A human nose-on-a-chip as a physiologically relevant *in-vitro* model for nasal drug delivery

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Summary
A dual-channel nose-on-a-chip (NOC) was developed incorporating an air-liquid interface model of human nasal epithelial cells as a novel platform for testing nasal drug delivery *in-vitro*. The novelty of the NOC lies in mimicking realistic deposition of drug aerosols in the apical donor microchannel, where the cells are exposed to a flow-induced shear stress of 0.23 Pa, mimicking the *in-vivo* nasal airflow during inhalation. In addition, a pulsatile fluid flow within the acceptor basolateral microchannel mimics the systemic circulation through the vasculatures in the human nasal mucosa. This dynamic microenvironment significantly influences the transport profile of ibuprofen (IBU-model drug) across the nasal epithelium. The pulsatile flow in the basolateral microchannel significantly increases the rate of drug transport and the delivery of aerosolized drug under flow conditions in the apical microchannel causes a further increase in the percentage of transported IBU. Furthermore, the barrier function of the nasal epithelium remains unaffected throughout the study, as assessed by the *in-situ* transepithelial electrical resistance measurements. Hence, this NOC highlights the significant effect of the biomechanical factors such as shear stress on nasal drug delivery assay results and the importance of incorporating these physiological dynamic conditions for *in-vitro* nasal drug testing. The miniaturized platform of the NOC with in-built electrochemical and impedance sensors is rapid and relatively economical for quantification of IBU transport and epithelial integrity, respectively. Importantly, these sensors are sufficiently accurate for the quantification of the IBU transport and comparable to current analytical methodologies.

Key Message
Nasal drug delivery testing using the physiologically relevant dynamic microenvironment recreated by the NOC demonstrated the impact of biomechanical factors such as shear stress and dynamic real aerosol deposition on drug permeability. Hence, NOC can serve as a potential alternative to the conventional static models and enhance *in-vitro in-vivo* correlations.

Introduction
In-vitro evaluation of nasal drug formulations includes investigating the toxicity and transport of drugs across the human nasal mucosa before reaching the target site to elucidate its pharmacological action [1]. Therefore, establishing a physiologically relevant *in-vitro* model of the human nasal mucosa barrier is of paramount importance for these assessments to accurately predict clinical outcomes. The current *in-vitro* models of the human nasal mucosa used for nasal drug testing were developed using the air-liquid interface (ALI) model of the nasal epithelial cells on Transwell/Snapwell inserts, where the transport of drugs across the epithelium is evaluated using drug solution or suspension under static conditions. These models fail to simulate the physiology of human nasal mucosa in terms of the aerosol deposition and dynamic biomechanical stimuli such as fluid and air flow over the mucosa. This can potentially affect the ability of these conventional approaches to accurately predict the efficacy of nasal therapeutics *in-vivo* [2]. In addition, these conventional assays typically require multiple steps including samples preparation before analyses and subsequent drug quantification. These steps are often time-consuming and require extensive use of solvents and consumables, as well as expensive analytical instruments. As a result, there is a need to develop novel nasal drug testing platforms with improved physiological relevance that could also act as an analytical tool that is time and cost-efficient. This study aims to develop a human nose-on-a-chip (NOC) model that mimics the realistic flow of aerosolised nasal formulations and deposition of particles on the nasal mucosa that enables testing of nasal formulations under *in-vivo*-inspired fluid dynamic conditions. In addition, the NOC is integrated with miniaturised analytical sensors for *in-situ* drug quantification and epithelial barrier function monitoring to study real-time drug transport across the epithelium and the potential effects on the barrier integrity. Ibuprofen (IBU) is used as a model drug in this study due to its effect in the management of Alzheimer's disease and the growing interest in dosing it via direct nose-to-brain drug delivery route [3].

Experimental methods

Fabrication of the NOC and its use to emulate the delivery of the nasal aerosol particles *in-vitro*

The design and fabrication of the NOC have been reported elsewhere [4]. Briefly, the NOC comprises of two microchannels made of polydimethylsiloxane, sandwiching a transparent polystyrene membrane (12 µm thickness, 0.4 µm pore size, 2×10^6 pores/cm^2). Slight changes are made in the design, including the insertion of a 2.5 cm long vacuum resistant Tygon® tube (OD = 2 mm, ID = 0.8 mm) horizontally at the inlet of the donor microchannel for aerosol deposition as shown in Fig. 1. Four platinum (Pt) wire electrodes are inserted into the microchannels (two electrodes in each microchannel) and carbon nanofibers (CNFs)-modified carbon electrode (CNFs-C) is integrated into the acceptor microchannel (Fig. 1). These electrodes enable *in-situ* transepithelial electrical resistance (TEER) and drug transport monitoring, respectively [4]. RPMI 2650 (CCL-30) cell line was cultured under ALI condition in the donor microchannel for 14 days as optimized previously [4] for tight junctions (TJs) formation and mucus secretion.

