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Xinyue Zhang , Stephanie Chow , Ho Wan Chan ,
Shing Fung Chow

PII: S0022-3549(26)00005-5
DOI: https://doi.org/10.1016/j.xphs.2026.104156
Reference: XPHS 104156

To appear in: *Journal of Pharmaceutical Sciences*

Received date: 16 November 2025
Revised date: 2 January 2026
Accepted date: 3 January 2026

Please cite this article as: Xinyue Zhang , Stephanie Chow , Ho Wan Chan , Shing Fung Chow , Overcoming translational barriers in nose-to-brain drug delivery for clinical applications, *Journal of Pharmaceutical Sciences* (2026), doi: https://doi.org/10.1016/j.xphs.2026.104156

Highlights

- Guidance on selecting excipients and nasal delivery devices, along with specific formulation and delivery strategies to optimize nose-to-brain transport.
- A comprehensive overview of common characterization techniques used to evaluate the nasal biopharmaceutics of drugs within the nose-to-brain delivery pathway.
- An in-depth discussion of potential influencing factors and major challenges that may affect the clinical translatability of intranasal drugs.
- Strategic recommendations to enhance the reliability and applicability of relevant characterization techniques in translational research.

Overcoming translational barriers in nose-to-brain drug delivery for clinical applications

Xinyue Zhang [a], Stephanie Chow [a], Ho Wan Chan [a,*,#], Shing Fung Chow [a,b,*]

[a] Department of Pharmacology and Pharmacy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

[b] Advanced Biomedical Instrumentation Centre, Hong Kong Science Park, Shatin, Hong Kong SAR, China

[*] *Co-corresponding authors*

[#] *Present address: Institute of Pharmaceutical Science, King's College London, London SE1 9NH, United Kingdom*

Shing Fung Chow BEng, PhD

Department of Pharmacology and Pharmacy

Li Ka Shing Faculty of Medicine

The University of Hong Kong

L2-08B, 2/F, Laboratory Block

21 Sassoon Road, Pokfulam, Hong Kong S.A.R., China

Email: asfchow@hku.hk

Tel: +852-39179026

Fax: +852-28170859

Abstract

Recent research has shown an increasing interest in nose-to-brain drug delivery due to its non-invasive nature and ability to transport therapeutics directly to the central nervous system. This approach offers significant advantages over traditional administration routes, such as the circumvention of the blood-brain barrier and avoidance of first-pass metabolism, thereby enhancing therapeutic efficacy while reducing systemic side effects. Despite promising preclinical findings, nose-to-brain delivery remains underrepresented in the pharmaceutical market, highlighting a critical gap between experimental research and clinical translation. This review critically examines the major challenges confronting nose-to-brain delivery systems, including formulation development, selection of nasal devices, and methodologies for evaluating the nasal biopharmaceutics of drugs during delivery. Furthermore, strategic recommendations and research priorities are outlined to address these barriers. By identifying and analyzing factors that contribute to the translational failure of nose-to-brain systems, it is believed that more effective delivery systems can be developed, ultimately revolutionizing treatment strategies for neurological diseases.

Keywords: Nasal drug delivery; Central Nervous System (CNS); Formulation; Drug transport; Biopharmaceutical characterization; *In vitro* model(s); *In silico* modeling; *In Vitro*/*In Vivo* (IVIVC) Correlation(s)

1. Introduction

The global burden of neurological diseases has markedly increased over recent decades, both in terms of mortality and disability. This trend is expected to continue as the population ages and grows.[1] In 2021, diseases related to the nervous system affected an estimated 3.40 billion individuals, accounting for 43.1% of the world's total population, and were responsible for 11.1 million deaths worldwide.[2] Central nervous system (CNS) disorders, such as stroke, Parkinson's disease, psychiatric conditions, Alzheimer's disease, brain tumors, epilepsy, meningitis, and insomnia, affect millions of patients and their families worldwide. However, the development of effective drug delivery systems for treating CNS disorders has remained largely stagnant, primarily due to the restrictive nature of the blood-brain barrier (BBB). This challenge results in high failure rates and necessitates prolonged, costly research efforts.[3, 4] Conventional treatment modalities, mainly oral or systemic administration, are often limited by severe systemic adverse effects and poor BBB penetration, thereby reducing therapeutic efficacy.[3] Beyond efficacy, many CNS disorders demand a rapid onset of action, for instance, in seizures and for neuroprotection during the hyperacute phase of ischemic stroke. Others require more convenient administration routes to improve patient compliance, such as in Parkinson's disease and psychiatric disorders. Additionally, reducing off-target side effects is critical for specific treatments to ensure safety during long-term use, as in dementia and insomnia.

Nose-to-brain (NTB) drug delivery presents a promising approach to address these challenges by enabling direct drug delivery to the CNS while circumventing the BBB. Although intranasal administration presents challenges such as relatively low maximum doses (due to the restricted volume of the nasal cavity), rapid mucociliary clearance (thereby limiting sustained drug release), and potential ciliary toxicity, this modality also offers several advantages, including convenience, painless and non-invasive administration, suitability for self-administration, and rapid onset of action.[5, 6] Although the precise mechanisms underlying NTB drug delivery remain an active area of research, two direct pathways are recognized as the most significant: the olfactory and trigeminal pathways.[4] Among these, the olfactory pathway is particularly notable as it is the shortest path from the nose to the CNS, thereby positioning the olfactory region as a primary target in current research.

The unique merits of NTB drug delivery have spurred increased research interest. However, such drug products remain underrepresented in the pharmaceutical market, highlighting a gap between experimental research and clinical translation. Apart from physiological factors such as mucociliary clearance and enzymatic degradation, drug formulation and nasal device design can also influence delivery outcomes. Moreover, the selection of characterization methods and their parameters may produce misleading results, contributing to translational failure. Current regulatory guidance from agencies such as the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) for nasal

products primarily focuses on general nasal product characterization (e.g., droplet size distribution and dose uniformity), with no specific framework for characterizing product attributes for NTB drug products.[7-9] This underscores the need for standardized guidelines to optimize characterization methodologies across nasal products intended for different delivery pathways. Many existing protocols in the literature inadequately capture the complex physiological fate of drug-loaded aerosols after emission from the nasal device, thereby limiting their predictive ability of *in vivo* drug biological fate and clinical performance. The emerging field of "nasal biopharmaceutics", i.e., the "scientific understanding of product and patient factors that determine the rate and extent of drug exposure following nasal administration" as defined by Forbes et al.,[10] provides insights for developing and translating nasal therapies. This is particularly pertinent for products intended for NTB drug delivery, given the multifaceted considerations in formulation strategies, device designs, and the intricate mechanisms governing NTB drug transport pathways.[10]

Previous review articles have independently summarized formulation strategies, nasal device designs, and characterization techniques for NTB drug delivery systems. However, a comprehensive and critical assessment of the unresolved challenges impeding translational progress in this area remains lacking.[11, 12] Accordingly, this review aims to provide strategic recommendations for the rational design of drug formulations and the selection of nasal delivery devices tailored for NTB drug products. It offers an exhaustive evaluation and analysis of key characterization methodologies, emphasizing their respective advantages and limitations, while also examining the experimental variables that influence their outcomes. Furthermore, this review discusses potential factors contributing to translational hurdles and proposes strategies to enhance the reliability and clinical relevance of characterization approaches, thereby facilitating the advancement of NTB drug products toward clinical applications.

2. Formulation strategies

Developing an ideal formulation for NTB drug delivery involves the judicious selection of appropriate dosage forms and excipients to maximize the brain-targeting delivery efficiency of the active pharmaceutical ingredient. **Table 1** lists considerations for nasal formulation development in NTB drug delivery. It is important to note that the overarching requirements for designing nasal formulations for local or systemic action and those intended for NTB delivery are broadly similar, with parameters such as pH and osmolality (for liquid formulations) being paramount. However, due to the differing targeted deposition sites (the turbinates versus the olfactory region) and the site of action (the nasal cavity or systemically vs. the brain), specific characteristic considerations, such as particle size and excipient selection, vary accordingly. This review concentrates exclusively on formulation strategies for NTB delivery.

Currently, liquid formulations (e.g., solutions, suspensions, and emulsions) are commonly employed among approved nasal drug products due to their ease of

manufacturing and lower irritancy to the nasal mucosa. They may also be particularly favorable when rapid drug action is desired, such as emergency treatment of seizures or acute ischemic stroke, as the step for powder dissolution or drug diffusion across semi-solids can be omitted. However, liquid formulations are inherently less physically and chemically stable and are more susceptible to microbial contamination. Consequently, the addition of preservatives, such as parabens, is often necessary, which may raise concerns about allergies. Alternatively, aseptic manufacturing processes can be employed, which may lead to increased production costs.

A critical limitation for NTB drug delivery is the short residence time of liquids within the nasal cavity, typically ~15 minutes, due to mucociliary clearance. This significantly constrains drug permeation across the nasal cavity and subsequent transport to the brain.[13] Semi-solid (e.g., gels and ointments) and powder formulations are preferred for NTB drug delivery, as they can adhere to nasal mucus and alleviate mucociliary clearance, thereby potentially prolonging drug retention in the nasal cavity and subsequently enhancing NTB drug transport.[14, 15] Powder formulations offer additional benefits by enhancing physicochemical and microbiological stability and increasing drug loading capacity. Semi-solid and powder formulations are therefore suitable for chronic disease treatment (e.g., dementia, Parkinson's disease, depression), where sustained drug levels in the brain are desirable. However, the scarcity of semi-solid and powder formulations in approved nasal drug products demonstrates that several limitations hinder their clinical translation. For semi-solid formulations, localized nasal application can be challenging for patients due to the small volume and unique anatomy of the nasal cavity. They may also pose risks of nasal obstruction or breathing difficulties. These issues could be overcome by stimuli-responsive *in-situ* gelling systems (**Section 2.2**), which are easier for patients to administer. Powder formulations are more complex and costly to produce, especially for NTB drug delivery, as particle size significantly affects deposition in the olfactory region. Moreover, powders often require specialized packaging and storage conditions to prevent moisture sorption and drug degradation, and they may induce mucosal irritation. The use of particle engineering techniques (e.g., spray drying and spray freeze drying) to engineer powders tailored for olfactory region deposition is currently under active investigation.

The performance of NTB drug delivery can be modulated by the use of excipients, as summarized in **Table 2**. Specifically, certain excipients modulate drug absorption across the nasal mucosa and facilitate subsequent transport into the brain. Certain excipients are specifically used to enhance drug transport across the nasal mucosa, including permeation enhancers, mucoadhesive agents, and enzyme inhibitors.[14, 16-18] Permeation enhancers are used to increase the nasal mucosal permeability of drugs with poor permeability (e.g., proteins or hydrophilic small molecules) by opening tight junctions, increasing membrane fluidity, and/or forming hydrophilic pores in the epithelium. Permeation enhancers are particularly preferable to be

incorporated into formulations for NTB delivery of drugs with emergency indications (e.g., seizures or acute ischemic stroke), as they promote rapid drug absorption into the brain and accelerate the onset of action.[18] Mucoadhesive agents are typically polymers that interact with mucus and modify its rheology, thereby reducing mucociliary clearance and prolonging nasal drug retention[14, 19]. First-generation mucoadhesive polymers interact non-specifically with mucus via non-covalent interactions[14, 19]. Chitosan is one of the most widely investigated first-generation mucoadhesive polymers for NTB delivery by virtue of its cationic nature, which allows it to bind electrostatically to negatively charged sialic and sulphonic acid groups in mucin.[20] Despite the popularity of first-generation mucoadhesive polymers, there is growing interest in second-generation polymers (e.g., lectins, thiolated polymers) that achieve specific mucosal adhesion via covalent bonding and thereby promote specific drug absorption across the nasal epithelium. For example, lectins, which specifically recognize sugar molecules and bind glycosylated membrane components on the nasal mucosa, have been demonstrated by several studies to enhance nasal drug absorption and NTB delivery efficiency.[21-23] Nasal drug residence time can also be extended with enzyme inhibitors, which reduce the rate of nasal drug metabolism.[18]

In addition, some excipients that are conventionally regarded as “inert” excipients, including fillers (e.g., mannitol, microcrystalline cellulose)[24] and preservatives (e.g., benzalkonium chloride),[25] may also affect drug absorption and NTB transport by their effects on mucociliary clearance, drug dissolution/release, and permeability across the nasal epithelium. Therefore, it is essential to study the influence of candidate excipients on various biological processes involved in NTB drug delivery using appropriate models (**Section 4**). Such assessment should be integrated with considerations of their safety profile (**Section 5.2**) and its physicochemical properties. This comprehensive assessment aids in selecting excipients that can optimize the NTB drug delivery efficiency.

As the field of NTB delivery advances, strategies to enhance delivery efficiency have evolved from the addition of conventional excipients to the development of novel technological approaches. Specific formulation and drug delivery strategies that have gained recent popularity for enhancing NTB drug transport are discussed below.

2.1 Nanotechnology

The integration of nanotechnology with NTB drug delivery represents a promising approach for treating CNS disorders. Various nanocarrier categories, including liposomes, nanoemulsions, magnetic nanoparticles, polymeric nanoparticles, and micelles, have been investigated for such purposes.[26-28] Nanoparticles offer several advantages, including enhancing drug solubility and permeability across the nasal mucosa, inhibiting rapid mucociliary clearance, and protecting therapeutic agents from enzymatic degradation. These effects collectively prolong residence time within the nasal cavity, facilitating improved drug absorption and therapeutic efficacy.[29, 30]

A systematic review by Pires and Santos has revealed that nanoparticle-based formulations significantly improve the median values of various metrics used to assess NTB drug delivery, including drug transport efficiency (%DTE), NTB drug transport percentage (DTP%), and log $B\%_{brain\ IN/IV}$, compared to conventional solution formulations (**Fig. 1**).[31] Beyond small molecules, nanocarriers can also be used to deliver nucleic acids such as ribonucleic acids (RNAs). Encapsulation of RNA into nanocomplexes confers tunable size, protection from degradation, and prevention of premature RNA release.[32] These strategies have been demonstrated to increase the accumulation of therapeutic RNA within the hippocampus, thereby opening new avenues for the interventions of neurodegenerative diseases.

