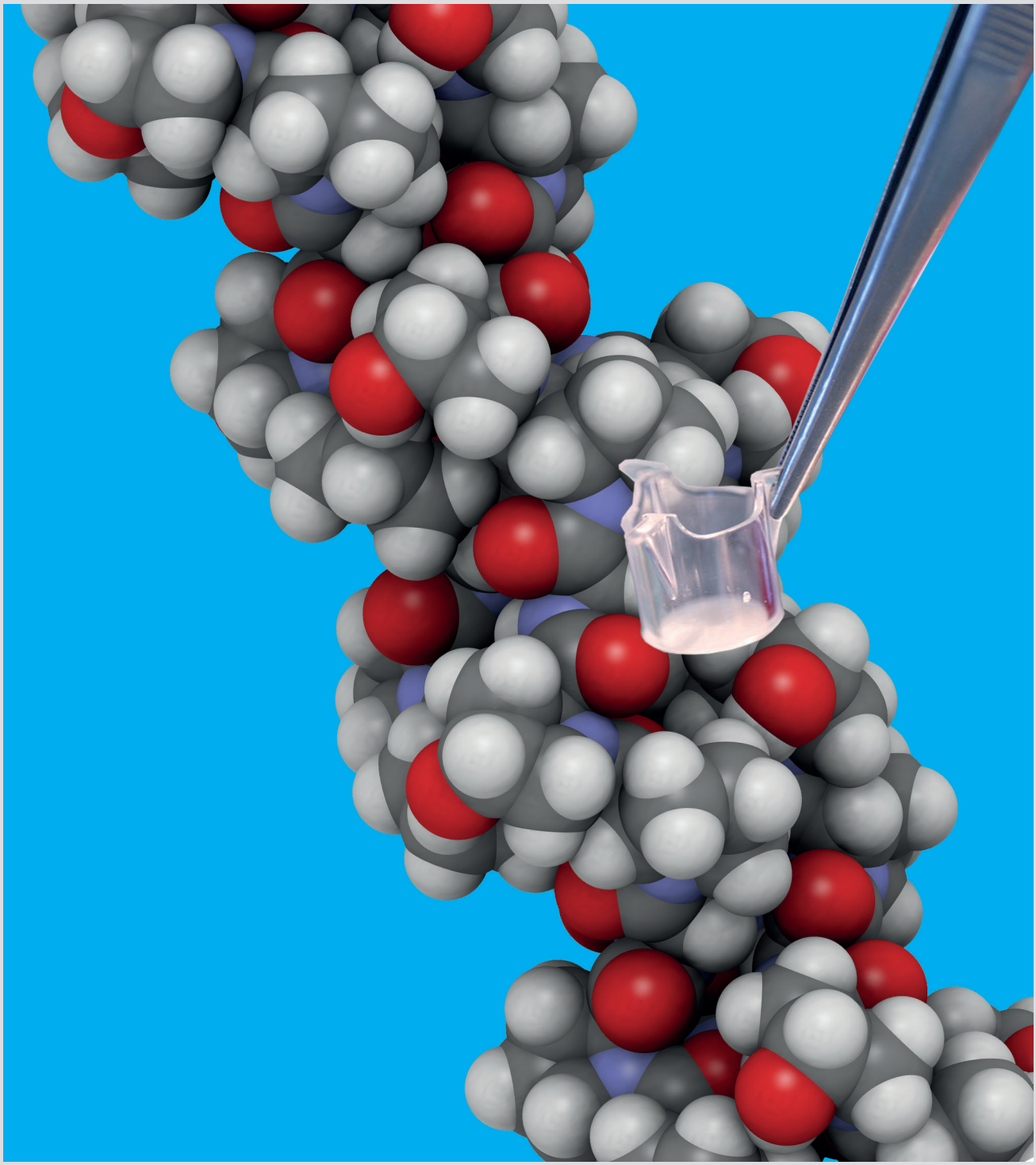
Application Note

Protein coatings facilitate the differentiation of reconstructed epithelia in ThinCert™ cell culture inserts



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1. Introduction

Key to the establishment of a significant *in vitro* model is the identification of all essential parameters characterising the corresponding *in vivo* situation. Furthermore, it must be assured that these critical parameters are maintained sufficiently *in vitro*. In the case of reconstructing epithelia, such crucial factors are the presence of an equivalent of the basement membrane as well as the supply with nutrients from both sides of the cultivated cell layer (apical and basolateral)[1,2].

