Article

Nanoparticle Intestinal Transport Characterization Using *In Vitro* Co-Culture Models

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Abstract: We co-cultured Caco-2 and HT29-MTX to ensure a tunable intestinal model and study the transport of two classes of nanoparticles. We exposed Caco-2/HT29-MTX of different seeding ratios, cultured on Transwell® systems, to non-cytotoxic concentration levels (20 μg/mL) of Si/SiO2 quantum dots and iron oxide (α-Fe2O3) nanoparticles. Transepithelial electric resistance was measured before and after exposure, and permeability was assessed via the paracellular marker Lucifer Yellow. At regular intervals during the 3-hour transport study, samples were collected from the basolateral compartments for detection and quantitative testing. Cell morphology characterization was done by phalloidin-FITC/DAPI labelling, and Alcian Blue/eosin staining was performed on insert cross-sections in order to compare the intestinal models and evaluate the production of mucins. Morphological alterations of the Caco-2/HT29-MTX (7:3 ratio) co-cultures were observed at the end of the transport study compared to the controls. The nanoparticle suspensions tested did not diffuse across the intestinal model and were not detected in the receiving compartments, due to their tendency to precipitate at the monolayer surface level and form visible aggregates. These preliminary results indicate the need for further nanoparticle functionalization in order to appropriately assess intestinal absorption in vitro.

Keywords: intestinal mucosa; co-culture intestinal model; Caco-2; HT29-MTX; nanoparticle transport; quantum dots; iron oxide nanoparticles.

1. Introduction

Oral drug administration is the preferred route when it comes to delivering most active compounds, due to many considerations, including patient comfort, reducing chances of infection and it being the least invasive route when compared to alternative delivery systems. The assimilation process via the gastrointestinal (GI) tract is a paramount condition for the delivery of any xenobiotic active compound to target tissues. In order to reach the system circulation, orally administered drugs usually have to pass through the small intestinal barrier. However, some active compounds are

especially vulnerable to the harsh GI environment and, therefore, require protection during transit in order to prevent degradation [1]. In this context, nanoparticles constitute novel candidates as future carrier-type agents [2]. Furthermore, human exposure to food products containing nanoparticulate materials is projected to increase in the near future, which calls for the development of a reliable screening solution [3].

The intestinal mucosa constitutes the major absorption site in vivo, with nutrient and xenobiotics having to penetrate two types of barriers – an acellular, mucus layer, respectively the intestinal epithelium. There is currently a wide array of in vitro intestinal models being used in studies that aim to estimate/predict drug absorption in vivo. Even though there has been a surge in the development of organotypic (3D) models [4–6] and organs-on-chip (body-on-a-chip) systems [7,8], the majority of transport studies rely on simpler, in vitro co-culture models using conventional cell lines. There are many advantages in terms of cost, good reproducibility and fidelity, yet the use of tumoral cell lines (which is common practice in most 2D in vitro intestinal models) raises several concerns regarding the ability of the models to reflect in vivo intestinal absorption in a pertinent manner. More often than not, tumoral cells are found to overexpress key proteins [9,10], and they generally exhibit an altered transcriptional regulation phenotype that may impact tissue permeability. For example, the Caco-2 adenocarcinoma cell line has been used extensively for the past couple of decades in nutrient and drug transport studies as an adequate in vitro model of the intestinal mucosa [11,12]. However, due to the over-expression of tight junction protein complexes [13–17], simple Caco-2 monolayers fail to provide a reliable estimation in terms of in vivo paracellular permeability of small hydrophilic compounds. To address this issue, Caco-2 cells are routinely co-cultured alongside HT29-MTX (goblet-like) cells [15] on Transwell® inserts.

This co-culture model allows for intercellular junction geometry modulation thereby fine-tuning the effective permeability (P_{eff}) of the monolayer by simply adjusting the initial cell seeding ratio [14]. The human adenocarcinoma line HT-29 preconditioned in methotrexate (MTX) has the added benefit of expressing mucins in culture [18] – the subsequently mucus layer produced constitutes an additional physical barrier [19], potentially impeding xenobiotic transport across the epithelium as would be the case in vivo conditions. Intestinal permeability correlates with the rate of compound transport across the mucosa which is calculated according to the following equation:

$$P_{eff} = \frac{dQ\,V}{dt\,A\,C_o}\ [cm/s], \quad (1)$$

where **dQ/dt** represents the apparent flow rate in time across the monolayer (mM/mL·s), **V** is the volume (mL) within the receiving compartment (BL), $\mathbf{C_0}$ is the initial concentration of compound (mM) in the donor compartment (AP), and **A** is the exposed tissue surface area (cm^2).

$$F = f_a\,(1 - E_G) \cdot (1 - E_H), \quad (2)$$

where fa is the absorbed fraction of the dose administrated (mass/dose), while considering first-pass metabolism of the compound in the gut wall (E_G) and liver (E_H). These are variables which current in vitro co-culture models can not account for.

