# Cultivation of hepatoma cell line HepG2 on nanoporous aluminum oxide membranes

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## Abstract

Nanoporous aluminum oxide membranes were prepared by anodic oxidation of aluminum for application as novel cell culture substrates. Self-supporting as well as mechanical stabilized nanoporous membranes were produced from aluminum plates and micro-imprinted aluminum foils, respectively. Membranes of two different pore sizes (70 and 260 nm) were selected to investigate cellular interactions with such nanoporous substrates using cells of hepatoma cell line HepG2. The membranes express excellent cell-growth conditions. As shown by SEM investigations the cells could easily adhere to the membranes and proliferate during a 4 day cell culture period. The cells exhibit normal morphology and were able to penetrate into the pores with a diameter of 260 nm by small extensions (filopodia). On mechanical stabilized aluminum oxide membranes it was observed that the cells even adhere to the walls of the small cavities. With the experiments it was demonstrated that the nanoporous aluminum oxide membranes are well-suited as substrates in cell culture model systems for metabolic, pharmacologic-toxicologic research, tissue engineering and studies on pathogens as well as bioartificial liver systems.

## Keywords

Nanoporous aluminum oxide, Cell culture, HepG2, Scanning electron microscopy

## 1. Introduction

In the last decade nanoporous aluminum oxide has become very attractive in various fields of research [1-5]. Due to anodic oxidation of aluminum in polyprotic acids, a layer of aluminum oxide is originating on the surface. This layer is characterized by parallel pores with orientations perpendicular to the sheet surface, whereas the aspect ratio (pore diameter vs. pore length) can reach values of 1 : 1000 and more. The pore diameter depends mainly on the applied voltage and can be varied between 10 and 450 nm with a narrow size distribution.

Predominantly, porous aluminum oxide is used as an important material in the fabrication of nanostructures. A number of studies were performed in which the nanoporous structure was used as a template in the production of nanowires or nanotubes from different materials, e.g. carbon [6, 7], metals [8, 9] or polymers [10]. Other applications include the usage of the porous membranes as a filter [11]. Beneath these more technical applications the unique properties and structure of nanoporous aluminum oxide also seems beneficial in biomedical fields like tissue engineering. For example, the development of drug delivery systems by immobilization of drugs in the pores with sustained release after implantation into the body could be possible [12]. The adhesion of substances to the pore walls was also used in some biosensor applications [13, 14]. Another approach is to use the filtration capacities of the nanoporous aluminum oxide for purification of DNA [15] or whole cells from blood [16]. Furthermore, alumina is already a well known material mainly in orthopedic surgery or for dental implants. Here it is normally used with smoothed surfaces and has already proven biocompatible [17].

Besides this, recent studies deal with the application of nanoporous aluminum oxide films on orthopedic implants since better cell ingrowth into the pores and consequently a stabilizing of the endoprosthesis is expected [18-20]. Here it was shown, that osteoblasts could adhere and interact with the porous membrane. Thus, it is apparent that nanoporous aluminum oxide is also usable as a cell culture substrate. Due to its special porous characteristic, particularly the establishing of co-cultures, i.e. culturing cells on both sides of the membrane, seems to be a promising application in some fields of tissue engineering. A self-supporting aluminum oxide membrane can act as a physical barrier and so the cultivation of different cells close to each other but without contact is possible. In this way, the cells can communicate only by soluble factors, which diffuse through the pores. As shown with porous polymeric membrane materials (PET, PTFE), such a co-culture set-up can preserve functions for example of liver cells over long periods and sophisticated culture systems can be developed [21, 22]. Nevertheless, compared to the materials used so far, nanoporous aluminum oxide has the great benefit, that the pore diameter is adjustable over a wide range. Consequently the diffusion of mediators through the membrane will be controllable. In addition, as already been shown, the pore size can have a considerable impact on cellular functions [23]. Further on, the membranes are optical transparent, which facilitates daily observations of cell growth and morphology by light microscopy.

In this paper, we report the fabrication of nanoporous aluminum oxide membranes with different pore diameters and their application in cell culturing. Besides, a new preparation process to produce mechanical stabilized nanoporous aluminum oxide membranes is described. Via mechanical prestructuring of aluminum foils the mechanical stability of the resulting nanoporous membranes is improved and thus they could be handled more easily during cell culturing. HepG2 hepatoma cells were

used to investigate cellular behavior and morphology with regard to pore diameter of such nanoporous substrates. The cell line HepG2 is derived from a hepatocellular carcinoma and shows some specific biochemical functions of hepatocytes [24]. Since the HepG2 cells are immortal and resistant to cryopreservation their usage is advantageous compared to primary liver cells in terms of availability, growth activity and quality control. Therefore, cell line HepG2 is an adequate model to evaluate the usability of nanoporous aluminum oxide membranes for conceivable co-culture experiments with primary hepatocytes as a contribution for liver tissue engineering.

## 2. Materials and Methods [*]

### *2.1. Preparation of nanoporous aluminum oxide membranes*

Nanoporous aluminum oxide membranes were formed by anodic oxidation of aluminum sheets in different polyprotic acids, according to [25-29]. Figure 1 shows a schematic diagram of the general production process. In the first step, the aluminum plates (150x100 mm², 99.99 % purity) were electropolished in a solution containing a 1:1 volume ratio of 96 wt.% sulfuric acid and 85 wt.% phosphoric acid at 60 °C and a voltage of 10 V. The duration of polishing depends on the surface smoothness and purity and was 1-2 h in average. After two washing steps in potassium hydroxide solution and distilled water, the aluminum plates were transferred into the anodization bath where they acted as anode. To obtain membranes with different pore diameter, 4 vol.% oxalic acid or 1 vol.% phosphoric acid were used as electrolyte. In the first

[*]Cell culture media and supplements were obtained from C.C.Pro (Oberdorla, Germany). All other chemicals were purchased from VWR International (Darmstadt, Germany), unless otherwise stated.

case, anodization was done over 20 h at a constant voltage of 40 V and a temperature of 8 °C. The anodic oxidation in phosphoric acid was performed over a period of 21 h at 150 V and 7 °C.

The pore diameter depends mainly on the applied anodization voltage. Some correlations regarding this are described elsewhere [2, 19, 25]. Briefly, there is a nearly linear relationship between applied voltage and pore size. The slope depends mainly on the electrolyte and its temperature. By varying voltage and electrolyte type and/or concentration, membranes with defined pore diameter could be fabricated.