Cell culture inserts with porous membranes have proven to fulfil both functions and are therefore optimum tools for reconstructing epithelia for transport, permeability or uptake studies. When cultivated on ThinCert™ cell culture inserts, epithelial cells develop characteristic features, such as the formation of tight junctions, the polarised localisation of proteins to the apical or basolateral membrane compartment[3], and the expression of differentiation markers (e.g. alkaline phosphatase), as well as the formation of microvilli. Finally, reconstructed epithelia gain full functionality including barrier function and vectorial transport[4].

Within this application note, several aspects of epithelial cell culture in ThinCert™ cell culture inserts are addressed and protocols for determining the epithelial barrier function are provided. Furthermore, advantages of ThinCert™ cell culture inserts pre-coated with collagen type I are detailed.

2. Results & Conclusion

2.1 The Lucifer Yellow assay for determination of the epithelial barrier function

Lucifer Yellow is a fluorescent dye normally neither taken up by living cells nor transported transcellularly. The dye can therefore be used to confirm that epithelial cells express tight junction proteins and the cell layer forms a diffusion barrier. **Figure 1** illustrates the major steps of the Lucifer Yellow permeability assay. As depicted in **figure 2**, the Lucifer Yellow assay reveals a typical time-dependent permeability profile of reconstructed Caco-2 epithelia with permeability being high after 2 days *in vitro* (div) and reaching a low level from 7 div onwards.

Applying collagen type I coated ThinCert™ cell culture inserts accelerates this process; however cells on non-coated inserts may 'catch up' with those on pre-coated membranes as early as 7 div. It is noteworthy, that this temporary advance on collagen I coated inserts later translates into a much earlier and higher expression of alkaline phosphatase – a late epithelial differentiation marker (see chapter **2.2** and **figure 3**).

2.2 Alkaline phosphatase – a marker for epithelial differentiation

In the context of epithelial cell culture, alkaline phosphatase (AP) is a helpful marker protein indicating that these cells have reached their terminal stage of differentiation.

In vitro, the intracellular AP content reaches its maximum after 28 days (**figure 3**). Here we could demonstrate that pre-coating ThinCert™ cell culture inserts with collagen type I strongly facilitates the differentiation of Caco-2 cells, thus accelerating their time-dependent increase in AP expression (**figure 3**). This pre-coating allows for shortening the cultivation period of Caco-2 cells from 21-28 days to 14 days, thereby still reaching the same level of AP activity and differentiation.

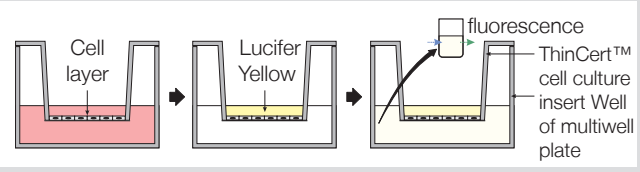

Figure 1: Lucifer Yellow assay for determination of the epithelial barrier function.
A: Epithelial cells are pre-cultivated on ThinCert™ cell culture inserts.
B: Cell culture medium is removed and membrane and cells are washed with HBSS/HEPES. Subsequently, 1 mM Lucifer Yellow in HBSS/HEPES is added to the upper compartment contains HBSS/HEPES.
C: After two hours of incubation a 200 µl sample is withdrawn from the lower compartment and the fluorescence (excitation 485 nm, emission 530 nm) detected using a spectrophotometer. The fluorescence signal withdrawn from no-cell containing inserts is set as 100 %.

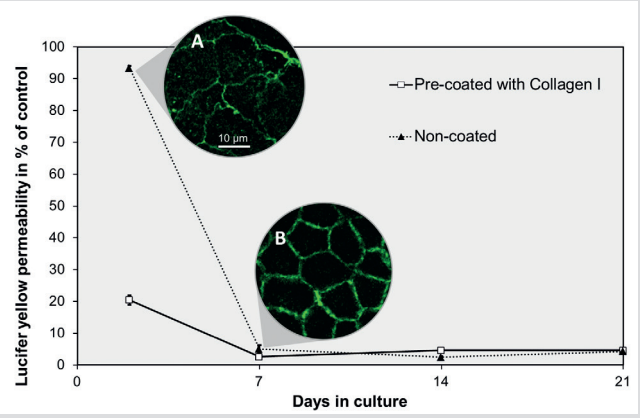

Figure 2: Lucifer Yellow permeability of Caco-2 epithelia cultivated on ThinCert™ cell culture inserts.
Caco-2 cells were cultivated on non-coated or collagen type I coated ThinCert™ cell culture inserts. After 2,7,14 and 21 days the permeability of the Caco-2 cell layer was assessed using the Lucifer Yellow assay. Whereas Caco-2 cells from equally good barriers on non-coated and coated inserts after 7, 14 and 21 days; collagen type I coating facilitates the barrier formation at earlier time points (2 days ***in vitro***). The onset of barrier formation correlates with the expression of the tight junction associated protein Claudin 1 (immuno-cytochemistry in A and B). Error bar = standard error of the mean.