Despite extensive research investigating the application of nanotechnology to enhance NTB drug delivery, a comprehensive understanding of the biological fate of nanoparticles during NTB drug delivery remains a knowledge gap in the literature, hindering the rational design and clinical translation of such drug delivery systems. A central research question is whether enhancements in brain bioavailability resulting from drug encapsulation into nanoparticles are primarily due to the enhanced NTB transport of intact nanoparticles or the released drug cargo. Conventionally, the uptake pathway of nanoparticles has been tracked using fluorescent nanoparticle labeling; however, the integrity of nanoparticles and the simultaneous entry of drug cargo are not guaranteed. Recent studies by Ahmad et al. and Li et al., in which intact nanoparticles were tracked using aggregation-caused quenching (ACQ) probes, suggest that intact nanoparticles mainly were “trapped” inside the nasal mucosa and cribriform plate, with only a small fraction of nanoparticles successfully reaching the trigeminal nerve and being transported directly into the brain.[33, 34] Conversely, the drug cargo would be released into the nasal mucosa, enter the olfactory bulb and the trigeminal nerves, and be rapidly transported into the brain. These findings do not support the notion that nanoparticles primarily reach the brain intact, raising questions about the performance of drug delivery systems where the integrity of the nanoparticle is essential (e.g., ligand-decorated drug delivery systems for regional or cellular-level targeting within the brain), despite some studies demonstrating that intranasal (IN) administration of ligand-decorated drug delivery systems could achieve brain cellular-level targeting.[35, 36] Further studies are necessary to elucidate the pathways and kinetics of brain entrance for intact nanoparticles after IN administration.

Another area requiring more investigation is the correlation between nanoparticle physicochemical properties (e.g., particle size, surface properties, particle morphology) and NTB drug delivery performance. For example, nanoparticles with positive zeta potential are expected to enhance mucoadhesion (as nasal mucus is negatively charged) and extend nasal residence time. Similarly, smaller particle sizes are more likely to penetrate the mucus layer and be transported across the nasal epithelium in larger quantities.[5] However, an analysis by Bourganis et al. failed to establish a clear relationship between particle size or zeta potential with %DTP, possibly attributed to confounding factors including (a) the design and architecture of

nanoparticles; (b) physicochemical properties of the drug (hydrophilic drugs may benefit greater from NTB drug delivery due to poor BBB permeability) and (c) drug release profile of the nanoparticles.[37] The lack of consensus on optimal physicochemical properties complicates early stages of product development by increasing reliance on trial-and-error work and increasing the risk of failure in later stages. Systematic studies are needed to elucidate how physicochemical properties of nanoparticles influence various biopharmaceutical processes critical to NTB drug delivery (e.g., mucoadhesion, nasal residence time, and drug and integral nanoparticle transport across the nasal epithelium and into the olfactory bulb, trigeminal nerve, and brain).

A notably under-investigated property of nanoparticles designed for NTB delivery is their interaction with nasal mucus. As nanoparticles are susceptible to rapid mucociliary clearance from the nasal cavity, two major surface modification strategies have emerged to overcome the nasal mucosal barrier. Muco-penetrating nanoparticles are coated with dense polymers, e.g., polyethylene glycol (PEG), to enhance nanoparticle diffusion and penetration across the mucus layer, while mucoadhesive nanoparticles are coated with mucoadhesive polymers (e.g., chitosan, lectin) to promote mucus adhesion.[38] Considering that mucus turnover is very rapid (~15 minutes) in the nasal cavity, relying solely on either strategy is risky: While mucoadhesion is necessary to promote cellular uptake, the nanoparticles may be cleared by mucus turnover before they can effectively traverse the mucosal barrier.[39] Therefore, rational design of nanoparticle-based NTB drug delivery systems requires a careful balance of mucoadhesion and muco-penetration properties to maximize nasal drug bioavailability.[39] While *in silico* molecular dynamic simulation tools have recently been developed to evaluate the interactions between nanoparticles and mucosal barriers,[39] further research is necessary to devise suitable strategies for balancing mucoadhesion and muco-penetration.

Given the complexity of these interactions, a Design of Experiments (DoE) approach is helpful to delineate the individual and interactive effects during formulation development. Alternatively, machine learning (ML) models leveraging existing literature can be developed to predict these effects. For example, Yousfan et al. developed an ML model for the brain drug uptake index based on physicochemical properties, *in vitro* drug release profile, and pharmacokinetic data.[40] The model identified that diminished brain-targeting efficiency benefits were observed with drugs of higher molecular weight and nanoparticles with rapid drug-release profiles. Simultaneously, drugs that are P-glycoprotein (P-gp) substrates would benefit from nanoparticle-mediated NTB delivery by virtue of the protection from P-gp clearance offered by nanocarriers. Notably, the model demonstrated good predictive accuracy when validated with *in vivo* studies. Nevertheless, it should be noted that these results may be confounded by the lack of standardization in the characterization and evaluation approaches, as well as in the reporting of physicochemical and pharmacokinetic data. Recommendations for standardizing *in vitro* and *in vivo* studies to evaluate nasal biopharmaceutics designed for NTB drug delivery are

presented in **Section 4** below. Although recommendations for standardizing nanoparticle physicochemical characterization fall outside the scope of this review, interested readers are encouraged to consult other review articles on this topic.[41, 42]

Other important aspects to consider for promoting the clinical translation of nanoparticle-based NTB drug delivery systems include manufacturing technology and safety profile. The engineering of nanoparticles for NTB drug delivery increasingly involves sophisticated designs aimed at achieving specific functions (e.g., enhancing nasal mucosal penetration or regional targeting within the brain). However, such designs often require fabrication steps that are either too costly, difficult to scale up, or suffer from poor reproducibility, which pose significant risks of failure for pilot- or industrial-scale production in later development stages and preclude these advanced systems from reaching clinical use. Researchers should adopt a scale-up mindset early in formulation development by employing scalable, reproducible unit operations whenever possible. This can be achieved through flow-based approaches (e.g., flash nanoprecipitation) for nanoparticle fabrication.[43] Such delivery systems may also encounter hurdles during clinical translation due to concerns about nasal mucosal toxicity and/or neurotoxicity. While the current literature suggests that nanoparticle-mediated NTB drug delivery systems are generally not toxic, this may reflect publication bias favoring successful studies.[44] An example contrary to this observation is the study by Mistry et al., which demonstrated that exposure of porcine olfactory mucosa to chitosan-modified polystyrene nanoparticles resulted in size-dependent tissue damage, with 20 nm nanoparticles causing greater damage than 100 nm or 200 nm nanoparticles, possibly because the smaller nanoparticles induced organelle dysfunction and increased oxidative stress.[45] Therefore, it is vital to conduct thorough toxicity evaluations of the most commonly used nanoparticles as nanocarriers to confirm their biocompatibility for NTB applications.

2.2 Stimuli-responsive *in-situ* gelling systems

As mentioned earlier, semi-solid formulations are preferred over liquid formulations, as they enhance drug retention in the mucosa and protect against enzymatic degradation and mucociliary clearance. However, their application is less convenient for patients compared to liquid formulations. Stimuli-responsive *in-situ* thermal gelling systems have gained popularity to address this dilemma, where liquid aerosols (generated by a nasal spray device) undergo a sol-gel transition (gelation) upon deposition in the nasal cavity, forming gels that enable sustained drug release, mucoadhesion, and enhanced nasal drug retention.[46, 47] As nasal drug retention is modulated by only changes in the polymer structure within the nasal cavity, this approach is suitable for enhancing NTB delivery of a wide variety of therapeutics, from small molecules (both lipophilic and hydrophilic) to biological drugs (e.g., proteins, peptides, and RNA), as long as the drug is compatible with the polymer.[48]

The *in-situ* gels can be broadly classified into physical and chemical crosslinking gels based on their triggering mechanisms.[48] Owing to the potential irritation and toxicity

of organic solvents and initiators during chemical crosslinking, physically crosslinked gels are generally preferred for NTB applications.[48, 49] Physical crosslinking can be activated by stimuli such as changes in pH, ionic strength, or temperature. Thermoresponsive systems are based on the micellization of thermoresponsive polymers, e.g., poloxamers P407 and P188, to achieve sol-gel transition around 28 – 34 °C. This allows the formulation to remain liquid during ambient storage, with gelation only activated upon contact with the warm nasal mucosa.[48] Ion-responsive systems typically employ anionic polymers (e.g., gellan gum), which form ionic interactions with the cations (Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}) present in nasal fluid, thereby inducing a sol-gel transition.[15, 50] pH-responsive systems utilize pH-responsive polymers, e.g., Carbopol, which has a pK_a of ~5.5. These formulations are generally stored at an acidic pH (~4–5.5) and undergo a sol-gel transition through the phase transformation of the pH-responsive polymer upon exposure to the less acidic environment of the nasal cavity (pH ~6.2).

Regardless of the stimuli that the system is designed to respond to, the *in-situ* gelling system should be carefully formulated such that (a) gelation can be readily activated upon contact with nasal fluid; (b) the gelling time (t_{gel}) allows adequate spreading of the formulation across the olfactory region while minimizing drainage; and (c) the formulation exhibits pseudoplastic rheology to facilitate ease of administration using a nasal spray device, with high viscosity restored upon deposition in the nasal cavity. However, there are no standardized methods to characterize the properties of the *in situ*-formed gel, leading to the use of techniques that may inadequately represent *in vivo* gelation conditions. For example, the gelation temperature (T_{gel}) of thermoresponsive gels is frequently determined using a “tube inversion” method, where T_{gel} is recorded as the temperature at which the gel no longer flows upon tilting. There is a need for emerging analytical techniques that better replicate the *in vivo* gelation conditions and establish acceptable thresholds for characterization parameters (e.g., t_{gel}, viscosity) to expedite the translational research of stimuli-responsive *in situ* gelling systems.[15]

2.3 Cell-penetrating peptides (CPPs)

The selective permeability of cell membranes presents a challenge to the effective translocation of drugs across the nasal mucosa, particularly for hydrophilic compounds and biologics.[51-53] Although permeation enhancers are commonly used to increase drug permeability across the nasal mucosa, their use raises safety concerns due to potential irreversible damage to the nasal epithelium.[54]

Cell-penetrating peptides (CPPs) have emerged as an alternative strategy to penetrate the epithelial tight junctions of the nasal mucosa. They facilitate the intracellular delivery of various types of therapeutics, including small molecules,[55] proteins,[56] and oligonucleotides,[57] by enabling efficient cellular penetration. In the context of NTB drug delivery, CPPs can enhance drug permeation across the nasal mucosa, NTB drug translocation, and intracerebral drug diffusion. The efficiency of CPP-mediated nasal mucosa penetration can be further optimized through chemical

modification, e.g., increasing positive charge via arginine enrichment to promote transcellular transport or incorporating hydrophobic amino acids such as tryptophan to augment mucus penetration.[51] While co-administration of CPPs can enhance NTB drug delivery, it is more common to conjugate CPPs directly to the therapeutic agent or nanocarrier. This approach could address the inherent instability of CPPs within the nasal milieu, where they are susceptible to oxidation, hydrolysis, and enzymatic degradation.[58-61] While CPPs can enhance the delivery of both small molecules and biological drugs, the enhancement is greater for biologics due to their large size, poor mucosal permeability, and high hydrophilicity.[51] For example, IN administration of methoxy poly(ethylene glycol)/poly(ε-caprolactone) (mPEG-PCL) micelles modified with TAT peptide (YGRKKRRQRRR) demonstrated increased intracerebral drug distribution compared to IN administration of unmodified mPEG-PCL micelles.[62] The Tat-modified micelles effectively delivered camptothecin, alone or in combination with *Raf-1* siRNA, to the brain, resulting in prolonged survival in intracerebral glioma-bearing rats. Moreover, these micelles facilitated the delivery of tumor necrosis factor-alpha (TNF-α) siRNA via the NTB pathway, which enhanced neuronal functional recovery in a transient middle cerebral artery occlusion (MCAO) rat model.[63-65]

Unlike traditional permeation enhancers, CPPs exhibit a more favorable safety profile for the nasal mucosa, as they can enter cells non-invasively.[66, 67] For instance, Xu et al. demonstrated that IN delivery of human acidic fibroblast growth factor conjugated to TAT over 5 weeks caused no observable pathological changes in tissues or organs, nor any differential expression in sensory neurons of the nasal epithelium.[66] Nasal ciliotoxicity assessments, conducted using *in situ* palate models and optical microscopy, further confirmed the safety of this approach. Interestingly, some CPPs possess both cell-penetrating and therapeutic properties, offering a direct approach to treating neurological diseases. For example, the anti-inflammatory cell-penetrating KAFAK peptide has demonstrated efficient brain uptake (**Fig. 2(a)-(f)**) and reduced pro-inflammatory cytokines (**Fig. 2(g)**) in a mouse model of traumatic brain injury (TBI) *in vivo*.[68]

As CPPs have only recently emerged as NTB drug delivery tools, several issues must still be addressed before their successful commercialization. Firstly, conventional CPPs used to enhance nasal mucosal permeation and NTB drug transport exhibit limited targeting specificity for specific tissues or cell types. This may result in suboptimal delivery to the diseased site, thereby reducing therapeutic efficacy and increasing the risks of systemic adverse effects due to off-target distribution. To address these challenges, strategies involving the combination of CPPs with cell- or tissue-targeting moieties have been proposed. For example, Kanazawa et al. modified the mPEG-PCL-Tat micelles with bombesin to achieve selective targeting to gastrin-releasing peptide receptor (GRPR)-positive glioma (**Fig. 2(h)-(l)**)[69] Secondly, while the primary mechanisms are generally recognized as direct translocation across the cell membrane and endocytosis, the specific pathways involved remain incompletely characterized.[70, 71] Multiple factors, including

the physical, structural, and physicochemical properties of the CPPs, their concentration, cell type, cell density, cell cycle stage, temperature, binding mode, and the nature of the cargo, can influence the uptake mechanisms.[51, 72-74] This complexity prevents the elucidation of the relationship between the CPP sequence and brain-targeting efficiency or intracerebral drug distribution patterns, which is necessary to guide the rational selection of CPPs for enhancing NTB drug delivery. Certain CPPs, especially recombinant CPPs or CPPs derived from viral or bacterial sequences, may elicit immune responses upon administration.[59] To mitigate this, one approach is to incorporate non-natural amino acids into T-cell epitopes within the peptide sequence,[75] which reduces T-cell stimulation and immunogenicity. Nevertheless, the primary challenge of CPPs lies in their high manufacturing costs due to the complexity of large-scale synthesis and quality control matters (e.g., bioassay, purity, and secondary structure). Addressing these challenges is crucial for the clinical translation of CPPs in NTB drug delivery.

