The role of mucus as an invisible cloak to transepithelial drug delivery by surface-engineered nanoparticles

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Abstract

Mucosal administration of drugs and drug delivery systems has gained increasing interest. However, nanoparticles intended to protect and deliver drugs to epithelial surfaces require transport through the surface-lining mucus. Translation from bench to bedside is particularly challenging for mucosal administration since a variety of parameters will influence the specific barrier properties of the mucus including the luminal fluids, the microbiota, the mucus composition and clearance rate, and the condition of the underlying epithelia. Besides, after administration, nanoparticles interact with the mucosal components, forming a biomolecular corona that modulates their behavior and fate after mucosal administration. These interactions are greatly influenced by the nanoparticle properties and therefore different designs and surface-engineering strategies have been proposed. Overall, it is essential to evaluate these biomolecule-nanoparticle interactions by complementary techniques using complex and relevant mucus barrier matrices.

Key words

Nanoparticle formulation strategies, corona formation, digestive tract, respiratory tract, luminal content, methodologies, analysis

1 Introduction

Numerous attempts are made to deliver small and large molecular weight drugs by mucosal administration. To mediate sufficient delivery of the active drug molecule, encapsulation or association in microparticulate or nanoparticulate structures are often pursued to protect the drug and to direct the delivery to target site. As biotechnology has improved over the last decades, research on transmucosal delivery of biologics and other macromolecules using non-injectable drug delivery systems (DDS) has gained increasing interest [1] aiming to avoid the risk of pain, high costs and compliance issues related to the use of injectables [2,3]. However, drugs and DDS administered by non-injectable routes of administration must overcome several biobarriers in order to reach their target. The ability of a DDS to deliver the active drug molecule to the site for absorption may be hampered by hostile environments as resembled in the intestinal tract lumen with high enzymatic and hydrolytic activity, since this may lead to degradation of both the DDS and the macromolecular cargo [4]. Further, the limited liquid volume lining especially the respiratory tract, but also to some extent the gastrointestinal tract, may affect the dissolution of the DDS, the drug and relevant functional excipient(s) at the luminal surface of the mucosa from where the absorption is intended [5]. The use of dosage forms of large size, including microparticles, requires dissolution or disintegration to allow for diffusionof dissolved constituents or particles small enough to diffuse into and/or through the mucus. In interplay with the luminal content, the mucus constitutes a complex barrier to diffusion of even nano-sized DDS and molecules towards the epithelial surface. The mucus is a dynamic multicomponent matrix of which little is still known regarding the specific composition at different sites in the human body. Its barrier properties are highly influenced by health conditions and patient age. Even fluctuations in the luminal content during feed or fasted state, the overall volume interacting with the mucus from the luminal side, secretions from the underlying epithelium, the microbiota as well as will affect the mucus function as a biobarrier to transmucosal absorption of drugs. Even oral DDS may

affect the properties of the mucus. Nanoparticle-mediated drug delivery comprises multiple applications of nanoparticulate structures as DDS to mediate absorption of an active drug molecule. Irrespective of whether the strategy is to deliver the DDS to the epithelial surface with subsequent release of the drug molecule, or to aim for cellular uptake and potential transcytosis of the DDS, researchers have proposed multiple approaches to engineer nanoparticles in terms of size, shape and surface properties. In this process, it must be realized that although the effect of such engineering seems promising overcoming one specific barrier, e.g. the epithelium, the overall impact of the engineering is influenced by other biobarriers such as the mucus. In the following sections, the role of mucus as a cloak to (surface-engineered) nanoparticles will be addressed specifically in relation to the digestive and respiratory tract.

2. Lumen, mucus and epithelium: interactive players in the digestive and respiratory tracts

After mucosal administration, the drug molecule or nano-sized DDS must sustain in the lumen and translocate through the mucus layer before reaching the surface of the epithelium from where transepithelial absorption may occur. The permeation across an epithelium occurs by translocation via the tightly regulated narrow paracellular space, and/or by transport first through the apical plasma membrane (for intracellular delivery) and possibly also through the basolateral part of the plasma membrane (for transcellular delivery) to reach the systemic circulation. These interactive players will have an impact on the drug and particle diffusion, as well as on the mucosal barrier properties.

2.1. Lumen contents and effects on mucosal barrier properties

The volume, dynamics and composition of the luminal fluids; i.e. the fluid in contact with the surface of the mucus, are important parameters to consider when designing a DDS for a specific route of administration. The fluid will interplay with the mucus potentially modulating the barrier properties of the mucus. Further, the components of the lumen will likely interact with the DDS prior to its encounter with the mucus and may alter the surface properties of the DDS.

2.1.1. Digestive tract fluid

The luminal fluid in the small intestine is a dynamic mixture of enzymes, lipids, bile, bacteria, cellular debris and shed mucus, with significant changes to the composition following food intake (Table
1). The bile salt content increases 4–5 times, lipid content up to 10 times, enzyme content up to 5 times leading to a subsequent fatty acid and electrolyte content increase [6–8]. Another aspect of food intake is the peristaltic movement in the bowel following processing of the food that will lead to the removal of most of the loosely adhered mucus. Therefore, the interactions between exogenous compounds and the epithelial membrane would increase after food intake. However, it has recently been shown that postprandial levels of lipids created a more dense mucus barrier, and reduced transmucosal drug permeation [9,10]. This may be related to increased levels of lipids being incorporated into the hydrophobic domains of the mucus, thereby affecting the rheological properties of the barrier [11]. Finally, the rheological properties of the mucus will limit the interaction between the innermost firmly adhered mucus and exogenous compounds, despite the absence of the loosely adhered mucus layer. In addition to affecting the mucus barrier, the composition of exogenic compounds found in the intestinal fluid may affect the delivery propensity of the DDS following drug release [12], e.g. by affecting the solubility of the cargo [13],

or by creating micelles, thereby affecting the amount of drug available for interaction with the cell membrane [14].

The secretion of electrolytes increases the ionic strength of the intestinal fluid from < 200 mOsm/kg preprandial to > 400 mOsm/kg postprandial [15,13]. Thus, the electrostatic interactions between mucins and exogenous compounds such as DDS will be partially shielded due to the higher density of electrolytes [16,17]. Furthermore, increases in ionic strength has also been shown to reduce the viscosity of mucus, where di- and trivalent ions exert the greatest effect [11]. Interactions between the mucins and ions may further limit the availability of charged sialic acids, which form the bulk of the interactive barrier. This is further emphasized at lower pH values, albeit this scenario is most commonly encountered in gastric mucus [11]. Finally, while bile salts are known to be important constituents in the formation of intestinal micelles [18], and have been shown to be involved the delivery of a multitude of drugs across the mucosal barrier [19,20], only limited interaction of bile salt with bovine, porcine and rat mucus have been reported [21,22].

2.1.2. Respiratory tract fluid

The lung lining fluid in the alveoli region consists of a thin hypophase covered by a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)-rich surfactant film. Pulmonary surfactant is an essential lipid-protein complex that forms a monolayer at the air-liquid interface and serves to reduce the surface tension of the air-liquid interface to stabilize the respiratory units, i.e. alveoli, during breathing [33]. Briefly, the phospholipid species in mammalian pulmonary surfactants mainly include zwitterionic phosphatidylcholine (PC, 60–70% by mass), anionic species such as phosphatidylglycerol (PG) and phosphatidylinositol (PI) (8–15% by mass) and neutral lipids mostly cholesterol (3–8% by mass) (Table 2). The specific surfactant proteins (SP), which are approximately 10% by mass, can be classified into two families: SP-A and SP-D (hydrophilic) and SP-B and SP-C (hydrophobic) [34,35]. DPPC is the lipid considered as primarily responsible for the surface tension reducing properties of the surfactant, while the precise lipid composition ensures the appropriate thermodynamic properties, surface active function and stability of surfactant membranes at a wide range of environmental temperatures [36,37]. SP-A is the most abundant surfactant protein, which is mainly involved in innate defense mechanisms at the alveoli together with SP-D. In addition, SP-A is also a film-association surfactant protein, which can accelerate the adsorption of surfactant phospholipids at the air-liquid interface [38]. SP-B and SP-C are thought to be crucial for lipid packaging, re-organization, and adsorption to the air-liquid interface during breathing [39].

Pulmonary surfactants are mainly secreted by AT-II cells in the lung epithelium. However, the surfactants synthesis and secretion in the lung are not exclusively restricted to the AT-II cells in the distal part of the respiratory tract. There is evidence that it likely also take place in more proximal parts of the respiratory tract, for instance in Clara cells and possibly even in the tracheal epithelium [40,41]. Consequently, recent studies have shown that a surfactant film is present at the air-liquid interface in both alveoli and upper respiratory tract [42].

The surfactants present in the upper respiratory tract may also be supplemented by alveolar surfactants through the mucociliary escalator. However, it should be noted that the composition of pulmonary surfactants in the central part of the respiratory tract is different from that in the alveolus. For example, SP-B and SP-C only exist in the alveolus, while only few SP-A and SP-D are sparsely observed [38]. It is also reported that surfactant-derived phospholipids are located between the sol and gel phases of central respiratory tract mucus [43]. The roles of surfactant lipids in the central respiratory tract upon DDS administration may include: 1) regulation of the

proportion of the sol and gel phases of the mucus leading to changes in mucus viscosity, 2) induction of changes in mucus penetration or adhesiveness as a result of interaction with the DDS and/or the mucus, 3) increased ciliary function leading to enhanced mucociliary clearance of DDS by means of the displacement of particles into the hypophase [44].