At the end of the anodization process the nanoporous membranes had to be lifted off the remaining aluminum sheets. This was initiated by reducing the voltage in defined steps [30]. The disrupting of the membrane from the aluminum sheet was assisted by the production of hydrogen on the pore grounds. Due to the accumulation of gas bubbles at the interface of the porous membrane and the aluminum, the membrane could be easily detached from the underlying substrate. After this preparation step, the produced self-supporting nanoporous membranes had no open pores at both sides. To achieve open porosity, the membranes were finally etched in 5 vol.% phosphoric acid at room temperature and were subsequently cleaned in distilled water. For the investigations described in the following, two representative membranes, referred to as membrane 1 and 2, were selected as depicted in Table 1. Membrane 1 had a pore diameter of 76 nm, whereas membrane 2 had a pore diameter of 263 nm.

By the described preparation process, membranes with areas of 50 $cm^2$ and more were achieved. To establish a defined seeding area for cells, the nanoporous membranes were cut with an Nd-YAG-Laser at a wavelength of 1064 nm into round pieces with a diameter of 12.5 mm. Prior to cell seeding the membranes were

cleaned in distilled water and sterilized with ultraviolet light (UV) in a laminar flow box during an one hour period.

The thickness of the resulting nanoporous membrane is mainly influenced by the anodization time [25]. Due to the application of the described preparation process a minimal membrane thickness of about 45 µm was feasible. Because of their brittleness, thinner membranes are not suited very well for cell cultivation. However, in terms of using nanoporous aluminum oxide membranes as a substrate for co-culture applications, a smaller thickness would be preferable because of better diffusion conditions for molecules across the membrane. Thus, we developed a concept for improving the mechanical stability of nanoporous aluminum oxide membranes with thicknesses less than 45 µm.

### *2.2. Preparation of mechanical stabilized nanoporous aluminum oxide membranes*

By anodizing aluminum, the formation of the nanoporous oxide layer follows exactly the profile of the aluminum surface. Hence, aluminum oxide membranes on a structured aluminum substrate can be produced [31]. Further on, it is possible to create nanoporous aluminum oxide with thinner free-standing areas on a supporting aluminum foil [32].

In our experiments prestructuring of the aluminum foil was done by thermo-mechanical stamping. The stamping was performed on a 200 µm thick aluminum foil (diameter 35 mm, 99.99 % purity) using a special stainless steel stamp. On this stamp, an array of rectangular features (1x1 mm², height 180 µm) with a spacing of 1.5 mm was fabricated by milling. Therewith an arrangement of rectangular cavities

could be produced on the surface of the aluminum foils. The prestructuring was done at a temperature of 220 °C, a force of 10 kN and a duration of 15 minutes using a universal testing machine (Zwick 1446, Zwick, Germany). Subsequent to the surface structuring, an anodization process similar to that described above was carried out.

In the electropolishing step it was possible to affect the thickness of the whole aluminum foil and consequently the thickness of the nanoporous aluminum oxide layer on the ground of the cavities. Due to electropolishing, an average maximal thickness of approximately 165 µm within the unstamped area and an average minimum thickness of 28 µm within the produced cavities were achieved. Afterwards, the foils were fixed in a special specimen holder in the anodization bath in such a way, that only the structured side was exposed to the electrolyte. Anodization was done at a constant voltage of 40 V in 4 vol.% oxalic acid solution at 5 °C or at 150 V in 2 vol.% phosphoric acid at 9 °C. In both cases the process was stopped after 10 hours. Hence, the ground of the stamped areas consisted of nanoporous aluminum oxide, whereas in the unstamped regions a layer of porous oxide laid on the supporting bulk aluminum (Fig. 2).

In comparison to the classical anodization process the separation of the nanoporous membrane from the aluminum substrate is not necessary anymore. So the membranes were removed from the holder, etched in 5 vol.% phosphoric acid and finally were cleaned with distilled water. Cutting of the membranes was not required because the seeding area for cell culturing was given by the dimensions of the foil. Prior to cell seeding the slices were sterilized by UV in the same way described above. In the following, the used mechanical stabilized membranes were denoted as membrane 3 and 4, respectively (Table 1). Membrane 3 had a pore diameter of 63 nm, whereas membrane 4 had a pore diameter of 234 nm.

### *2.3. HepG2 cell culture*

HepG2 cells were cultured in RPMI medium supplemented with 10 vol.% fetal calf serum (FCS), 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin, at 37 °C and 5 % $CO_2$ in a humidified incubator. They were grown to confluence in 25 cm² tissue flasks. Subsequently, they were harvested with Trypsin/ EDTA solution in phosphate buffered saline (PBS), collected by centrifugation and resuspended in fresh media supplemented as above. The cells were counted in a Neubauer counting chamber and the cell concentration was calculated. Cells were seeded onto the different membranes placed in a tissue culture dish at a density of $5 \cdot 10^5$ cells/ml. The cells were allowed to attach to the membranes within 120 minutes. Known from our experience, during this time a maximal number of cells could attach to the substrate. Afterwards, the membranes were washed with PBS buffer to remove unattached cells. Fresh RPMI medium was added and the culture was continued up to 4 days. The culture medium was changed every day. Daily cell counting and observation of cell morphology was done with a light microscope (Axiovert 25 CFL, Zeiss, Germany). After the 4 day culture period, the membranes were prepared for scanning electron microscopy (SEM).

### *2.4. Preparation for SEM investigations*

Investigations of the pore geometry and cell morphology were done by high resolution scanning electron microscopy (HR-SEM) using a JEOL SEM (JSM 6700). Prior to SEM investigations the samples were prepared as follows.

At the end of the culture period the cells on the nanoporous aluminum oxide membranes were fixed with 2 vol.% glutardialdehyde (Sigma-Aldrich, Taufkirchen, Germany) in PBS over 2 hours. The cells on the self-supporting membranes were then stained in 1 vol.% osmium tetroxide in PBS for 45 minutes. Thereafter they were dehydrated through a series of acetone concentrations (10, 30, 50, 70, 90 and 100 vol.%) for 10 minutes, respectively. Final desiccation was done using a critical point dryer (CPD030, BAL-TEC, Liechtenstein). In the case of the structured membranes the staining step with osmium tetroxide was omitted and the dehydration was done in a series of ethanol concentrations (10, 30, 50, 70, 90 and 100 vol.%) for 10 minutes, respectively. Desiccation was carried out with two mixtures of hexamethyldisilazane/ethanol (1:2, 2:1) and pure hexamethyldisilazane for 3 minutes each, followed by air drying.

Finally, the samples were placed on SEM specimen holders. In order to improve the contrast for SEM investigations, the samples were sputter coated with a thin platin layer. Based on SEM pictures, pore diameter and porosity of the nanoporous membranes were determined and quantified by optical image processing (analySIS® 3.1, Soft Imaging System, Germany).