To simulate the airflow inside the device, the apical donor microchannel outlet was connected to a vacuum pump and the inlet was connected to a Flow Meter 4040 (TSI instruments Ltd, UK) to adjust the airflow rate to 0.5 L/min. Given the dimensions of the donor microchannel cross-section (1 × 1 mm), this flow rate results in shear stress of 0.23 Pa on the cells as calculated by $\tau = 6\mu Q/h^2 w$ [5], where τ is the shear stress (Pa), µ is the dynamic viscosity of air (kg.m^{-1}.s^{-1}), Q is the airflow rate (m^3.s^{-1}), h is the channel height (m), and w is the channel width (m). This applied shear stress for drug deposition simulates the shear stress in the inferior and middle turbinates of the human nasal cavity *in-vivo* (0.2 up to 1.2 Pa) during breathing [6-8].

To deposit the drug aerosols in the NOC, the donor inlet was connected to a PARI LC® Sprint jet nebulizer, driven by a PARI Turbo Boy S compressor (Starnberg, Germany) to generate aerosols while 0.5 L/min air flow rate was applied. This nebulizer generates aerosol particles of similar size (mass median diameter = 3.5 µm) to the PARI Sinus, which is used for efficient sinus targeting and aerosol administration into the nasal cavity [9]. To visualize the deposited particles in the NOC, Hank's balanced salt solution (HBSS) was loaded with sodium fluorescein (flu-Na) with 2.5 mg/mL concentration. After deposition of the aerosolized particles to the apical donor microchannel, microscopic images were taken with an x20 objective of a Nikon Elipse TI time-lapse microscope. To study the drug transport by NOC, ibuprofen (IBU) was added to HBSS (2 mg/mL) as a model drug. This concentration of IBU was selected based on the standard solubility of the drug in PBS, which did not show toxic effects on nasal epithelial cells *in vitro* [10]. The drug transport was studied under three different settings: (I) the apical microchannel was filled with 35 µL of IBU-HBSS and the basolateral microchannel was filled with phosphate buffer (PBS; 0.1 M, pH 7) under static conditions; (II) a pulsatile flow of PBS at the rate of 0.5 mL/min through the basolateral microchannel was applied while the apical microchannel was filled with IBU-HBSS; and (III) the aerosolized IBU-HBSS was deposited in the donor microchannel with 0.5 L/min airflow rate in addition to the pulsatile flow of PBS in the basolateral microchannel.

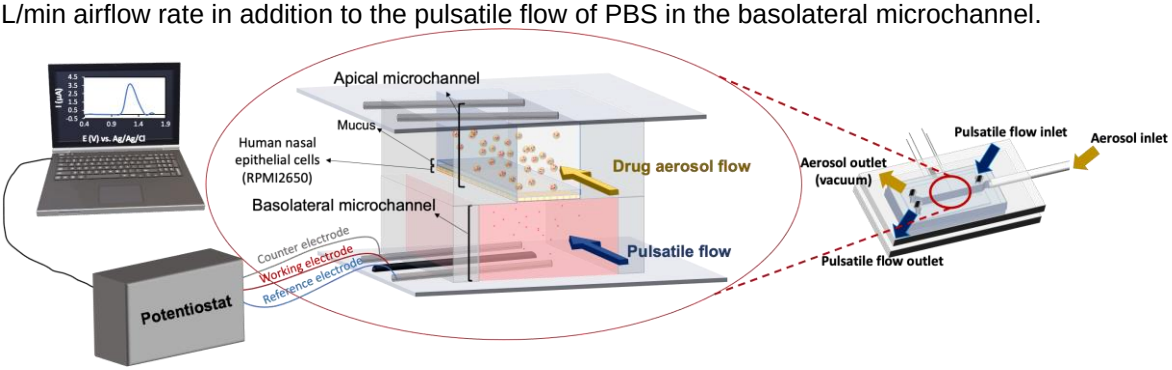


Figure 1. The schematics of NOC structure, deposition of the aerosol particles in the donor microchannel, and connection of the electrodes to a potentiostat to monitor the drug transport in real-time.

In-situ TEER measurements by the NOC

Pre-warmed cell culture was injected into the donor and acceptor microchannels and the NOC was incubated for 15 min for equilibration. One pair of the Pt electrodes (one electrode in the donor and one in the acceptor channel) were connected to a potentiostat (PGSTAT204, Metrohm Autolab, Netherlands) and small amplitude (0.01 VRMS) AC excitation signal (1 mA) with a frequency sweep (10^{-1} – 10^6 Hz) was applied and the amplitude and phase response of the resulting current were recorded. An equivalent electrical circuit representing the resistance and capacitance of the cell layer was then fitted to the recorded data using NOVA 2.1.2 software (Metrohm Autolab, The Netherlands) to determine the

electrical resistance of the cell layer. The measurement was repeated for the second pair of the Pt electrodes.

