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Published in:
European Journal of Pharmaceutics and Biopharmaceutics

Link to article, DOI:
[10.1016/j.ejpb.2019.08.011](https://doi.org/10.1016/j.ejpb.2019.08.011)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Jørgensen, J. R., Jepsen, M. L., Nielsen, L. H., Dufva, M., Nielsen, H. M., Rades, T., Boisen, A., & Müllertz, A. (2019). Microcontainers for oral insulin delivery – In vitro studies of permeation enhancement. *European Journal of Pharmaceutics and Biopharmaceutics*, 143, 98-105. <https://doi.org/10.1016/j.ejpb.2019.08.011>

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1 **Microcontainers for oral insulin delivery – *in vitro* studies of permeation enhancement**

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14 **Abstract**

15 Oral delivery of peptides is challenging due to their low uptake through the small intestinal epithelium. Tight
16 junctions, connecting the enterocytes, impede permeability, often necessitating the use of permeation enhancers
17 in the formulation. Loading of peptide and permeation enhancer into micro-scale devices, such as
18 microcontainers, can potentially confine the effective absorptive area through unidirectional release and thereby
19 enhance absorption. This concept is investigated by *in vitro* permeation studies of insulin across Caco-2 cell and
20 Caco-2/HT29-MTX-E12 co-culture monolayers mimicking the intestinal absorption barrier. The importance of
21 proximity between the microcontainers and the barrier is assessed, by keeping the amounts of insulin and sodium
22 caprate fixed throughout all experiments, while collectively orienting the unidirectional release towards the cell
23 monolayers. Increasing the distance is observed to have a negative effect on insulin permeation matching a one-
24 phase exponential decay function, while no difference in insulin transport is observed between Caco-2 and co-
25 culture monolayers. Although there are no signs of cytotoxicity caused by the microcontainer material, reversible
26 cell deterioration, as a consequence of high local concentrations of sodium caprate, becomes evident upon
27 qualitative assessment of the cell monolayers. These results both suggest a potential of increasing oral
28 bioavailability of peptides by the use of microcontainers, while simultaneously visualising the ability of
29 regaining monolayer integrity upon local permeation enhancer induced toxicity.

30 Keywords: oral peptides; micro devices; permeation enhancers; sodium caprate (C₁₀); Caco-2 cells.

31 1. Introduction

32 Oral delivery of macromolecules has been a major aim in drug delivery ever since the discovery of insulin in the
33 1920s (Moroz et al., 2016). The field has seen significant progress within the last decade, resulting in several
34 oral peptide formulations advancing to phase II and III clinical trials. However, only two oral dosage forms for
35 systemic delivery of peptides with molecular sizes higher than 500 Da have reached the market, namely;
36 Neoral[®]/Sandimmune[®] (Cyclosporine A, Novartis) and Minirin[®] (Desmopressin, Ferring Pharmaceuticals)
37 (Aguirre et al., 2016). Regardless of which peptide or protein is attempted for oral delivery, the main challenges
38 come down to their relatively large size, hydrophilicity and chemical predisposition to degradation; all together
39 leading to low oral bioavailability (Moroz et al., 2016; Smart et al., 2014). Protecting peptides from both pH and
40 enzyme catalysed degradation in the stomach has largely been achieved by enteric coatings, leaving the intestinal
41 environment as the main focus of developing delivery strategies (Felton and Porter, 2013; Thakral et al., 2013).
42 As dissolution of the enteric coating makes the peptide accessible to intestinal enzymes, further enzymatic
43 protection is often incorporated in oral formulations. Locally decreasing the pH below the optimal conditions for
44 enzymatic activity by co-release of citric acid or direct inhibition using competitive peptidase inhibitors are two
45 of such approaches (Bernkop-Schnürch, 1998; Welling et al., 2014).

46 However, increasing the fraction of native peptide reaching the enterocytes through gastric protection and
47 enzyme inhibition will not necessarily lead to higher bioavailability, as the permeability of peptides is hindered
48 by their aforementioned physicochemical properties. Overcoming the challenge of gaining peptide transport
49 across the enterocytes and into the bloodstream has been thoroughly investigated by the use of permeation
50 enhancers (Maher et al., 2016). By their interaction with the protein complexes forming the inter-enterocyte
51 connections, known as tight junctions, the paracellular transport of larger hydrophilic molecules can be
52 facilitated (Lindmark et al., 1998; Taverner et al., 2015). Several other interesting approaches in the field of
53 macromolecular absorption enhancement have likewise shown promising properties, such as liposome
54 formulations, cell-penetrating peptides and microneedle-based delivery devices (Abramson et al. 2019; Nielsen
55 et al., 2014; Parmentier et al., 2010; Traverso et al. 2015). Nevertheless, commercially available peptides for oral
56 delivery are currently limited to a mass of 1.2 kDa (Aguirre et al., 2016). Consequently, there is a need for novel
57 strategies for systemic delivery upon oral administration of larger macromolecules, such as the 5.8 kDa dipeptide
58 hormone, insulin, and glucagon-like peptide-1 (GLP-1) agonists both used in the treatment of diabetes mellitus.

