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Functionality of MDCK kidney tubular cells on flat polymer membranes for biohybrid kidney

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The prerequisite for the development of a biohybrid artificial kidney, is a substrate for confluent growth of renal cells forming an epithelial monolayer without any leaks. Conventional cell culture supports cannot be adapted for this purpose, because they lack adequate mechanical properties and thermal stability. From two suitable materials, polysulfone and polyacrylonitrile, two permeable polymeric membranes have been produced that were, according to ISO 10993-5, not cytotoxic. Cloned Madin Darby Canine Kidney (MDCK) cells (an established renal cell line) were cultured on the surface of the plastic materials, and on conventional cell culture supports. With all materials, assays of mitochondrial and lactate dyhydrogenases exhibited similar proliferation and the viability of the MDCK cells. Transmission electron microscopy showed the expression of a normal morphology of kidney tubular cells. Perfect barrier function, consequent on the formation of intercellular junctions in a confluent tight epithelium, was visualized in electron micrographs, and quantified by measurement of the transepithelial resistance. The uniformity of the cells grown was demonstrated in samples by electron microscopy and in the whole epithelium by intravital impedance analysis. It was concluded that polymeric membranes produced from polysulfone or polyacrylonitrile are appropriate substrates in the design of biohybrid kidney devices.

1. Introduction

Conventional haemodialysis cannot completely replace the function of the natural organ which may result in negative clinical consequences during the long-term treatment of patients [1, 2]. In addition, shortages in the availability of donor organs for orthotopic liver and kidney transplantation has stimulated the development of biohybrid liver and raised the question of developing a biohybrid kidney employing renal epithelial cells [3, 4]. The biohybrid organ, which incorporates both synthetic materials and living cells, should assume the function of the natural organ.

Materials used as a cell culture substrate in the biohybrid kidney have to meet a number of requirements, such as sufficient transport properties for the exchange of oxygen, nutrients, and waste products, while ensuring isolation of the cells from the host immune system. Moreover, the substrate has to support the attachment and proper function of renal cells. The latter must form an epithelial layer that can separate different extracellular fluid compartments, and can perform passive and active transport functions [5].

Conventional cell culture supports are not suitable, because mechanical fragility and thermal instability render infeasible their long-term use in artificial organs. Hence, we produced polymer membranes of polysulfone (PSU) and polyacrylonitrile (PAN). Owing to their excellent mechanical properties and thermal stability, these materials are often used in artificial kidneys [6]. In this work, we demonstrate the ability of the polymer membranes to support the attachment, proliferation and function of Madin Darby canine kidney (MDCK) renal epithelial cells.

2. Materials and methods

2.1. Materials

Polyacrylonitrile (PAN) and polysulfone (PSU) asymmetric membranes prepared by the phase inversion technique were prepared as flat membranes

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formed by a bicomponent-process, as described previously [7]. Asymmetric membranes, which have different pore diameters on their two sides, were produced because of the analogy to the fine structure of the glomeruli [5]. PAN and PSU are known to elicit only small immunological responses [6].

MDCK cells of clone C7 were plated on PAN or PSU membranes or on Millicell-HA culture plate inserts (Millipore Products Division, Bedford, USA; 0.6 cm^2 surface area). The cells were cultured as described by Gekle *et al.* [8], i.e. in a humidified incubator at 37 °C in air with 5% CO_2 floating in plastic wells which contained Minimum Essential Medium (MEM) with Earle's salts, amino acids, *N*-acetyl-L-alanine-L-glutamine, and 2.2 $g\,l^{-1}$ $NaHCO_3$ (MEM FG0325, Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum, 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin.

2.2. Cytotoxicity assays

PSU and PAN membranes were incubated in 10 ml medium without phenol red for 1, 2, 3 and 7 d. According to the recommendations of ISO 10993-5, the extracts were brought into contact with subconfluent NIH-3T3 fibroblasts. After 24 h incubation, XTT assay (Boehringer Mannheim, Germany), measuring the activity of the mitochondrial dehydrogenases [9, 10], and neutral red assay, estimating the integrity of the cell membrane, were carried out.

