Chapter 7

# Challenges and barriers

Elizabeth Nance[1,2,3,4] and Michael McKenna[1]

[1]Department of Chemical Engineering, University of Washington, Seattle, WA, United States; [2]Department of Radiology, University of Washington, Seattle WA, United States; [3]Center on Human Development and Disability, University of Washington, Seattle, WA, United States; [4]Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA, United States

## 7.1 Overview

Depending on the route of administration, the intended target site, and the desired action of a therapeutic, a nanoparticle delivery vehicle must overcome a multitude of barriers to have the intended on-target, on-site effect. This chapter focuses on these barriers, which are broadly grouped into surface, en-route, and cellular barriers. En-route barriers are further subdivided into (1) the barriers that exist in the process of nanoparticle absorption across an epithelium to transport to an endothelial barrier and (2) the barriers that exist for a nanoparticle to passage across an endothelial barrier to a target cell within an organ. This chapter also discusses the challenges presented by the physical and physiological barriers at each step of a nanoparticle's route to a target site. Lastly, we highlight key findings in the field for how nanoparticles can be designed to overcome these barriers.

## 7.2 Surface barriers

Nanoparticles administered into the human body via transdermal, oral, inhalation, intranasal, ocular, vaginal, or rectal delivery first encounter an epithelial layer. The ability of a nanoparticle to deliver an active drug molecule to a site for absorption often requires traversing this epithelial layer. This passage can be hampered by hostile environments, for example, as resembled in the intestinal tract lumen where there is high enzymatic and hydrolytic activity.

### 7.2.1 Skin

For transdermal nanodrug delivery systems, the first barrier encountered is the skin. The human skin is the largest organ in our body, with a surface area of 1.8−2.0 $m^2$ in the average adult. It is composed of three main layers: the epidermis, dermis, and hypodermis (subcutaneous layer).[1] The skin protects the body against environmental factors and regulates heat and water loss from the body. For drug delivery, the skin is an important route when topical, regional, and systemic effects are desired, and it is also useful for avoiding hepatic first-pass metabolism.[2] Drug permeation through the skin is usually limited by the stratum corneum, which is about 15−20 cell layers thick. Nanoparticles applied topically to the skin can access several routes of delivery, which include passage (1) across the intact stratum corneum, (2) through the hair follicles with the associated sebaceous glands, or (3) via the sweat glands.[3]

The most used and investigated nanocarriers for dermal/transdermal drug delivery include liposomes, transfersomes, ethosomes, niosomes, dendrimers, lipid and polymer nanoparticles, and nanoemulsions. However, transdermal delivery systems have been limited to certain carriers of a range of size, molecular weight, lipophilicity, and charge preference. To overcome the skin barrier, hydrophilic molecules diffuse predominantly "laterally" along surfaces of the less abundant water-filled interlamellar spaces or use the free space between a lamella and a corneocyte outer membrane. Transcellular diffusion is generally considered minimal for transdermal drug transport.[4] In regions of narrow aqueous transepidermal pathways, the presence of poor cellular and intercellular lipid packing coincides with wrinkles on the skin surface, and they are the sites of lowest skin resistance to the transport of hydrophilic entities. Transport through the follicles has recently become of greater interest, although follicular orifices occupy only 0.1% of the total skin surface area.[5] Investigations in ex vivo porcine skin and in vivo human skin have revealed that polystyrene nanoparticles

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accumulate preferentially in the follicular openings in both species.[6–8] This distribution was increased in a time-dependent manner, and follicular localization was favored by smaller particle sizes. The current consensus in the field is that nanoparticles smaller than 20 nm can penetrate or permeate intact skin, while nanoparticles between 20 and 45 nm can penetrate damaged skin.[9] Larger particles can be translocated or may be stored in skin appendages.[2,9]

The skin carries a negative surface charge due to the presence of phosphatidylcholine and negatively charged groups on carbohydrates found in mammalian cells. Therefore, cationic compounds have a positive effect on skin permeation and nanoparticles with predominant positive charge would promote transdermal permeation.[10] Shape has also been identified as a factor that influences skin penetration. Rod-shaped particles have shown higher permeation in the skin than spherical particles.[11] Additional favorable physicochemical characteristics for transdermal delivery include a molecular weight less than approximately 500 Da and an affinity for both lipophilic and hydrophilic phases.[12]

## 7.2.2 Luminal space in the gastrointestinal and respiratory tracts

Nanoparticles administered to the respiratory or gastrointestinal tracts interact first with the luminal fluid. The luminal fluid is in contact with the surface of the mucus and varies in volume, dynamics, and composition. The luminal fluid in the small intestine is a dynamic mixture of enzymes, lipids, bile, bacteria, and cellular debris, with significant changes to the composition following food intake.[13] All of these components can interact with or influence the stability of a nanoparticle, altering the physicochemical properties of the nanoparticle. Further, the volume of the luminal fluid may affect the dissolution and distribution of the nanotherapeutic to the underlying mucosal layer. Importantly, the presence of enzymes in the luminal fluid can degrade a nanoparticle and the drug carried within the nanoparticle, prior to reaching the drug's site of action. The use of enteric coatings on nanoparticle formulations can provide protection from enzymatic degradation while transiting the gastrointestinal or respiratory tracts.[14]

## 7.2.3 Passage across the mucosal layer to underlying epithelium

For delivery to the nose, lung, eye, vaginal tract, rectum, or gastrointestinal tract, or administration methods that access these organs (e.g., intranasal, inhalation, oral delivery), a nanoparticle will encounter a mucosal layer prior to reaching the underlying epithelium.[15–18] The mucus constitutes a complex barrier to diffusion of nanoparticles toward the epithelial surface. Mucus is a viscoelastic hydrogel composed of large glycoproteins, predominantly of the mucin family.[19,20] Mucus production amounts to an average of 1 kg/day in an adult human. Mucus serves as an adhesive barrier to most foreign entities and also introduces steric hindrance and enzymatic activity that can impede or degrade a delivery vehicle.[19] The barrier properties, as discussed in this section, are variable throughout the body (Table 7.1) and also vary with patient age and health condition. The current understanding of the barrier properties of mucus are discussed in a recent review[21] and include an expert analysis on the effect on transport and therapeutic outcome.

The ability of nanoparticles to overcome the mucosal barrier and penetrate the mucosal layer has been extensively studied in recent years.[18,21,39,40] In general, the use of multiple particle tracking has shown that mucus has a pore cutoff size of 500 nm.[41] Spherical nanoparticles larger than 500 nm become sterically trapped, independent of surface chemistry. For smaller particles, surface chemistry can significantly impact nanoparticle mobility, retention, and transport to the underlying epithelium.[41–43] Mucus can bind nanoparticles and proteins via hydrophobic interactions.[44] Charged groups of the mucin proteins can also interact with charged particles and immobilize them in mucus.[45] The charge density in the mucus mesh depends on local ionic strength and pH. Under hypertonic conditions, charge interactions between mucus and particles will be partially shielded by ions in the fluid, reducing interactions below the levels seen in hypotonic fluids.[45] Negatively charged carboxylate- and sulfate-modified particles showed a higher transport rate than near neutral or positively charged amine-modified particles. The amine nanoparticle transport is limited by particle aggregation and electrostatic adhesive interaction with mucin fibers. The interactions of particle and mucus made by electrostatic–ionic interactions, van der Waals interactions, hydrophobic forces, and hydrogen bonding also influence nanoparticle retention at the mucosal surface.