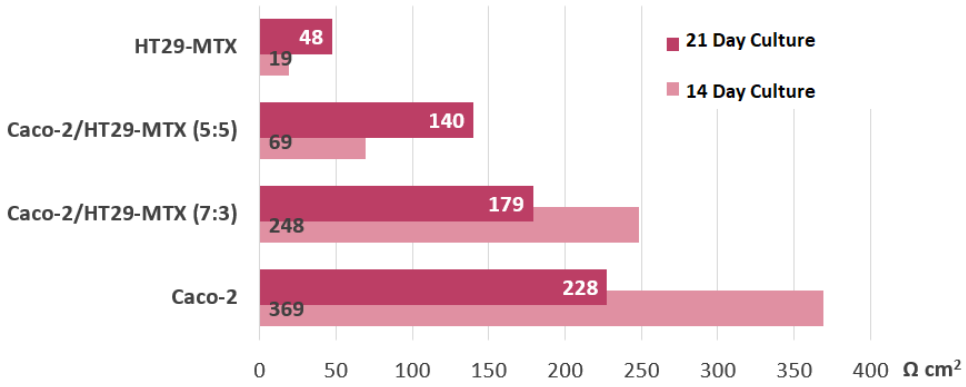
Although Caco-2/HT29-MTX co-cultures are routinely used in transport studies, the model is yet to be fully characterized, especially when compared to the well-established Caco-2 monoculture model. The present study aims to add to the already established body of work in this direction [13,21], while assessing the potential of two types of nanoparticles for oral drug delivery or screening perspectives. We established several Caco-2/HT29-MTX cultures by altering the initial seeding ratios (10:0, 7:3, 5:5, 0:10). The cells were cultured using Transwell® systems and were allowed to develop into stable monolayers for 21 days before exposing them to non-cytotoxic concentration levels (20 μg/mL) of Si/SiO2 quantum dots and iron oxide (α-Fe2O3) nanoparticles. Transepithelial electric resistance (TEER) was measured before and after exposure, and monolayer permeability (Peff) was assessed via the paracellular marker Lucifer Yellow. At regular intervals during the 3-hour transport study, samples were collected from the basolateral compartments for detection and quantitative testing. Cell morphology characterization was done by phalloidin-FITC/DAPI labelling, and Alcian

89 Blue/eosin staining was performed on insert cross-sections in order to compare the intestinal models
90 and evaluate the production of mucins.

91 **2. Results**

92 The intestinal models tested in the present study recorded similar TEER values to those reported
93 in the literature. After 14 days in culture, the Caco-2 cell line alone produced a very compact
94 monolayer ($TEER_{\mu}$ = 369 Ω cm²); measurements taken a week after revealed a steep drop to 228 Ω
95 cm², and a similar phenomenon was observed in the case of the 7:3 (seeded ratio) Caco-2/HT29-MTX
96 co-cultures. This would indicate a tissular integrity alteration of around 38%, as seen in Figure 1. Co-
97 cultures initially seeded at equal ratios and goblet cell-like monocultures evolved in a predictable
98 scenario of steadily increasing TEER in time. Nonetheless, after 21 days the TEER values overall still
99 maintained a similar differential rate of increase across models, corresponding to the increasing ratio
100 of Caco-2 initially seeded.

101 However, TEER measurements one week apart showed that the co-cultures with the higher ratio
102 of Caco-2 (7:3), as well as the Caco-2 monolayers alone, were lower than expected. One possible
103 explanation of this is the fact that, unlike HT29-MTX, the Caco-2 cell line has higher requirements in
104 terms of cell culture media and cellular expansion is more likely inhibited in a post-confluent setting.
105 Other studies also indicate the degradation of TEER after a certain time in culture has passed, thereby
106 resulting in more permeable monolayers for compounds that employ the paracellular route.



