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PAPER

Macroporous silicon chips for laterally resolved, multi-parametric analysis of epithelial barrier function†

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This study describes a novel assay to visualize the macromolecular permeability of epithelial and endothelial cell layers with subcellular lateral resolution. Defects within the cell layer and details about the permeation route of the migrating solute are revealed. The assay is based on silicon chips with densely packed, highly ordered, dead-ended pores of μm -diameters on one side. The cells under study are grown on the porous side of the chip such that the pores in the growth surface serve as an array of femtolitre-sized cuvettes in which the permeating probe accumulates at the site of permeation. The pattern of pore filling reveals the permeability characteristics of the cell layer with a lateral resolution in the μm range. Coating of the chip surface with a thin layer of gold allows for impedance analysis of the adherent cells in order to measure their tightness for inorganic ions at the same time. The new assay provides an unprecedented look on epithelial and endothelial barrier function.

Introduction

Epithelial and endothelial tissues cover all inner and outer surfaces of the human body and act as a diffusion barrier between two adjacent compartments. By virtue of their location at the interface they control the flux of metabolites and xenobiotics between these compartments. Obvious examples are the intestinal epithelium, the bladder epithelium or the endothelial cells in brain capillaries that form the blood–brain barrier. In these interfacial cell layers the intercellular diffusion pathway is sealed by special cell-to-cell junctions — so-called tight junctions — that are characteristically found in barrier

forming tissues.^{1–3} Limited diffusion along the intercellular pathway ensures that the transport of most solutes is mediated and controlled by specific transport proteins in the membrane. Besides this fundamental physiological importance of epithelial and endothelial barrier function in controlling tissue homeostasis, they also play a major role in pharmacokinetics as they largely define the distribution of drugs within the body. Most drugs that are administered orally or intravenously have to pass one or more tissue barriers to become available in the target tissue in relevant concentrations. Thus, permeation across cellular barriers is a key aspect in drug targeting and drug development.⁴

In order to learn about a compound's ability to pass barrier forming tissues and to study the barrier forming cell junctions, several *in vitro* assays have been developed in the past. Epithelial barrier function is commonly measured and described by two quantities: the transepithelial electrical resistance (TER) and the permeation rate of labelled tracer compounds (P_E).⁵ For both assays the cells are grown on permeable filter membranes that separate two fluid compartments. For TER measurements electrodes are introduced in the upper and the lower fluid compartments with the epithelial cell layer sandwiched in between. Measuring the integral electrical resistance of the cell layer (TER) provides a quantitative indicator for the cell layer's tightness towards the permeation of ions — most notably Na^+ , K^+ and Cl^- .⁶ For P_E measurements a labelled probe, like, for instance, fluorescence-labelled dextrans or radio-labelled sucrose, is added to one compartment and the concentration of this probe in the other compartment is determined as a function of time. The higher the flux of probes from the donor to the

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acceptor compartment, the higher is the permeability P_E of the cell layer. In TER and P_E assays it is very common to use filter inserts with a growth area of 1 cm^2 or even larger. Thus, both readouts, TER and P_E , integrate over large cell sheets but do not provide information on defects in these extended cell layers. They are both prone to artefacts as minor defects in the cell layer may significantly alter the integral result of the experiment. Moreover, they do not provide information on the permeation route of the probe under study, *i.e.* the trans- or paracellular permeation pathway.

Devices for spatially resolved electrical measurements have been developed that are capable of reporting the local electrical properties of the cell layer with subcellular resolution.^{7–9} However, so far no experimental approach has been described to study the macromolecular permeability of 2D tissues with lateral resolution such that defects within the cell layer and the transport route across the cell layer become available.

This study describes a novel strategy to determine the permeability and transport characteristics of 2D tissues with lateral resolution. It is based on growing the cells of interest on macroporous silicon chips. The pores in the silicon substrate are much smaller than the diameter of the cells, densely packed and only open to the side facing the cell layer. Thus, they serve as a highly ordered array of femtolitre cuvettes in which the permeating compound can accumulate at the site where it permeates across the cell layer. Imaging the filling of the pores reveals leaks within the cell layer and the permeation pathway across the cell monolayer. This study shows proof-of-principle experiments of the concept that is illustrated in Scheme 1.