### *7.2.3.1 Eye*

In the eye, the cornea and conjunctiva epithelia are covered by surface mucins. Ocular mucins are both secreted and cell surface-associated, and are distributed throughout the corneal and conjunctival epithelia, goblet cells, and the lacrimal apparatus.[15] They have heterogeneous functions at the ocular surface including (1) clearance of allergens, pathogens, and debris; (2) lubrication; (3) antimicrobial activity; (4) surface protection against abrasive stress (boundary lubrication); and (5) formation of an apical cell surface barrier.[15,46] A topically applied drug delivery system can be rapidly removed from the ocular surface through blinking, which shears anything off the tear film

**TABLE 7.1** Overview of properties of mucus that influence nanoparticle penetration and retention, where reported in literature.

| Type of mucus | Average thickness (μm) | Reported thicknesses (μm) | Clearance time | Clearance rate (mm/min) | pH |
|---|---|---|---|---|---|
| Respiratory | | | 10–20 min[22] | 2–10[22–24] | |
| Nasal | | | 8–9 min[25] | 5–11[24] | 6. 5[26] |
| Airway | 15 [27] | 7–30[28,29] | | 1–4[23] | 7.0–9.0 (Trachea)[30] |
| Bronchial | 55 [31] | 50–60[31] | | 2–3[24] | 7.0–9.0[30] |
| Gastrointestinal | | | 2–6 h (Mouth to colon)[32] | | |
| Gastric | 189 [17] | 160–200[17] | | | 1.0–2.0[32,33] |
| Duodenum | 170 [17] | 140–200[17] | | | 2.5–6[32,33] |
| Jejunum | 123 [17] | 115–125[17] | | | 4.5–6.5[32,33] |
| Ileum | 480 [17] | 400–500[17] | | | 7.0–8.0[32] |
| Colonic | 830 [17] | 700–900[17] | ~Hours to days (for oral administration route)[32] | | 7.0–8.0[32] |
| Ocular | | | 5–10 min[34] | | 7.0–7.4[32,35] |
| Mucus layer | 0.035 [36] | 0.02–0.05[36] | | | |
| Tear film | 5 [37] | 3–7[37] | | 1 μL/min34 | ~Slightly basic[36] |
| Female cervical vaginal tract | | | ~1–3 h[18] | | 3.5–4.5 (Healthy women)[38]<br>5.4–8.2 (Infertile women)[18] |

The values for the average and reported thickness of mucus are total thickness, which includes the thickness of both the loosely adherent and firmly adherent layers.

layer of the eye and results in a short drug retention time.[34] The flow of lacrimal fluid moves the drug to the nasolacrimal duct from the ocular surface in a few minutes, with a lacrimal turnover rate of approximately 1 μL/min.[47] Typically, less than 5% of the drug administered is retained on the ocular surface as a result of the corneal epithelium barrier and nasolacrimal duct drainage.[48,49] The cornea itself is approximately 500 μm thick.[50] The healthy corneal epithelium is lipophilic in nature with tight junctions, which leads to limitation of the permeation of hydrophilic molecules.[51] Chitosan has been the most widely explored nanoparticle coating to increase residence time in the precorneal region.[52] For intravitreal delivery, poly(ethylene glycol) (PEG) coatings on nanoparticles can increase distribution and retention in the eye.[53] For posterior segment delivery, nanoparticle residence time and localization depend on the size and surface properties. Particles in the range of 20−2000 nm have been retained at the site of administration for at least 2 months.[54,55] Positively charged nanoparticles can penetrate the vitreal barrier and reach the inner limiting membrane of the eye.[56] Negatively charged particles can penetrate the whole retina and reach the outer retinal layers such as the photoreceptor layer and the retinal pigment epithelium (RPE). In the presence of disease, negatively charged particles can reach even further to the choroid region due to disruption of the RPE.[57]

#### 7.2.3.2 Nose

Nasal delivery has conventionally been restricted to topically or locally acting therapeutic agents for the treatment of a nasal issue. More recently, nasal delivery has received increased attention as a substitute for oral and parenteral routes for several systemic therapeutic agents. The volume and surface area of the human nasal cavity is 15−20 mL and 150−200 $cm^2$, respectively.[58] The nasal mucosa lines the entire nasal cavity from the nostrils to the pharynx. A dynamic layer of mucus overlies the nasal epithelium (the outermost layer of cells of the nasal mucosa). The nasal submucosa underlies the basement membrane. This layer is made up of glands, mucus, nerves, an extensive network of blood vessels, and cellular elements like blood plasma. The entire mucosa is highly concentrated with blood vessels and contains large venouslike spaces.[59] Nanoparticles have been used for intranasal vaccine delivery,[60] to induce systemic and mucosal immunity, and for treatment of neurological disorders.[61] Although studies identifying particle size are not conclusive, the most commonly used size ranges are 40−200 nm.[62] Cationic particles increase residence time in the nasal cavity compared to anionic platforms. Cationic chitosan nanoparticles and polymer nanoparticles have demonstrated the ability to cross epithelium via tight junction disruption, and uptake in the brain.[63,64] However, cationic phospholipid nanoparticles used for vaccine delivery have not taken up in the brain, or crossed airway epithelium,[65] suggesting that nanoparticle composition might play a role in transport across the nasal mucosal barrier.

#### 7.2.3.3 Lung

An inhaled nanotherapeutic in the lungs encounters airway surfaces lined by ciliated epithelial cells and covered with an airway surface layer. The airway surface layer has a mucus layer that entraps inhaled particles and foreign pathogens, and a low viscosity periciliary layer (PCL) that lubricates airway surfaces and facilitates ciliary beating for efficient mucus clearance.[16,24] Normal respiratory mucus is composed of ∼1% mucins, ∼1% salt, ∼1% other proteins, and ∼97% water.[66,67] The hydration status of respiratory mucus is principally regulated by the export of $Cl^-$ through the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) and $Ca^{+2}$-activated chloride channels, and by the influx of $Na^+$ through the epithelial $Na^+$ channel.[68] By regulating these two processes, the epithelium controls the amount of water on the airway surface, which influences the thickness of the mucosal barrier. In normal, healthy lungs, after secretion and hydration of mucins, a thin layer of mucus (e.g., 2−5 μm thick in the trachea) is formed above the cilia from the bronchioles to the upper airway to protect the epithelium. The thickness of this mucosal layer is altered in the presence of disease, which can significantly impair nanoparticle transport to the airway epithelium.[27,28] Inhalation of drug-loaded nanoparticles has been a promising approach for the treatment of diseases such as asthma, chronic obstructive pulmonary disease, CF, and lung cancer. Nanoparticles with a primary or agglomerate particle size between 10 and 100 nm will deposit more efficiently in the alveolar region compared to particles with an agglomerate particle size between 100 and 1000 nm.[69−72] A large body of literature has focused on making nanoparticles mucoadhesive through the incorporation of cationic or thiolated surfaces that interact with negatively charged glycosylated or cysteine-rich hydrophobic domains of airway mucin fibers. Additionally, nanoparticle formulations with hydrophobic core polymers could interact with mucins through hydrophobic interactions. Nanoparticles that are mucoadhesive were thought to be retained for longer duration in the lungs due to the ability of these charged nanoparticles to change mucus rheology through multivalent mucus−particle interactions, which decreases mucociliary clearance (MCC) rates.[73] However, mucoadhesive nanoparticles are unable to reach the underlying epithelium due to entrapment in the mucus gel layer, which is rapidly cleared. Therefore, more recent findings have focused on the penetration and retention of nanoparticles that are mucus-penetrating.[40] Mucus-penetrating particles possess a dense brush layer of PEG on

the particle surface.[74–76] When particles are mucus-penetrating, they can reach the underlying PCL that is cleared less rapidly and diffuse to the epithelial surface.