59 Perhaps counter-intuitively, the large surface area ($>30\text{ m}^2$) of the small intestine (Helander and Fändriks, 2014)
60 might be a disadvantage when delivering peptides. As peptide and excipients are released, they are diluted in the
61 fluid along the epithelium often leading to fast absorption of the permeation enhancer (Buckley et al., 2018).

Potentially, this might lead to local permeation enhancer concentrations below their effective threshold and further to a reduced degree of peptide-permeation enhancer co-localisation. Spatial proximity has recently been shown to be crucial for the gastric uptake of the GLP-1 analog, semaglutide, which is governed by the permeation enhancer sodium *N*-[8-(2-hydroxybenzoyl) aminocaprylate] SNAC being present at a very confined area under and around the site of tablet disintegration (Buckley et al., 2018). Increasing the amount of drug and excipients is a way of compensating for the dilution effect in the gastrointestinal tract (GI-tract), however, higher doses will result in more expensive formulations, as well as larger dosage forms. Alternatively, the dilution could be reduced by confining the effective absorptive area of the intestine by the use of micro-fabricated delivery systems capable of unidirectional release. Such micro-devices have previously shown the potential to increase oral bioavailability of small molecules in rodents compared to controls of the same dose either in solution or as powder in capsules (Chirra et al., 2014; Mazzoni et al., 2017). Moreover, another study reported a tendency of the cylindrical-shaped microcontainers to become embedded in the intestinal mucus in rats (Nielsen et al., 2016). As this behaviour could minimise the release of the encapsulated microcontainer content into the intestinal lumen and thus lower the risk of enzymatic degradation, such unidirectionally releasing devices are of significant interest for oral delivery of peptides (Ahmed et al., 2002; Banerjee and Mitragotri, 2017; Whitehead et al., 2004).

In this study, the concept of utilising microcontainers to improve insulin permeation was investigated across Caco-2 cell culture and mucus-secreting Caco-2/HT29-MTX-E12 co-culture monolayers. Unidirectional release was optimised by collectively orientating the openings of the microcontainers towards the cell monolayers, while the amounts of permeation enhancer and insulin were kept constant throughout all the studies. This allowed for the assessment of the direct effect of proximity on insulin transport, by manipulating the distance between the monolayers and the point of release from the microcontainers. Distances similar to the thickness of mucus along the GI-tract of laboratory animals were chosen (0.2 – 2 mm) (Atuma et al., 2001; Varum et al., 2012). For all the studies, the medium chain fatty acid, sodium caprate (C₁₀), was used, due to its ability to enhance paracellular permeation (Lindmark et al., 1998; Maher et al., 2009). Furthermore, the importance of insulin and C₁₀ co-localisation was evaluated by loading microcontainers either with a mixture of the two components (1:1 w/w) or by loading the single components into separate microcontainers prior to the transport studies.

92 2. Materials and methods

93 2.1. Materials

94 Silicon wafers (4" (100) *n*-type) were obtained from Okmetic (Vantaa, Finland). SU-8 2075 and SU-8 Developer
95 were acquired from Micro Resist Technology (Berlin, Germany). Human recombinant insulin, bovine serum
96 albumin (BSA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Triton™ X-100, Dulbecco's
97 Modified Eagle's Medium (DMEM), penicillin-streptomycin, L-glutamine and MEM non-essential amino acid
98 solution (100x) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Capric acid sodium salt (C₁₀)
99 was obtained from abcr (Karlsruhe, Germany), fetal bovine serum from PAA Laboratories (Pasching, Austria)
100 and trifluoroacetic acid (TFA) from Carl Roth (Karlsruhe, Germany). Hanks' Balanced Salt Solution (HBSS,
101 calcium, magnesium, no phenol red), sodium bicarbonate solution and Hoechst 33342 solution were acquired
102 from Thermo Fisher Scientific (Waltham, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-
103 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) were
104 obtained from Promega (Madison, WI, USA). Paraformaldehyde (PFA) 16% (w/v) aqueous solution was
105 provided by Alfa Aesar (Haverhill, MA, USA). All other chemicals and solvents were of at least analytical grade
106 and obtained from commercial suppliers. Ultrapure water purified by an Ultra Clear UV system (Evoqua Water
107 Technologies, Pittsburgh, PA, USA) was used throughout the studies.

