laboratory grade glass control. The first membrane was produced in-house, while the second was a commercially available membrane (Whatman® Anodisc 25, 0.1 µm). Both membranes had a mean pore diameter of 100 nm, but both had different inter-pore spacing and surface roughness. The cellular response of Vero cells to the two types of membranes, the HAP composites and a glass control substrate was evaluated over a 72 h cell proliferation assay. Cell adhesion and morphology on all membrane types and the glass control was investigated using optical microscopy (OM), Atomic Force Microscopy (AFM) and Field Emission Scanning Electron Microscopy (FESEM).

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (Castle Hill: NSW, Australia) and used without further purification. Milli-Q® water ($18.3 \text{ M}\Omega \text{ cm}^{-1}$) was used in all aqueous solution preparations and was produced from a Barnstead Ultrapure Water System D11931 (Thermo Scientific, Dubuque, IA). The Anodisc membrane (diameter 25 mm, pore size 0.1 µm) used for comparative purposes was supplied by Whatman® Anopore (UK). The high purity (99.99%) Aluminium (Al) sheets, (100 mm square and 0.25 mm thick) used to manufacture the in-house membranes were supplied by Alfa Aesar, USA.

2.2. Synthesis of Nano-HAP Powders

The three-step synthesis procedure is schematically presented in Figure 1, and begins with adding a 40 mL solution of 0.32M calcium nitrate tetrahydrate into a small glass beaker. The pH of the solution is then adjusted to 9.0 by slowly adding and mixing approximately 2.5 mL of ammonium hydroxide. Once the pH is stabilized, the solution is then exposed to ultrasonic irradiation for 1 h, with the processor set to 50 W and maximum amplitude.

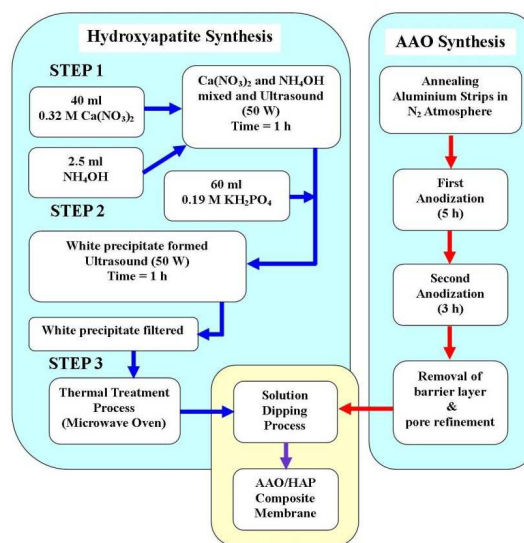


Figure 1. Schematic representation of the fabrication procedure used to produce an AAO/HAP composite membrane for cell culture.

After the first hour, a 60 mL solution of 0.19 M potassium di-hydrogen phosphate was slowly added drop-wise into the first solution while undergoing a second hour of ultrasonic irradiation. During the second hour, the pH of the solution was checked and maintained at 9.0 and the Calcium/Phosphate [Ca/P] ratio was maintained at 1.67. At the end of the second hour, the solution underwent centrifugation ($15,000 \text{ g}$) for 20 minutes at room temperature; the resultant white precipitate sample was collected and placed into a fused silica crucible (supplied by Rojan Advanced Ceramics Pty Ltd, Western Australia). The crucible was then placed into a standard domestic household microwave (1100W at 2450 MHz-LG® Australia); set at 100 % power and

the thermal treatment cycle set at 40 minutes. At the end of the heating cycle the sample ended up as a white agglomerated mass. After the sample had cooled, it was then ball milled until all the agglomerations were removed and all that remained was an ultrafine nano-HAP powder. This synthesis procedure is repeated until a sufficient amount of the nano-HAP powder was available for advanced characterisation and for the solution template wetting of AAO membranes to form the AAO/HAP composites.