3. Nasal delivery device selection

While most research has focused on developing suitable formulations for NTB drug delivery, fewer studies have focused on designing nasal delivery devices tailored for this purpose. However, nasal drug products are inherently a combination of formulation and nasal device, with interactive effects on their overall performance. Conventional nasal preparations, e.g., nasal drops and nasal sprays (**Fig. 3(a)** and **(b)**), which account for ~95% of the total number of nasal products approved by the US FDA, are designed to deliver drugs to the lower nasal cavity for local conditions like allergic rhinitis. Consequently, they achieve low deposition in the olfactory region (typically ~5%) and restrict direct NTB drug transport. Additionally, dripping of concentrated nasal drop/spray formulations after administration can cause an undesirable bitter taste.[76]

A variety of nasal delivery devices have been developed to enhance deposition within the olfactory region (or upper nasal space). Notably, the Precision Olfactory Device® (POD®; Impel NeuroPharma, Seattle, WA, USA) (**Fig. 3(c)**) and the Optinose® powder/Opti-Powder device (**Fig. 3(d);** OptiNose US, Yardley, PA, USA) were approved in drug-device combinations for migraine treatment by the US FDA. The POD® device is a propellant-powered device that, upon actuation, mixes the propellant with the formulation and expels it as a narrow aerosol plume. Following emission, residual propellant continues to push the aerosol through the nasal valve into the upper nasal space. The Opti-Powder device is powered by breath-driven Bi-Directional™ nasal technology, wherein the positive pressure created when the user exhales into the mouthpiece not only facilitates powder dispersion as aerosol but also elevates the soft palate, effectively preventing oral inhalation. The sealing nosepiece expands the nasal valve both mechanically and through the dynamic pressure transferred from the oral cavity, facilitating aerosol penetration into the upper posterior region of the nasal cavity. The pressure across the nasal palate is also balanced by the sealing mouthpiece such that the nasal palate is not “over-

elevated" and an open flow path is available between the two nasal passages behind the nasal septum to allow airflow to leave from the other nostril (hence the "bi-directional" airflow).[76, 77] Both devices have demonstrated significant enhancement of deposition in the upper nasal space/upper posterior region in *in vivo* nasal regional deposition studies in humans compared to conventional nasal spray products.[78, 79] The POD® device is particularly suitable for emergency uses (e.g., during a seizure attack or the hyperacute phase of ischemic stroke) when patients may be unconscious and require drug administration by caregivers or ambulance staff, as it does not require patient efforts to coordinate breathing or actively sniff to achieve dose actuation, unlike the breath-powered actuation mechanism for Opti-Powder. Results from proof-of-concept Phase 1 studies have also demonstrated the feasibility of delivering olanzapine powders using the POD® device for acute agitation, suggesting its potential for both powder and liquid formulations.[80]

It is worth noting that both the POD® and Opti-Powder devices target the "upper nasal space" and "upper posterior region", respectively (which comprises both the olfactory region and the upper turbinate lined with respiratory mucosa) instead of solely the olfactory region, as their goal is to take advantage of the vascular-rich and slower mucociliary clearance properties of the upper nasal space to enhance systemic drug absorption and minimize absorption variations due to dripping instead of achieving direct NTB drug delivery. Therefore, the extent to which the devices can enhance direct NTB drug delivery by increasing deposition strictly in the olfactory region remains to be investigated. Deposition data in the upper nasal space/upper posterior region from either device should not be compared with olfactory deposition data from other nasal delivery devices.

The Unidose (UDS) Powder Nasal Spray device (Aptar Pharma, Rueil-Malmaison, France) (**Fig. 3(e)**) was approved as Baqsimi® in combination with glucagon nasal powder for the treatment of severe hypoglycaemia. Although Baqsimi® was not designed for targeted glucagon delivery into the olfactory region, the UDS device was shown to achieve an olfactory region deposition efficiency of ~34% in an *in vitro* anatomical nasal cast model in combination with lactose powder [Median volume diameter ($D_{v,50}$) = 80 μm].[81] Combined with its commercial availability and ease of assembly in laboratories, the UDS has become popular for evaluating the feasibility of targeting the olfactory region with powder formulations designed for NTB drug delivery.[82, 83] Other devices claimed to be intended for NTB drug delivery and tested in clinical trials include the ViaNase™ (Kurve Therapeutics, Lynnwood, MA, USA) nebulizer, which generates an active vortex of nebulized droplets (with adjustable velocity and orientation to control droplet trajectories) within the 9–11 μm diameter range,[84-86] and the Naltos™ device (Alchemy Pharmatech, Manchester, UK), which is similarly propellant-powered as the POD® device using inert gas as the propellant.[85] However, their claims for NTB drug delivery remain unvalidated due to the lack of published *in vitro* or *in vivo* data on nasal regional deposition.

Although it is challenging to elucidate the effect of spray characteristics (e.g., plume geometry, spray angle, spray velocity) on olfactory region deposition based on the limited data available from commercial nasal devices, evidence from custom-made devices suggests that a narrow, nearly linear plume geometry, slower spray velocity, and deeper nosepiece insertion (which can be facilitated by modifying the design of the nasal device to include a narrower and longer tip) can enhance olfactory region deposition by minimizing impaction on the nasal valves and loss of drug-loaded aerosols to the lower turbinates.[87] Nevertheless, such studies have traditionally been difficult due to the difficulty of fabricating prototype nasal devices within the laboratory. With the recent advancements of 3D printing technology, various inhaler device designs have been actively explored to enhance the aerosol performance of dry powder inhaler formulations for oral inhalation.[88] It is recommended that researchers leverage 3D printing technology further to engineer innovative nasal device designs that enable more precise targeting of the olfactory region, e.g., by optimizing the geometry of the nasal tip or nosepiece.

Importantly, regulatory oversight for nasal delivery devices remains limited, particularly as devices shift away from non-specific regional targeting within the nasal cavity toward targeted delivery to the upper nasal space for superior systemic drug absorption or to the olfactory region for nose-to-brain delivery. The regulatory pathway for NTB drug products (drug-device combinations) is more complex than for conventional dosage forms (e.g., tablets), as additional regulatory requirements will apply to the nasal delivery device. For example, the EMA requires that all nasal delivery devices fulfil the general requirements as outlined in the Medical Device Regulation (EU) 2017/745.[8, 89] Furthermore, due to the unique designs of such devices, device-specific instructions to patients should be established during device development with clear guidance on actuation parameters (e.g., insertion depth, actuation angle, head positioning, breathing pattern), as they can critically affect *in vivo* nasal regional deposition profile, drug absorption, brain drug bioavailability, and therapeutic outcomes.[87] Optimal parameters can be derived from *in vitro* studies examining their effects on the *in vitro* nasal regional deposition profile (see **Section 4.1.2** below). Nevertheless, human use evaluation studies are essential to confirm whether nasal delivery devices can be used as intended by patients or their caregivers and to ensure consistent, safe, and effective therapeutic outcomes across diverse patient populations.

4. *In vitro and in silico* methods for evaluating nasal biopharmaceutics of nose-to-brain drug products

The guidelines and regulatory frameworks established by the EMA and FDA delineate *in vitro* testing procedures for the characterization of nasal spray products.[8, 9] The publication by Karpe et al. provides an in-depth account of the development and validation of analytical methodologies, aiming to compile the pertinent specifications and regulatory requirements of nasal spray products derived from authoritative guidelines and research.[90] However, it is noteworthy that current

regulations predominantly address nasal spray formulations, which mainly focus on local treatment, whereas the burgeoning field of NTB delivery is increasingly involved. Consequently, there is a pressing need to develop and standardize characterization methodologies specifically tailored to assess NTB delivery, thereby expanding the existing regulatory and analytical landscape to encompass these emerging drug delivery systems. As proposed by Forbes et al., a nasal biopharmaceutical framework should encompass the deposition of drug-containing aerosol particles in the nasal cavity and the dynamics of absorptive (e.g., systemic absorption and direct NTB drug transport) and non-absorptive (e.g., mucociliary) clearance (**Fig. 4**) and requires *in vitro* characterization methods that are predictive of *in vivo* performance.[10] Herein, we critically discuss the existing techniques for evaluating the nasal biopharmaceutics of NTB drug products, focusing on their limitations and potential research directions to improve *in vitro-in vivo* correlation (IVIVC).

4.1. Regional drug deposition in the nasal cavity

As the olfactory region is the primary site of NTB drug transport within the nasal cavity, regional drug deposition after IN administration is of great interest for NTB drug delivery. The gold standard for evaluating *in vivo* human nasal deposition is gamma scintigraphy following IN administration of radiolabeled drug products. However, conducting *in vivo* studies routinely is often infeasible due to their high costs and time requirements. Therefore, alternative *in silico* computational fluid dynamics (CFD) and *in vitro* anatomical models have been developed to predict *in vivo* nasal regional deposition. **Table 3** provides a comprehensive overview of the advantages and limitations of each method.

4.1.1. In silico computational fluid dynamics (CFD) models

In silico CFD models are constructed from anatomically accurate reconstructions of the human nasal cavity derived from computed tomography (CT) or magnetic resonance imaging (MRI) data. These models can be segmented into various regions of interest (ROI), such as the olfactory region, nasopharynx, paranasal sinuses, turbinates, and vestibule. This segmentation is particularly beneficial for NTB drug products, as the olfactory region can be easily designated as a specific ROI for focused analysis. CFD has several merits over conventional *in vitro* and *in vivo* experimentation. Firstly, the use of different geometries from specific patient populations (e.g., pediatric patients or patients undergoing nasal mid-vault surgery) can enhance accuracy in predicting nasal deposition profiles in these groups, for which *in vivo* studies are often impractical and *in vitro* anatomical models are not readily available. Moreover, CFD studies allow detailed simulations across various parameter settings, including aerosolized droplet characteristics from nasal sprays (such as spray cone angle, spray velocity, spray ovality, and droplet size distribution) and airflow within the nasal cavity, without requiring additional resources for *in vitro* or *in vivo* experimentation.

In silico studies examining the nasal regional deposition profile of nasal sprays suggest that a median droplet volume diameter of 10–25 μm would result in maximized deposition in the olfactory region. However, the predicted olfactory deposition efficiency remains relatively low, typically less than <10%.[91, 92] Additionally, CFD-simulated nasal regional deposition profiles have shown a good agreement with results obtained from *in vitro* anatomical models.[79] The exact droplet size within this range that maximizes olfactory region deposition varied across studies. These discrepancies are likely attributed to differences in nasal geometry used to construct the anatomical models, variations in nasal spray actuation parameters, the nasal spray device used, and the inspiratory airflow profile during testing. Since nasal geometries are typically constructed from imaging data of individual subjects, concerns arise regarding the generalizability of *in silico* CFD simulation results derived from these geometries. Simulation studies in idealized geometries obtained by "averaging" the realistic geometries from a group of patients could enhance data generalizability and expedite nasal product development.[93] Interestingly, nasal regional deposition results from *in silico* CFD studies based on nasal spray products seemingly cannot be extended to nasal powders, as deposition studies of nasal powder formulations using *in vitro* anatomical models have demonstrated significantly higher drug deposition in the olfactory region (~15 – 50%), including studies wherein the average powder size was ~300 μm, which is an order of magnitude higher than the desired droplet/aerosol size range as mentioned above.[94-96] Simulation of the aerodynamic diameter distribution of aerosolized nasal powders is more complex than nasal spray products, as it is simultaneously affected by the actuation parameters of the nasal powder spray and the inherently heterogeneous particle size distribution of powder particles. *In silico* studies for nasal powder products are therefore of significant interest, not only to validate the results of *in vitro* deposition studies, but also to provide valuable guidance for optimizing powder properties (e.g., particle size) to maximize deposition in the olfactory region.

As previously mentioned, *in silico* simulations of nasal region deposition profiles have demonstrated equivalence with *in vitro* anatomical models, primarily due to their reliance on identical nasal geometries. However, limited data are available comparing *in silico* data with *in vivo* nasal deposition data. Further research is necessary to validate the capability of *in silico* models to predict *in vivo* deposition within the olfactory region accurately. Furthermore, most *in silico* studies assume steady flow and cannot account for unsteady flow in realistic breathing profiles (e.g., rapid sniffing).[97, 98] Given that airflow dynamics significantly affect total nasal deposition, further CFD studies utilizing dynamic flow modeling are necessary to predict olfactory region deposition efficiency under realistic breathing profiles. The development of CFD models that can simultaneously predict both nasal and pulmonary deposition would also be valuable in predicting inadvertent pulmonary drug exposure.[97]

4.1.2. *In vitro* anatomical models

While the drug mass fraction of particles/droplets with aerodynamic diameter above 10 μm provides an overall account of drug deposition inside the nasal cavity,[99] NTB products require special consideration in that deposition in the olfactory region of the nasal cavity (instead of other regions, e.g., the turbinates) is desired to utilize direct NTB drug delivery pathways. Therefore, evaluating the *in vitro* deposition profile within the nasal cavity using anatomically accurate nasal models that replicate the intricate structure of the human nasal cavity and can be segmented into multiple nasal regions is essential for precise assessment of drug deposition, especially in the context of NTB drug delivery.

Traditionally, plasticized cadaver heads were used as they bear the closest resemblance to nasal geometry; nevertheless, the advent of 3D printing has considerably improved the convenience in manufacturing anatomically correct nasal casts. Various models differ in complexity and structural details and can be classified into simplified geometry models, sophisticated but incomplete geometry models, and sophisticated, comprehensive geometry models.[100] Simplified geometry models typically represent only the basic structural framework, often consisting of one or two parts, and are therefore generally unsuitable for deposition studies related to NTB drug products. Sophisticated models are constructed from CT or MRI images of the nasal cavity and generally resemble its key regions and volumes. “Incomplete” models omit some paranasal sinuses, while completed geometry models include all paranasal sinuses, thereby bearing a greater resemblance to the actual human geometry. However, the increased geometric complexity of the nasal cast would also result in longer printing times, more intricate design requirements, and slower, less convenient analysis of drug deposition after the cast is disassembled. The trade-off between precision and efficiency thus warrants significant consideration. As the paranasal sinuses have been demonstrated to have minimal effect on airflow in healthy patients, a sophisticated but incomplete geometry may already be sufficient for evaluating olfactory region drug deposition.[100] As mentioned above, mucus may significantly affect nasal regional deposition.[101] The coating of nasal casts with artificial mucus is therefore desirable to closely mimic the *in vivo* environment. The “ideal” artificial mucus should have a rheological profile (pseudoplastic behavior), biochemical composition (~1 – 5% mucin, 90 – 95% water, lipids, proteins, etc.), and bilayer structure (upper gel layer and lower periciliary liquid layer) similar to that of human nasal mucus. However, currently available compositions of artificial mucus can only replicate its viscoelastic properties using aqueous solutions of mucin or galactomannan gum, and further work is needed to develop more realistic artificial nasal mucus formulations. The coating (e.g., pipetting or brushing artificial mucus onto the cast interior or complete filling of the internal cast surface) and cleansing procedures also require optimization to ensure the film is applied evenly and reproducibly across the cast and is removable after each experiment. The choice of material for constructing the nasal model is also a critical factor. Commonly used materials include thermoplastics (e.g., polypropylene and polyoxymethylene), photopolymers (e.g., VeroClear, Stereocol®, Watershed®, and FullCure 720), and

various elastomers and flexible materials (e.g., rubber and silicone).[100] The adhesion properties, surface texture, and potential surface charges of these materials can influence particle deposition patterns, and the material should also be compatible with the rinsing solvent.