108

109 2.2 Fabrication and filling of microcontainers

110 The microcontainers were fabricated with the parameters previously described (Nielsen et al., 2012) based on the
111 published method by Tao et al. (2006). Briefly, the epoxy-based negative photoresist, SU-8, was dispensed and
112 spin coated onto silicon wafers followed by a baking step and lastly UV exposure using a chromium mask
113 creating the base of the microcontainers. These steps were then repeated with the use of a different chromium
114 mask in order to generate the walls of the microcontainers after which the non-polymerised SU-8 was removed
115 from the wafer. The silicon wafers were then cut into chips ($12.8 \times 12.8 \text{ mm}^2$) each holding 625 microcontainers.
116 The exact dimensions of the individual microcontainers have previously been determined with an average cavity
117 diameter of 188 μm and a volume capacity of 7.5 nL (Mazzoni et al., 2017). Prior to filling, a shadow mask was
118 aligned on top of the microcontainer chip in order to minimise the amount of powder being distributed between
119 the microcontainers, as previously illustrated (Abid et al., 2017). A powder mixture of insulin and C₁₀ (1:1 w/w)
120 was distributed on top of the shadow mask and subsequently loaded into the individual microcontainers by
121 centrifuging the microcontainer chip in a flat-bottomed Falcon™ tube using a Heraeus Megafuge 16R

122 Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at $3,720 \times g$ for 30-40 s at 21 °C. Any excessive
123 powder, between the microcontainers, was afterwards removed by pressurised air while simultaneously covering
124 the microcontainer openings with a flat silicon chip. Additionally, to investigate the importance of co-
125 localisation, insulin and C₁₀ were individually filled into each half of the microcontainer chip. This was done by
126 first covering one half of the shadow mask with a layer of tape prior to centrifuging with insulin, upon which the
127 shadow mask was removed and cleaned using pressurised air. Subsequently, the shadow mask was now placed
128 with the tape covering the insulin-filled microcontainers, followed by a second centrifugation step with C₁₀.
129 Scanning electron microscopy (SEM) was used to visualise both empty and filled microcontainers using a
130 Hitachi TM3030 tabletop microscope (Hitachi High-Technologies Europe, Krefeld, Germany) with 15 kV
131 accelerating voltage.

132

133 2.3 *In vitro* permeation studies with insulin

134 Caco-2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured under conditions and with
135 growth medium as previously described (Larsen et al., 2008). Both Caco-2 cells alone and 1:1 co-cultures of
136 Caco-2 cells and mucus-secreting HT29-MTX-E12 cells (Inserm, Paris, France) were cultured on polycarbonate
137 Transwell® filters with a surface area of 4.67 cm² and 0.4 µm pore size (Corning Costar from Sigma-Aldrich, St.
138 Louis, MO, USA). The Caco-2 cells were used from passage 26-48, and the HT29-MTX-E12 cells from passage
139 55-63. Both Caco-2 and co-culture monolayers were allowed to mature for 21-28 days prior to insulin
140 permeation studies. All studies were conducted from the apical to the basolateral side at 37 °C using 10 mM
141 HEPES-buffered HBSS (hHBSS) containing BSA (0.05% w/v). Solubilisation of both insulin and C₁₀ in the
142 hHBSS was ensured by adjusting the pH of both the donor and receptor compartment to 7.4, i.e. above the pKa
143 of C₁₀ of 6.5-7.2 (Kanicky et al., 2000; Maher et al., 2016) and more than two units above the isoelectric point of
144 insulin of 5.3 (Iyire et al., 2016). The cells were washed two times with hHBSS after which 1.50 mL and 2.60
145 mL hHBSS were added to the apical and basolateral side, respectively. The study was initiated by gently placing
146 a chip of microcontainers either directly on top of the monolayer ($d = 0.0$ mm) or at defined distances ($d = 0.2$,
147 0.5, or 2.0 mm). Placement of the microcontainers upside down was possible without loaded powder falling out,
148 due to its centrifugal compaction. The fixed distances were achieved by using custom-made
149 polytetrafluoroethylene (PTFE) carvings (Fig. 1).

