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Review

Surface-modified nanocarriers for nose-to-brain delivery: from bioadhesion to targeting

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Abstract: In the field of nasal delivery, one of the most fascinating applications is the delivery of drugs directly to the central nervous system bypassing the blood brain barrier. This approach would provide a series of benefits, such as dose lowering and direct targeting of potent drugs, ultimately reducing their systemic side effects. Recently, clinical trials have explored the nasal administration of insulin for the treatment of Alzheimer's disease, with promising results. The use of nanomedicines could provide further options for making nose-to-brain delivery reality. In particular, apart from the selection of devices able to deposit the formulation in the upper part of the nose, surface modification of these nanomedicines appears the key strategy to optimize the delivery of drugs from the nasal cavity to the brain. In this review, nanomedicines delivery approaches based on surface electrostatic charges, mucoadhesive polymers, as well as chemical moieties targeting nasal epithelium, will be discussed and critically evaluated for nose-to-brain delivery.

Keywords: Nose to brain delivery; Nanoparticles; Pharmaceutical nanotechnology; Mucoadhesion; Mucus penetrating particles; Targeting; CNS disorders; Neurodegenerative diseases; Alzheimer's disease; Parkinson's disease.

1. Pharmaceutical Nanotechnologies for Nose-to-Brain Delivery

Among routes of drug administration that may represent an alternative to the parenteral and oral ones, nasal delivery has undoubtedly received less attention compared to others, such as the pulmonary or transdermal delivery routes. Conventionally, nasal drug administration has been associated to the treatment of minor local ailments such as rhinorrhea, nasal congestion, nasal infections and allergic or chronic rhinosinusitis [1]. However, nasal delivery shows a high number of clear advantages, such as ease of administration, non-invasiveness, good patient compliance, rapid onset of action, relatively large and permeable absorption surface, reduced enzymatic activity and avoidance of hepatic first-pass metabolism. Therefore, an increasing number of products exploiting

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the nose as site of administration for the systemic delivery of small and large molecules (including peptides, proteins and vaccines) are being developed and are reaching the market. In this context, nasal delivery is one of the most versatile routes of administration with applications going from smoke cessation (nicotine, Nicotrol® NS, Pfizer, USA) to flu vaccination (live attenuated influenza vaccine, FluMist® Quadrivalent, Astra Zeneca, USA), from pain management (fentanyl, Intstanyl®, Takeda, Japan and Pecfent/Lazanda®, Archimedes Pharma Ltd., UK; butorphanol tartrate spray, Mylan Inc., USA) to postmenopausal osteoporosis (salmon calcitonin, Fortical®, Upsher-Smith, USA), from the treatment of migrane (zolmitriptan, Zomig®, AstraZeneca, UK; sumatriptan, Imigran, GSK, UK and Onzetra™ Xsail™, Avanir Pharmaceuticals, USA) to that of endometriosis (nafarelin, Synarel®, Pfizer, USA) or prostate cancer (buserelin, Suprecur®, Sanofi-Aventis, France) [2].

Seemingly, however, the best is yet to come, as the nasal cavity offers a unique opportunity for the delivery of pharmaceutically active ingredients (APIs) to the central nervous system (CNS). Considering the increasing incidence of brain diseases and neurological disorders associated with the aging population, achieving efficient drug delivery to the brain is one of the priorities of modern pharmaceutical sciences. However, brain delivery of drugs is a complex challenge, as the CNS is protected by the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB), two formidable structures providing a selective brain permeability to circulating molecules. These physical, metabolic and transporter-regulated barriers tremendously limit the number of pharmacologically active substances able to access the CNS at therapeutic concentrations [3]. Several approaches have been proposed to improve brain delivery across BBB [3,4], including nanoparticulate drug carriers targeting specific transporters present on the BBB [5-7]. Unfortunately, the percentage of injected drug dose reaching the brain even with BBB targeting or permeation enhancing strategies is below 5%, typically less than 1%, with the remaining 95-99% of the drug off-target and potentially responsible for systemic side effects. Furthermore, in the case of nanocarriers, the CNS chronic toxicity and immunogenicity of polymers, surfactants and other components must be carefully evaluated, especially considering the prolonged treatments required for CNS diseases [8].

An increasing number of studies suggests that intranasal drug delivery enables brain delivery of both small and large molecules actually bypassing the BBB via the nerves present in the nasal cavity, *i.e.* the olfactory and trigeminal nerves. In particular the olfactory 'neuroepithelium', present exclusively in the nasal cavity, is the only part of the CNS that is in direct contact with the external environment. Consequently, it is a unique access port to the brain [9]. On the other hand the trigeminal nerve has been demonstrated to be significantly involved in the nose-to-brain (N2B) delivery of certain substances, especially towards the posterior region of the brain [10,11]. As a consequence, drugs can reach the CNS following nasal administration via three main pathways, namely: A) the olfactory nerve pathway, which innervates the olfactory epithelium of the nasal mucosa and terminates in the olfactory bulb, B) the trigeminal nerve pathway, which innervates, through its ophthalmic and maxillary branches, both the respiratory and (to a lesser degree) the olfactory epithelium and terminates in the brainstem and olfactory bulb, respectively, and C) the vascular pathway. Among these, the olfactory and trigeminal nerve pathways provide brain delivery either via a slow intracellular axonal transport (hours or even days) or fast perineural paracellular transport (minutes) from the sub-mucosal space to the cerebrospinal fluid (CSF) compartment [12,13]. The vascular pathway provides a secondary, indirect mechanism of delivery, whereby the drug is firstly absorbed into systemic circulation and subsequently transported to the brain across the BBB [14]. Figure 1 outlines the nasal innervation and the three brain-targeting pathways of nasal delivery.

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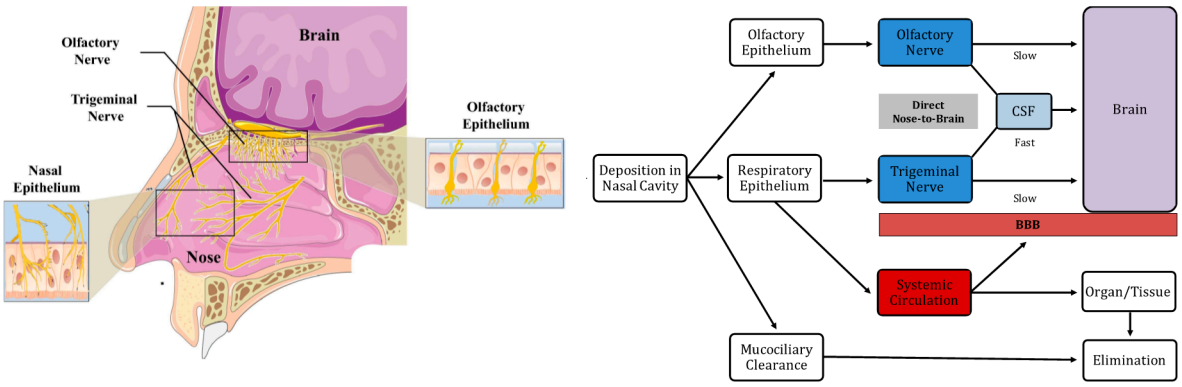

Figure 1. (**a**) Nasal innervation; (**b**) Nose-to-brain (N2B) pathways of drug delivery (modified from [15] and [16]).

Hence, nasal delivery has been proposed for the treatment of various central nervous system conditions, such as migraine [17], sleep disorders [18], viral infections [19], brain tumors [20,21], multiple sclerosis (MS) [22], schizophrenia [23], Parkinson's disease (PD) [24], Alzheimer's disease (AD) [25] and even for the treatment of obesity [26]. However, several limitations can be envisaged such as small nasal cavity volume, limited amount of formulation that can be administered, poor olfactory region deposition from conventional nasal devices, short mucosal contact time due to the mucociliary clearance, poor bioavailability of hydrophilic and/or large molecules, mucosal irritation and lack of validated translational animal models [27]. All these may negatively affect the potential of the nose-to-brain transport, to the point that some authors around the mid 2000's doubted whether such approach could be exploited therapeutically in humans [28,29].

Since then, a number of nasal devices specifically able to deposit nasal formulation in the olfactory region of the nasal cavity, such as ViaNase atomizer (Kurve Technologies, USA), pressurized Precision Olfactory Device (Impel Neuropharma, USA) and the liquid and powder Exhalation Delivery Systems (OptiNose, USA), have been designed and are now available for the development of new medicinal products [30].

Preclinical studies in animals increasingly use specific indexes to quantify the efficiency of brain delivery following administration, such as nose-to-brain drug targeting efficiency (DTE, Equation 1) and direct transport percentage (DTP, Equation 2) [31]. Drug targeting efficiency index provides the exposure of the brain to the drug after nasal administration relative to that obtained by systemic administration:

$$DTE = \frac{\left(AUC_{Brain}/AUC_{Blood}\right)_{IN}}{\left(AUC_{Brain}/AUC_{Blood}\right)_{IV}} \cdot 100, \quad (1)$$

where AUC_{Brain} and AUC_{Blood} are the area under the concentration *vs.* time curves of the drug in the brain and in the circulation (blood, plasma or serum) after intranasal (IN) and intravenous (IV) administration. DTE values can range from 0 to +∞, with values above 100% representing a more efficient brain targeting after IN administration *vs.* IV, whereas values below 100% indicate the opposite.

Direct transport percentage index calculates the estimated fraction of the dose reaching the brain via direct nose-to-brain pathways over the cumulative amount of drug reaching the brain after intranasal delivery:

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$$DTP = \frac{B_{IN} - B_x}{B_{IN}} \cdot 100, \quad (2)$$

where B_{IN} is the brain AUC following intranasal administration, and B_x is the fraction of the same AUC due to the drug crossing the BBB from systemic circulation calculated according to Equation 3:

$$B_x = \frac{B_{IV}}{P_{IV}} \cdot P_{IN}, \quad (3)$$

where P_{IN} and P_{IV} are the blood AUC after intranasal and intravenous administration, respectively. DTP positive values up to 100 indicate a contribution of the direct nose-to-brain pathways to brain drug levels, whereas DTP equal to 0 (or even negative) indicates a preferential brain accumulation after IV administration of the drug. This quantitative preclinical pharmacokinetics data associated with pharmacodynamics results allows for the creation of advanced translational PK am PK-PD models able to predict CNS concentrations in humans [32].