Imaging of substrate permeation has been combined with an *in situ* approach to follow the electrical properties of the same cell layer by impedance analysis as an equivalent to TER measurements. For this an additional layer of gold was sputter-coated on top of the macroporous chip in order to serve as a chemically inert electrode underneath the cells for impedance analysis. Together with a counter electrode dipping into the medium from above (Scheme 1) we were able to study epithelial barrier properties by integral impedance readings and imaging of laterally resolved substrate permeation.

Scheme 1 Schematic of the laterally resolved permeation assay. Barrier forming epithelial cells are grown on a macroporous silicon substrate coated with a thin film of gold for impedance analysis. The permeating probe (yellow = transcellular, red = paracellular path) is collected and detected at the site of permeation after accumulation in the pores. Please note that for real silicon chips the inner walls of the pores are coated with gold to an unknown depth.

Experimental

Preparation of silicon chips with macroporous surfaces

Macroporous silicon chips were prepared as described elsewhere before.¹⁰ The procedure yielded macroporous silicon surfaces with an average pore diameter of $1.3\text{ }\mu\text{m}$, a lattice constant of $4.2\text{ }\mu\text{m}$ and a pore depth of $10\text{ }\mu\text{m}$. The total volume of an individual pore is thus in the order of 10 fl . The final substrates with an insulating SiO_2 layer as the topmost material were sputter-coated (Bal-Tec SCD 050, Liechtenstein) with a 100 nm thick layer of gold. The circular electrode area with a diameter of 2 mm (surface area $\sim 0.03\text{ cm}^2$) was created by photolithography using the positive photoresist Microposit SC 1827 that was applied to the chip by spin-coating at 3500 rpm using a home-made spin coater. After a soft bake, exposure to UV light through a corresponding mask, development of the UV exposed areas and subsequent iodine/iodide-based etching of gold, the remaining photoresist was removed by washing with acetone. The chips were then thoroughly washed with deionized water and dried at ambient conditions.

Cell culture

All experiments were performed using the epithelial cell line MDCK II (Madin-Darby canine kidney, strain II). The cells were kept under ordinary cell culture conditions with 5% CO_2 (v/v) and $37\text{ }^\circ\text{C}$ in a humidified atmosphere. We used MEM-Earle medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Biochrom), 4 mM L-glutamine, $100\text{ }\mu\text{g mL}^{-1}$ penicillin and $100\text{ }\mu\text{g mL}^{-1}$ streptomycin (Biochrom). The culture medium was exchanged twice a week. Routine subcultivation was pursued using standard trypsinization techniques (0.25% w/v trypsin, 1 mM EDTA). First, the confluent cell monolayer was washed twice with PBS (phosphate buffered saline without divalent cations). To facilitate cell detachment, the cells were incubated in PBS supplemented with 1 mM EDTA for 10 min prior to trypsin treatment. Enzymatic cell detachment was stopped by adding an excess of serum-containing culture medium. After centrifugation ($110 \times g$, 10 min) the cells were resuspended and seeded.

To perform the laterally resolved permeation assay with cell-covered macroporous chips, the silicon substrates were placed into ordinary Petri dishes. Prior to inoculation, the clean and dry surfaces were sterilized by exposure to an argon plasma (30 s). Macroporous chips and flat reference surfaces were inoculated with $5 \times 10^5\text{ cells cm}^{-2}$ in serum-containing medium without any additional surface coating.