Real-time nasal drug transport monitoring by the NOC

After the introduction of IBU into the NOC, the IBU transfer across the epithelium to the acceptor microchannel was quantified *in-situ*. For this, one of the Pt wires in the acceptor channel was replaced with a silver/silver chloride wire electrode. This electrode with the CNFs-C and the Pt electrode in the acceptor microchannel were connected to the potentiostat. The transported IBU into the basolateral channel was quantified based on electrochemical analysis by performing square wave voltammetry within 0.8 – 1.4 V potential range with 5 mV step potential, 20 mV modulation amplitude, 10 Hz frequency, and 0.1 s interval time as optimized previously for detection of IBU [4]. The assay was conducted up to 4 h as this is the widely accepted time for evaluating respiratory drug delivery *in vitro* [1,10, 11] as the biological properties and viability of the cells are maintained throughout the assay. To validate the IBU quantification by the NOC, a sample collected from the acceptor microchannel at the end of the experiment was analyzed by high-performance liquid chromatography (HPLC) as reported previously [10]. Briefly, the mobile phase was 60% V/V acetonitrile solution in water with pH adjusted at 3.5 using acetic acid. The analyses were conducted by a Shimadzu Prominence UFLC system equipped with the SPD-20A UV-Vis detector (Shimadzu Corporation, Japan) and C18 column (5 µm, 250 × 4.6 mm). The detection was performed at 254 nm wavelength and 1.5 mL/min mobile phase flow rate, 100 µL injection volume, and retention time of 4.5 min.

Results and discussion

Deposition of nasal formulation aerosols in the NOC

The microscopic image obtained from the apical donor microchannel of the NOC after deposition of the HBSS aerosols loaded with flu-Na is shown in Fig. 2. The obtained images were analyzed with Image J software to measure the area of each particle and calculate the diameter of the particle. The frequency distributions of the measured particles size were obtained and a range of particles size between 1 – 30 µm was determined. This can mimic the aerosol deposition in regions of the human nasal cavity such as the olfactory region, where deposition of particles with 8 – 12 µm size is reported [12]. Delivery of nasal drugs to the olfactory region is essential for direct nose-to-brain drug delivery. This results in skipping blood-brain barrier and efficiently targeting brain tissue to treat neurological disorders [10].

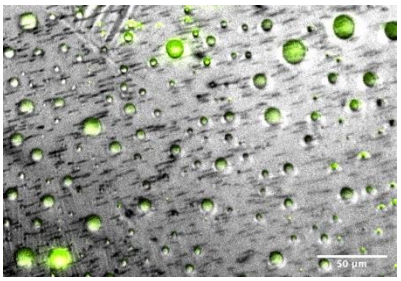


Figure 2. Microscopic image taken from HBSS aerosols deposited into the donor microchannel of the NOC when 0.5 L/min airflow rate was applied. The scale bar shows 50 µm.

The evaluation of the mucosal barrier function of the NOC under dynamic conditions

The *in-situ* TEER measurements by the Pt electrodes in the NOC allowed for monitoring the barrier properties of the modelled nasal epithelium before and after exposing the cells to the flow of the aerosolised formulations. It was found that the TEER measured before and after exposure of the cells to the flow of aerosolised particles was not statistically different (373.48 ± 73 $\Omega.cm^2$). Therefore, the integrity of the nasal epithelium in the NOC under the simulated dynamic conditions was not negatively affected under the time scale studied. Hence, we can confidently study the nasal formulations under the *in-vivo*-relevant dynamic conditions without affecting the barrier function of the epithelium.

Real-time nasal drug transport monitoring by the NOC

Fig. 3a shows the time-course of cumulative IBU mass detected in the acceptor microchannel by the CNFs-C sensor in three different conditions: (I) static condition in NOC, (II) static apical and pulsatile flow in the basolateral microchannel, and (III) aerosol flow in the apical and pulsatile flow in the basolateral microchannels. To evaluate the effects of the dynamic pulsatile flow conditions in the basolateral microchannel on the rate of the IBU transport, the obtained profiles of the static NOC and dynamic basolateral microchannels were statistically compared based on the Moore and Flanner fit factors [11]. According to this analysis, the difference (f1) and similarity (f2) factors were calculated to compare the obtained profiles. It is the FDA requirement to evaluate the fulfilment of both f1 and f2 factors to consider two profiles different or similar [11]. The f1 and f2 factors were calculated as 53.76 and 47.72, respectively. Since $f1 \geq 10$ and $f2 \leq 50$, the difference between the two profiles is statistically

different [11]. The basolateral flow resulted in a significant increase in the rate of drug transport over time. This can be associated with the increase in the convective mass transfer coefficient in the basolateral microchannel as a result of the fluid flow conditions [4]. Similarly, the profile of the IBU mass transport was compared between the dynamic basolateral only and dynamic apical and basolateral conditions. For these two profiles, f1 and f2 were calculated as 87.66 and 36.17, respectively, showing a statistically significant difference.

