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REVIEW

Surface Modification of Lipid-Based Nanoparticles

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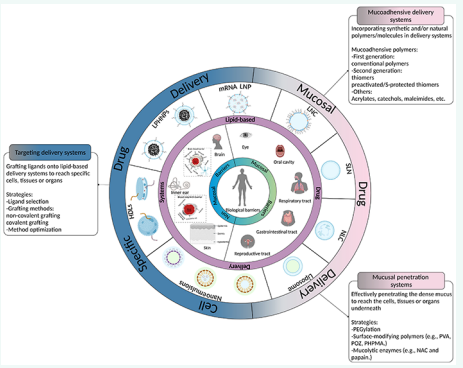
ABSTRACT: **There is a growing interest in the development of lipid-based nanocarriers for multiple purposes, including the recent increase of these nanocarriers as vaccine components during the COVID-19 pandemic. The number of studies that involve the surface modification of nanocarriers to improve their performance (increase the delivery of a therapeutic to its target site with less off-site accumulation) is enormous. The present review aims to provide an overview of various methods associated with lipid nanoparticle grafting, including techniques used to separate grafted nanoparticles from unbound ligands or to characterize grafted nanoparticles. We also provide a critical perspective on the usefulness and true impact of these modifications on overcoming different biological barriers, with our prediction on what to expect in the near future in this field.**



KEYWORDS: *lipid nanoparticles, targeting, surface modification, ligands, characterization, mucus, grafting, chemical reaction*

1. INTRODUCTION

Nanomedicines to enhance drug efficacy and/or safety have been developed and are currently commercialized. Among the vast array of nanomedicines, lipid-based nanoparticles are some of the most popular. For instance, liposomes were used to formulate Doxil, an FDA-approved product based on a nanomedicine, to improve the safety profile of doxorubicin.[1,2] Recently, lipid-based nanoparticles have been in the spotlight as one of the essential components of mRNA vaccines against COVID-19;[3] however, these nanoparticles are the result of decades of research and development efforts. Most likely, these vaccines would not have been efficient without the protection of the encapsulated mRNA and increased intracellular delivery that lipid-based nanoparticles provide. Lipid nanoparticles present several advantages that make them attractive when nanocarriers are needed. Lipid nanoparticles are biodegradable and biocompatible and present low immunogenicity. Their lipid components allow efficient incorporation of lipophilic drugs, which is critical as the proportion of new lipophilic drugs increases.[4] The size and surface properties of these nanoparticles can be easily tuned by using lipids with different physicochemical characteristics, such as their molecular weight, charge, and lipophilicity. Another attractive feature is the possibility of modifying the surface of the lipid nanoparticles with targeting moieties to achieve selective, cell-specific targeting.

In this review, we focus on strategies that have been developed in recent years to improve lipid nanoparticle performance: (i) improving mucus interactions, (ii) specifically targeting cells, (iii) crossing barriers, and (iv) modifying the drug release. We also address how to tailor the surface of lipid nanoparticles and characterize them, as these measures represent challenging steps that are not always treated with the attention required for clinical translation.

2. LIPID-BASED NANOPARTICLES

Over the last several decades, the array of lipid-based nanoparticles has rapidly expanded. In this section, the main types of lipid nanoparticles will be briefly introduced, that is, nanoemulsions, liposomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs),[5−8] lipid nanocapsules (LNCs), lipid−polymer hybrid nanoparticles (hybrids), and high-density lipoproteins (HDLs) (Figure 1). Nanoemulsions are colloidal dispersions prepared by combining water, oils, and surfactants, with the most important nanoemulsions being self-nanoemulsifying drug delivery systems for oral drug delivery.[9] Liposomes are composed of a phospholipid bilayer that surrounds an aqueous core, thereby being suitable for