Immunocytochemical staining

To visualize the intracellular distribution of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ *via* immunofluorescence staining we washed the confluent cell layers with PBS^{++} (PBS supplemented with 0.5 mM Mg^{2+} and 1 mM Ca^{2+}) and fixed them with 4% (w/v) paraformaldehyde for 10 min . After an additional washing step the cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS^{++} for 5 min . Unspecific binding sites were blocked by incubation with a 3% (w/v) BSA solution in PBS^{++} for 20 min before $50\text{ }\mu\text{g mL}^{-1}$ of the anti- $\text{Na}^+\text{-K}^+\text{-ATPase}$ monoclonal mouse antibody (Millipore, Billerica, USA) were applied. After washing and a

second blocking step with 3% (w/v) BSA the primary antibody was labelled by a 45 min incubation with $2\ \mu\text{g mL}^{-1}$ of a polyclonal goat anti-mouse IgG secondary antibody (Millipore, Billerica, USA) conjugated to tetramethylrhodamine isothiocyanate (TRITC). Diluted antibody solutions were prepared in PBS⁺⁺ containing 0.5% BSA.

Laterally resolved permeation assay

In order to visualize a cell layer's macromolecular permeability with subcellular resolution, we used 4 kDa dextran conjugated to fluorescein isothiocyanate (FITC) (0.5 mM in PBS⁺⁺; Sigma, Germany) as permeating probe. FITC-dextran is frequently used to probe the paracellular permeation pathway as they cannot diffuse across the plasma membrane. To avoid starvation effects along the experiment on the microscope stage, $1\ \text{g L}^{-1}$ D-glucose was added to the CO₂-independent experimental buffer solution PBS⁺⁺. Confluent cell layers grown on the macroporous silicon chips were incubated with this solution for varying times before the accumulation of the probe in the pores was imaged by an upright confocal laser scanning microscope (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). Images were recorded using either a $10\times$ (NA = 0.3) or $63\times$ (NA = 0.9) water immersion objective. The fluorophore was excited at 488 nm and emission was detected at 508 nm.

To simulate transcellular permeation and the impact of single cell defects on epithelial barrier function, we made use of saponin (Sigma, Germany), an amphipathic glycolipid that is known to permeabilize cell membranes. Saponin was dissolved in PBS⁺⁺ (plus $1\ \text{g L}^{-1}$ D-glucose) to final concentrations of 0.1% (w/v) or 0.03% (w/v). These solutions were added to the cell layers 30 min after the incubation with the fluorescent dextran probe had been started. Probe accumulation in the pores was again monitored using confocal laser scanning microscopy at different time points while the breakdown of barrier integrity was also monitored by simultaneous multi-frequency impedance readings.

Impedance-based monitoring of cell spreading and barrier formation

Tempering of the macroporous silicon chips provided insulating SiO₂ as the topmost layer which allowed us to coat the surface with a 100 nm layer of gold and to use this gold film as a well-behaved, almost ideally polarizable electrode for impedance analysis. A low-impedance gold dipping electrode served as counter electrode (Scheme 1). Impedance analysis of the cell-covered macroporous electrodes was performed at 61 designated frequencies equally spaced on a logarithmic scale between 1 Hz and 1 MHz using the continuous wave impedance analyzer SI-1260 (Solartron Instruments, Farnborough, UK). The amplitude of the sensing AC voltage was 10 mV (rms). Experiments were performed in a humidified incubator at 37 °C. Impedance measurements were used to monitor (i) attachment and spreading of the cells, (ii) the expression of an epithelial barrier function and (iii) loss of membrane integrity.

Monitoring cell spreading by capacitance readings

The measured complex impedance was broken up into real (resistance) and imaginary (reactance) contributions along the

entire spectrum. The frequency-dependent capacitance of the system was then calculated from the reactance.¹¹ Capacitance readings that have been recorded above a certain threshold frequency are sensitive to cell attachment and spreading as only high frequency current couples capacitively through the cell membranes instead of flowing around the cell bodies. When the cell membranes flatten out on the electrode, the overall capacitance drops from values of a cell-free to those of a cell-covered electrode and mirrors the time course of cell spreading. For electrode sizes used here, capacitance readings at an AC frequency of 20 kHz are well-suited for this purpose.