Despite the increasing availability of 3D printers, commercially available nasal casts are more convenient for researchers and drug developers who have limited access to 3D printers or expertise in 3D printing. Until recently, as shown in **Fig. 5(a)**, the Koken nasal cast model (originally intended as an educational tool) was the only commercial nasal model available for purchase. While it has been previously applied to evaluate *in vitro* nasal drug deposition, it has now fallen out of favor (especially for NTB-intended products) as (a) the olfactory region cannot be segmented, hindering accurate quantification of olfactory drug deposition; (b) deposition pattern can only be visualized and quantified using colored or fluorescent dyes, which may not accurately reflect deposition of the API; and (c) the dimensions of the Koken cast exceed the typical anatomical range and may misestimate regional drug deposition.[102]

More recently, the Alberta Idealized Nasal Inlet (AINI), developed by Copley in Nottingham, UK, has received rapid popularity for *in vitro* nasal deposition studies. This model is based on an idealized nasal geometry averaged from the anatomical features of 10 normal adults. Its advantages include (a) construction from aluminum, which is resistant to organic solvents for rinsing; (b) it can be segmented into four distinct regions: the vestibule, turbinates, olfactory region, and nasopharynx, allowing regional drug assay; (c) demonstrated good correlation with *in vivo* results obtained through gamma scintigraphy;[103] and (d) facilitation of simultaneous quantification of nasal regional and lower airway deposition when combined with a cascade impactor, e.g., the Next Generation Impactor (**Fig. 5(b)**). However, it is important to emphasize that AINI should not be used to replace simplified nasal inlets (e.g., the glass expansion chamber or Kiel nasal inlet) when evaluating the drug mass fraction of particles with aerodynamic diameter <10 μm, unless proper validation has been performed. This caution is warranted because the segmentation of the AINI and particle bouncing within the device may result in underestimation of deposition, which could impact regulatory compliance.[104] Particle bouncing can be minimized by using a glycerol-surfactant (Tween 20 or Brij® L23) coating, which has been shown to mitigate particle bounce similarly to that of mucin-based artificial nasal mucus.[105] However, the lengthy coating procedure (~75 minutes) is inconvenient for high-throughput evaluation. Furthermore, while the AINI is a convenient tool during early product development, it is based on an idealized nasal geometry of normal adults. Results should not be generalized to special populations (e.g., individuals with anatomical deformities or pediatric patients), for whom *in vitro* deposition evaluation using 3D-printed nasal casts tailored to their respective geometries is considered more appropriate. Notably, no systematic investigation has been conducted to compare *in vitro* deposition data from the AINI model with *in vivo* imaging modalities and establish an IVIVC for nasal powder formulations.[10]

Regardless of the *in vitro* anatomical model(s) chosen, the setup should facilitate a comprehensive evaluation of various factors that could affect nasal regional deposition, particularly patient-related factors associated with product use, e.g., nosepiece insertion angle and depth, actuation force (for spray pump products), head orientation, inspiratory flow rate, etc. The inspiratory profile adopted in *in vitro* deposition studies has varied significantly among studies, with a few studies employing a physiologically unrealistic inspiratory flow rate of 60 or 90 L/min.[106, 107] We recommend conducting *in vitro* deposition studies at flow rates of 0, 7.5, 15, and 30 L/min, corresponding to the patient holding their breath and performing slow, moderate, and rapid nasal breathing, respectively. If resources permit, slow and fast, realistic breathing profiles should be used in lieu of a fixed inspiratory flow rate to obtain more physiologically relevant deposition profiles.[108] The conduct of studies at varied (rather than a single) inspiratory flow rates or profiles is critical for drafting instructions for use for patients (e.g., if a slow inspiratory profile results in optimal olfactory region deposition, patients should be asked to breathe gently through the nostril). It is also recommended to use automatic actuation systems to minimize human variations in nasal product actuation parameters and improve reproducibility. However, it is worth noting that the conditions tested *in silico* and/or *in vitro* may be challenging for patients to replicate in a real-world setting. For example, Seifelnasr et al. recommended that patients administer nasal sprays at a nozzle angle ranging from 5° to 10° counterclockwise from the nostril normal to maximize olfactory deposition efficiency.[109] While this can be achieved using automated actuation systems, patients may find it challenging to follow these instructions in practice. We suggest implementing a DoE approach to systematically investigate the effects of factors on olfactory deposition efficiency (or other related deposition metrics, such as total nasal drug deposition). Firstly, the DoE approach not only elucidates the individual effects of each factor but also their interactive effects, which affect particle deposition (e.g., actuation force and inspiratory flow rate may have synergistic effects on particle velocity). Secondly, mapping the response surface facilitates the determination of a normal operating range where satisfactory olfactory deposition efficiency can be achieved. This approach accommodates slight deviations from ideal conditions, ensuring consistent therapeutic performance across most patients.

4.2. *In vitro* drug release

A major goal of formulation strategies, such as drug encapsulation into nanoparticles and thermoresponsive gels, is to achieve controlled and sustained drug release in the brain. Therefore, it is imperative to evaluate *in vitro* drug release in physiologically relevant conditions. Similarly, nasal powders must be wetted and then dispersed in nasal fluid for the drugs to diffuse across the epithelium for effective absorption. An ideal *in vitro* drug release setup should, at a minimum, mimic (a) the limited volume of the nasal fluid and (b) the physiological environment of the nasal cavity (i.e., the nasal mucosa is practically exposed to an air-liquid interface). Conventional apparatuses used for dissolution studies of oral dosage forms, e.g., the United States Pharmacopoeia (USP) apparatuses 1 (basket) and 2 (paddle), are

considered unsuitable, as the large receptor medium volumes do not reflect physiological conditions accurately.[110] Even if non-compendial apparatus (e.g., small, jacketed beakers) are used to mimic the volume of the nasal fluid, sample and separation methods are still inadequate, as they do not replicate the physiological aerosol dispersion conditions at the air-liquid interface (ALI) of the nasal mucosa. The Franz vertical diffusion cell, initially developed for *in vitro* release/permeation testing of topical products, has become increasingly popular for evaluating nasal products. This is due to its ability to simulate the administration of formulations onto a mucosal surface at the ALI. The formulation is applied to the donor compartment, which is separated from the acceptor compartment (with volume <15 mL to correspond to the human nasal fluid volume) with an inert membrane. The wetted membrane (or the addition of a minimal volume of nasal fluid to the apical compartment) enables the hydration (and, if applicable, gelation) of powder formulations, thereby mimicking the humid conditions in the nasal cavity. The Franz cell ensures uniform powder distribution across the entire membrane surface by evenly spraying powder particles, thereby reducing aggregation and stacking, which leads to more accurate *in vitro* drug release data.[111] Furthermore, the same Franz cell setup can be used for *ex vivo* permeation experiments by replacing the artificial membrane with an excised nasal mucosa. A similar model based on using a cell culture insert for separating powders from the nasal fluid and a 3D-printed dissolution chamber was developed by Inoue et al., which allowed both separate studies of dissolution and permeation (across Calu-3 cell monolayers) and the combined assessment through direct administration of powders onto the Calu-3 cell monolayer.[112] Alternatively, side-by-side horizontal diffusion cells have been demonstrated to be suitable apparatuses for *in vitro* drug release studies of nasal formulations, wherein stirring could be conducted in the apical compartment to mimic ciliary beating.

Apart from the choice of apparatus, various parameters for *in vitro* drug release studies require optimization to mimic nasal physiological conditions as closely as practicable. Firstly, the release medium should have a composition and pH similar to that of human nasal fluid. While phosphate-buffered saline (PBS) at pH 7.4 is commonly used for release studies, its ionic composition and pH differ significantly from human nasal fluid (which is mainly based on chloride but not phosphate salts and has a pH of ~5.5 – 6.5).[110] Furthermore, mucus and lipids are core components of human nasal fluid and are absent in both PBS and common simulated nasal fluid (SNF) buffers.[24] Zhao et al. recently developed a novel SNF composition [8.47 g/L sodium chloride (NaCl), 2.61 g/L potassium chloride (KCl), 0.43 g/L calcium chloride ($CaCl_2$), 0.24 g/L calcium glycerophosphate, 0.2% (*w/v*) Intralipid, and 2% (*w/v*) mucin, adjusted to pH 6.4]. This formulation exhibits physicochemical properties (e.g., viscosity, surface tension, pH, and drug solubility) similar to those of human nasal fluid, while being substantially more cost-effective.[113] If sink conditions for poorly water-soluble drugs cannot be attained using SNF, a small amount of organic solvent can be added to ensure sink conditions while avoiding an artificial

overestimation of *in vitro* drug release. The temperature should also be maintained at ~34°C to replicate *in vivo* nasal conditions precisely.[9] A particular challenge in standardizing the release studies is the variation in total testing duration, as these studies did not account for mucociliary clearance. Formulations without mucoadhesive properties are rapidly cleared by mucociliary clearance within 15–30 minutes; therefore, the experimental duration of drug release studies is typically limited to 1–2 hours. However, for formulations with mucoadhesive properties, longer durations are necessary to capture the release characteristics. Currently, limited models can simultaneously examine the effects of mucociliary clearance and drug release (see **Section 4.5**); therefore, further research efforts are recommended.

4.3. Nasal residence time

Non-absorptive clearance mechanisms, such as mucociliary clearance and enzymatic degradation, significantly limit the nasal drug residence time and pose a challenge for NTB drug transport. As various formulation strategies (e.g., nasal powders, stimuli-responsive gels, incorporation of mucoadhesive agents) are employed to enhance NTB drug delivery by resisting mucociliary clearance and increasing nasal retention time and drug absorption, the study of nasal residence time (or mucociliary clearance rate) of aerosol particles deposited in the nasal cavity is of significant interest. The results of mucociliary clearance or nasal drug residence time studies also inform the selection of the duration of *in vitro* drug release studies (**Section 4.2**) or permeation studies (**Section 4.4**). Techniques for predicting nasal drug residence time and/or mucociliary clearance rate are summarized in **Table 4**. The mucociliary clearance rate of aerosol particles in humans can be directly tracked by *in vivo* gamma scintigraphy (similar to regional deposition). Alternatively, strong dyes or saccharin can be incorporated into the formulation to determine the mucociliary transit time, which is the time when the dye color disappears from the nasal cavity or a sweet taste is perceived by the individual, respectively. Similarly, *in vivo* mucociliary clearance can be tracked in animals using direct imaging techniques (e.g., fluorescent imaging)[114] or by swabbing or washing the nasal cavity after intranasal administration of the drug product.[115, 116] However, it is more common to assess mucoadhesion or nasal drug residence *ex vivo* using excised nasal tissues from animals, e.g., rabbit,[117] sheep,[118] and porcine nasal mucosa[119]. Several techniques could be used to assess nasal drug residence,[11] including the “falling liquid film” technique (i.e., continuous flow of buffer through the nasal mucosal tissue after deposition of drug aerosol particles),[120] “wash-off” technique (wherein the mucosal tissue is manually washed up to and down to quantify detached drug particles),[121] or evaluation of mucoadhesive (tensile) strength or rheology.[119] However, such studies rarely specified the regional origin of the excised nasal mucosal tissues. The mucociliary clearance in the olfactory mucosa relies on the movement of the mucus blanket instead of cilia beating, as the olfactory cilia do not beat. Therefore, the olfactory mucosa may have different mucociliary clearance rates compared to the respiratory mucosa, even if smaller patches and islets of respiratory mucosa can be found in the olfactory mucosa.[10, 122] *Ex vivo* tissue-based studies

may therefore misestimate nasal drug residence time if the formulation can achieve high deposition in the olfactory region. Furthermore, inter-individual differences may limit inter-study comparisons.[123]

To adhere to the 3R (replacement, reduction, and refinement) principles of animal research and enhance data comparability, various non-tissue-based *in vitro* methods have been developed to evaluate mucoadhesion, including direct quantification of entrapped particles in simulated nasal mucus (e.g., by fluorescence microscopy or drug assay),[124-126] indirect quantification of mucin bound to the formulation,[20, 120] displacement of the particles on agar/mucin gel, dynamic vapor sorption (DVS), and performing ellipsometry, tensile strength, and rheology measurements of a mixture of the formulation with simulated nasal mucus/SNF.[123, 127] It is strongly recommended to use several mucoadhesion characterization techniques simultaneously to accurately predict the nasal drug residence achieved by specific drug delivery systems, as certain excipients may not universally demonstrate increased mucoadhesion across all methods. Ivarsson and Wahlgren demonstrated that no clear correlation could be obtained between ellipsometry, tensile strength, and rheology measurements. Several commonly used mucoadhesive polymers (as described in **Section 2**) demonstrated enhanced mucoadhesion in tensile-strength and rheology tests but not in ellipsometry tests.[127] In contrast, chitosan exhibited mucoadhesive properties only in the ellipsometry test, possibly due to its more direct interaction with mucin. Trenkel et al. proposed that combining rheology measurements, adhesiveness on mucin-agar gels, and dynamic vapor sorption (DVS) could be a valuable tool for predicting the nasal residence time of nasal powders.[123] Such use of complementary techniques was necessary as rheology measurements (of mixtures of the powder dispersion with SNF or mucin) cannot reflect the physiological scenario in which aerosolized powder particles deposit onto the nasal mucosa and are subsequently wetted by mucus, while DVS is valuable in evaluating discrepancies in observed trends between rheology measurements and displacement on agar-mucin plates: Powders with hydroxypropyl cellulose demonstrated the least displacement on agar-mucin plates compared to those formulated with other excipients despite limited effect on viscoelastic properties of SNF, which was confirmed by DVS to be due to its limited hygroscopicity (and therefore formation of a more stable gel layer on agar-mucin plates). Critically, unlike *in vitro* deposition, drug release, or permeation methods, no correlation data were available between mucoadhesion or nasal drug residence time predicted by these non-tissue-based methods and *in vivo* nasal drug residence time or absorption. More research should be dedicated to establishing such correlations and informing the selection of the most suitable technique(s) for different types of formulations, as the use of complementary approaches can be resource-intensive.

4.4. Drug permeability across the nasal mucosa

The drug permeation rate across the nasal mucosa is an important indicator of nasal drug absorption and systemic and/or brain drug bioavailability. While *in-situ* perfusion

models most closely resemble *in vivo* conditions, they are complex to establish and raise ethical concerns; hence, they are rarely employed for evaluating drug permeability across the nasal mucosa.[128, 129] Instead, permeability assays are commonly conducted to predict nasal drug absorption and bioavailability. These assays can be performed using *ex vivo* nasal mucosal tissue-based models, *in vitro* cell-based models, and non-cell-based models. **Table 5** provides a comprehensive overview of the advantages and limitations of each method.