2.2. Composition and barrier properties of the mucus layer

After administration at mucosal surfaces, the drug molecule in solution, assembled or incorporated into a DDS should overcome the mucus absorption barrier, which covers almost every internal surface of the body. In broad terms, the mucus layer is a hydrophilic and viscous liquid, which is practically impermeable to compounds with specific properties, and is secreted by goblet cells found in the epithelium of the mucosal membranes [47]. The function of the intestinal mucus barrier is to limit the access of exogenous (e.g. pathogens, drugs) and endogenous compounds (e.g. luminal fluid components) to the epithelial surface while simultaneously acting as a lubricant preventing mechanical damage caused by e.g. food transit through the intestinal tract [48,49]. The main roles of respiratory mucus include to act as protective barrier against the external environment, to maintain the hydration of the respiratory tract and the barrier function of the epithelium, and to regulate the immune response, cell proliferation and differentiation [50]. The continuous secretion of mucins allows for the gel-forming capabilities of mucus [51,52]. Mucins are long, thread-like, complex glycoconjugates with size of 200 kDa to 200 MDa and consist of a linear peptide backbone, to which hundreds of carbohydrate side-chains are O-linked, but also with additional N-linked glycans. Further, each mucin monomer may be linked by cysteine bridges to several other monomers resulting in a mass of > 100 MDa, which makes mucins some of the largest known proteins [51]. The lubricating and protective effect as well as the high mass of the mucins can be attributed to the extensive O-glycosylation of each monomer [53,54]. The glycosylation pattern is complex and extremely diverse [55], which offers a high degree of resistance to microbial proteases and facilitates broad-spectrum bacterial attachment and subsequent clearance [56,57]. The O-glycosylation takes place in specific domains within the mucin structure, known as PTS domains, which are repeated structures of 8–169 amino acids [58] with a high prevalence of proline, threonine and serine (typically at least 45% [54]), conjugated to polysaccharide side chains (2–20 monosaccharides [58]). These PTS domains represent 50–90 % of the mucin weight [58,59]. Mucins are also characterized by the von Willebrand domains that allow for oligomerization through cysteine knots, cleavage of membrane bound mucins and gel-forming capabilities in secreted mucins [52,58]. In addition, hydrophobic domains found in the non-PTS regions allow for hydrophobic interactions. Due to free carboxylate and sulfonate groups found in the glycosylated PTS domain, the mucus exhibits an overall negative charge capable of electrostatic and hydrogen bonding interactions to foreign entities such as drug molecules or DDS [60]. Most mucins also have high sialic acid content, leading further to a strongly negative surface believed to be an important determinant of the viscosity and elasticity of mucus [50].
The mucin network acts as a size-exclusion filter for particles and large compounds to pass, and it is often referred as the steric barrier of the mucus. The mesh spacing of the native mucin hydrogel is found to be very heterogenic ranging from hundreds nanometers to micrometers due to different anatomical origin, the inherent heterogenicity of the mucus network and the different methods used to determine the mesh [48,61]. Thus, the mucus is often considered as a triple barrier to macromolecular drug and particle permeation: 1) a steric barrier, where the mucin network acts as a size exclusion filter; 2) an interactive barrier, because of the multiple low-affinity interactions, and 3) a dynamic barrier, due to the continuous secretion of mucins from the underlying epithelium and flow of lumen fluids [60]. Further, the rheological properties of mucus

(non-Newtonian and shear-thinning) limit passage of foreign entities due to an unstirred region on the epithelial surface that acts as a rate-limiting step especially for the diffusion of lipophilic components [11]. As increased shear may be applied, the unstirred region becomes thinner and the entangled fibers of the mucus network are pulled apart, creating a slippage plane with a decreased viscosity as compared to the mucus closer to the epithelial surface [48].

2.2.1. Digestive tract

Despite the intestinal tract being the primary site for drug absorption of orally administered drugs, the mucus barrier in the human small intestine is only poorly described and is highly affected by the intestinal motility and intestinal fluids composition [48]. The thickness, composition and pH of the mucus in the gastrointestinal (GI) tract vary with region of interest, as well as with species. The actual thickness of the mucus layer depends on a relation between several types of biomolecules: the glycocalyx, the firmly adhered mucus and the loosely adhered mucus. The glycocalyx is the innermost epithelial membrane-associated part and consists of 1.5–2 μm of dense, membrane-bound mucins and other membrane-adherent glycans [48,62].
Due to the location and composition, the glycocalyx may be considered as part of the firmly adherent mucus layer [62]. The firmly adhered mucus constitutes the barrier physically attached to either the glycocalyx or the epithelial membrane [63] and cannot be removed by interaction with bile, acid, ethanol or hyperosmotic electrolyte solutions in rats [64], but can be damaged by pepsin, a commonly encountered GI enzyme, as well as a variety of surfactants and stronger acids [63,65]. The loosely adhered mucus comprises the mucus facing the luminal side, and is not physically attached to the cell membrane. Furthermore, the loosely adhered mucus is easily removed by gentle aspiration [63], but is regrown to its initial state in approximately one hour [52]. Due to the dynamics of the loosely adhered mucus, the determination of the exact thickness may prove difficult. As such, reported values of the thickness of the mucus barrier throughout the human digestive tract vary between studies (Table 3) and it is seldom reported whether several mucus layers are distinguished or just treated as one. The total mucus thickness is reported in the range of 10–250 μm across all regions of the human digestive tract, and differs from the intestinal mucus thickness of laboratory animals (i.e. human mucus may exhibit twice the thickness of what is observed in rats across all regions [64]). Other mucus properties also differ between human and laboratory animals. Comparable rheological properties of porcine mucus to human mucus have been demonstrated [66], but porcine mucus has been reported to be hypertonic (> 400 mOsm/ kg [66]) and thus may interact differently with charged compounds as compared to human mucus, which has been hypothesized to have isoosmolality [67]. Furthermore, the turnover rate of the loosely adhered intestinal mucus (i.e. the time necessary to return to its initial stage if removed) has been reported to be faster in humans as compared to rodents [68]. Also, the pH of mucus in the GI tract varies depending on site. As such, the acidic contents of the stomach fluid and secretion of bicarbonate at the epithelial membrane result in a pH gradient starting at pH 1–2 at the surface of the mucus facing the lumen in the upper part of the GI tract, and increasing to approximately pH 7 at the epithelial surface. Further through the tract, the pH increases in alignment with the intestinal fluid, peaking at a pH of 7–8 in the terminal ileum [49]. The pore size of intestinal mucus has been investigated mainly using porcine mucus as model due to its availability. Pore sizes ranging from 20 to 200 nm were reported using atomic force microscopy or scanning electron microscopy [69–71]. Whereas the exact composition of the GI mucus remains unknown and depends on the exact region, the rough composition is approximately 90–98 % water, 2–5 % mucins and 1–3 % lipids, proteins, macromolecules as well as trace amounts of electrolytes and DNA [88]. Furthermore, foreign entities such as cell debris, pathogens and

components of the luminal fluid are scattered within the mucus [88]. As previously described, the mucins represent the core structure of the mucus layer. The intestinal mucins are classified into secreted mucins (e.g. MUC2, MUC5A, MUC5B, MUC6) and membrane-bound mucins (e.g. MUC1, MUC4, MUC13, MUC16) [58,89]. Continuous shedding of mucins throughout the GI tract occurs at a rate of 1–100 μm/s [66]. This shedding prevents severe degradation and physical erosion of the firmly adhered mucus by intestinal peptidases [63,90], as well as overgrowth of both naturally occurring bacteria and pathogens [48]. The GI tract is lined with up to 1×10^{14} bacteria of more than 2000 species [47], and they serve a multitude of functions such as influencing the degradation of mucus and exogenous compounds [91,92], maintaining homeostasis [47], and protecting against pathogens [93]. The microbiota has also been proven to affect the biotransformation of drugs [32] or even exerting therapeutic effects in itself [94,95]. These organisms are therefore seldom pathogenic by definition, but may become pathogenic if the mucus barrier is damaged [96]. It has been proven that commensal organisms cannot enter the firmly adhered mucus layer in the colon of mice [85]. Indeed, most pathogens are trapped within the mucosal mesh, transported to the luminal surface by mucosal shedding and subsequently cleared. Also, the secretion of antimicrobial compounds from epithelial Paneth cells protects the epithelium from pathogens [47,93]. Some pathogens can, however, migrate through the mucus barrier by degrading the mucus enzymatically, by flagella-mediated motility, or by simply avoiding the mucus barrier through pockets that are not completely covered by mucus (Figure 1) [96]. In an infected state, the mucus barrier exhibits altered physicochemical properties such as charge and viscosity by e.g. modulating the glycosylation [97] or degradation [96] of mucins (Table 4), therefore affecting the mucus-DDS interactions. However, studies addressing the impact of the mucus as a drug delivery biobarrier under pathological conditions are very limited. Bacterial infections may also result in altered mucosal membrane permeability, which may even further limit the effectiveness of the membrane barrier towards exogenous entities [96]. Similarly, inflammatory diseases like Crohns disease (CD), ulcerative colitis (UC) and inflammatory bowel disease are known to alter the mucosal barrier in the intestinal tract. A defective adherent mucus layer results in increased contact between pathogens and antigens, resulting in increased inflammatory responses in UC [52,79,85,98]. This is interesting, as healthy adherent mucus has been shown to be devoid of bacteria in mice [85]. A similar hypothesis has been proposed for CD, for which a defective epithelium, a disrupted mucus layer and increased presence of microorganisms has been associated with increased intestinal permeability [99]. However, the mechanism has largely been attributed to defects within the intestinal epithelium (e.g. in the TJ proteins), and the disease has further seen remission by the use of antibiotics [99].