Monitoring epithelial barrier formation

Formation of an epithelial barrier makes the cell layer resistant to the passage of ions. Thus, barrier formation can be monitored non-invasively from recordings of the real part of the electrical impedance (resistance) with a time resolution in the order of seconds. The monitoring frequency has to be sufficiently low in order to guarantee current flow around the cells through the junctions. For the electrode sizes used here, the monitoring frequency was adjusted to 200 Hz to zoom in on junction formation.

Monitoring loss of tissue integrity

In order to monitor the permeabilization of the membranes and the associated loss of tissue integrity by saponin with highest sensitivity, we used an intermediate monitoring frequency of 2 kHz. Impedance readings at this frequency mirror contributions from both the trans- and the paracellular pathways.

Results and discussion

Substrate characterization

Fig. 1 provides a set of three scanning electron micrographs (SEM) that show the substrates at three different stages of preparation: (A) after etching the pores and producing the final oxide layer by tempering in air at 900 °C, (B) after subsequent sputter-coating of the Si/SiO₂ surface with a 100 nm thin gold layer and (C) after photolithographic layouting of the gold film to produce the final electrode design with well-defined electrode sizes in the mm range. Fig. 1C shows the edge of the layouted electrode. The pre-etched squares are entirely lined with gold. The gold lining, however, only reaches into the pores for 1–2 μm (micrographs not shown). After photolithography the gold surface shows a nanoscale roughness which does not interfere with the impedimetric measurements.

Cytocompatibility of the macroporous surfaces

The cytocompatibility of the macroporous surfaces is an unconditional prerequisite to perform cell-based assays with physiological relevance. It seems very likely that the topography of the growth surface might affect cell-substrate interactions which in turn may have an impact on cell differentiation. We therefore studied (i) the kinetics of cell spreading on the macroporous surfaces, (ii) the time course of epithelial barrier formation as an indicator for epithelial differentiation and (iii)

Fig. 1 Scanning electron micrographs of macroporous silicon substrates (top view). (A) Uncoated substrate. The pyramidal pre-etched areas form a pit which tapers into a circular pore. The lattice constant is 4.2 μm and the pore diameter is 1.3 μm . (B) Complete surface coverage with gold after sputter deposition. (C) Perimeter of the electrode after photolithographic processing. Micrographs were recorded by nanoAnalytics GmbH, Münster (Germany).

the intracellular distribution of $\text{Na}^+\text{-K}^+\text{-ATPase}$ as another indicator for the expression of a polarized epithelial phenotype.

(i) Kinetics of cell spreading. There are several parameters to quantify the interactions of cells with an *in vitro* surface. One of them is the *rate of cell spreading* as it is directly proportional to the adhesion energy between cell and surface.¹² Cell spreading kinetics are accurately available from time-resolved capacitance readings when the cells are allowed to attach upon a conducting growth surface.¹³

Using the setup shown in Scheme 1 we followed the time course of attachment and spreading of initially suspended MDCK II cells by capacitance readings at an AC frequency of 20 kHz. Fig. 2A compares the averaged time course of the normalized capacitance for macroporous gold electrodes (open symbols) and flat reference electrodes of the same size (filled symbols) when the same number of MDCK II cells (10^6 cells cm^{-2}) was seeded on either surface at time zero. The seeding density was high enough to ensure complete coverage of the surface after attachment and spreading without any cell proliferation. Thus, the time course of the capacitance mirrors the time course of cell spreading on either substrate and not proliferation. In all experiments we only observed minor differences in attachment and spreading kinetics between macroporous and flat surfaces. The rate of cell spreading (slope of capacitance curve) was slightly lower on macroporous compared to flat surfaces. However, after approx. 400 min cell spreading was complete on either surface. This data indicates that the topography of the surface does indeed affect cell surface interactions to a measurable degree but the observed differences are minor and do not interfere with the suitability of the chips for physiological experiments using established epithelial and endothelial cell layers.

(ii) Time course of epithelial barrier formation. A hallmark of epithelial differentiation is the expression of barrier forming cell–cell contacts (tight junctions) that occlude the paracellular cleft and make the cell layers more or less tight for hydrophilic molecules and ions.³ When epithelial cells are seeded on gold film electrodes the process of barrier formation is conveniently monitored by time-resolved impedance readings.

