Polymeric microcontainers improve oral bioavailability of furosemide

Nielsen, Line Hagner; Melero, Ana; Keller, Stephan Sylvest; Jacobsen, Jette; Garrigues, Teresa; Rades, Thomas; Müllertz, Anette; Boisen, Anja

Published in:
International Journal of Pharmaceutics

Link to article, DOI:
[10.1016/j.ijpharm.2016.03.050](https://doi.org/10.1016/j.ijpharm.2016.03.050)

Publication date:
2016

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Nielsen, L. H., Melero, A., Keller, S. S., Jacobsen, J., Garrigues, T., Rades, T., Müllertz, A., & Boisen, A. (2016). Polymeric microcontainers improve oral bioavailability of furosemide. *International Journal of Pharmaceutics*, 504(1-2), 98-109. <https://doi.org/10.1016/j.ijpharm.2016.03.050>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Polymeric microcontainers improve oral bioavailability of furosemide

Line Hagner Nielsen^{a,*}, Ana Melero^b, Stephan Sylvest Keller^a, Jette Jacobsen^c, Teresa Garrigues^b, Thomas Rades^c, Anette Müllertz^{c,d}, Anja Boisen^a

^aDepartment of Micro- and Nanotechnology, Technical University of Denmark, Ørstedes Plads 345B, 2800 Kongens Lyngby, Denmark

^bDepartment of Pharmacy and Pharmaceutical Technology, University of Valencia, Valencia, Spain

^cDepartment of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, Copenhagen, Denmark

^dBioneer:FARMA, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, Copenhagen, Denmark

*Corresponding author: Department of Micro- and Nanotechnology, Technical University of Denmark, Ørstedes Plads 345B, 2800 Kongens Lyngby, Denmark. Tel: +4545256843, Fax: +4545887762. E-mail address: lihan@nanotech.dtu.dk (L. Hagner Nielsen).

Abstract

Microcontainers with an inner diameter of 223 μm are fabricated using the polymer SU-8, and evaluated *in vitro*, *in situ* and *in vivo* for their application as an advanced oral drug delivery system for the poorly water soluble drug furosemide. An amorphous sodium salt of furosemide (ASSF) is filled into the microcontainers followed by applying a lid using Eudragit L100. It is possible to control the drug release *in vitro*, and *in vitro* absorption studies show that the microcontainers are not a hindrance for absorption of ASSF. *In situ* perfusion studies in rats are performed with ASSF-filled microcontainers coated with Eudragit and compared to a furosemide solution. The absorption rate constant of ASSF confined in microcontainers is found to be significantly different from the solution, and by light microscopy, it is observed that the microcontainers are engulfed by the intestinal mucus. An oral bioavailability study in rats is performed with ASSF confined in microcontainers coated with Eudragit and a control group with ASSF in Eudragit-coated capsules. A relative bioavailability of 220% for the ASSF in microcontainers compared to ASSF in capsules is found. These studies indicate that the microcontainers could serve as a promising oral drug delivery system.

Keywords

Micro devices, furosemide, oral delivery, delivery systems, oral bioavailability, intestinal perfusion

1 Introduction

Oral pharmaceutical products represent approximately 70% of the value of the US pharmaceutical market, and oral delivery has for many decades been the preferred administration route for drugs, and continues to be so (Colombo et al., 2009; Perioli et al., 2012). However, there is a tendency for new drug compounds currently in the pipelines of the pharmaceutical industry to have a poor solubility in water and maybe even a low intestinal permeability, meaning that they are classified as class II or IV in the Biopharmaceutics Classification System (BCS) (Agrawal et al., 2014; Amidon et al., 1995; Lipinski, 2001). This trend complicates utilising the oral route for drug delivery purposes. Consequently, many approaches to overcome these obstacles, such as the use of lipid based drug delivery systems, permeation enhancers and nanoparticles, have been suggested (Agrawal et al., 2014; Colombo et al., 2009; Ensign et al., 2012). Sometimes, it can even be necessary to employ more advanced drug delivery systems allowing the potential for targeted and/or sustained delivery in the gastro-intestinal (GI) tract. Advanced drug delivery devices may have the potential to make the treatments more safe and efficient along with more convenient (Ensign et al., 2012). Micro fabricated drug delivery devices have been proposed as advanced drug delivery systems being able to increase the oral bioavailability of drugs (Chirra and Desai, 2012). Of these micro devices, microcontainers have been suggested as promising new advanced oral drug delivery systems (Hariharasudhan D Chirra et al., 2014). Primarily, this is due to the fact that the size and shape of the microcontainers can be controlled very precisely whereby polydispersity, as seen e.g. for many micro- and nanoparticles, is avoided (Randall et al., 2007). Microcontainers are polymeric, cylindrical devices in the micrometre size range (Figure 1) (Nielsen et al., 2014, 2012). A major advantage is that these devices allow for unidirectional release, as only one side of the microcontainer is open, compared to more conventional microparticles where release can occur from the whole surface area (Eaimtrakarn et al., 2001; van Hoogevest et al., 2011). Moreover, the drug can be protected inside the cavity of the microcontainer from the harsh environment of the stomach until release is desirable e.g. in the small intestine (where most drugs are absorbed) (Ahmed et al., 2002; Ainslie et al., 2009; Eaimtrakarn et al., 2001; Tao et al., 2003). A hydrogel with acyclovir entrapped inside micro reservoirs, thereby facilitating unidirectional release of the drug, has previously been described. The unidirectional release resulted in a high drug concentration at the micro reservoir-epithelial cell interface and allowed for increased *in vitro* permeation of acyclovir across a Caco-2 cell monolayer. Furthermore, the micro reservoirs led to increased oral bioavailability of acyclovir in mice (Hariharasudhan D. Chirra et al., 2014). Other studies have also demonstrated that the unidirectional release of drug from a micro reservoir delivery system resulted in an increased local concentration of the drug in close proximity of the targeted epithelium (Ahmed et al., 2002; Ainslie et al., 2009). The materials for fabrication of drug delivery systems can vary from well-known polymers such as poly-L-lactic acid (PLLA) (Nielsen et al., 2015) and poly(lactic-co-glycolic acid) (PLGA) (García-Díaz et al., 2015; Randall et al., 2007) to naturally occurring protein polymers such as silk fibroin (Wenk et al., 2011). SU-8 is an epoxy based polymer, and is reported suitable as implant material and also as a biocompatible polymer for biomolecular encapsulation (Nemani et al., 2013). In this paper, SU-8 was used as a model polymer for fabrication of the microcontainers, as it has been described in previous research (Ahmed et al., 2002; Nielsen et al., 2014, 2012).

Figure 1: Scanning electron microscope (SEM) image of a microcontainer made of SU-8.

There are two crucial steps for successful delivery of orally administered drugs; drug dissolution and drug absorption (Avdeef and Tsinman, 2008). Early prediction of the intestinal absorption of a compound is essential for further development, making the ability of *in vitro* methods to provide this information extremely important. Cell culture models are one of many methods used to study intestinal absorption (Patel et al., 2006; Pretorius and Bouic, 2009). The Caco-2 cell line is an epithelial cell line originating from a human colonic adenocarcinoma, which represents many of the characteristics and functions found in the epithelium of the small intestine (tight junctions, microvilli, growth factor receptors, and major drug metabolizing enzymes) (Berginc et al., 2012; Hidalgo et al., 1989; Hilgers et al., 1990). The Caco-2 cell model has become a useful *in vitro* tool to predict permeability and absorption of drugs across the small intestinal membrane, and is a valuable set-up to be employed during the development of oral drug delivery systems (Hidalgo et al., 1989). Even though, Caco-2 cells can be a good *in vitro* model for permeation, it lacks the mucus layer, covering the epithelial linings. The mucus layer is two layered; closest to the epithelial cell surface is a firmly adherent mucus layer and on top of that, a loosely transient adherent mucus layer. Both layers vary in thicknesses, ranging from 16-29 μm for the firmly adherent layer throughout the small intestine, whereas the loosely adherent layer is reported to have a thickness of varying from 123-480 μm in the small intestine (Ensign et al., 2012). The main role of the mucus layer is to protect and lubricate the epithelial lining, and for drugs, the mucus layer may be a physical barrier to absorption. The mucus has variable turnover time in the GI tract, and the turnover time in the intestine is reported not to be longer than 2 h (Crater and Carrier, 2010; Ensign et al., 2012; C.-M. Lehr et al., 1991; Lehr, 2000). For being able to study the interaction of the drug delivery system with the intestinal membrane and also the mucus layer, *in situ* intestinal perfusion studies are ideal (Lennernas, 1998; Lennernas, 2014; Lozoya-Agullo et al., 2015; Song et al., 2013). The single-pass intestinal perfusion model and closed-loop rat perfusion are both equally useful for obtaining information about drug absorption and interaction between the intestinal membrane and a drug delivery system. However, the closed-loop model has the advantage of measuring the absorption across a large part of the small intestine (Doluisio et al., 1969; Lozoya-Agullo et al., 2015). Absorption data from intestinal perfusion in rats are found to correlate well with human absorption data (Fagerholm et al., 1996; Lozoya-Agullo et al., 2015).