2.2.2. Respiratory tract

Like the GI tract mucus, respiratory tract mucus is a complex mixture secreted by epithelial goblet cells in the upper respiratory tract. The composition, structure and thickness of lung lining matrix vary from site to site. In the respiratory tract, the sol and gel mucus layers are surmounted by a surfactant film at the air-liquid interface. The main constituents of respiratory tract mucus are water (95–99%) and mucins, with small quantities of salts, enzymes and anti-enzymes, oxidants and antioxidants, exogenous bacterial products, endogenous antibacterial secretions, cell-derived mediators and proteins, plasma derived mediators and proteins, and cell debris such as DNA. Comparable to intestinal mucus, the mucus in the upper respiratory tract is considered to form a liquid bilayer: a more viscous mucus gel layer on top of a periciliary liquid/sol layer [107]. The “gel-on-brush” model has been used to describe the mucus barrier [108]. In the upper respiratory tract, the inhaled particles are trapped in the sticky gel layer and are removed from the airway by

mucociliary clearance (MCC), whereas the sol layer lubricates the beating cilia [50]. MCC exists in the conducting region, where the movement of the cilia transports the mucus with the inhaled particles towards the pharynx/larynx. The reported tracheal mucociliary clearance rate in young healthy subjects range from 4 to 20 mm/min [109]. However, the value of the mucus transport velocity varies largely according to human health condition. The thickness of lung lining matrix is reported to range from 0.05–0.08 μm in the alveolar zone (mainly lung surfactant layer) to 5–10 μm in the central lung which is composed of the mucus layer and the surfactant layer [5,110,111]. Pores in human respiratory tract mucus vary from tens to hundreds of nm, with many pores under 100 nm [112]. In equine bronchial mucus, large pores are observed in combination with very small pores, ranging from 100 nm to several micrometers [113]. There are only a few studies on the rheological properties of airway mucus due to difficulty in collection of tracheobronchial mucus from healthy human lung airways. The viscosity was measured in the range of 12–15 Pa・s, with a relaxation time of 40 s and an elastic modulus of 1 Pa, representing an optimal rheological profile for MCC [11]. It should be noted that the commonly used methods to collect airway mucus are endotracheal tube, screens and specimen brush. However, it is difficult to assess how representative of 'normal' the mucus is when collected using the above methods which can mechanically stimulate the airway epithelial secretory cells. The major respiratory airway mucins are MUC1, MUC4, MUC5AC, MUC5B, and MUC16. Among them, MUC1, MUC4 and MUC16 are membrane-tethered mucins, which have a hydrophobic domain that anchors the mucin in the plasma membrane [114]. MUC5AC and MUC5B are the major secreted mucins, which are stored intracellularly in secretory granules and are released at the apical surface of the cell in response to stimuli. The pH of the respiratory tract liquid matrix under normal physiological condition is near neutral pH. However, it could be ~ 0.5 units lower due to the dysfunction of ion channels in lung diseases such as chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) [115]. Under healthy conditions, respiratory tract mucus protects the epithelial lining by entrapping foreign debris, bacteria and viruses and clearing them from the airway by ciliary movement. While extensively investigated in the digestive tract [76,96,116,117], the microbiota in the respiratory tract has been neglected. The latter may be attributed to the hypothesis that the lungs are sterile domains, which has been disproven in current research [118]. As such, the role of the pulmonary microbiota is largely unknown, but may be expected to exert a similar role as the microbiota found at other mucosal surfaces. In addition, the interaction between mucins and respiratory pathogens is also more complicated than mere entrapment. It is found that mucins play an integrated role in the host response to pathogens [50]. The conversion from healthy to pathologic mucosa in the lungs occurs by multiple mechanisms that change its hydration and biochemical constituents. These include abnormal secretion of salt and water, increased production of mucins, infiltration of mucus with inflammatory cells, and altered bronchovascular permeability. Accumulation of mucus with a different chemical composition is often observed and results from a combination of overproduction and decreased clearance. Persistent accumulation can lead to further infection and inflammation by providing an environment for microbial growth that also may influence the mucus barrier properties. As examples, the mucus compositions in patients with different diseases are outlined in Table 5. Effects of asthma are characterized by increases in epithelial mucin stores as the surface epithelial mucous metaplasia with modest hyperplasia and increased numbers of subepithelial bronchial microvessels that become leaky during inflammation. In COPD, increased mucin stores occur because of surface epithelial mucous metaplasia and some hyperplasia, together with increases in the volume and number of the submucosal glands. In cystic fibrosis, epithelial mucin stores are similar to normal levels (possibly because of increased secretion), but submucosal glands are very prominent. In addition, the increased numbers of inflammatory cells in the respiratory tract epithelium and lumen can be observed in all respiratory tract diseases.

Products of inflammatory cell death include DNA and actin polymers, which are important constituents of pathologic mucus. All these altered components are likely to have an impact on the interaction with the drug and DDS delivered to pathological lung.

2.3. Epithelia as barriers to drug delivery

Due to extensive folding (villi in the intestines and alveoli in the airways), the digestive and respiratory tract both exhibit a high surface area of approximately 200 m^2 and 160 m^2 for the small intestines and the lungs, respectively [119]. This large surface area available for drug absorption makes both administration routes ideal for delivery of large doses, assuming that the drug can be delivered in sufficient amounts to the site of absorption, remain stable and avoid clearance until absorbed across the mucosa composed of the epithelium surface-lined with mucus and luminal fluid. Although the epithelia in both the digestive and respiratory tract are similar in the sense of being primarily monolayered and (partly) surface-lined by mucus and an aqueous surfactant-containing layer, different cell types and barrier conditions are relevant to consider for the specific mucosal barrier properties.

2.3.1. Digestive tract

The largest area available for absorption in the digestive tract, the intestinal epithelium (Figure 2a), consists primarily of four cell types; absorptive enterocytes, mucus-producing goblet cells, hormone-producing endocrine cells, and Paneth cells secreting antimicrobial compounds [47]. Whereas other cells are also present in the small intestine, these typically fulfill other roles than barrier function (e.g. transportive M cells and immune related macrophages and dendritic cells). In the stomach, the cell composition is different, and consists primarily of mucus-producing foveolar cells, enzyme secreting chief cells, acid secreting parietal cells and enterohormone secreting cells [123]. Therefore, mucus is present throughout the digestive tract, albeit the structural composition and thickness varies greatly (see Section 2.2). In order to permeate across the epithelium, drugs or DDS would have to permeate across either through the cells forming the epithelium (transcellular route) or through the channels making up the intercellular space (paracellular route), as previously mentioned. Inherently, the paracellular space is too tight to allow for diffusion of macromolecules and nanoparticles. The radius of the space regulated by tight junctions (Figure 2b) has been reported to be < 10 A with the exact properties being dependent on site, species and region [99,124]. These tight junctions effectively prevent the permeation of any drugs larger than 15 A (approximately 3.5 kDa) [125] and nanoparticulate structures. However, many compounds can interact with the tight junctions, and thereby reversibly increase the size of the pore [119]. These tight junction modulators can facilitate the paracellular transport of compounds with a mass of up to 10 kDa [119]. It should be noted, however, that overall the paracellular route likely allows for a permeation rate for molecules slower than the transcellular route due to the reduced absorptive area of the junctions compared to the cell membrane [126]. On the other hand, the cell plasma membrane constitutes a tight, lipid bilayered membrane consisting primarily of phospholipids, cholesterol, carbohydrates and aminoglycans, with a thickness of 40–100 A [127,128]. Due to the high lipid content and the presence of surface-linked glycans, the epithelial membrane exhibits highly hydrophobic characteristics with a net negative charge [129,130], which makes it effectively impermeable to large and (especially positively) charged drug molecules and DDS [129]. Cellular uptake of larger structures such as nanoassemblies and nanoparticles may occur by endocytotic processes, followed by cellular trafficking and potentially transcytosis.

2.3.2. Respiratory tract

Approximately 40 different cell types are present in the respiratory tract. Ciliated cells account for approximately 50% of the cell population in the human tracheal epithelium (Figure 3). Mucus producing goblet cells are present only in the upper respiratory tract. Clara cells are a subset of secretory cells present in human tracheal epithelium, which have the capacity of self-renewal and maintain and repair the bronchioles [131,132]. Mucus is not excreted in the alveolar region, where alveolar epithelial type I (AT-I) and type II (AT-II) cells constitute the major cellular population. AT-I cells are relatively large and thin, covering approximately 90% of the alveolar surface area, which are mainly responsible for gas exchange, ions and protein transport. AT-II cells are smaller and only cover 3% of the alveolar surface area and are responsible for the synthesis and secretion of pulmonary surfactant [133]. Additionally, it is reported that each of the 500 million alveoli in human lungs is routinely 'patrolled' by 12–14 alveolar macrophages [134] and more than 90% of alveolar macrophages are located at or near alveolar septal junctional zones [135].

3. Drug delivery system designs for mucosal administration

Drugs and DDS for mucosal administration must overcome multiple challenges for obtaining sufficient drug bioavailability. One of the main bottlenecks for the transmucosal delivery of a drug is sufficient, and sufficiently fast, diffusion of the DDS or released drug through the mucus. Thus, the drug passage through the mucosal barrier is influenced by a combination of several factors including size, shape and surface properties (Figure 4), and it is difficult to evaluate the effect of one single contribution.

3.1. Size

The mucin fibers form a crosslinked and entangled network responsible for the size filtering property of mucus. The mesh spacing of this network would define a size cut-off above which the diffusion of particles is sterically impeded. This mesh pore size has been measured to be in the order of nanometers (see Section 2.4), so DDS intended for mucosal diffusion should be in the nano-size range. However, the diffusion of nanoparticles through the mucus is not only conditioned by the mesh pore size. Even particles small enough to pass through the mesh pores are still somewhat affected by the rheological properties of the mucus. The Brownian diffusion of small particles is affected by the drag exerted by the water in the mucus (microrheology) to a greater extent than larger particles [11], effectively meaning that smaller particles do not necessarily permeate mucus better or faster than slightly larger particles. Several studies have tried to determine the size threshold for drug and nanoparticle penetration in healthy [9,70,112,138–144] and diseased state [145–148] mucus. It is generally observed that the diffusion rate through mucus decreases with increasing particle size, and this size-dependency is highly dependent on the surface chemistry of the nanoparticles (Figure 5). For example, a study using small silica nanoparticles of different sizes in fresh native porcine jejunal mucus found that 10 and 50 nm particles diffused better that 100 or 200 nm particles, and 200 nm particles did not permeate irrespective of the surface coating [138]. Other authors set the cut-off size between 200 and 500 nm [70]. *In vitro* studies using models of different complexity also showed a significant size-dependency in nanoparticle uptake via enterocytes and M-cells, which was strongly impacted by the mucus layer [140,149]. In studies of nanoparticles intended for lung delivery, different authors have used particle tracking techniques to determine that both 100 and 200 nm particles

rapidly penetrated healthy human airway mucus when PEGylated, whereas 500 nm particles were essentially immobile [112,139]. However, after aerosol deposition only 100 nm particles were observed to penetrate into the mucus [139].