#### 7.2.3.4 Gastrointestinal tract

Following oral delivery, a nanotherapeutic will encounter a heterogenous mucosal environment as it transits through the gastrointestinal (GI) tract. The thickness of mucus layer in the human intestine ranges from 100 to 900 μm (gastric compartment to colon) and consists of an outer, loosely adherent layer, and an inner, thinner, and more strongly adherent layer. The inner, strongly adherent layer has been estimated to be 116 μm thick in the colon.[17,41,77] The outer loosely attached layer is thinner in the small intestine (~100–170 μm) but thicker in the colon (600–800 μm).[17,41,78] In the intestinal tract, the ionic strength, ionic composition, and pH have all been shown to vary significantly depending on the location in the intestine, feeding status, and meal contents. Osmolality and ionic strength can fluctuate from hypotonic to isotonic to hypertonic within short distances in the gut after a meal.[79] Therefore, for orally delivered nanotherapeutics, particle interactions with the mucosal layers will depend partially on, among other factors, feeding state. Gut transit time will also play a role in nanoparticle transport across the mucosal layer because the GI tract is continuously motile and in various stages of activity.[80] As discussed in Section 7.2.3.7, gut transit time varies across species, therefore translation of results for nanoparticle retention from one study to the next should take into account the species and model used for the specified study.

Although there are many different sites within the GI that might be targeted with nanotherapeutics, there are general considerations that apply to the ability of nanoparticles to overcome or interact with the mucosal layer throughout the GI tract. There is a size limit to cross the intestinal mucosal barrier because the range of mesh pore spacing of the mucus is 50–1800 nm.[81] Hydrophobicity and surface charge play a key role in interaction with GI mucus. Particles with hydrophobic surfaces are generally considered mucoadhesive. Similarly, particles with positive surfaces, such as those coated or made with chitosan, show increased interaction and adherence to the luminal mucus gel.[82] Additionally, as with delivery to the lung, mucus-penetrating particles have shown greater ability to reach the underlying adherent layer, diffuse to the epithelial surface, and transverse the epithelium to reach systemic circulation.[83] Given the wide range of conditions in the GI tract, design of nanoparticles to leverage transport to a specific region of the GI tract should be considered. The reader is referred to several excellent reviews that discuss the various barriers, including gut transit time and feed state, to developing drugs for effective oral delivery.[39,84–87] For recent developments in targeting nanoparticles to specific sites in the GI tract, the reader is referred to a review by Ensign et al.[88]

#### 7.2.3.5 Vaginal tract

In the vaginal tract, cervicovaginal mucus (CVM) can have a significant impact on the penetration, distribution, and residence time of nanoparticle-based systems for vaginal drug delivery applications. Mucus produced at the cervix bathes and coats the vaginal walls, mixing with vaginal epithelial cells and vaginal transudate, and serves as a physical barrier to protect the vagina against infection. Outside the period of ovulation, the composition of CVM is composed mostly of water (~90%–95%) with gel-forming glycoproteins, lipids, soluble proteins, enzymes, and various immune factors.[19] During ovulation, cervical mucus becomes watery and mucin proteins align to allow sperm to pass more readily through the cervix into the uterus. However, ovulatory mucus is produced in more copious amounts, thus facilitating clearance and impeding drug absorption. Mucins in CVM from nonovulatory women and women on hormonal contraceptives form a tight meshwork, acting as a barrier to protect the epithelium.[89] Nonovulatory human CVM was recently found to have pores in the range of 50–1800 nm, with an average of 340 ± 70 nm.[81]

#### 7.2.3.6 Colorectal delivery

For certain applications, such as treatment of colorectal cancer and colitis, or rectal protection against sexually transmitted diseases, colorectal delivery may be utilized. Additionally, colorectal delivery can allow drugs to reach systemic circulation without degradation due to stomach acid or digestive enzymes and avoids the hepatic first-pass metabolism, discussed in Section 7.3.1. The colon absorbs 1.4–1.8 L of water per day, a process which is driven by active transport.[86] A wide range of nanoparticles sizes, from 100 to 500 nm are capable of penetrating the colorectal mucosal barrier,[83] as long as the particles are mucus-penetrating. Interestingly, what might be a more important factor than the nanoparticle design is the tonicity in which the nanoparticles are delivered. Further studies by Maisel et al. show that the ion composition of the fluid significantly affects nanoparticle penetration across the colorectal mucosal layer.[90] Nanoparticle retention and distribution was improved when nanoparticles were administered in moderately hypotonic enemas, whereas hypertonic and isotonic enemas reduced retention and limited distribution, even of mucus-penetrating particles, in colorectal tissue.[90]

#### 7.2.3.7 Mucosal variance across species

Many nanotherapeutics are tested preclinically in animal models. Therefore, it is important to acknowledge the mucus barrier shows large species variation. In the nose, the total mucosal area is correlated to the nasal surface area. The nasal surface area: body weight ratio of humans is 2.5 $cm^2$/kg, whereas in animals it ranges from 7.7 to 46 $cm^2$/kg for rats, rabbits, pigs, dogs, and monkeys.[91] In the GI, the rat intestinal mucus layer is 10-fold thicker or more in all segments of the intestine compared to the thickness in humans.[17,81,92] The variation in mucosal barrier properties and GI transit time differs species to species. The rat and mouse have a long GI transit time of 20−30 h, whereas the canine is approximately 6−8 h.[32] In the vaginal tract, the immunological and hormonal differences between murine models and humans alter the barrier properties of the mucosal layer,[39] as well. Each of these species-based differences can introduce a translational barrier for nanotherapeutics that must overcome the mucosal layer to be effective.

### 7.2.4 Mucociliary clearance

The understanding of mucus layer thickness, function, and clearance times at various mucosal surfaces is important to the development of nanoparticles, since they must penetrate mucus at rates markedly faster than mucus renewal and clearance in order to overcome the barrier. Mucus is continuously secreted, then shed and discarded or digested and recycled. The fastest turnover is typically observed at surfaces with the thinnest mucus layer. In the eye, the tear turnover rate under normal physiological conditions is in the range of 13%−20% per minute, leading to nearly complete clearance of most molecules and particulates from the eye within minutes.[93] In the nose, cilia lining epithelium coated with nasal mucosa create motions which drain mucus from the nasal passage to the throat, where the mucus is swallowed and digested by stomach enzymes. The activity level of cilia in the nasal cavity is dependent on temperature, where in cold temperatures cilia become less active. The mucus flow rate is about 7−12 mm per minute, and the mucus layer is renewed approximately every 20 min.[22,94,95] In the respiratory tract, the coordinated interaction of the mucus layer and PCL on the surface of the respiratory tract results in mucociliary clearance (MCC). MCC rates of 100−300 μm/s have been measured in the human trachea,[23] and the luminal gel layer of respiratory tract mucus is replaced every 10−20 min, leading to efficient clearance of inhaled particulates.[22] The sol phase of respiratory mucus is thought to be cleared much less rapidly than the more solidlike luminal gel layer. In the GI, peristaltic forces lead to quick turnover times of the mucus, on the order of 4−6 h.[96−98] In the vagina, mucus is cleared by intra-abdominal pressure as well as abdominal motions, which squeeze the walls of the vagina together.[99] The typical clearance time in the human cervical vaginal tract remains unclear, but is likely on the order of a few hours. Therefore, due to these differences in MCC along with differences in mucosal properties, the design of a nanoparticle to overcome mucosal barrier properties and mucosal clearance is dependent on target location and necessary site of action.

### 7.2.5 Absorption across an epithelial layer

If the target site requires access to systemic circulation, nanoparticles must absorb across the epithelium. Nanoparticle design factors that influence absorption across an epithelium include (1) physical and chemical stability of the particles and drug at the mucosal site, (2) residence times in regions of particle uptake, (3) interaction with mucosal contents and (4) transport through mucus, as discussed previously in this chapter, and (5) adhesion to epithelial surfaces. Nanoparticles can permeate across an epithelium by translocation via the tightly regulated narrow paracellular space, or by transport first through the apical plasma membrane (for intracellular delivery).[100,101] There is some evidence to support transport through the basolateral part of the plasma membrane (for transcellular delivery) to reach systemic circulation.[102] General mechanisms of nanoparticle uptake in cells are discussed in Section 7.4.

