The hemocompatibility and the reabsorption function of TiO₂ nanotubes biomembranes

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A novel high-intensity TiO₂ nanotubes array membrane was fabricated via electrochemical anodization of highly pure titanium foil and an open-ended TiO₂ nanotubes array membrane was obtained by HF gas etching at the bottom of the nanotubes. Pig tubular epithelial cells (LLC-PK1) and vascular endothelial cells (ECV304) were cultivated on the open-ended TiO₂ nanotubes surface through a mixing implantation method and TiO₂ nanotubes biomembrane materials with physiological function were successfully produced. Hemocompatibility of glass slides, pure titanium, TiO₂ nanotubes without cells and TiO₂ nanotubes with culture cells were investigated by the plasma recalcification time method and reabsorption of sodium and potassium were measured by custom-designed devices. The results show that the hemocompatibility of the TiO₂ nanotubes array membrane with culture cells was superior to the control group, and the biomembrane has an excellent reabsorption function. This demonstrates that a TiO₂ nanotubes array membrane has excellent physiological function and is an ideal candidate material for biological dialysis.

TiO₂ nanotube arrays, hemocompatibility, reabsorption function, biomembrane

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In recent years, global morbidity rates of chronic kidney disease have gradually increased and account for nearly 10% of the world population. Thus, it follows heart, cerebrovascular disease, tumor and glycaemia as another fatal disease imperiling human health. Treatment of end-stage renal disease patients by renal transplant is hindered by a shortage of donor organs and traditional hemodialysis is still the most effective treatment regimen. However, despite the fact that this treatment can prolong the lives of end-stage renal failure patients, it is expensive, inconvenient, and confers significant morbidity and mortality [1]. Further, although conventional hemodialysis therapy may replace hemofiltration function, it does not perform the reabsorption, excretion, metabolic and endocrine functions of renal tubules. Studies have shown that these serious complications are the main cause of death [2].

At the end of the last century, Humes HD's research

group was the first to use MDCK (Madin-Darby canine kidney epithelial cells) to structure the Bioartificial renal tubule assist device and preliminary studies showed that it could realize renal tubule function [3,4]. Over the next few years, scientists confirmed the method of RAD (bioartificial renal tubule assist device) construction and perfected the RAD's renal tubule function through extensive experiments and clinical trials [5–7]. In China, Wangxiaoyun's Research Group [8] adopted the LLC-PK1 cell line to structure RAD and measured its function, with results showing that RAD possesses the reabsorption function. Chengxiangmei's Research Group [9,10] adopted the method of mixed cells planting to structure a bioartificial renal tubule, with preliminary results indicating that ECV304-hNanog and HKC-hNanog can adhere and proliferate in polysulfone fibres, and that these two types of cell can express their own physiology functions. Thus, it was demonstrated that the construction of multifunction bioartificial nephrons by mixed cells planting is feasible. This provides a new idea for de-

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veloping miniaturized and multifunctional artificial kidneys.

Currently, despite significant progress in research into biological artificial renal devices, dialysis efficiency is not perfect because of limitations in membrane materials. In the ideal situation, an artificial kidney depends on capillary pressure for the ultrafiltration driving force. To meet this requirement, the pore density of a dialysis membrane should reach $5 \times 10^{10}/\text{cm}^2$ [11]. Traditional membranes, such as cellulose and polymer membranes, are typically produced by extrusion and solvent casting techniques [12], leading to the maldistribution of pore sizes and low pore density ($< 10^9/\text{cm}^2$), which causes poor penetrability of substances and the transportation of species such as β_2 -microglobulin. A membrane with uniform pores can reduce resistance to fluid flow while maintaining molecular selectivity, compared to poly-disperse porous materials. Also, the control of pore geometry can potentially provide novel opportunities to optimize molecular transport rates [13].