150 A control group was included in which a chip of empty microcontainers was placed directly on the monolayer
151 together with a solution of 0.1 mM insulin and 3 mM C₁₀ (1:1 w/w) in hHBSS. These concentrations were
152 calculated based on a maximal loading capacity of 1.6 mg of insulin:C₁₀ (1:1 w/w) powder mixture per

153 microcontainer chip and an apical volume of 1.50 mL. The permeation study was then carried out with orbital
154 shaking (Compact Shaker KS 15 A, Edmund Bühler, Bodelshausen, Germany) of 75 rpm for 2 h at 37 °C with
155 basolateral sampling of 100 µL at 15, 30, 45, 60, 90 and 120 min. Each sample was replaced with 100 µL
156 preheated hHBSS to maintain a basolateral volume of 2.60 mL. Samples were stored at -20 °C until
157 quantification by reversed phase high-performance liquid chromatography (RP-HPLC). Cumulated transported
158 percentages of the total amount of insulin were calculated by including the apical concentration at 120 min. All
159 experiments were performed in triplicates and repeated over three passages (n = 3). The Caco-2 cell monolayers
160 were washed twice with hHBSS after the final sampling and evaluated regarding effects on the monolayers.
161 These evaluations were carried out both immediately after the permeation studies, as well as after subsequent
162 incubation periods of 24 h in growth medium at 5% CO₂ and 37 °C.

163

164 *2.4 Insulin quantification by RP-HPLC*

165 The permeation study samples were analysed by RP-HPLC-UV with 20 µL injection volume, using a Dionex
166 UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Kinetex XB-C18
167 column (100 × 4.60 mm, 5 µm, 100 Å; Phenomenex, Torrance, CA, USA). Elution was done with two mobile
168 phases: A: TFA in water (0.1% v/v) and B: TFA in acetonitrile (0.1% v/v) with a gradient of 0-3 min A-B (75:25
169 v/v) to A-B (20:80 v/v), 3-3.5 min A-B (20:80 v/v) to A-B (75:25 v/v), and 3.5-4.5 min A-B (75:25 v/v) and a
170 flow rate of 1.0 mL/min at 22 °C. Quantification of insulin was determined as the area under the curve (AUC) of
171 the UV-absorbance peak at 214 nm with retention time of 2.7 min. A new insulin standard curve ranging from 2-
172 100 µg/mL (LOD = 0.25 µg/ml) was prepared for each day of quantitative analysis.

173

174 *2.5 Caco-2 monolayer integrity and viability*

175 Transepithelial electrical resistance (TEER) was measured across the monolayers to assess their integrity using
176 an Epithelial Volt/Ohm Meter (EVOM) (World Precision Instruments, Sarasota, FL, USA) with Endohm
177 chambers. Measurements were performed before and after the transport studies, as well as upon recovery after
178 24 h of incubation under the same conditions as during culturing (Larsen et al., 2008). Cell viability after 2 h of
179 transport was evaluated for the cells having a chip of microcontainers loaded with insulin-C₁₀ powder mixture at
180 $d = 0.0$ mm. For this, a cell metabolic assay (MTS/PMS) was implemented and compared to a control group of
181 Caco-2 cells only being exposed to exchange of growth medium to hHBSS. A solution of MTS (240 µg/mL) and

182 PMS (2.4 µg/mL) in hHBSS was prepared immediately before use and 1.5 mL was added to the apical side of
183 the wells. The plate was then protected from light and incubated at 37 °C with orbital shaking of 75 rpm for 1 h.
184 Samples of 100 µl were taken in triplicates from each well and transferred to a 96-well plate for absorbance
185 measurements at 492 nm using a Labsystems Multiskan MS 352 Microplate Reader (Labsystems, Finland). Both
186 integrity and viability assays were performed in triplicates and repeated over three passages (n = 3).

187

188 2.6 Staining and visualisation of Caco-2 cells

189 Visualisation of cell nuclei was performed either immediately after the transport studies or upon the 24 h
190 recovery period. The Caco-2 cell monolayer only being exposed to hHBSS for 2 h was visualised for
191 comparison. Fixation of the cells was achieved by incubation of the monolayers in a paraformaldehyde solution
192 in hHBSS (4% w/v) for 15 min at room temperature. The cells were then permeabilised with a Triton™ X-100
193 aqueous solution (0.1% v/v) for 10 min after which any excess membrane protein binding sites were blocked
194 with a BSA solution in hHBSS (3% w/v) for 30 min, both at ambient temperature. Hoechst 33342 staining
195 solution (1 µg/mL), prepared in hHBSS immediately before use, was applied to the cells for 15 min at room
196 temperature while protected from light. The Transwell® filters were then cut out, placed on microscopy slides
197 and visualised at an excitation wavelength of 405 nm using an LSM 700 scanning confocal microscope (Carl
198 Zeiss, Oberkochen, Germany) with EC Epiplan Neofluar 10×/0.25 HD objective. Images were processed using
199 ImageJ version 1.52a (National Institute of Health, Bethesda, MD, USA).

200

201 2.7 Statistics

202 All data were handled using GraphPad Prism version 8.1.2 and expressed as mean ± standard deviation (SD)
203 unless otherwise stated. Comparisons of insulin transport were based on the slopes derived by linear regression
204 analysis of the transport profiles and defined as significant at p-values below 5% ($P < 0.05$) and very significant
205 at p-values below 1% ($P < 0.01$).