## 7.3 En-route barriers

Once a nanotherapeutic has absorbed across an epithelial layer it passes into systemic circulation. However, if administered orally, a nanoparticle must first overcome the first-pass effect. In this section, we will cover barriers introduced by the first-pass effect, circulation, passage across an endothelial layer, and transit through a tissue extracellular space (ECS) (Fig. 7.1).

### 7.3.1 First-pass effect following oral delivery

Extensive hepatic first-pass metabolism is one of the principal reasons for poor oral bioavailability of drugs. Hepatic first-pass (referred to as first-pass effect or first-pass metabolism) occurs when a drug absorbed from the GI tract is metabolized by enzymes within the liver to their water-soluble form, which facilitates excretion through the kidneys.[103] Enzymes that can act on a drug are GI lumen enzymes, gut wall enzymes, bacterial enzymes, and hepatic enzymes. Metabolism of a drug to its water-soluble form prevents the required amount of drug to reach systemic circulation, which can result in the need for higher doses of drug to achieve a minimum effective plasma concentration.

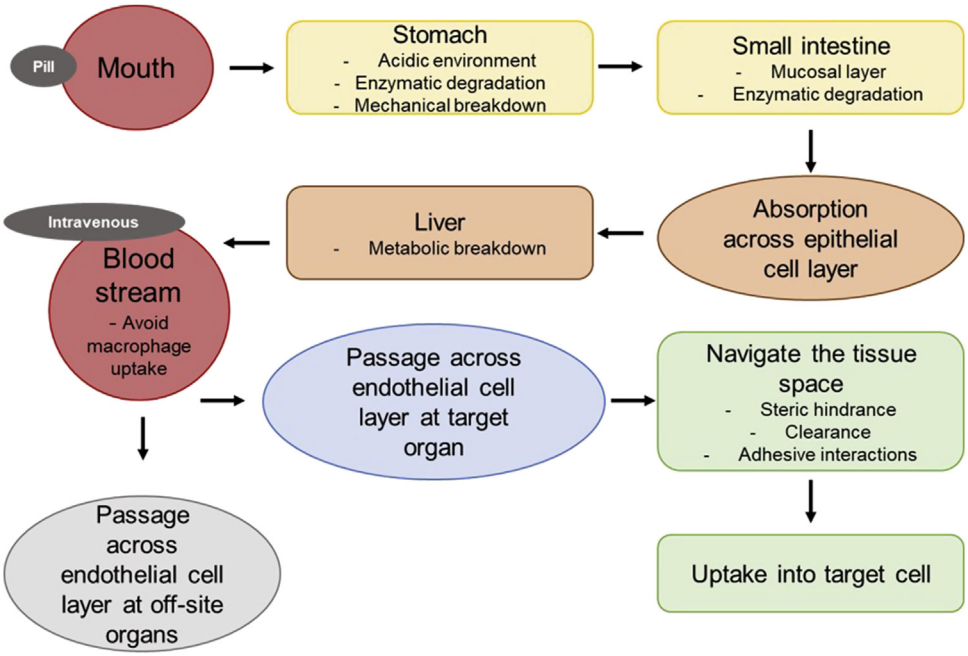


**FIGURE 7.1** Overview of biological barriers encountered by a nanoparticle when administered orally or through a route that reaches systemic administration.

When this happens, dose-related side effects can occur—the resulting metabolites may possess equal pharmacological activity or may have modified activity leading to increased or decreased effect, and the metabolites produced can be toxic compared to the parent drug. Nanoparticles have been commonly used to overcome the first-pass metabolism of most drugs through protection of the drug from enzymatic degradation by incorporation into the nanoparticle core, or through the use of surface coatings.[88] Surface coatings can decrease hepatic first-pass clearance by increasing transport in Peyer's patches, small patches of lymphatic tissue in the intestine, or uptake in M-cells, specialized epithelial cells associated with the immune system in the gut.[85,104] Using nanoparticles to target the intestinal lymphatic system can be useful for improving oral bioavailability of drugs that are susceptible to a high-degree of first-pass metabolism, such as steroids.[105–108]

## 7.3.2 Circulation

A large amount of focus in the nanotechnology field has centered around the barriers faced when nanoparticles are administered to or reach systemic circulation. These barriers include opsonization and subsequent sequestration by the mononuclear phagocyte system (MPS) (also referred to as the reticuloendothelial system (RES)), nonspecific distribution, hemorheological or blood vessel flow limitations, pressure gradients, enzymatic degradation, and cellular internalization.[109,110] Additionally, while in circulation, nanoparticles can become destabilized, have leakage or displacement of cargo, undergo degradation or disassembly, or have premature detachment of target ligands.[111] Nanoparticle size and surface chemistry or surface coating are the most common properties to tune to increase circulation time and reduce opsonization and MPS clearance. For example, highly cationic nanoparticles are rapidly cleared from circulation to a greater extent than anionic nanoparticles.[112] Neutral nanoparticles, as well as those with a slight negative charge, show significantly prolonged circulating half-lives.[113] Nanoparticle circulation studies have defined optimal size ranges to avoid excretion from filtration through the liver, spleen, and kidney. Generally, smaller than 10 nm unmodified nanoparticles are filtered by the kidney,[114] nanoparticles between 10 and 200 nm are captured by Kupffer cells in the liver and splenic macrophages,[115] and particles larger than 200 nm are retained in the red pulp of the spleen.[115–117] In addition, some studies have suggested that nanoparticles should be greater than 120 nm to avoid nanoparticle entrapment in the disse and hepatic parenchymal space.[118]

### *7.3.2.1 Nanoparticle opsonization and the mononuclear phagocyte system*

The MPS consists of a system of phagocytic cells, predominantly resident macrophages, in the spleen, lymph nodes, and liver (Fig. 7.2). The MPS can sequester nanoparticles immediately after systemic injection.[119] Sequestration can occur when nanoparticles become opsonized. Opsonization involves the adsorption of plasma proteins,

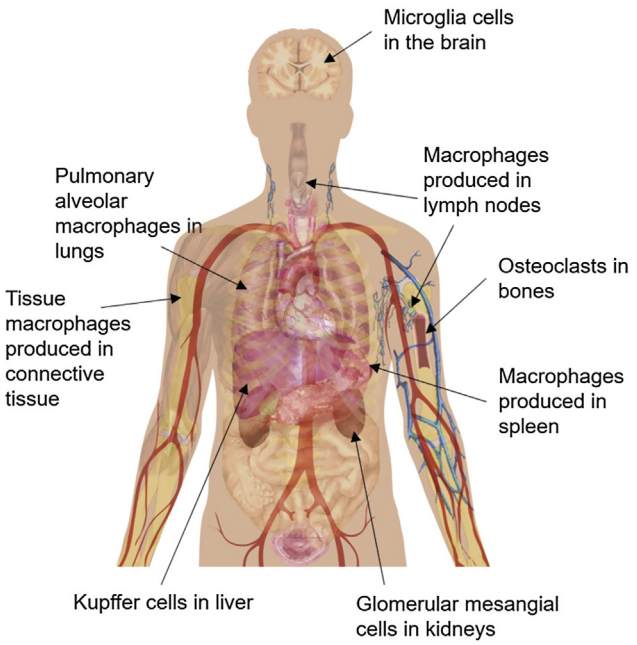


**FIGURE 7.2** Phagocytic cells that can internalize nanoparticles in circulation or in tissue.

including immunoglobulins, complement proteins, albumin, apolipoprotein, and fibrinogen, onto the surface of circulating nanoparticles.[120] There are many factors that can influence the process of opsonization, including nanoparticle size, surface charge, hydrophobicity, and surface chemistry.[121] Once proteins are adsorbed to the surface of a nanoparticle, the nanoparticle can undergo attachment to specific receptors on the surface of phagocytes, which leads to nanoparticle internalization. Nanoparticles are then transported to phagosomes and fused with lysosomes.[122] Opsonization can mask active-targeting ligands on the surface of nanoparticles, resulting in a reduction in specificity. The opsonization of nanoparticles and subsequent clearance by MPS could also initiate severe immunological reactions.