3.2. Shape

The effect of size limitations in the ability of particles to permeate through mucus must be considered in relation to the shape of the particle. The clear majority of studies with nanoparticles have so far been limited to spherical particles. However, it has been shown that shape affects biological responses to nanoparticles [151,152]. Also in nature shape has a critical role, in particular in pathogenesis of intestinal bacteria with high mobility in mucus [153]. It is demonstrated that the helical shape of the *Campylobacter jejuni* [154] and *Helicobacter pylori* [155] aids the transit through the intestinal mucus promoting the intestinal colonization and *in vivo* pathogenesis. Being inspired by these findings, Yu and coworkers hypothesized that the shape of nanoparticles may also be critical in the mucus-penetrating ability of particles [156]. Using the multiple-particle tracking method, they found that nanorods of 80 × 240 nm of dimension diffuse faster than their spherical counterparts of 80 and 140 nm in diameter in fresh intestinal mucus. This enhanced diffusivity led to deeper mucus penetration and longer retention time in the GI tract after oral administration in fasted rats *in vivo*. Molecular dynamics simulations revealed that the superior mucus-penetrating ability of the nanorods was due to the rotational motions facilitated by the shear flow and the mesh structure of mucus. Mitragotri's group has also observed an enhanced *in vitro* uptake and transport across mucus-containing intestinal monolayers of rod-shaped nanoparticles of about 390 nm in hydrodynamic diameter compared to spheres or discs [157]. The role of particle geometry after deposition onto the pulmonary epithelium has also been investigated. *In vitro* studies using different primary and immortalized alveolar model cells without mucus demonstrated that different geometries exhibited different time- and cell-specific uptake patterns [158]. On the other hand, the mucociliary clearance velocity of particles trapped in the pulmonary mucus was shown to be independent of size, shape and surface properties of particles using *ex vivo* and *in silico* approaches [159]. This lack of difference is mainly caused by the lack of immediate penetration of deposited aerosol particles through the mucus blanket.

3.3. Surface charge

In addition to the steric barrier, mucus can form low-affinity interactions with drugs and nanoparticles, preventing them for further entry into the body. As described in Section 2 the carboxyl and sulfate groups of the oligosaccharide chains provide mucus with a net negative charge [48,60]. Also, properties of the intestinal fluid such as the secretion of electrolytes during fed state, or the presence of zwitterionic phospholipids in the respiratory lining fluid would contribute to the mucus interactive barrier. Consequently, the diffusion behavior of particles in mucus is likely influenced by electrostatic interactions. Positively charged nanoparticles interact strongly with mucus and other biological components [138]. In fact, positively charged polymers such as chitosan are often used to design systems with mucoadhesive properties [160]. These interactions between cationic particles and mucus are also seem to alter the viscoelasticity of mucus [138]. Negatively charged particles are more slippery owing to the repulsive forces [138,161], whereas neutral nanoparticles were observed to diffuse faster through the native intestinal mucus, since their neutral surface reduce hydrophobic and electrostatic interactions [71,162]. The electrostatic interactions can be altered by buffer conditions such as pH and ionic

strength. This fact is of particular interest in the GI tract where pH varies dramatically throughout the tract [49], or in vaginal delivery where the cervical mucus pH changes during the ovulatory cycle [163]. It has been demonstrated that the human immunodeficiency virus or negatively charged nanoparticles are trapped in CV mucus at acidic pH, but not at neutral pH where the native negative surface charge is neutralized [161,164]. A similar behavior has been observed in reconstituted mucin hydrogels. At low pH, charged particles have strong interactions with the mucin network, whereas at neutral pH, the hydrogel permeability is high [17,165]. Apart from the modulation of the attractive and repulsive forces, the pH also can change dramatically the viscosity of mucus, and therefore the mucosal membrane permeability. In studies on reconstituted pig gastric mucin the bulk viscosity increases 1000 times when decreasing pH from 6 to 4 [166]. Changes in viscosity of native pulmonary sputum have been also observed [167], whereas CV mucus viscosity exhibit minor changes throughout a broad range of pH [168]. The particle mobility in mucus is also affected by the ionic strength [17]. However, the net surface charge is not enough to predict mucosal passage of molecules and particles. Spatial arrangements of charge are also crucial in determining the interactions with the mucin chains [169]. Bernkop-Schnurch's group recently presented an innovative approach using carrier systems that change zeta potential while the carrier is migrating in mucus [170–172]. A negative surface charge has been proved beneficial for mucus penetration, whereas positively charged particles are more likely to interact with negatively charged epithelial membranes and trigger endocytic transport mechanisms. The group has developed polymeric nanoparticles and self-emulsifying drug delivery systems (SNEDDS) that have anionic phosphate groups on the surface allowing for faster diffusion across the negative mucus network. Upon reaching the intestinal epithelium, the alkaline phosphatase activity of the intestinal enterocytes cleaved off the phosphate groups, shifting the surface charges to positive and thus enhancing cellular uptake. However, *in vivo* studies have to be performed to validate this approach. Following a similar idea of addressing the diffusion and absorption barriers, Shan and coworkers used zwitterion- based nanoparticles [173]. The neutral and hydrophilic coating of lipid-based zwitterions allowed for excellent mucus diffusion similar to the PEGylated controls. These particles possessed high affinity to the epithelial cellular membrane, significantly increasing (4.5-fold) the cellular uptake, compared to the PEGylated counterpart. These results were also confirmed *in vivo* after oral gavage of diabetic rats.

3.4. Surface engineering

Besides the electrostatic interactions, hydrophobic interactions can serve a substantial filtering mechanism to diffusion of drugs and nanoparticles through the mucus. This hydrophobic barrier is mainly present due to the non-glycosylated regions of the mucins and the significant amount of lipids bound or adsorbed in the mucus [48,49]. Lipophilicity was shown to be the most important physicochemical characteristic influencing the diffusion of a series of model drugs in native and biosimilar intestinal mucus [142,143]. Also hydrophobic nanoparticles such as carboxylated polystyrene (PS) beads, in spite of their negative charge, are highly retained by the mucin hydrogel as they form multiple hydrophobic adhesive interactions [17,141,174]. Thus, the choice of material and its surface properties such as hydrophilicity or crosslinking density would be crucial parameters for the development of a mucus-penetrating DDS. In nature, viruses that are capable of overcoming the mucus barrier have specific surface characteristics. They exhibit a densely charged capsid that creates a hydrophilic and near-neutral surface charge and minimizes hydrophobic and electrostatic interactions, diffusing essentially unhindered through mucus [174]. Inspired by these viruses, researchers have proposed different surface engineering strategies that create a hydrophilic coating and reduce particle adhesion to mucus. The group of Hanes

contributed to the pioneering approach of producing mucus-penetrating particles by coating the nanoparticles with poly(ethylene glycol) (PEG) [112,141,175–177]. PEG is routinely used in drug delivery coating to increase the circulation time and reduce the uptake by the reticuloendothelial system [178]. Lai and coworkers first demonstrated that this uncharged and hydrophilic polymer also reduced the hydrophobic interactions with the mucus components [141]. Large particles of 200 and 500 nm in diameter coated with PEG diffused through fresh undiluted CV mucus, whereas non-coated carboxylated PS particles exhibited very low mobility. Further studies demonstrated that low molecular weight PEG (up to 5 kDa) and high surface density (dense brush conformation) were necessary to achieve high diffusion rates and improved mucosal distribution *in vivo* [175,176,179] (Figure 6). Bernkop-Schnurch's group proposed an alternative approach to PEG coating. Biomimicking the virus capsid, they developed a series of highly dense charged particles bearing positive and negative charges that were able to efficiently diffuse in mucus. These virus-inspired polyelectrolyte particles had a near-neutral overall surface charge that minimized the electrostatic and hydrophobic adhesive interactions with mucus [71,162,180]. A negatively charged polymer (polyacrylic acid or chondroitin sulfate) was self-assembled with a positively charged polymer (chitosan or polyallylamine) by ionic gelation resulting in near-neutral particles. These particles showed high diffusion in mucus, comparable with that of a highly diffusive PEGylated particle [71]. A recent work has combined both strategies, namely the PEGylation strategy and the virus-mimicking strategy, to generate mucus-penetrating particles [181]. Low molecular weight PEG (5 kDa) was conjugated to the anionic polymer chondroitin sulfate and self-assembled with the cationic polymer chitosan to render slightly negative nanoparticles for insulin delivery. Despite their relatively large size around 500 nm, the PEGylated nanoparticles showed high permeation through mucus, showing the potential of combining synergistically different surface engineering approaches.

3.5. Targeting ligands

Active targeting strategies have been proposed to increase nanoparticle diffusion through mucus, mucoadhesion and/or cellular translocation by covalently coupling specific targeting ligands to the surface of the nanoparticles. A promising approach to increase nanoparticle diffusion through mucus is the immobilization of mucolytic enzymes on the surface of the nanoparticles. Compared with the extensive mucus disruption of co-administered mucolytic agents, the effect of these mucolytic enzymes is localized since they are covalently bound to the nanoparticles. Thus, Muller *et al*. developed a mucus-penetrating system based on the enzyme papain grafted to polyacrylic acid (PAA) nanoparticles by covalent bonding [182]. The mucolytic activity of papain that decreased the mucus viscosity, combined with the repulsive forces of the negatively charged PAA resulted in highly diffusive nanoparticles, both *in vitro* and *in vivo*. In a follow-up study, Pereira de Sousa and coworkers demonstrated that nanoparticles decorated with bromelain exhibited higher performance in permeating intestinal mucus compared to the papain-decorated nanoparticles [183]. Lectins are often used as ligands in targeted drug delivery to improve mucoadhesion and nanoparticle residence time, followed by enhanced particle translocation through specific cell interactions [184]. Lectins are glycoproteins that specifically bind carbohydrate residues. Lectins are known to interact strongly with mucins [185], and are also explored as cell-targeting moieties as they are involved in cell recognition and adhesion processes through sugar residues such as N-acetyl-D-glucosamine located on the surface of intestinal M-cells or alveolar epithelial cells [120,186]. Wheat germ agglutinin (WGA) has been extensively used for this purpose. It exhibits low immunogenicity and good enzymatic stability [184]. For example, Makhlof and coworkers conjugated WGA to cationic liposomes for targeted delivery of calcitonin. WGA-modified