2.3. Proliferation assays

Several small chambers, the bottom of which was formed by the different substrate materials (Surface area 0.6 cm^2), were filled with 500 µl of a suspension (80 000 cells ml^{-1}) of MDCK cells. The proliferation was determined by colorimetric assays (Boehringer Mannheim, Germany) after 1, 2, 3, 6 and 9 d. One assay (MTT) measured the activity of mitochondrial dehydrogenases, and the other (LDH) measured that of the cytosolic lactate dehydrogenase.

2.4. Transmission electron microscopy (TEM)

After fixation with 3% glutaraldehyde, the cells were rinsed with cacodylate buffer (pH 7.2) and contrasted with OsO_4 (25% in PBS) applied for 15 min at 4 °C. The samples were rinsed three times with PBS buffer and dehydrated in a graded series of ethanol (70; 80; 90; 96; and three times with 100%). After embedding in Epon, ultrathin sections were made with an Ultracut E Microtom (Reichert-Jung, Germany). Poststaining was carried out with 4% uranylacerate. Then samples were rinsed and dried, stained with lead citrate (5%) and washed again, as before. The examinations were performed with an EM 410 transmission electron microscope (Phillips, The Netherlands).

2.5. Electrophysiology

After 7 d of cultivation, the transepithelial resistance of confluent monolayers was investigated in four-electrode Ussing-type chambers made of acrylic glass. Thus the exposed area was 0.6 cm^2. In all experiments, the temperature was maintained at 37 °C.

Measurement of the transepithelial impedance in the frequency range of 1.3 Hz–65 kHz was performed as described previously [11]. Data were fitted with an electrical model [12], which represented the epithelial cells by a resistor, R_P, in parallel to a capacitor, C_P. Any ohmic non-epithelial contribution was represented by an additional series resistor, R_S. The impedance, Z, of this equivalent circuit is a complex function of R_P, C_P, R_S, and the frequency, f, of the stimulus. In a Nyquist plot, this function forms a semicircle with its center on the abscissa. If a cultured monolayer consists of uniform cells, the impedance locus recorded, fits well to the semicircle [11]. By contrast, if the cells grow heterogeneously, the impedance locus measured deviates from the semicircle of the model [13].

Results are expressed as means $\pm$ S.E.M. Statistical comparisons were performed by a Student's t-test for unpaired variates. Significances are denoted by symbols: $*$, $p < 0.05$, $**$, $p < 0.01$, $***$, $p < 0.001$; ns, not significantly different.

3. Results

Cell viability and proliferation, ultrastructure and electrophysiological properties were investigated in MDCK monolayers grown on the smooth side of PAN or PSU polymer membranes, as well as in MDCK monolayers grown on a standard cell culture support (MC).

3.1. Cell viability and proliferation

Standard cytotoxicity assays, performed with up to 7 d extraction, revealed that the membranes of PSU and PAN were non-toxic. The investigation of MDCK cell proliferation with LDH assay, shown in Fig. 1a, indicated that with all substrates, cell growth reached the plateau phase after 6 d culture. LDH activity was similar for all substrates. It was slightly enhanced in monolayers grown on PAN membranes. Because the quantity of LDH released after lysis of cells is known to be proportional to the cell number, it was concluded that all materials provide sufficient support for establishment of a cellular monolayer on the different membrane surfaces. An assay of mitochondrial dehydrogenases was applied as a further measure of MDCK cell proliferation on the different materials. The results, shown in Fig. 1b, demonstrated that the metabolic activity of cells increased up to day 7 for all types of membranes. While the measured activity remained almost constant during the next 2 d for PSU and PAN membranes, cells cultured in Millipore supports membranes exhibited a further increase in the metabolic activity up to day 9.