## 3. Results and discussion

### *3.1. Membrane Characterization*

Characterization of nanoporous aluminum oxide membranes was done by evaluation of SEM pictures. Table 1 depicts the pore diameter, porosity and thickness of the different membranes. Thickness was determined by light microscopy and SEM using membrane cross sections.

Due to the different parameters of the anodization processes, two kinds of self-supporting membranes were produced (Fig. 3). The anodic oxidation of aluminum in oxalic acid resulted in pore diameters of 76 nm (Fig. 3A). However, if phosphoric acid was used as electrolyte the pore diameter was in the range of 263 nm (Fig. 3B). SEM pictures of the membrane surfaces reveal a pore arrangement, which was more or less hexagonal.

Preparation parameters of mechanical stabilized aluminum oxide membranes and their properties are also shown in Table 1 (membrane 3 and 4). The pore diameter and porosity are comparable to the self-supporting membranes produced under the same parameters. However, the pore diameters of the mechanical stabilized membranes are somewhat smaller. This is related to slightly different temperature and flow conditions in the special designed specimen holder used to fix the structured alumina foils in the anodization bath. Nevertheless, prestructuring of the aluminum substrate had no influence on the development of the nanoporous aluminum oxide and its pore structure. The nanoporous membrane follows the shape of the underlying substrate, so that even the walls of the cavities are lined by a continuous nanoporous layer (Fig. 4).

### *3.2. Cell morphology on self-supporting nanoporous aluminum oxide membranes*

SEM micrographs of HepG2 cells on the self supporting membranes 1 and 2 are shown in Fig. 5 and 6. As cell counting revealed in both cases, about half of the initial number of seeded cells attach to the membrane. Nevertheless, the cells proliferate during the culture period and cell number was doubled until day 4. However, in order

to correlate pore diameter and cell growth, the experiments will be extended to get statistically proved results.

The overall cell morphology was comparable to cells cultured in normal tissue culture flasks. The cells were distributed homogeneously over the nanoporous substrate. They appeared flat and possessed microvilli (Fig. 5A, 6A). Due to the small pore diameter of membrane 1 (76 nm), the cells could not penetrate into the pores (Fig. 5B). However, if membrane 2 with a pore diameter of 263 nm was used as culture substrate, it was observed, that the cells developed membrane protrusions (filopodia) which were able to penetrate into the pores of the underlying aluminum oxide (Fig. 6B). It seems that the cells use the pores as anchorage points to adhere onto the membrane. As shown in Fig. 7 the small filopodia even stick to the pores after SEM preparation and the possible accompanied shrinking process. In some cases it was observed that they were branched and anchored at two different pores simultaneously resulting in an intensive cell-substrate interaction. Additional studies will explore how the cells adhere to the nanoporous aluminum oxide membrane, i.e. to demonstrate whether the whole cell is spread out on the substrate or if it develops specific adhesion points. It could be noted that the pores were not occluded by any cellular products or ingredients of the culture medium. Thus, a diffusion of nutrients from the backside of the membrane to the cells is possible. This effect could have a positive influence on cell polarity or growth and will also be investigated further.

### *3.3. Cell morphology on mechanical stabilized aluminum oxide membranes*

HepG2 cells seeded on mechanical stabilized aluminum oxide membrane 3 and 4 are shown in Fig. 8 and 9, respectively. Fig. 8 shows an overview of membrane 4 in order to demonstrate the spatial distribution of the cells in and around the cavities.

For both membranes, no difference in cell distribution and cell adhesion was visible at this low magnification. The cells adhered to the entire substrates. They were distributed homogenously and proliferated during the 4 day culture period.

At higher magnification (Fig. 9), the cell adhesion and morphology can be characterized in more detail. The adhesion and morphology of cells on membrane 3 (pore size 63 nm, Fig. 9 A, B) as well as membrane 4 (pore size 234 nm, Fig. 9C, D) was comparable to the results described before. At higher magnification microvilli were observed on the cell surface. Cells cultured on membrane 4 developed filopodia which could penetrate into the pores (diameter 234 nm, Fig. 9D), whereas this was not observed for cells cultured on membrane 3 (pore diameter 63 nm, Fig. 9B). To subsume, the previous findings of cell morphology of HepG2 on the self-supporting aluminum oxide membranes were confirmed. Additionally, the SEM micrographs show that the cells could even adhere to the walls of the cavities at both membranes (Fig. 9A, C). They also assemble in larger clusters following the shape of the cavity edges. Such cavities could provide small culture wells for the seeding of cells [31] and conceivably support the development of functional tissues.

However, in order to ascertain differences in cell growth compared to the self-supporting substrates, more systematic experiments are needed. In this context it has to be considered that there is a possible difference in cell function and cell growth at the bottom of the cavities and on the surrounding nanoporous aluminum oxide layer, respectively. The continuous pores of the aluminum oxide in the prestructured areas result in a supply of nutrients from both cell sides. This could have a beneficial effect on cell functions, especially for polar cells like hepatocytes. However, this is not possible on the remaining substrate because here the pores end on the underlying aluminum. Further investigations of cellular function on both substrate areas could demonstrate this.

By decreasing the features on the stamp, the fabrication of smaller cavities is feasible. This could improve the three dimensional growth of cells in the cavities because the supply of nutrients is provided from both sides of the membrane resulting in smaller diffusion paths. Furthermore the establishing of co-cultures benefits from the thin aluminum oxide membrane on the ground of the cavities.

In the experiments described above, the leakage of aluminum ions from the substrate and their influence on cell behavior was neglected. It has been reported that only non-toxic amounts of aluminum ions leak out from nanoporous aluminum oxide membranes [18]. Further on, it has to be noted, that the supporting aluminum has a natural oxide layer which prevents the liberation of aluminum ions.

## 4. Summary

In this report, we have described the cultivation of hepatoma cell line HepG2 on self-supporting and mechanical stabilized nanoporous aluminum oxide membranes. It was shown that the cells adhere to the membranes and proliferate during the culture. More cell counting data will be determined in order to correlate pore diameter and/or the different production processes of the membranes to cell growth. Results will be given in a forthcoming paper.

As SEM investigations revealed, the cells were distributed homogenously over the entire substrates and aggregate in larger cell clusters. Furthermore, the cells showed a normal morphology and developed microvilli. If aluminum oxide membranes with pore diameters larger than 230 nm were used, the cells develop filopodia which penetrate into the pores, resulting in an intensive cell-substrate interaction. Further work is aimed to investigate the pore diameter at which the penetration of the pores by filopodia starts.