liposomes showed effective cellular adhesion and 20-fold enhancement of calcitonin bioavailability after oral administration in rats compared to the non-targeted liposomes [187]. These liposomes were also administered via pulmonary delivery, demonstrating an improved bioadhesion to lung epithelia and prolonged therapeutic efficacy of the encapsulated peptide drug [188]. Other lectins such as tomato lectin or peanut agglutinin have been also explored for targeted delivery to mucosal sites [189,190]. Different ligands for various receptors expressed in the epithelial cells have been proposed for increasing the cellular uptake after mucosal administration. One of the most exploited metabolic pathways for targeted oral delivery is the vitamin B12 uptake mechanism. Vitamin B12 (cobalamin) binds intrinsic factor molecules secreted in the intestine, and the resulting complex is internalized by receptor-mediated endocytosis in the ileum. Chalasani and coworkers conjugated vitamin B12 to dextran nanoparticles for oral delivery of insulin [191]. After administration to diabetic rats by oral gavage, vitamin B12-conjugated nanoparticles triggered high and prolonged glucose reduction, with a bioavailability of approximately 30%. Vitamin B12 has been also conjugated to other DDS such as solid lipid nanoparticles, micelles or ceramic nanoparticles with an enhanced absorption both *in vitro* and *in vivo* [192–194]. In addition to the increased cellular uptake, data suggest that vitamin B12-conjugated nanoparticles undergo a non-lysosomal internalization pathway, resulting in a dramatic increase of drug intestinal absorption [195]. In addition to vitamin B12, other ligands such as folate, integrin or transferrin, have been also explored for targeting epithelial receptors after mucosal administration [196–198]. In the recent years, cell-penetrating peptides (CPPs) have demonstrated the potential to enhance the mucosal delivery of biopharmaceuticals and DDS [2,121,199]. These peptides have been used for surface modification of nanomedicines to enhance gene delivery to the lungs [200,201]. The group of Saltzman modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles with different CPPs (mTAT, BPrPp and MPG) through a PEGylated phospholipid linker [200]. These formulations showed up to 4.5-fold improved intracellular uptake and transfection efficiency *in vitro*. In a follow-up study, it was demonstrated that MPG-conjugated nanoparticles were successfully delivered to the lungs of mice, where they associated to around 30% of lung cells (macrophages and alveolar epithelial cells), compared to 5% for the non-targeted formulation [201]. However, this formulation exhibited modest effect with < 1% of gene correction. Thus, future work needs to address the interaction of these moieties with the pulmonary milieu since most of these CPP have positive charges that might interact strongly with the oligosaccharide chains of the pulmonary mucus, therefore shielding their cell-penetrating ability. The use of monoclonal antibodies for targeted delivery of drugs through mucosal administration has been also investigated, since antibodies show high specificity and are not trapped in the mucus mesh [202]. Oral delivery by use of antibody-based nanoparticles is, however, challenged by the presence of proteolytic enzymes along the GI tract degrading approximately 60% of the administered antibody [203]. Antibody-decorated formulations have been tested for selective delivery of microbicides to HIV susceptible cells present in the vaginal mucosa [204]. These formulations showed improved selectivity and higher intracellular drug concentration; however, the *in vivo* feasibility of this approach has still to be demonstrated.

4. Interaction of nanoparticles with biological matrix components

As described, current studies in drug delivery focus on the design of novel nanoparticles to overcome the mucus barrier by minimizing the interactions with the complex mucosal components. Besides the mucus barrier, there are other biological components that might interact with the nanoparticle and modulate transmucosal translocation of the DDS or the encapsulated drug. As a result from these interactions, a biomolecular corona is formed on the nanoparticle

surface, constituting a major element of their biological identity and affecting behavior and fate after mucosal delivery [205,206]. From a nanomedicine perspective, it is essential to characterize and understand these bio-nano interactions in the complex biological matrix environment to design and develop more efficient DDS. The ongoing controversial discussions on the use of nanotechnology regarding risk assessment and nanotoxicology further emphasize the demand for carefully elucidating the biological fate of nanomedicines after dosing [207,208]. The development of advanced analytical techniques allows for detailed insight into bio-nano interactions, which promote further understanding of the actual fate of nanoparticles in the biological environment.

4.1. Methods for characterization of mucus-nanoparticle interaction

The interaction of particle and mucus made by electrostatic interactions, van der Waals interactions, hydrophobic forces, or hydrogen bonding influences the nanoparticle mobility at the mucosal surface. Different methods have been developed to determine the mucus nanoparticle interactions including multiple-particle tracking (MPT), quartz crystal microbalance with dissipation monitoring (QCM-D), and membrane-supported mucus diffusion systems, among others. Here, some of these commonly used methods are introduced and an overview of their advantages and limitations are summarized in Table 6. For detailed information about these and other techniques, readers are referred to other reviews [209,210].

4.1.1. Multiple-particle tracking (MPT)

Multiple-particle tracking (MPT), sometimes referred as single- particle tracking (SPT), has been widely used to investigate the diffusion behavior of nanomedicines in complex fluids and biological specimens [211]. This technique enables simultaneous real-time tracking of displacement of multiple individual particles with nanometer spatial resolution by using video microscopy. The post-acquisition analysis of the particle trajectories provides the dynamics of the single-particle diffusion, and thus the classification of different populations, but also provides information about the environment in which the particles are moving such as the mucus mesh network or mucus micro-rheology [11,144]. The fundamental principles of MPT have been thoroughly described elsewhere [211]. Briefly, the trajectories of fluorescently labeled particles in mucosal fluids are acquired. The analysis of the trajectories is mostly done in terms of the mean square displacement (< MSD >) over successive timescales. The mode of transport is represented by an exponential anomalous exponent (α). For $\alpha < 1$ restricted diffusion of particles is indicated and generally termed “sub-diffusive” transport. Values of α between 0.2 and 0.9 reflect the varying degrees of hindrance to particle movement; whereas α values < 0.2 represent particle immobilization. As an example, Hanes' group has assessed the particle diffusion of PEGylated mucus- penetrating nanoparticles through a range of mucus such as respiratory mucus [112,148], GI mucus [177], rhinosinusitis mucus [147] or CV mucus [141,176].

4.1.2. Quartz crystal microbalance with dissipation monitoring (QCM-D)

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a highly sensitive technique for real-time studies of the dynamic behavior of a layer of mucus deposited on a crystal surface. This technology based on piezoelectric effect measurements allows for the simultaneous monitoring of changes in frequency (Δf) and energy dissipation factor (ΔD), which are related to the mass adsorption onto the sensor surface and viscoelastic property of the adsorbed layer, respectively. Therefore, QCM-D is a powerful technique to elucidate interactions on various

surfaces [212]. QCM-D has been adapted to investigate the interaction of polymers and nanoparticles with mucin by immobilizing mucin on the surface of gold-coated crystals [213–215]. Different purified mucins have been tested, from commercial sources and from native gastric mucus. It has been demonstrated that the source of the mucin [215] and the buffer conditions (pH and ionic strength) [214], greatly influence the viscoelastic properties of the mucin layer and consequently result in different mucin-nanoparticle interactions. Traditionally, QCM-D has been applied to evaluate the mucoadhesive properties of polymeric materials and nanoparticles [160,213]. Recently, the QCM-D technique has been optimized to study also the mucus penetration of the nanocarriers [214,215]. QCM-D can record the different overtones of the oscillating system by sequential multi-frequency measurement. Each overtone has a specific penetration depth, thereby measuring the behavior at different depths of the mucin layer. Thus, if the nanoparticle only adsorbed onto the mucin surface, the overtone response would be very different from that of a nanoparticle penetrating the mucus. On the contrary, complete penetration through the mucin layer would lead to a similar overtone behavior.

4.1.3. Rheology

The mucin-polymer interaction (mucoadhesion of polymers) has been widely identified by means of rheological measurements using rheological synergism parameter [216]. The rheological response of a gel-mucin mixture should be larger than the sum of the individual contributions from the gel and the mucin. Through the monitoring of rheological synergism and other viscoelastic properties such as the storage modulus and the loss modulus, the particle-mucus interactions can be determined [209]. On the other hand, mucus viscoelastic properties can be affected upon interaction with the nanoparticle formulations. Thus, das Neves *et al*. studied the rheological properties of simulated vaginal fluid after the addition of polymeric nanoparticles with different surface properties [161]. Muller *et al*. investigated the mucolytic potential of papain-modified nanoparticles by using rheological measurements. They found that the presence of papain on the surface and inside the particles strongly decreased the viscosity of the mucus, which significantly promoted the particle diffusion across the mucus layer [183].

4.1.4. Membrane-supported mucus diffusion system

The standard method for studying drug permeation through biobarriers such as epithelia and mucus *in vitro* is by using membrane-supported biomatrices such as the TranswellR system. In this model, donor and receptor compartments are separated by the biobarrier. By monitoring the flux of the investigated drug or nanoparticle to the receptor chamber, the apparent permeability coefficient (P_{app}) can be derived. Different groups have adapted this routine assay to evaluate the diffusion of macromolecules and DDS through a mucus layer. Friedl and coworkers studied the diffusion on different SNEDDS formulations through native porcine intestinal mucus [217]. A 0.33 cm_2 Transwell membrane insert was covered with 50 mg of intestinal mucus, resulting in a mucus layer of ca. 900 µm thickness. SNEDDS formulations were added to the donor chamber, and the permeated amount was monitored in the receptor chamber at specific time points. Similarly, Groo *et al*. deposited porcine intestinal mucus on a 1.12 cm_2
Snapwell membrane insert to study the diffusion of different formulations of paclitaxel-loaded solid lipid nanoparticles [218]. The membrane- supported mucus diffusion system also allows the parallel comparison of the diffusion of drugs through mucus alone, or through a combined model of mucus and an epithelial monolayers [143]. In our lab, we evaluated the effect of size and physicochemical properties of different peptide drugs on the diffusion through the so-called cell-

free mucus barrier model using porcine and biosimilar mucus. The parallel comparison with the standard Caco-2 cell monolayer and with the combined model also allowed independent evaluation of the barrier properties of the different components of the intestinal mucosa.