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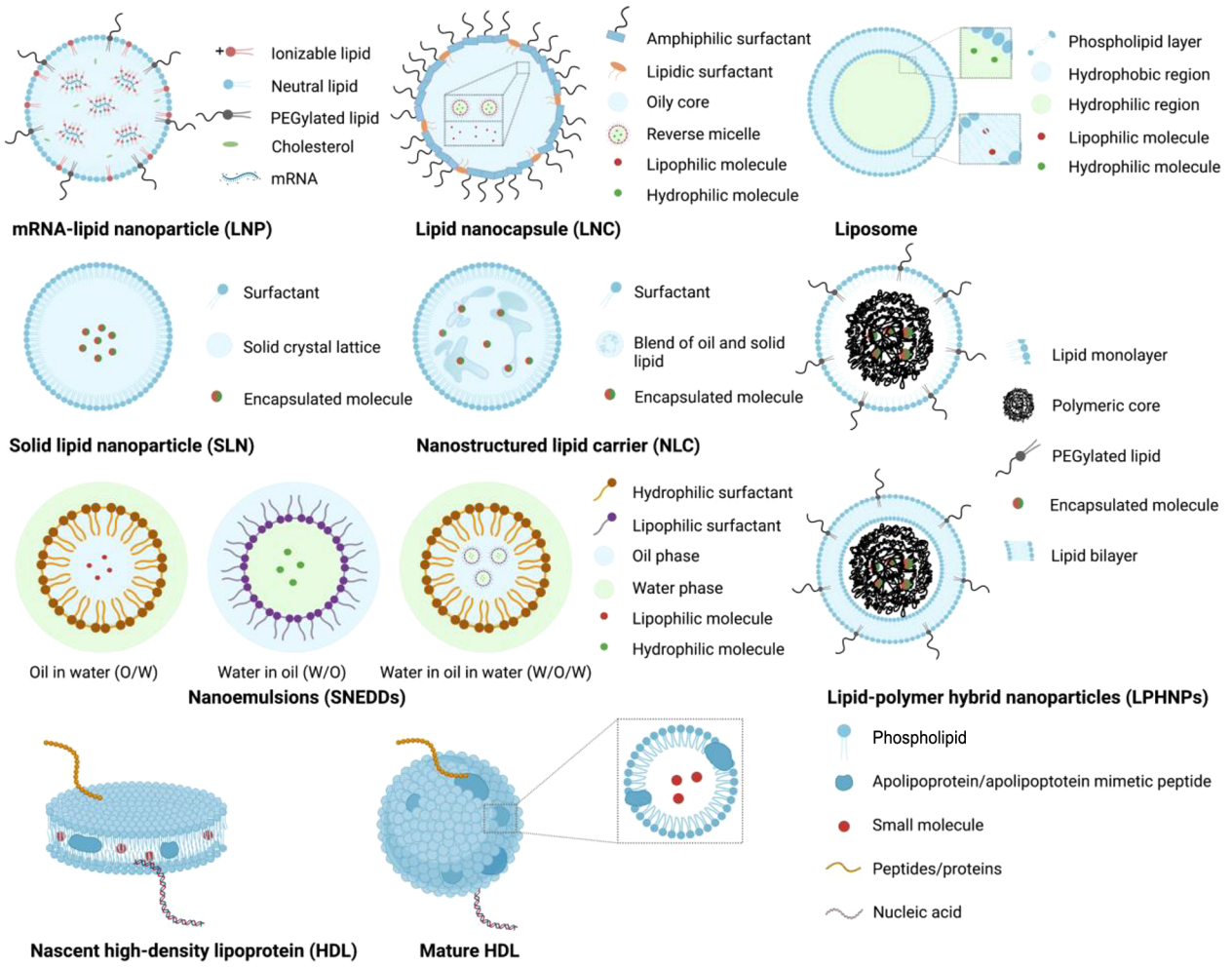