Opsonization can be reduced or avoided through controlling the surface coating of nanoparticles. For example, PEG has been widely utilized to shield particle surfaces from protein interaction,[123] effectively making them stealthlike in the body. PEG is a biocompatible, hydrophilic, biologically inert polymer that has been approved by the US Food and Drug Administration (FDA) for internal use, for a number of applications. PEGylation involves the grafting of PEG to the surface of nanoparticles. Ethylene glycol units form tight associations with water molecules, leading to formation of a hydrating layer[124] that hinders protein adsorption and subsequent clearance by the MPS. The density of PEG engraftment on the surface of nanoparticles can affect the effectiveness of the PEG layer.[75,125] While PEG is the most commonly utilized polymer for coating nanoparticles, other materials including polaxamers, polyvinyl alcohol, poly(amino acid)s, and polysaccharides[126] and zwitterionic compounds[127] have also been used to reduce nanoparticle opsonization. More recent advances have utilized cell membrane coatings (both lipidic and protein components) isolated from red blood cells or leukocytes on nanoparticles to reduce protein adsorption.[128,129]

### 7.3.2.2 Alterations of nanoparticle physicochemical properties in circulation

The adsorption of proteins to the surface of nanoparticles can potentially destabilize nanoparticles and lead to the premature release of their payloads.[130] When a drug is released prematurely in circulation, it eliminates the favorable pharmacokinetics of the nanocarrier and is then available to induce toxicity or susceptible to enzymatic degradation and clearance. Plasma proteins can also bind to or displace the encapsulated drug. The abilities of proteins to destabilize nanostructures are often measured and can vary protein to protein.[131] For example, polymeric micelles (e.g., PEG-b-poly(propyl methacrylate-co-methacrylic acid)/poly(amidoamine) and PEG-b-poly(D,L-lactide)) were tested with a series of proteins (e.g., albumin, α- and β-globulins, γ-globulins). Although most proteins were found to contribute to micelle destabilization, a more significant effect was observed for α- and β-globulins.[130] However, destabilization of nanoparticles may also result from interactions with other components in blood (e.g., blood cells) and the degradation of the polymeric constituents of the nanoparticle through hydrolysis or enzymatic activity.[132]

### 7.3.2.3 Blood vessel flow limitations and pressure gradients

Blood vasculature varies throughout the body based on blood flow needs and nutrient demand of tissues. The properties of blood vessels influence nanoparticle flow and partitioning to the endothelial surface. The capillary is the smallest of the blood vessels, ranging from 5 to 40 μm in diameter.[133] The capillary has a high surface area to volume ratio, maximizing the potential for blood−tissue exchange. Furthermore, the vessel walls of most capillaries in the body consist of an endothelial cell layer, just one-cell thick, thereby minimizing transport times across the vessel wall.[134] There is a hydrostatic pressure that exists between capillary and tissue, and an osmotic pressure that is the difference in protein concentration in blood and the interstitial space. The net difference in these two pressures causes fluid flow from capillaries into tissue, driving convective transport of macromolecules into the interstitial space.[135] Blood flow is dependent on the volume of blood,

viscosity of blood (as determined by composition), blood vessel length and diameter, and blood vessel curvature and branching, all of which can be influenced by age and health status.[136] Blood flowing within blood vessels is composed of blood cells, which make up 45% of total blood volume and are primarily red blood cells. Red blood cells accumulate preferentially within the center of the blood vessel, creating a cell-free layer closer to the endothelium, leading to small particle accumulation in this space.[137]

#### *7.3.2.4 Nanoparticle margination in blood flow*

In addition to blood vessel and blood flow characteristics, nanoparticle fluid dynamics in blood vessels is an important nanoparticle design consideration. In particular, margination dynamics influences nanoparticle association with vessel walls, which favors particle−cell binding and receptor−ligand interactions in active targeting strategies and enables extravasation through the fenestrated vasculature in most target organs. Margination is the lateral drift of nanoparticles to endothelial walls and has been shown to be influenced by nanoparticle size and shape.[138] Larger nanoparticles are subjected to a greater magnitude of drag force from fluid flow, which leads to lower interaction with blood vessel endothelium. Spherical geometries exhibit minimal lateral drift and are less likely to marginate to vessel walls and establish contact/binding points with endothelial cells. Nonspherical nanoparticles are more prone to tumbling and oscillatory effects in vasculature, which increases the likelihood of nanoparticle−cell wall contact and potential extravasation through fenestrations in vasculature.[139] Recent research has begun to explore the role of particle stiffness on nanoparticle margination in blood flow. Discher et al. have shown that increased flexibility of nanoparticles can increase circulation, through avoiding interaction and uptake by macrophages.[140] Top-down particle fabrication approaches, such as Particle Replication in Nonwetting Templates (PRINT), nanoimprint lithography, and thermostretched template−induced methods are providing ways to control nanoparticle stiffness.[141,142] From these techniques, the field has generally found that "soft" nanoparticles have longer circulation times in vivo compared to "hard" nanoparticles.[143,144]

### 7.3.3 Splenic clearance

Nanoparticles in blood circulation are susceptible to splenic filtration. The structure of the spleen enables the removal of older erythrocytes from the blood circulation as well as blood-borne microorganisms, cellular debris, and nanoparticles.[145] Many of these entities are captured by splenic macrophages in the marginal zone of the spleen. The maximal slit size in the spleen is estimated between 200 and 500 nm in width, but the spleen can also filter nanoparticles smaller than 100 nm.[132] Some studies have shown this can lead to a higher uptake of nanoparticles per unit mass than nanoparticle uptake in the liver.[117,146,147] Nanoparticles larger than 100−200 nm are incapable of crossing the endothelial slit of splenic sinuses, and instead are filtered off and retained in the red pulp. In the red pulp, these nanoparticles are internalized by red pulp macrophages and slowly destroyed.[115,116] Particle removal by splenic filtration tends to increase with size and is maximal for particles larger than 400 nm.[117,148] As nanoparticle size increases, Kupffer cell capture decreases, and splenic capture is enhanced.