107

108 **Figure 1.** TEER evolution before initiating the transport study.

109 Regardless of whether the models were exposed to nanoparticle suspension or not, at the end of
110 the experiment all groups displayed significantly lower TEER values (see Figure 2), which suggests
111 that other variables at play affect monolayer integrity. Interestingly, when compared to the
112 monocultures, the co-culture models recorded the highest alteration levels, but *only* if they were
113 exposed to either type of nanoparticle suspension during this time.

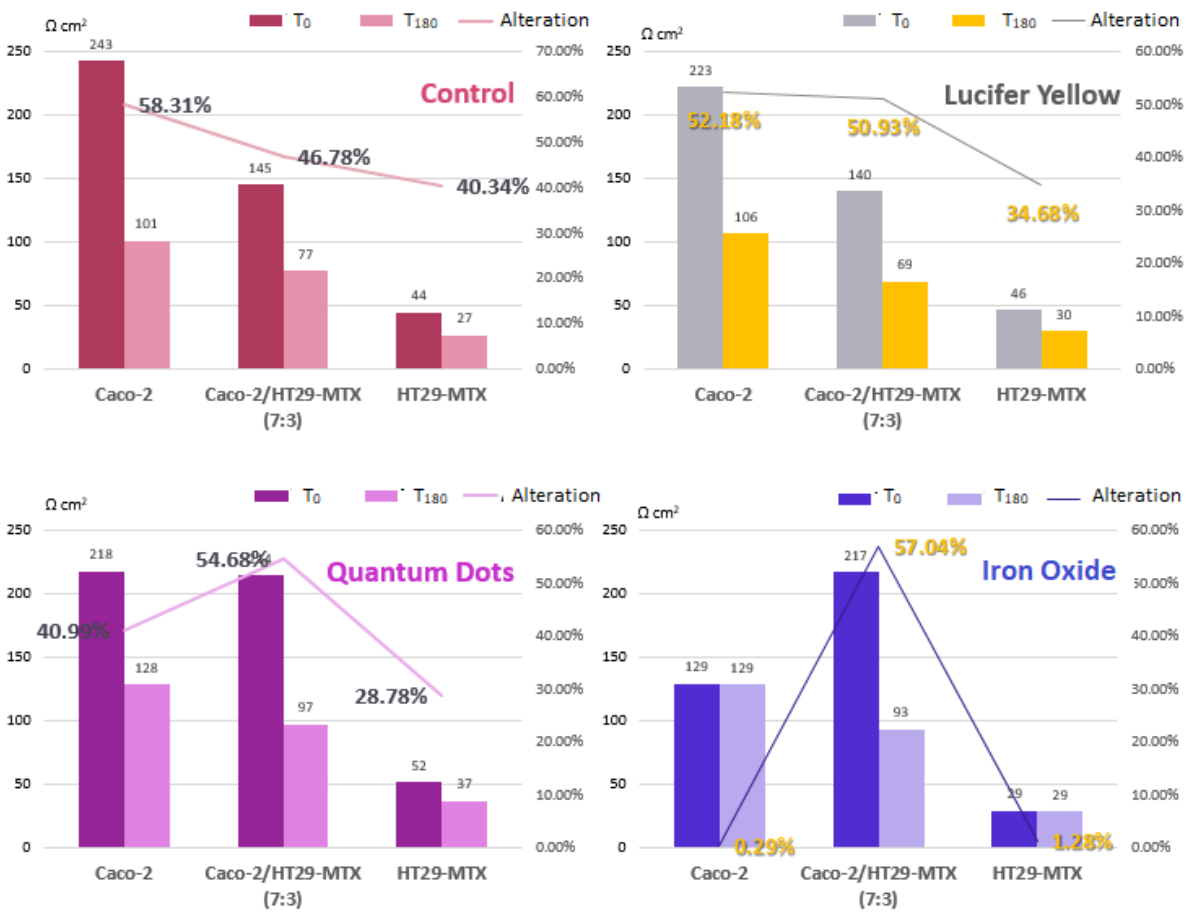


Figure 2. TEER alteration levels – comparison between three intestinal models (Caco-2, HT29-MTX and co-culture), before initiating the transport study (T_0) and after three-hour exposure to the nanoparticles (T_{180}).