4.4.1. *Ex vivo* nasal mucosa models

Ex vivo nasal mucosa models retain the nasal epithelial architecture and more closely mimic the physiological nasal microenvironment than *in vitro* cell-based models, thereby providing more physiologically relevant insights into drug permeability and absorption. The human nasal mucosa bears the highest physiological relevance; however, obtaining a substantial number of human nasal tissue samples is challenging. Moreover, patients undergoing nasal surgery often present with lesions or compromised tissue, which can further increase the likelihood of lesion development in excised human nasal mucosa. Such lesions may adversely affect the accuracy and reproducibility of experimental results. Consequently, animal mucosal tissues are frequently used as alternatives. Among these, bovine,[130] porcine,[131] goat,[132] sheep,[120, 133] rabbit,[134] and rat nasal mucosa are common choices.[135] Bovine, porcine, and sheep nasal mucosa exhibit the highest physiological similarity to human nasal mucosa, rendering them particularly suitable in experimental studies.[136-138] *Ex vivo* permeability studies are generally conducted using either horizontal or vertical diffusion chambers, wherein the mucosa or membrane is positioned between the donor and receptor compartments, which are oriented accordingly. Commonly employed models include the Franz diffusion cell and the horizontal Ussing chamber, as shown in **Fig. 6**.

Data reproducibility and comparability in permeation studies using *ex vivo* nasal mucosa models are more challenging than when using *in vitro* cell-based models due to the greater inherent variability of biological tissues. Therefore, it is imperative to minimize variations in tissue properties (e.g., anatomical origin and tissue thickness). The tissue should be freshly excised within 4 hours post-mortem to maximize tissue viability and minimize structural damage before use.[12, 139, 140] The olfactory region should be preferably selected for *ex vivo* permeation studies of NTB products. Tissue thickness is also a critical confounding factor, as the diffusion flux is inversely proportional to tissue thickness according to Fick's diffusion law, and may contribute to differences in drug permeability across various regions of the nasal cavity.[141] While selecting tissue of similar thickness from the same animal is preferred to reduce variability, it may not always be possible due to biological or logistical constraints. An alternative approach is normalizing the obtained results by correcting for thickness-related deviations. Zhao et al. proposed a method to normalize *ex vivo* permeation curves by simulating standardized permeation curves using a diffusion model in a simulated Franz diffusion cell geometry with a

standardized mucosal barrier thickness of 0.80 mm.[142, 143] Normalization was successful in differentiating P_{app} differences between model compounds and reducing both inter-individual and, especially, intra-individual P_{app} variability. Other experimental considerations that should be standardized in *ex vivo* permeation studies (e.g., composition and pH of the receptor medium, incubation temperature) are similar to *in vitro* release studies and are described above (**Section 4.2**).

4.4.2. *In vitro* cell-based models

While *ex vivo* nasal mucosa models most closely resemble *in vivo* conditions, *in vitro* cell-based models are increasingly favored to minimize the use of animals and adhere to the 3R principles of animal research. The "ideal" *in vitro* cell-based model for permeability assays should have the following characteristics to mimic the nasal mucosa anatomy and physiology closely:[144, 145]

1. Retain the organotypic properties [i.e., cilia beating, mucus expression, tight junction formation, and "leaky" epithelium (Transepithelial electrical resistance (TEER) ~100 Ω cm^2)], major cell type distribution (differentiated ciliated epithelial cells, goblet cells and basal cells), and functional expression of ATP binding cassette (ABC) drug transporter proteins [e.g., P-glycoprotein (P-gp), multidrug resistance associated protein (MRP)1, MRP2, and breast cancer resistance protein (BCRP)] of the human (olfactory) nasal mucosa
2. Can be easily, rapidly, and sustainably cultivated at reasonable costs
3. High data reproducibility
4. Ability to detect effects of formulation composition variations (e.g., dosage form, excipients, dual-drug combinations, etc.)
5. Good correlation with *in vivo* bioavailability and *ex vivo* nasal mucosal permeability

Both primary and immortalized cell lines have been used in *in vitro* permeability assays. A key advantage of primary cell cultures is that they are morphologically and functionally very similar to the nasal mucosa, as primary nasal epithelial cells are fully differentiated into ciliated, goblet, and basal cells, with functional cilia beating, mucus secretion, tight junction formation, and the expression of ABC drug transporter proteins, including P-gp, MRP, and BCRP. ABC drug transport proteins have been shown to mediate the efflux of xenobiotics from cells actively, thereby severely limiting drug absorption across the epithelial barrier.[146] Although the functional expression of ABC drug transport proteins has been well-characterized in the BBB and the intestinal barrier,[147,148] their expression in the human nasal mucosa has been relatively recently elucidated. Notably, the expression of MRP1 in the human nasal mucosa is very high and greater than that of the Caco-2 epithelial barrier model. At the same time, that of P-gp and BCRP is weak and substantially lower than that of the Caco-2 model.[149, 150] Nevertheless, the functional activity of P-gp and BCRP was demonstrated successfully in primary nasal airway epithelial ALI cell cultures, wherein P-gp- and BCRP-substrate efflux was substantially reduced or even completely abolished by their respective inhibitors.[150] Therefore, it is vital to

ensure the functional expression of these key transporters in *in vitro* nasal epithelial models to enable accurate prediction of their effects on drug permeability and bioavailability.[150, 151]

Commercially available primary nasal airway epithelial ALI cell cultures, such as EpiAirway™ (MatTek Corporation, Ashland, MA, USA) and MucilAir™ (Epithelix, Plan-les-Ouates, Switzerland), are increasingly favored due to their convenience and greater reproducibility. Notably, MucilAir™ has been extensively characterized as a nasal epithelial barrier model, comprising a tight, polarized, pseudo-stratified nasal epithelium with fully differentiated ciliated, goblet, and basal cells, and functional expression of P-gp and BCRP proteins.[150] MucilAir™ also demonstrated superior correlation to *in vivo* nasal drug absorption in rats compared to EpiAirway™.[152] Therefore, MucilAir™ is regarded as a more relevant model of the nasal epithelium and is commonly used as a benchmark against immortalized cell line models. However, these commercially available systems are often more costly. Critically, primary human nasal cells may be of limited relevance to NTB drug transport, as they are unlikely to originate from the olfactory mucosa due to its small area and difficult access. Therefore, an alternative approach is to collect primary nasal cells from *post-mortem* animal olfactory mucosa, where the olfactory region is more accessible. Olfactory mucosal primary cells have been successfully isolated from animals such as rats and pigs for the purpose of evaluating drug and nanoparticle permeation.[153] In particular, Ladel et al. revealed that porcine olfactory epithelial cells have lower barrier integrity (as indicated by TEER values), increased transepithelial permeability, and higher mucus secretion than respiratory epithelial cells of the exact origin, highlighting the importance of using an equivalent tissue type to investigate drug transport in olfactory mucosa.[154] Nevertheless, developing *in vitro* primary nasal cell-based models requires considerable technical expertise to accurately identify the nasal region and collect representative primary nasal cells. There is inherent variability between cells from different batches.[145] Furthermore, primary cells are typically limited to a maximum of four passages before senescence or differentiation compromises their utility, which precludes their adoption in large-scale studies.[145]

Due to the complexity and high costs associated with obtaining primary nasal cell cultures, immortalized cell lines are more frequently used as *in vitro* models for permeability studies. These cell lines can be easily and sustainably cultivated, thereby facilitating large-scale testing and research. Commonly used cell lines include those derived from normal bovine turbinates (BT), rat nasal squamous carcinoma (NAS 2BL), human normal bronchial epithelium (16HBE14o-), human lung adenocarcinoma (Calu-3), and human nasal anaplastic squamous cell carcinoma of the nasal septum (RPMI 2650).[145] However, ALI cultures of RPMI 2650 and Calu-3 cells have been more extensively characterized as *in vitro* models of the nasal epithelial barrier and will be discussed in more detail here. The use of ALI instead of liquid-liquid interface (LLI) conditions is essential as both cell lines display closer correlation to the human nasal mucosa when cultured at ALI conditions: LLI RPMI 2650 cultures do not develop tight junctions, resulting in TEER values (~25 –

30 Ωcm^2) significantly lower than those observed in human nasal mucosa tissues (~60 – 180 Ωcm^2), while LLI Calu-3 cells formed a less differentiated simple cuboidal epithelium with lower mucus secretion, shorter and fewer microvilli, and drastically higher TEER values (400 – 1700 Ωcm^2) compared to human nasal mucosa.[155-157]

In terms of their anatomical and physiological relevance to the nasal mucosa, both RPMI 2650 and Calu-3 have their respective strengths and limitations. Although Calu-3 is derived from the lungs, it can be differentiated under ALI culture into mucus-secreting goblet cells. While some studies report the presence of cilia in Calu-3 ALI cultures, this has yet to be consistently demonstrated and should not be expected, given the bronchial submucosal origin of these cells.[158] In contrast, RPMI 2650, despite its nasal origin, is composed exclusively of epithelial cells and is unable to secrete mucus or form cilia. Nonetheless, the "leaky" epithelial barrier formed by RPMI 2650 cells under ALI culture results in TEER values that more closely resemble those obtained in *ex vivo* human nasal mucosa tissues compared to Calu-3 cells, which form a tighter epithelial barrier.[159] Furthermore, under ALI culture, Calu-3 cells form monolayers that more closely resemble the structure of the human nasal epithelium. In contrast, RPMI 2650 cells form multilayers with thicknesses similar to those of the nasal mucosa. In terms of bidirectional drug transport, the functional expression of P-gp, MRP1, MRP2, and BCRP transporters in RPMI 2650 cells was diminished when cultured at ALI conditions for RPMI 2650 cells, while only the functional activity of P-gp was retained in ALI-cultured Calu-3 cells.[156, 160] Despite their limitations in mimicking nasal mucosal characteristics, the P_{app} values obtained in both RPMI 2650 and Calu-3 cells (when cultured under ALI conditions) have demonstrated strong, comparable correlations with MucilAir™ and *ex vivo* nasal mucosa models.[20, 123, 124] Both cell models also possess the ability to differentiate variations in permeability due to differences in formulation composition.[159] Despite the limited data available on systemic drug availability after IN drug administration, studies have also demonstrated that *in vivo* drug bioavailability can potentially be reliably predicted from *in vitro* ALI cell cultures. More data are currently available on Calu-3 cells compared to RPMI 2650 cells.[128, 152, 159] A comparison of MucilAir™, RPMI 2650, and Calu-3 cells as *in vitro* cell-based permeability models is provided in **Table 6**.

Various challenges remain to improve the reliability of *in vitro* cell-based models and the correlation between *in vitro* drug permeability data and the biological fate of nasal drug products for NTB delivery. Firstly, culture conditions (e.g., passage number, airlift timepoint, duration of ALI culture, serum content in cell culture media, seeding density, membrane pore size and pore density, etc.) could significantly affect the morphological properties, barrier integrity, and functional expression of drug transporters, resulting in variations in experimental permeability values. For example, using advanced minimum essential media (MEM) with 2.5% FBS rather than MEM with 10–15% FBS resulted in ALI RPMI 2650 cultures with more microvilli and cilia-like structures.[161] Discrepancies in cilia presence across studies in ALI Calu-3 cultures were attributed to potential variations in cell passage number, as cells at

passages 20–40 were used in studies reporting ciliogenesis in ALI Calu-3 cultures.[158] Therefore, it is essential to develop standardized protocols to minimize inter-laboratory and inter-experiment variability. Extensive characterization of critical parameters for ALI RPMI 2650 cultures has been conducted by Barlang et al., whereas similar characterization has not yet been performed for Calu-3 cells.[162] Secondly, the grand challenge of developing an immortalized cell line that allows sustained culture while retaining as many organotypic properties of the human nasal epithelium as possible remains to be addressed. Bendas et al. recently developed an immortalized "P1" cell line from porcine nasal mucosa that (a) remained in a stable phenotype over 30 passages; (b) was differentiable into ciliated epithelial cells, goblet cells, and basal cells under ALI culture; (c) had similar thickness (~100 μm) to the human nasal mucosa epithelium; (d) TEER values (~190 – 510 Ω cm^2) that closely resemble *in vivo* conditions than primary nasal cells; and (e) yielded P_{app} values of selected compounds that more closely resemble ones obtained in *ex vivo* human and porcine nasal mucosa tissues compared to MucilAir™.[163] However, this cell line is currently not commercially available, and its ability to differentiate between model compounds of varying permeability has yet to be validated. Future efforts should be dedicated to establishing a similar immortalized human cell line. Finally, currently available (albeit limited) IVIVC data are established for systemic, but not brain, bioavailability. Drugs exhibiting higher nasal permeability may have limited benefit from NTB delivery, as a larger fraction of these drugs would likely be rapidly absorbed into the systemic circulation and subsequently enter the brain by crossing the BBB (instead of direct NTB transport). Further studies correlating P_{app} values with *in vivo* NTB drug delivery metrics (e.g., %DTP & %DTE) would significantly accelerate the prediction of bioavailability enhancement and efficacy during the pre-clinical development of drug products.

4.4.3. Non-cell or tissue-based models

Both *in vitro* cell-based and *ex vivo* permeability assays are highly time-consuming and labor-intensive, which limits their high-throughput capabilities. Henriques et al. recently developed a novel non-cell-based model for evaluating nasal drug permeability based on the Parallel Artificial Membrane Permeability Assay (PAMPA), which has previously been applied to predict intestinal drug absorption and permeability across the BBB.[164-166] Instead of the typical setup in *in vitro* cell-based models where cells were applied onto the cell insert membrane, a phosphatidylcholine-based artificial lipid-oil-lipid membrane [2% (*w/v*) phosphatidylcholine in dodecane] was coated onto the porous hydrophobic filter to mimic the lipid composition of the human nasal aspirate. The donor solution contained mucin [0.5% (*w/v*)] to mimic the nasal mucus. The P_{app} values obtained using the nasal-PAMPA model showed good correlation with those obtained using an *in vitro* RPMI 2650 cell model, and the nasal-PAMPA model discriminated between the effects of drug formulation and solid state on drug permeability. Since the assay requires only one day to complete and does not require training in cell culture techniques, the nasal-PAMPA model could offer a more convenient and high-

throughput method for assessing nasal drug permeability. However, the nasal-PAMPA model assesses solely passive diffusion and does not consider active transport processes, which may limit its applicability to certain specific NTB drug-delivery strategies (e.g., nanoparticles that may be transported across the nasal epithelium via endocytosis).