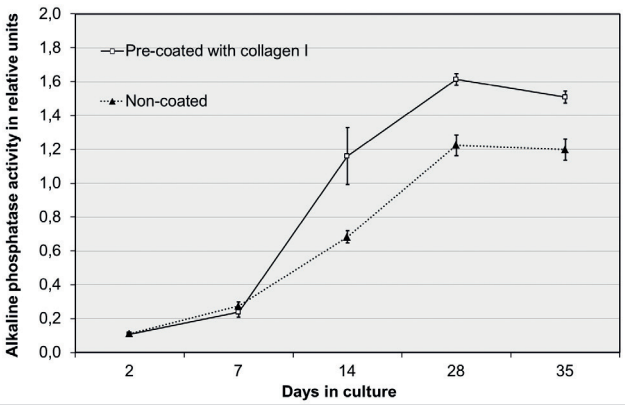

Figure 3: Alkaline phosphatase (AP) activity in lysates from Caco-2 cells cultivated on ThinCert™ cell culture inserts.
Caco-2 cells were cultivated on non-coated or collagen I coated ThinCert™ cell culture inserts and cell lysates analysed for alkaline phosphatase activity after several days ***in vitro***. When cultivated on pre-coated inserts, Caco-2 cells develop higher levels of AP activity at earlier time points. Thus, the pre-coating of ThinCert™ membranes with collagen type I helps to shorten the cultivation period required for full epithelial differentiation from 28 div to 14 div. Error bar = standard error of the mean.

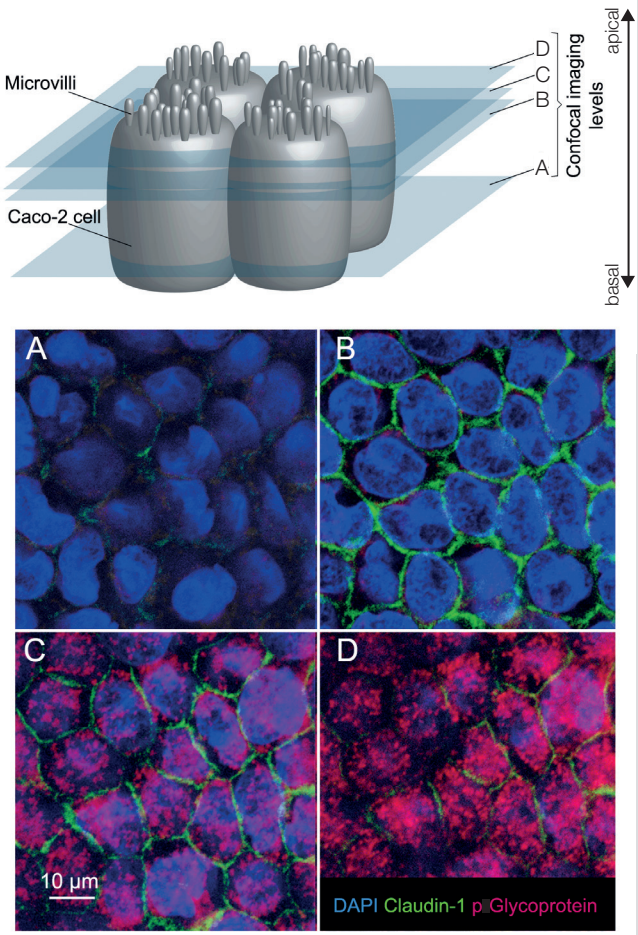

Figure 4: Polarisation of Caco-2 cells on ThinCert™ cell culture inserts.
Caco-2 cells were cultivated on collagen I coated ThinCert™ cell culture inserts and stained for Claudin-1 and p glycoprotein. Confocal analysis revealed fully polarised epithelial cells with p glycoprotein being expressed in the apical membrane compartment above the Claudin-1 positive membrane segment.