Further qualitative assessments corroborate the TEER results. Comparative characterization of the monolayers after exposure via Alcian blue (Figure 3) and F-actin staining (Figure 4) revealed morphological alterations following nanoparticle exposure.

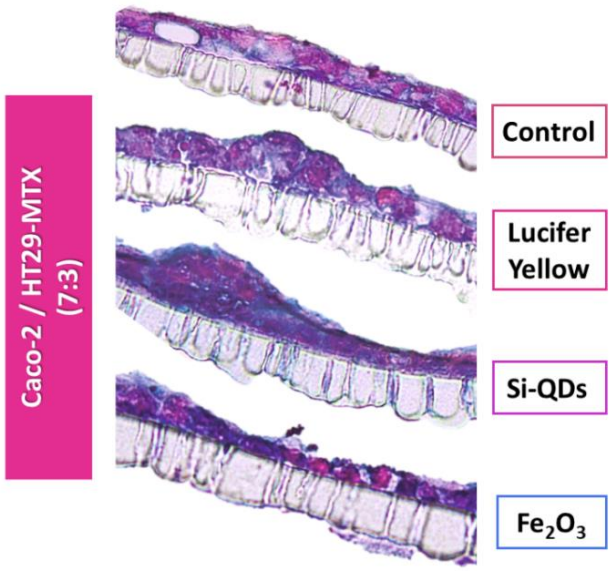


Figure 3. Insert cross-sections of the **Caco-2/HT29-MTX** intestinal model after the three-hour transport study. The cells were stained with 1% Alcian blue/0,1% eosin in order to detect mucin production. Monolayer disruptions of the experimental groups are also visible.

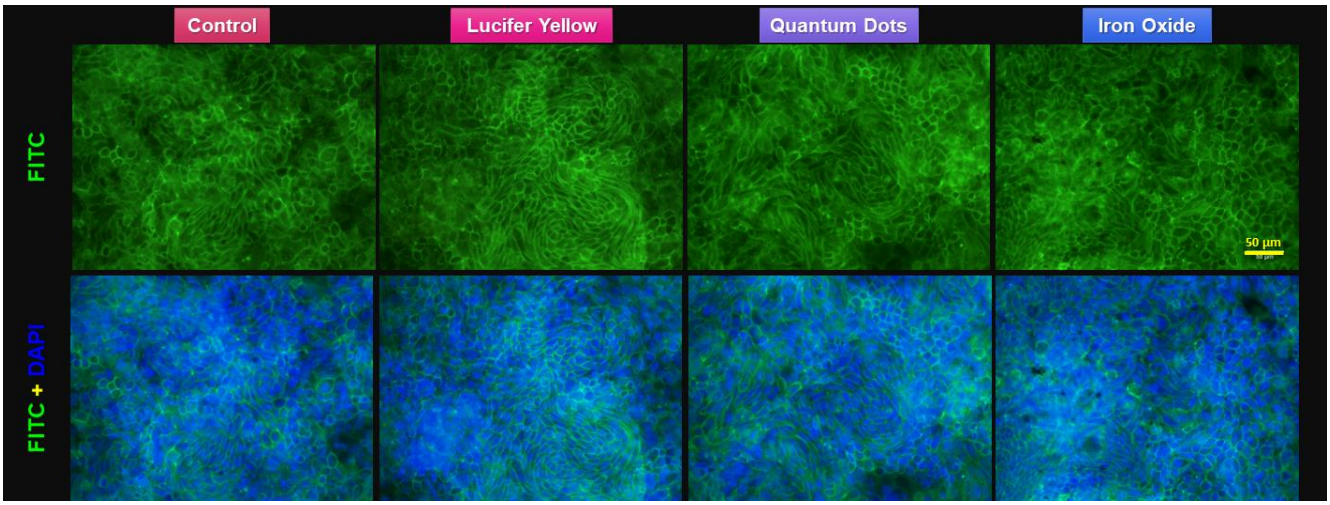


Figure 4. Fluorescence staining of the actin cytoskeleton of **Caco-2/HT29-MTX** monolayer after the three-hour transport study. F-actin is marked with fluorescein (FITC)-phalloidin (green). Nuclear counterstain with DAPI (blue).