Figure 1. Structures of different types of lipid-based nanoparticles. Created with BioRender.com

encapsulating lipophilic, hydrophilic, and amphiphilic cargoes.[10] Liposomes present the advantage of the size and change tunability and can be used to encapsulate hydrophobic and hydrophilic cargos. SLNs and NLCs can be classified as solid core nanoparticles. They both have a strong protective effect on the incorporated therapeutics.[6,11] LNCs present a lipoprotein-like structure organized with an internal liquid or semiliquid oily core and an external PEGylated rigid membrane.[12] Hybrids are core−shell lipid-based nanostructures that combine a polymeric core encapsulating a payload and a phospholipid shell (e.g., a lipid-polyethylene glycol (PEG) layer). Hybrids can display the merits of both systems (liposomes and polymeric nanoparticles).[13−18] HDLs are dynamic natural nanoparticles, presenting an ultrasmall size (8−12 nm in diameter), biocompatibility, nonimmunogenicity, biodegradability, and intrinsic targeting properties to different recipient cells.[19,20] HDLs are considered promising drug delivery systems that can efficiently deliver imaging agents, small molecules, peptides/proteins, and nucleic acids to specific organs/tissues.[20] The detailed preparation techniques, physicochemical properties, and pharmacological applications of these lipid nanoparticles have been reviewed elsewhere.[15,20−22]

3. MODIFICATION OF NANOPARTICLE SURFACE TO INTERACT WITH MUCUS

Mucus is a complex biopolymer-based hydrogel located on the epithelial mucosae, that is, the eyes, nasal cavity, oral cavity, airways, gastrointestinal tract, and reproductive tract. Mucus is constantly secreted by goblet cells and submucosal glands and is eventually shed from the surface of mucosal tissues; it is a dynamic layer covering the surface of the epithelial membrane.[23,24] It has multiple functions, such as lubrication and reduction of dehydration of the epithelia, allowing carbon dioxide, oxygen, and nutrient exchanges with the underlying epithelium and limiting the entrance of pathogens and foreign substances into the body.[25,26]

Mucus consists of mucins, lipids, proteins, ions, salts, cells, cell debris, and water. Mucins, heavily glycosylated proteins, are the major structure-forming components of mucus, conferring its characteristic gel-like, cohesive, and adhesive properties.[27] There are 21 mucin-related genes whose expression and functions vary in mucosal tissues.[28,29] Depending of the mucosal tissue, mucus pH varies. For instance, nasal and lung mucus is neutral,[30] while vaginal mucus is acidic (pH 3.5−4.5).[31] The gastric lumen exhibits a large pH gradient (from ∼1 to ∼7), even in the same mucus layer.[32,33] Mucin proteins are aggregated in a highly acidic environment, which increases their viscoelasticity.[34] The thickness of mucus can also vary depending on the mucosal surface. Both pH and thickness of the mucus are