2.3 P glycoprotein expression and epithelial polarisation

P glycoprotein 1 is an ABC-transporter expressed in many epithelia, transporting a wide variety of substrates across extra- and intracellular membranes[5].
P glycoprotein is usually expressed in the apical membrane compartment of epithelial cells. In this study p glycoprotein, together with the tight junction protein Claudin-1, were used as markers for determining the polarisation of the reconstructed Caco-2 epithelium. From 7 div on, Claudin-1 expression was detectable without gaps across the entire cell layer. Applying confocal microscopy, Claudin-1 exhibited localisation in the upper third of the lateral cell membrane (**figure 4 B and C**), whereas the apical membrane compartment was characterised by strong p glycoprotein expression (**figure 4 C and D**).

2.4 Formation of microvilli

In vivo, intestinal epithelia carry a seam of thousands of microvilli, the so called “brush border.” These microscopic membrane protrusions increase the surface area of cells and are involved in various functions such as secretion, absorption and cellular adhesion[6]. With the electron microscopical identification of microvilli at the apical surface of Caco-2 cells cultivated on ThinCert™ (**figure 5**), the picture of a fully differentiated epithelium is finally completed. Microvilli could be detected at 7 div in cultures on pre-coated as well as non-coated inserts.

2.5 Conclusion

As proven by several independent read-outs, Collagen type I coated ThinCert™ cell culture inserts provide helpful tools for the reconstruction of polarised and functional epithelia from immortalised cells. The biological coating allows for an enhanced formation of the epithelial barrier and differentiation of epithelial cells with formation of microvilli and polarised protein localisation.

3. Methods and Material

Table 1 lists the material used in the context of this application note with supplier and ordering information.

3.1 Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. For the experiments described within, 24 well ThinCert™ cell culture inserts with translucent 0.4 µm PET membranes were used. For propagating the Caco-2 cells DMEM medium supplemented with 10 % FCS, 4 mM L-alanyl-L-glutamine and 1x MEM-amino acids was used. 30.000 Caco-2 cells in 200 µl medium were seeded in each 24 well insert and the wells of the receiver plate filled with 600 µl medium. Cell culture was carried out at 37 °C and 5 % CO_2, with cell culture medium changed every second day.

Table 1: Material

Description	Supplier	Ordering number
Alexa Fluor 488 goat anti rabbit IgG antibody	Life Technologies	A11008
Alexa Fluor 546 goat anti mouse IgG antibody	Life Technologies	A11003
Alkaline buffer solution	Sigma	A5533
Anti-Claudin 1 antibody (polyclonal)	Abcam	ab15098
Anti-P Glycoprotein antibody (monoclonal)	Abcam	ab10333
CELLSTAR® 24 well cell culture plate	Greiner Bio-One GmbH	662160
CELLSTAR® 96 well cell culture plate	Greiner Bio-One GmbH	655160
Complete Lysis-M, EDTA-free	Roche Diagnostics	04719964001
DMEM medium	Biochrom	F0435
Fetal calf serum	Biowest	S1810-500
HBSS w/o phenol red	SAFC® global	55037C
HEPES buffer 1 M	Sigma	H0887
L-alanyl-L-glutamine	Biochrom	K0302
Lucifer Yellow	Sigma	L0144
MEM-amino acids, 50x	Biochrom	K0363
PBS	Biochrom	L1825
ThinCert™ cell culture insert 24 well, collagen type I coating	Greiner Bio-One GmbH	662654

Table 2: Primary and secondary antibodies and applied dilutions

Antibody	Dilution and diluent
Mouse anti human p glycoprotein	1 : 100 (1 µg/ml)
Rabbit anti human Claudin-1	1 : 250 (0,8 µg/ml)
Alexa 488 goat anti rabbit IgG	1 : 250 (8µg/ml)
Alexa 546 goat anti mouse IgG	1 : 250 (8µg/ml)

3.2 Lucifer Yellow assay

For determining the barrier function of reconstructed Caco-2 epithelia, the cell culture medium was removed from the insert and well and the membrane washed with HBSS/HEPES. 1 mM Lucifer Yellow in HBSS/HEPES was added to the upper compartment while the lower compartment contained HBSS/HEPES. After two hours of incubation, a 200 µl sample was withdrawn from the lower compartment and the fluorescence detected using a spectrophotometer (excitation 485 nm, emission 530 nm). Fluorescence signals from inserts containing no cells were set to 100 %.

3.3 Alkaline phosphatase assay

For alkaline phosphatase detection, culture medium was removed and the 24 well inserts were placed into the wells of a 12 well plate, thus enabling a “sitting position” on the well bottom in lieu of “hanging” from the well-top.