Fig. 2B compares the time course of the normalized resistance (real part of impedance) at an AC frequency of 200 Hz when the same number of initially suspended MDCK II cells was seeded

Fig. 2 Adhesion and differentiation kinetics of MDCK II cells grown on flat (●) or macroporous (○) gold film electrodes monitored by impedance measurements. (A) Time course of the normalized electrode capacitance C_{norm} recorded at an AC frequency of 20 kHz that mirrors the time course of cell spreading. (B) The time course of the normalized resistance R_{norm} at 200 Hz traces the establishment of epithelial barrier function. Measured values of C and R were normalized to the first value of the measurement. The inoculation was 10^6 cells cm^{-2} . Mean $\pm$ SDM, $n = 4$; $T = 37^\circ\text{C}$.

on macroporous (open symbols) or flat (filled symbols) gold electrodes at time zero. The cells were seeded to confluence so that no cell proliferation was necessary to form a continuous monolayer and the expression of tight junctions could start immediately after the cells had spread out. The experimental data mirrors the formation of epithelial tight junctions on either growth substrate. Data in Fig. 2B shows that barrier formation is delayed by approx. 120 min on macroporous compared to flat surfaces but the final resistance values for the established cell layers are not significantly different. The observed delay in barrier formation is a direct consequence of the slower cell spreading which has to be complete before cell differentiation sets in. Thus, except for the described delay the macroporous culture surface does not interfere with the expression of a barrier forming epithelial phenotype.

(iii) Polar expression of $\text{Na}^+\text{-K}^+\text{-ATPase}$. Epithelial cells are well-known to show a polarized expression of certain membrane proteins. Once established this polarized distribution is maintained by the cytoskeleton and the tight junctions. $\text{Na}^+\text{-K}^+\text{-ATPase}$ is an ubiquitous protein that is exclusively targeted to the lateral membrane in MDCK II cells.¹⁴ We studied the localization of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in MDCK cells that were grown on macroporous silicon chips by means of immunocytochemical labelling and subsequent confocal laser scanning microscopy. Fig. 3 compares optical *xy*- and *xz*-sections of confluent MDCK monolayers immuno-stained for $\text{Na}^+\text{-K}^+\text{-ATPase}$ when they were either grown on regular culture substrates (A) or on macroporous silicon surfaces (B). The optical *xy*-sections already indicate what becomes most obvious from *xz*-sections: on either surface the $\text{Na}^+\text{-K}^+\text{-ATPase}$ is targeted predominantly to the lateral membrane of the cells, whereas apical and basal membranes show only a minor fluorescence labelling independent of the substrate. Comparison of Fig. 3 (A) and (B) does not reveal any significant differences in the staining pattern and, thus, localization of the protein under study.

We conclude from these immunocytochemical stainings, the time course of epithelial barrier formation and cell spreading kinetics for flat and macroporous growth surfaces, that the latter can be considered as equally cytocompatible as other *in vitro* surfaces that are used in tissue culture.

Fig. 3 Confocal fluorescence micrographs (*xy*- and *xz*-sections) of MDCK II cells immuno-stained for $\text{Na}^+\text{-K}^+\text{-ATPase}$ (A) on a regular culture substrate and (B) on a macroporous silicon substrate. Reflection of the emitted light at the SiO_2/Si interface causes periodic intensity fluctuations beneath the basal membranes of the cells.