During the 3-hour transport study, neither Si-quantum dots, nor iron oxide nanoparticles permeate across the monolayer insert and reach the receiving compartment (data not shown). The paracellular marker Lucifer Yellow passively diffused and reached the basolateral compartment where it was detected (Figure 5). Lucifer Yellow transport rate correlates with TEER measurements – the progressive integrity loss of the monolayers and, implicitly, the loosening of tight junction complexes established by Caco-2 cells would result in the increase of effective permeability even without considering their initial seeding ratio.

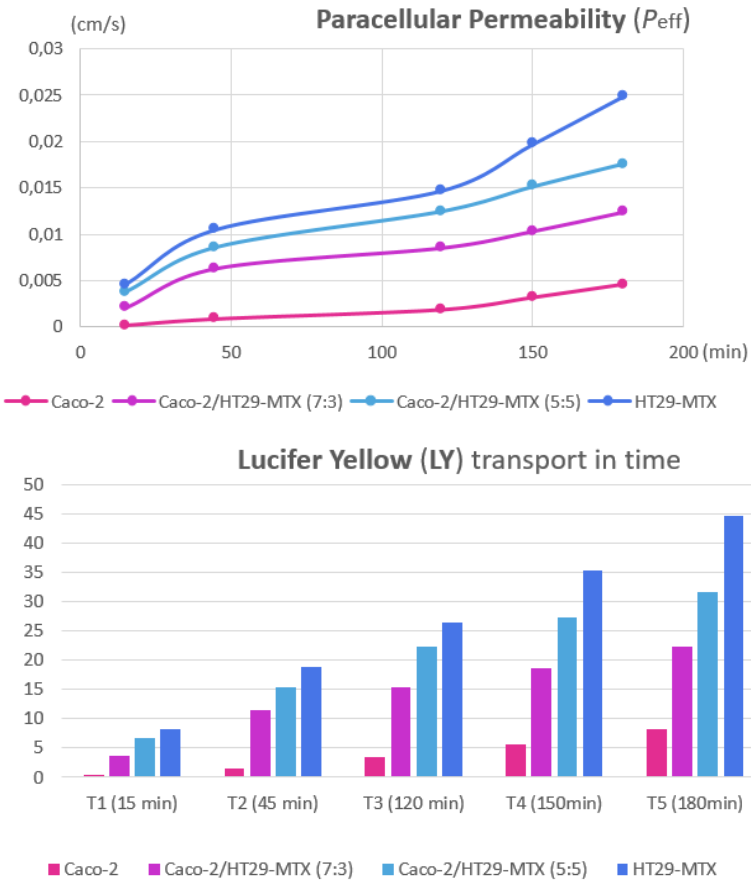


Figure 5. Effective permeability increases across all intestinal *in vitro* variants (top panel). Lucifer Yellow transport in time (bottom panel) shows the relative amount (in percentages) of paracellular marker which was detected at five measurement points during the experiment.

3. Discussion

Drug permeability coefficients have been historically assessed using simple Caco-2 cultures. This classical intestinal model does present itself with many limitations, one of which is an abnormally high transepithelial electrical resistance (TEER). TEER measurements ensure a consistent monitoring of tissular integrity due to it being a highly sensitive, non-invasive technique. TEER values also inversely correlate with paracellular permeability, making it the method of choice for intestinal transport studies [22]. *In vivo* studies reveal different TEER values between different regions of the human gut; post confluent Caco-2 monocultures are known to generate signals that vary between 150 and 500 Ω cm^2, whereas *in vivo* TEER recordings place the intestinal epithelium in a realistic range of 12-69 Ω cm^2 [14,22]. This significant difference can be explained by the fact that Caco-2 cells in culture form many more tight junctions.

After 14 days in culture, the Caco-2 cell line alone produced a very compact monolayer ($TEER_\mu$ = 369 Ω cm^2); measurements taken a week after revealed a steep drop to 228 Ω cm^2, and a similar phenomenon was observed in the case of the 7:3 (seeding ratio) Caco-2/HT29-MTX co-cultures. This would indicate a tissular integrity alteration of around 38%. Co-cultures initially seeded at equal ratios and goblet cell-like monocultures evolved in a predictable scenario of steadily increasing TEER in time. Nonetheless, after 21 days the TEER values overall still maintained a similar differential rate of increase across models, corresponding to the increasing ratio of Caco-2 initially seeded.