Furthermore, some clinical trials on brain delivery in humans of nasally delivered drugs, such as insulin for the treatment of Alzheimer's disease [33,34], oxytocin for autism [35], schizophrenia and major depressive disorder [36] and davunetide for mild cognitive impairment [37,38] and progressive supranuclear palsy [39], clearly demonstrate that the N2B delivery has been considered since 2012 a viable and promising clinical approach by several pharmaceutical companies [40].

Despite all these advancements, the delivery of drugs presenting unfavorable physico-chemical and biopharmaceutical characteristics such as rapid chemical or enzymatic degradation, poor solubility, low permeability and low potency requires a formulation able to promote the mechanisms of transport of the drug to the brain, without disrupting the structure and physiologic function of the nasal epithelium.

Pharmaceutical nanotechnologies appear as an ideal formulative strategy for the N2B delivery of these "problematic" substances, including peptide and proteins. In fact, nano-sized (1-1000 nm) drug delivery systems can:

- Protect the encapsulated drug from biological and/or chemical degradation;
- Increase apparent drug water-solubility;
- Enhance residence time at the site of absorption;
- Promote mucosal permeation and/or cellular internalization;
- Control the release kinetics of the encapsulated drug;
- Achieve targeted drug delivery through surface modification with specific ligands;
- Reduce drug distribution to non-target sites, minimizing systemic side effects.

All these features appear desirable for an efficient N2B delivery and are potentially critical to enable the therapeutic application of drugs that without a proper carrier would not reach the CNS at sufficient concentrations to elicit a pharmacological response.

Therefore, almost all types of pharmaceutical nanocarriers have been studied for nose-to-brain delivery, including and not limited to nanocrystals [41,42], micelles [43,44], liposomes [45], solid lipid nanoparticles (SLN) [46,47], nanostructured lipid carriers (NLC) [48,49], polymeric nanoparticles [50-52], albumin nanoparticles [53], gelatin nanoparticles [53], dendrimers[54], mesoporous silica nanoparticles [55], nanoemulsions [56].

In consequence of such an intense research activity in this field, several reviews have been published recently on the use of nanoparticles for the N2B delivery, covering both general [57-61] and specific disease [62,63] or vector-related topics [64-66]. Hence, the present review does not aim to provide an exhaustive report on the various applications of nanoparticles administered nasally

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allowing for a direct drug delivery to the brain. In contrast, it collects and appraises critically some facts and figures related to the leading strategies of nanoparticle design for nose-to-brain delivery. In particular, the review will focus on nanoparticles physico-chemical characteristics and surface modification with mucoadhesive, penetration enhancing or targeting moieties, able to affect and promote the brain delivery of therapeutically active substances.

2. Influence of Physico-chemical Properties in Nanoparticles Nose-to-Brain Delivery

Many papers have described enhanced delivery to the brain after nasal administration of nanoencapsulated drugs in comparison with simple drug solutions. However, few studies draw attention on the precise mechanism through which nanoparticles enhance drug transport to the brain. Different scenarios can be depicted, from the simplest one, where the nanocarriers just interact with the mucus layer and release the drug in the mucus or at the mucus/epithelial cell interface, to the most "challenging" that see the drug-loaded nanoparticles crossing the mucosal barrier, being uptaken by neurons and translocated along the axons of trigeminal and olfactory nerves to reach the brain, where the drug is released. In the middle, there is the possibility of nanoparticle uptake into the respiratory epithelium and/or through olfactory neuroepithelium, where the payload is released and then the drug diffuses along perineural spaces to reach the CNS. It is clear that the fate of particles depends on the physicochemical characteristics of the nanoparticles themselves. Indeed, composition, size, surface charge, shape and surface hydrophobicity/hydrophilicity have an impact on nanocarrier interaction with the biological environment. In the case of nose-to-brain delivery, these features influence the interaction with the mucus, the uptake in the epithelial and neuroepithelial cells, the translocation to the brain by diffusion along the axons and the release kinetics of the drug. In this context, the elucidation of the role of physicochemical properties of NP is essential to be able to design both efficient and safe carriers.

In order to clarify the role of NP characteristics such as particle size, surface charge, hydrophobicity on their fate, some authors have studied nanoparticles transport either *in vitro* across olfactory cells monolayer, *ex vivo* across excised nasal mucosa, or *in vivo* on rat /mouse models.

In a recent paper Gartziandia *et al*. [67] have compared the permeability of nanoparticles having different physico-chemical properties across rat olfactory mucosa primary cells monolayers. A fluorescent probe (DiR; 1-1'dioctadecyl-3,3,3',3' tetranethylindotricarbocyanine) was loaded to track the particles: previous studied demonstrated the absence of probe release in the transport buffer. The authors found significant differences in nanoparticle permeation as a function of the constituting material: nanostructured lipid carriers (NLC) penetrated to a higher extent compared to PLGA nanoparticles having the same zeta potential (-23 mV). The change of the surface potential of NLC from negative to positive by chitosan coating determined an almost 3 folds increase in the transcellular transport compared to the uncoated NLC. Finally, the surface functionalization using cell-penetrating peptides (in particular Tat) further enhanced nanoparticle transport. While the role of chitosan can be explained considering an electrostatic interaction with the negatively charged cells, the different performance observed deserves further investigation before attributing it to nanoparticle constituents, *i.e.* polymeric *vs.* lipid particles. Indeed, the particles analyzed had different size (*approx.* 100 nm for NLC and 220 nm for PLGA nanoparticles) and different surfactants were used for their preparation: while NLC were made using polysorbate 80 and poloxamer (*i.e*. PEG moieties could be found on NP surface), PVA was used as surfactant for PLGA nanoparticles. This can contribute to the differences found considering the mucus-penetrating properties of PEG (see also section 4.1) and the mucoadhesive properties of PVA-coated particles, reported to interact with mucus constituents by hydrogen bonding and/or hydrophobic interactions [68].

Musumeci and collaborators [69] prepared PLGA, PLA and chitosan nanoparticles using polysorbate 80 (Tween 80) as surfactant and rhodamine as fluorescent probe. They found a higher uptake in olfactory ensheathing cells (extracted from rat pups olfactory bulbs) for PLGA NP (132 nm, -15.8 mV) compared to chitosan (no surfactant, 181 nm, +34 mV) and PLA (152 nm, -30 mV)

nanoparticles. In this case, the higher uptake of PLGA nanoparticles compared to the others was explained by the authors considering the lower absolute superficial charge, but the presence of PEG moieties on PLGA and PLA particle surface could have also contributed to the obtained result. However, it is difficult to compare the data from the two previously cited studies since different cells were used. It is known that the type and the physiological status of the cell highly influence its behavior toward nanoparticle uptake [70].

Mistry *et al*. [71] adopted a more complex barrier, *i.e.* excised porcine olfactory epithelium mounted on Franz-type diffusion cells, to compare the behavior of carboxylate-modified fluorescent polystyrene nanoparticles measuring 20, 100 and 200 nm in size (ζ potential: approx. -42 mV) with surface-modified nanoparticles obtained by coating with chitosan (48, 163 or 276 nm; ζ potential approx. +30 mV) or polysorbate 80 (ζ potential approx. – 21 mV). None of the tested particles crossed the nasal epithelium after 90 minutes, but polysorbate 80-coated (PEGylated) particles penetrated deeper in the tissue compared to uncoated and chitosan-coated nanoparticles. On the other hand, the number of particles present at the epithelial surface was higher in case of chitosan coated particles, and histological images suggested a localization within the mucus layer. No clear trend was found concerning the influence of nanoparticle size on drug uptake into the tissue.

The same nanoparticles were also evaluated *in vivo* on a mice model [72]. 15 µl of formulation containing either 105 nm polystyrene nanoparticles (-42 V), 163 and 276 nm chitosan-coated NP (ζ potential +30 and +23 mV respectively) or 107 and 180 nm polysorbate 80 coated NP (ζ potential -21 and -24 mV respectively) were applied daily for 3 days. All nanoparticles were transported to some extent across the mucosa (both olfactory and respiratory) via a transcellular route. The presence of polysorbate 80 coating did not enhance tissue uptake as compared to the uncoated particles, despite the claimed mucus penetrating properties of PEG. The authors explained these results considering that the PEG chains were not covalently bound to the particles, and that a precise length and density of PEG chains is needed to obtain a relevant mucus penetrating effect [73,74]. A significant difference was found between 107 and 180 nm polysorbate 80 coated NP (ζ potential -21 and -24 mV respectively), and the lower accumulated amount measured for the bigger particles was attributed to a slower diffusion inside the mucus network. Chitosan-coated particles were mainly retained inside the mucus and lower amounts were found in the tissue in comparison with uncoated and polysorbate 80-coated nanoparticles. Despite the long application time (4 days) nanoparticles were never found in the olfactory bulb, regardless the size or the superficial properties.

Ahmad [75] studied the permanence of a nanoemulsion made with Labrafac®WL1349/Labrafac®CC and Solutol®HS15 in the nasal cavity of rats from 0.5 to 16 hours after the application of 100 µl of formulation. In particular, nanoemulsions with droplets of 80, 200, 500 and 900 nm (NE80, NE200, NE500 and NE 900) were compared. The droplets were tracked with environment-responsive probes giving a fluorescent signal when dispersed in the nanocarrier matrix that is quenched immediately after release. It was found that the smaller the droplet size, the higher the retention in the nasal cavity. The even longer retention obtained with positively charged chitosan-coated nanodroplets (size 108 nm) was attributed to the electrostatic interaction with the mucus and with the negatively charged epithelial membranes. Further evaluations were done with NE80, NE900 uncoated nanoemulsions and the chitosan-coated nanoemulsion 108 nm in size. After 1 h from the nasal instillation a large number of nanoemulsion droplets were present in the nasal mucosa, the highest signal was found for the chitosan-coated nanoemulsion, followed by NE80 and NE900 uncoated nanoemulsions. In the trigeminal nerve the translocation was size-dependent being ranked NE80 > Chitosan coated > NE900. Accumulation in the brain (and in particular in the olfactory bulb) was also assessed: only minimal numbers of NE droplets entered the brain after 1 h, and these were only visualized in case of particles with small size (NE80 and 108 nm, chitosan coated), in agreement with the *in vivo* results obtained by Mistry [72].