With the development of nanoscience and technology, nanoscale materials have gradually become available for improving biomedical devices. For instance, Roy's research group [13] used nanoporous silicon membranes as a dialysis membrane to construct of an implantable bioartificial kidney and obtained favorable results. A TiO_2 nanotube membrane is an ideal candidate material for biomedical applications because of its superior biocompatibility and uniform pores. Recent studies suggest that titanium dioxide nanotube arrays may be a promising candidate for osteocyte and stem cells as well as vascular implants [14]. According to research on adsorption of key blood proteins, adhesion and activation of platelets and whole blood clotting kinetics, TiO_2 nanotubes have excellent biocompatibility [15]. In addition, these nanotubes surface properties elicit low levels of monocyte activation and cytokine secretion and improve osteoblast attachment, function, and proliferation [16,17]. Studies with vascular cells suggest that TiO_2 nanotubes may enhance endothelial cells motility, proliferation, and function, while decreasing vascular smooth muscle cells proliferation [18]. The nanotube density of a TiO_2 nanotubes array membrane made by the electrochemical anodic oxidation method can approach more than $10^{10}/\text{cm}^2$, and they are easy and simple to handle. We can obtain membranes with different diameters and thickness [19–26] and can produce an open-ended TiO_2 nanotube membrane via opening the bottom of the nanotubes by appropriate processing methods [27]. Paulose's Research Group [28] fabricated titania nanotube arrays over 1000 μm in length by an anodic oxidation method and measured the permeability. To date however, studies on the TiO_2 nanotubes membrane with physiological function using the mixed cell planting method are not available. Our group has researched the effect of surface characteristics of TiO_2 nanotube arrays on in early stage porcine renal tubular epithelial cell growth in the early stage [29]. On that basis, we continue to research the hemocompatibility and physiological function of membranes after

culturing cells. The results are expected to provide an experimental basis for constructing bioartificial kidney units with physiological function in the future.

1 Materials and methods

1.1 Fabrication of TiO_2 nanotube membrane

Prior to anodization, to remove surface mechanical scratches, titanium foil samples (0.5 mm thick, 99.8% purity, 10 mm $\times$ 80 mm) were progressively polished using abrasive papers (600#, 800#, 1000#, 1200#, metallographic) and then ultrasonicated for 10 min with acetone, distilled water and ethanol, and blow-dried under N_2 .

The electrolyte was prepared by mixing glycol (100 mL) with distilled water (2 mL) and NH_4F (0.32 g). Anodization was performed with a two-electrode configuration with titanium foil as the working electrode and platinum as the counter electrode under 56 V at room temperature for 30 h.

1.2 Open-ended TiO_2 nanotube membrane fabrication

TiO_2 nanotube membranes were detached from reactive Ti foil by ultrasound in ethanol for 80 s. The TiO_2 nanotube membrane was covered with nanowires due to the long period of anodic oxidation. Due to the inner stress, these nanowire fibers on the surface form coherently, resulting in blocking of the surface. In order to remove these nanowire fibers, we placed the prepared membrane in ethanol and ultrasonicated it for several minutes [20,22]. Ultrasonication was also convenient for detaching the membrane from the Ti foil in entirety. The membrane was then placed in a beaker and ultrasound continued in distilled water with a miniwatt ultrasonic device. The membrane was immersed in ethanol after ultrasound to prevent it from breaking. An open-ended TiO_2 nanotube membrane was obtained by etching the closed bottom of the TiO_2 nanotube membrane with hydrofluoric vapor. The etching device is shown in Figure 1. 40% HF was placed in a plastic container and a metal net placed on the container opening with a distance of

Figure 1 Schematic of process for etching the bottom of the TiO_2 nanotubes membrane.

2–3 cm between the HF and the metal net. The TiO₂ nanotube membrane was placed on the metal net and etched for about 15 min. The etched membrane was then placed in distilled water to rinse for 10 min and after rinsing, was dehydrated with ethanol and placed in hexamethyl disilazane for 20 min to prevent it from breaking. It was then removed and dried in air.