However, TEER measurements taken one week later showed that the co-cultures with the higher ratio of Caco-2 (7:3), as well as the Caco-2 monolayers alone, were lower than expected. One possible explanation of this is the fact that, unlike HT29-MTX, the Caco-2 cell line has higher requirements in terms of cell culture media and cellular expansion is more likely inhibited in a post-confluent setting. Other studies also indicate the degradation of TEER after a certain time in culture has passed, thereby resulting in more permeable monolayers for compounds that employ the paracellular route. This hypothesis does not account for the higher alteration levels recorded by the co-culture models that were part of the experimental groups. This preliminary result suggests that even though the nanoparticle suspensions were designed to not reach cytotoxic levels for either cell line, the co-culture model somehow makes them more vulnerable and more responsive to them.

One possible explanation is given by other authors [23] who attribute the cytotoxic effects of some nanoparticulate materials to their inherent tendency to precipitate on top of the cells and form visible aggregates. We noticed a similar phenomenon occurring, prompting further work in order to properly functionalize them and increase their solubility in buffer solutions. Iron oxide nanoparticles are often challenging to work with in this regard – for example, cellular intake of superparamagnetic iron oxide nanoparticle variants (UPSIO NPs) required further functionalization with an oleic acid coating [24].

In conclusion, the current study partly achieved its goals concerning the characterization of *in vitro* intestinal model Caco-2/HT29-MTX in a transport study setting. Further quantitative testing for the purpose of evaluating nanoparticle toxicity is required, as well as preventing aggregation.

4. Materials and Methods

4.1. Nanoparticles

Si-Quantum dots (Si/SiO_2 QDs) and iron oxide(α-Fe_2O_3) used in this study were produced at the Laser Department from the National Institute of Lasers, Plasma and Radiation Physics, Bucharest-Măgurele. The production process and characterization of the nanomaterials are detailed in previous work [25,26]

4.2. Cell Culture conditions

In a preliminary phase, Caco-2 (CRL-2102) and HT-29 (HTB-38) cell lines were grown separately in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% PSA. The cultures were maintained in humid atmosphere at 37°C, 5% CO_2 and were routinely subcultured once a week with trypsin-EDTA (0.25%, 0.53 mM). For several months HT-29 cells had to undergo treatment with methotrexate (MTX) according to the original protocol developed by *Lesuffleur et al.* [27].

Subsequently to the stabilization of HT29-MTX (mucus-secreting) clones, co-cultures were initiated. The cells were seeded on 12-well plates with Transwell® inserts (with polycarbonate membranes, 3 μm pore size) at a final density of 100,000 cells per well, regardless of the final seeding ratio (Caco2:HT29-MTX): 10:0, 7:3, 5:5, 0:10.

4.3. Transport Study

We exposed Caco-2, HT29-MTX and co-cultures to non-cytotoxic concentration levels (20 μg/mL) of Si/SiO2 quantum dots and iron oxide (α-Fe2O3) nanoparticles, and to Lucifer Yellow (50 ug/mL). HBSS was chosen as transport buffer. Particle quantification was done by measuring absorbance/fluorescence levels for each (Iron oxide NPs: Abs. 325/500; Si-QDs: Ex/Em 325/644; LY: Ex/Em 405/535) using the Flex Station 3 Multireader. TEER monitoring was performed using a Millipore® Millicell Electrical Resistance (ERS) system. Measurements were performed in three different points of each well, and final TEER was calculated after this formula:

$$\mathbf{TEER_{final}} = (TEER_{mean}\ [\Omega] - TEER_{blank}\ [\Omega]) \times A_{well}\ (1.12cm^2)\ [\Omega\ cm^2]$$

At the end of the experiment, a select part of Transwell inserts were removed and fixed în 4% PFA (paraformaldehyde) for 24 hours before being paraffinized, sectioned (5 μm thick cross-sections) and stained with 1% Alcian Blue-8GX and 0.1% eosin. The procedure was done according to the manufacturer kit (Bio-Optica) instructions. Images were captured using Olympus BX43 (XC30 software).

Other cells cultured on inserts were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 – 2% bovine serum albumin for 40 minutes. The F-actin was stained for 30 minutes with 10 μg/ml phalloidin-FITC (fluorescein isothiocyanate) and the nuclei were counterstained with 2 μg/ml DAPI (4',6-diamino-2-phenylindole). The fibroblasts were examined with an inverted fluorescence microscope Olympus IX71 (Olympus, Tokyo, Japan).