In the current study, the amorphous sodium salt of furosemide (ASSF) was utilised as a model drug. Furosemide is a class IV compound in BCS. Hence, it has a poor aqueous solubility and a low intestinal permeability. Furosemide is a weak acid with pK_a values of 9.9 and 3.5 (Matsuda et al., 1990), therefore it is possible to utilise the salt form of the drug. ASSF has previously been shown to significantly improve the solubility and dissolution rate of furosemide when compared to the drug in the commonly available crystalline acid form (Nielsen et al., 2013a). The poor intestinal absorption of furosemide is not only influenced by a low solubility, but is further complicated by the occurrence of site-specific absorption,

partly in the stomach, but especially in the upper part of the small intestine, leading to a considerable inter- and intra-individual variation in oral drug bioavailability (20-60%) (Granero et al., 2010; Iannuccelli et al., 2000). The intestinal absorption of furosemide is reported to be highly influenced by the dosage form (Granero et al., 2010), and therefore, there is a further need to improve furosemide absorption and specifically reduce the variation in bioavailability. This task could be accomplished through the use of an advanced drug delivery system such as microcontainers. A previous study showed that microwells fabricated with the biopolymer poly-L-lactic acid (PLLA), filled with ASSF, and coated with the pH-sensitive polymer Eudragit L100 made it possible to control the release of ASSF (Nielsen et al., 2015).

The aim of the current study was to evaluate microcontainers *in vitro*, *in situ* and *in vivo* as a potential oral drug delivery system for a poorly water-soluble drug (Figure 2). The release and absorption of ASSF confined in microcontainers were investigated using Caco-2 cells. Furthermore, intestinal perfusion studies in rats were carried out to explore any interaction between the microcontainers and the small intestinal mucus. Finally, the oral bioavailability of ASSF in rats was determined with and without confinement in microcontainers.

Figure 2: Schematic of the studies carried out. A) Microcontainers fabricated on silicon and filled with amorphous sodium salt of furosemide (ASSF) and coated with Eudragit L100 to be tested for *in vitro* release. B) After filling the microcontainers with ASSF the transport of ASSF through Caco-2 cells was studied. C) Showing the microcontainers fabricated on silicon with a fluorocarbon coating followed by ASSF confined in the microcontainers and coated with Eudragit L100. The microcontainers were afterwards *in situ* and *in vivo* tested in rats.

2 Materials and methods

2.1 Materials

Furosemide (>98% purity) was purchased from Fagron Nordic (Copenhagen, Denmark), while taurocholic acid sodium salt hydrate (sodium taurocholate), 2-(N-morpholino)ethane sulfonic acid (MES), bovine serum

albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Eudragit L100 was obtained from Evonik Industries (Darmstadt, Germany) and Hank's Balanced Salt Solution (HBSS) and sodium bicarbonate (7.5% w/v) solution were purchased from Life Technologies Europe (Naerum, Denmark). Phosphatidylcholine (Lipoid S PC, purity $\geq 98\%$) was acquired from Lipoid AG (Ludwigshafen, Germany). Sodium dihydrogen phosphate monohydrate, potassium dihydrogen phosphate and di-sodium hydrogen phosphate dehydrate were obtained from Scharlau (Sentmenat, Spain), whereas sodium azide, sodium chloride, and potassium dihydrogen phosphate were acquired from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), 90 U/mL penicillin, 90 $\mu\text{g/mL}$ streptomycin, non-essential amino acids, L-glutamine were sourced from Sigma-Aldrich Danmark A/S (Broendby, Denmark), while foetal bovine serum (FBS), was obtained from Gibco, Fisher Scientific (Slangerup, Denmark). Scintillation cocktail (Ultima Gold™) and ^{14}C -mannitol (58.8 mCi/mmol) were purchased from PerkinElmer Inc. (Boston, MA, USA). T-75 culture flasks and Transwell® permeable supports (filters) (in polycarbonate with a 0.4 μm pore size and with a diameter of 2.4 cm) were acquired from Corning Costar, Fisher Scientific (Slangerup, Denmark). Silicon wafers (4-in 100 n-type) were purchased from Okmetic (Vantaa, Finland). SU-8 2075 and SU-8 Developer were provided by Microresist Technology GmbH (Berlin, Germany). Chromium masks were designed using L-Edit® from Tanner EDA (Monrovia, CA, USA) and supplied by Delta Mask B.V. (GJ Enschede, the Netherlands). Ketoprofen (Imalgene®) was obtained from Merial® laboratories (Barcelona, Spain) and Medetomidine hydrochloride (Domtor®) was from Esteve Veterinaria® (Barcelona, Spain). Deionised water was obtained from an SG Ultra Clear water system (SG Water USA, LLC, Nashua, NH, USA) and was freshly produced in all cases. All other chemicals used were of analytical grade.

2.2 Manufacturing of SU-8 microcontainers

The microcontainers were fabricated as described previously with two steps of photolithography employing the negative epoxy-based photoresist, SU-8 (Nielsen et al., 2014, 2012; Tao and Desai, 2007). The microcontainers were fabricated on silicon wafers (Figure 2A and 2B), and for the *in situ* and *in vivo* studies, the microcontainers were further fabricated on a fluorocarbon coating deposited on top of the supporting silicon wafer by plasma polymerisation (Figure 2C). This enabled dry removal of the fabricated SU-8 structures from the support substrate in order to obtain individual microcontainers (Keller et al., 2007). The microcontainers had an inner diameter and height of $223\pm 3\ \mu\text{m}$ and $270\pm 3\ \mu\text{m}$, respectively, resulting in a volume of $10.6\pm 0.5\ \text{nL}$. For the *in vitro* studies the silicon wafers supporting the fabricated microcontainers were cut into chips of $12.8\times 12.8\ \text{mm}^2$ with arrays of 25×25 containers with a pitch of 450 μm being contained on each chip.

2.3 Preparation of ASSF

The preparation of ASSF was carried out as described previously (Nielsen et al., 2013a). Briefly, water and ethanol (96%) were mixed in a ratio of 10:1 v/v, and crystalline furosemide was added at a concentration of 0.4 w/v%, together with 5 M NaOH in a molar ratio of 1:1 with furosemide. The solution was spray dried on a Büchi Mini Spray-Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland) to obtain ASSF.

2.4 Filling of ASSF into the microcontainers

ASSF powder was manually distributed on the microcontainer chip. The excess drug in between the microcontainers was then removed with pressurised air, leaving ASSF-filled microcontainers. The chip with

microcontainers was weighed before and after filling to determine the amount of drug filled into the microcontainers. This was determined to be 7.5 ± 1.5 mg of ASSF per chip corresponding to 12.0 ± 2.4 $\mu\text{g}/\text{container}$.

2.5 Spray coating of the drug-filled microcontainers with Eudragit L100

A spray coating system (ExactaCoat, Sono Tek, USA) equipped with an ultrasonic nozzle actuated at 120 kHz (Keller et al., 2013) was used to deposit Eudragit L100 (dissolved to a 2 wt% solution in isopropyl alcohol) on the cavity of the drug-filled microcontainers in a set-up similar as previously described (Nielsen et al., 2015). The generator power was set to 1.5 W, and the polymer solution was pumped through the nozzle at a flow rate of 100 $\mu\text{L}/\text{min}$. Nitrogen gas at a pressure of 10 mbar was used to direct the beam of droplets onto the microcontainers, and the distance between nozzle and substrate was 40 mm with the beam diameter on the substrate being approximately 4 mm. The lateral movements of the nozzle were controlled by an x-y stage and the nozzle path was defined in the equipment software. The nozzle was moved line-by-line at a speed of 25 mm/s, and the coating was repeated 60 times to obtain a coating thickness in the μm range. The thickness of the layer was measured using a surface profilometer (KLA-Tencor Alpha-Step IQ, CA, USA).