2.3. Fabrication of In-House Nano-Porous AAO Membranes

Fabrication of the in-house membranes begins by annealing 50 mm x 20 mm x 0.25 mm Al strips in a nitrogen atmosphere at 500 °C for 5 hours in a standard tube furnace. During annealing, re-crystallisation takes place and in the process the mechanical stresses created during the formation of the strips is released and leads to a better stress-free membrane. After annealing, the strips were then allowed to cool down to room temperature in the nitrogen atmosphere. At room temperature the strips were first washed in acetone and then etched in a 3.0 M sodium hydroxide solution for 5 minutes before being thoroughly washed in Milli-Q® water. After drying a thin layer of polymer was applied to one side of the strip to protect it from the effects of the anodization process. Once the polymer had set, the strip was ready for the first step of the two-step anodization procedure. During the first step, each strip was anodized using a voltage of 60 V in an electrolyte solution consisting of 0.3 M oxalic acid for 5 hours. At the end of the first step, the strip was 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 hour to remove the thin oxide layer formed on the non polymer coated surface. Removal of this oxide layer exposes a highly periodic and indented surface which forms the nucleation sites for pores formed during the second anodization step. The second anodization was performed under the same conditions as the first, the only difference being the shorter anodization period of 3 hours. During the 3 hour period, a regular, honeycomb array of nanometre-sized pores were formed in the resulting oxide layer. The pores in the second oxide layer were then widened by chemical etching in a 5% solution of phosphoric acid at 35 °C for 15 minutes. The strip was then washed in Milli-Q® water and then allowed to dry before a thin layer of Acrifix® 192 polymer was applied to the anodized side of the Al strip. The Acrifix® layer served as a physical support for the membrane during the removal of the Al substrate with an acidic solution mixture composed of 0.1 M copper chloride and 7% hydrochloric acid. Next, the barrier layer and Acrifix® 192 support layer was removed from the membrane by etching in a 0.3 M solution of phosphoric acid. The resulting off white coloured membrane was sterilized by immersing in a 30% solution of hydrogen peroxide at 60 °C for 15 minutes. This was followed by quickly dipping the membrane into a solution of Milli-Q® water for 10 seconds to remove any residual hydrogen peroxide from the membrane surface and then placed under ultraviolet light for 2 hours before being stored in airtight containers. Figure 2 (a) presents a FESEM micrograph of a typical AAO membrane fabricated

in-house using the two-step anodization procedure.

2.4. Dip Coating of Nano-Porous Membranes

Both types of nano-porous membranes were pre-soaked in a 1 % sodium dodecyl sulfate (SDS)/aqueous solution for 1 h. The membranes were then soaked in a saturated HAP for 1 h and then placed into a vacuum oven (Napco® 5831 E series, USA), which was then pumped down to 85 kPa and maintained at room temperature (24 °C) where they remained overnight. The following day the membranes were removed from the vacuum oven and stored in airtight containers until required.

2.5. Characterization of Nano-Porous Membranes and Cell Cultures

All nano-porous membranes were examined using a field emission scanning electron microscopy (FESEM). The micrographs were taken using the Zeiss Neon 40EsB FIBSEM (Carl Zeiss, Oberkochen, Germany) located at the Centre for Materials Research (CMR) at Curtin University of Technology. The field emission electron gun provided both high brightness and high resolution (0.8 nm). Samples were mounted on individual substrate holders using carbon adhesive tape before being sputter coated with a 2 nm layer of platinum to prevent charge build up using a Cressington 208HR High Resolution Sputter coater. Micrographs were taken at various magnifications ranging from 2 to 5 kV using the SE2 and In-Lens detectors. 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 cells could be viewed by optical microscopy, the cells on each respective substrate were fixed using a 1:1 solution of acetone and methanol and then stained using an aqueous solution containing 1% Fuchsin acid. After 1 h the excess stain was rinsed off using Milli-Q® water, dried and then mounted onto microscope slides ready for optical microscopy investigation at various magnifications (4x, 10x, 20x and 40x). All substrates used in cell studies that were going to be studied using FESEM needed to be specially prepared. The preparation consisted of an initial washing in 30 % ethanol followed by sequential washing/drying of the samples using progressively increasing concentrations of ethanol washes (2 washes of 50%, 70%, 80%, 90%, and 95%). The final wash was carried out in a solution of 100% ethanol for 30 minutes. After drying, the substrates were then treated with a 50:50 solution of ethanol and amylacetate for 30 minutes. This was then followed by 2 immersions in amyl acetate over a period of 1 h before being placed into a critical point dryer. Finally, the substrates were mounted on FESEM stubs before being sputter-coated with a 2 nm layer of platinum metal for imaging purposes. The samples were then ready for FESEM investigations.