Differently, a recent paper reported significant brain accumulation of PLGA nanoparticles after nasal administration in rats. The authors prepared rhodamine-loaded PLGA nanoparticles (surfactant: polysorbate 80, size 118 nm, ζ potential -26 mV) and chitosan-PLGA nanoparticles (213nm, +69 mV) and analyzed the brain distribution of fluorescence after 8, 24 or 48 h from the

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intranasal administration. The results evidenced that both positively and negatively charged particles could reach the brain and were localized mainly in the cytosol of the neural cells. Different localization (caudal *vs.* rostral area) could be obtained by modifying the surface charge. A particle distribution as a function of the post-application time was also described, with slower brain uptake for positively charged particles, attributed to both mucus-nanoparticle interaction in the nasal cavity and a different nose-to-brain pathway. The authors hypothesized that the slower translocation of positive particles was due to an intra-neuronal pathway (trigeminal nerve), whereas the extra-neuronal pathway, relying on bulk flow transport, was responsible for the rapid transport of negatively charged particles. Despite the interesting results, no experimental evidence, with the exception of the different timing of brain appearance, supported the hypothesis and the involvement of different (systemic) pathways could not be excluded.

A specific brain distribution as a function of the properties of the nanocarriers was also described by Kanazawa *et al.* [76] by using peptide-based carriers. An arginine-rich oligopeptide (designed to have adhesiveness and transmissibility) was conjugated with either a hydrophobic moiety (stearic acid) or a hydrophilic one (PEG-PCL block copolymer) in order to obtain two stable micellar formulations. An Alexa-dextran complex (MW 10,000 Da) was used as fluorescent probe to asses biodistribution. The stearate-peptide and PEG-PCL-peptide micelles were 100 and 50 nm in size and had a ζ potential of +20 and +15 mV, respectively. After intranasal application in rats, the two carriers determined a much higher uptake in the nasal mucosa and in the brain as compared to Alexa-dextran alone. The hydrophobic stearate-peptide determined a markedly higher fluorescence in the nasal epithelium compared to the hydrophilic PEG-PCL-peptide, but a lower fluorescence in the trigeminal nerve. Additionally, when analyzing the intracerebral distribution pattern of Alexa (Figure 2) as a function of the time post-intranasal administration, a significant difference between the two carriers could be appreciated: hydrophobic stearate-peptide showed a strong fluorescence in the forebrain after 15 minutes, 30 minutes and 1 hour and no transport to the hindbrain was evidenced. In contrast, upon PEG-PCL-peptide administration, a spreading of the fluorescence after 30 minutes and 1 hour was evident, indicating a distribution of Alexa to the entire brain. This result, together with the higher trigeminal fluorescence, suggests that PEG-PCL-peptide nanocarriers penetrated across the nasal mucosa and transported the probe to both the olfactory bulb (forebrain) and to the hindbrain via the olfactory and trigeminal nerves.

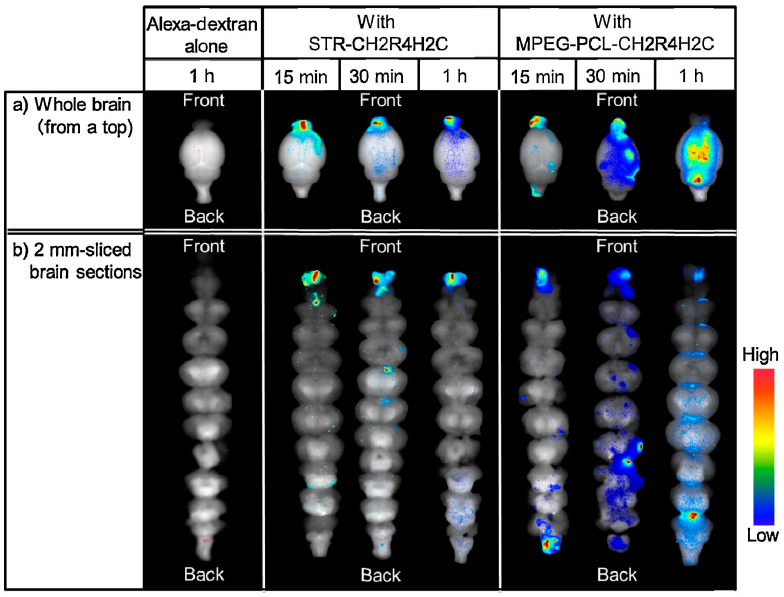

Figure 2. Dynamics of Alexa-dextran in brain tissue following NASAL administration of hydrophobic (STR-CH2R4H2C) and hydrophilic (MPEG-PCL CH2R4H2C) surface nanocarriers in: **(a)** Whole brain and **(b)** 2 mm-sliced brain sections (reprinted with permission from [76]).

Gabal *et al*. [77] evaluated the impact on N2B delivery of the nanoparticle surface charge by preparing anionic and cationic NLC having very similar size (175 nm and 160 nm respectively) and opposite ζ potential values (-34 and +34 mV respectively), encapsulating the same amount of drug (ropinirole HCl, EE% 53 and 50%, respectively) and characterized by a very similar release kinetics in vitro. These nanoparticles were dispersed in a thermosensitive gel made of poloxamers (407 and 188) and HPMC and administered intranasally in albino rats. Animals were sacrificed 3 to 360 minutes after the intranasal application and drug levels were measured in plasma and brain to calculate the pharmacokinetic parameters. Overall, there was no significant difference between negative and positive nanoparticles and both performed much better than a drug solution. However, the actual contribution of the nanocarrier is not clear since the improved bioavailability appears mainly due to the increased residence time given by the gel and/or to the presence of a penetration enhancer (sodium deoxycolate) in the nanoparticle formulation. Indeed, the authors also compared the toxicity of anionic NLC, cationic NLC and the nanocarriers dispersed in the gel after 10 µl application daily for 14 days in rats. The results highlighted the highest toxicity for cationic NLC, a lower toxicity for anionic NLC while no histopathological alterations were found in animals treated with gels loaded either with cationic or with anionic particles. The authors attributed this finding to a direct protective mechanism of poloxamer 188 against oxidative stress and inflammation. However, the hindered nanoparticle diffusion through the gel network and the consequent limited interaction between nanoparticles and epithelium may be considered the real reasons of the reduced toxicity evidenced.

The potential toxicity of nanoparticles on the mucosa and also to CNS structures is a critical point, related to the physicochemical properties, to be taken into account in nose-to-brain applications. In agreement with the above-mentioned results, also other authors described a higher toxicity of positively charged nanoparticles: chitosan-coated nanoparticles applied in a pH 6.0 citrate buffer vehicle had a size-dependent damaging effect on the excised porcine olfactory epithelium: 20 nm nanoparticles caused substantial tissue damage as compared to 100 and 200 nm nanoparticles [71]. This effect can be partially attributed to the buffer, but also to the high relative surface area, combined with the presence of the positive surface charge. However, it is worth mentioning that toxicity studies on animal models have evidenced a substantial safety of nanoparticles made or coated with chitosan [78] suggesting that *in vivo* the presence of a thicker mucus can reduce the toxic effect, probably by reducing the interaction between nanoparticles and epithelium.

Extensive nanotoxicological literature showed the capability of pollutants and metal nanoparticles to reach the brain parenchyma after nasal instillation or inhalation and, in some cases, to elicit toxic effect on the CNS [79-81]. A careful selection of the excipients used is thus mandatory and rapid and efficient biodegradation within the absorption tissue appears the best strategy to avoid unwanted accumulation and potential CNS toxicity of the innovative nanocarrier.

3. Mucoadhesive Nanoparticles

One of the physiological factors having a significant impact on nasal delivery and on N2B transport is mucociliary clearance. This physiological protection mechanism of the respiratory system allows for an efficient and rapid elimination of noxious substances, particulate matter and microorganisms trapped in the mucus layer (10-15 µm thick) during air intake (clearance $t_{1/2}$ 20 minutes in humans). However, this appreciably limits the residence time of substances administered inside the nasal cavity. As a consequence, traditional nasal formulations exploited excipients able to increase viscosity and/or provide bioadhesion, such as hydrophilic polymers, to reduce mucociliary clearance, prolong formulation residence time, improve systemic bioavailability and reduce nasal absorption variability [82]. The mucus lining the nasal epithelium is secreted by goblet cells present in the epithelium itself and mainly by submucosal glands present in the lamina propria. It is is composed of about 90-95% water, 2-5% mucins, 1% salts and variable amounts of cellular products and debris such as DNA, albumin, immunoglobulins, lysozyme, lactoferrin, and lipids [73,83]. In

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particular, the highly glycosylated proteins mucins (10-40 MDa) are the major determinant of mucus properties. These glycoproteins, because of their molecular weight, hydration, crosslinking and entanglement, confer to the nasal secretion viscosity and elasticity, typical of a non-Newtonian thixotropic gel. In addition, mucins present a high sialic acid and sulfate content, providing a strong negative charge to the polymer chains contributing to the rigidity of their networks [73]. Mucoadhesion has been described in the case of polymeric excipients as a combination of events necessary to explain the adhesion process: hydration of polymer chains, intimate contact with the mucus, diffusion and entanglement with mucin fibers, dynamic creation and disruption of labile bonds, such as disulfide bridges, electrostatic attractive forces, hydrophobic interactions, hydrogen and van der Waals bonds [84]. Several natural (gums, alginates, starch and gelatin), semisynthetic (cellulose derivatives, such as methyl-, hydroxylpropyl-, hydroxypropylmethyl- and carboxymethylcellulose) and fully synthetic polymers (polyacrylates, polymethacrylates, crospovidone) have been used to improve nasal delivery of drugs [83]. In the case of N2B transport, for example, chitosan and low molecular weight pectins were shown to prolong the residence time of nasal formulations in the olfactory region in man [85]. Sodium hyaluronate improved brain delivery of a high molecular weight hydrophilic model compound (fluorescein-labeled 4 kDa dextran) after nasal administration in rats [86].