Permeation assay with lateral resolution: proof of concept

Pre-experiments with cell-free surfaces. It is another prerequisite for the performance of the assay that the fluorescence-labelled permeation probe readily enters the pores from the bulk phase and that pore filling is not hampered by entrapments of air in the pores. Thus, we studied the lateral fluorescence intensity distribution across the cell-free chip when it was incubated with the experimental buffer containing the fluorescence-labelled permeation probe. Before the macroporous surface got in contact to the fluorophore containing liquid, it was treated in an argon plasma for 30 s to render the surface hydrophilic. Shortly after adding the fluid to the plasma-treated surface all pores got filled homogeneously as indicated in the typical optical *xy*-section provided in Fig. S1A (ESI†). Within the time resolution of confocal microscopy we could not identify any delayed pore filling or any significant lateral inhomogeneity that would indicate incomplete filling due to entrapment of air. From these experiments we conclude that fluorescent tracers will be readily captured within the pores at the site where they cross the cell layer. There is no noticeable access resistance to the pores that would limit the resolution of the approach. The good wetting of the surface and accessibility of the pores is most likely due to the initial plasma treatment which renders the SiO_2 surface hydrophilic.

Paracellular probe permeation across epithelial cell layers. The 4 kDa FITC-dextran is a membrane-impermeable probe that only diffuses across intact epithelial cell layers on paracellular pathways (compare Scheme 1, red circles). Thus, it is a good model compound to test whether or not the permeation pathway can be imaged with the help of macroporous chips as used here. After a confluent and mature cell monolayer had been established within several days, the cell-covered chip was incubated with PBS^{++} (plus 1 g L^{-1} D-glucose) containing 0.5 mM of the 4 kDa FITC-dextran. The probe was allowed to diffuse across the cell monolayer before filling of the pores underneath the cells was imaged by confocal fluorescence microscopy.

Fig. 4A provides an optical *xy*-section of a cell-covered substrate when the focal plane of the microscope was set to the substrate surface. The image demonstrates that the FITC-dextran probe was predominantly found in the pores that are located at the periphery of individual cells beneath the cell-cell contact sites. The bodies of most cells appear as dark areas whereas the cell borders are labelled by the fluorophore in the pores underneath them. Out-of-focus light from the bulk phase above the cells is efficiently eliminated from the image by the confocal pinhole. Thus, the pattern of fluorescence intensity correctly reports on the permeation pathway of the dextran model probe for most cells.

Fig. 4B and 4C provide magnifications of the dashed boxes in Fig. 4A. The magnified images demonstrate a detail of the assay in its current form that needs to be addressed in future developments as it limits the resolution. The fluorescence intensity distribution in Fig. 4B indicates that the probe has permeated along the cell junctions at the periphery of the cell in the centre of the image. The pores underneath the cell body are hardly visible indicating that no permeation marker has

Fig. 4 (A) Confocal fluorescence micrograph (*xy*-section) of a macro-porous substrate covered with a confluent MDCK II cell layer after incubation with FITC-dextran in the extracellular buffer (4 kDa, 0.5 mM, 8 h). The focal plane was set to the substrate surface. (B) and (C) provide magnified images of areas that are delineated by dashed boxes in (A). (D) Fluorescence intensity along the path labelled in part (C) by dashed lines. The red line represents a cubic spline through the maxima of the line profile (see text).

accumulated here. For the cell highlighted in Fig. 4C most pores underneath the cell body are visible and obviously filled with the fluorescent probe to different degrees. As the dextran probe cannot diffuse across the plasma membranes due to its size and hydrophilicity, it can only enter the pores underneath the centre of the cell body by diffusion from the cell periphery along the diffusion pathway between cell membrane and substrate surface. The line profile (Fig. 4D) of the fluorescence intensity between the dashed white lines in Fig. 4C from the periphery of the cell through the centre onwards to the other side of the cell confirms this interpretation. The maxima of the oscillating pattern represent fluorescence inside the pores, whereas the minima indicate the ridges between pores. The red line is a cubic spline connecting only the maxima of the oscillating raw data, thus, representing the fluorescence intensity line profile of the pore filling. Fluorescence intensity of the pores decreases towards the centre and increases again towards the periphery at the other side of the cell, indicating a gradual filling of the central pores by diffusion from the periphery. A closer attachment of the cells to the substrate surface might reduce or eliminate this problem and thereby increase the resolution of the assay.

Transcellular permeation through leaks in epithelial cell layers.