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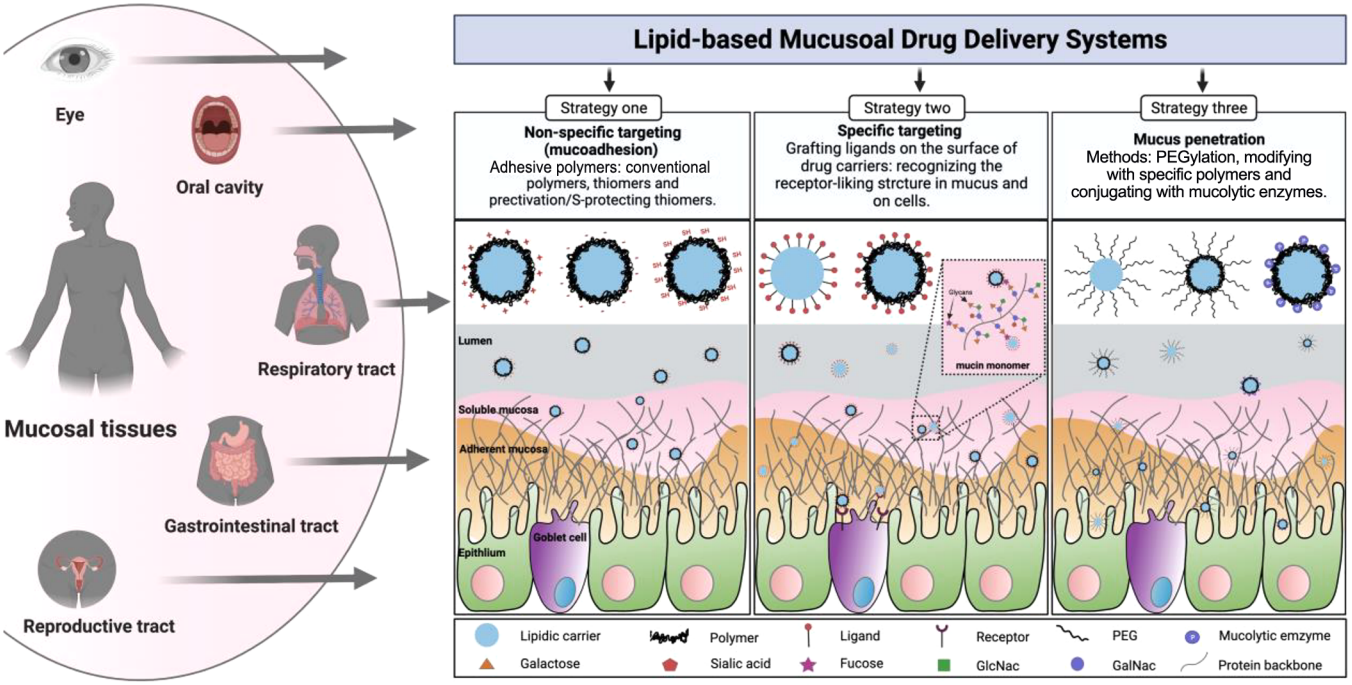

Figure 2. Graphic scheme of the mucosal delivery strategies of lipid-based nanocarriers. Created with BioRender.com.

Table 1. Summary of the Merits and Limitations of Mucoadhesive Polymers Used in Nonspecific Mucosal-Targeting Drug Delivery Systems

mucoadhesive polymers	merits	limitations
first generation		
conventional polymers	well-studied mucoadhesive polymers; natural sources; fewer required synthesis procedures; cost-effective; nontoxic	weak noncovalent forces (van der Waals or ionic interactions); short mucus retention time and rapid elimination; nonspecific adhesion to mucus; insufficient mucoadhesiveness; easily affected by the mucus turnover
second generation		
thiomers	strong covalent force (disulfide bond); strong mucoadhesion; long mucus retention time	relatively complex chemical synthesis; potential toxicity; oxidation instability and pH-dependent reactivity
preactivated/S-protected thiomers	more reactive covalent bond (disulfide bond); improved stability against oxidation; excellent mucoadhesion; extended mucus retention time; unaffected by pH levels	complex chemical synthesis; time-consuming and expensive; missing data regarding the safe use of these polymers
others		
acrylates, catechols, maleimides, *etc.*	strong covalent force (disulfide bond); strong mucoadhesion; long mucus retention time	complex chemical synthesis; insufficient data regarding different mucosal surfaces

associated with lesions of tissues/organs. The deficiency or overexpression of mucin proteins is associated with many pathological conditions, including ulcerative colitis, cancer, dry eye syndrome, and various respiratory diseases.[35,36] The composition and physicochemical properties of mucus in different body parts and in different diseases have been reviewed in detail elsewhere.[23,25,33,37]

We shall first describe how mucus can be targeted (Section 3.1) and then explain how lipid nanoparticles can be modified to interact with (Section 3.2) or penetrate (Section 3.3) the mucus (Figure 2).