When these polymers are main constituents or surface-modifiers of nanocarriers, the underlying adhesion mechanisms do not change, but the carrier's high surface-area-to-volume ratio translates into an extensive interface for more stable and prolonged interactions with the mucus compared to larger structures. Besides, size below 500 nm allows nanoparticles to fit in the low viscosity aqueous spaces left in-between the mesh of the mucins network, further enhancing the interaction with the mucus at molecular level. Finally, positive and hydrophobic surfaces may contribute to maximize nanoparticle adhesion towards the mucus, as a consequence of electrostatic attraction and hydrophobic interactions with mucins' negatively charged and hydrophobic domains, respectively [84]. Some surface modifications, however, reduce mucoadhesion, a characteristic exploited by mucus penetrating nanocarriers as discussed in next section of this review (Figure 3).

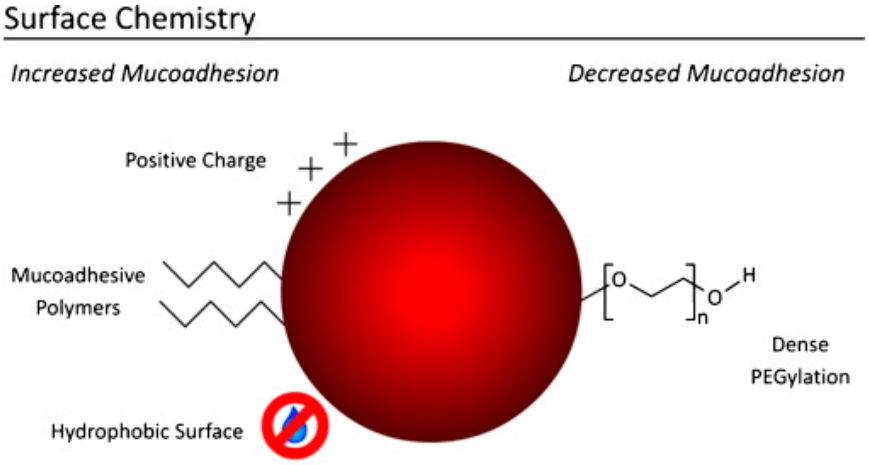

Figure 3. Surface chemistry of nanoparticles affecting mucoadhesion (modified with permission from [84]).

For the above reasons, mucoadhesive nanocarriers have been extensively studied for the N2B delivery of drugs.

Already in the year 2000, Betbeder and coworkers proposed Biovector™ nanoparticles for the nasal delivery of morphine to exploit the direct pathway between the olfactory mucosa and the CNS. These maltodextrin-based cationic nanoparticles surrounded by a phospholipid bilayer (average size 60 ± 15 nm) prolonged morphine antinociceptive activity when co-administered with the opioid drug in mice in comparison to morphine solution. Interestingly, the result was not replicated when

morphine was administered with a penetration enhancer such as sodium deoxycholate. Despite morphine was not actually encapsulated in the nanoparticles, it appears that nanoparticles improved specifically nose-to-brain transport of the opioid, while blood levels were not significantly affected. This was likely due to the interaction of positive nanoparticle with the nasal mucus layer that may have increased the formulation residence time and absorption from the olfactory region that is especially developed in mice [87].

In another approach, the group of Silvia Guterres and Adriana Pohlmann developed an amphiphilic methacrylic copolymer-functionalized poly(ε-caprolactone) nanocapsules as mucoadhesive system for the N2B delivery of the atypical antipsychotic drug, olanzapine [88]. Nanocapsules showed pH dependent size and surface charge values, ranging from 324.3 to 235.2 nm and from +55 and +22.7 mV. Nanocapsule/mucin interaction was demonstrated based on increase in particle size and reduction in nanoparticle concentration using Nanoparticle Tracking Analysis (NTA). The polymer mucoadhesion was tested both in terms of peak force and work of adhesion and of prolongation of olanzapine-loaded nanocapsules residence time on porcine nasal mucosa. *In vivo* studies performed in rats not only confirmed a 1.55 folds higher accumulation of olanzapine in the brain after nasal administration of mucoadhesive nanoparticles in comparison to a drug solution as control, but amphiphilic methacrylic copolymer-functionalized poly(ε-caprolactone) nanocapsules loaded with olanzapine outperformed the controls (drug solution and blank nanocapsules) also in the pre-pulse inhibition model of cognitive impairment symptoms typical of schizophrenia. Interestingly, the authors noted that brain accumulation was higher than those reported in literature for olanzapine-loaded PLGA nanoparticles and attributed the improvement to the cationic mucoadhesive coating of the nanocapsules [88].

Notwithstanding the possibilities provided by new synthetic polymers, polysaccharides have been amongst the most popular materials used to obtain mucoadhesive nanocarriers. In fact, polysaccharides appear an ideal choice for the production of nanoparticles to be delivered nasally, not only because of their mucoadhesive properties, but also for a number of unique characteristics, such as biomimetic mucosal recognition, well-documented biocompatibility and biodegradability and ease of chemical modification with targeting moieties. In this regard, polysaccharides can be incorporated into pharmaceutical nanocarriers in three ways: by absorption to preformed nanoparticles, by copolymerization or covalent grafting leading to surface modification or by directly manufacturing polysaccharide-based nanoparticles [89]. For example, albumin nanoparticles obtained by coacervation and thermal cross-linking, were prepared in presence of β-cyclodextrin derivatives in order to develop an innovative nasal drug delivery system for the anti-Alzheimer drug tacrine. The inclusion of β-cyclodextrin derivatives affected drug loading and modulated nanoparticle mucoadhesiveness. Finally, drug permeation behavior across sheep nasal mucosa from tacrine-loaded nanoparticles modified with β-cyclodextrin derivatives was increased compared to nanoparticles based on albumin alone, but it was lower than that obtained with tacrine solution. This can be explained considering the prolonged release of tacrine observed for nanoparticles [90].

Surface-engineered nanostructured lipid carriers coated with *Delonix regia* gum (DRG-NLC) as a natural mucoadhesive polymer were proposed for the efficient N2B delivery of ondansetron (OND), a centrally active drug used for the management of chemotherapy induced nausea and vomiting. The mucoadhesive formulation of OND was produced by high pressure homogenization using glycerol monostearate and Capryol® 90, as solid and liquid lipids, while soybean lecithin and poloxamer 188 were used as stabilizers. The nanostructured lipid carriers were subsequently coated by dispersing OND loaded nanoparticles in a DRG 0.75% w/v aqueous solution. The optimal nanoparticle formulation showed an average size of 92 nm, a polydispersity index (PDI) of 0.36, negative surface charge (-11 mV) and acceptable encapsulation efficiency (40% EE, 5.6% drug loading). Mucoadhesion was assessed *in vitro* by determining a percentage of binding efficiency between DRG-NLC and mucin (72%). *In vivo* studies carried out in rats showed a brain targeting efficiency (DTE) of 506% and a direct transport percentage (DTP) of 97% for the intranasal administration of OND-loaded DRG-NLC using as control the IV injection of a commercial OND IV formulation (Emeset®). This study, despite the remarkable results, lacked a nasal control (non-

mucoadhesive formulation) and did not clarify what was the impact of the presence of free gum, of non-encapsulated drug, as well as of the processing of nanoparticles (freeze-drying) on the *in vivo* brain distribution results obtained after DRG-NLC nasal delivery [91].

Alginate nanoparticles were produced for the N2B delivery of venlafaxine, a serotonin and norepinephrine reuptake inhibitor, to treat depression. Alginate nanoparticles were prepared by ionotropic gelation with calcium ions and subsequent cross-linking with a polycation, such as low molecular weight chitosan glutamate. Optimized nanoparticles showed an average size slightly below 175 nm and positive surface charge (+37.4 mV), PDI 0.391, with a high encapsulation efficiency (81%) and a drug loading of nearly 27%. Mucoadhesion was not studied directly, but *ex vivo* permeation through porcine nasal mucosa was found to be more than double over 24 hours, when comparing the venlafaxine-loaded alginate nanoparticles to a drug solution. Forced swimming test and locomotor activity test were used as behavioral test to assess the efficacy of the nasal administration of the antidepressant nanoformulation using as controls the drug solution administered intranasally and a suspension, obtained by crushing commercial tablets, administered orally. The alginate nanoparticle formulation performed better than the other formulations in the treatment of depressed animals, even if climbing and immobility parameters were not restored to levels of naive animals. It has also to be noted that, considering the volume of nanoparticle suspension administered nasally (100 µl), the possible inhalation or swallowing of part of the nanoformulation could not be excluded. This would eventually provide confounding effects to the final results. In any case, pharmacokinetics studies conducted using venlafaxine IV administration as reference and venlafaxine solution administered nasally as control, showed reduced blood levels and increased brain concentration for the antidepressant drug formulated in alginate nanoparticles. In particular, DTE and DTP calculated for the venlafaxine-loaded nanocarrier were 426% and 76%, respectively. An increase in absorption as a consequence of reduced nasal mucociliary clearance, enhanced mucosal permeation and the modulation of P-gp efflux transporters are some of the factors explaining the pharmacokinetics data [92]. It is remarkable, however, that the nanoparticle formulation increased the DTP value only from 63 to 76%, when compared to the nasal solution and that a significant contribute to the DTE increase came from BBB crossing of the drug after systemic absorption. This is not unexpected, considering that the antidepressant drug studied is already marketed as immediate and controlled release tablets and can cross the BBB. As such, the real improvement of the N2B delivery will consist mainly in the reduction of systemic side effects of the drug.

Among polysaccharides, chitosan, the glucosamine and N-acetyl glucosamine co-polymer obtained by the deacetylation of chitin, has been demonstrated one of the most promising and versatile materials for N2B delivery. In fact, chitosan not only is biocompatible, biodegradable, mucoadhesive and positively charged at nasal slightly acidic pH, but is also an efficient permeation enhancer able to transiently open the tight junctions of epithelial cells in mucosal tissues [78,93]. Interestingly this feature was demonstrated to be retained when chitosan was used as main constituent or surface coating for nanocarriers [84,94].