206

207

208

209 3. Results and discussion

210 3.1 Filling of microcontainers with insulin and C₁₀

211 Drug and permeation enhancer loading were carried out in one microcontainer chip (12.8 x 12.8 mm²) at a time
212 each holding 625 microcontainers. Utilising a swinging bucket centrifuge, ensured powder settlement
213 perpendicular to the axis of rotation, thereby compacting the powder into the microcontainers. Combined with a
214 shadow mask, this filling method is especially useful for filling of expensive powders, due to the minimisation of
215 powder accumulation between the microcontainers, which is otherwise difficult to retrieve. Microcontainer chips
216 were successfully filled either with the insulin and C₁₀ powder mixture, or with the individual powders (Fig. 2).
217 HPLC analysis confirmed efficient filling of insulin and C₁₀ at a 1:1 weight ratio upon loading of the powder
218 mixture resulting in 1.2 ± 0.2 mg of the powder mixture per microcontainer chip, equivalent to an average
219 microcontainer load of 1.0 µg of insulin.

220

221 3.2 Distance dependency studies

222 Insulin transport was initially monitored across Caco-2 cell monolayers using varied distances (0-2 mm) between
223 the monolayer and the microcontainers loaded with a mixture of insulin and C₁₀ (1:1 w/w). The effect of an
224 equivalent amount of insulin and C₁₀ in solution, 0.1 mM and 3 mM respectively, was also tested, together with
225 an empty microcontainer chip. The TEER was measured before and after the 2 h transport experiments as well as
226 upon a subsequent 24 h incubation period for the cell monolayers suffering the most significant loss of integrity,
227 due to direct contact with the microcontainers ($d = 0.0$ mm), (Fig. 3).

228 Based on the TEER values, the loss of cell integrity induced by C₁₀ increased with decreasing distances between
229 microcontainers and cell monolayer. Although the solution only resulted in a drop in TEER to 71% of the initial
230 value, the same amount released from microcontainers with $d = 0.0$ mm caused a drop to 27%. Regarding the
231 effect of specific concentrations of permeation enhancers, substantial lab-to-lab variability is reported in
232 literature (Maher et al., 2009), yet complete recovery of the TEER value has previously been shown after a
233 decrease to only 10% of the initial TEER triggered by a 8.5 mM solution of C₁₀ (Brayden et al., 2015). For the
234 current distance-dependency study, the highest risks of irreversible loss of integrity and cell damage were
235 expected in the experiments with $d = 0.0$ mm, as this likely would result in the highest local concentrations of
236 C₁₀. Incubating these Caco-2 monolayers for 24 h in growth medium subsequent to exposure, however, proved
237 their capability of regaining 86% of their initial TEER value. Insulin transport monitored across the Caco-2

monolayers followed the anticipated trend, i.e. insulin transport rates increased with decreasing distances between monolayer and microcontainers. Despite the fact that insulin permeation is influenced by the interplay of several mechanisms, (e.g. dissolution-/diffusion rates of insulin and C₁₀, and the rate of cell monolayer integrity loss), a relatively simple analysis could be used to fit the data. Plotting the transport rate constants, obtained by linear regression of the transport profiles over 2 h, as a function of the distance (d) identifies the mathematical correlation as a one-phase exponential decay regression (Fig. 4).

Upon release with $d = 0.0$ mm, 18% of insulin was transported to the basolateral side after 2 h. Even at $d = 2.0$ mm, a significant increase in insulin transport of more than 2-fold was observed compared to the solution (Fig. 6). From the rate constant-distance correlation, it is obvious that mucus, acting as a spacing layer, is likely to have a negative impact on the bioavailability. Based on the exponential equation: $Y = 8.29e^{-5.28x} + 0.57$, a distance increase of 0.13 mm results in a 50% decrease of the insulin transport rate through the Caco-2 monolayer, calculated as $\ln(2)/5.28$. While the thickness of the adherent mucus layer is relatively uniform (25-55 μm) throughout both rat and pig small intestines (Varum et al., 2012), larger variations of the non-adherent layer have been observed in rats. Thicknesses from 120-200 μm in the duodenum and jejunum to about 500 μm in the ileum and almost 1000 μm in the colon have been reported (Atuma et al., 2001). Although mucus potentially will have a negative effect on insulin absorption, it serves as a protecting layer throughout the GI-tract partly by minimising the risk of pathogen absorption (Cornick et al., 2015). However, as parallel uptake of e.g. pathogens is a common concern regarding the use of permeation enhancers, further evaluation of the monolayer integrity was performed using confocal laser-scanning microscopy.