4.1.5. Other methods

Additionally, some advanced techniques have also been explored to investigate the nanoparticle-mucus interactions and diffusion. For instance, diffusion nuclear magnetic resonance (NMR) and small angle neutron scattering (SANS) have been employed to investigate the effect of enzyme-decorated nanoparticles on the mucus network [219]. Other authors used the fluorescence recovery after photobleaching (FRAP) technique to study the diffusion coefficient of molecules and virus-like particles in human cervical mucus [174]. In order to study the depth of diffusion, the rotating tube technique has been used [170,180]. This easy-to-implement technique allows the quantification of the penetration depth of the nanoparticles. Besides, the change of the nanoparticle surface properties after mucus interaction is often evaluated via size and zeta potential measurements. As an example, mucoadhesive nanoparticles that interact strongly with mucus showed aggregation upon incubation with diluted mucus [180].

4.2. Methods for characterization of nanoparticles-biomolecules corona

Interaction of nanoparticles with elements of the complex and dynamic biological matrix residing in the lumen as well as in the mucus often results in a biomolecule corona formed on the DDS surface. To date, many studies have focused on the protein corona formed after incubation with blood components due to the abundant and convenient availability [220,221]. However, the composition and characteristics of the biomolecule corona differs depending on the surface properties of the nanoparticles [222], and on the interactive players found at the mucosal site, which differ from the ones found in plasma [206–208]. Here, we briefly describe the methods commonly used for characterization of the biomolecular corona by means of composition and kinetics. Most of these methods have been optimized for the plasma protein corona although they can be easily adapted for the study of mucosal components.

4.2.1. Composition and structure of corona

The first step to analyze the corona composition is the isolation of nanoparticle-biomolecules corona complexes, typically through a differential sedimentation centrifugation [223]. Following protein detachment, the corona protein pattern can be visualized by gel electrophoresis and quantitatively analyzed using label-free liquid chromatography– high-resolution mass spectrometry (LC-MS) [223]. Bioinformatic methods such as proteomic and lipidomic analysis can be used to identify the composition of the biomolecular corona [224,225]. This protocol can also be used to obtain time-resolved protein corona profiles [205,225]; as the corona formation is a dynamic process. However, it should be noted that the approach can only determine the composition of ‘hard’ corona (i.e. strongly adsorbed biomolecules), lacking of the information on the composition of ‘soft’ corona due to the dynamic process of corona formation (i.e. the most abundant biomolecules are likely to bind first, but will be displaced with time by the biomolecules with high affinity [205]) and the complexity of sample preparation in the protocol. In addition, the structure (e.g. thickness) of particle–‘hard’ corona complexes can be investigated by using dynamic light scattering, transmission electron microscopy, and fluorescent correlation spectroscopy.

4.2.2. The dynamic and kinetic aspects of corona formation

As mentioned above, the corona is not at thermodynamic equilibrium. Fundamental understanding of the dynamic process is actually a prerequisite to correlate the nature of corona with its biological consequence. The more abundantly associated biomolecules do not necessarily have the most profound effect, whereas less abundant biomolecules with high affinity and specificity for a particular receptor may instead be a key player. The kinetics of the nanoparticle-biomolecule binding and evolution can be monitored by using dynamic light scattering (DLS), zeta potential, and UV-Vis measurements [208,222], among others. However, these techniques lack of information on adsorption properties such as affinity, maximum adsorption, adsorption constant rate, and desorption constant rate. The study of these adsorption processes is critical for the understanding of the formation and dynamic evolution of the biomolecular corona and its subsequent biological impact. Cedervall *et al*. developed an approach to study these parameters by combining different non-perturbing methods: i.e. size-exclusion chromatography gel filtration (SEC), isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) [226]. Other methods based on established techniques such as infrared and Raman spectroscopies, fluorescence correlation spectroscopy, or QCM-D are also used to study adsorption kinetics and biomolecules-nanoparticles interactions. The changes in the conformational structure of the proteins after adsorption on the nanoparticles have been studied by circular dichroism, infrared spectroscopy and NMR. The efforts on connecting the nature of corona to biological impact also led to the emergence of cut-edge techniques. For example, a flow cytometry-based methodology has been developed to detect the molecular motifs presented for biological recognition on the nanoparticle surface in biological milieu without isolation and purification processes [227]. Langhammer's group developed a novel technique based on nanoplasmonic sensor surface that enables real-time in situ analysis of corona formation without the risk of sample aggregation or the need for purification processes [228,229].

4.3. Effect of corona on nanoparticle properties

Immediately after administration, the DDS immediately interact with the biological environment and their surfaces are covered by individual biological components, which will differ depending on the administration site. This biomolecular corona has serious implications for the physicochemical properties of the nanoparticles, and influences their *in vivo* biological fate. After oral administration, the DDS encounter different GI fluids that vary in composition along the tract (see Section 2). These include zwitterionic phospholipids, bile salts and enzymes that can adsorb into the nanoparticle's surface and modulate their passage through the intestinal mucosa. It has been demonstrated that the diffusion of latex beads through intestinal porcine mucus was dramatically enhanced by the adsorption of bile salts [19]. These biosurfactants shifted the surface charge of the nanoparticles toward more negative values, reducing the interactions with the mucus network through repulsion forces. The composition of the intestinal fluids dramatically varies between fasted and fed state, having an impact on the interactions with the nanomedicines. The exposure to lipids had an impact on the mucus diffusion of PS particles [9], and silica nanoparticles agglomerated in fed-state simulated intestinal fluid [230]. This sensitivity to lipids can be alleviated by surface coating the nanoparticles with PEG [9], demonstrating once more that PEGylation is a good strategy to reduce the interactions with the mucosal components. Also, the digestive enzymes present during digestion may have an effect on the nanoparticles' fate. Walczak and coworkers showed that these enzymes affected the protein corona formed on the surface of PS

nanoparticles, consequently increasing their *in vitro* mucus translocation [231]. The respiratory tract lining fluid is the first biological matter that nanoparticles encounter after inhalation and deposition in the deep lung. This layer is mainly composed of a surface-active lipid-protein mixture, known as pulmonary surfactant (Section 2). The interactions of these components with the inhaled nanomaterials are of increasing interest, since they likely modulate the bioavailability and final fate of the nanomedicines and therefore their therapeutic and toxicological effect [206,232,233]. Some of these works focused on the identification of this biomolecular corona formed around the nanoparticles after deposition in the deep lung. A high amount of lipids, mainly glycerophosphocholine, was found to adsorb onto particles and was the same for different nanoparticles investigated [224]. The most abundant proteins found in the adsorbed corona did not reflected the concentration of the native protein surfactant [224]. These abundant proteins were the same regardless of the surface properties of the nanoparticles [224,225], however there was a marked difference in the total composition of the protein corona [224]. The formation of the biomolecular corona has been demonstrated to have biological consequences. Ruge *et al*. demonstrated that the adsorption of SP-A, SP-D and surfactant lipids modulated the nanoparticle uptake by alveolar macrophage (AM) [234]. Recently, these authors exploited this interaction for the design of macrophage-targeted nanoparticles [235]. Thus, PEGylated polymeric nanoparticles were decorated with mannose residues, which are known to interact with lectin proteins such as SP-A with high affinity. These mannose-decorated nanoparticles showed an increased uptake by AM both *in vitro* and *in vivo* only after interaction with the pulmonary surfactant containing SP-A. This study demonstrates that the characterization of the biomolecular corona is crucial for the understanding of bio-nano interactions and the advancement of the design of efficient DDS for mucosal administration.

4.3.1. Shielding of targeting ligands

The biomolecular corona is especially relevant for nanoparticles with grafted targeting ligands [221,236]. The protein corona formed around the nanoparticle surface establishes a barrier that hinders the interactions between the ligand and the cell surface, thus reducing or even completely losing the active targeting property [237,238]. Indeed, such decorated DDS often show promising results *in vitro*, but disappointing *in vivo* conclusions [239]. This *in vitro-in vivo* discrepancy is partially due to the use of *in vitro* incubating media that only partly mimic the *in vivo* biological environment. Different strategies have been proposed to bypass the shielding of the targeting ligands due to the protein corona. PEGylation of nanoparticle surface can mitigate the negative impact of the protein corona on the cellular targeting by reducing the protein binding [240]. However, this strategy may be not enough to preserve the targeting specificity [238]. Dai *et al*. showed that PEG molecules should not be longer than the ligand linker; otherwise, the PEG molecules interfered with ligand-receptor recognition [240]. The blocking of the active groups present on the surface of the nanoparticles with short molecules such as 2-mercapto ethanol [241], or zwitterionic amino acids such as cysteine [242], resulted in successful approaches to inhibit the corona formation and to preserve the active targeting capabilities. As mentioned, the vast majority of reported research focuses on the study of protein corona upon plasma or blood contact. Although considerable emphasis has gone into the development of non-injectable DDS for mucosal delivery in the past decade [243], studies involving the mucosal biomolecular corona are still scarce.

5. Expert opinion

Designs of more or less complex nanoparticles intended to protect and deliver drugs to epithelial surfaces for absorption or for uptake of the particle into the epithelium require transport through the surface-lining mucus layer. From the scientific literature, it is clear that the desired effects of nanoparticles with specific geometries and surface properties, e.g. targeting ligands, are highly impacted by the presence of the mucus biomatrix. Throughout the body, this matrix is highly variable in terms of composition, thickness and clearance rate and is also affected by natural variations related to e.g. intake of food, motility and health/disease state conditions. Thus, in many cases, the designed nanoparticulate DDS successfully evaluated in simplified *in vitro* and *ex vivo* systems do not perform sufficiently *in vivo*. One of the main reasons is that the biomolecule corona on the nanoparticle critically affects the characteristics of the drug delivery system to a degree that overall regulates the drug delivery potential of the designed nanoparticle. Interactions with components of the luminal fluids and with the mucus may thus lead to altered kinetics of the nanoparticle transport to the desired site. This includes 1) decreased level and rate of nanoparticle entry into the mucus, 2) insufficient diffusion rates in the mucus, 3) diffusion parallel to the epithelial surface rather than diffusion towards the epithelial surface. All of the above contribute to *in vivo* clearance by the dynamic nature of the mucus before reaching the epithelial surface. Also, the corona may affect epithelial uptake of the nanoparticles, e.g. by shielding targeting moieties, and even influence drug release rate and amount at the epithelial absorption site. It is a delicate balance to foresee the effects of the inevitable interactions with the variety of biological components that nanoparticle drug delivery systems are exposed to after dosing. To achieve this, sets of complementary tools must be implemented in the research to analyze the composition, the appearance, as well as the impact that this biomolecular corona has on mucus and epithelia interactions. Advanced analytical tools must be used in combination with relevant complex biosimilar samples of mucus to advance designs of nanoparticles drug delivery systems to overcome the mucus cloak.