2.6 Scanning electron microscopy

The morphology of the microcontainers (drug-filled microcontainers with and without coating) was examined using scanning electron microscopy (SEM). The investigations were carried out using a Nova 600 NanoSEM from FEI (Eindhoven, the Netherlands). Imaging was performed in low-vacuum mode at a pressure of 0.6 mbar and an operation voltage of 5 kV. Prior to examination, the samples were mounted onto metal stubs and were tilted by 15-30°.

2.7 Preparation of media

For the drug release from the coated microcontainers, a medium simulating the composition of the fasted gastric fluid followed by a medium simulating the conditions of the fasted intestinal fluids were employed. The medium simulating the fasted gastric fluid with a pH of 1.6 was prepared in accordance with Vertzoni et al. (Vertzoni et al., 2005), whereas the medium simulating the fasted intestinal state (pH 6.5) was prepared as described previously (Nielsen et al., 2013a, 2013b).

For the cell transport studies, a simulated intestinal medium containing 5 mM bile salt and 1.25 mM phospholipid at pH 6.5 was employed as a transport medium. The medium was prepared in HBSS buffer pH 6.5, a stock solution of which was prepared by mixing HBSS together with 10 mM of MES buffer, 7.5% sodium bicarbonate and BSA, made to volume with water and pH adjusted to 6.5. For preparation of the simulated intestinal medium, an appropriate quantity of a stock solution of phosphatidylcholine in chloroform (100 mg/mL) was first evaporated under a steady stream of nitrogen in order to form a lipid film. The volume of a stock solution of sodium taurocholate hydrate (6 mg/mL) required to achieve a concentration of 5 mM bile salt was then added together with the stock solution of HBSS, pH 6.5. The medium was stirred at 37°C overnight, followed by adjustment to pH 6.5 and then to a final volume with buffer (Nielsen et al., 2013a; Patel et al., 2006). The basolateral medium for the Caco-2 cell studies consisted of a HBSS buffer at pH 7.4 (prepared by mixing a stock solution of HBSS buffer with HEPES buffer, 7.5% sodium bicarbonate, and BSA). After solubilisation, pH was adjusted to pH 7.4 (Patel et al., 2006).

2.8 Release of ASSF from microcontainers

The *in vitro* release of ASSF from the microcontainers was tested using a μ -Diss profiler (pION INC, Woburn, MA, USA) in a similar set-up as described earlier (Nielsen et al., 2015, 2014). Experiments were carried out at 37°C employing a stirring rate of 100 rpm. The path length of the *in situ* UV probes was 1 mm, and each channel of the profiler was calibrated with its own standard curve prior to the experiments. The microcontainers were filled with ASSF and coated with Eudragit L100 as described above. The chips with microcontainers were attached to cylindrical magnetic stirring bars (using carbon pads) placed in the bottom of sample vials, and covered with 10 mL of stimulated gastric medium pH 1.6 for 120 min followed by simulated intestinal medium at pH 6.5 for 180 min. The *in situ* UV probes situated in each sample vial scanned and detected the absorbance of released/dissolved ASSF for a total duration of 300 min. The experiment was performed in 5 replicates.

2.9 Transport studies with ASSF

The Caco-2 cell line (HTB-37) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) (DSMZ GmbH, Braunschweig, Germany) and used between passage numbers 2 and 8. The cells were cultured as described by Larsen et al. (Larsen et al., 2008). Transport experiments were performed on polarised cells after 18-20 days in culture on permeable supports.

Apical to basolateral transport of ASSF either applied as bulk powder or filled into microcontainers across the Caco-2 cell layer was investigated, using simulated intestinal medium pH 6.5 (see section: 'Preparation of media') as transport medium. Transepithelial electrical resistance (TEER) was measured at room temperature prior to each transport experiments in a tissue resistance measurement chamber (Endohm, World Precision Instruments, Berlin, Germany) combined with a voltohmmeter (EVOM, World Precision Instruments). TEER values were also measured after the ASSF experiments (at 120 min). The ASSF transport was determined over a 120 min time period with cell plates circulating at 95 rpm on a KS15 Edmund Bühler Compact Shaker (Holm and Halby, Broendby, Denmark) coupled with a thermostatic water bath kept at 37°C. The filter-grown cell cultures were initially washed with pre-heated (37°C) HBSS buffer; subsequently, the transport experiments were initiated. The transport of ASSF as bulk powder or filled into microcontainers was determined by applying the powder or microcontainer chips in custom-made baskets (Figure 3) hanging above the cell layer, in a similar set-up as previously described by Sander et al. (Sander et al., 2013). For the bulk ASSF, 6.5 ± 1.5 mg was weighed and added to the baskets hanging above the cell layer. The drug-filled microcontainers (containing 7.5 ± 1.5 mg of drug) were added to the baskets with the cavity of the microcontainers immersed in apical medium. The simulated medium at pH 6.5 was utilised as the apical medium and HBSS pH 7.4 as the basolateral medium in volumes of 2.5 and 1.5 mL, respectively. In each experiment, controls with either medium alone or empty basket plus medium were also employed. Samples of 100 μ L were withdrawn from the basolateral compartment after 15, 30, 45, 60, 90, and 120 min and were replaced with pre-heated HBSS buffer at pH 7.4. The samples were diluted two-fold with acetonitrile and centrifuged at 15,000 rpm for 15 min, following supernatants were transferred to HPLC vials. Samples were kept at -20°C until HPLC-UV analysis. The transport studies were carried out in replicates of 10-19.

Figure 3: A) Set-up of the transport studies with the ASSF-filled microcontainers. B) Custom-made stainless steel baskets utilised for the Caco-2 cell transport studies.

After completion of the transport studies of ASSF, the viability of the cells was investigated using ^{14}C -mannitol and measuring the permeability. The cell cultures were washed twice with HBSS buffer and after leaving the cells for 10 min at room temperature, the TEER was measured. Subsequently, the permeability experiments with mannitol were initiated by adding 1 mL of simulated medium containing $17.5\ \mu\text{M}$ ^{14}C -mannitol ($1\ \mu\text{Ci}$) to the apical compartment and 1.5 mL HBSS buffer pH 7.4 to the basolateral compartment. The cells were incubated at 37°C at a circulating speed of 95 rpm. Sample volumes of $100\ \mu\text{L}$ were withdrawn from the basolateral compartment after 15, 30, 45, and 60 min, and after completion of the ^{14}C -mannitol transport study, apical samples ($20\ \mu\text{L}$) were also withdrawn. The samples were transferred to scintillation vials to which 2 mL of scintillation cocktail was added and the content of ^{14}C -mannitol was determined using a Tri-Carb® 2910TR liquid scintillation analyser (PerkinElmer, Boston, MA, USA).

2.10 HPLC and data analysis for cell transport studies

The amount of ASSF in samples from the transport studies was quantified by HPLC-UV using the method previously described (Nielsen et al., 2013a), with the exception that a Phenomenex Luna C18 column with the dimensions of $2.1 \times 50\ \text{mm}$, $3.5\ \mu\text{m}$ was used.

For the data analysis, flux (F) [$\text{mol}/\text{cm}^2/\text{s}$] for the ASSF and apparent permeability (P_{app}) [cm/s] for ^{14}C -mannitol across the Caco-2 cells were calculated at steady state rate of permeation according to Eqs. 1 and 2, respectively.

$$F = \frac{dQ}{dt} \cdot \frac{1}{A} \quad (1)$$

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_0} = \frac{F}{C_0} \quad (2)$$

where dQ/dt [mol/s] is the rate of drug permeation, A [cm^2] is the area of the inserts ($2.4\ \text{cm}^2$), and C_0 [mol/cm^3] is the initial donor concentration of ^{14}C -mannitol.