Wang and co-workers loaded estradiol in chitosan nanoparticles produced by ionotropic gelation for nasal administration in view of the treatment of Alzheimer's disease. Chitosan nanoparticles were obtained using tripolyphosphate anions (TPP) and chitosan with Mw 50,000 Da in order to obtain nanoparticles with particle size about 270 nm with positive surface charge (+25 mV). Encapsulation efficiency was around 60% (estradiol concentration 1.9 mg/ml). Smaller (below 100 nm) or larger (500 nm) nanoparticles could be obtained with Mw 6000 Da and 200,000 Da chitosan, respectively, but they were not considered suitable for the application. *In vivo* studies in rats showed a DTE of 320% and DTP of 68% by measuring estradiol concentration in cerebrospinal fluid (CSF) after IN or IV administration of the estradiol-loaded chitosan nanoparticles. The results were explained by the ability of chitosan nanoparticle to bind mucins and the improvement of paracellular transport [95]. However, in these experiments the nasal cavity was isolated from the respiratory and gastrointestinal tracts, a surgical practice reducing the likelihood of interfering absorption from other organs but altering retention time and hence absorption from the nasal mucosa. In addition,

paracellular transport is not expected to affect significantly the mucosal permeation of highly lipophilic steroid hormones like estradiol.

Similar chitosan nanoparticles obtained by ionotropic gelation with TPP have been proposed by several authors for the for the N2B delivery of very different drugs: rivastigmine (size 164 nm, PDI 0.42, EE% 85; DTE 355%, DTP 71%) [96] and thymoquinone (size 103 nm, PDI 0.40, EE%; DTE 3321% DTP 97%)[97] for Alzheimer's disease, bromocriptine (size 161 nm, PDI 0.44, EE% 84; DTE 630%, DTP 84%) [98], ropinirole (size 171 nm, PDI 0.39, EE% 70; superior brain/blood ratio compared to IN solution in γ-scintigraphy studies) [99], rasagiline (size 151 nm, PDI 0.38, EE% 96; DTE 325%, DTP 69%) [100] and pramipexole (size 292 nm, PDI 0.29, EE% 93; superior to IN and oral solution in pharmacodynamics studies) [101] for Parkinson's disease, tapentadol (size 201 nm, PDI 0.20, EE%; DTE 321%, DTP 69%) for chronic pain management [102]. Despite the different physico-chemical characteristics of the studied drugs, the results shared striking similarities.

Chitosan presents a number of desirable features but also some limitations, for example it is insoluble at physiologic pH and it is protonated only in acidic conditions, features potentially interfering with bioadhesion. Hence, several authors adopted nanocarriers based on chitosan derivatives. Trimethyl chitosan (TMC), for example is a water soluble permanently positively charged chitosan derivative and was used to encapsulate the analgesic neurotransmitter leucine-enkefaline (Leu-Enk) by ionotropic gelation. TMC nanoparticles (size 443 nm, PDI 0.32, EE% 78) were able to increase 35 times the permeability of the peptide across porcine nasal mucosa and produced a significant increase of the antinociceptive effect in mice after nasal administration during the hot plate and the acetic acid induced writhing tests [103]. Thiolated chitosan can increase mucoadhesion via the formation of covalent bonds, such as disulphide bridges, between the thiol groups and mucus glycoproteins [104]. Cyclobenzaprine (CB) and tizanidine (TZ), two central acting muscle relaxant used for pain management, were loaded into thiolated chitosan nanoparticles obtained by gelation with sodium alginate and showed enhanced permeation and reduced toxicity in the RPMI2650 cell model of human nasal epithelium, increase brain uptake (DTE 2461 and 8523% , DTP 96 and 99% for CB and TZ, respectively) and significantly enhanced antinociceptive activity for both drugs when compared to non-thiolated nanoparticles administered nasally [105,106]. In another study, in which the antidepressant selegiline HCl was encapsulated in thiolated chitosan nanoparticles (215 nm, EE% 70), authors claimed improved anti-inflammatory and neuroprotective effects for the thiolated polysaccharide itself. In *in vivo* experiments in depression-induced rats, thiolated nanoparticles were found to be superior to control chitosan nanoparticles when administered at the same dose (10 mg/kg), while providing non-significant differences in the pharmacodynamics effect compared to controls when the dose administered was halved (5 mg/kg) [107]. In another research, Di Gioia and co-workers assessed the ability of chitosan derivative nanoparticles to deliver dopamine to the striatum. Glycol chitosan, a water-soluble derivative of chitosan, was used to manufacture nanoparticles by ionotropic gelation with TPP along with sulfobutylether-β-cyclodextrin to improve dopamine loading. When administered nasally as a single dose nanoparticles did not modify brain levels of the neurotransmitter, but repeated intranasal administration significantly increased dopamine levels in the ipsilateral striatum [108].

It is worth mentioning that in almost all these promising studies, the nanocarriers improved the performance of drugs that already showed N2B transport when administered nasally with traditional liquid formulations such solutions.

In alternative formulation approaches chitosan is used as a surface modifier of carriers made of other materials. The water soluble antipsychotic chlorpromazine HCl was loaded into chitosan grafted PLGA nanoparticles in view of nasal treatment of schizophrenia to provide brain targeting and sustained release of drug, decrease the dose and administration frequency and reduce side effects. PLGA nanoparticles were prepared with a combined self-assembly/nanoprecipitation method in presence of dextran sulphate, followed by the grafting of the chitosan on PLGA nanoparticles surface. The selected formulation, characterized by an average size 464 nm (PDI 0.19) and 37% encapsulation efficiency, showed good mucoadhesion on sheep nasal mucosa and provided a permeation of chlorpromazine of 9% over 4 hours, controlled by the release kinetics of the PLGA nanoparticles (40%

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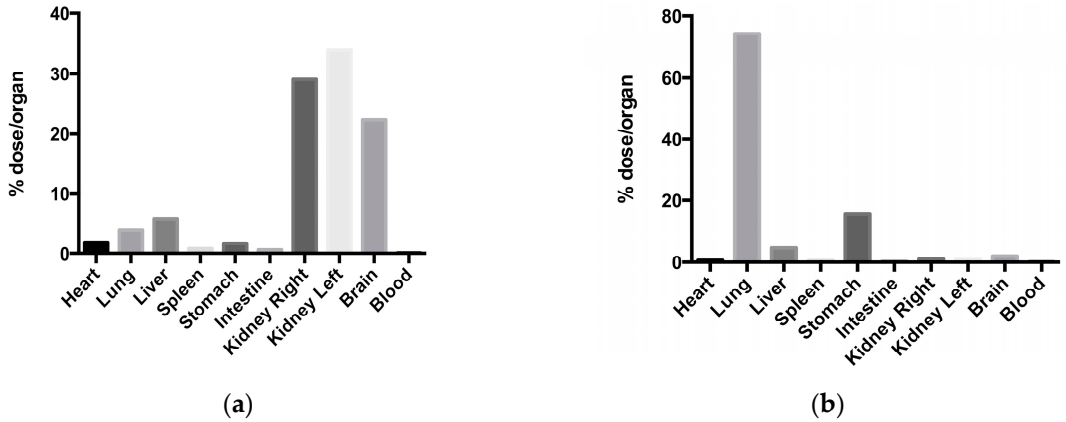
release over 48 hours) [109]. Liposomes coated with a chitosan derivative were proposed for the nasal administration of ghrelin. Ghrelin is a centrally acting peptide hormone able to stimulate food intake and for this reason a potential drug for the treatment of cachexia the wasting pathologic syndrome associated to some chronic diseases, such as cancers, hearth or kidneys failure. Ghrelin loaded liposomes were prepared by the lipid film re-hydration/extrusion technique followed by coating with N-([2-hydroxy-3-trimethylammonium]propyl) chitosan chloride (size 194 nm, PDI 0.19, EE% 56). The chitosan coated liposomes bound mucin more efficiently than uncoated anionic liposomes (63% *vs.* 40%) and improved permeation through a Calu-3 cell monolayer used as model of the upper airways epithelial barrier (10.8% *vs.* 3.6% anionic liposome *vs.* 0% free peptide) [110]. Finally, Clementino and co-workers developed hybrid chitosan/lipid nanocapsules for the N2B delivery of simvastatin. Statins have been suggested as potential neuroprotective drugs, in reason of their pleiotropic effects, *i.e.* anti-inflammatory, antioxidant and immunomodulatory actions [111,112]. The particles obtained by the self-assembly of a mixture of phospholipids and liquid lipids in presence of chitosan aqueous solution. The nanocapsules obtained not only showed small particle size (200 nm), positive surface charge and high encapsulation efficiency, but were shown to be efficiently biodegraded by enzymes present in nasal secretions such as lysozyme to provide a more efficient release of the drug at the deposition site on nasal mucosa. Gamma scintigraphy studies evidenced a significantly higher brain accumulation of the isotope (above 20% of the administered radioactivity) after IN administration of simvastatin loaded nanocapsules in rats in comparison to the IN administration of a ^{99m}Tc-labelled suspension of the statin (Figure 4) [113].



(**a**) (**b**)

Figure 4. Fraction of total radioactivity recovered per organ 90 minutes after the nasal administration in rats of (**a**) ^{99m}Tc-labelled simvastatin-loaded nanoparticles; (**b**) ^{99m}Tc-labelled simvastatin suspension [113].

In recent years, several studies have proposed the inclusion of nanoformulations, often micro/nanoemulsions, lipid or polymer nanoparticles, within mucoadhesive gels (xantan gum, chitosan) [114,115] or *in situ* gelling preparations obtained with thermosensitive (poloxamers 407) [116], pH sensitive (Carbopol 934) [117] and ion sensitive (gellan gum) [118,119] polymers. Even if the approach is seemingly appealing (combination of nanoparticle encapsulation/protection within viscous medium able to prolong residence time), the gel matrix viscosity might result in hindered nanoparticles migration, poor interaction with the nasal mucosa as well as low drug release. In addition, the superiority over more traditional formulation solutions is open to question.

In summary, mucoadhesive nanoparticles have been one of the most explored platforms for the N2B delivery of drugs. Results are supporting in all cases a superiority of the mucoadhesive nanoformulation over drug solutions administered nasally and/or intravenously, although a limited number of studies focus on direct comparison with potential competing formulation approaches such as liquid or even solid formulations containing mucoadhesive or permeation enhancing excipients.