1.3 Cell culture and passage

Porcine renal proximal tubule cell lines (LLC-PK₁) were obtained from the Cell Resource Center, IBMS (Institute of Basic Medical Sciences), CAMS (Chinese Academy of Medical Sciences)/PUMC (Peking Union Medical College), and cultured in M199 with 3% FBS (Fetal bovine serum), 100 U/mL penicillin and streptomycin. Human umbilical vein endothelial cells were obtained from the Center for Culture Collection in Wuhan University, China.

The culture dish was placed under a phase-contrast microscope to observe the growth of cells, and passage was carried out when the cells covered 90% of the bottom of the culture dish. The old medium was washed away by pipettor, and PBS used to rinse it once or twice. The 2 mL of TE (0.25% Trypsin-0.02% EDTA) were then added to the culture dish and it was placed in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. The culture dish was removed and gently shaken after 5 min and observed under the phase-contrast microscope. The 3 mL of medium was added immediately if the majority of cells appeared to be in contraction and roundout. The cell suspension was moved to a centrifuge tube and centrifuged at 1000 r/min for 5 min, and the supernatant removed and passaged with a 1:4 ratio. The growth medium was exchanged every 2–3 days.

1.4 Culture cells on materials

The prepared TiO₂ nanotube membrane was placed into a 24-well plate and 1 mL cell suspension added to each well ($5 \times 10^4/\text{cm}^2$). It was then placed in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. The medium was changed every two days. Culture time was set according to the content of the experiment.

1.5 Plasma recalcification time

A uniformly sized glass slide was prepared with a titanium and TiO₂ nanotube membrane, prior to each experiment and immersed in ethanol for 10 min, then rinsed with distilled water and dried in air. About 0.1 mL of plasma and 0.1 mL of a 0.025 mol/L aqueous solution of CaCl₂ were mixed uniformly. The 20 μL of the mixed liquor was introduced onto the glass slide, titanium, TiO₂ nanotube membrane and TiO₂ nanotube membrane containing culture cells after 3 d. The plasma solution was monitored for clotting by manually dipping a stainless steel wire hook into the plasma solution

to detect fibrin threads. The clotting time was recorded as the first sign of fibrin formation on the hook [30]. At least three experiments were carried out on each substrate surface and the plasma recalcification time reported was the mean value.

About 20 μL of the mixed liquor was introduced onto the TiO₂ nanotube membrane without cells and the TiO₂ nanotube membrane with culture cells after 3 d. Scanning electron microscope samples were made after water was evaporated, and images were taken on a Sirion Scanning Electron Microscope (SEM) at 5 kV after the surface was sputter coated in gold.

1.6 Reabsorption of sodium and potassium

The materials were placed in a 24-well plate, and 1 mL cell suspension ($5 \times 10^4/\text{cm}^2$) was added to each well and medium added to the blank group. Individual cell suspensions comprising ECV304 cells, LLC-PK₁ cells, LLC-PK₁ cells and ECV304 cells were mixed in equal volumes and then placed in the 24-well plate in a humidified incubator in an atmosphere of 5% CO₂ at 37°C.

The membrane was fixed to the aluminum plate with holes and then put into custom made devices (as shown in Figure 2) with the surface with cells oriented upwards. A perfusion solution was infused into the top and bottom space countercurrently at a rate of 2.5 mL/min. After the plasma had circulated once through the system, 1 mL samples were taken from the bottom reservoir as the initial concentration. After 1 h, 1 mL samples were taken from the bottom reservoir as samples, and the concentration of sodium and potassium were analyzed.

To demonstrate that biomembranes had reabsorption function, ouabain (0.1 mmol/L) was added to the perfusion solution and the solute concentration analyzed. The concentration of sodium and potassium were measured by atomic absorption spectrophotometer. At least three experiments were carried out on each substrate surface and the reported plasma recalcification time was the mean value.