In terms of using thin nanoporous aluminum oxide membranes as substrates for co-cultivation, a concept to enhance their mechanical stability was applied successfully. It was demonstrated that the membrane thickness can be reduced to about 20 µm. The realization of membrane regions with a thickness down to 5 µm seems achievable. Small membrane thicknesses would be preferred in co-culture experiments because of better diffusion conditions of molecules across the membrane. Then, communication of cells between both sides of the membrane can be controlled independently by pore size and membrane thickness. In addition, the optical transparency of the membranes is a significant feature for cell culturing and daily observation of cell morphology.

Altogether, the results presented herein are encouraging to extend the cell culture experiments to more sophisticated cells, e.g. primary hepatocytes. Hence, the unique membrane structure could have substantial benefit in various tissue engineering approaches or in pharmaceutical research.

**Acknowledgement**

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## References


[1] Martin CR. Nanomaterials: a membrane-based synthetic approach. Science 1994;266:1961-1965

[2] Hanaoka TA, Heilmann A, Kröll M, Kormann HP, Sawitowski T, Schmid G, et al. Alumina membranes - template for novel nanocomposites. Appl. Organomet. Chem. 1998;12:367-373

[3] Li AP, Müller F, Birner A, Nielsch K, Gösele U. Hexagonal pore arrays with a 50-420 nm interpore distance formed by self-organization in anodic alumina. J Appl Phys 1998;84:6023-6026

[4] Lee W, Scholz R, Nielsch K, Gösele U. A template-based electrochemical method for the synthesis of multisegmented metallic nanotubes. Angew Chem Int Ed 2005;44:6050-6054

[5] Masuda H, Asoh H, Wabe M, Nishio K, Nakao M, Tamamura T. Square and triangular nanohole array architectures in anodic alumina. Adv Mater 2001;13:189-192

[6] Zhao J, Gao QY, Gu C, Yang Y. Preparation of multi-walled carbon nanotube array electrodes and its electrochemical intercalation behaviour of Li ions. Chem Phys Lett 2002;358:77-82

[7] Lysenkov D, Abbas H, Mu□ller G, Engstler J, Budna KP, Schneider JJ. Electron field emission from carbon nanotubes on porous alumina. J Vac Sci Technol B Microelectron Nanometer Struct 2005;23:809-813

[8] Choi J, Sauer G, Nielsch K, Wehrspohn RB, Gösele U. Hexagonally arranged monodisperse silver nanowires with adjustable diameter and high aspect ratio. Chem Mater 2003;15:776-779

[9] Lahav M, Sehayek T, Vaskevich A, Rubinstein I. Nanoparticle nanotubes. Angew Chem Int Ed 2003;42:5576-5579

[10] Steinhart M, Wendorff JH, Greiner A, Wehrspohn RB, Nielsch K, Schilling J, et al. Polymer nanotubes by wetting of ordered porous templates. Science 2002;296:1997

[11] Yamaguchi A, Uejo F, Yoda T, Uchida T, Tanamura Y, Yamashita T, Teramae N. Self-assembly of a silica-surfactant nanocomposite in a porous alumina membrane. Nat Mater 2004;3:337-341

[12] Sawitowski T. New drug delivery systems - examples of applied nanotechnology. Proc Intern Conf Microtech 2000;1:343-346

[13] Heilmann A, Teuscher N, Kiesow A, Janasek D, Spohn U. Nanoporous aluminum oxide as novel support material for enzyme biosensors. J Nanosci Nanotechnol 2003;3:375-379

[14] Takhistov P. Electrochemical synthesis and impedance characterization of nano-patterned biosensor substrate. Biosens Bioelectron 2004;19:1445-1456

[15] Vlassiouk I, Krasnoslobodtsev A, Smirnov S, Germann M. "Direct" detection and separation of DNA using nanoporous alumina filters. Langmuir 2004;20:9913-9915

[16] Mihailescu GH, Pruneanu S, Neamtu S, Olenic L. Alumina membranes used as molecular filters for human red blood cells and bovine serum albumin. Stud Uni Babes-Bolyai, Phys, Special Issue 2001

[17] Hulbert SF. The use of alumina and zirconia in surgical implants. In: Hench LL, Wilson J, editors. An introduction to bioceramics. Singapore: World Scientific; 1993. p 25-40

[18] Karlsson M, Palsgard E, Wilshaw PR. Initial in vitro interaction of osteoblasts with nano-porous alumina. Biomaterials 2003;24:3039-3046

[19] Briggs EP, Walpore AR, Wilshaw PR, Karlsson M, Palsgard E. Formation of highly adherent nano-porous alumina on Ti-based substrates: a novel bone implant coating. J Mater Sci Mater Med 2004;15:1021-1029

[20] Popat KC, Swan EEL, Mukhatyar V, Chatvanichkul KI, Mor GK, Grimes CA, Desai TA. Influence of nanoporous alumina membranes on long-term osteoblast response. Biomaterials 2005;26:4516-4522

[21] Murakami S, Ijima H, Ono T, Kawakami K. Development of co-culture system of hepatocytes with bone marrow cells for expression and maintenance of hepatic functions. Int J Artif Organs 2004;27:118-126

[22] Endo M, Enosawa S, Ozaki M, Tsuruoka S, Hiramatsu S, Kim K, et al. Artificial mimicking of physiological active transport by a membrane co-cultured with two different cells: Hepatic origin HepG2 and renal origin PCTL-MDR. Artif Organs 2002;26:806-811

[23] Karlsson M, Johansson A, Tang L, Boman M. Nanoporous aluminum oxide affects neutrophil behaviour. Microsc Res Tech 2004;63:259-265

[24] Takagi M, Fukuda N, Yoshida T. Comparison of different hepatocyte cell lines for use in a hybrid artificial liver model. Cytotechnology 1997;24:39-45

[25] O'Sullivan JP, Wood GC. The morphology and mechanism of formation of porous anodic films on aluminum. Proc Royal Soc Lond A 1970;317:511-543.