3.1. Mucus-Targeting Strategies. Mucus targeting is defined here as the ability to adhere to a mucosal surface by both nonspecific and specific strategies. Mucosal-targeted drug delivery systems are obtained by incorporating synthetic and/or natural polymers/molecules into (nano)formulations, thus prolonging drug release at the targeted mucosal sites and/or enhancing drug absorption by mucosal cells. Nonspecific mucus targeting results from mucoadhesive polymer interactions with the targeted mucosal sites, mostly via electrostatic and sulfhydryl interactions.[38] Specific mucus targeting occurs when ligands (e.g., lectins, peptidic ligands and bacterial invasins) specifically recognize and interact with molecules composing the mucus or those that are present at the surface of mucus-secreting cells.[39−41]

3.1.1. Nonspecific Targeting (Mucoadhesion). Enhancing the mucoadhesion of lipid nanoparticles using bio/mucoadhesive polymer-based excipients was proposed in 1947[42] and has currently become the most popular strategy in the development of nonspecific mucosal-targeting delivery systems. The mechanism behind mucoadhesive polymer and mucosal site interactions is a complex and not well-understood process. The main physicochemical mechanism of this strategy is the entanglement of the polymer chain into the mucus network through hydrogen bonding, van der Waals forces, electrostatic forces, and hydrophobic interactions.[42,43] Polymer adhesiveness is affected by various factors, such as molecular weight, cross-linking, spatial conformation, surface charge, and the hydrogen-

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bonding capacity of the polymer.[44,45] In general, ideal mucoadhesive polymers are characterized by a high molecular weight, their 3D spatial arrangement, exposure of all active groups on the polymeric chain, a cross-linked network, a strong anionic or cationic charge, and strong binding functional groups (such as hydroxyl, amide, carboxyl, sulfate, and thiol).[46] Two generations of adhesive polymers have been reported to date[47] (Table 1).

The mucus layer is charged due to mucin glycoproteins, which initially led to the development of the first generation of mucoadhesive polymers (also known as conventional mucoadhesive polymers). They extend the retention time of delivery systems based on noncovalent bonds (e.g., hydrogen bonds and hydrophobic and electrostatic interactions) between their functional groups and the mucin glycoproteins. Anionic polymers present with carboxyl and sulfate functional groups, such as poly(acrylic acid), hyaluronic acid (HA), pectin, and alginate,[48] which bind to mucus via hydrogen bonds.[49] In addition to hydrogen bonding, cationic polymers primarily bind to mucosal sites via ionic interactions between amino functional groups and the sialic acid and sulfonic acid substructures of mucin glycoproteins in mucus.[50] Generally, noncovalent bonds formed between conventional mucoadhesive polymers and mucus exhibit an insufficient strength and structural stability, depending on the variation in the mucosal environment, such as ion concentrations, pH values, and the temperature. Moreover, because of a high mucus turnover, the insufficient mucoadhesiveness of the first generation of mucoadhesive polymers resulted in a short mucus retention time and rapid elimination (Table 1).