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4. Beyond bioadhesion: mucus penetrating and penetration enhancing nanocarriers

4.1. Mucus penetrating nanocarriers

The popular mucoadhesive approach has been in recent years challenged by the evidence of the multiple barrier properties provided by the presence of the mucus layer. The mucus physiologic function relies on it acting as a dynamic semipermeable barrier via two major mechanisms working together: interaction filtering and size filtering (Figure 5) [120].

Interaction filtering occurs for molecules, supramolecular structures and particles, independently of their size. This phenomenon limits diffusion through the mucus via direct non-specific interactions, such as electrostatic, hydrogen and hydrophobic binding, with glycosylated, non-glycosylated regions of mucins as well as with lipid components of mucus. These interactions have been evidenced for charged and/or hydrophilic molecules, lipophilic drugs, peptides and proteins [121].

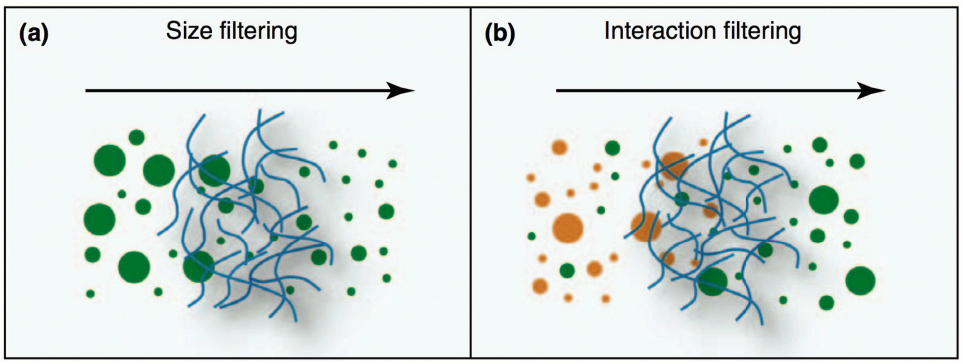

Figure 5. Major mechanisms hindering particles from diffusing through mucus: **(a)** Size filtering, only particles smaller than the mesh openings of the mucin fibers network are allowed to cross, whereas larger objects are blocked; **(b)** Interaction filtering, particles behavior is different according to surface properties: particles (orange) interacting strongly with the components of the mucus gel are trapped, whereas particles (green) showing weak interactions are allowed to cross (reproduced with permission from [121]).

Mucus is a dense molecular network with a characteristic mesh spacing that prevents larger particles diffusion through it. This mesh spacing has been reported to vary from 20 up to 200-500 nm from various authors [120,122].

Hence, it has been hypothesized that the use of nanocarriers with a sufficiently small size and coated with polymers that minimize interactions with mucins such as poly(ethylene) glycol (PEG) may increase mucus diffusivity and favor the close contact with the underlying epithelium. Interestingly, already in 2004 the group of Maria José Alonso observed that when fluorescent labelled PEG-PLA nanoparticles were administered intranasally in rats, the presence of PEG, smaller size (175 nm) and higher PEG coating density were the properties favoring the accumulation of particles within rat nasal mucosa [123]. Later, a group at John Hopkins University demonstrated using multiple particle tracking that 100 and 200 nm particles coated with high density of low MW PEG (2 or 5 kDa) were able to rapidly penetrate human respiratory [124] and chronic rhinosinusitis mucus [125] and designed mucus penetrating PEG-PLGA nanoparticles [126,127]. However, those results were not replicated in experiments in which PEG-coated magnetic nanoparticles despite showing decreased adhesion to mucus components failed to penetrate through native respiratory mucus even in presence of a magnetic field [128].

Diazepam and midazolam, two benzodiazepines in use for the treatment of *status epilepticus*, were encapsulated in PLGA nanoparticles coated with poloxamers 407 (Pluronic® F127), a block co-polymer surfactant previously reported to enhance mucus penetration of nanoparticles [125]. In both research works optimal particles showed size below 200 nm, moderately negative surface potential (ζ potential between -15 and -30 mV), controlled drug permeation kinetics across sheep nasal mucosa,

direct brain transport (DTP around 60%)and superior brain targeting efficiency compared to drug solution after nasal administration in rats (235-260 % *vs.* 125-160% DTE) [51,129]. In these papers, however, the authors did not claim or study the mucus penetration of the produced particles, but the nanocarrier characteristics match those of mucus penetrating carriers.

Recently, Sekerdag and collaborators proposed lipid/PEG-PLGA nanoparticles as mucus penetrating carriers of farnesylthiosalicylic acid (FTA) for a non-invasive nose-to-brain treatment of glioblastoma. The nanoparticles produced (size 163 nm, PDI 0.19, surface charge -12 mV, EE% >95%) were demonstrated after nasal administration to significantly reduce tumor volume (55% reduction as evidenced by MRI analysis) induced by implanting RG2 cells in the rat brain. The antitumor effect obtained was comparable to that obtained by nanoparticles administered intravenously. The biodistribution studies evidenced that the percentage of the dose attaining the brain was 0.04% ID/g for both formulations, but in the case of the nasal administration the amount of the dose accumulating in liver and spleen was significantly reduced, suggesting a superior safety profile for the nasal administration of the nanocarrier [130].

It is interesting to note that in some cases PEG-coated nanoparticles have been used as mucoadhesive carriers [131], but this is due to the fine tuning of the particle size and coating characteristic necessary to obtain particles able to slip through the mucus layer [132]. Furthermore, PEG coating has been associated with reduced interactions with epithelial cells and as a consequence reduced uptake, for the same exact reasons related to mucus penetration, *i.e.* reduced interactions with proteins and biomolecules [133,134]. It appears clear that mucus penetrating particles have not been yet sufficiently studied in nose-to-brain delivery and that more robust studies have to demonstrate the validity of the concept in terms of improved targeting of the CNS compared to other surface modification approaches.

4.2. Penetration enhancing nanocarriers

In alternative to mucus penetrating "stealth" particles, some authors have developed particles in which components can act as penetration enhancers. In reality, this classification is often superimposed to others (chitosan for example acts both as mucoadhesive agent and as penetration enhancer), but for the purpose of this review penetration enhancing particles will be considered those made of components, often surfactants, claimed to have the power to alter the barrier properties of the nasal mucosa.

For example, zolmitriptan and sumatriptan, two selective serotonin agonists in use for the treatment of acute migraine, were encapsulated into micelles composed of PEG 400, benzyl alcohol, Vitamin E TPGS, Pluronic® F127 and Trascutol P®. In particular, Transcutol P®, *i.e.* diethylene glycol monoethyl ether, and Vitamin E TPGS were used to solubilize and enhance the absorption of drugs through the nasal mucosa [135]. The micelles exhibited size below 25 nm, significantly increased triptan delivery to the brain (up to 3-7% of the administered dose) compared to IN or IV drug solution and did not show signs of local toxicity also after prolonged nasal administration in comparison to controls (28 days) [136,137].

Olanzapine-loaded nanocubic vesicles, obtained by incorporating the surface-active triblock copolymer poloxamer 188 (consisting of a hydrophobic polypropylene oxide block capped with hydrophilic polyethylene oxide moieties) in phosphatidylcholine bilayers, were compared to the corresponding liposomes in biodistribution studies in rats after nasal delivery. The nanocubic vesicles (size 363 nm, PDI 0.088, EE% 67) improved absolute bioavailability (37.9 *vs.* 14.9%) and DTE (100 *vs.* 80%) compared to the control liposomal formulation. The improvement was attributed to the presence of poloxamers in the formulation imparting both higher elasticity and penetration enhancing properties to vesicles [138]. In this study, one of the few using LC-MS/MS to quantify the drug in the biodistribution studies, the nanocarrier nasal delivery was not superior to the IV control in delivering the drug to the brain, as evidenced by the value of DTE. Also in the work of Albdelrahman and co-workers elasticity of spanlastics nanovesicles was claimed to be the mechanism improving the N2B delivery of the antipsychotic drug risperidone. Spanlastics vesicles, produced by

injecting a Span 60 and risperidone ethanol solution in a PVA aqueous solution (size 103 nm, PDI 0.34, EE% 64), exhibited relatively high Newtonian viscosity, improved permeation through *ex vivo* nasal sheep mucosa and improved brain accumulation of the drug in comparison to drug solution (DTE 469% *vs*. 217 %). However, DTP was superior for the nasal solution (55 % *vs*. 79 %) as spanlastics significantly improved systemic absorption of the drug [139].

Gelatin nanostructured lipid carriers were used to deliver to the brain the neurotrophic factor, basic fibroblast growth factor (bFGF), suggested protecting dopaminergic neurons in Parkinson's disease, via the non-invasive nasal route. The gelatin nanoparticles were prepared by a water-in-water emulsion in presence of poloxamer 188 and phospholipids, crosslinking with glyceraldehyde following by freeze-drying (size 172 nm, PDI 0.210, ζ-potential -38 mV, EE% 87). The nasal administration of the gelatin NLC significantly increased exogenous bFGF in olfactory bulb and striatum without influencing the integrity to nasal mucosa. The surface modified nanocarriers outperformed control gelatin nanoparticles also in studies with hemiparkinsonian rats inducing functional recovery after IN but not after IV administration. This was attributed to the presence of poloxamers 188 and its ability to reduce the barrier of mucus layer altering its viscosity and to enhance permeation by interacting and perturbing lipid membranes and/or modulating tight junctions [140]. Polysorbate 80 (Tween 80) was claimed to have similar effects in SLN loaded with rosmarinic acid obtained using glyceryl monostearate (GMS) and hydrogenated soy phosphatidylcholine. These particles were designed to manage the symptoms of Huntington's disease. Polysorbate 80 coated SLN improved behavioral abnormalities and attenuated the oxidative stress in 3-nitropropionic acid treated rats to greater extent that rosmarinic acid administered intranasally or the same nanoparticles injected intravenously [141].

Fatty acids have been traditionally indicated as absorption promoting agents in nasal delivery [142]. Recently, zolmitriptan has been formulated in novasomes, *i.e.* nanovesicular fatty acid enriched structures. The optimized novasomes formulated with a combination of Span® 80, cholesterol and stearic acid (size 150 nm, PDI 0.48, ζ-potential -38 mV, EE% 93), showed an enhanced brain accumulation (C_{max} 1.27 % ID/g) with a direct transport percentage of 99.2% when compared with the intravenous drug solution. The effects were attributed to the potential disruption of the nasal membrane and to the ability of these vesicles to "squeeze" themselves through the olfactory epithelium opening [143]. The actual contribution of this last mechanism to the nose-to-brain delivery is far from being supported by actual data demonstrating such ability to transfer across the nasal mucosa of elastic vesicles. Similarly, the occlusive effect claimed by other authors for nasally delivered alprazolam SLN appears highly unlikely [144].