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References

[1] Wan, Z.-L.; Guo, J.; Yang, X.-Q. Plant Protein-Based Delivery Systems for Bioactive Ingredients in Foods. *Food Funct.*, **2015**, *6*, 2876–2889.

[2] Schimpel, C.; Teubl, B.; Absenger, M.; Meindl, C.; Fröhlich, E.; Leitinger, G.; Zimmer, A.; Roblegg, E. Development of an Advanced Intestinal in Vitro Triple Culture Permeability Model to Study Transport of Nanoparticles. *Mol. Pharm.*, **2014**, *11*, 808–818.

[3] Walczak, A.P.; Kramer, E.; Hendriksen, P.J.M.; Tromp, P.; Helsper, J.P.F.G.; Van Der Zande, M.; Rietjens, I.M.C.M.; Bouwmeester, H. Translocation of Differently Sized and Charged Polystyrene Nanoparticles in in Vitro Intestinal Cell Models of Increasing Complexity. *Nanotoxicology*, **2015**, *9*, 453–461.

[4] Chen, Y.; Lin, Y.; Davis, K.M.; Wang, Q.; Rnjak-Kovacina, J.; Li, C.; Isberg, R.R.; Kumamoto, C.A.; Mecsas, J.; Kaplan, D.L. Robust Bioengineered 3D Functional Human Intestinal Epithelium. *Sci. Rep.*, **2015**, *5*, 1–11.

[5] Leushacke, M.; Barker, N. Ex Vivo Culture of the Intestinal Epithelium: Strategies and Applications. *Gut*, **2014**, *63*, 1345–1354.

[6] Costello, C.M.; Hongpeng, J.; Shaffiey, S.; Yu, J.; Jain, N.K.; Hackam, D.; March, J.C. Synthetic Small Intestinal Scaffolds for Improved Studies of Intestinal Differentiation. *Biotechnol. Bioeng.*, **2014**, *111*, 1222–1232.

[7] Sarmento, B.; Andrade, F.; da Silva, S.B.; Rodrigues, F.; das Neves, J.; Ferreira, D. Cell-Based in Vitro Models for Predicting Drug Permeability. *Expert Opin Drug Metab Toxicol*, **2012**, *8*, 607–621.

[8] Sakolish, C.M.; Esch, M.B.; Hickman, J.J.; Shuler, M.L.; Mahler, G.J. Modeling Barrier Tissues In Vitro: Methods, Achievements, and Challenges. *EBioMedicine*, **2016**, *5*, 30–39.

[9] Fanning, A.S.; Van Itallie, C.M.; Anderson, J.M. Zonula Occludens-1 and -2 Regulate Apical Cell Structure and the Zonula Adherens Cytoskeleton in Polarized Epithelia. *Mol. Biol. Cell*, **2012**, *23*, 577–590.

[10] Selga, E.; Noé, V.; Ciudad, C.J. Transcriptional Regulation of Aldo-Keto Reductase 1C1 in HT29 Human Colon Cancer Cells Resistant to Methotrexate: Role in the Cell Cycle and Apoptosis. *Biochem. Pharmacol.*, **2008**, *75*, 414–426.

[11] Artursson, P.; Palm, K.; Luthman, K. Caco-2 Monolayers in Experimental and Theoretical Predictions of Drug Transport. *Adv. Drug Deliv. Rev.*, **2012**, *64*, 280–289.

[12] Lechanteur, A.; Almeida, A.; Sarmento, B. Elucidation of the Impact of Cell Culture Conditions of Caco-2 Cell Monolayer on Barrier Integrity and Intestinal Permeability. *Eur. J. Pharm. Biopharm.*, **2017**, *119*, 137–141.

[13] Hilgendorf, C.; Spahn-langguth, H.; Regårdh, C.G.; Lipka, E. Caco-2 versus Caco-2 / HT29-MTX Co-Cultured Cell Lines : Permeabilities Via Diffusion , Inside- and Outside-Directed Carrier-Mediated Transport. *J. Pharm. Sci.*, **2000**, *89*, 63–75.