257

3.3 Qualitative assessment of Caco-2 monolayers

An MTS/PMS viability assay demonstrated a relative monolayer metabolic activity of $93 \pm 2\%$ immediately after transport studies with $d = 0.0$ mm, compared to monolayers, which had only been subjected to exposure of hHBSS. This indicated either a negligible cytotoxic effect of C₁₀ distributed along the monolayer or alternatively more profound effects at local areas. Confocal laser-scanning microscopy images were obtained after staining the Caco-2 cell nuclei with Hoechst 33342 in order to qualitatively assess the local effect caused by C₁₀ during the transport studies (Fig. 5).

Fig. 5A-C show the Caco-2 cell nuclei density immediately after transport studies with $d = 0.0, 0.2$, and 0.5 mm, respectively. Areas, corresponding to microcontainer openings, of complete absence of Caco-2 cells, were apparent after the 2 h permeation study with $d = 0.0$ mm (Fig. 5A). Despite this pronounced toxic effect on the

268 Caco-2 cells, the relatively low drop in viability compared to the drop in TEER can be explained by the confined
269 environments of high C_{10} concentrations. Considering a mean diameter of the microcontainer cavities of 188 μm ,
270 the total area of 625 microcontainers only corresponds to 3.6% of the total area of the monolayer. Likewise,
271 areas of low Caco-2 cell nuclei densities were visible after a release at $d = 0.2$ mm (Fig. 5B). The toxic effects of
272 C_{10} , and surfactants in general, are well known at concentrations even below their critical micelle concentration
273 (Maher et al., 2009). In this case the cytotoxicity manifests itself as local disruptions of the monolayer, resulting
274 in the relatively high transport rates for the short microcontainer-monolayer distances ($d = 0.0$ - 0.2 mm), as seen
275 in Fig. 4. However, despite a TEER value decrease to 60% of the initial value after 2 h permeation study with d
276 $= 0.5$ mm, no variation in the monolayer integrity was observed when visualising the cell nuclei (Fig. 5C),
277 compared to monolayers being subjected to 2 h exposure of hHBSS (Fig. 5F). This indicates that the 4.2-fold
278 increase in insulin flux, observed with $d = 0.5$ mm, compared to the 0.1 mM insulin and 3 mM C_{10} solution, was
279 triggered by paracellular transport across an intact monolayer, rather than by local deterioration of the barrier.
280 No distinguishable impressions of microcontainers were visible on the cell monolayers after exposure to the
281 solution in combination with a chip of empty microcontainers placed directly on the monolayer (Fig. 5E).
282 Permeation enhancing and cytotoxic effects must therefore be a consequence of high local concentrations of C_{10} ,
283 rather than an effect caused by microcontainer material itself. Cell proliferation and/or migration of cells to the
284 compromised areas of the cell monolayers with $d = 0.0$ mm was clearly visible upon 24 h incubation in growth
285 medium, as the areas of complete absence of nuclei had restored some extent of integrity (Fig. 5D) in accordance
286 with the recovering TEER values. A feature unlikely to happen had the whole cell monolayer been exposed to a
287 cytotoxic C_{10} concentration similar that of the local areas under the microcontainers (Chao et al., 1999; Sakai et
288 al., 1998; Shima et al., 1999).

289 There is an on-going debate regarding the risks of utilising permeation enhancers for oral formulations
290 (McCartney et al., 2016). Certainly, these considerations also need to be taken into account when promoting
291 permeation enhancement through confined high concentrations. The risk of co-absorption is of concern as
292 opening of tight junctions and/or cell membrane perturbation could facilitate the concurrent systemic uptake of
293 e.g., pathogens (McCartney et al., 2016). Although the results obtained from the cell integrity and viability
294 assays did not immediately give rise to any concerns, the confocal images of the monolayers upon 2 h exposure
295 to high local concentrations of C_{10} clearly depict the reasons for this debate. Studies on Caco-2 cell monolayers,
296 however, often overestimate toxic effects, due to their lack of *in vivo* complexity, such as mucus, heterogenic
297 cell type composition, co-factors, and peristalsis altogether resulting in reduced repair functions (McCartney et
298 al., 2016; Swenson et al., 1994). The magnitude of the observed toxic effects are therefore likely to be reduced in
299 the GI-tract, however, further measures might be necessary in case local tissue damage is still observed *in vivo*.

300 Reducing the amount of C₁₀ in the microcontainers or loading of alternative permeation enhancers could resolve
301 potential cytotoxic effects. Permeation enhancers of interest might simply show a broader range between
302 efficient- and cytotoxic concentrations or alternatively work by peptide specific complexation resulting in
303 increased hydrophobicity and thus transcellular uptake. The latter mechanism has previously claimed to be the
304 cause of enhancement by SNAC (Ding et al., 2004; Malkov et al., 2005). As the absence of mucus on Caco-2
305 cell monolayers is one of the main differences compared to *in vivo* conditions, transport studies were also
306 conducted with mucus-secreting co-cultures.