In general, penetration enhancing nanocarriers studies lack of a fundamental control that is administration of a conventional formulation of the same drug containing the penetration enhancer alone. Only demonstrating a superior nose-to-brain delivery over this formulation of the use of nanocarriers would be fully warranted.

5. Targeting the Nasal Epithelium for Optimizing the Nose to Brain Delivery

Undoubtedly, one of the most fascinating aspects of the pharmaceutical nanotechnology is the so called "active" targeting of nanoparticles, *i.e.* the recognition of cells or tissues affected by the disease by means of surface ligands able to interact specifically with receptors and/or other biomolecules present on the biologic target. To deliver specifically the therapeutic dose of the drug where it has to act, avoiding side effects and with optimal therapeutic efficacy, has been the Grail Quest of pharmacotherapy since the dawn of modern pharmaceutical sciences [145].

As pointed out by an excellent paper by Alexander Florence, several obstacles (often underestimated or disregarded by scientist) are present *in vivo* for targeted nanocarriers (nanocarrier aggregation, premature drug release from the carrier, uptake by reticuloendothelial system, delivery off-target, degradation) and those have actually hindered the successful translation of targeted approach of drug delivery systems to clinical settings [146].

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In the case of nose-to-brain delivery the approach is interestingly not directed to the delivery of the particles themselves to a specific cell or receptor within the CNS, but to the interaction with cells of the nasal region more likely to favor the translocation to the brain, *i.e.* the olfactory epithelium. Several surface ligands have been proposed, but two strategies emerge as the most studied in recent years: lectins and cell penetrating peptides targeted nanocarriers.

5.1. Lectin-modified nanocarriers

Lectins are proteins or glycoproteins extracted from plants able to recognize and bind with high level of specificity glycan arrays of glycosylated lipids and proteins present on the surface of different cell types. For this reason, since 1988 they have been proposed as targeting ligands for drug delivery systems, initially in order to obtain absorption enhancement in the gastrointestinal tract [147]. However, the potential of lectins for nose-to-brain delivery has been demonstrated qualitatively and quantitatively in two seminal works by Broadwell and Balin [148] and Thorne *et al*. [149]. In those works, it has been demonstrated that wheat germ agglutinin (WGA)/horseradish peroxidase conjugate (62 kDa) can bind the cell surface of olfactory sensory cells, undergo adsorptive endocytosis and anterograde axonal transport and eventually accumulate in the olfactory bulb at concentrations (140 nM) more than 100 folds that those obtained by IV administration of the same conjugate and 700 folds those attained by the enzyme not conjugated to WGA. In fact, WGA obtained from *Triticum vulgare* specifically bind to N-acetyl-D-glucosamine and sialic acid residues abundantly present in nasal structures. As a consequence, WGA has been the most explored targeting ligand for nanocarriers for nose-to-brain delivery.

WGA was conjugated to PEG-PLA nanoparticles to obtain 85-90 nm targeted nanoparticles loaded with the fluorescent dye 6-coumarin. WGA targeted nanoparticles administered intranasally improved both blood (1.4 folds) and brain (2 folds) concentrations of the fluorescent dye when compared to plain PEG-PLA nanoparticles, without appreciable nasal ciliotoxicity [150]. Other studies in which WGA-conjugated PEG-PLA were radiolabelled with ^{125}I revealed that that nanoparticles after nasal administration in rats were rapidly (5-30 min) transported to the CNS via the extracellular pathway along olfactory and especially trigeminal nerves, while cerebrospinal fluid appeared to contribute to a small extent to the process [151].

The same group from the Fudan University of Shanghai (PR China) incorporate vasoactive intestinal peptide (VIP), a neuroprotective peptide potentially useful in a number of neurodegenerative disorders including AD in WGA conjugated PEG-PLA nanoparticles (size 100-120 nm, EE% 70, drug loading% 1.4). In biodistribution studies, 30-50% of radioactively labelled VIP was found in the CNS and WGA-conjugated targeted improved brain targeting (5.66-7.71 folds increase) compared to non-targeted PLA nanoparticles (3.57-4.74 folds increase) and control VIP solution administered nasally in mice. Furthermore, in an *in vivo* model of cholinergic impairment, VIP loaded WGA-NP induced improvement in spatial memory of rats at lower doses compared to non-targeted nanoparticles (12.5 *vs.* 25 μg/kg) [152]. In a subsequent work, WGA-targeted PEG-PLA nanoparticles were loaded with quantum dots in order to develop specific brain imaging agents for CNS diseases. Once again, the brain targeting capacity of the WGA was demonstrated with a relative fluorescence intensity detected three hours after nasal administration in mice (40 mg/kg, 5 μl each nostril) ranked as follows: brain ≥ lung > liver > kidney > heart > spleen (Figure 6) [153].

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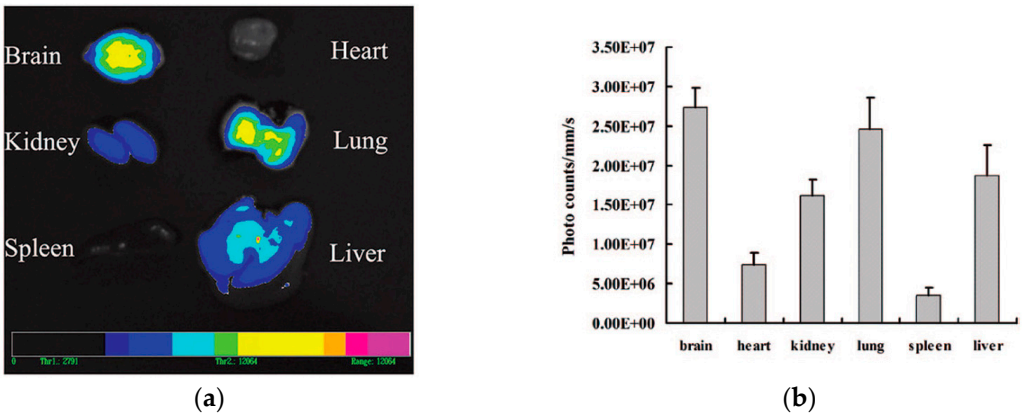

(**a**) (**b**)

Figure 6. Distribution of WGA-conjugated quantum dots-loaded nanoparticles in various organs following intranasal administration 3 h after dosing: **(a)** optical image **(b)** quantification of luminescence signals (adapted with permission from [153]. Copyright 2008 American Chemical Society).

PEG-PLA or PEG-PLGA nanoparticles were conjugated to other lectins, such as *Solanum tuberosum* lectin (STL), binding to N-acetylglucosamine [154], Ulex europeus agglutinin I (UEA I) and odorranalectin (OL), both binding to L-fucose largely present on the olfactory epithelium [155,156]. STL-functionalized PEG-PLGA nanoparticles loaded with haloperidol (size 132 nm, PDI 0.174, ζ-potential -14 mV, EE% 73, DL% 0.85) increased the brain tissue haloperidol concentrations after nasal administration by 1.5–3-fold compared to non-STL-functionalized particles and other routes of administration [157].

Since one of the major problems of lectins is their immunogenicity, being odorranalectin the smallest peptide with lectin-like activity, it was identified as a potential targeting ligand with reduced immunogenicity. OL-conjugated PEG-PLG nanoparticles were loaded with peptide urocortin reported to be able to restore nigrostriatal function in PD (size 115 nm, PDI 0.193, EE% 75, DL% 0.14). The *in vivo* studies in hemiparkinsonian rats evidenced a better improvement of the symptoms related to the dopaminergic lesion for OL-conjugated nanoparticles compared to control treatments and this was shown to be related to a partial but not complete recovery of monoamine neurotransmitters levels [158].

Despite this recent advancement, the use of lectins still faces criticism related to the potential toxicity and immunogenicity, making mandatory a toxicological assessment of the targeted carrier both systemically and locally towards the nasal epithelial surface and the CNS [159].

5.2. Cell penetrating peptides as surface ligands for targeted nanocarriers

Cell penetrating peptides (CPPs) are short cationic sequences of amino acids able to cross cell membranes and translocate to intracellular space similarly to their model, *i.e.* the HIV transactivator of transcription (Tat) protein [160]. CPPs have been shown to be unspecific to cell-type and able to translocate various cargos (small molecules, proteins, nucleic acids, nanocarriers) across different biological barriers such as intestinal wall, BBB, skin [161].

These characteristics make CPPs attractive as ligands for nose-to-brain delivery of nanocarriers. In the already cited study by Gartziandia, thw conjugation of CPPs (Tat or Penetratin) to the surface of polymer and especially of lipid-based nanoparticles improved their ability to cross an *in vitro* primary rat olfactory cell monolayer model [67]. In another study, PEG-PLA nanoparticles functionalized with low molecular weight protamine were found to accumulate in 16HBE14o- human bronchial epithelial cells at a greater extent than unmodified particles and after loading with the fluorescence dye coumarin-6 and nasal administration to rats increased fluorescence signal in various brain structures more than two folds compared to unmodified particles[162]. In a similar set of experiments, micelles obtained using Tat conjugated to methoxy poly(ethylene glycol)-poly(ε-

caprolactone) (mPEG-PCL) amphiphilic block copolymers and loaded with coumarin accumulated more than original micelles in C6 rat glioma cells and in rats bearing C6 tumors intracranially. In the latter *in vivo* experiment, no difference was found in fluorescence brain distribution 1h after nasal administration between micelles. However, after 4 hours, while fluorescence signal decreased for unmodified micelles, it increased in animals treated with Tat conjugated mPEG-PCL micelles, probably due to their greater ability to penetrate intracellularly [163]. In subsequent works, the same group from Tokyo University of Pharmacy and Life Sciences loaded the above-mentioned micelles with camptothecin in view of treating brain tumors and with small-interferin RNA (siRNA) for the suppression of genes involved in CNS disorders. Camptothecin-loaded Tat-mPEG-PCL micelles (size 88.5 nm, ζ-potential +10 mV, EE% 62) showed C6 higher cytotoxic effect on rat glioma cells *in vitro* compared to micelles not modified with the CPP and, more interestingly, after nasal administration prolonged survival of rats bearing intracranial C6 glioma (including 1 long term survivor) compared to the control micelles and drug solution (1.2 mg/kg, once-a-day for one week) [164]. In the case of siRNA, the nucleic acid was condensed with Tat-mPEG-PCL to obtain polyplexes of 50-100 nm. Brain distribution studies after nasal administration, carried out loading Alexa-dextran 10 kDa as model of siRNA in micelles, evidenced an increase in brain accumulation in comparison to controls (IV Tat-mPEG-PCL micelles or IN mPEG-PCL micelles) due to Tat-promoted permeation of the nasal mucosa and increased uptake within olfactory and trigeminal nerves [165].