2.11 Closed-loop intestinal perfusion in rats

Animal protocol number 2015/VSC/PEA/00084 was utilised for these studies of the Ethical Committees of the University of Valencia and the Valencian Regional Government. Female Wistar rats (220-270 g) were anaesthetised by intraperitoneal (IP) injection using a mixture of ketoprofen and medetomidine hydrochloride in a concentration of $1.5\ \text{mL}/\text{kg}$ and $0.5\ \text{mL}/\text{kg}$, respectively. The intestinal perfusion surgeries were performed in a similar set-up as described in the literature (Doluisio et al., 1969; Lozoya-

Agullo et al., 2015). Briefly, it consists of preparing the small intestine as a closed compartment. A midline abdominal incision following the *linea alba* was made, and the small intestine was exposed. The bile duct was ligated in order to avoid drug enterohepatic circulation and the presence of bile salts in lumen. Two incisions were created in the intestine, the first at the beginning of the duodenal segment, and the second at the end of the ileum segment, just before the cecum, and the entire small intestine was cannulated through these incisions. Care was taken to avoid disturbance of the intestinal blood supply. In order to remove all intestinal contents, the small intestine was thoroughly flushed with pre-warmed (37°C) physiologic solution consisting of NaCl aqueous solution (9g/L) containing Na₂HPO₄·2H₂O and NaH₂PO₄·2H₂O both in water, and pH adjusted to 6.9. The catheters were then connected to a glass syringe using a stopcock three-way valve, and the intestine was carefully placed back into the peritoneal cavity with covering the abdomen with cotton wool pads to prevent peritoneal liquid evaporation and heat loss. The samples tested were 10 mL of ASSF solution (10 mg/mL) in 100 mM phosphate buffer pH 6.5, and Eudragit-coated ASSF-filled microcontainers dosed directly to the intestinal tubing at the same time as dosing 10 mL of 100 mM phosphate buffer, pH 6.5. As a control, empty microcontainers were dosed together with 10 mL of phosphate buffer. For acquiring a sample, the luminal content was pushed out from one syringe to the other using the stopcock valves and a sample of 200 µL was taken out. This procedure was done alternatively from the proximal syringe to the distal one, assuring the mixing of the solution in the intestinal lumen. The samples were collected every 5 min for a period of 30 min. After 30 min, the solution was emptied from the intestine and the volume was measured and a heart puncture was performed to collect the blood from the rat. The blood (to obtain the plasma) and the perfusion samples were centrifuged at 2500 rpm for 10 min. Following the 30 min of perfusion studies, the intestine was taken out of the rat, and placed on microscope slides. A light microscope (Eclipse E800, Nikon Instruments Europe) and Nikon ACT-1 (version 2.70) was used as software for visualising the microcontainers on the intestinal membrane.

Water flux throughout the experiment can be significant and was therefore accounted for. A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each tested compound, the initial volume (V_0) was determined on groups of four rats, while the endpoint volume (V_t) was measured on every animal used. The drug concentration in the samples was corrected as:

$$C_t = C_e \cdot \left(\frac{V_t}{V_0} \right) \quad (3)$$

where C_t represents the concentration in the absence of water reabsorption at time t , and C_e the experimental value. The absorption rate coefficients (k_a) of the compounds were determined by nonlinear regression analysis of the remaining concentrations in the intestinal lumen (C_t) vs. time:

$$C_t = C_0 \cdot e^{-k_a t} \quad (4)$$

These absorption rate coefficients were then transformed into permeability values using the relationship:

$$P_{app} = k_a \cdot \frac{R}{2} \quad (5)$$

where R is the effective radius of the intestinal segment, calculated from the area/volume relationship where the volume and length were measured at each experiment (Lozoya-Agullo et al., 2015).

2.12 Oral bioavailability study in rats

All animal care and experimental studies were approved by the Animal Welfare Committee, appointed by the Danish Ministry of Justice, and were carried out in compliance with EC Directive 2010/63/EU, the Danish law regulating experiments on animals and NIH Guidelines for the Care and Use of Laboratory Animals. The experiments were performed under the following license: 2012-15-2934-00186 and the experimental animal work was performed at the Department of Experimental Medicine, University of Copenhagen, Denmark, which is accredited by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC). Male Sprague–Dawley rats (314–342 g) were purchased from Taconic (Bomholt, Denmark) and acclimatised for a minimum of 7 days in groups of two on wooden bedding (Tapvei, Korteinen, Finland) in plastic cages on standard feed (Altromin 1324, Altromin Spezialfutter, Lage, Germany). The rats had free access to fresh tap water (also during the experiments) in an air-conditioned building with controlled environmental parameters (humidity 40–60%, temperature $20\pm1^{\circ}\text{C}$, light from 6am–6pm). Before entry into the experiments, the animals were fasted for approximately 6 h and randomly assigned to receive one of the treatments. One day prior to the *in vivo* experiments, Eudragit-coated ASSF-filled microcontainers were filled into gelatine capsules, size 9 (Torpac, NJ, USA) with a drug dose corresponding to 4.5 mg ASSF (equal to approximately 15 mg/kg dosed to the rats). As a reference 4.5 mg ASSF was filled into the capsules and the capsules were coated with Eudragit L100. The microcontainers were filled into the capsules by manually gently scrapping the microcontainers of the fluorocarbon coated chips. The bottom of the capsules were placed in a stand and filled via a funnel. The capsules (one capsule per rat) were dosed with a dosing syringe (Torpac, NJ, USA) to the bottom of the oesophagus of the rat. Blood samples (200 μL) were taken from the tail vein by individual venipuncture and collected into EDTA coated tubes. Samples were collected at 10, 20, 30, 45 min, and 1, 2, 3, 5, 7, and 24 h after drug administration. Plasma was harvested immediately by centrifugation at 3600 g (12 min) and stored at -20°C until further analysis. The *in vivo* studies were performed in 5 and 6 replicates for the microcontainers and the ASSF powder, respectively.

2.13 Liquid chromatography–mass spectrometry (LC-MS) analysis

Plasma samples were processed using 30 μL of the plasma spiked with 300 μL of acetonitrile to precipitate proteins. The mixtures were centrifuged at 3600 g for 15 min at room temperature. After centrifugation, the produced supernatants were transferred to HPLC vials and analysed by LC-MS, using the method described previous (Nielsen et al., 2013a).

2.14 Pharmacokinetic analyses

The pharmacokinetic parameters following oral administration of ASSF were obtained by non-compartmental analysis. The area under the curve (AUC) of the plasma concentration versus time curves was determined by the linear log trapezoidal method from time $t = 0$ min either to $t = 180$ min or to the last plasma concentration measured at $t = 1440$ min after dosing. AUC values were used to calculate the relative bioavailability. The maximum furosemide plasma concentration (C_{max}) and time to peak concentration (T_{max}) were determined from the individual plasma concentration versus time curves.

2.15 Statistics

The *in vitro* and *in situ* data are expressed as mean $\pm$ standard deviation (SD), whereas the *in vivo* data are stated as mean $\pm$ standard error of mean (SEM). Where appropriate, statistical analysis was carried out using

Student t-tests using GraphPad Prism version 6.05. *P*-values below 5% ($p < 0.05$) were considered statistically significant.

3 Results and discussion

The use of advanced drug delivery systems for oral delivery may be necessary, especially when delivering BCS class II and IV drugs, but also if delivering peptides or proteins by the oral route (Twibanire and Grindley, 2014). It is therefore important to initially investigate the *in vitro* and *in situ* properties of the delivery system, in particular with respect to the release of the drug from the system in simulated media. It is furthermore of importance to study the intestinal absorption of the drug prior to animal testing.

3.1 Drug-filled microcontainers

In Figure 1, an empty microcontainer fabricated in SU-8 is shown. Following the fabrication, the SU-8 microcontainers were filled with ASSF (Figure 4A). For the *in vitro* release, *in situ* perfusion and bioavailability studies, the microcontainers were further spray coated with a lid of the pH-sensitive polymer Eudragit L100 (Figure 4B) in order to provide controlled release, and additionally to maximize drug retention within microcontainers until the absorptive environment of the small intestine was reached. The Eudragit layer was found to be $9.7 \pm 0.3 \mu\text{m}$ ($n = 5 \pm \text{SD}$) thick, with consistent thickness over the entire chip area.