2.6. Cell Culturing and Growth

2.6.1. Cell Seeding and Culture

The cell line used in this *in vitro* study was the

Cercopithecus aethiops, (African green monkey) Kidney (Vero) epithelial, and were 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) [28]. A standard cell culturing procedure used in previous AAO cell line studies by the authors is presented and discussed in reference [29]. All cell-culturing procedures were carried out in triplicate to ensure consistency in the study.

2.6.2. Cell Adhesion and Morphology Studies

Apart from sterilization prior to cell seeding all nano-porous membranes and glass controls were used without surface pre-treatments. The study started by first making up a series of sample sets composed of both types of nano-porous membranes and glass controls. Each set was made up by adding 2 samples of each nano-membrane type and 2 glass controls to a 6 well culture plate (N° 657-160) supplied by CellStar® Greiner Bio-One, Germany. Then a 3 mL solution of Vero cells (approximately 3×10^5 cells/mL) suspended in culture medium and 10% FCS were transferred to each well of the culture plate using a pipette. Once the wells were filled, the plates were then incubated at 37 °C under a 5 % CO₂ atmosphere while gently being oscillated. After 24 h of incubation, the plates were removed and respective membranes and glass controls were washed and then placed into a fixing solution composed of a 1:1 solution of methanol: acetone. After 10 minutes, the membranes and glass controls were removed from the fixing solution and allowed to air dry at room temperature. Then the cells on the respective membranes and glass controls were soaked for 1 h in an aqueous solution containing 1% Fuchsin acid. After soaking, the membranes and glass controls were removed from the staining solution and the stain was rinsed off using Milli-Q® water and allowed to air dry at room temperature. After the membranes and glass controls had dried they were mounted onto microscope slide before a cover slip was added. The samples were then ready for the optical microscopy studies such as cell adhesion and cell morphology. The cell adhesion procedure was carried out in triplicate to ensure consistency.

2.6.3. Cell Proliferation Studies

The number of viable Vero cells proliferating over the surface of each respective membrane and glass control were quantified over a 72 h period using a standard cell counting technique. The assay procedure consisted of overlaying a grid onto a random location of the photographic image of the substrate surface and then counting the number of cells present in that area. A minimum of 10 random locations per substrate was examined and then the mean \pm standard deviation of cells present on each substrate was calculated.

3. Results and Discussions

The surface topography and open porosity of both the

in-house manufactured nano-porous AAO membrane and a commercially available alumina membrane (Whatmann® Anodisc) were investigated using FESEM. Typical surface micrographs of both membranes are presented in Figure 2. The most significant difference seen between Figures 2 (a) and (b) is the very rough surface of the anodisc membrane compared to the smooth and undulating in-house membrane. Surveys of the anodisc surface terrain revealed a mean pore diameter of 120 ± 45 nm and a mean distance between pores ranged from between $0.32 \mu\text{m}$ to $0.37 \mu\text{m}$. Examination of the in-house membrane revealed highly ordered, close packed hexagonal arrays of uniformly sized pores consisting of pores with a mean diameter of 104 ± 12 nm and a mean inter-pore distance of 150 ± 14 nm. Further details of the structure and surface topographical features of both membrane types can be found in a recent and comprehensive materials study of the membranes by Poinern *et al* [29, 30].

Optical and FESEM microscopy investigations of Vero cells cultivated on the in-house AAO and anodisc membranes after 24 hours reveal good cell adhesion that is comparable to the glass control. Optical images presented in Figures 3(a), (c) and (e) reveal that the cells have a flattened polygonal morphology and have extensively covered the surface of all three substrate surfaces. Cell attachment was also confirmed by FESEM micrographs taken of the three substrate types as seen in Figures 3(b), (d) and (e). Similar cell attachment was also seen for both membrane types that had been treated in the HAP solution. The HAP coated membranes examined all revealed cell attachment and widespread coverage as seen in Figure 4(d) for the anodisc/HAP composite. Further examination using FESEM microscopy of the HAP coated membranes revealed the presence of numerous microvilli over the surface of the cells as seen in Figures 4(e) and (f). These microvilli were also seen on the surface of cells attaching to the untreated membranes as seen in Figures 4(b) and (c). The presence of these microvilli clearly indicates that the cells are actively involved in adsorption, cellular adhesion and secretion [31]. An enlarged view of a typical cell with microvilli (indicated by blue arrows) is presented in Figure 5(b).