In addition to charged mucin glycoproteins, the cysteine-rich mucinous layer containing active sulfhydryl groups can spontaneously react with other sulfhydryl groups to form disulfide bonds. In thiolated polymers or thiomers that were reported in the late 1990s,[47] the introduction of immobilized thiol groups on mucoadhesive polymers resulted in significant improvements in their mucoadhesiveness.[48] Notably, thiolation prolonged the mucoadhesion time by up to 140-fold in comparison with that of unmodified chitosan.[51] The mucoadhesive capacity of thiomers depends on the rate and degree of disulfide-bond formation between the thiolated polymer and the mucus layer,[48] which is controlled by the degree of thiolation, the type of the sulfhydryl ligand, and the neighboring groups to the thiol group.[52] Since the reactive form is not actually a thiol but a thiolate anion, the pK_a value of sulfhydryl ligands has a great impact on the reactivity of thiomers. Generally, the lower the pK_a value of the thiol group of the chosen ligand is in the synthesis process, and the more thiolate anions are at a physiological pH, thereby leading to a higher reactivity of thiomers.[53] Additionally, anionic neighboring groups on the backbone of polymers accelerate the formation of disulfide bonds and vice versa.[52] Thiomers usually require a relatively complex chemical synthesis process. Bonengel et al. comprehensively summarized synthesis approaches and processes for thiomers.[53] Briefly, the synthesis approaches for thiomers mainly include amide, amidine, or amine-bond formation and the conversion of hydroxyl groups into thiol groups, which depends on the chemical structure of their parent polymers. From 1998 to the present, extensive work has demonstrated the effectiveness of thiomers in mucosal delivery. The safety of some thiomers, such as thiolated chitosan, thiolated HA, and thiolated poly(acrylic acid), has also been demonstrated in various clinical trials[52,53] (ClinicalTrials.gov identifier: NCT01887873). Nevertheless, the application of thiomers still faces some challenges, such as pH-dependent reactivity and oxidation instability (Table 1).[47] Generally, at pH values above 6, reactive thiol groups can form disulfide bonds with proteins in the mucus layer, resulting in a strong adhesion.[54] However, some mucosal sites, such as the stomach or the proximal small intestine, have a pH below 5, which results in a failure of thiomer adhesion in such sites.[55] Thiomers are stable in the dry state, whereas intermolecular and intramolecular disulfide bonds are formed in aqueous solutions containing oxidants, such as oxygen, at pH above 5.[53]

More recently, preactivated S-protected thiomers have been shown to form covalent bonds between thiol-bearing polymers and pyridinyl structures.[56] On average, the mucus layer contains almost twice as many free thiol groups as disulfide bonds.[57] The protection of thiol groups prevents premature oxidation prior to the contact with the mucus layer, whereby more active thiol groups can be in close contact with the mucosal membrane for a long time.[58] In addition, preactivated thiomers rapidly and quantitatively interact in a pH-independent manner through thiol disulfide exchange reactions.[58] The preactivation/S protection of the thiol groups of mucoadhesive polymers was introduced in 2012 by Iqbal et al.[59] who preactivated poly(acrylic acid)-cysteine (thiomer) by conjugation with 2-mercaptonicotinic acid. Preactivated poly(acrylic acid)-cysteine conjugates with a molecular weight of 450 kDa exhibited a striking mucoadhesive capacity that was 960-fold higher than that of the unmodified polymers.[59] Since then, the mucoadhesive properties of many other preactivated S-protected polymers, including preactivated chitosan (chitosan-thioglycolic acid-mercaptonicotinic acid),[60] S-protected glycol chitosan (glycol chitosan-*N*-acetylcysteine-2-mercaptonicotinic acid and glycol chitosan-glutathione-2-mercaptonicotinic acid),[56] S-protected pectin (pectin-cysteine-mercaptonicotinic acid),[61,62] preactivated gelatin (gelatin-thiobutylamidine-2-mercaptonicotinic acid),[63] S-protected alginate (alginate-cysteine-2-mercaptonicotinic acid),[64] and S-protected cross-linked poly(acrylic acid) (cross-linked poly(acrylic acid)-cysteine-2-mercaptonicotinic acid),[65] have been developed and used at different mucosal delivery sites (e.g., gastrointestinal, vaginal, and buccal delivery). Selecting these preactivated S-protected polymers with outstanding advantages as excipients for nontargeted mucosal drug delivery will be a major trend in the near future. In addition, at a very early development phase, many aspects will need to be improved and solved, that is, their complex synthesis and lack of toxicity data in vivo (animals and humans) (Table 1).

In addition to thiomers/preactivated thiomers, mucoadhesive polymers can be modified with other chemical functions to enhance their mucoadhesive properties. Bianco-Peled's group reported acrylated polymers, which underwent a Michael-type addition reaction between an acrylate end group on a polymer and sulfide groups in the cysteine-rich terminus of glycoproteins on the mucosal surface, thus exhibiting a strong mucoadhesion.[66] Using a similar mechanism, Khutoryanskiy et al. showed that polymers functionalized with maleimide groups had good mucoadhesive properties.[67] Catechol conjugated to mucoadhesive polymers (e.g., chitosan) also greatly improved their mucoadhesion by forming covalent bonds with the thiolate of cysteine in mucin.[68] It should be mentioned that these materials were introduced nearly 10 years ago, but their practical applications are limited.