Figure 4: SEM images of a microcontainer A) filled with ASSF, and B) filled with ASSF and coated with Eudragit L100 in a thickness of $9.7 \pm 0.3 \mu\text{m}$ (measured using a surface profilometer).

3.2 Release of ASSF from coated microcontainers

In order to obtain the best *in vitro-in vivo* correlation, it is important to evaluate the release of drug from an oral delivery system in conditions simulating the GI tract as closely as possible (Kostewicz et al., 2014). Therefore, the release of ASSF from the Eudragit-coated microcontainers was studied in simulated gastric and intestinal media over periods of 120 min and 180 min, respectively (corresponding to the average transit time for drug *in vivo* in the stomach and intestine). The aim was to have a controlled drug delivery system, which could prevent release in the gastric environment, but facilitate a fast release in the absorptive upper small intestinal region, in order to provide the opportunity for maximal oral drug bioavailability. In Figure 5, it can be seen that no release was observed in the first 120 min (gastric medium, pH 1.6) and when the medium was changed to intestinal medium (pH 6.5), a rapid release of ASSF from the microcontainers accompanying dissolution was observed. Of the initially incorporated ASSF, $88.2 \pm 1.4\%$ was released and dissolved within the 180 min period of exposure to the simulated intestinal medium. The rapid release of ASSF following the pH shift was expected given that Eudragit L100 is known to dissolve above pH 6. Also, ASSF has been shown to have a very high dissolution rate in simulated intestinal medium (Nielsen et al., 2013a). The release data therefore show that the Eudragit coating is sufficiently thick to

serve as an effective lid for the microcontainers, protecting the drug from being released at pH 1.6, but providing no hindrance to a fast drug release at pH 6.5. It was found that the microcontainers were not a significant hindrance for a fast release compared to unconfined ASSF (bulk). After 2 min of dissolution 94.4±4.6% of the ASSF in bulk form was dissolved, whereas 81.7±3.1% of ASSF confined in microcontainers was released and dissolved. At the endpoint of the experiment (after 180 min), 85.9±2.2% of ASSF was released from the microcontainers. A small but significant difference (p -value 0.0253) was noted in comparison to the ASSF in bulk form, where 94.0±5.1% of the drug was dissolved at 180 min. From previous studies, it is known that ASSF is physically stable at storage conditions for 291 days, but during dissolution and thereby also release from the microcontainers, ASSF converts to a trihydrate quickly upon wetting. This will be the case for both ASSF in powder bulk and for ASSF confined in microcontainers (Nielsen et al., 2013a, 2013b). The *in vitro* release characteristics of ASSF from the microcontainers are encouraging in terms of developing the microcontainers as an oral drug delivery system, as most of the ASSF confined in the microcontainers was released quickly and the characteristics of the release curves were very similar over the whole time period to the curves from the dissolution of ASSF in bulk form. The drug can, with a combination of the microcontainer and the Eudragit layer, be protected inside the device through the harsh environment of the stomach and then a fast release in the upper part of the intestine was obtained.

Figure 5: Release-time profiles obtained from SU-8 microcontainers filled with ASSF and coated with Eudragit L100 in gastric medium pH 1.6 (from 0-120 min) and intestinal medium pH 6.5 (120-300 min). Data are presented as mean±SD (n=5).

3.3 Flux of furosemide across the Caco-2 monolayer

Furosemide is a known substrate of efflux transporters such as the P-glycoproteins, and there is also a significant paracellular contribution to the passive uptake of furosemide (Granero et al., 2010). The weakly acidic properties of furosemide result in the permeability being highly variable with pH (Pade and Stavchansky, 1997). Pade et al. presented values of apparent permeability (P_{app}) of 0.012×10^{-5} cm/s at pH 7.2, whereas a P_{app} value of 0.51×10^{-5} cm/s was reported at pH 5.4 (Pade and Stavchansky, 1997). The P_{app} values for furosemide in the literature is highly variable and values as low as 0.029×10^{-5} cm/s (at pH 6.5) have been reported (Hilgendorf et al., 2000). In Figure 6, no apparent lag-time for the permeation of furosemide in bulk or in microcontainers was observed across the monolayer of Caco-2 cells. The flux of

ASSF was calculated from the slope of the curves in the time interval of 15-120 min. At pH 6.5, the flux across the Caco-2 cell monolayer was calculated to be $1.13 \pm 0.95 \times 10^{-4}$ and $1.47 \pm 0.71 \times 10^{-4}$ $\mu\text{mol}/\text{cm}^2/\text{s}$, for ASSF in bulk and microcontainers, respectively. There was no significant difference in the flux of bulk and confined ASSF (p -value 0.2569). When developing the microcontainers as an oral drug delivery system, it is a positive indication that there is no difference in the transport characteristics of the drug when dosed as bulk or confined in microcontainers. The Caco-2 cell culture model is well developed and absorption through the cell monolayer is found to be a key parameter in estimating the *in vivo* performance of a drug after oral administration (Amidon et al., 1995; Sjogren et al., 2013). The *in vitro-in vivo* correlation seem to improve even further when the cell studies are performed in medium closely mimicking the properties of human intestinal fluid (as was done here) (Lind et al., 2007; Patel et al., 2006).

Figure 6: Transport of furosemide applied as ASSF powder or confined in microcontainers across a Caco-2 cell culture model in apical to basolateral direction. The experiments were performed using custom-made baskets. Data are presented as mean+SD, (bulk ASSF n=13) and mean-SD, (ASSF in microcontainers n=19).

The viability of the cell layer after the 120 min of ASSF experiments was examined using a mannitol assay together with TEER values. The P_{app} values for the mannitol can be found in the supplementary data (Table A.1) where the controls refer to cell culture filters where no ASSF had been transported. There was a significant difference between the permeability of mannitol where bulk ASSF has been applied and the controls (p -value 0.0079), whereas no significant differences were observed between the mannitol permeability pre-treated with ASSF in microcontainers and the controls (p -value 0.1694), or the ASSF in microcontainers and bulk ASSF (p -value 0.1003). Furthermore, a drop in TEER values could be observed for the Caco-2 cells for all the treatments (supplementary data, Table A.1). It should however be noted that it has previously been reported that drops in TEER values can be observed when utilising a simulated intestinal medium (Ingels et al., 2002). For the bio-relevance of the studies reported here, it was decided to utilise simulated intestinal medium even though it can cause a reduction in the integrity of the cell layer, as mimicking the conditions in the intestine is important when studying new formulation effects and delivery systems (Berginc et al., 2012; Patel et al., 2006). In the present studies, the mannitol permeability was found to be a 100-fold higher compared to values reported in the literature ($0.3\text{-}0.5 \times 10^{-6}$ cm/s) (Kumar et al., 2010). It is speculated that furosemide may have a slightly toxic effect on the cells as the mannitol

permeability is highest for the bulk ASSF (supplementary data, Table A.1) and the TEER values were also found lowest for this treatment – although this is not found reported in the literature.

3.4 *In situ* closed-loop rat intestinal perfusion

The *in situ* perfusion studies were essential to investigate if there was any interaction between intestine/intestinal mucus and the microcontainers. Earlier, intestinal perfusion studies have been used to investigate absorption mechanisms e.g. for drug suspensions (Xu et al., 2014), and they are in general appropriate models for observing mechanisms of absorption and interactions. Furthermore, good *in vitro*–*in vivo* correlation has been found between perfusion and oral absorption data in rats and thereby it has been concluded that *in situ* intestinal perfusion is useful in predicting *in vivo* pharmacokinetic characteristics in rats (Xu et al., 2014).