### 7.3.4 Renal clearance

The kidney is capable of rapidly removing molecules from the vascular compartment with minimal catabolism or breakdown. Nanoparticles smaller than 10 nm can be excreted by renal clearance in the kidney, with smaller nanoparticles exhibiting faster excretion rates.[114] The renal molecular weight cutoff size is ~48 kDa (for some polymers such as PEG and dextran). Circulating nanoparticles will enter the glomerular capillary bed via the afferent arteriole. This bed is composed of three layers, including the fenestrated endothelium, the glomerular basement membrane (GBM) which is negatively charged, and podocyte extensions of glomerular epithelial cells.[149] Glomerular filtrate flows through the fenestrate, across the GBM, and through filtration slits formed by the spaces between podocyte extensions. This filtration slit introduces the primary size barrier for nanoparticles and has a pore size of 4.5−5 nm.[114] Nanoparticles that are less than 6 nm can be freely filtered, independent of molecular charge. Filtration of particles in the range of 6−8 nm is dependent on charge interactions between the particle and the negative charges of the GBM.[150] Based on this, positive particles are more readily filtered than negatively charged particles of equal size. Particles larger than 8 nm do not undergo glomerular filtration. Following glomerular filtration, nanoparticles enter the proximal tubule of the kidney where they can be resorbed into the luminal space for excretion. However, the brush border of the proximal tubule epithelial cells is negatively charged, so positively charged nanoparticles are more readily resorbed compared to negatively charged nanoparticles.[151,152]

### 7.3.5 Hepatic clearance

For nanoparticles that do not undergo renal or splenic clearance, the hepatobiliary system represents the primary route of excretion. The liver serves as a site of phagocytosis, catabolism, and biliary excretion of circulating

nanoparticles.[153] Nanoparticles in the size range of 10–200 nm can be rapidly captured by the liver. Phagocytosis mainly occurs through uptake by Kupffer cells, which have ciliated borders and stellate branches that act as mechanical traps for the removal of nanoparticles in blood.[114] Kupffer cells possess receptors for selective endocytosis of opsonized particles, including receptors for complement proteins. In addition to Kupffer cells, hepatocytes can clear nanoparticles via endocytosis and enzymatic breakdown. Hepatocytes are within the pathway for biliary excretion, and therefore particles processed by these cells are potentially excreted into the bile.[151] Kupffer cells are part of the MPS and rely exclusively on intracellular degradation for particle removal. Nanoparticles that are excreted via the biliary system are catabolized through hepatocytes. However, the phagocytic capacity of hepatocytes is much less than that of Kupffer cells. Interestingly, although uptake of particles from the blood to the liver may occur relatively quickly, hepatic processing and biliary excretion of these particles is relatively slow, often resulting in prolonged retention of NPs within the liver parenchyma itself.[114]

### 7.3.6 Endothelial barriers

Blood vessels throughout the body vary slightly in structure, but share the same general features. Importantly, in all blood vessels, an intact layer of healthy endothelial cells is essential for normal blood vessel function. Endothelial cell barrier function is attributed to the close alignment of endothelial cells in the vessel wall such that movement of water, proteins, and blood cells between the intravascular and interstitial compartments is controlled.[154] The endothelial barrier is formed by a layer of endothelial cells joined laterally by cell–cell junctions. The basolateral aspect of this layer is attached to a basement membrane composed of collagen, fibronectin, laminin, and glycosaminoglycans (GAGs). The permeability of blood endothelium is multifold, and the restrictiveness of transport depends on the organ, whether the endothelium is continuous or noncontinuous, and whether it is fenestrated or not.

#### *7.3.6.1 General endothelium structure*

Blood endothelium can be fenestrated, meaning the endothelial layer contains small holes, approximately 60–80 nm in diameter,[155] that allow diffusion of molecules and proteins.[156] Blood endothelium can also be discontinuous, with larger gaps than in fenestrated endothelium.[157] Discontinuous endothelia are found primarily in the liver and spleen where large macromolecules must easily cross the endothelium.[158] Similar to other epithelia, endothelial cells rest on a basement membrane. The basement membrane of endothelium provides structural support and also inhibits diffusion due to the presence of negatively charged domains on proteoglycans. The thickness of the basement membrane does vary throughout the body.[159]

Nanoparticles can penetrate the endothelium through several pathways. Depending on size, particles can passively diffuse through fenestrations or gaps in fenestrated or discontinuous endothelial layers.[160] Additionally, nanoparticles can passage across endothelial layers through transcellular, paracellular, or receptor-mediated pathways.[161] Paracellular transport occurs between endothelium cells, and transcellular transport occurs through endothelial cells. Nanoparticles can be actively targeted by grafting the surface or the shell of the nanocarriers with specific ligands or antibodies to molecules expressed on the endothelium. More recently, researchers have found that nanoparticles in the size range of 50–100 nm, a common size used for drug delivery, can take advantage of the transcellular caveolar pathway to traffic across endothelium.[162,163] In the presence of disease, the endothelium can become injured or dysfunctional, which can lead to discontinuities or breaks in the endothelial lining. The disruption of normal endothelial function and structure can lead to increased passive transport of nanoparticles across the endothelium.[154,164]

#### *7.3.6.2 Blood–retinal barrier*

The blood–retinal barrier (BRB) is a specialized transport barrier between the blood and the retina that has tight junctions between the monolayer of retinal pigmented epithelial cells and retinal capillary endothelial cells of the retinal circulation.[165] As a result of the anatomic position of the BRB, it effectively limits the transportation of molecules from the choroidal blood circulation to the posterior segment of the eye.[48] Moreover, the BRB also plays an important role in controlling the environment of the neural retina compared to the high blood flow and leaky walls of choroidal vasculature. In the choroidal vasculature, molecules easily enter into the choroidal extracellular gap, but have difficulty passing through the retinal pigmented epithelial layer.[166] Therefore, nanoparticle design would need to be tailored toward the specific barriers based on the intended site of action in the eye.

#### *7.3.6.3 Blood–brain barrier*

One of the most restrictive and exclusive barriers in the body is the blood–brain barrier (BBB). The BBB is a description of the structural interface that exists between the brain tissue and circulating blood. The BBB is continuous and not fenestrated.[167] There are about 400 miles of blood vessels in the adult brain, and almost all of these vessels consist of a layer of endothelial cells that line the capillary wall, with pericytes embedded in the basement membrane of the capillary, and astrocyte end-feet

ensheathing the capillary.[168] The astrocytes provide biochemical support to the endothelial cells, which in part regulates vasodilation and constriction of the blood vessels. Paracellular transport is utilized for ions and solutes that depend on a gradient of concentration. Transcellular transport of lipophilic molecules occurs primarily via passive diffusion. The balance between paracellular–transcellular transport is often the metric used to define the degree of permeability in a healthy BBB.[169] Hydrophilic molecules, like proteins and peptides, rely on specific transport through interaction with specific receptors on the surface of endothelial layers. Importantly, the brain endothelium expresses a family of efflux pumps, known as ATP-binding cassette transporters that can actively efflux foreign entities, including nanoparticles, into the blood.[170]

Nanoparticle physicochemical properties can be tailored toward a mechanism of passage across the BBB. It is generally thought that nanoparticles must be low molecular weight and lipophilic or amphiphilic to passively cross the BBB. Nanoparticles can pass through brain endothelial cells via transcytosis, accessed by activating receptors for transferrin and low-density lipoproteins.[171–173] Active targeting of these receptors has been achieved with peptides, proteins, or antibodies conjugated to the surface of nanoparticles. One well-studied mechanism for transport across the BBB is nanoparticles coated with polysorbate 80 (P80, also known as Tween 80). Nanoparticles coated with P80 absorb apolipoprotein E to the surface, which induces a receptor-mediated transcytosis process across the brain endothelium.[174,175] Other surfactants and surface coatings are being studied to determine if similar mechanisms occur. Additionally, targeting ligands such as transferrin have been commonly used to increase uptake across the BBB.[176] Nanoparticles can be transported through endothelial cells by endocytosis, as well, where content can be released in the cytoplasm and then exocytosed to the endothelium abluminal (brain parenchyma) side.[177] There are also recent findings that show nanoparticles can open tight junctions between endothelial cells, which leads to localized permeabilization of the BBB.[178] In the presence of injury or disease, the BBB is often impaired, which can result in increased nanoparticle uptake,[179–182] although the mechanism of this uptake is still being explored.