[26] Masuda H, Fukuda K. Ordered metal nanohole arrays made by a two-step replication of honeycomb structures of anodic alumina. Science 1995;268:1466-1468

[27] Jessensky O, Müller F, Gösele U. Self-organized formation of hexagonal pore structures in anodic alumina. J Electrochem Soc 1998;145:3735-3740

[28] Thompson GE, Furneaux RC, Wood GC, Richardson JA, Goode JS. Nucleation and growth of porous anodic films on aluminium. Nature 1978;272:433-435

[29] Thompson GE, Wood GC. Porous anodic film formation on aluminium. Nature 1981;290:230-232

[30] Rigby WR, Cowieson DR, Davies NC, Furneaux RC. An anodizing process for the production of inorganic microfiltration membranes. Trans. Inst. Met Finish 1990;68:95-98

[31] Swan EEL, Popat KC, Grimes CA, Desai TA. Fabrication and evaluation of nanoporous alumina membranes for osteoblast culture. J Biomed Mater Res A 2005;72:288-295

[32] Heilmann A, Teuscher N, Gumbsch P. Patent pending 30.10.03, DE 103 49 471.5

**List of Legends**

**Figure 1** Schematic diagram of the anodization process

**Table 1** Used membranes and corresponding parameters (*membrane thickness in prestructured areas)

**Figure 2** Scheme of a mechanical stabilized nanoporous aluminum oxide membrane

**Figure 3** Pore size histograms of self-supporting aluminum oxide membrane 1 (A) and membrane 2 (B)

**Figure 4** Cross section of a mechanical stabilized aluminum oxide membrane

**Figure 5** HepG2 cells on self-supporting aluminum oxide membrane 1 (pore diameter 76 ± 10 nm). Overview (A) and magnification of a cell border (B).

**Figure 6** HepG2 cells on self-supporting aluminum oxide membrane 2 (pore diameter 263 ± 29 nm). Overview (A) and magnification of a cell border with filopodia (B).

**Figure 7** SEM micrograph of filopodia endings and their interaction with self-supporting aluminum oxide membrane 2 (pore diameter 263 ± 29 nm).

**Figure 8** HepG2 cells on mechanical stabilized nanoporous aluminum membrane 4 (pore diameter 234 ± 43 nm). Overview of a rectangular cavity.

**Figure 9** HepG2 cells on mechanical stabilized nanoporous aluminum membrane 3 (pore diameter 63 ± 10 nm) and membrane 4 (234 ± 43 nm). Edge of a cavity (A, C) and magnification of a cell border (B, D).

**Fig.1.**

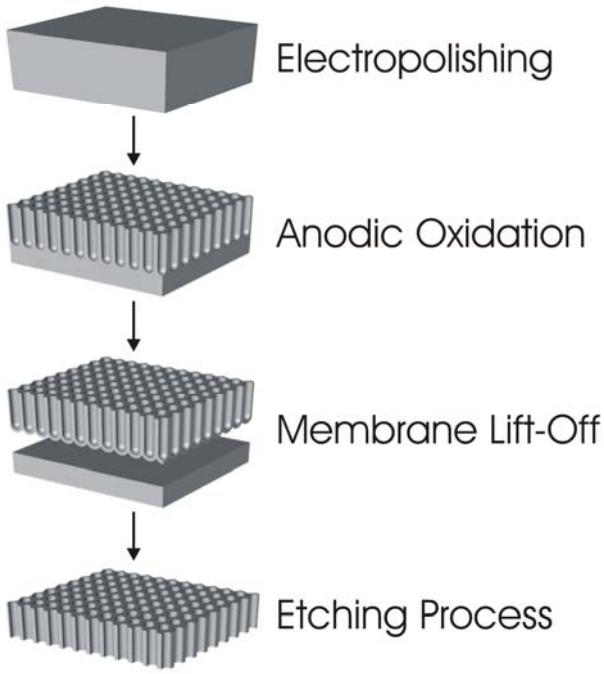


**Table1.**

| Membrane | Type | Electrolyte | Voltage [V] | Pore diameter [nm] | Porosity [%] | Membrane thickness [µm] |
|---|---|---|---|---|---|---|
| 1 | self-supporting | 4 vol.% oxalic acid | 40 | 76 ± 10 | 43 | 44 |
| 2 | self-supporting | 2 vol.% phosphoric acid | 150 | 263 ± 29 | 33 | 48 |
| 3 | mechanical stabilized | 4 vol.% oxalic acid | 40 | 63 ± 10 | 30 | 21* |
| 4 | mechanical stabilized | 2 vol.% phosphoric acid | 150 | 234 ± 43 | 38 | 35* |