It was observed that after 30 min, the microcontainers were engulfed by the intestinal mucus when visualising the intestine and microcontainers under a light microscope (Figure 7). In Figure 7A-D, empty microcontainers are visualised and it can be seen that both the empty and the coated microcontainers interacted with the intestinal mucus; the microcontainers were completely covered by the intestinal mucus. The intestinal mucus acts as a steric and interaction barrier (Nordgård et al., 2014), and it seems as mucus was entangling the microcontainers and the drug is then released from the microcontainers before the microcontainers can be cleared away. To protect the mucosal epithelium the constant fast turnover of the mucus layer serves to remove potentially damaging compounds introduced to the GI tract, and therefore the microcontainers provide a transient mucoadhesion and thereby prolong the contact time for epithelial uptake of furosemide. However, furosemide was released within minutes from the microcontainers (Figure 5) (Ensign et al., 2012; C. M. Lehr et al., 1991), and this is probably why there was an increased absorption of furosemide through the intestinal wall. For investigations into the effect of interactions of the microcontainers with the intestinal mucus for the absorption of furosemide, the absorption rate constants were calculated for ASSF in microcontainers coated with Eudragit L100 and for ASSF in solution. The absorption rate constant for the ASSF confined in the microcontainers was calculated to be $1.91 \pm 0.22 \text{ h}^{-1}$, whereas the value was $1.23 \pm 0.47 \text{ h}^{-1}$ for the solution, creating a significant difference (*p*-value: 0.0249) between the two groups tested (Figure 8). This correlates well with the observation that the microcontainers interact with and adhere to the intestinal wall, thereby increasing the absorption rate of ASSF. When dosing the solution of furosemide, the furosemide is likely to be quickly flushed further down in the intestine (where absorption is not occurring) due to the continuing flow of fluid through the small intestine (Sjögren et al., 2014). It has earlier been reported that, unlike microparticles that experience a high intestinal and peristaltic shear, planar microdevices experience a low shear stress and remain in the upper intestine for a longer time (Hariharasudhan D. Chirra et al., 2014). Even though the microdevices described in the literature had other dimensions (200 μm in diameter and 4 μm in depth) than the microcontainers described here, it can be speculated that it is the same phenomenon that is observed also for non-planar devices as utilised in this study (Hariharasudhan D. Chirra et al., 2014). This leads to the argument that microcontainers could be a promising oral drug delivery system also for other types of drugs than poorly soluble drugs, e.g. for peptides, proteins and low permeable drugs. Furosemide is a loop diuretic and widely used orally for hypertension and oedema (Goud et al., 2011; Matsuda et al., 1990), and therefore a rapid absorption will often be beneficial for the patients, but in case of other drug candidates where a prolonged release may be required other polymers than Eudragit L100 can be utilised as coating agents.

Figure 7: Microcontainers in intestinal mucus following *in situ* intestinal perfusion studies. A) and B): Empty microcontainers without drug and coating. C) and D): Microcontainers filled with ASSF and coated with a lid of Eudragit L100.

Figure 8: Calculated absorption rate constants from the *in situ* intestinal perfusion studies for the Eudragit L100 coated drug-filled microcontainers and for furosemide solution. Data are mean $\pm$ SD, n=4-5.

3.5 Oral bioavailability in rats

To investigating the potential of microcontainers as an oral drug delivery system further, it was studied how the microcontainers would influence the oral bioavailability of ASSF. The mean plasma concentration versus time profiles of ASSF in microcontainers and ASSF in Eudragit-coated capsules are presented in Figure 9, and the non-compartmental pharmacokinetic parameters are provided in Table 1. For the first 180 min, no significant difference was observed for the AUC between ASSF confined in Eudragit-coated microcontainers and ASSF in capsules coated with Eudragit L100 (p -value: 0.8599). Additionally, no significant difference was observed between the two groups in terms of T_{max} and C_{max} (p -values of 0.310 and 0.8199, respectively). After 180 min however, a prolonged absorption for ASSF from microcontainers

was seen, and this difference continued for the 1440 min the studies were carried out for (Figure 9 and Table 1). This is reflected in a significant difference in the $AUC_{0-1440\text{min}}$ between the two test groups (p -value: 0.0020), which resulted in a relative oral bioavailability of ASSF from the microcontainers of $220.2 \pm 43.2\%$ compared to the capsules. This correlates well with the *in situ* studies where the microcontainers were adhering to the mucus and releasing the ASSF in a prolonged fashion compared to the powder in the capsules. These results suggest that by the use of microcontainers, absorption of drugs can be prolonged and the bioavailability can be increased.

Figure 9: Plasma furosemide concentrations after dosing to rats of i) microcontainers filled with ASSF, coated with Eudragit L100 and filled into gelatin capsules and ii) ASSF powder in capsules with Eudragit coating. The data is presented as mean $\pm$ SEM with $n=6$ for microcontainers and $n=5$ for capsules.

Table 1: Non-compartmental pharmacokinetic parameters of ASSF following oral administration to rats

	Eudragit coated microcontainers (mean $\pm$ SEM)	Eudragit coated gelatin capsules (mean $\pm$ SEM)
$T_{\max}$ (min)	34.2 $\pm$ 9.7	48.0 $\pm$ 7.3
$C_{\max}$ (µg/mL)	1.9 $\pm$ 0.48	1.7 $\pm$ 0.68
$AUC_{0-180\text{ min}}$ (µg min/mL)	179.6 $\pm$ 33.3	152.2 $\pm$ 39.2
$AUC_{0-1440\text{ min}}$ (µg min/mL)	1696.4 $\pm$ 299.7*	326.1 $\pm$ 55.0*
Relative bioavailability (%) ¹	220.2 $\pm$ 43.2	-

¹Relative bioavailability was determined as: $100 \times (AUC_{mc}/dose_{mc}) / (AUC_{eudragit}/dose_{eudragit})$

* Significant difference (p -value: 0.0020)

4 Conclusion

Microcontainers fabricated in the polymer SU-8 were evaluated *in vitro*, *in situ* and *in vivo* for their properties as advanced oral drug delivery systems for poorly soluble drugs, using furosemide as a model

drug. When filling the microcontainers with ASSF, it was found that drug release could be controlled by coating the cavity of the microcontainers with Eudragit L100 as no release was measured in simulated gastric medium, whereas rapid release in the simulated intestinal medium was observed. Furthermore, no significant difference was seen for the transport and flux of ASSF in Caco-2 cells, neither in the bulk form nor in the microcontainers. The *in situ* perfusion studies showed that the microcontainers were engulfed by the intestinal mucus resulting in an increased absorption rate for the confined ASSF compared to furosemide solution. In the oral bioavailability study in rats, differences of AUC were observed from 180 to 1440 min leading to a relative bioavailability of 220% of the ASSF in microcontainers compared to ASSF in capsules. These results indicate that it is possible to utilise the microcontainers as an oral drug delivery system, here shown for a poorly soluble model drug, but with perspective of serving as an oral drug delivery system for other drugs as well.

Acknowledgement

The authors would like to acknowledge the Villum Kann Rasmussen Foundation and the Danish Research Council for Technology and Production (FTP), Project DFF - 4004-00120B for financial support.

Moreover, the Danmarks Grundforskningsfonds and Villum Fondens Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN) is acknowledged.

Supplementary information:

Table A.1: The apparent permeability (P_{app}) for ^{14}C -mannitol across the Caco-2 after 120 min of incubation with ASSF either as bulk or filled into microcontainers. TEER values from the Caco-2 monolayer before and after experiments with ASSF in bulk form, confined in microcontainers and the controls (only medium). Data are presented as mean $\pm$ SD, n=10-19.

	P_{app} mannitol [$\times 10^{-5}\text{cm/s}$]	TEER values [$\text{Ohm}\cdot\text{cm}^2$]	
		<i>Before experiment</i>	<i>After experiment</i>
Bulk ASSF	3.81 $\pm$ 0.94	337.2 $\pm$ 112.5	137.5 $\pm$ 12.0
ASSF in microcontainers	3.26 $\pm$ 0.90	370.1 $\pm$ 140.7	159.1 $\pm$ 42.9
Control	2.76 $\pm$ 0.83	387.4 $\pm$ 151.4	200.6 $\pm$ 38.2