## 7.3.7 Extracellular matrix navigation

Once the barriers associated with endothelium have been overcome or bypassed, or if the route of administration is direct injection into the tissue of interest, nanoparticles must then be able to navigate the spaces that exist between cells to achieve sufficient distribution and induce a therapeutic outcome in distant diseased cells. There are multiple obstacles that can limit the effectiveness of this extracellular transport, including the tortuous geometry of ECSs, interactions with cellular surfaces, and interactions with the extracellular matrix (ECM). The ECM is a highly heterogeneous network of proteins and macromolecules that come together to form meshlike structures between cells.[183,184] This network presents both steric and adhesive barriers to any drug delivery vehicle attempting to travel the ECS. Sterically, the ECM hinders free movement by presenting additional physical structures that must be navigated. Adhesive interactions are brought about by the transient binding of nanoparticles to ECM-associated components or nonspecific interactions with fixed charges on the ECM. To better understand how these physical and adhesive interactions come about, it is important to have a grasp of both ECM composition and structure.

### 7.3.7.1 ECM Composition

The composition of the ECM is highly variable, both spatially—from tissue to tissue and even within tissues—and temporally—differing throughout development, disease progression, and wound healing.[184–188] This fluidity relates directly to the tissue-specific functions of the ECM and the roles it plays in pathological processes and wound healing. The dynamic nature of the ECM makes it impossible to define a constant chemical makeup. However, there does exist some underlying compositional principles that transcend all ECM structures. In general, the ECM is composed of some combination of two main classes of macromolecules: fibrous proteins and proteoglycans.[187–189]

Fibrous proteins (including collagens, elastin, fibronectin, and laminins) constitute the main structural elements of the ECM, providing tensile strength, regulating cell adhesion, and directing tissue development.[184,190,191] Collagen, the most abundant of these proteins, possesses the ability to assemble into supramolecular complexes, such as fibrils and networks, depending on the resident tissue and the current needs of the local cellular environment.[192] Collagen fibers possess a near-neutral surface charge at physiological pH and thus predominantly act as physical barriers to extracellular nanoparticle transport.

Proteoglycans are composed of GAG chains covalently linked to a specific protein core (with the exception of hyaluronic acid) and form the basis of higher order ECM structures. The primary functions of proteoglycans (providing compressive resistance and trafficking cellular signals) can be attributed to the hydrodynamic and biochemical characteristics of their GAG components. GAGs are long, negatively charged, linear chains of disaccharide repeats. The fixed negative charges present on GAGs allow them to attract positively charged ions and form osmotically driven hydration layers. These hydrodynamic properties are utilized for specific roles in multiple tissues and are known to be abundant in cartilage and

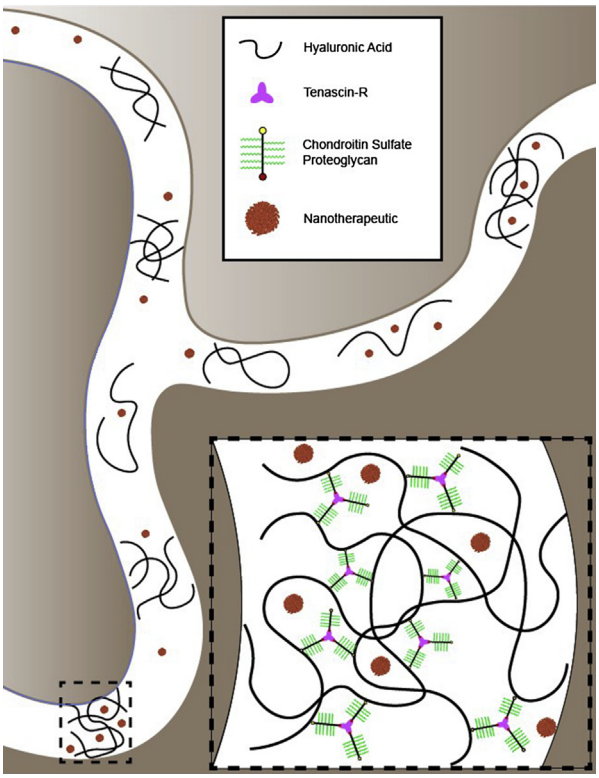


**FIGURE 7.3** Nanoparticle-based therapeutics (orange) attempting to navigate the extracellular matrix (ECM) of the brain. The ECM of the brain is composed of hyaluronic acid, chondroitin sulfate proteoglycans, and glycoproteins such as tenascin-R.

neural ECM.[193,194] As noted, the ECM of the brain is a good example of a matrix rich in proteoglycans. Brain ECM is composed primarily of a hyaluronic acid backbone cross-linked by chondroitin sulfate proteoglycans (a distinct class of proteoglycans) and glycoproteins, such as tenascin R (Fig. 7.3). Any nanoparticle-based drug delivery vehicles attempting to navigate the brain ECM, such as those depicted in Fig. 7.3 (displayed as orange spheres), are consequently subject to potential repulsive or attractive forces brought about by electrostatic interactions with negative charges present on the chondroitin sulfate GAG chains, as well as hydrophobic interactions with proteoglycan core proteins. Similar to the previously mentioned fibrous proteins, proteoglycans also present steric obstructions to free nanoparticle movement within the ECS, while also altering local viscosity gradients. The extent at which these physical and adhesive interactions interplay is dependent on the ECM structure.

#### *7.3.7.2 ECM Structure*

Due to the variation in ECM composition and function throughout the body, the ECM can take on a wide range of structures. In load-bearing tendons, for example, the ECM can assemble with highly ordered and specialized axial and longitudinal organization.[195,196] On the other end of the spectrum, the ECM can be entirely amorphous. Typically, GAG-rich structures, such as the ECM of loose connective tissue, take on a fluidlike state.[194,197] ECM components can either be bound to cells or free floating within the ECS, depending on both the local composition and cellular environment.[183] As an additional layer of complexity, ECM structures can change frequently and are known to rearrange in the presence of disease, throughout development, and during aging.[187,188]

The ECM presents a unique challenge to any nanoparticle attempting to move within the ECSs of tissue environments. The composition of the ECM can alter local viscosity regimes, and its presence can lead to an enhanced drag on any particles subject to steric interactions with its structure. It also presents additional obstructions that must either be navigated through or steered around, increasing the mean free path a particle must take to travel a given distance. Nanoparticles are also prone to adhesive interactions with the ECM. These interactions are brought about by electrostatic and hydrophobic interactions with various components of the ECM. Collectively, these steric and adhesive barriers can significantly hinder the tissue penetrative ability of particles and should always be accounted for when developing nanoparticle-based therapeutics.

## 7.4 Cellular barriers

In many cases, nanoparticles must internalize into cells to have the intended therapeutic effect. When this is the case, nanoparticles must cross the cell membrane, traffic within the cell to the target intracellular compartment, and release the payload in a timely fashion, while avoiding premature degradation or exocytosis. In this section, we will discuss common cellular uptake mechanisms, and trafficking in and toward intracellular compartments to target subcellular organelles.

### 7.4.1 Uptake mechanisms

Nanoparticles must traverse the cell membrane lipid bilayer, which is typically 4 nm thick. Low molecular weight (<1 kDa), hydrophobic molecules are capable of simple diffusion through the lipid bilayer membrane of cells; however, microscale and nanoscale supramolecular constructs require active uptake mechanisms. Nanoparticle surface charge is a major determinant of cellular internalization, with charge-based uptake highly dependent on cell type. With different surface modifications, nanoparticles can be taken up via specific (receptor-mediated) endocytosis or nonspecific endocytosis.[198] Additionally, size,[199] shape,[200] and particle rigidity[201] are key parameters, especially for internalization of nanoparticles via phagocytosis.