[14] Béduneau, A.; Tempesta, C.; Fimbel, S.; Pellequer, Y.; Jannin, V.; Demarne, F.; Lamprecht, A. A Tunable Caco-2/HT29-MTX Co-Culture Model Mimicking Variable Permeabilities of the Human Intestine Obtained by an Original Seeding Procedure. *Eur. J. Pharm. Biopharm.*, **2014**, *87*, 290–298.

[15] Walter, E.; Janich, S.; Roessler, B.J.; Hilfinger, J.M.; Amidon, G.L. HT29-MTX/Caco-2 Cocultures as an in Vitro Model for the Intestinal Epithelium: In Vitro-in Vivo Correlation

with Permeability Data from Rats and Humans. *J. Pharm. Sci.*, **1996**, *85*, 1070–1076.

[16] Artursson, P.; Karlsson, J. Correlation between Oral Drug Absorption in Humans and Apparent Drug Permeability Coefficients in Human Intestinal Epithelial (Caco-2) Cells. *Biochem. Biophys. Res. Commun.*, **1991**, *175*, 880–885.

[17] Van Itallie, C.M.; Anderson, J.M. Architecture of Tight Junctions and Principles of Molecular Composition. *Semin. Cell Dev. Biol.*, **2014**, *36*, 157–165.

[18] Kitamura, H.; Cho, M.; Lee, B.H.; Gum, J.R.; Siddiki, B.B.; Ho, S.B.; Toribara, N.W.; Lesuffleur, T.; Zweibaum, A.; Kitamura, Y.; Yonezawa, S.; Kim, Y.S. Alteration in Mucin Gene Expression and Biological Properties of HT29 Colon Cancer Cell Subpopulations. *Eur. J. Cancer*, **1996**, *32*, 1788–1796.

[19] Lechanteur, A.; das Neves, J.; Sarmento, B. The Role of Mucus in Cell-Based Models Used to Screen Mucosal Drug Delivery. *Adv. Drug Deliv. Rev.*, **2017**.

[20] Lennernäs, H. *Intestinal Permeability and Its Relevance for Absorption and Elimination*; **2007**; Vol. 37.

[21] Mahler, G.J.; Shuler, M.L.; Glahn, R.P. Characterization of Caco-2 and HT29-MTX Cocultures in an in Vitro Digestion/Cell Culture Model Used to Predict Iron Bioavailability. *J. Nutr. Biochem.*, **2009**, *20*, 494–502.

[22] Srinivasan, B.; Kolli, A.R.; Esch, M.B.; Abaci, H.E.; Shuler, M.L.; Hickman, J.J. TEER Measurement Techniques for In Vitro Barrier Model Systems. *J. Lab. Autom.*, **2015**, *20*, 107–126.

[23] Soto, K.; Garza, K.M.; Murr, L.E. Cytotoxic Effects of Aggregated Nanomaterials. *Acta Biomater.*, **2007**, *3*, 351–358.

[24] Kenzaoui, B.H.; Vilà, M.R.; Miquel, J.M.; Cengelli, F.; Juillerat-Jeanneret, L. Evaluation of Uptake and Transport of Cationic and Anionic Ultrasmall Iron Oxide Nanoparticles by Human Colon Cells. *Int. J. Nanomedicine*, **2012**, *7*, 1275–1286.

[25] Stan, M.S.; Memet, I.; Sima, C.; Popescu, T.; Teodorescu, V.S.; Hermenean, A.; Dinischiotu, A. Si/SiO2quantum Dots Cause Cytotoxicity in Lung Cells through Redox Homeostasis Imbalance. *Chem. Biol. Interact.*, **2014**, *220*, 102–115.

[26] Radu, M.; Munteanu, M.C.; Petrache, S.; Serban, A.I.; Dinu, D.; Hermenean, A.; Sima, C.; Dinischiotu, A. Depletion of Intracellular Glutathione and Increased Lipid Peroxidation Mediate Cytotoxicity of Hematite Nanoparticles in MRC-5 Cells. *Acta Biochim. Pol.*, **2010**, *57*, 355–360.

[27] Lesuffleur, T.; Barbat, A.; Dussaulx, E.; Zweibaum, A. Growth Adaptation to Methotrexate of HT-29 Human Colon Carcinoma Cells Is Associated with Their Ability to Differentiate into Columnar Absorptive and Mucus-Secreting Cells. *Cancer Res.*, **1990**, *50*, 6334–6343.

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