4.5. Integrated models

The preceding sections provided a comprehensive overview and critique of models commonly applied to study the individual nasal biopharmaceutical processes involved in NTB delivery. However, these models cannot fully reflect the *in vivo* drug biopharmaceutics during NTB delivery, as these dynamic biological processes co-occur and are intricately intertwined. Therefore, there is growing interest in developing integrated *in silico* and *in vitro* models that can simultaneously study multiple nasal biopharmaceutical processes. For example, the combined study of regional aerosol deposition with drug release and permeation after aerosol deposition would more accurately reflect *in vivo* drug transport across the olfactory nasal mucosa after IN administration of the formulation. Maaz et al. successfully developed an *in vitro* model for such studies by combining a 3D-printed nasal cast with a Snapwell cell culture insert holder in the olfactory region section of the nasal cast, thereby facilitating the simultaneous investigation of regional aerosol deposition within the nasal cavity and drug release and permeability across ALI RPMI 2650 cultures from drug-loaded aerosols that deposit in the olfactory region.[167] However, such models still rely on “static” cell culture and cannot mimic the dynamic nature of the human nasal mucosa. Therefore, advanced new approach methods (NAMs) have been developed to overcome this challenge. These include advanced *in silico* models based on CFD and mathematical models that can comprehensively predict aerosol deposition, clearance, and absorption within the nasal cavity, as well as human nasal mucosa-on-a-chip models that could quantitatively and instantaneously monitor nasal biopharmaceutical processes.[168, 169] The mucosa-on-a-chip tools integrate cell cultures with real-time electrochemical sensing in the acceptor compartment to monitor drug permeability and perform *in situ* TEER measurements to evaluate barrier integrity. This enables high-throughput data readout compared to traditional characterization approaches, such as drug assays and TEER measurements, which utilize high-performance liquid chromatography (HPLC) and Voltohmmeters, respectively.[170-172] The donor chamber design can be optimized to mimic the realistic wall shear stress exerted onto the nasal cavity during aerosol administration. While these NAM models will require further validation *in vivo*, it is anticipated that they could allow the prediction of pharmacokinetic profiles and brain-targeting efficiency associated with NTB drug delivery. This approach has the potential to minimize or even eliminate reliance on animal testing, thereby expediting the drug development process.

5. Biological performance of NTB drug delivery systems

Despite the abundance of *in silico*, *in vitro,* and *ex vivo* models available (as described in **Section 4**) to predict the drug delivery efficiency of NTB drug delivery systems, *in vitro* and/or *in vivo* pharmacokinetic, efficacy, and toxicity studies remain indispensable to evaluate their biological performance. Given the extensive variety of disease models developed for various neurological conditions, a detailed discussion of considerations for *in vivo* efficacy studies is beyond the scope of this review. Relevant insights and comprehensive analyses can be found in other specialized reviews.[173-175]

5.1. Pharmacokinetic and brain drug distribution studies

In vivo pharmacokinetic studies are performed to evaluate the brain-targeting efficiency of the drug product designed for NTB drug delivery. These studies are most often performed by randomizing animals to be administered with the candidate formulation by IN administration or intravenous (IV) injection of an appropriate formulation containing the same drug, followed by sampling of the brain tissue and blood to obtain their respective pharmacokinetic parameters [e.g., maximum drug concentration (C_{max}), T_{max}, area under the curve (AUC), etc.]. Rodents are most frequently used because they are relatively accessible and inexpensive, and their physiology is well-characterized.[176] Brain-targeting efficiency is typically evaluated using the following metrics:

- DTE, calculated using the formula $\mathrm{DTE\%} = \frac{(\mathrm{AUC_{brain,IN}})/(\mathrm{AUC_{blood,IN}})}{(\mathrm{AUC_{brain,IV}})/(\mathrm{AUC_{blood,IV}})} \times 100$ [wherein AUC is the area under the curve, or total drug exposure over time (in the brain or blood), for the duration of the study (AUC_{0-t}), and IN and IV indicate the administration route (intranasal or intravenous, respectively) to which the AUC values correspond to], evaluates the brain-targeting efficiency of IN over IV administration. A DTE% >100% suggests that IN delivery achieves better brain targeting than IV administration.
- DTP, calculated using the formula $\mathrm{DTP\%} = \frac{\mathrm{AUC_{brain,IN}} - (\frac{\mathrm{AUC_{brain,IV}}}{\mathrm{AUC_{blood,IV}}} \times \mathrm{AUC_{blood,IN}})}{\mathrm{AUC_{brain,IN}}} \times 100$, evaluates the drug fraction *directly* transported to the brain via the olfactory and trigeminal pathways by subtracting the contribution of indirect drug transport from $AUC_{brain,IN}$. A DTP% >0 indicates drug transport across the direct pathways after IN administration.
- $B\%_{brain\ IN/IV}$, sometimes referred to as "comparative bioavailability", is a measure of brain drug accumulation through the IN route over the IV route and is calculated by the formula $B\%_{brain\ IN/IV} = \frac{AUC_{brain.IN}}{AUC_{brain,IV}} \times 100$. $B\%_{brain\ IN/IV}$ >100 indicates better brain drug accumulation through IN administration relative to IV administration.

While the "minimum" thresholds to demonstrate brain targeting after IN drug product administration are DTE%, DTP%, and $B\%_{brain\ IN/IV}$ values of >100, >0, and >100, respectively, higher values are desirable to reduce dose requirements and minimize

systemic adverse effects. However, there are no established guidelines or standards for analyzing pharmacokinetic data in NTB drug delivery systems, resulting in significant heterogeneity between studies and precluding direct head-to-head comparisons of findings. Significant inter-study differences in methodologies can be present in either the drug formulation selected for IV injection, the IN administration technique, brain sampling techniques, or the calculation method of brain targeting metrics. It is necessary to develop a standardized protocol for *in vivo* pharmacokinetic studies in rodents evaluating the brain-targeting efficiency of NTB drug delivery systems. The following recommendations, primarily based on previous studies by Pires and Santos, Wang et al., and Dhuyvetter et al., could serve as preliminary guidance:[31, 177, 178]

1. A plain drug solution instead of or in addition to a drug nanoparticle formulation should be administered by IV injection and DTE%, DTP%, and $B\%_{brain\ IN/IV}$ values should be calculated based on data from IV drug solution whenever possible, as nanoparticle encapsulation can markedly alter the intrinsic pharmacokinetic properties of the drug; if it is desired to specifically compare between IN and IV administration of the same nanoparticle-based formulation, the metrics should be referred by alternative nomenclature to avoid confusion.
2. The dosing method for IN administration should cover the entire nasal cavity. While techniques have been developed to specifically administer the formulation onto the olfactory mucosa of the mouse or rat, this is not representative of the clinical scenario, as it is practically impossible to achieve complete olfactory region drug deposition in humans and will exaggerate brain-targeting metrics (since a fraction of the drug will be transported into the brain via the non-direct systemic absorption route).
3. Validation parameters for the analytical method should be provided to ensure the reliability of the reported data.
4. The olfactory bulb should be separated from the rest of the brain during sampling and analyzed separately. As mentioned in **Section 2.1** above, it is common to observe drug accumulation in the olfactory bulb with limited entry into the rest of the brain. Failing to separate them will result in exaggerated calculations.
5. AUC_{brain} and AUC_{blood} should be calculated from the *unbound* fraction of drugs in the blood and the brain, respectively, as the unbound fraction is most relevant in terms of efficacy and safety. Drugs with low protein binding in the blood should not be assumed to have similarly low protein binding in the brain, as demonstrated by Wang et al., where the unbound fraction of HIV-1 replication inhibitor DB213 in the blood and brain were close to 100% and ~5%, respectively.
6. Linearity of the pharmacokinetic data (i.e., no saturation of the individual absorption, distribution, metabolism, and elimination processes) should be

validated. Otherwise, brain-targeting metrics may misrepresent the actual brain-targeting efficiency due to direct NTB drug transport, and

7. The calculation method of the pharmacokinetic ratios should be clearly reported. Some studies used different calculation formulas or AUC_{∞} to estimate DTE%, resulting in apparently poor correlation with DTP% (when both metrics were calculated from the same data).

In vivo pharmacokinetic studies typically measure drug concentration in whole brain homogenates and therefore assume that the drug is evenly distributed within the brain after IN formulation administration, which is not accurate, as drugs initially enter the brain via either the olfactory bulb or brain stem (via the trigeminal nerve) before being transported to other regions of the brain. Understanding intracerebral drug distribution is valuable when pathological changes are more likely to occur within a specific region of the brain (e.g., the hippocampus for Aß plaques in Alzheimer's disease). Imaging techniques are preferred for providing spatiotemporal information on drug distribution. Commonly applied techniques include MRI, CT, single-photon emission CT, positron emission tomography, gamma scintigraphy, and optical (fluorescent/bioluminescent) imaging. Interested readers are referred to the review by Veronesi et al. on the respective merits and limitations of various imaging techniques, as well as considerations for choosing an appropriate method for *in vivo* studies of drug distribution within the brain.[179]

While *in vivo* pharmacokinetic studies in rodents can reasonably demonstrate that brain targeting can be achieved with IN administration of the formulation, extending these results to predict pharmacokinetic profiles in humans is highly challenging. Firstly, the olfactory epithelium accounts for approximately 50% of the nasal cavity surface area in rodents but only approximately 8% in humans (**Table 7**).[180] Consequently, direct NTB transport (and hence brain targeting efficiency) will be overestimated in rodents. Secondly, due to the small nasal opening in rodents, IN formulation administration techniques differ from those of the clinical drug product (e.g., liquids are commonly instilled into the nasal cavity of mice using autopipettes instead of being aerosolized as droplets by nasal spray devices).[177] The IN instillation technique also typically requires the rodent to be held in a supine position (i.e., with the head facing upward), which differs from the typical head position of humans during nasal product administration. These differences would likely result in different drug deposition profiles within the nasal cavity. Thirdly, rodents may require anesthesia for accurate IN drug dosing, which may alter mucociliary clearance and nasal airflow, thus affecting drug deposition and absorption.[181]

Several strategies can be applied to enhance the predictive accuracy of brain-targeting in pharmacokinetic studies. One approach is the use of large animals in addition to (or in lieu of) rodents, e.g., dogs, rabbits, and non-human primates, owing to their closer anatomical and physiological similarity to humans (**Table 7**).[182-191] Aerosols can also be administered to large animals using nasal delivery devices designed for human applications, which closely mimic aerosol entry and deposition

in the human nasal cavity. However, challenges in acquisition, high costs, and ethical considerations limit their use. Rabbits and dogs are more readily accessible than non-human primates and have nasal anatomical features that more closely resemble those of humans, making them valuable models for *in vivo* studies.[176, 192] Nevertheless, limited *in vivo* pharmacokinetic studies of NTB drug products in large animals have been reported. Interspecies differences in nasal anatomy, mucociliary clearance rates, breathing patterns, and airflow dynamics remain to be elucidated.[186] The head orientation position (i.e., supine, vertical, or head-down) of the animal and insertion depth of the nasal device further influence regional aerosol deposition.[181, 193-195] More research is required to evaluate which large animal is most suitable for predicting brain-targeting efficiency, balancing the need for physiological similarity to humans with resource and cost limitations, and to develop standardized protocols for conducting *in vivo* pharmacokinetic studies in large animals. Regardless of the selected animal species, various biological factors such as age, weight, gender, and health status can affect pharmacokinetics and drug distribution within the brain. Notably, some neurological diseases (e.g., Alzheimer's disease or stroke) are known to compromise the integrity of the blood-brain barrier, resulting in more significant indirect NTB drug transport compared to healthy animals (i.e., lower DTE% and DTP%).[196, 197] For such conditions, it is recommended to use animal models of disease alongside healthy animals for pharmacokinetic studies, thereby enabling more accurate predictions of the brain-targeting effect in patients following IN drug administration.

Another emerging approach is the development of advanced physiologically based pharmacokinetic (PBPK) models in rodents that could be scaled to humans. A typical multi-compartment drug metabolism and pharmacokinetic (DMPK)-based model in rodents, as described in studies by Stevens et al. and Wang et al., is presented in **Fig. 7(a)** below.[178, 198] The multi-compartment model comprises two absorption compartments corresponding to direct and indirect NTB transport, respectively. Unbound drug concentrations in the plasma and the brain are represented by the central and brain compartments, respectively, with an additional peripheral compartment accounting for the distribution of the drug from the blood to peripheral organs. Allometric scaling principles can then be applied to the first-order rate constants to predict the pharmacokinetic profile in humans. Stevens et al. successfully applied PBPK modelling to pharmacokinetic profiles obtained from IN and IV remoxipride administration in rats to predict the human plasma pharmacokinetic profile.[198] The PBPK model can be integrated with pharmacodynamic readouts in rodents to form a pharmacokinetic-pharmacodynamic model, which can then be scaled to predict pharmacodynamic responses in humans. Nevertheless, the study by Stevens et al. remains a sole example in the literature for predicting NTB brain-targeting efficiency in humans using rodent pharmacokinetic data, most likely due to the lack of human data for validation. The multi-compartment model described in **Fig. 7(a)** also fails to account for differences between rodents and humans in several biological processes that may affect NTB drug delivery (e.g.,

regional deposition within the nasal cavity, non-absorptive mucociliary and enzymatic clearance). Therefore, the use of physiologically based *biopharmaceutical* modelling (PBBM), a subset of PBPK modelling wherein models of the nasal biopharmaceutical processes described in **Fig. 4** are integrated into the PBPK model, has recently been advocated to enable more accurate *in vivo* brain concentration profile predictions and inform dose selection of nasal products designed for NTB drug delivery (**Fig. 7(b)**). The characterization data obtained using the techniques described in **Section 4** can be incorporated as input parameters for the PBBM model (**Table 8**). While PBBM models have yet to be developed for NTB drug delivery systems, extensive efforts have already been dedicated to typical nasal spray products, in which *in silico* models were used to predict drug dissolution, absorption, and clearance (DAC) within the nasal cavity.[199, 200] Dutta et al. recently developed one of the most comprehensive CFD-DAC-pharmacokinetic models to date. This model can predict regional drug deposition and simulate post-deposition transport (including mucociliary clearance, gastrointestinal tract absorption of the swallowed dose, dissolution, and nasal tissue absorption).[201] The predicted pharmacokinetic profiles of a triamcinolone acetonide nasal spray product at different doses, generated using the model, showed reasonable agreement with clinical pharmacokinetic data. Further research in this area is warranted to expedite the development of NTB drug products.

5.2. Safety/toxicity studies

As discussed in **Section 2**, various strategies have been employed to enhance the efficiency of NTB drug delivery, which requires the use of different excipients. However, whilst most excipients have demonstrated a good safety profile in oral or topical routes of administration, minimal safety data is available for nasal administration, as evidenced by the limited number of excipients listed to be safe for nasal administration in the US FDA's inactive ingredient database. Notably, some excipients that were generally considered non-toxic in the literature, e.g., chitosan, have been shown to induce local nasal toxicity potentially.[45] Consequently, it is suggested to conduct thorough toxicity screening of candidate excipients prior to commencing formulation studies.