307

308 3.4 Impact of co-localisation with permeation enhancer and mucus on insulin transport

309 Insulin transport studies across Caco-2/HT29-MTX-E12 co-culture monolayers were carried out to address the
310 potential negative impact of mucus on insulin permeation, when placing the microcontainer chips directly on the
311 monolayer ($d = 0.0$ mm). In parallel, the importance of co-localisation was evaluated by the use of
312 microcontainer chips where half of the microcontainers were filled with insulin and the other half with C₁₀. The
313 latter evaluation was likewise carried out on both Caco-2 and co-culture monolayers with $d = 0.0$ mm (Fig. 6).

314 Loading insulin and C₁₀ on each half of the microcontainer chip triggered a 4-5 fold decrease of the insulin
315 transport over 2 h, compared to microcontainers with the powder mixture (1:1 w/w), on both monolayer types
316 with $d = 0.0$ mm. A similar importance of co-localisation has previously been observed when simply controlling
317 the degree of co-localisation of 4 kDa fluorescein isothiocyanate-dextran and C₁₀ by intestinal instillation in rats
318 either together or at staggered time points (Wang et al., 2010). The microcontainer chips with insulin and C₁₀
319 individually filled, however, still resulted in a 10-fold increase in insulin transport across the Caco-2 monolayer
320 compared to the equivalent mass of insulin and C₁₀ in solution (0.1 mM and 3 mM, respectively), most likely
321 caused by diffusion of insulin to the areas of high local C₁₀ concentrations.

322 No differences in insulin transport were observed between the mucus-secreting co-culture monolayers and Caco-
323 2 cell monolayers. This could simply be due to the weight of the microcontainer chip causing penetration thereby
324 bypassing the mucus layer. However, neither was any difference in insulin transport observed between Caco-2
325 cell monolayers and co-culture monolayers from the separately loaded microcontainers, even though the insulin
326 molecules inevitably would need to diffuse along the monolayers in order to undergo transport. This implies that
327 the hydrodynamic size of insulin (1.5 – 3.0 nm) (Jensen et al., 2014) is sufficiently below the mesh spacing of
328 the mucus, secreted by the HT29-MTX-E12 cells, in order not to be retained. Other studies have previously
329 determined limited diffusivity for peptides of molecular mass above 12.4 kDa in porcine intestinal mucus

330 (Bernkop-Schnürch and Fragner, 1996) where the pores in such mucus have been determined to range from 100
331 nm to several micrometers by cryo-SEM (Boegh and Nielsen, 2015). The mucus layer *in vivo* is, however, still
332 likely to negatively influence absorption as removal of mucus on rat ileal segments has previously been found to
333 significantly increase insulin transport (Aoki et al., 2005). Although the authors claimed the mucus to
334 predominantly function as an enzymatic barrier, the thickness of the mucus might additionally lead to increased
335 distances between microcontainers and enterocytes.

336 Furthermore, it might be unlikely to imagine an *in vivo* scenario in which capsule disintegration will result in
337 unanimous optimal orientation of microcontainers, however, our *in vitro* results strongly indicate the importance
338 of continuous initiatives to improve these parameters. *In situ* intestinal perfusion studies have previously shown
339 the propensity of microcontainers to become partly embedded in the mucus, thereby shortening the distance to
340 the absorptive barrier lower than that of the mucus thickness (Nielsen et al., 2016). While this might compensate
341 for some degree of suboptimal orientation, further initiatives in order to increase the tendency of unidirectional
342 release towards the barrier remain an important focus of microcontainers as well as for other oral peptide
343 delivery devices (Abramson et al., 2019; Mazzoni et al., 2019; Vaut et al., 2019).

344

345 4. Conclusion

346 The concept of improving intestinal permeation of insulin by the use of unidirectionally releasing
347 microcontainers in combination with sodium caprate (C_{10}) was investigated in *in vitro* transport studies across
348 Caco-2 cell monolayers and mucus-secreting co-culture monolayers. Decreasing the distance between the point
349 of unidirectional release and the barrier resulted in enhanced insulin permeation, but also increased local
350 cytotoxic effects observed by confocal microscopy. Close proximities (0.0-0.2 mm) triggered local reversible
351 deteriorations of the barrier, while distances of 0.5-2.0 mm seemed to prompt non-destructive paracellular
352 permeation enhancement. To which extent local epithelial deterioration is acceptable needs further evaluation in
353 more complex barrier models, in order to assess the true potential of using unidirectionally releasing micro
354 devices in combination with permeation enhancers for oral delivery of insulin.