Reference list

- Agrawal, U., Sharma, R., Gupta, M., Vyas, S.P., 2014. Is nanotechnology a boon for oral drug delivery? *Drug Discovery Today* 19, 1530–1546. doi:10.1016/j.drudis.2014.04.011
- Ahmed, A., Bonner, C., Desai, T.A., 2002. Bioadhesive microdevices with multiple reservoirs: a new platform for oral drug delivery. *Journal of Controlled Release* 81, 291–306. doi:10.1016/s0168-3659(02)00074-3
- Ainslie, K.M., Lowe, R.D., Beaudette, T.T., Petty, L., Bachelder, E.M., Desai, T.A., 2009. Microfabricated Devices for Enhanced Bioadhesive Drug Delivery: Attachment to and Small-Molecule Release Through a Cell Monolayer Under Flow. *Small* 5, 2857–2863. doi:10.1002/smll.200901254
- Amidon, G.L., Lennernas, H., Shah, V.P., Crison, J.R., 1995. A Theoretical Basis for a Biopharmaceutic Drug Classification - the Correlation of in-Vitro Drug Product Dissolution and in-Vivo Bioavailability. *Pharmaceutical Research* 12, 413–420. doi:10.1023/a:1016212804288
- Avdeef, A., Tsinman, O., 2008. Miniaturized rotating disk intrinsic dissolution rate measurement: Effects of buffer capacity in comparisons to traditional Wood's apparatus. *Pharmaceutical Research* 25, 2613–2627. doi:10.1007/s11095-008-9679-z
- Berginc, K., Trontelj, J., Kristl, A., 2012. Bio-relevant media to assess drug permeability: Sodium taurocholate and lecithin combination or crude bile? *International Journal of Pharmaceutics* 429, 22–30. doi:10.1016/j.ijpharm.2012.03.015
- Chirra, H.D., Desai, T.A., 2012. Multi-Reservoir Bioadhesive Microdevices for Independent Rate-Controlled Delivery of Multiple Drugs. *Small* 8, 3839–3846. doi:10.1002/smll.201201367
- Chirra, H.D., Shao, L., Ciaccio, N., Fox, C.B., Wade, J.M., Ma, A., Desai, T. a, 2014. Planar microdevices for enhanced in vivo retention and oral bioavailability of poorly permeable drugs. *Advanced healthcare materials* 3, 1648–54. doi:10.1002/adhm.201300676
- Chirra, H.D., Shao, L., Ciaccio, N., Fox, C.B., Wade, J.M., Ma, A., Desai, T. a., 2014. Planar Microdevices for Enhanced In Vivo Retention and Oral Bioavailability of Poorly Permeable Drugs. *Advanced Healthcare Materials* 3, 1648–1654. doi:10.1002/adhm.201300676
- Colombo, P., Sonvico, F., Colombo, G., Bettini, R., 2009. Novel Platforms for Oral Drug Delivery. *Pharmaceutical Research* 26, 601–611. doi:10.1007/s11095-008-9803-0
- Crater, J.S., Carrier, R.L., 2010. Barrier Properties of Gastrointestinal Mucus to Nanoparticle Transport. *Macromolecular Bioscience* 10, 1473–1483. doi:10.1002/mabi.201000137
- Doluisio, J., Tan, G., Billups, N., Diamond, L., 1969. Drug absorption II: Effect of Fasting on Intestinal Drug Absorption. *Journal of Pharmaceutical Sciences* 1200–1202.
- Eaimtrakarn, S., Itoh, Y., Kishimoto, J., Yoshikawa, Y., Shibata, N., Takada, K., 2001. Retention and transit of intestinal mucoadhesive films in rat small intestine. *International Journal of Pharmaceutics* 224, 61–67. doi:10.1016/s0378-5173(01)00738-4
- Ensign, L.M., Cone, R., Hanes, J., 2012. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. *Advanced Drug Delivery Reviews* 64, 557–570. doi:10.1016/j.addr.2011.12.009
- Fagerholm, U., Johansson, M., Lennernas, H., 1996. Fagerholm U et al 1996.pdf. *Pharmaceutical Research* 13, 1336–1342.
- García-Díaz, M., Foged, C., Nielsen, H.M., 2015. Improved insulin loading in poly(lactic-co-glycolic) acid (PLGA) nanoparticles upon self-assembly with lipids. *International Journal of Pharmaceutics* 482, 84–91. doi:10.1016/j.ijpharm.2014.11.047
- Goud, R., Swarupa, G., Suresh, K., Pal, S., Manjunatha, S.G., Nambiar, S., Nangia, A., 2011. Novel Furosemide Cocrystals and Selection of High Solubility Drug Forms. *Journal of pharmaceutical sciences* 101, 664–680. doi:10.1002/jps
- Granero, G.E., Longhi, M.R., Mora, M.J., Junginger, H.E., Midha, K.K., Shah, V.P., Stavchansky, S., Dressman, J.B., Barends, D.M., 2010. Biowaiver Monographs for Immediate Release Solid Oral Dosage Forms: Furosemide. *Journal of Pharmaceutical Sciences* 99, 2544–2556. doi:10.1002/jps.22030
- Hidalgo, I.J., Raub, T.J., Borchardt, R.T., 1989. Characterization of the Human-Colon Carcinoma Cell-Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. *Gastroenterology* 96, 736–749.

- Hilgendorf, C., Spahn-Langguth, H., Regardh, C.G., Lipka, E., Amidon, G.L., Langguth, P., 2000. Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: Permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. *Journal of Pharmaceutical Sciences* 89, 63–75. doi:10.1002/(sici)1520-6017(200001)89:1<63::aid-jps7>3.0.co;2-6
- Hilgers, A.R., Conradi, R.A., Burton, P.S., 1990. Caco-2 Cell Monolayers as a Model for Drug Transport across the Intestinal-Mucosa. *Pharmaceutical Research* 7, 902–910. doi:10.1023/a:1015937605100
- Iannuccelli, V., Coppi, G., Leo, E., Fontana, F., Bernabei, M.T., 2000. PVP solid dispersions for the controlled release of furosemide from a floating multiple-unit system. *Drug Development and Industrial Pharmacy* 26, 595–603. doi:10.1081/ddc-100101274
- Ingels, F., Deferme, S., Destexhe, E., Oth, M., Van den Mooter, G., Augustijns, P., 2002. Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *International Journal of Pharmaceutics* 232, 183–192. doi:10.1016/s0378-5173(01)00897-3
- Keller, S., Haefliger, D., Boisen, A., 2007. Optimized plasma-deposited fluorocarbon coating for dry release and passivation of thin SU-8 cantilevers. *Journal of Vacuum Science & Technology B: Microelectronics and Nanometer Structures* 25, 1903. doi:10.1116/1.2806960
- Keller, S.S., Bosco, F.G., Boisen, A., 2013. Ferromagnetic shadow mask for spray coating of polymer patterns. *Microelectronic Engineering* 110, 427–431. doi:10.1016/j.mee.2013.03.029
- Kostewicz, E.S., Abrahamsson, B., Brewster, M., Brouwers, J., Butler, J., Carlert, S., Dickinson, P.A., Dressman, J., Holm, R., Klein, S., Mann, J., McAllister, M., Minekus, M., Muenster, U., Mullertz, A., Verwei, M., Vertzoni, M., Weitschies, W., Augustijns, P., 2014. In vitro models for the prediction of in vivo performance of oral dosage forms. *European Journal of Pharmaceutical Sciences* 57, 342–366. doi:10.1016/j.ejps.2013.08.024
- Kumar, K.K., Karnati, S., Reddy, M.B., Chandramouli, R., 2010. Caco-2 cell lines in drug discovery- an updated perspective. *J Basic Clin Pharm* 1, 7.
- Larsen, M., Brodin, B., Frølund, B., Uhd, C., 2008. Transport of amino acids and GABA analogues via the human proton-coupled amino acid transporter , hPAT1 : Characterization of conditions for affinity and transport experiments in Caco-2 cells. *European Journal of Pharmaceutical Sciences* 35, 86–95. doi:10.1016/j.ejps.2008.06.007
- Lehr, C.M., 2000. Lectin-mediated drug delivery: The second generation of bioadhesives. *Journal of Controlled Release* 65, 19–29. doi:10.1016/S0168-3659(99)00228-X
- Lehr, C.M., Poelma, F.G.J., Junginger, H.E., Tukker, J.J., 1991. An Estimate of Turnover Time of Intestinal Mucus Gel Layer in the Rat Insitu Loop. *International Journal of Pharmaceutics* 70, 235–240. doi:10.1016/0378-5173(91)90287-x
- Lehr, C.-M., Poelma, F.G.J., Junginger, H.E., Tukker, J.J., 1991. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. *International Journal of Pharmaceutics* 70, 235–240. doi:10.1016/0378-5173(91)90287-X
- Lennernas, H., 1998. Human Intestinal Permeability. *Journal of Pharmaceutical Sciences* 87, 403–410.
- Lennernäs, H., 2014. Regional intestinal drug permeation: Biopharmaceutics and drug development. *European Journal of Pharmaceutical Sciences* 57, 333–341. doi:10.1016/j.ejps.2013.08.025
- Lind, M.L., Jacobsen, J., Holm, R., Mullertz, A., 2007. Development of simulated intestinal fluids containing nutrients as transport media in the Caco-2 cell culture model: Assessment of cell viability, monolayer integrity and transport of a poorly aqueous soluble drug and a substrate of efflux mechanisms. *European Journal of Pharmaceutical Sciences* 32, 261–270. doi:10.1016/j.ejps.2007.08.002
- Lipinski, C.A., 2001. Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods* 44.
- Lozoya-Agullo, I., Zur, M., Wolk, O., Beig, A., González-Álvarez, I., González-Álvarez, M., Merino-Sanjuán, M., Bermejo, M., Dahan, A., 2015. In-situ intestinal rat perfusions for human Fabs prediction and BCS permeability class determination: Investigation of the single-pass vs. the Doluisio experimental approaches. *International Journal of Pharmaceutics* 480, 1–7. doi:10.1016/j.ijpharm.2015.01.014
- Matsuda, Y., Tatsumi, E., Thermal, S., 1990. Physicochemical characterization of furosemide modifications.