To simulate transcellular permeation through the plasma membranes, we applied the FITC-dextran probe together with rather low concentrations of saponin. Saponins are a family of glycosylated steroids from plants that are known to solubilize lipids from the cell membrane and create holes in the membrane when present beyond a threshold concentration. In contact to red blood cells, for instance, saponins lead to haemolysis.¹⁵ Low concentrations provided a sequential permeabilization of individual cells within the monolayer, such that we could study the impact of single cell defects in the cell layer that increase with time.

Fig. 5A to 5D show microscopic images of the pore filling pattern when confluent MDCK cells are exposed to a saponin concentration of 0.1% (w/v). As before the FITC-labelled dextran is added to the incubation buffer to serve as a permeation marker. The images show a constant field of view along the time course of exposure. After 8 min of exposure (Fig. 5A) only individual, isolated pores show a weak fluorescence. At the sites indicated by arrows a few adjacent

Fig. 5 (A–D) Optical *xy*-sections recorded at different time points along the exposure of MDCK cells to 0.1% (w/v) saponin. The focal plane was adjusted to the substrate surface. (E) Time course of the electrical impedance at a sampling frequency of 2 kHz for confluent MDCK cells exposed to 0.1% and 0.03% (w/v) saponin.

pores are starting to fill. After 40 min there are extended areas in which adjacent pores emit bright fluorescence (Fig. 5B). Apparently, the saponin treatment has permeabilized the membranes of these individual cells. The fluorescence pattern is very distinct from the one shown in Fig. 4. Whereas in Fig. 4 the brighter pores are located at the periphery of the cell indicating paracellular diffusion, saponin induces homogeneous filling of the pores underneath an affected cell due to probe permeation across the permeabilized membranes (compare Scheme 1, yellow solute). After 60 min of exposure (Fig. 5C) the areas with filled pores have increased considerably. Not only the pores are filled in these areas. The images also document out-of-focus light from fluorophores inside the cytoplasm (blurred background). Apparently, the permeating probe has diffused across the apical membrane, through the cytoplasm and across the basal membrane to accumulate inside the pores. But the permeabilization of cells within the monolayer is not homogeneous. Some cells are either more resistant than others or they did not get hit by a saponin micelle. This information on the lateral distribution of barrier breakdown is not available from the well-established integral permeation assays and it demonstrates that single cell defects within epithelial or endothelial cell sheets can be sensitively detected. Moreover, using the established integral assay we could only speculate about the permeation pathway of the compound under test whereas the use of macroporous growth substrates unravels the details of cell layer permeabilization.

When intact cells are surrounded by permeabilized cells, the pores underneath the intact cell bodies start to fill due to lateral diffusion of the probe from the periphery through the cell–substrate junction as described above (thick arrow). When intact cells are surrounded by other intact cells this lateral diffusion from the sides is clearly reduced. Pores underneath these cell bodies remain unlabelled. After 140 min of exposure the field of view shows that most of the pores are homogeneously filled with the model probe (Fig. 5D). The cell membranes are now almost entirely permeabilized and the cell layer cannot provide any diffusion barrier anymore. Whether it will be possible to distinguish locally different transport rates within the lower membrane of a single cell is ongoing research and currently limited by the lateral diffusion of the probe in the cleft between cell membrane and substrate.

Fig. 5E compares the time courses of the normalized impedance at an AC frequency of 2 kHz when MDCK cells that were grown to confluence on macroporous silicon chips were exposed to two different saponin concentrations. Upon exposure to 0.03% (w/v) saponin (arrow) in cell culture medium, the course of the impedance does not indicate any impact on epithelial barrier function or membrane integrity. When the concentration is raised to 0.1% (w/v) the integral impedance readout reports a massive loss of barrier integrity starting shortly after saponin has been added to the culture medium. At the end of the observation time the measured impedance approaches the impedance of a cell-free electrode indicating complete loss of membrane integrity.