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Table 1: Composition and properties of human intestinal fluid at fasted and fed state.

Component	State	Duodenum	Jejunum	Ileum
Constituents				
Total bile salt concentration (mM)	Fasted	1.6-2.8 [15,23–26]	2.2-3.4 [25,27,28]	n/a
	Fed	10.7-14.4 [23,24]	8 ± 0.2 [28]	n/a
Protein content (mg/mL)	Fasted	3.1 [15]	1-2.1 [27,28]	n/a
	Fed	6.1 ± 3.3 [24]		n/a
Phospholipids (mM)	Fasted	0.3-6 [8,26]	0.2-3 [8,28]	n/a
	Fed	2.6-6.3 [23,24]	3 ± 0.3 [28]	n/a
Glycerides (mM)	Fasted	1.7 ± 1.1 (DG), 5.9 ± 1.9 (MG) [23]	n/a	n/a
	Fed	4.7 ± 5.2 (TG), 6.5 ± 6.3 (DG) 8.1 ± 5.7 (MG) [24]	2.2 (MG), 4.4 (DG) [28]	n/a
Fatty acids (mM)	Fasted	n/a	0.9 [28]	n/a
	Fed	39-52 23]	13.2 [28]	n/a
Enzyme activity				
Proteases and peptidases (U/mL)*		20-100 (I), 60-1000 (EP), 150-1500 (LP) [6,7]		
Lipases (U/mL)*		100-1000 (I), 500-6000 (EP), 400-4000 (LP) [6,7]		
Amylases (U/mL)*		50-250 (I), 150-1000 (EP), 150-500 (LP) [6,7]		
Properties				
Total fluid volume (mL)*	Fasted Fed	43-184 [25,29]	43-212 [6,25,29]	43-105 [6,25,29]
pH	Fasted	5.6-7.0 [8,15,25,26]	6.5-7.8 [8,25,30,31]	7.5-7.8 [30,31]
	Fed	6.23 ± 0.52 [24]		
Osmolality (mOsm/kg)	Fasted	137-236 [15,25,26]	200-300 [8,25]	
	Fed	291-534 [15,24]		
Buffer capacity (mM/pH)	Fasted	5-28 [15,23,24]	2-3 [28]	
	Fed	18-30 [15,23,24]	13-15 [28]	
Surface tension (mN/m)	Fasted	32-34 [15,23,24]	27-29 [28]	
	Fed	27-29 [15,23,24]	26-28 [28]	
Bacterial content (CFU/g)		1×10^4 [6,32]	1×10^4 [6]	1×10^6-10^8 [6,32]

Fed state values were acquired 30-60 minutes after food intake when applicable. *Some references did not discriminate between intestinal regions. Abbreviations: Fa: Fasted state. Fe: Fed state. PC: Phosphatidylcholine. TG: Triglycerides. DG: Diglycerides. MG: Monoglycerides. I: Interdigestive. EP: Early postprandial. LP: Late postprandial. n/a: not available

Table 2: Composition, properties and function of lung surfactant fluid.

Component		Amount	Properties	Functions
Lipids	*PC*	60-70% [34,33]	More abundant compound is DPPC (40-50%), unsaturated PC: 17-25% [35]	Formation of the surfactant film, decrease the surface tension [34]
	PG	8-15% [34]	Anionic phospholipids	Little is known about the role of the other lipid components. Cholesterol modulates the organization and dynamics of lipid phases. [33]
	PI			
	PE	3-5% [45]		
	Cholesterol	3-8% [34]	Neutral lipids	
Proteins	*SP-A*	5-6% [45], 3-5% [34]	32kD, hydrophilic, consists of 18 SP-A monomers (octadecamer or six trimers), organized by means of covalent disulfide bridges and noncovalent interactions in the shape of a bouquet of tulips [35,46].	SP-A and SP-D are the important components of the pulmonary host defense system [38,46]. SP-A is also a film-association surfactant protein, which can accelerate the adsorption of surfactant phospholipids at the air-water interface [33].
	SP-D	~0.5% [45]	43kD, hydrophilic, consists of 12 SP-D monomers, three of which are joined to form a trimer. Four trimmers form a cross-shaped molecule [35,46].	
	SP-B	1-1.5% [45] 0.5-1% [34]	8kD, hydrophobic, positively charged, dimer[35,46]	Critical role in the formation and stabilization of pulmonary surfactant films [35,36,46].
	SP-C	1-1.5% [45] 0.5-1% [34]	4kD, hydrophobic [35,46]	

PC: Phosphatidylcholine. PG: Phosphatidylglycerol. PI: Phosphatidylinositol. DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. SP-A/B/C/D: Surfactant protein A/B/C/D.

Table 3: Thickness of the mucus barrier (in μm) in the gastrointestinal tract in humans and laboratory animals used in standard experimental settings.

Species	Stomach	Small intestine	Large intestine	Rectum
Human	106-175 [72–75]	10-162 [75,76]	9-218 [76–79]	88-155 [77,78]
Pig	51-222 [80,81]	25-53 [80,81]	14-56 [80,81]	40-58 [80,81]
Rat	124-277 [73,80,82]	73-480 [80,82]	63-830 [80,82]	115 [80]
Rabbit	31-69 [80]	30-38 [80]	48-65 [80]	n/r
Mouse	100-140 [83]	200-450 [68,83]	10-150 [83–87]	n/r

As not all studies discriminated between loosely adhered mucus and firmly adhered mucus, the two mucus layers were treated as one for all species. n/r; not reported.

Table 4: Examples of the effects of various intestinal disease states on the mucus barrier.

Condition	Type of disease	Effect on mucus	Ref
Consortia from feces	Infection	Increased susceptibility to degradation by glycosidases. Increased activity for colitis ulcerosa patients	[100,101]
Shigella flexneri and enteroaggregative *Escherichia coli*	Infection	Mucin degradation, hemagglutination and serum resistance (limiting the effect of bactericidal serum)	[102]
Vibrio cholerae	Infection	Promoted mucus gel penetration, detachment and spread of infection	[103]
Clostridium perfringens	Infection	Removal of almost all types of mucin O-glycans, thereby altering the physicochemical properties of the mucin	[104]
Heliobacter Pylori	Infection	Increased pH by converting urea to ammonia, and thus reducing viscoelasticity of the mucus and making it more permeable	[105]
Clostridium dificile	Infection	Can thrive in a firmicute-depleted environment caused by antibiotic treatment. Degradation of MUC2 to gain access to host cell surface	[106]
Ulcerative colitis	Autoimmune disease	Decreased adherent mucus thickness. Less mucus is associated with higher degree of inflammation	[77]
Crohns disease	Autoimmune disease	Increased adherent mucus thickness	[77]
Carcinoma	Cancer	Upregulation in MUC2 and/or MUC1 by tumor cells may promote tumor growth and shield the cells from the immune system	[89]

Table 5. Respiratory tract mucus composition and thickness in healthy and diseased state. The number of Xs indicates the relative abundance of the constituents in each disease state. Modified from [110].

Component	Healthy state	Asthma	COPD	Cystic fibrosis
Mucin	XX	XXXXX	XXXX	XXXX
Plasma proteins	X	XXXX	XX	XX
Inflammatory cells	X	XXX	XXX	XXXXX
DNA		X	XX	XXXXX
Actin		X	XX	XXXXX
Bacteria			XX	XXXXX
Mucus thickness		*X*	*XXX*	*XXXXX*

Table 6. Comparison of selected techniques for the characterization of nanoparticle-mucus interactions.

Techniques	Advantages	Limitations
MPT	Robust method, hundreds of individual particles can be tracked and analyzed; able to reveal the different modes of particle diffusion	Labeling of the particles are needed; the information based on the static condition; mucus usually need to be diluted due to the microscopic limitations; limited availability of human mucus
QCM-D	Labeling-free technique; insight into the nature of the interaction between mucin and nanoparticles; measurements under biologically relevant conditions	Lack of information on the interaction of nanoparticles with other components in mucus; unable to provide information on diffusion or penetration
Rheology	Ability to determine how the particles or excipients to influence the viscoelastic properties of the mucus	Dehydration during measurement may greatly influence rheological properties; difficulty in applying the technique to pathologically heterogeneous samples
Membrane-supported mucus diffusion system	Direct information on diffusion rates for penetration of nanoparticles through any kind of mucus, easy to mimic in vivo situation	Studies on bulk samples in complex mucus matrix; difficulty in gaining detailed/mechanistic understanding

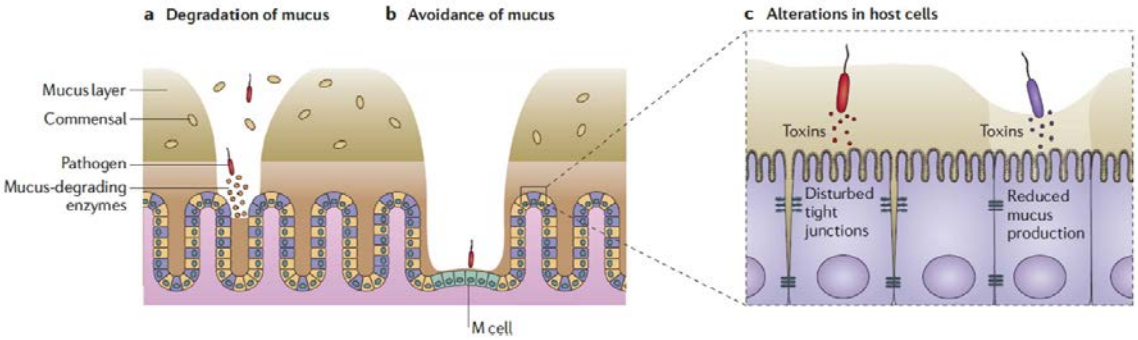

Figure 1: Effects of microorganisms on the intestinal mucus barrier. Pathogens degrade or migrate through the mucus barrier (a) or locate in areas with reduced mucus coverage (b), which limits the barrier capabilities towards otherwise harmless bacteria. As a result, the epithelial membrane may be compromised, which likely leads to reduced barrier capabilities of the membrane and reduced mucus production. Images from [96] with permission.