355

356 Acknowledgements

357 Lene Grønne Pedersen and Mette Frandsen are acknowledged for their efforts regarding cell culturing. Stephan
358 Sylvest Keller and Lasse Højlund Thamdrup are both thanked for manufacturing of microcontainers. Lasse
359 Johansson is acknowledged for production of the PTFE carvings and Daniel Bar-Shalom is recognised for
360 initiating ideas leading to the method for filling the microcontainers. This work was supported by the Danish
361 National Research Foundation (DNRF122) and Villum Foundation (Grant No. 9301), Center for Intelligent Drug
362 delivery and sensing Using microcontainers and Nanomechanics (IDUN).

363

364 Declaration of interest

365 The authors have no competing interests.

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 502

503 **Figures**

505 Fig. 1. Left: Illustration of the permeation study setup using polytetrafluoroethylene (PTFE) carvings to control
 506 the distance between the microcontainer chip and the Caco-2 cell monolayer. Right: Micrograph of a
 507 microcontainer chip elevated 0.5 mm by a PTFE carving, with depiction of dimension, d , ensuring exact
 508 microcontainer-monolayer distance. Visualised using a Dino-Lite Premier AM7013MZT digital microscope
 509 (AnMo Electronics Corporation, Taiwan).

510

512 Fig. 2. Representative SEM images of microcontainers. A: empty, B: loaded with insulin and C_{10} (1:1 w/w), C:
 513 loaded with insulin, D: loaded with C_{10}

514

515

516 Fig. 3. TEER values of the Caco-2 cell monolayers after the 2 h permeation study (left of the dotted line) and
 517 after subsequent 24 h recovery (right of the dotted line) with different distances (0-2.0 mm) between the
 518 microcontainer chips and the cells. The solution consisted of 0.1 mM insulin and 3 mM C₁₀ (1:1 w/w) in
 519 combination with an empty microcontainer chip with direct contact to the monolayer. Control cells were
 520 exposed to 2 h in fresh 10 mM hHBSS. Expressed as percentages of initial TEER values; mean + SD (n ≥ 3).
 521 Absolute values of the initial TEER was 278 ± 17 Ω cm² (mean ± SD, n = 7). **P* < 0.1, *****P* < 0.0001 compared
 522 to control TEER after transport and *ns*: not significant, *P* > 0.05, compared to control TEER after recovery based on a
 523 Tukey's multiple comparisons one-way ANOVA test.

524

525

526 Fig. 4. Exponential decay regression of the insulin transport rate as a function of the distance between the
 527 microcontainer chip and Caco-2 cell monolayer. Expressed as mean $\pm$ SD ($n \geq 3$). A one phase decay (least
 528 squares) fitting analysis was done using GraphPad Prism resulting in the equation: $Y = 8.29e^{-5.28x} +$
 529 $0.57, R^2 = 0.904$.

530
 531 Fig. 5. Representative confocal laser-scanning microscopy images of Caco-2 cell monolayers with Hoechst
 532 33342 nuclei staining. A: Monolayer upon 2 h permeation study with microcontainers at direct contact, B:
 533 Monolayer upon 2 h permeation study with microcontainers fixed at a 0.2 mm distance, C: Monolayer upon 2 h
 534 permeation study with microcontainers fixed at a 0.5 mm distance, D: Monolayer after 24 h incubation upon 2 h
 535 permeation study with microcontainers at direct contact, E: Monolayer after 2 h exposure to a solution of 0.1
 536 mM insulin and 3 mM C_{10} with a chip of empty microcontainers, F: Control monolayer upon 2 h exposure to
 537 hHBSS. Images have been adjusted for brightness/contrast and smooth processed using ImageJ. Scale bars
 538 represent 100 μm ($n = 2$).

539

540 Fig. 6. Combined plot of all eight insulin transport profiles of insulin from microcontainers across Caco-2 or
541 Caco-2/HT29-MTX-E12 co-culture monolayers. Microcontainers were filled with insulin and C₁₀ either;
542 premixed (1:1 w/w), indicated with filled symbols, or individually, indicated with half-filled symbols, and
543 compared with a solution of 0.1 mM insulin and 3 mM C₁₀ (1:1 w/w) in combination with an empty
544 microcontainer chip, indicated with ⊗. Distance between the microcontainers and the Caco-2 cell monolayer
545 was varied between 0-2 mm for the microcontainers loaded with premixed insulin and C₁₀. All studies were
546 carried out in 10 mM hHBSS with pH 7.4 and 0.05% (w/v) BSA at 37 °C. The graphs are expressed as mean +
547 SD (n = 3). Increased number of passages (n = 7) for direct Caco-2 monolayer-microcontainer contact (0.0 mm)
548 were carried out to ensure comparability across passages.