3.1.2. Specific Targeting. Using mucoadhesive polymer-based excipients to increase the nanomedicine residence time

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Table 2. Mucosal-Targeting Lipid-Based Drug Delivery Systems (Nonspecific and Specific)

routes	mucosal-targeting ligand/mucoadhesive polymers	therapeutics	drug delivery systems	refs
buccal cavity	Carbopol 980 and polycarbophil	Ibuprofen	mucoadhesive gel with NLCs	85
	carboxymethyl chitosan	Cucurbitacin B	mucoadhesive film with phospholipid-bile salt mixed micelles	86
	chitosan (CS)	Genistein	mucoadhesive tablet with nanoemulsions	83
	Cys-PEG-SA	Cyclosporine A	Cys-PEG-SA-coated NLCs	87
	Polycarbophil and poloxamer 407	Curcumin	mucoadhesive gel with SLNs	88
	six types of mucoadhesive polymers	Curcumin	lyophilized sponges with SLNs	89
eye	CS	–	CS-coated SLNs	80
	chitosan-*N*-acetylcysteine (CS-NAC)	Coumarin-6	CS-NAC-coated NLCs	90, 91
	chitosan oligosaccharide (COS)	Coumarin-6	COS-coated SLNs	92
	positive charge	dexamethasone acetate, polymyxin B sulfate	nanoemulsions	93
gastrointestinal tract	CS	Alendronate	CS-coated liposomes	94
	CS	Amphotericin B	CS-coated NLCs	81, 95
	CS	Insulin	CS-coated SLNs	96, 97
	CS	Melatonin	CS/lecithin nanoparticles	98
	CS	Silybin	CS-coated SLNs	99
	CSKSSDYQC (CSK)	Salmon calcitonin	CSK-modified SLNs	74
	N-trimethyl chitosan-grafted palmitic acid (TMC-g-PA) mucoadhesive copolymer	Resveratrol	TMC-g-PA-coated SLNs	100
	polyethylene glycol 400, polyvinyl alcohol, and CS	Curcumin	mucoadhesive polymer-coated NLCs	101
	pectin	Calcitonin	pectin-liposome nanocomplexes	102
	S-protected chitosan-thioglycolic acid (CS-TGA-MNA)	Salmon calcitonin	CS-TGA-MNA-coated liposomes	103
	WGA	Oridonin	WGA-modified lipid-polymer hybrid nanoparticles (LPNs)	39
	WGA	–	WGA-modified liposomes	69
respiratory tract	alginate, CS	Fluticasone propionate	alginate/CS-coated solid lipid microparticles (SLMs)	104
	delonix regia gum (DRG)	Ondansetron hydrochloride	DRG-coated NLCs	105
	poloxamer 407 mixed with other mucoadhesive polymers	Almotriptan malate	mucoadhesive gel with SLNs	106
	sodium alginate polymer	Salmeterol Xinafoate	alginate-coated SLMs	107
	WGA, carbopol (CP)	Calcitonin	CP-WGA surface-modified liposomes	84
vaginal tract	CS	Clotrimazole	CS-coated liposomes	82
	glycol chitosan (GC)	*Chlamydia trachomatis* antibody	GC-coated LPNs	108
	hydroxyethyl cellulose	siRNA	lyophilized sponges with lipoplexes	109

could present some limitations, such as off-target mucoadhesion or premature inactivation of these polymers due to slime shedding.[24] By contrast, grafting molecules onto the surface of drug carriers that specifically recognize receptor-like structures of the mucus and underlying cells allows specific targeting by nanomedicines. This targeting is not only restricted to mucus binding but may also trigger nanoparticle internalization in the cell epithelium by endocytosis.[69] Typically, the ligands can be lectins, peptides, or bacterial invasins.[39−41]