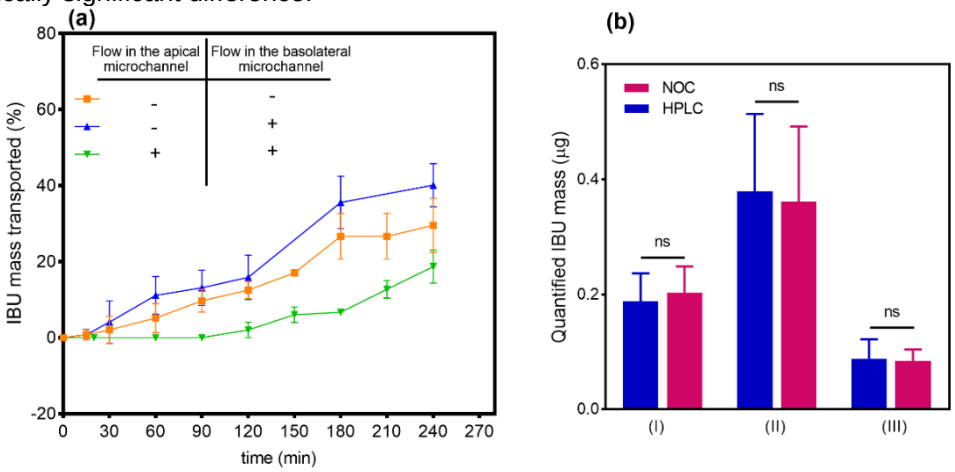


Figure 3. (a) The IBU mass transported across the RPMI 2650 cell layer under ALI in the NOC after deposition of the aerosolized IBU-HBSS at different static and dynamic conditions. (b) The IBU mass in the bottom channel of the NOC detected electrochemically by the CNFs-C electrode integrated into the acceptor microchannel versus IBU quantifications by HPLC for (I) static condition in NOC, (II) static apical and pulsatile flow in the basolateral microchannel, and (III) aerosol flow in the apical and pulsatile flow in the basolateral microchannel (n = 3, mean ± StD).

When the IBU aerosol was deposited on the epithelium under the dynamic condition, IBU transport rate was significantly slower compared to transport rates in both the static conditions when IBU solution was administered. The slower IBU transport rate may be related to the lower IBU mass that was delivered to the cells by the aerosolised particles compared to the other two experiments, where the donor microchannel was filled with IBU solution under static conditions. Importantly, IBU aerosols deposited on the nasal epithelium requires an additional step of wetting and diffusion across the mucus layer prior to drug absorption. This is also in agreement with the observation of a gradual increase in the rate of IBU transport 2 hrs after start of the experiment.

Further, the *in-situ* drug quantification by the NOC was validated versus HPLC. Fig. 3b shows the IBU mass detected by the NOC and HPLC at the endpoint of the three experiments (4 h). For the experiments under static condition, dynamic basolateral microchannel, and dynamic apical and basolateral microchannels, no significant difference ($P = 0.7258$, 0.8761, and 0.3639, respectively) was found between the measurements obtained using the NOC and HPLC methods, the latter is currently used as the gold standard analytical technique in the pharmaceutical industry. This data shows the reliability of the NOC for drug quantifications.

Conclusions

A novel NOC platform was successfully developed with capabilities to emulate the dynamic microenvironment of the human nasal mucosa to enhance the physiological relevance of the *in-vitro* nasal drug tests to *in-vivo* clinical performance. The simulation of *in-vivo*-inspired shear stress in the apical donor microchannel of the NOC, as well as the circulating pulsatile flow in the basolateral acceptor microchannel, were found to significantly affect nasal drug transport studies. The NOC with its improved *in-vitro in-vivo* relevance facilitated drug quantification and TEER assessments using the in-built analytical capability and could potentially be used as a rapid and economic alternative to conventional analytical testing by HPLC. Furthermore, the CNFs-C electrode used in the NOC has great potential to be used for quantification of different drug compounds to broaden the applicability of the NOC for testing different drugs. However, this platform may need optimization of the electrode composition and supplied voltage depending on how different the electroactivity of the test drugs can be. Future research will investigate the simulation of localized therapies in the dynamic microenvironment of the NOC and assess its performance against *in-vivo* results.

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