Despite the impressive traslocation abilities, CPPs have some drawbacks such as sequestration in endosomes, limited intracellular localization profiles and lack efficient transport to the cytoplasm [160]. These drawbacks have prompted the development of different delivery approaches.

For example, the group of Paolo Giunchedi from University of Sassari designed a drug delivery system for N2B delivery of the complex of the 29-amino-acid peptide derived from the rabiesvirus glycoprotein (RVG) with siRNA interfering with the expression of BACE1, the β secretase responsible for the processing of amyloid precursor protein in β amyloid peptide, the main constituent of extracellular plaques hallmark of AD. The RVG-siRNA complex was loaded in SLN coated with chitosan and preliminary experiments showed an improved crossing of CaCo-2 cells monolayer by RVG-siRNA formulated in SLN and especially in chitosan-coated SLN, compared to the naked complex. In this approach, the mucoadhesive nanocarrier has to protect the complex and help it cross the nasal mucosal barrier to allow interaction of RVG cell penetrating peptide with acetylcholine receptors located at the trigeminal nerve ending and olfactory bulb. This approach has to still be validated with *in vivo* experiments [166].

5.3. Other targeting approaches

Several other ligands have been indicated to be potentially useful to enhance nose-to-brain delivery and in this section some examples are provided of the most interesting approaches alternative to lectins and cell penetrating peptides.

One of the early approaches for targeted drug delivery, suggested also for other administration routes, has been the use of viral vectors. Frenken and Solomon adopted filamentous bacteriophages as vector for the nasal administration of anti-β amyloid (Aβ) antibodies designed for the monitoring of amyloid plaques of living AD patients. The bacteriophage f88 was genetically engineered to encode on its surface protein III, a single-chain antibody constructed from variable sections of light and heavy chains of anti- Aβ IgM 508 antibody. After three daily nasal administrations of the filamentous bacteriophage vector, amyloid plaques were successfully targeted and visualized fluorescent-labeled antiphage antibodies in the olfactory bulb and the hippocampus region of transgenic mice carrying a double mutation in the amyloid precursor protein (APP). The crossing of the nasal barrier was attributed to the linear structure of the phage, previously demonstrated to provide penetration properties in various membranes. The lack of spreading to other brain sections suggested a transport via the olfactory neurons. The vector was proven inert and non-toxic, despite one of the possible drawbacks of viral vectors could be the trigger of immune defense mechanisms such as the activation

of microglia scavenger cells [167]. Interestingly, the approach is the object of a filed patent application [168].

Lactoferrin (Lf), a natural iron-binding cationic glycoprotein of the transferrin family, has been used as targeting ligand on the surface of nanocarriers as Lf receptor has been demonstrated to be highly expressed on the surface of respiratory epithelial cells as well as on neurons and brain endothelial cells [168]. For this reason, lactoferrin modified PEG-PCL nanoparticles were developed to enable brain delivery following intranasal administration of the neuroprotective NAP peptide, a fragment of the activity-dependent neuroprotective protein. Nanoparticles were prepared using emulsion/solvent evaporation technique followed by conjugation with thiolated Lf (size 88 nm, PDI 0.22, ζ-potential -24 mV, EE% 48, DL% 0.62). The Lf targeted nanoparticles not only increased brain accumulation more than two folds compared to unmodified nanoparticles, but showed improved neuroprotective effects in an AD model, *i.e.* mice intracerebroventricularly co-injected with ibotenic acid and β-amyloid$_{1-40}$, as shown by behavioral experiments such as the Morris water maze task. This was found to be related to an amelioration of the impaired cholinergic neurotransmission via a reduction in acetylcholinesterase activity and reduced depletion of choline acetyltransferase [169,170]. The same targeting strategy was successfully adopted for rotigotine-loaded PEG-PLGA nanoparticles for PD treatment [171] and for mPEG-PLA nanoparticles encapsulating α-asarone, a drug extracted from the traditional Chinese medicine herb *Acorus tatarinowii* Schott and recently proposed for the treatment of epilepsy [172].

PEG-PLA nanoparticles loaded with the analgesic peptide α-cobrotoxin were modified with OX26 antibodies to target transferrin receptors present in the BBB after nasal administration (size 96 nm, PDI 0.11, ζ-potential -33 mV, EE% 82). Results showed that brain delivery of the peptide labelled with fluorescein isothiocyanate was enhanced by the intranasal delivery of nanoparticles in comparison with intramuscular administration and that the enhancement was more pronounced in the case of antibody targeted nanoparticles. The peptide solution could barely penetrate the brain. Despite the authors attributed this to an appreciable capacity of the nanoparticles to cross the BBB, the actual mechanism of transport hypothesized, *i.e.* intact nanoparticles crossing the nasal mucosa, entering capillaries and finally crossing untouched the BBB, was not supported by clear experimental evidence [173].

Finally, in an interesting approach from the group of Rodney Ho of the University of Washington (USA) liposomes targeted with the integrin-targeting ligand Arg–Gly–Asp (RGD) were coupled with a pressurized olfactory drug (POD) delivery device to improve nose-to-brain administration of the analgesic opioid fentanyl. RGD can increase binding and enhance permeability across epithelial cells expressing $\alpha_v\beta_3$ integrins, thus fentanyl-loaded liposomes integrating the palmitoylated peptide (size 96 nm, PDI 0.11, EE% 80) were used to enhance residence time and absorption of the nasally administered opioid. The POD intranasal delivery device is a new device enabling preferential deposition of the aerosolized formulation on the olfactory region. Experiments demonstrated that RGD-conjugated liposomes could withstand the aerosolization with POD device without size change, phospholipid bilayer disruption or impairment of the targeting. Interestingly, when liposomes were administered to rats using POD device, fentanyl plasma concentrations as well as those measured in the brain 5 minutes post-administration were inferior (although not significantly different) to those obtained using free fentanyl. However, when the analgesic effect was measured, the fentanyl-loaded RGD liposomes provided a slightly slower onset of action and lower but more prolonged analgesic effect [174]. The decisions to combine the use of a device that is designed for an optimal deposition in human nasal cavity and the adoption of small animals for *in vivo* studies appear questionable. A larger animal model such as sheep would suit more the testing of nasal devices.

6. Future perspectives of nose-to-brain delivery with nanocarriers

Nose-to-brain delivery is a fascinating scientific topic. In the quest to achieve a non-invasive, efficient, safe and potentially disruptive innovation in the field of treatment of CNS disorders and

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brain diseases, the application of nanocarriers appears an asset, with several advantages but also few risks to be addressed early-on in the medicinal product development. Despite the promising results with several drugs, different materials and targeting approaches, to the best of our knowledge not a single one is actively developed by a pharmaceutical company to transfer the technology from the laboratory to the clinical stage. Several reasons are behind the lack of translational research successes for nanomedicines, some of which are related to the manufacturing scale-up, safety and quality challenges related to these non-biological complex medicinal products in general. Some reasons are however to be pinpointed in some shortcomings of the scientific works that in several cases highlighted the potential overlooking the weak points of the approach or the more pertinent controls necessary to demonstrate superiority over "traditional" formulation approaches. Furthermore, too often the drugs selected are already able to cross the BBB at some extent and/or produce CNS pharmacological effects, thus for these nanoencapsulation improve the only performance. Nanomedicines, especially in the case of a sensitive application such as the therapy of CNS conditions, are not required to *improve*, but to *enable* therapies that would not be possible without the application of nanoencapsulation [175].

For these reasons, in the planning of future nose-to-brain research protocols applying nanocarriers for the formulation of the drug, the rigorous and methodical pharmaceutical scientist should take in to account the following points in order to demonstrate the superiority of the designed nanomedicines and provide the data necessary for further development of a medicinal product:

- Select a potent drug with unfavorable physico-chemical characteristics for N2B;
- Design particles with biocompatible, biodegradable, GRAS materials;
- Adopt a robust, validated and up scalable fabrication method;
- Determine drug release from nanocarrier in biorelevant conditions;
- Establish early-on the safety and biodegradability pattern of the nanocarrier;
- If possible/advisable, adopt particles with size 100-400 nm, as smaller particles are more likely to enter the CNS with consequent concerns related to the nanotoxicology of those materials;
- Develop bioanalytical methods able to detect the drug instead of fluorescent or radioactive labels in biodistribution studies, if possible;
- Develop methods allowing to track the particles in the tissues in order to differentiate free drug and nanomaterial biodistribution;
- Carry out the *in vivo* experiments perfusing the organs before dissection, in order to eliminate blood contamination from the analytical quantitation;
- Establish the pharmacokinetics of free and nanoencapsulated drug, applying multiple and relevant controls (IV and IN administered solutions or formulations including absorption promoting excipients);
- Determine relevant parameters such as drug targeting efficiency (DTE) and direct transport percentage (DTP);
- Establish the therapeutic proof of concept through pharmacodynamics studies in a disease model as close as possible to the human condition;
- Combine PK and PD data to critically predict the feasibility of the treatment in terms of drug dose, amount of formulation to be administered, posology etc.;
- Select the candidate formulation for pre-clinical/clinical development.

In conclusion, nose-to-brain delivery evolved from a series of interesting observations looked upon with skepticism to a promising, although challenging, field of research. Nanomedicines appears to be a pivotal tool to enable the brain delivery of potent drug unable to cross the BBB and when used as such they will fulfill the potential demonstrated in the many scientific studies conducted so far.

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