3.2. Ultrastructure

Transmission electron microscopy of MDCK cells after 7 d of culture are shown in Fig. 2. Overall, it can

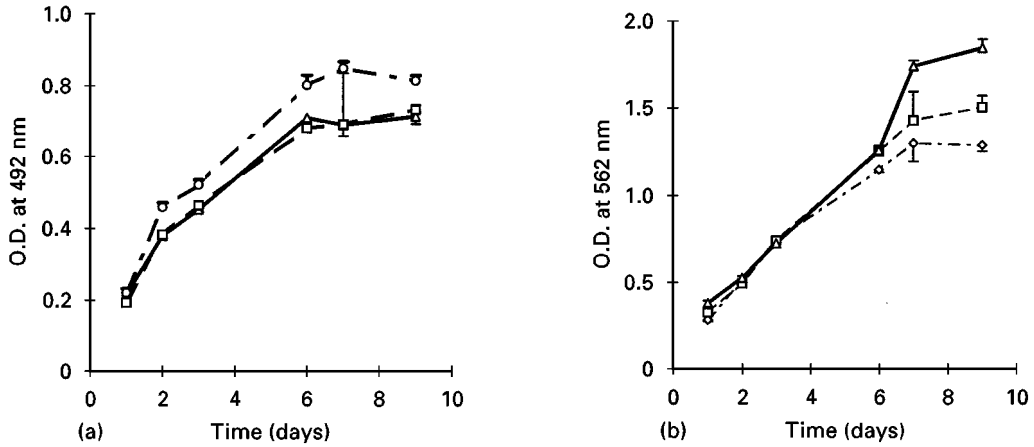
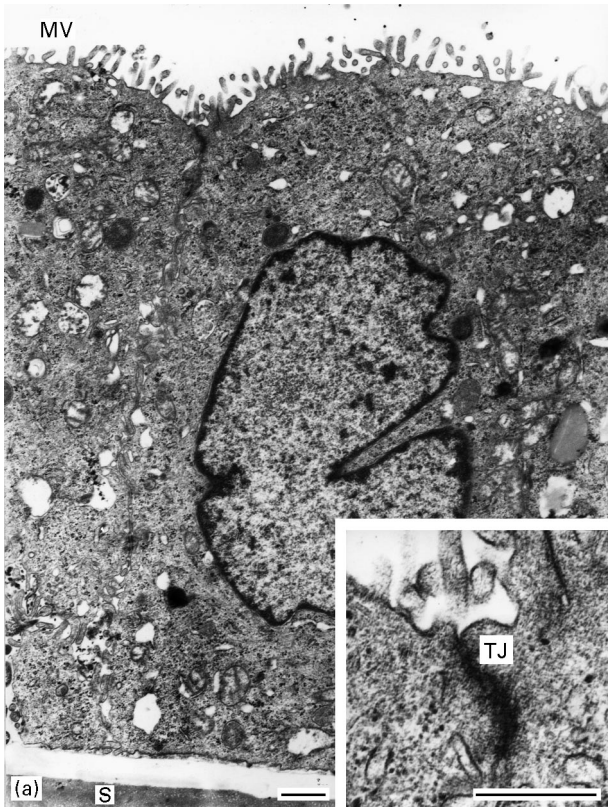


Figure 1 Proliferation and metabolic activity of MDCK-C7 cells cultured for 9 d on different support materials: (△) MC, (□) PSU and (○) PAN smooth side. (a) The increase of cellular LDH with the time of culture. The optical density (OD) of the assay is directly proportional to the cell number. (b) MTT assay showing mitochondrial dehydrogenase activity (measured as optical density, OD).