The net movement of substances was calculated as

Net movement of the substance = adjacent ions concen-

Figure 2 Schematic of reabsorption of sodium and potassium device. 1, Top inlet; 2, top outlet; 3, bottom inlet; 4, bottom outlet; 5, base plate of device; 6, silicone rubber slice; 7, biomembrane.

tration difference $\times$ solution volume.

2 Results

2.1 Surface characterization of titania nanotube membrane

Figure 3 shows the FE-SEM images of the titania nanotube membrane made by the anodic oxidation method, where Figure 3(a) is the top surface of the TiO_2 nanotube membranes detached from Ti foil by ultrasound. As shown in the figure, the surface of the TiO_2 nanotube membrane retained a slight cover of nanowire fiber fragments. Residual fragments were removed after ultrasound by miniwatt ultrasound. Ultimately, we obtained a TiO_2 nanotube array that was vertically oriented, with a uniform diameter and the surface was glossy and smooth. Figure 3(b) shows the side view of the TiO_2 nanotube membrane. It can be seen that the length of the nanotubes were about $150\ \mu\text{m}$ when anodized for 30 h under 56 V. Figure 3(c) shows the bottom of the TiO_2 nanotube membrane. The bottom of the film were hexagonally sealed with closely spaced and regular, dense block layers taking the shape from the hexagonal seal. On the block layer, there was a TiO_2 nanotube array with uniform distribution, perpendicular to the substrate.

Because of the TiO_2 nanotube array formation from Ti foil substrate, the shape of the single TiO_2 nanotube was similar to a test tube, with the top having an opening and bottom being closed. If the bottom is not open, substances

will not pass through the membrane, and we cannot detect the function of permeability and reabsorption. Therefore, to detect the permeability function, we adopted HF vapor etching of the bottom layer of the TiO_2 nanotubes. Figure 3(d) shows the back side of the TiO_2 nanotube after etching by HF vapor. As shown in the figure, after 15 min etching, the bottom of the TiO_2 nanotubes was essentially open, producing an open-ended TiO_2 nanotube membrane.

2.2 Biocompatibility of the TiO_2 nanotube membrane

When blood is in contact with a foreign surface, coagulation will take place, leading to thrombogenesis and a foreign body response. Measurement of plasma recalcification time is a common way to test *in vitro* static clotting time. When Ca^{2+} is in contact with an anticoagulated platelet-poor plasma test system, prothrombin is activated by the endogenous blood coagulation system and gives rise to thrombin. Thrombin, in turn, will initiate insoluble fibrin formation from fibrinogen. The duration of the process is recorded as the plasma recalcification time [31].

Figure 4 shows the plasma recalcification times of the glass slide, titanium and TiO_2 nanotube membrane were 7, 11, and 28 min, respectively, and there was no obvious plasma recalcification phenomenon on the TiO_2 nanotube membrane with culture cells. Titanium has high biocompatibility, so its plasma recalcification time was longer than the glass slide. Plasma recalcification time of the TiO_2 nanotube membrane was clearly longer than for titanium,

Figure 3 FE-SEM images of TiO_2 nanotubes array membrane. (a) Top surface; (b) cross-section; (c) back side; (d) back side after etching.

Figure 4 Plasma recalcification time for each group.

indicating that the TiO₂ nanotube membrane has superior hemocompatibility. When ECV304 cells were cultured on the TiO₂ nanotube membrane, there was no plasma recalcification throughout the experiment, which ended when all the water in the plasma was evaporated. ECV304 cells have multifold functions in anticoagulation, synthesizing and secreting renal substances. The plasma recalcification time of the TiO₂ nanotube membrane with cells was far longer than in the other three samples, indicating that ECV304 cells could confer excellent anticoagulation properties to the TiO₂ nanotube membrane, and illustrating that ECV304 cells could grow on the TiO₂ nanotube membrane and perform its biological function well.