150 µl lysis buffer (Lysis-M, prepared according to manufacturer’s manual) was added to each insert and the inserts incubated for 10 min at room temperature (slightly shaking). Subsequently, the lysis buffer was pipetted up and down and the inserts incubated for another 10 min. 45 µl alkaline buffer and 45 µl phosphatase substrate solution (4 mg/ml in distilled water, stored at -20 °C) were pipetted into the wells of a 96 well plate and 10 µl substrate transferred to this solution. Following, the absorbance at 405 nm was measured (5 measurements with a 3 minutes interval, calculation of an average value).

3.4 Immunocytochemistry

For immunocytochemical staining the cell culture medium was removed and the 24 well inserts placed within a 12 well plate, thus enabling them to touch the plate bottom as described in the paragraph above. Cells were fixed with ice-cold ethanol (100 %) for 10 min and washed twice with PBS. Afterwards, non-specific binding sites were blocked with 10 % FCS in PBS for 1.5 hours. Cells were washed three times 5 min with PBS and incubated with the primary antibody for 1 h at room temperature (see **table 2**) followed by another set of washes with PBS. The secondary antibody (**table 2**) was applied and incubated for 1 h at room temperature. After a final series of washing steps with PBS (3 x 5 min), the insert membrane was detached using a scalpel and mounted onto a microscopy slide using Fluoroshield mounting medium with DAPI.

3.5 Electron microscopy

For electron microscopy, the cell culture medium was removed and cells were fixed with 3 % formaldehyde (pH 7.2) for 10 minutes at 37° C. Cells were washed three times for 10 minutes at 37° C with PBS followed by another fixation step with 2.5 % glutaraldehyde for 2 h on ice. Insert membranes were cut out using a scalpel, washed in PBS and incubated with the following solutions: osmium tetroxide (1 % in PBS), water, uranyl acetate (1 % in distilled water), water, ethanol 50 %, ethanol 70 %, ethanol 95 %, ethanol 100 %, ethanol 100 % (on molecular sieve, twice), ethanol-EPON 1:1-mixture and EPON 100 % (twice). Membranes were embedded into EPON in embedding molds and polymerisation was carried out at 60 °C. Ultra-thin sections were prepared applying semi-thin sectioning with a diamond knife. Finally samples were sputtered with gold for 1 minute and FIB-SEM electron microscopy was carried out using an Auriga 40 microscope from Zeiss.

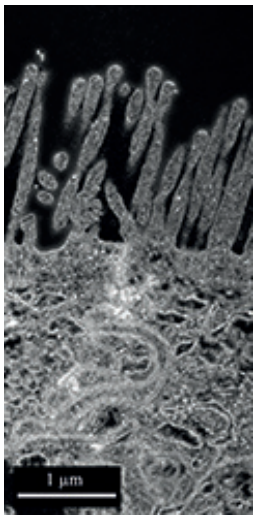

Figure 5: Electron microscopy.
The electron microscopical analysis reveals the formation of microvilli at the surface of Caco-2 cells cultivated on ThinCert™ cell culture inserts.

6. References

[1] Guguen-Guillouzo C, Guillouzo A. Isolated and cultured hepatozytes. 1986. Paris, Les Éditions INSERM, John Libbey, Eurotext: 1-12.

[2] Chambard M, Verrier B, Gabrion J, Mauchamp J. Polarisation of thyroid cells in culture: evidence for the basolateral localisation of the iodide „pump“ and of the thyroid-stimulating hormone receptor-adenyl cyclase complex. J Cell Biol. 983 Apr;96(4):1172-7.

[3] Letschert K, Komatsu M, Hummel-Eisenbeiss J, Keppler D. Vectorial transport of the peptide CCK-8 by double-transfected MDCKII cells stably expressing the organic anion transporter OATP1B3 (OATP8) and the export pump ABCC2. Mol Pharmacol. 2005 Oct;68(4):1031-8.

[4] Kopplow K, Letschert K, König J, Walter B, Keppler D. Human hepatobiliary transport of organic anions analyzed by quadruple-transfected cells. Mol Pharmacol. 2005 Oct;68(4):1031-8.

[5] Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res. 2001 Jul;42(7):1007-17.

[6] Krause JW. Krause‘s Essential Human Histology for Medical Students. 2005 Jul. Universal-Publishers.

7. Additional literature

--> Application Note „Immunocytochemistry“ (Ordering number F073100)
--> Application Note „Skin models“ (Ordering number: F074062)
--> Application Note „Co-culture“ (Ordering number: F074059)
--> Application Note „TEER and impedance measurements“ (Ordering number: F073037)
--> ThinCert™ Bibliography (Ordering number: F073905)

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