- International Journal of Pharmaceutics 60, 11–26.
- Nemani, K. V., Moodie, K.L., Brennick, J.B., Su, A., Gimi, B., 2013. In vitro and in vivo evaluation of SU-8 biocompatibility. *Materials Science and Engineering C* 33, 4453–4459. doi:10.1016/j.msec.2013.07.001
- Nielsen, L.H., Gordon, S., Holm, R., Selen, A., Rades, T., Mullertz, A., 2013a. Preparation of an amorphous sodium furosemide salt improves solubility and dissolution rate and leads to a faster T-max after oral dosing to rats. *European Journal of Pharmaceutics and Biopharmaceutics* 85, 942–951. doi:10.1016/j.ejpb.2013.09.002
- Nielsen, L.H., Gordon, S., Pajander, J.P., Ostergaard, J., Rades, T., Mullertz, A., 2013b. Biorelevant characterisation of amorphous furosemide salt exhibits conversion to a furosemide hydrate during dissolution. *International Journal of Pharmaceutics* 457, 14–24. doi:10.1016/j.ijpharm.2013.08.029
- Nielsen, L.H., Keller, S.S., Boisen, A., Mullertz, A., Rades, T., 2014. A slow cooling rate of indomethacin melt spatially confined in microcontainers increases the physical stability of the amorphous drug without influencing its biorelevant dissolution behaviour. *Drug Deliv. and Transl. Res.* 4, 7.
- Nielsen, L.H., Keller, S.S., Gordon, K.C., Boisen, A., Rades, T., Mullertz, A., 2012. Spatial confinement can lead to increased stability of amorphous indomethacin. *European Journal of Pharmaceutics and Biopharmaceutics* 81, 418–425. doi:10.1016/j.ejpb.2012.03.017
- Nielsen, L.H., Nagstrup, J., Gordon, S., Keller, S.S., Østergaard, J., Rades, T., Müllerertz, A., Boisen, A., 2015. pH-triggered drug release from biodegradable microwells for oral drug delivery. *Biomedical Microdevices* 17, 1–7. doi:10.1007/s10544-015-9958-5
- Nordgård, C.T., Nonstad, U., Olderøy, M.Ø., Espevik, T., Draget, K.I., 2014. Alterations in mucus barrier function and matrix structure induced by guluronate oligomers. *Biomacromolecules* 15, 2294–300. doi:10.1021/bm500464b
- Pade, V., Stavchansky, S., 1997. Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model. *Pharmaceutical Research* 14, 1210–1215. doi:10.1023/a:1012111008617
- Patel, N., Forbes, B., Eskola, S., Murray, J., 2006. Use of simulated intestinal fluids with Caco-2 cells in rat ileum. *Drug Development and Industrial Pharmacy* 32, 151–161. doi:10.1080/03639040500465991
- Perioli, L., D’Alba, G., Pagano, C., 2012. New oral solid dosage form for furosemide oral administration. *European Journal of Pharmaceutics and Biopharmaceutics* 80, 621–629. doi:10.1016/j.ejpb.2011.12.011
- Pretorius, E., Bouic, P.J.D., 2009. Permeation of Four Oral Drugs Through Human Intestinal Mucosa. *Aaps Pharmscitech* 10, 270–275. doi:10.1208/s12249-009-9207-4
- Randall, C.L., Leong, T.G., Bassik, N., Gracias, D.H., 2007. 3D lithographically fabricated nanoliter containers for drug delivery. *Advanced Drug Delivery Reviews* 59, 1547–1561. doi:10.1016/j.addr.2007.08.024
- Sander, C., Nielsen, H.M., Jacobsen, J., 2013. Buccal delivery of metformin: TR146 cell culture model evaluating the use of bioadhesive chitosan discs for drug permeability enhancement. *International Journal of Pharmaceutics* 458, 254–261. doi:10.1016/j.ijpharm.2013.10.026
- Sjogren, E., Westergren, J., Grant, I., Hanisch, G., Lindfors, L., Lennernas, H., Abrahamsson, B., Tannergren, C., 2013. In silico predictions of gastrointestinal drug absorption in pharmaceutical product development: Application of the mechanistic absorption model GI-Sim. *European Journal of Pharmaceutical Sciences* 49, 679–698. doi:10.1016/j.ejps.2013.05.019
- Sjögren, E., Abrahamsson, B., Augustijns, P., Becker, D., Bolger, M.B., Brewster, M., Brouwers, J., Flanagan, T., Harwood, M., Heinen, C., Holm, R., Juretschke, H.-P., Kubbinga, M., Lindahl, A., Lukacova, V., Münster, U., Neuhoﬀ, S., Nguyen, M.A., Peer, A. Van, Reppas, C., Hodjegan, A.R., Tannergren, C., Weitschies, W., Wilson, C., Zane, P., Lennernas, H., Langguth, P., 2014. In vivo methods for drug absorption - comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects., *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*. doi:10.1016/j.ejps.2014.02.010

- Song, S., Tian, B., Chen, F., Zhang, W., Pan, Y., Zhang, Q., Yang, X., 2013. Potentials of proniosomes for improving the oral bioavailability of poorly water-soluble drugs 9045, 1–12. doi:10.3109/03639045.2013.845841
- Tao, S.L., Desai, T.A., 2007. Aligned arrays of biodegradable poly(epsilon-caprolactone) nanowires and nanofibers by template synthesis. *Nano Letters* 7, 1463–1468. doi:10.1021/nl0700346
- Tao, S.L., Lubeley, M.W., Desai, T.A., 2003. Bioadhesive poly(methyl methacrylate) microdevices for controlled drug delivery. *Journal of Controlled Release* 88, 215–228. doi:10.1016/s0168-3659(03)00005-1
- Twibanire, J.D.K., Grindley, T.B., 2014. Polyester Dendrimers: Smart Carriers for Drug Delivery. *Polymers* 6, 179–213. doi:10.3390/polym6010179
- van Hoogevest, P., Liu, X.L., Fahr, A., 2011. Drug delivery strategies for poorly water-soluble drugs: the industrial perspective. *Expert Opinion on Drug Delivery* 8, 1481–1500. doi:10.1517/17425247.2011.614228
- Vertzoni, M., Dressman, J., Butler, J., Hempenstall, J., Reppas, C., 2005. Simulation of fasting gastric conditions and its importance for the *in vivo* dissolution of lipophilic compounds. *European Journal of Pharmaceutics and Biopharmaceutics* 60, 413–417. doi:10.1016/j.ejpb.2005.03.002
- Wenk, E., Merkle, H.P., Meinel, L., 2011. Silk fibroin as a vehicle for drug delivery applications. *Journal of Controlled Release* 150, 128–141. doi:10.1016/j.jconrel.2010.11.007
- Xu, Y., Wang, Y., Li, X.M., Huang, Q., Chen, W., Liu, R., Chen, B., Wei, P., 2014. Study on the release of fenofibrate nanosuspension *in vitro* and its correlation with *in situ* intestinal and *in vivo* absorption kinetics in rats. *Drug Development and Industrial Pharmacy* 40, 972–979. doi:10.3109/03639045.2013.794828