Figure 5 shows the FE-SEM images of plasma recalcification samples. Figure 5(a) shows the TiO₂ nanotube membrane without cells; Figure 5(b) shows the TiO₂ nanotube membrane with cultured cells after 3 d. As shown in the figure, there was some agglomerated substance on the TiO₂ nanotube membrane without cells, which was fibrin formed from fibrinogen. There was no agglomerate substance on the TiO₂ nanotube membrane with cells, and it appeared to be smooth in the images. There was evidence of a small surface mark but this was not fibrin and may have been a shrinkage crack formed when the plasma was dried during

the experiment.

2.3 Reabsorption of sodium and potassium

To detect the resorption function of the TiO₂ nanotubes biomembrane, we compared the LLC-PK₁ cells group, mixed group and blank group, respectively. Figure 6(a) shows the reabsorption of sodium by the single LLC-PK₁ cells group, mixed cells group and blank group were 6.3, 5.1, and 1.3 mg/cm², respectively, and the single LLC-PK₁ cells group and mixed cells group were obviously higher than the blank group. The reason the reabsorption by the mixed cells group was less than the single LLC-PK₁ cells group should relate to the LLC-PK₁ cell quantities. The cell quantities of the two groups were identical when constructing the biomembranes, so the amount of LLC-PK₁ cells in the mixed group was half that in the single group. The function of sodium reabsorption is performed mainly by LLC-PK₁ cells and, as a result, the reabsorption of the mixed cells group was lower than in the single LLC-PK₁ cells group. When ouabain was added to the perfusion solution, reabsorption of sodium in the single LLC-PK₁ cells and mixed cells groups significantly decreased to 1.3 mg/cm², almost equal to the blank group. There was no change in the blank group. Ouabain, an inhibitor of Na⁺, K⁺-ATPase, can restrain LLC-PK₁ cell function. Because there were no cells in the blank group, there was no influence when ouabain was added to the perfusion solution. Sodium reabsorption sharply decreased in the cells group after addition of ouabain, demonstrating that ouabain inhibited the function of the cells, indicating that transport of sodium was performed by cells, and demonstrating that the TiO₂ nanotube biomembrane has physiological function.

Likewise, the reabsorptions of potassium by the single LLC-PK₁ cells group, mixed cells group and the blank group were 0.5, 0.4, and 0.04 mg/cm², respectively, so the single LLC-PK₁ cells group and mixed cells group were obviously higher than the blank group, and the mixed cells group reabsorption was lower than the single LLC-PK₁ cells group (Figure 6(b)). When ouabain was added to the perfusion

Figure 5 FE-SEM images of plasma recalcification. (a) No cells; (b) culture cells after 3 d.

Figure 6 Sodium and potassium resorption of each biological artificial kidney unit. (a) Sodium; (b) potassium.

solution, reabsorption of potassium by the single LLC-PK₁ cells and mixed cells groups significantly decreased to 0.05 and 0.04 mg/cm², respectively, reaching the level of the blank group. This demonstrates that the biomembrane has excellent function for sodium and potassium resorption.

3 Conclusion

Vertically oriented, uniform diameter TiO₂ nanotube membranes were produced by a simple anodization process, and we could control the parameters of the nanotubes. Open-ended TiO₂ nanotube array membranes were obtained by HF vapor etching with an optimal time of 15 min. A plasma recalcification experiment showed that the TiO₂ nanotube membrane has excellent hemocompatibility and ECV304 cells could grow on the TiO₂ nanotube membrane and displayed anticoagulation functionality. The results of sodium and potassium reabsorption showed that the TiO₂ nanotube biomembrane has excellent reabsorption characteristics and cells could perform their physiological function on the membrane. Reabsorption was significantly increased for the same effective area, compared with the traditional dialyser. The results demonstrate that a TiO₂ nanotubes array membrane is an ideal candidate material for constructing an artificial kidney with physiological function.

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