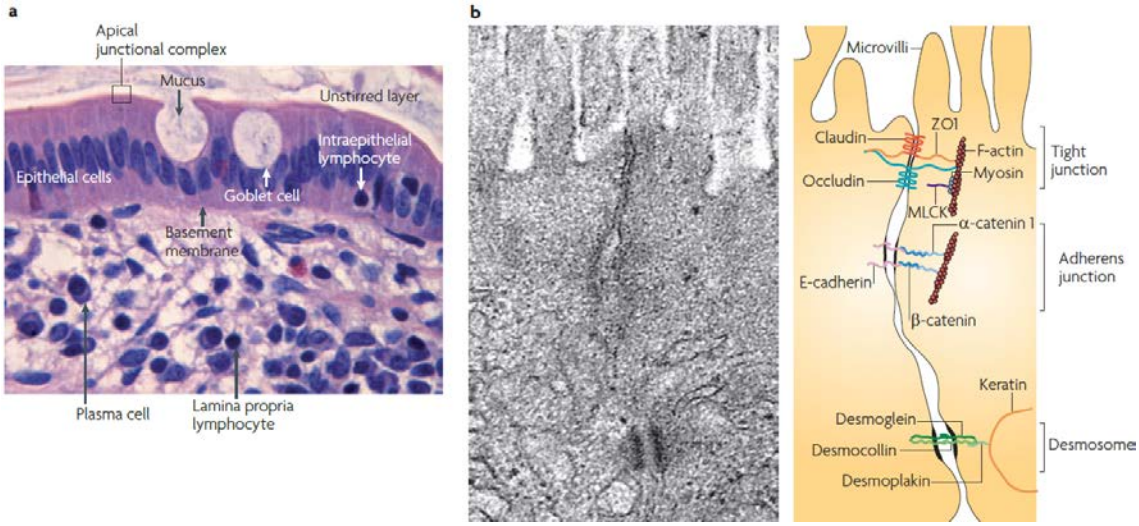

Figure 2: Morphology of the intestinal mucosal barrier. **a)** micrograph of the mucosal barrier with the apical surface of an epithelium covered by a mucus layer and a resulting unstirred hydrophilic barrier close to the epithelial membrane. The epithelial membrane consists primarily of absorptive cells, which drugs would need to penetrate from the apical side for intracellular drug delivery and exit through the basolateral membrane to reach the systemic circulation. **b)** micrograph (middle) and illustration of the interconnection between two epithelial cells leading to the formation of the tight junction regulated water channel. Images from [99] with permission.

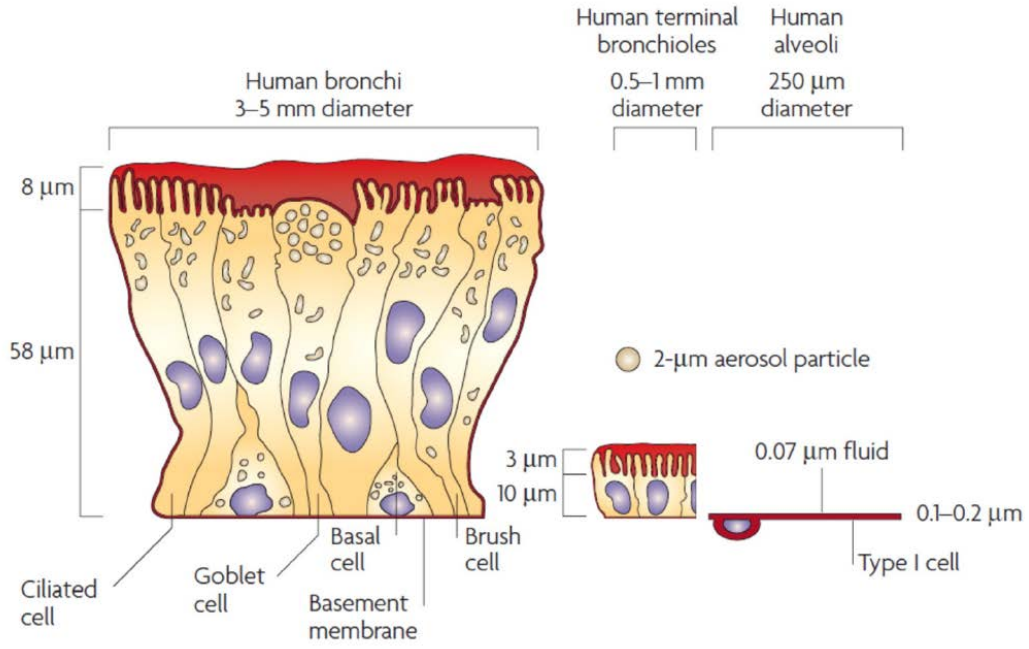

Figure 3: The lung epithelium at different sites within the lungs. From [133] with permission.

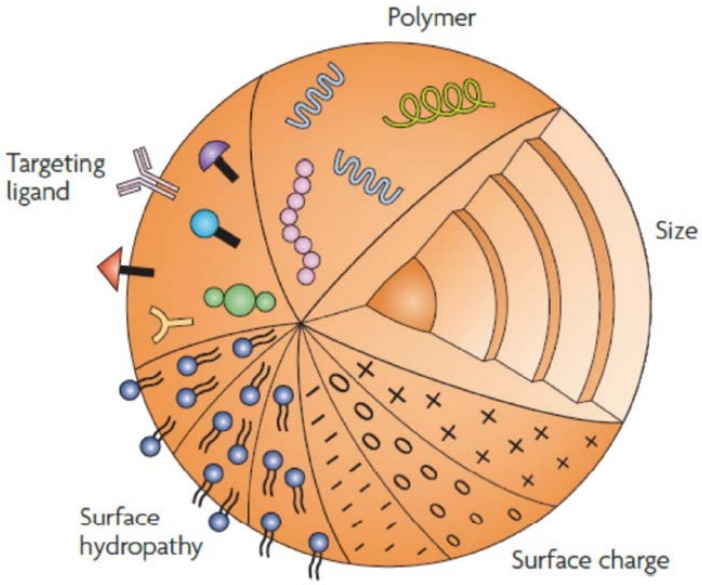

Figure 4: Illustration of the main properties that can modulate the interaction with mucosal components. Modified from [134] with permission.

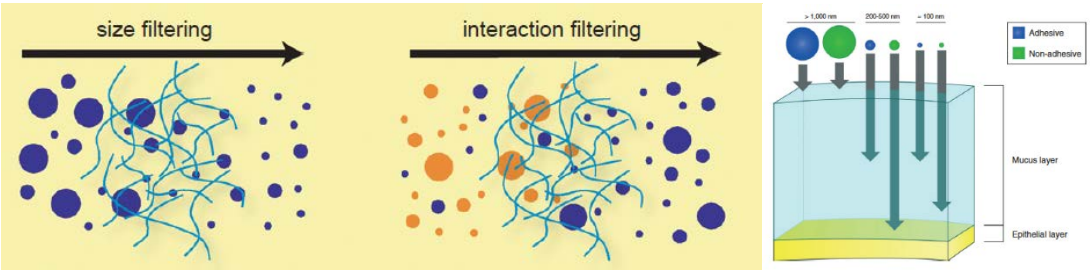

Figure 5: Illustration of the components of the mucosal static barrier. Left: Particles are filtered depending on size, as the mucus mesh only allows permeation of particles smaller than approximately 100 nm. Right: Particles are filtered dependent on physicochemical properties (charge, hydrophobicity). Sialic acid groups in the mucins will interact with positively charged groups, and hydrophilic glycan side chains will repulse hydrophobic groups. Modified from[17] and from [147].

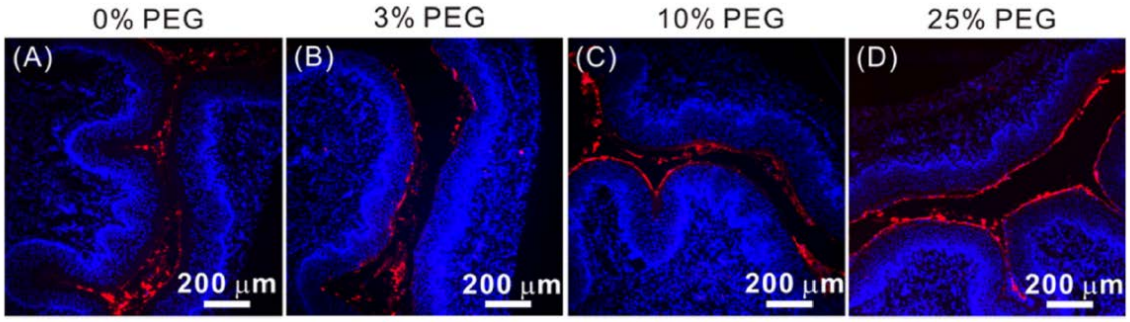

Figure 6: Distribution of PLGA nanoparticles with different PEG coatings in mouse vaginal mucus *in vivo.* The nanoparticles are fluorescently labeled (red), and the nuclei of the vaginal tissue stained with Hoechst (blue). The uncoated particles aggregated and did not penetrate the mucus barrier whereas the highly dense PEG surface coatings reached the vaginal epithelium. Modified from [173] with permission.