To minimize *in vivo* testing, toxicity studies are often conducted *in vitro* or *ex vivo* using models similar to permeability studies, e.g., ALI culture of primary (MucilAir™) or immortalized (Calu-3 and RPMI 2650) cells, and *ex vivo* nasal mucosa tissue culture using Franz vertical diffusion cells. Nasal mucosal toxicity can be determined using cell viability/cytotoxicity assay kits (e.g., MTT and LDH assay kits),[202, 203] real-time impedance or TEER measurements,[202] permeability markers,[204] and/or microscopic ultrastructural examinations.[203] The safe concentration must not be determined based on a single measurement technique due to several reasons. Firstly, the excipient or drug delivery system tested may interact with the principles of action of cell viability assay kits. For example, MTT assays failed to detect cell damage towards RPMI 2650 ALI cultures caused by 1% (*w/v*) methylcellulose

solution, which was postulated to be due to the highly viscous methylcellulose solution retarding the exocytosis of MTT formazan crystals.[202] Secondly, different culture types may exhibit counterintuitive responses to cell injury as measured by specific markers. While conventional permeability markers, e.g., fluorescein sodium and lucifer yellow, usually exhibit enhanced transport in damaged epithelial barriers due to chemical injury, Zhao et al. unexpectedly observed a *reduction* in fluorescein yellow transport in 10% (*v/v*) isopropyl alcohol-injured porcine nasal mucosa despite microscopic evidence of epithelial disruption, which was attributed to the occlusion or closure of paracellular pathways.[142] Therefore, caution is recommended when deriving epithelial integrity from permeability marker data. Apart from direct cellular injury, the excipient should also not interfere with normal ciliary function. MucilAir™ (or other primary nasal cell cultures) and *ex vivo* nasal mucosa tissue cultures are thus particularly valuable for toxicity studies, as they also provide additional information on ciliotoxicity through the observation of the ciliary beating frequency (CBF) using high-speed microscopy.[203, 205, 206] The challenge nevertheless remains to correlate *in vitro* data obtained from *in vitro* or *ex vivo* nasal toxicity studies with human *in vivo* toxicity data. Efforts to develop a framework to assess the safety of excipients for NTB drug delivery, similar to the Safety Assessment of Excipients model proposed for pulmonary drug delivery,[207] should be undertaken to guide the judicious selection of excipients for NTB drug delivery formulations. Apart from ciliotoxicity or direct nasal mucosal injury, deposition of foreign particles on the sensitive nasal mucosa may cause mucosal irritation. The slug mucosal irritation assay, wherein an increase in mucus formation in slugs is correlated with an elevated incidence of itching, stinging, or burning sensations in humans, can serve as a surrogate to predict patient acceptability of the formulation before proceeding into clinical trials.[123, 208]

For a comprehensive toxicity evaluation of the prototype drug product, it is recommended that *in vitro* toxicity studies be performed in both nasal- and brain-related cell lines (e.g., RPMI 2650, SH-SY5Y human neuroblastoma, C6 human astrocyte) to assess the formulation's biocompatibility in both the nasal cavity and the brain. Toxicity evaluation in pulmonary cell lines (e.g., A549) is recommended when respiratory toxicity is a concern. However, given that *in vitro* or *ex vivo* toxicity studies have limited physiological relevance, *in vivo* studies remain the “gold standard” for confirming the preclinical safety profile of the formulation, in accordance with the ICH guidelines. Nevertheless, per the 3R principles of animal research and with the US FDA recently announcing plans to reduce and ultimately replace animal testing, more efforts should be dedicated to the development of NAMs, e.g., complex human organoid and organ-on-a-chip models, that can capture the physiology and toxicology of NTB drug delivery, which could accelerate clinical translation of drug products developed for NTB delivery. The adverse-effect profiles from *in vivo* or *in vitro*/*ex vivo* studies incorporating NAMs can be linked to the PBPM models (as described in **Section 5.1** above) to ensure that plasma and brain drug concentrations remain within the therapeutic window and demonstrate treatment

efficacy without severe adverse effects, although future research is required in this area.[209]

6. Conclusions

Numerous preclinical studies have substantiated that NTB delivery can facilitate direct drug transport into the brain and improve therapeutic outcomes in CNS disorders. Consequently, many candidate drugs targeting CNS disorders, particularly those necessitating rapid onset of action, exhibiting high risks of systemic toxicity, or poor BBB permeability, can potentially benefit from NTB delivery. This innovative administration pathway holds promise not only for neurodegenerative disease therapeutics but also for analgesics, anticancer agents, psychotropic medications, and neuroprotective compounds, thereby broadening the therapeutic landscape for CNS-related conditions. However, a translational gap remains between NTB delivery research and its clinical application. This review assesses current advances in formulation strategies, nasal delivery device design, and characterization techniques, highlighting limitations that hinder translation and outlining directions to overcome them. Herein, we summarize the key unmet translational needs:

- Mechanistic studies examining how formulation properties (e.g., nanoparticle size and surface charge; CPP sequence and secondary structure) influence the biological fate of the drug and carrier after IN administration
- Design of novel nasal devices that effectively target the olfactory region
- Comprehensive evaluation of how formulation properties, nasal device actuation parameters, and patient characteristics affect nasal biopharmaceutical processes, especially the *in vivo* nasal regional deposition profile
- Standardization of *in silico*, *in vitro*, and *in vivo* protocols/techniques for evaluating nasal biopharmaceutical processes to improve their bio-relevance for NTB drug delivery
- Development and validation of advanced *in silico* (e.g., CFD-PBPK models) or *in vitro* (e.g., organ-on-a-chip) NAMs with high predictive accuracy for pharmacokinetics, efficacy, and safety of NTB drug delivery systems in humans

We urge the scientific community to dedicate concerted efforts towards addressing the aforementioned unmet needs and fill the translational gap in NTB drug delivery systems. We anticipate that these efforts will yield an evidence-based roadmap that guides the rational development of NTB drug delivery systems across the translational valley of death, ultimately benefiting millions of patients with CNS disorders.

CRediT authorship contribution statement

Xinyue Zhang: Conceptualization, Investigation, Writing – original draft, Visualization. Stephanie Chow: Visualization. Ho Wan Chan: Conceptualization, Writing – review &

editing. Shing Fung Chow: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the Health and Medical Research Fund (Reference number: 21223381), Health Bureau, Hong Kong SAR Government, the Health@InnoHK program, Innovation and Technology Commission, Hong Kong SAR Government, and the University of Hong Kong (Project number:109001171). The graphical abstract and Figs. 2–5 were created with the aid of BioRender.

Data availability

No data was used for the research described in the article

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Figure 1 Comparison of nose-to-brain drug delivery efficiency between nanoparticle-based systems (nanosystems) and solutions by log drug targeting efficiency (DTE)% (a), direct transport percentage (DTP)% (b), and log comparative brain bioavailability (intranasal vs. intravenous) ($B\%_{brain\ IN/IV}$) (c). Brackets signal potential outliers. Data correspond to individual values plus median ± quartiles. Statistical analysis was performed using the Mann-Whitney U test to compare intranasal administration of nanosystems with that of drug solutions as a control. $^{++}$: $p < 0.01$; $^{++++}$: $p < 0.0001$; IN: Intranasal. Reproduced from Pires and Santos with permission.[31]

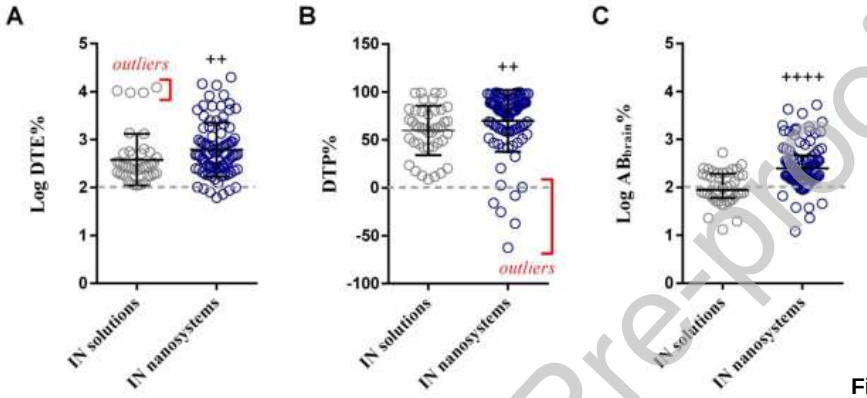

Figure 2 (a)-(c) and (d)-(f) are images from a representative section of the olfactory bulb and cerebral cortex, respectively, obtained from mouse brains perfused and stained with DAPI to visualize cell nuclei 4 hours after treatment with intranasal administration of 12 mmol (24 µL × 500 µM) rhodamine B (RITC)-KAFAK. RITC-KAFAK, DAPI, and merged fluorescent images were shown in (a) & (d), (b) & (e), and (c) & (f). Scale bars: 100 µm. (g) Levels of key proinflammatory cytokines in the brain in sham-injured, vehicle-treated mice (Sham), TBI-injured, vehicle-treated mice (TBI Vehicle), TBI-injured mice treated with intranasal administration of 12 mmol (24 µL × 500 µM) KAFAK (KAFAK IN), or TBI-injured mice treated with intraperitoneal injection of 16.4 mg/kg KAFAK (KAFAK IP). Data shown represent mean pg/mg tissue ± 2 SD, n = 5/group, * $p < 0.05$, ** $p < 0.01$). Figures (a) - (g) are adapted from Yanamadala et al. under CC-BY 4.0 license.[68] (h)-(k) represent confocal laser scanning microscopy images (20× magnification) of C6 cells transfected with (h) coumarin-loaded PEG-PCL, (i) PEG-PCL-Tat, (j) Bombesin (Bom)/mPEG-PCL-Tat, and (k) Bom/PEC-PCL-Tat pretreated with stearoyl-modified Bom at coumarin concentrations of 2.5 mg/mL. Blue and green fluorescence represent Hoechst-stained nuclei and coumarin, respectively. (l) Intracerebral distribution of coumarin-loaded micelles in orthotopic glioma-grafted rats following intranasal

administration. Rats were inoculated with C6 glioma and intranasally administered coumarin-loaded PEG-PCL-Tat or Bom/PEG-PCL-Tat micelles (Dose = 20 μg coumarin) 14 days after inoculation, and sacrificed 4 h after intranasal administration. Each brain was enucleated and divided into the C6 glioma-inoculated side and the non-treated normal side. Figures (h)-(l) are adapted from Kanazawa et al. with permission.[69]

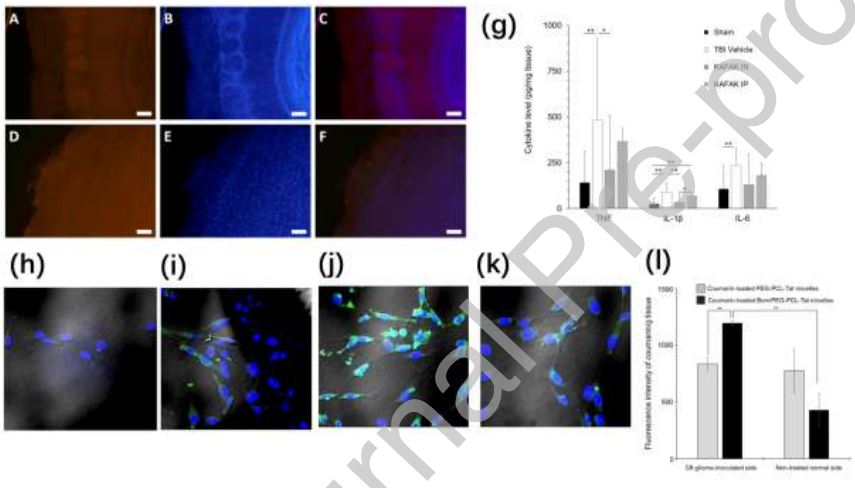

Figure 3 Examples of nasal devices for intranasal (including NTB) drug delivery. (a) Nasal drops; (b) Nasal sprays; (c) Precision Olfactory Device®; (d) Opti-Powder device; (e) Unidose nasal device.

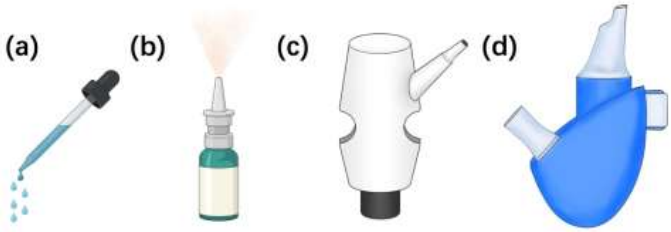

Figure 4 Nasal biopharmaceutical processes involved during NTB delivery.

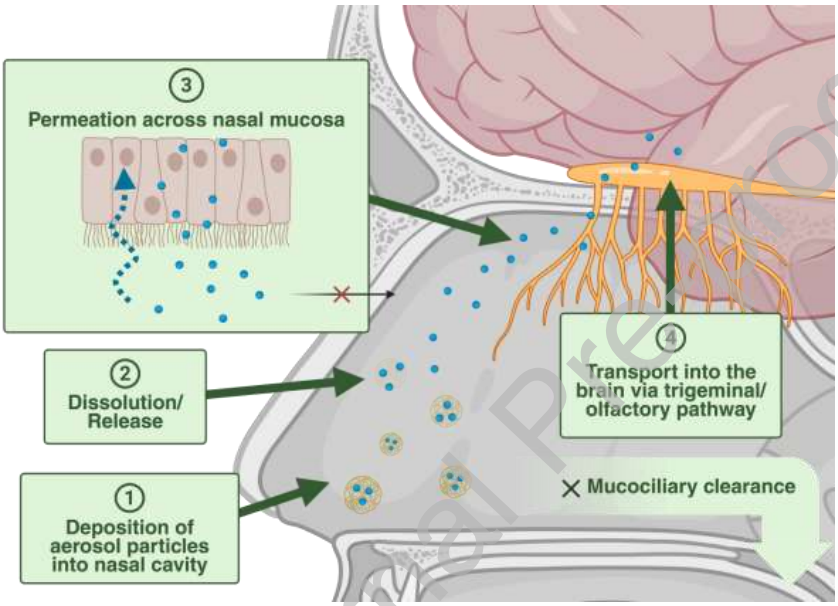

Figure 5 Examples of *in vitro* anatomical models for predicting nasal regional deposition. (a) Koken nasal cast model; (b) AINI coupled with NGI.