It is important to note that the use of PEG to overcome previously discussed barriers can often hinder or limit nanoparticle interactions with cell membranes, reducing total nanoparticle uptake. Recent research has focused on the incorporation of labile bonds in the PEG chain, so that nanoparticles become unPEGylated upon reaching the target cells.[202] This can be controlled to lead to increased rates of membrane destabilization, transport of the loaded cargo inside the cells, and release of the nanoparticle from the endosome.[203]

Nanoparticles can uptake into cells via several mechanisms. Phagocytosis is mainly conducted by specialized mammalian cells (like monocytes, macrophages, and neutrophils) as discussed previously in the chapter, and referenced in Fig. 7.2. Phagocytosis occurs for particles that have undergone opsonization or for solid particles with diameters >750 nm.[204] In this case, the cell membrane forms an internal phagosome containing the nanoparticle. Phagocytic cells, particularly macrophages, tend to show a strong preference for rigid particles. Shape also influences nanoparticle internalization in phagocytic cells, based on the aspect ratio of the particle when it first comes into contact with a cell.[205,206] Several studies have demonstrated the ability of a macrophage to internalize an ellipsoid particle within a few minutes when the cell contacts the pointed first, whereas the same ellipsoid particle takes over 12 h to internalize if the cell contacts the flat side first.[205] The mechanism of this effect originates in the complexity of the actin structure required to initiate uptake and was, to an extent, independent of particle size.

Endocytosis is a form of active transport in which a cell takes in objects by enclosing them in vesicles or vacuoles pinched off from its cytoplasmic membrane. The known endocytic processes that enclose nanoparticles in membrane vesicles in an energy-dependent manner are mainly via phagocytosis, pinocytosis, and caveolae-dependent or clathrin-mediated endocytosis.[207,208] Smaller particles ranging from a few to several hundred nanometers are internalized by pinocytosis or macropinocytosis, which occurs in almost all cell types.[209] Macropinocytosis involves fluid-phase uptake via membrane protrusions on the cell surface, and often occurs for particles larger than several hundred nanometers. Energy-dependent clathrin-mediated endocytosis is probably the primary characterized mechanism for the cellular uptake of nanoparticles, in which cargo is deposited in small endocytic vesicles (usual diameter < 100 nm) that fuse with early endosomes. Nanoparticles can also uptake via caveolin-mediated endocytosis, which involves specific receptor binding.[102,163,207] Caveolae/lipid rafts, consisting of plasma membrane invaginations of 50−80 nm in size, contain cholesterol, sphingolipids, and caveolins. For endothelial cells, the caveolae-mediated endocytosis is the important cellular uptake pathway for nanoparticles. More recently, a plethora of additional mechanisms have emerged that use clathrin- and caveolin-independent pathways, many of them relying on the cholesterol-dependent clustering of lipid-anchored proteins into diverse microdomains. It is important to remember that the heterogeneity of nanoparticle surfaces and potential polydispersity in nanoparticle size will require multiple uptake pathways to be involved in internalization in the cell.

### 7.4.2 Intracellular trafficking

Phagosomes formed from internalization of nanoparticles via phagocytosis are ferried through the cytoplasm. Actin becomes depolymerized from the phagosome, allowing the vacuole membrane to become accessible to early endosomes.[210] The vacuolar membrane will mature through a series of fusion and fission events, eventually fusing with late endosomes and ultimately lysosomes to form a phagolysosome. This process can take anywhere from a half hour to several hours depending on the surface properties of the ingested particle.[211]

Once a nanoparticle is internalized via macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis, the nanoparticle will then be trafficked within the cell. Macropinocytosis leads to the formation of a macropinosome, which is thought to eventually fuse with lysosomes or recycle its content to the surface. Clathrin-mediated endocytosis of a nanocarrier leads to the formation of an early endosome, which is acidified and fuses with prelysosomal vesicles containing enzymes.[208] This gives rise to a late endosome and then a lysosome, an acidic and enzyme-rich environment prone to nanocarrier and drug degradation. Unless a lysosomal delivery is desired, strategies for cytosolic drug delivery by this route focus on the drug escape from the endosome as early as possible. Caveolae-mediated endocytosis of a nanocarrier gives rise to a caveolar vesicle that can be delivered to a caveosome, avoiding a degradative acidic and enzyme-rich environment.

Internalization mechanisms and intracellular trafficking introduce additional barriers to effective therapeutic delivery with nanoparticles, given the harsh and highly degradative endosomal and lysosomal environments. Therefore, research has focused on strategies aimed at promoting endosomal escape or lysosomal avoidance altogether.[132] Several innovative "charge-conversion" strategies aimed at site-specifically switching the charge of nanoparticles in response to environmental stimuli, such as pH, have been utilized for protein-based nanocarriers,[212] polymers, and liposomes. Membrane-destabilizing peptides have been used to induce endosomal escape. Cationic polymers, such as poly(ethylene imine) (PEI) and poly (L-lysine) (PLL), have also been incorporated in nanoparticle design to release therapeutics from endosomal

compartments. The cationic charge of the nanoparticle interacts with the outer negatively charged surface of the endosomal membrane, resulting in membrane flipping and consequent destabilization.[213] The proton-sponge effect has been induced by polymers containing protonatable secondary and/or tertiary amine groups. With these polymers, protons are absorbed, which results in an influx of water into the endosomal compartment that leads the compartment to swell. With continual swelling, the endosomal compartment eventually ruptures, releasing its contents.[214]

### 7.4.3 Translocation to intracellular organelle

Incorporating multiple intracellular stimulus and tailored physiochemical materials properties of nanoparticles can release the drug to the intracellular organelles, such as cytosol, nucleus, mitochondria, Golgi, and endoplasmic reticulum (ER).[198] However, nanoparticles utilized for gene therapy face additional challenges. These include instability of the genetic material RNA and DNA-based therapies, and in the specific case of plasmid DNA, the need to translocate into the nucleus. Endosomal compartmentalization can degrade genetic material, but DNA that does survive endosomal escape can still be degraded by cytoplasmic nucleases. Nanoparticle encapsulation of DNA can help avoid early degradation, but will still need to enter the nucleus to be therapeutically effective. The nuclear envelope encases the cell genome and consists of a structure that is fluid with the ER. Nuclear pore complexes are locations where the inner and outer membranes of this envelope fuse together and are the sites of macromolecule trafficking for molecules smaller than 40 kDa in molecular weight. Therefore, most nucleic acid delivery systems are impermeable through nuclear pore complexes due to their large size.[215] To facilitate nuclear targeting through active transport, nuclear localization signal peptides have been developed to allow DNA nuclear entry. These peptides are short clusters of amino acids that can bind to DNA either through noncovalent electrostatic interaction or by covalent attachment, thereby increasing their entry into the nucleus and subsequent therapeutic benefit within the cell.

### 7.4.4 Exocytosis

While cellular uptake of nanoparticles is well researched, less is known about the elimination of nanoparticles from cells. Current techniques are often limited in differentiating between excretion of nanoparticles and degradation of nanoparticles. Studies that have explored cellular excretion mechanisms vary depending on the cell type and the nanoparticle type. Macrophages can exocytose up to 44% of iron oxide nanoparticles that are 15 nm or 30 nm in size within 7 days of internalization.[216] However, HeLa cells can rapidly exocytose 50% of 8 nm quantum dots within 100 min.[217] In general, exocytosis has been higher for smaller spherical particles.[218] Exocytosis of single-walled carbon nanotubes and gold nanoparticles suggests that an optimum size for exocytosis is 25 nm. Spherical particles are exocytosed less readily than rodlike particles, indicating a role for shape to play in this process, as well.[218] An extensive review of nanoparticle exocytosis discusses the effect of physicochemical properties and nanoparticle surface modifications of mechanisms of nanoparticle excretion from cells.[219]

## 7.5 Conclusions

There are many competing design considerations that influence a nanoparticle's path in vivo and eventual fate. To take into account the multiple interacting aspects of physiology and nanomaterial properties, the direction of nanoparticle applications should move toward the use of design mapping.[179,220] Synthesis of pathophysiological barriers and nanoparticle physicochemical properties into a broader system view creates an integrated approach to future work using nanotechnology to treat various diseases.

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