**Fig.2.**

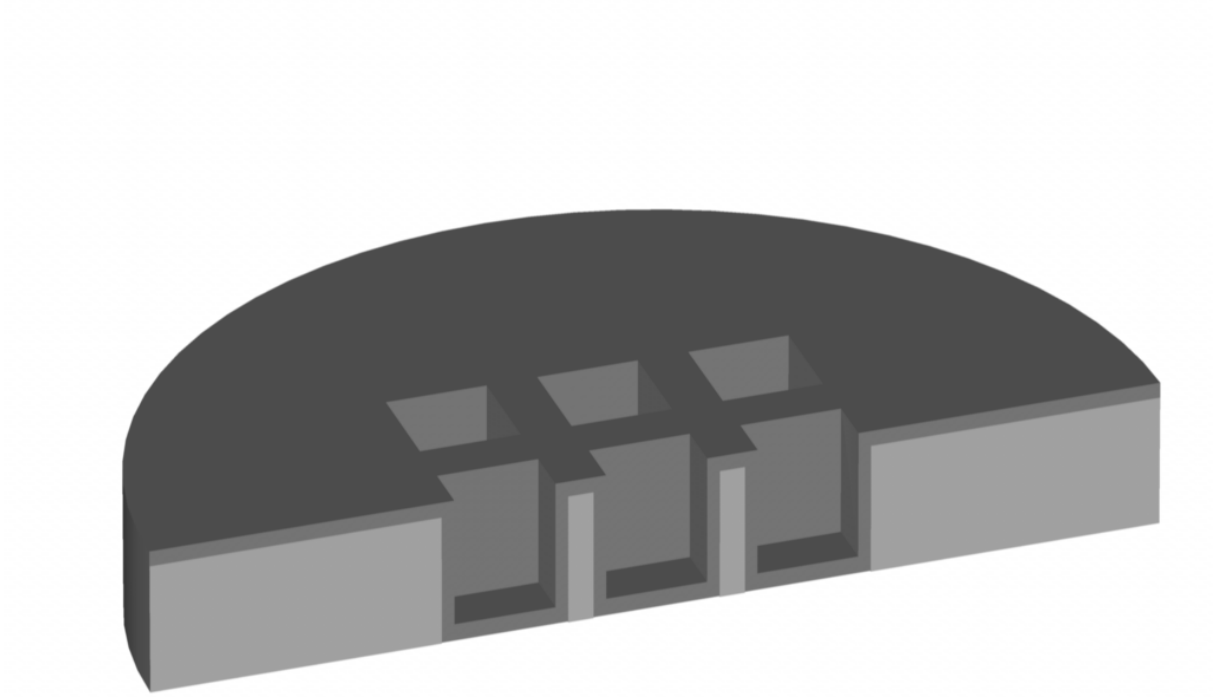

**Fig.3.**

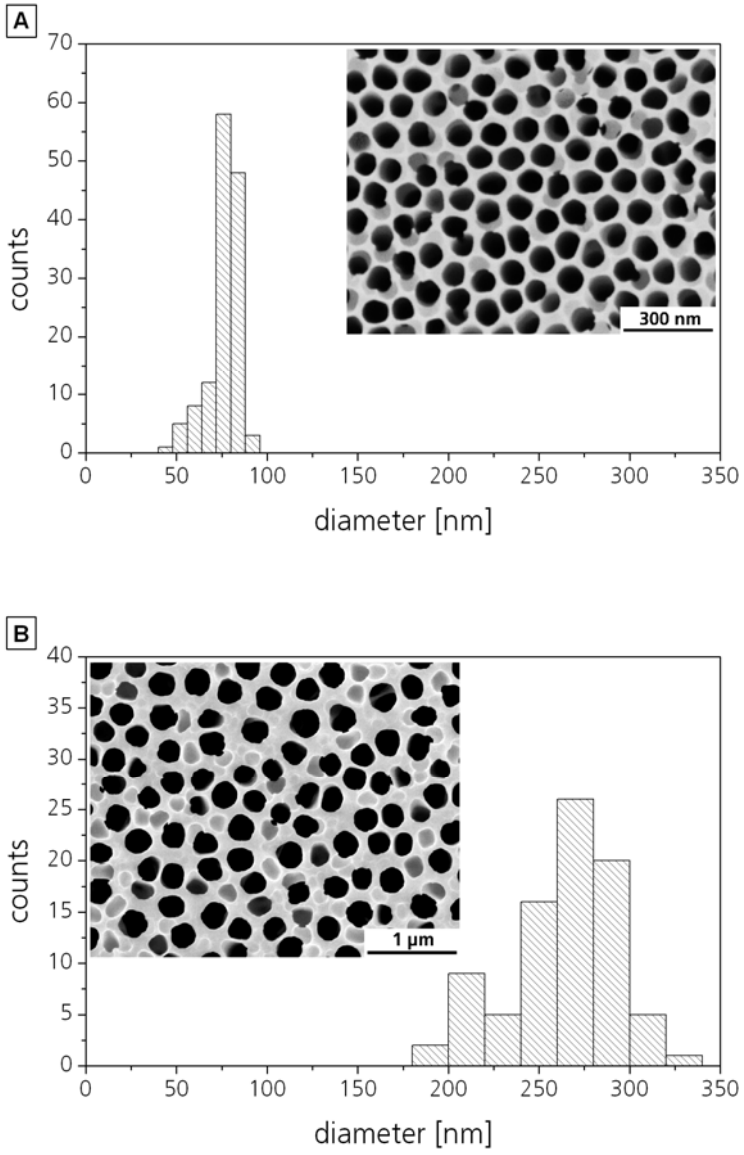
A
counts
diameter [nm]
300 nm
B
counts
diameter [nm]
1 µm

**Fig.4.**

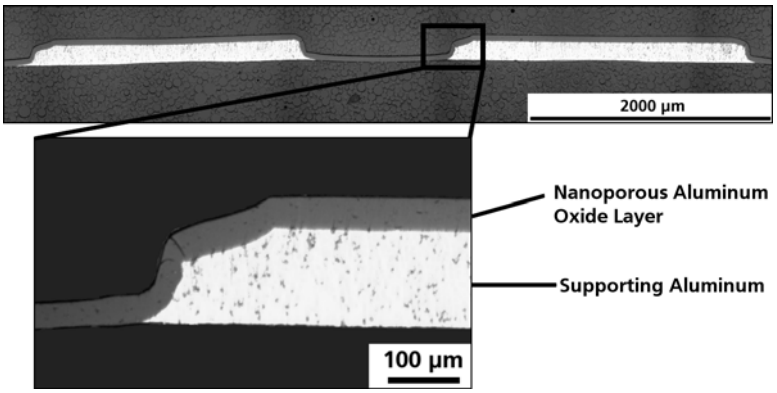
2000 µm
Nanoporous Aluminum Oxide Layer
Supporting Aluminum
100 µm