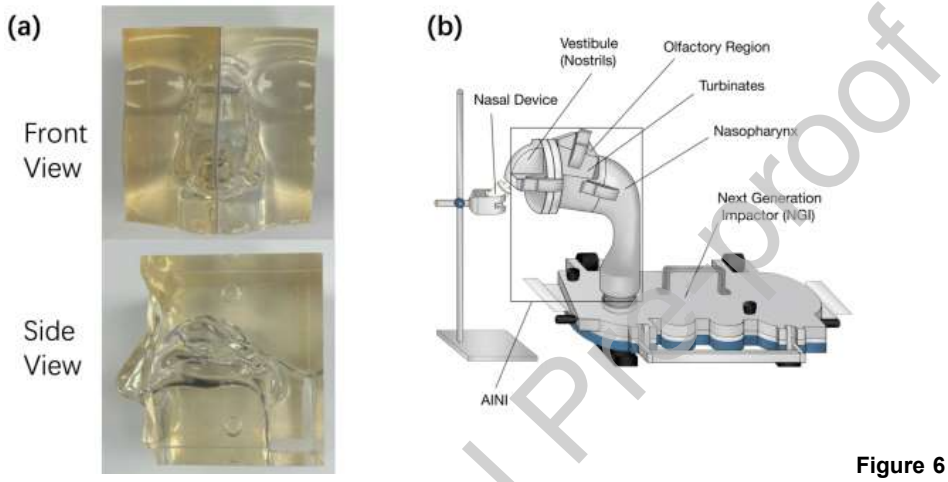

Figure 6 Examples of apparatuses used for *in vitro* evaluation of nasal drug permeation. (a) Vertical Franz diffusion cell; (b) horizontal Ussing chamber.

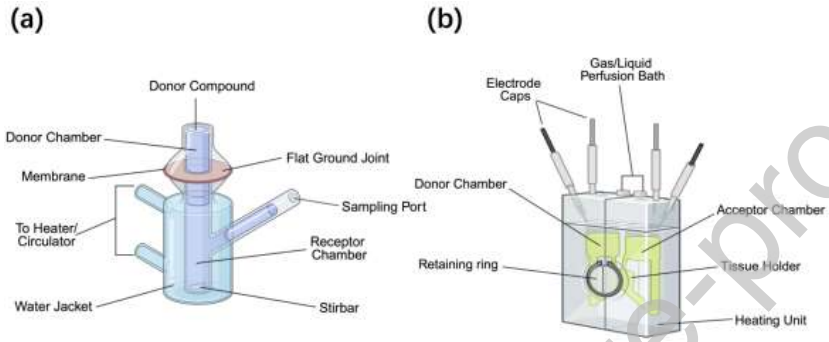

Figure 7 Examples of PBPK models for NTB drug delivery. (a) A typical multi-compartment DMPK model in rodents. ABS: Absorption compartment. Adapted from Stevens et al. with permission.[198] (b) An illustrative PBBM model. MCC: Mucociliary clearance. Adapted from Forbes et al. under CC-BY 4.0 license.[10]

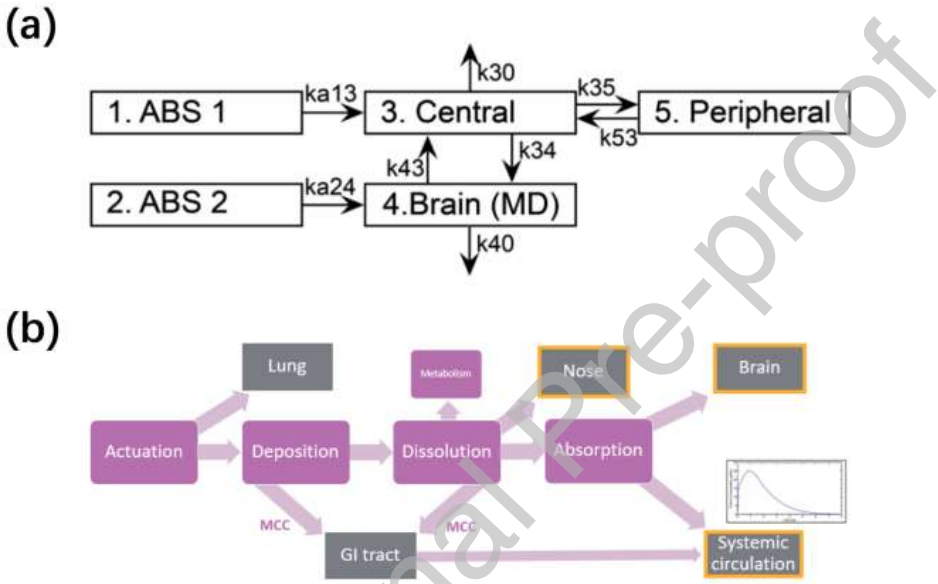

Table 1 Considerations during nasal formulation development for nose-to-brain delivery.[11, 16, 26]

Product characteristics	Characterization techniques	Therapeutic impacts	Recommended requirements

Liquid formulations (Solutions/suspensions)			
pH	pH meter	Biocompatibility with nasal tissue	4.5 – 6.5
Osmolality	Osmometer	Biocompatibility with nasal tissue	Preferably ~280 mOsmol/kg
		Influence on drug retention and absorption	Up to ~600 mOsmol/kg acceptable
Administration volume	N/A	Minimize dripping and patient discomfort	25 – 150 (max. 200) µL/nostril
Rheology	Rheometer	Enhance nasal retention without obstructing nasal airflow	Moderately viscous
Surface tension	Wilhelmy plate method	Enhance spray deposition in nasal cavity	Thixotropic behavior
Particle size (for suspensions/emulsions)	Dynamic light scattering Laser diffraction	Apparent solubility enhancement, nasal permeation, olfactory drug transport	<100 nm
Surface charge (for suspensions/emulsions)	Electrophoretic light scattering Zeta particle tracking analysis	Mucoadhesive properties	+ve surface charge (interaction with -ve-charged mucin)
Semi-solid formulations (Nasal gels)			

Gelling temperature	Oscillatory rheometers and temperature ramp tests	Enhance nasal retention	Gelling temperature: ~34°C (Above room temperature but below nasal cavity temperature)
Gelation time	Visual observation	Influence on drug retention Formulation uniformity	Approximately <30 seconds
Gel strength	Oscillatory rheometers	Enhance nasal retention	~5000 – 10000 Pa
Viscosity	Rheology	Enhance nasal retention	0.1 – 10 Pa·s
Mucoadhesion	Tensile tests Flow-through methods	Enhance nasal retention and permeation	~1200 – 9400 dyne/cm^2
Swelling ability	Immersion in simulated nasal fluid	Enhance nasal retention Prevent clotting	Moderate swelling ability
		Powder formulations	
pH (after powder dissolution)	pH meter	Biocompatibility with nasal tissue	4.5 – 6.5
Administered dose	N/A	Patient comfort	< 25 mg/nostril
Flowability	Angle of repose Carr's compressibility index	Consistent powder dispersion and fluidization	Hausner Ratio < 1.2 Carr's compressibility index <

	Shear cell testing Hausner Ratio	Smooth filling during manufacturing	15%
Moisture content	Thermogravimetric analysis Karl-Fischer titration	Powder dispersion, flowability and stability	~1 – 2 %
Particle morphology	Scanning electron microscopy	Higher surface area may be beneficial for mucoadhesion and rapid dissolution	Higher surface area
Solid-state form	Powder X-ray diffraction Differential scanning calorimetry	Amorphous powders may result in increased dissolution rate and more rapid absorption	Amorphous powders
Particle size	Aerodynamic particle sizer Laser Diffraction	Enhance deposition in nasal cavity Reduce lung exposure	10-45 μm

Table 2 Functions and examples of excipients used in nose-to-brain delivery.[13, 15, 17-20]

Excipient type	Function	Examples
Mucoadhesive agents	Enhance mucoadhesion and prolong n nasal drug residence	Cellulose Derivatives; Polyacrylates; Starch; Chitosan; Lectins; Thiolated polymers
Permeation/absorption enhancer	Enhance nasal mucosal drug permeability	Chitosan; Surfactants (e.g., Polysorbates, Bile Salts); Cyclodextrins; Fatty Acids (e.g., Oleic Acid)
Enzyme inhibitors	Reduce nasal enzymatic clearance	Boroleucine; Bestatin; Amastatin; Phospholipids; Fusidic Acids
Fillers	Increase the bulk weight of nasal powders	Microcrystalline Cellulose; Mannitol
Preservatives	Prevent microbial contamination	Benzalkonium Chloride; Benzyl Alcohol

Table 3 Advantages and limitations of characterization models for assessing drug deposition profile within the nasal cavity.[10, 79, 93, 100-102]

Models	Advantages	Limitations
In silico CFD simulations	● Customized to various geometries to suit specific patient populations ● Flexible tuning of testing parameters (e.g., spray angle, spray flow rate) ● Eliminate reliance on laboratory materials and animal studies	● Increased computational costs with finer meshes ● Limited applicability to nasal powders ● Expertise required to develop the simulation procedure ● Limited IVIVC data
In vitro anatomical models	● Suitable for evaluating various nasal dosage forms (including nasal powders) ● Commercially available ● Fast screening tool	● Coating procedure required ● Some nasal casts have poor IVIVC

Table 4 Advantages and limitations of methods for determining/predicting human nasal residence time.[114-116, 119-127]

Models	Advantages	Limitations
In vivo gamma scintigraphy in humans	● Direct evaluation of total nasal drug clearance	● Resource-intensive due to the use of radiolabeled materials ● Safety concerns associated with administration of radiolabeled materials to humans
In vivo particle tracing by dyes or saccharin taste	● Simpler and cheaper than gamma scintigraphy ● Good correlation between predicted mucociliary transport time with clearance half-life[192]	● Constant monitoring may be inconvenient for the human volunteer ● Patients may have a high taste threshold or do not taste the saccharin ● Saccharin taste tests cannot be performed consecutively (sweet taste disappears after ~4 hrs)
In vivo gamma scintigraphy or particle tracing in animals	● Less costly and resource-intensive compared to clinical studies	● Ethical concerns ● May misestimate human mucociliary clearance due to inter-species physiological differences
Ex vivo mucoadhesion evaluation techniques (e.g., “falling liquid film”, “wash-off” and texture analyses techniques)	● Reduce the use of laboratory animals	● Lack of comparative data between techniques ● Cannot account for other processes of mucociliary clearance (e.g., cilia beating or movement of the mucus blanket) ● Lack of reproducibility and comparability due to tissue variability
In vitro evaluation techniques	● Eliminate the use of animals	● Complementary use of multiple techniques

(e.g., rheology measurements, quantification of mucin-bound particles)	• High reproducibility	necessary to ensure accuracy • Limited correlation data with *in vivo* drug residence time

Table 5 Advantages and limitations of characterization models for assessing nasal drug permeability.[136-140, 144-150, 164-166]

Models	Advantages	Limitations
In vitro cell-based models	● Cost-effective for immortalized cells ● Primary nasal airway epithelial models are commercially available ● Higher data reproducibility	● High cost (for commercial models, e.g., MucilAir™) ● Finite passage capacity for primary cells ● Cannot retain all organotypic properties of the nasal epithelium
Ex vivo nasal mucosa models	● Retain the nasal epithelial architecture and organotypic properties	● Lack of reproducibility and comparability due to tissue variability ● Ethical concerns ● May be difficult to access fresh excised tissue
Non-cell or tissue-based models	● Time-efficient ● No need of training in cell culture techniques	● Cannot account for active transport processes

Table 6 Comparison of MucilAir™, RPMI 2650 cells, and Calu-3 cells as *in vitro* cell-based permeability models.[128, 152, 156, 158-160]

Name	Origin	Cell type	Layer structure	Transporter functional expression	Incubation period under air-liquid interface culture before experimentation[a]	TEER values under air-liquid interface culture[b]
RPMI 2650 cells	Human nasal squamous cell carcinoma	Immortalized cell	Leaky multilayers	/	Long incubation time (around 3 weeks)	~41 – 270 Ωcm^2
Calu-3 cells	Human lung adenocarcinoma	Immortalized cell	Tighter monolayers with mucus secretion (Ciliogenesis has been reported but inconsistently demonstrated)	P-gp	Long incubation time (around 3 weeks)	~300 – 500 Ωcm^2
MucilAir™	Human nasal tissue	Primary cells (Mix of basal cells, goblet cells, ciliated cells)	Tight multilayers with mucus secretion and cilia	P-gp BCRP	Ready to use	~316 – 650 Ωcm^2

[a] The incubation period is influenced by multiple parameters, such as cell seeding density and cell passage number.

[b] TEER values are influenced by multiple parameters, such as cell seeding density, incubation duration, and cell passage number.

Table 7 Interspecies comparison of nasal anatomical and functional parameters.[182-191]

Species		Nasal volume (mL)	Surface area (cm^2)	Clearance half-life (min)	Administered volume per nostril (μL)	Nasal length (cm)	Fraction of air passing through olfactory region (%) [a]	Fraction of nasal surface area covered by olfactory mucosa (%)
Human		20	160	15	150	7.5	3	3
Dog		20	221	20	207	10	15	30
Monkey		8	62	10	58	5.3	9	5
Rabbit		6	61	10	58	5.2	47.4	15.4[b]; 15.8[c]
Rodent	Mouse	0.03	2.8	1	3	0.5	/	45-50
	Rat	0.4	14	5	13	2.3	20	

[a] At a state of resting breathing

[b] For the left nasal passage

[c] For the right nasal passage

Table 8 Potential inputs for PBBM models (**Fig. 5**) and their typical characterization methods and data ranges. Adapted with modifications from Forbes et al. under CC-BY 4.0 license.[10]

Model Input	Measurement method	Typical range/units
Regional deposition	CFD modelling, *in vitro* nasal casts and gamma scintigraphy	% of dose per region (e.g., olfactory region, turbinates, vestibule and nasopharynx for AINI)
Dissolution under volume-limited conditions	Simulated nasal fluid dissolution in e.g., Transwell systems or Franz dissolution cells	% dissolved with time (mins to hrs)
Mucus thickness/viscoelasticity	Imaging (confocal microscopy), histology, rheology, microrheology	5 – 20 µm, 1 – 1000 cP
Absorption/permeation	Cell models (e.g., RPMI 2650, Calu-3, MucilAir) and *ex vivo* nasal tissue	10^{-6} to 10^{-3} cm/s
Mucociliary clearance half-life	Gamma scintigraphy and *in vivo* studies	15 – 30 min (varies by region)
Enzymatic degradation (e.g., esterases)	Biochemical assays and LC-MS/MS profiling	Half-life: Mins to hrs Enzymatic activity varies by region

Graphical Abstract

Declaration of Interest Statement

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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