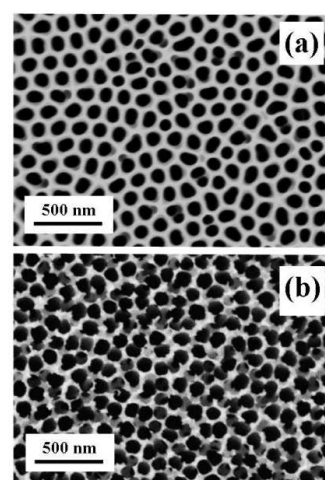


Figure 2. FESEM micrographs of typical porous surface terrains of (a) in-house AAO membrane and (b) Whatman® anodisc membrane.

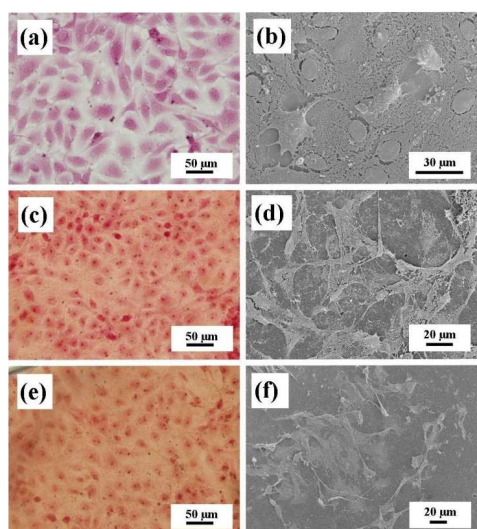


Figure 3. Optical and FESEM microscopy of Vero cell proliferation at the 24 h period: Optical images (a) cells on glass substrate (control); (c) Whatman® Anodisc membrane and (e) in-house AAO membrane. FESEM images of cell attachment: (b) Cells on glass substrate; (d) seen on Whatman® Anodisc membrane and (f) vero cells on in-house AAO membrane

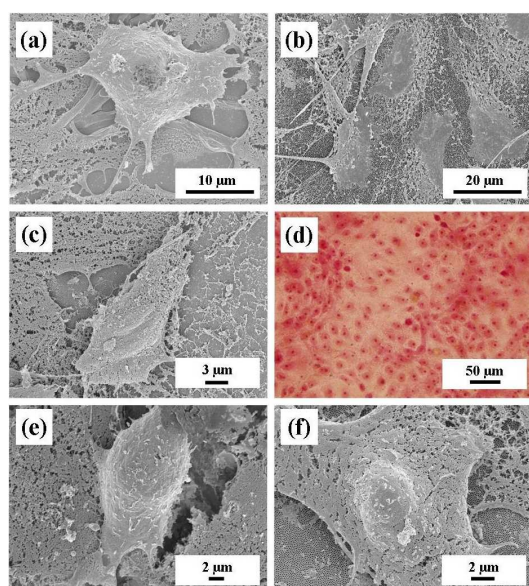


Figure 4. FESEM microscopy of single Vero cells at the 24 h period: a) glass substrate (control); (b) Whatman® Anodisc membrane; (c) in-house AAO membrane. (d) An optical image of cells attaching to an Anodisc/HAP composite membrane. Single cells anchored on (e) Anodisc/HAP composite membrane and (f) in-house AAO/HAP membrane.

Examination of the FESEM micrographs also clearly indicated the presence of filopodia at the cell boundaries as seen in Figure 4. The long thin filopodia (<100 nm in diameter) can be seen spreading out from individual cells to cover the surrounding substrate. The filopodia can be seen anchoring the cell to the underlining ECM and substrate surface. This type of cell attachment was seen for all substrates tested. An enlarged view of a typical cell on the in-house AAO membrane is presented in Figure 5(a). The sprawling filopodia (indicated by red arrows) can be seen attaching to the underlining nano-porous membrane structure. In spite of not

having the nanometre scale topographical features of the two nano-porous membranes and their respective composites, cells on the glass control substrates also had pronounced filopodia. And as the case of the membranes and composites, filopodia could be seen attaching to the surrounding ECM and the underlining glass substrate. Optical observations also revealed that the cells on all substrates were uniformly distributed over the entire surface of each respective substrate.