Comparing the microscopic and the impedimetric time course for the loss of barrier function, it is noteworthy that integral impedance readings report very sensitively on the onset of membrane permeabilization. Immediately after addition of

saponin the impedance drops significantly. In contrast, the non-integral permeation assay reports only a small fraction of permeabilized cells early on in the experiment. This comparison illustrates that single cell defects may significantly alter the readout of integral detection techniques. This is true for both, integral electrical measurements (TER) and integral permeation experiments (P_E).

Conclusion

Macroporous silicon chips with an uppermost SiO₂ or gold layer have proven to be an entirely cytocompatible material with no significant influence on cell spreading or cell differentiation. Even though we have focused on MDCK II cells in this study, several other cell lines have been successfully grown on these chips with the same result. Thus, the general concept of a laterally resolved permeation assay can be transferred to any model epithelium or endothelium—even primary cultured cells. In principle, the assay can be applied to all cellular barriers that are of interest with respect to substrate permeation. By means of the well-developed silicon technology macroporous surfaces can be arranged in an array-like format in the future to allow for multi-spot detection and even medium throughput. The assay is not only applicable to tracer molecules that permeate passively across the cell layer by diffusion but also to actively transported solutes. Future developments will address other readout techniques to quantify the filling of the pores. The combined measurement of macromolecular permeability on the one hand and electrical tightness of the same cell layers on the other provides a more detailed analysis of barrier modulation. The assay returns changes in epithelial barrier function with high time resolution from impedance readings and lateral resolution from analysis of the pore filling pattern. Thus, the new chip may pave the way for an entirely new perspective in screening campaigns with respect to drug targeting and bioavailability.

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References

- 1 S. Aijaz, M. S. Balda and K. Matter, *Int. Rev. Cytol.*, 2006, **248**, 261–298.
- 2 E. Steed, M. S. Balda and K. Matter, *Trends Cell Biol.*, 2010, **20**, 142–149.
- 3 J. Wegener and H.-J. Galla, *Chem. Phys. Lipids*, 1996, **81**, 339–348.
- 4 A. Braun, S. Hammerle, K. Suda, B. Rothen-Rutishauser, M. Gunthert, S. D. Kramer and H. Wunderli-Allenspach, *Eur. J. Pharm. Sci.*, 2000, **11**, S51–60.
- 5 J. Wegener, A. Hakvoort and H. J. Galla, *Brain Res.*, 2000, **853**, 115–124.
- 6 D. W. Powell, *Am. J. Physiol.*, 1981, **241**, G275–288.
- 7 M. Fromm, S. M. Krug, S. Zeissig, J. F. Richter, R. Rosenthal, J. D. Schulzke and D. Gunzel, *Ann. N. Y. Acad. Sci.*, 2009, **1165**, 74–81.
- 8 A. H. Gitter, L. Victor, M. Bertog, H. D. Foss, J. D. Schulzke and M. Fromm, *Ann. N. Y. Acad. Sci.*, 1998, **859**, 285–289.

-
- 9 A. H. Gitter, F. Wullstein, M. Fromm and J. D. Schulzke, *Gastroenterology*, 2001, **121**, 1320–1328.
 - 10 A. Birner, R. B. Wehrspohn, U. M. Gösele and K. Busch, *Adv. Mater.*, 2001, **13**, 377–388.
 - 11 J. Wegener, *Impedance Analysis of Cell Junctions.*, Wiley-VCH, Weinheim. 2008.
 - 12 T. Frisch and O. Thoumine, *J. Biomech.*, 2002, **35**, 1137–1141.
 - 13 J. Wegener, C. R. Keese and I. Giaever, *Exp. Cell Res.*, 2000, **259**, 158–166.
 - 14 L. Shoshani, R. G. Contreras, M. L. Roldan, J. Moreno, A. Lazaro, M. S. Balda, K. Matter and M. Cerejido, *Mol. Biol. Cell*, 2005, **16**, 1071–1081.
 - 15 E. Baumann, G. Stoya, A. Volkner, W. Richter, C. Lemke and W. Linss, *Acta Histochem.*, 2000, **102**, 21–35.