**Fig.5.**

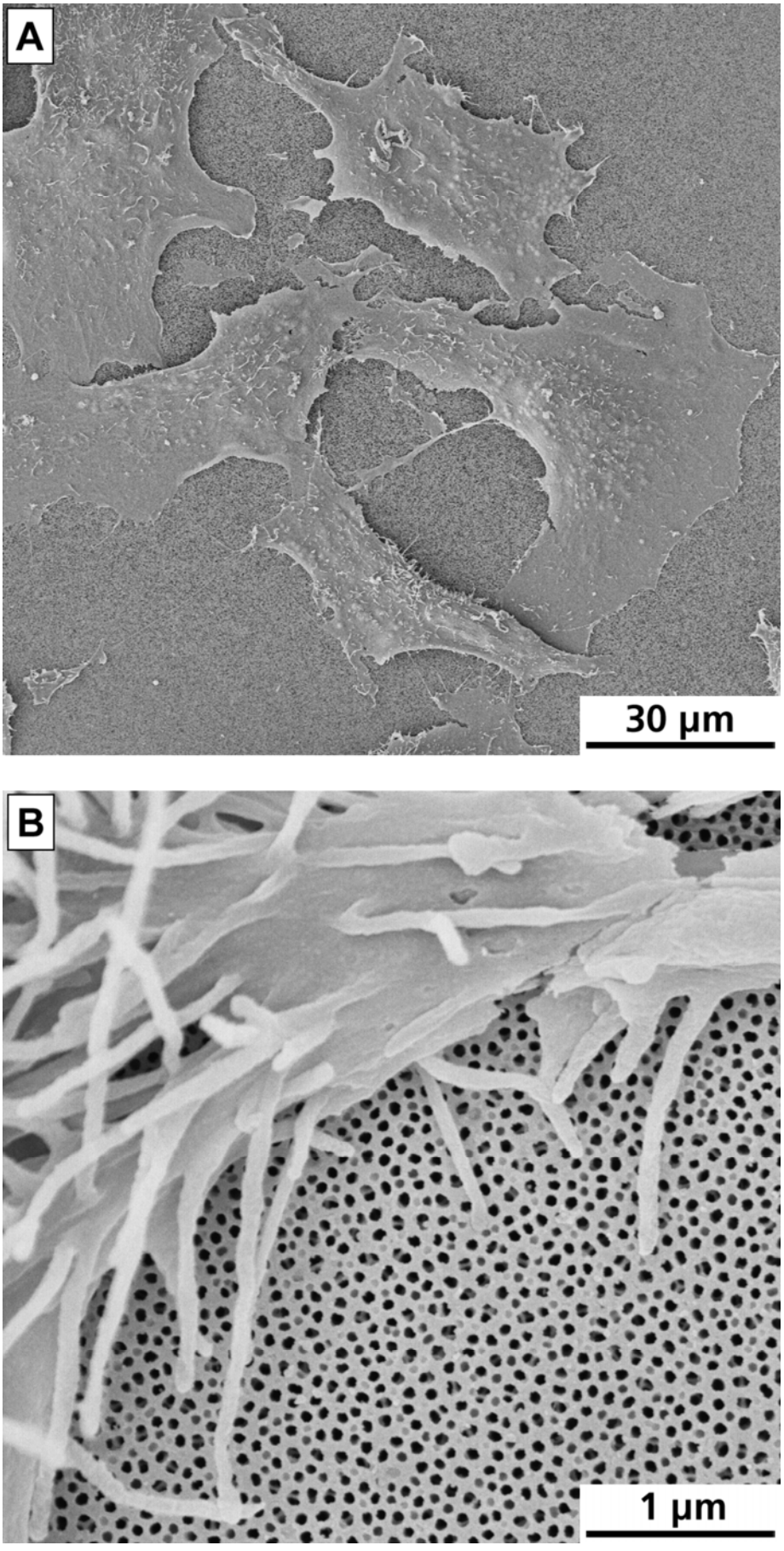

**Fig.6.**

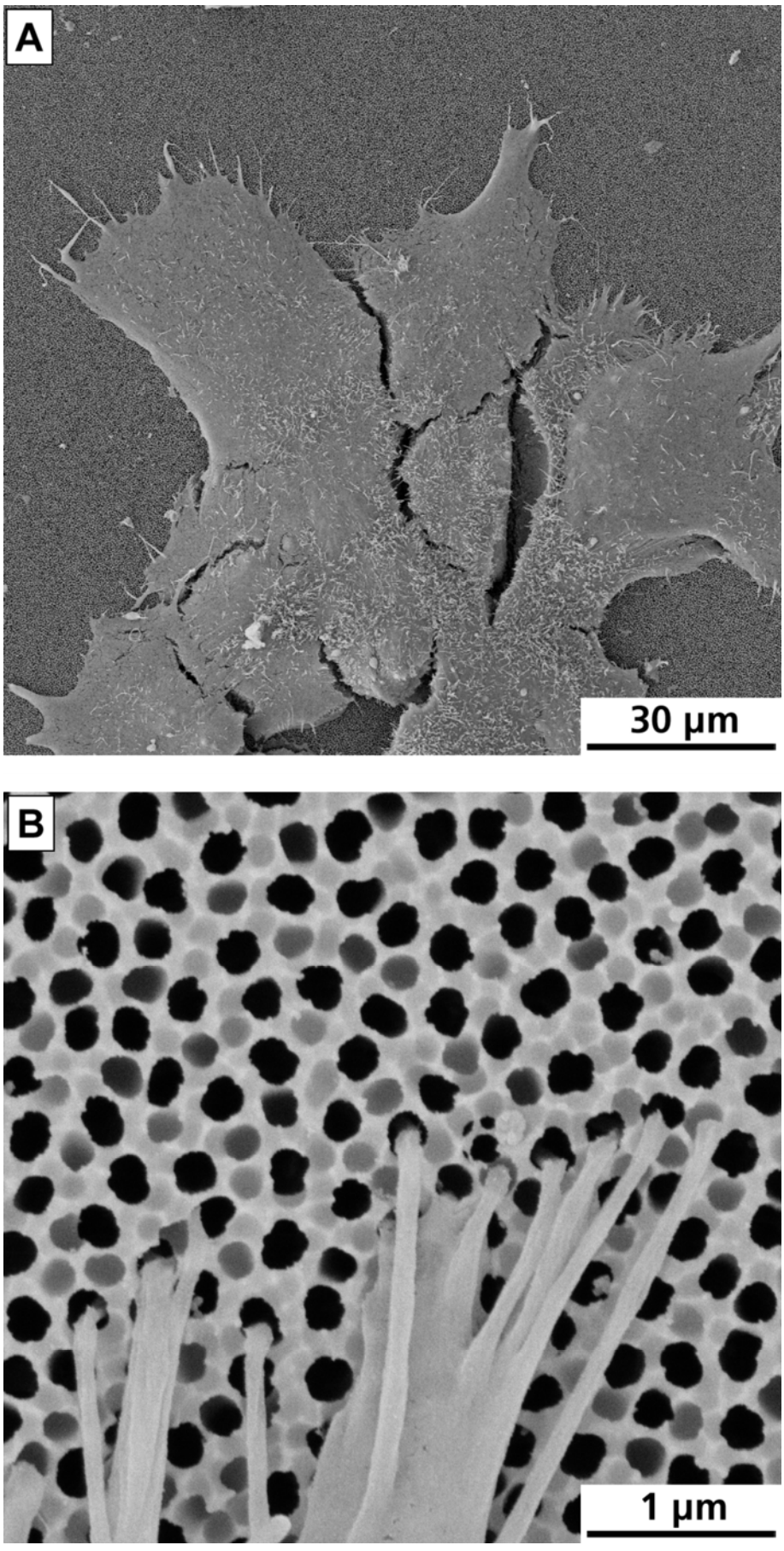