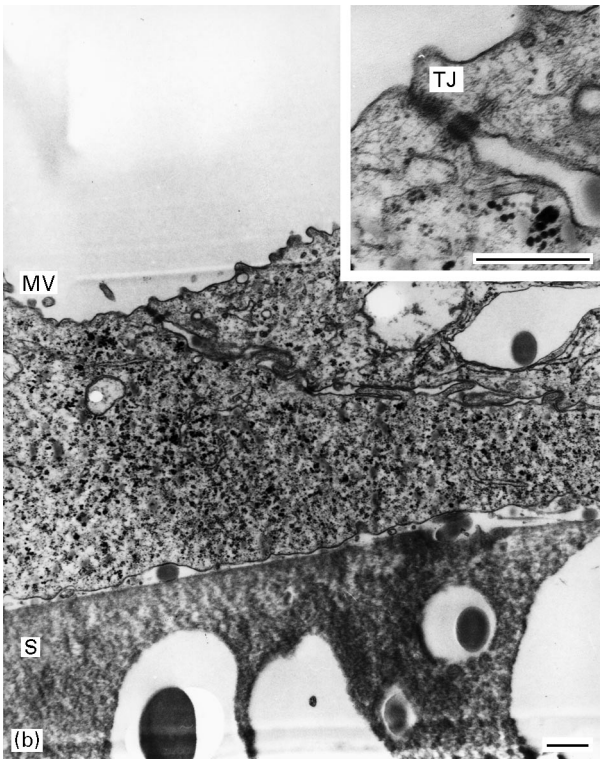


Figure 2 Transmission electron micrograph showing MDCK cells after 7 d of culture on the different materials. (a) PSU, (b) PAN, (c) MC. Attachment of cell membrane to the underlying support (S) is rare with PSU, but frequent with PAN and MC. However, with all substrates, tight junctions (TJ) and microvilli (MV) are found, bar 0.5 μm. Insert shows magnitude tight junction, bar 0.5 μm.

be stated that the expression of a normal morphology of kidney tubular cells was observed on all membranes investigated. This was concluded from the presence of microvilli, polar cell orientation, and the presence of tight junctions, features which are typical for a normal function of kidney cells. In more detail, it was observed that MDCK cells seemed to have denser cell–cell contacts and less tight cell–membrane

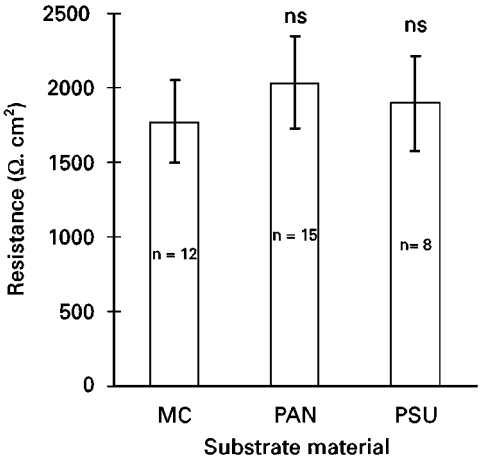


Figure 3 Transepithelial resistance of MDCK cells grown on different substrates. The mean resistance of monolayers grown on a polymer membrane (either PAN or PSU) was not different from that of monolayers grown on Millipore filters (MC). ns, not significant.

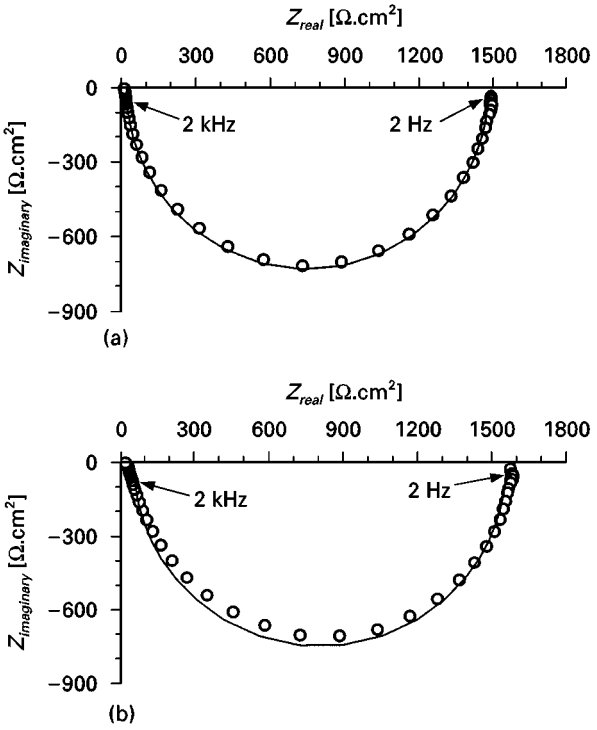


Figure 4 Nyquist diagram of the impedence of MDCK-C7 monolayers grown on (a) Millipore filters or (b) PAN cell supports. Data (○) are shown for the 48 frequencies used (between 1.3 Hz and 65 kHz). The electrical model (——) fits well. Numerical values are given in Table I.

contacts on PSU as shown in Fig. 2a. In this figure, a tight junction, as well as an adherent junction, can be seen. Further, many microvilli were detected on the apical side of the cells expressing an apical-to-basolateral orientation. In contrast, MDCK cells on PAN (Fig. 2b) and on MC (Fig. 2c) expressed much tighter cell–membrane contacts. However, it was observed that, in the case of PAN, the cell–cell contacts were less well organized.