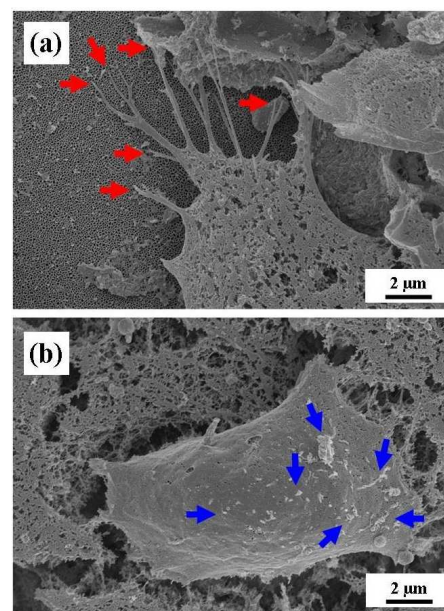


Figure 5. FESEM micrographs of individual Vero cells on the in-house AAO membrane at the end of 24 h period: (a) filopodia positions indicated by red arrows and (b) microvilli positions indicated by blue arrows.

The cell attachment interactions in this study have clearly demonstrated that the Vero cells have accepted the various substrates as suitable biological platforms for attachment. The subsequent production of ECM and cellular interaction confirm that the cells are performing normal cell functions. All substrates tested have confirmed biological suitability for cell attachment but did not provide any biological information regarding the long-term viability of cell survivability. Therefore, a cell proliferation assay over a 72 h period was carried out to determine the long-term survival of cells on the respective substrates. During the assay there was no evidence of infection or toxicity effects occurring to the cells over the test period. The results of the proliferation assay are presented in Figure 6.

A direct comparison between the various substrates used during the 72 h proliferation assay is presented in Figure 6. At the end of the 4 h period, the number of viable cells adhering to the glass control 1035 cells/mm², which was 6.6 % greater than the in-house AAO membrane. The Whatman® anodisc membrane had 11.5 % more cells than the glass control and the anodisc/HAP composite had 4.9 % less cell compared to the control. While the lowest cell number counts recorded was for the in-house AAO/HAP composite were 35.9 % below the control. During the following days, cell proliferation on all substrates continued to increase with cells uniformly covering

the substrate surfaces. By the end of the 24 h period the number of viable cells on the in-house AAO membrane was 14.9 % greater than the glass control, while anodisc membrane was 6.3 % greater than the control. The in-house AAO membrane continued to have greater numbers of viable cells compared to the control for the remainder of the assay period. At the end of the 72 h period the in-house AAO membrane cell numbers were 16.6 % greater than the control, while the anodisc membrane had 8.8 % more cells compared to the control. In the case of the two composites, at the 24 h period the anodisc/HAP composite membrane recorded 2.8 % more cells than the control.