**Fig.7.**

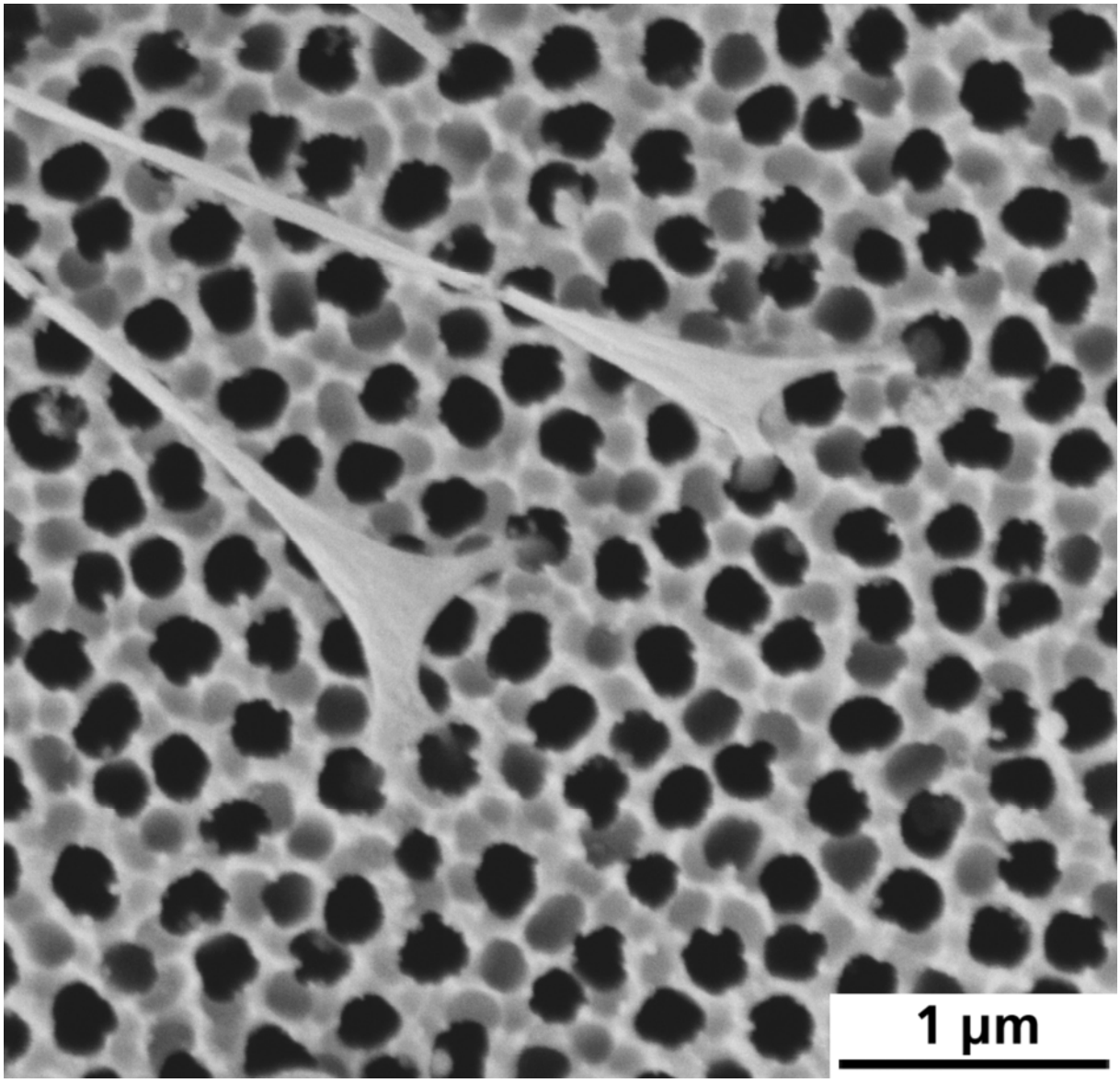

**Fig.8.**

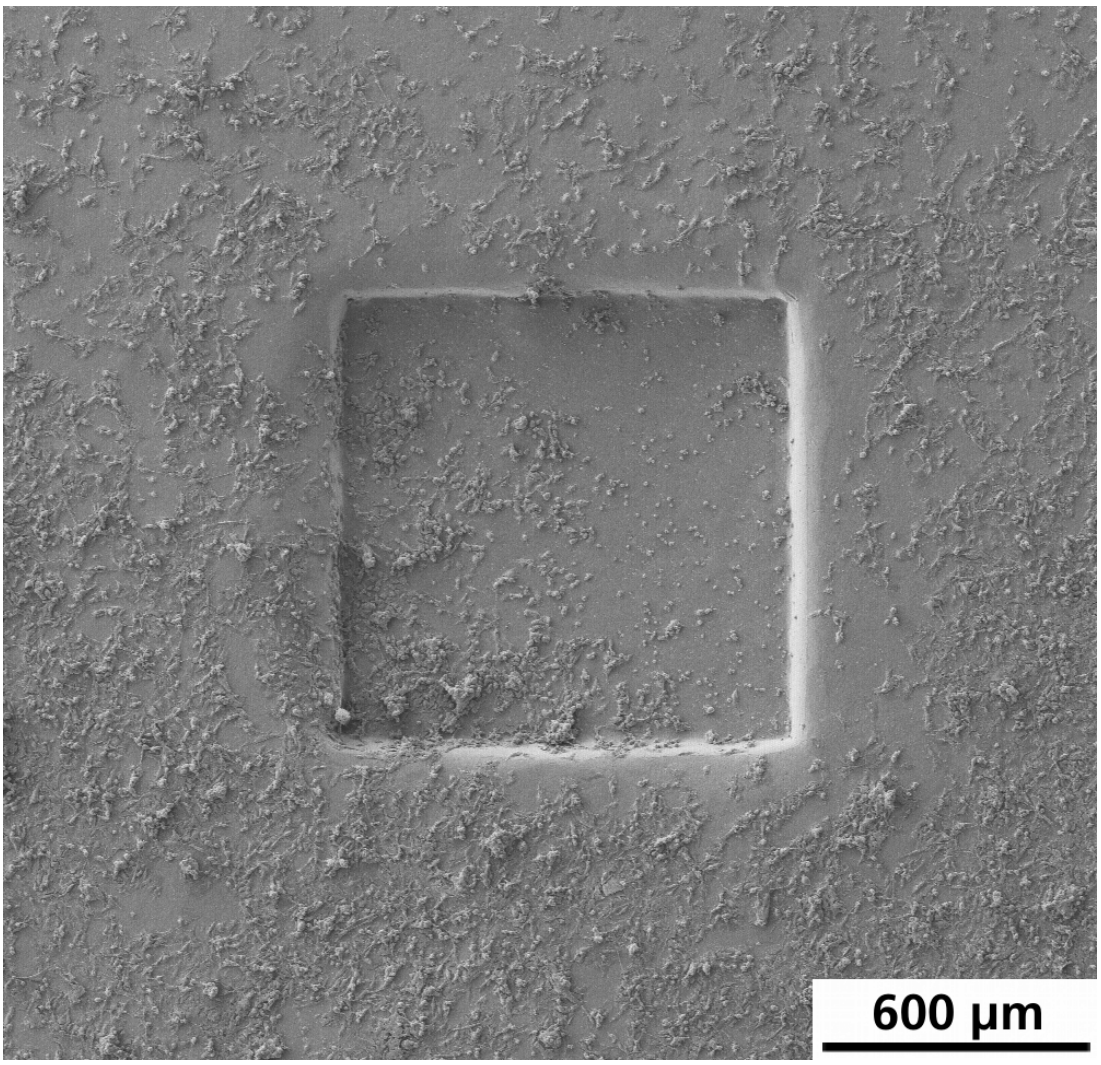

**Fig.9**