3.3. Transepithelial resistance and impedance

The transepithelial resistance of cultured MDCK cells grown on PAN or PSU supports was not different from that of cells cultured on Millipore filters, our reference material (Fig. 3). The magnitude, exceeding 1 kΩ cm² in all monolayers, suggests that they might be classified as a tight epithelium. This result indicates an overall barrier function typical for distal tubular epithelia.

Plotted in a Nyquist diagram, the impedance of monolayers grown on Millipore filters described an almost perfect semicircle (Fig. 4a, Table I). Because a single time constant ($R_P \cdot C_P$) sufficed, the cells of the epithelium can be considered to be uniform. The impedance loci of monolayers grown on PAN supports (Fig. 4b, Table I) exhibit a higher deviation from the semicircular curve typical of a homogeneous distribution of time constants. However, the deviation still being small, the cells can be described as being uniform.

The relatively small (as compared to R_P) series resistance R_S of MDCK-C7 cells grown on Millipore filters or PAN supports, indicates that cellular or extracellular elements between filter and the epithelium proper, for instance extraneous cells beneath the monolayer forming the mucosal surface, contribute only a little to the transepithelial resistance.

TABLE I Electrical parameters describing the impedance of MDCK-C7 monolayers grown on Millipore filters (4) or PAN cell supports (4). The model consists of a single *RC*-circuit (one resistor R_P and one capacitor C_P in parallel), in series to a resistor R_S. The degree of the fit to the 48 data points measured is given by χ^2

	R_P(Ω cm²)	R_P(Ω cm²)	C_P(mF cm²)	χ^2(Ω² cm⁴)
Millipore	23.5 ± 0.3	1465 ± 33	1.72 ± 0.01	7395 ± 173
PAN	52.6 ± 2.9	1562 ± 56	1.21 ± 0.03	46185 ± 2717
p	**[a]	ns	***[b]	***[b]

[a] $p < 0.01$.
[b] $p < 0.001$.

4. Discussion

Except for one study using polysulfone (PSU) [14], the two polymers tested in the present work have not been examined for possible usage in a biohybrid kidney. The experimental data presented in this study show that polymer membranes manufactured from polyacrylonitrile (PAN) or PSU [7] provide non-toxic substrates that support the culture of MDCK renal epithelial cells. Attachment, viability, and proliferation as well as ultrastructure and electrophysiological properties of the epithelial cells grown on PAN or PSU are similar to cells grown in standard cell culture systems.

The proliferation measured with LDH assay, indicated the presence of cellular monolayers on all membranes after 6 d of culture. Investigation of the metabolic activity of cells with MTT assay demonstrated that cells still increased their metabolic activity after reaching confluence, indicating some further changes in the functional state of cells. However, it was also evident that the metabolic activity of cells was slightly higher if they were cultured on Millipore

filters. The morphological studies revealed that MDCK cells were able to form epithelial-like layers on all membranes verified by the presence of tight junctions and microvilli. Here, it was observed that materials with less-pronounced cell–membrane contact, such as PSU, seemed to improve the expression of cell–cell contacts. If these stronger intercellular connections have any positive impact on the cellular function, it remains to be calculated in further studies.

Morphological and functional properties of the barrier function were studied by electron microscopy and electrophysiological methods. While electron microscopy allows a very detailed ultrastructural description of a small piece of the cell layer, the electrophysiological methods relay information on the overall functional properties of the whole cell layer. Measurement of the transepithelial resistance indicates confluence within 7 days of culture, and a barrier function of the monolayer. Impedance analysis shows the uniformity of the layer and the absence of a significant series of resistances that might inhibit transepithelial transports.

5. Conclusion

The present results indicate the applicability of PAN and PSU for the development of a biohybrid artificial kidney device.

Acknowledgments

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