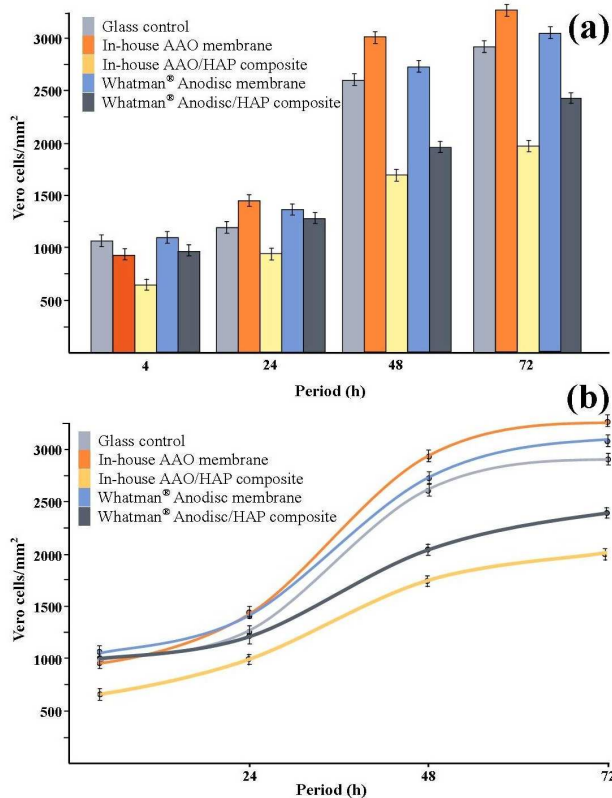


Figure 6. (a) Number of viable attached Vero cells on various substrates showing significant differences in cell attachment and cell proliferation on the various substrates and (b) comparisons between the numbers of viable Vero cells on the various membranes.

But from this period onwards the composite recorded significantly lower cell numbers and by the end of the assay period its cell numbers were 15.4 % lower than those of the control. Meanwhile, the performance of the in-house AAO/HAP composite continued to be the poorest and by the end of the assay period it had cell numbers 30.9 % below those of the control. However, at the end of the 3 day proliferation assay the highest number of viable cells were found on the in-house AAO membrane followed by the anodisc membrane and then the glass control. The assay has confirmed that both nano-porous membranes were superior to the control. However, this was not the case for the HAP based composite membranes which were both recorded fewer cell numbers at the end of the assay as seen in Figure 6. In the case of the

Anodisc/HAP composite cell numbers were 15.4% lower than the glass control, while the AAO/HAP composite recorded cell numbers 30.9% lower than the glass control. The results clearly demonstrated that the in-house AAO/HAP composites were the least productive membranes.

The present study indicates that both the in-house AAO membrane and the Whatman® anodisc membranes were capable of being used successfully as a cell culture substrate for the Vero cell line. The study also revealed that by the 48 h period the number of viable cells on the two nano-porous membranes had started to level out and mirror the glass control, see Figure 6(b). This levelling out characterized the formation of a confluent layer over the surface of the respective nano-porous membranes and glass control. Both optical microscopy and FESEM analysis of types of nano-porous membranes has retained histological characteristics consistent with the glass control and the cell proliferation rates on each membrane result in a confluent layer. In the case of the HAP composites the cell proliferation rates were much lower than their respective untreated membranes. The reduced cell numbers on both composite membranes has indicated that the Vero cell line is not as responsive to the presence of HAP in the composite. This is not too surprising since HAP is the main inorganic component of natural bone tissue and is involved in bone remodelling by bone cells. The significance of this study in respect to the HAP composites is that it has confirmed the biocompatibility of non-bone related cells to this new composite. The results of both the cell adhesion and cell proliferation assays have confirmed that the Vero epithelial cell line can attach grow and colonize nano-porous membranes composed of aluminium oxide and their respective HAP composites. The studies have also confirmed that a non-bone related cell line can survive on HAP, a material normally associated with bone cells. This result confirms a tissue scaffold composed of a nano-porous aluminium oxide/HAP composite can be used without any detrimental effects on the Vero cell line. With the only difference between the HAP composites and the untreated nano-porous membranes being lower cell proliferation numbers over the 72 h assay period.

4. Conclusion

The importance of this preliminary study in progressing potential tissue engineering applications using AAO membranes and composites incorporating HAP is that it has established the biomedical potential for culturing the African green monkey Kidney (Vero) epithelial cell line. All membranes and composite membranes were used without any further surface modification and showed no cytotoxic effects over the 72 h cell proliferation assay. Microscopy investigations revealed that cells attached to both membrane types and their respective HAP composites. Examination of the microscopy images revealed the presence of numerous microvilli over the surface of the cells and the presence of filopodia which were clearly seen anchoring the cells to the underlining substrate surface. The number of viable cells at

the end of the 72 h cell proliferation assay revealed that the in-house AAO membrane and the commercially available membrane had viable cell numbers greater than the glass control. The number of viable cells on the HAP composites was significantly less than their respective non-HAP equivalents and the glass control. However, the 72 h assay did confirm that the HAP composites were biocompatible and could sustain cell adhesion and proliferation.

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