Lectins are naturally occurring proteins from plants or bacteria that have a strong affinity for protein- and lipid-associated carbohydrates.[70] Plant-derived lectins are the most extensively studied ligands for mucosal targeting, as they recognize and bind to a particular array of sugars in mucins. Among plant lectins, wheat germ agglutinin (WGA) and *Ulex europaeus agglutinin I* (UEA-1) show the strongest mucoadhesion.[39] However, many lectins are toxic or immunogenic and are susceptible to proteolytic degradation.[71] To overcome these limitations, researchers have focused on the development of lectinomimetics (lectin-like molecules). These molecules are smaller, less toxic, and/or less immunogenic than natural lectins but still recognize their intended target.[69]

Compared to proteins, peptides have shorter sequences and lower immunogenicity and are easier to synthesize.[70,72] To date, only the peptide CSKSSDYQC (CSK) has been proven to target mucus-secreting cells (goblet cells).[41,73] Encouraging results have been obtained with CSK-decorated formulations[41,73,74] specific for mucosal targeting using peptidic ligands.

Bacterial invasins can also be used as ligands for mucosal-targeted delivery. Recent studies have shown that many bacterial pathogens strongly influence the formation, integration and function of the mucus layer, especially in the gastrointestinal tract.[75] Some mucosa-associated bacteria (e.g., *Yersinia*, *Listeria*, and *Salmonella*) are able to bind or degrade specific mucin glycans as a nutrient source.[75] Nikitas et al. found that *Listeria monocytogenes* can cross the intestinal barrier through the interaction between its surface protein internalin A and E-cadherin receptors that are expressed on goblet cells.[40] Of note, general information on ligand grafting strategies will be thoroughly discussed in Section 4 of this review.

3.2. Lipid-Based Nanoparticles in Mucosal Targeting.

Nanomedicines with mucus targeting properties have been widely developed to deliver therapeutics to different mucus-based regions, including ophthalmic, buccal, respiratory, gastrointestinal, and vaginal mucosa. Examples of polymeric

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Table 3. Impact of Pathology on Mucus Properties

diseases	mucus characteristics
cystic fibrosis	increased viscoelasticity, a higher concentration of physical entanglements and a thicker mucus due to impaired chloride ion channels;[135] both MUC5AC and MUC5B levels are increased[136]
chronic obstructive pulmonary disease	patients showed a 3−6-fold increase in the number of goblet cells in the airways and increased concentrations of the MUC5AC and MUC5B mucins in the lumen of small airways[137,138]
asthma	patients presented with a 2-fold increase in the number of goblet cells in the airway surface epithelium;[139] in addition, the MUC5AC and MUC5B levels increased 2-fold and approximately 7-fold, respectively[140]
cancer	mucin expression and composition are also altered in different types of cancers;[141] for instance, the overproduction of mucins has been correlated with cancer pathogenesis, in particular in adenocarcinomas[142]
dry eye syndrome	patients express less MUC5AC mucin[143]
inflammatory bowel disease	reduced thickness of the mucus layer, decreased viscosity, decreased MUC2 synthesis, and a depleted goblet cell population[144]

nanoparticles developed as mucosal-targeting nanoparticles can be found in excellent reviews.[76−78] Both nonspecific and specific targeting lipid formulations have also been developed. Examples of lipid-based drug delivery systems targeting mucus organized according to their routes of administration are summarized in Table 2. Most lipid-based formulations have been modified for nonspecific mucus targeting, while examples of specific mucosal-targeting lipid-based nanoparticles are limited.

Chitosan and its derivatives are thus far the most commonly used mucoadhesive polymers.[79] Chitosan and chitosan derivative-modified lipid nanoparticles (e.g., SLNs,[80] NLCs,[81] liposomes,[82] and nanoemulsions[83]) have shown a longer residence time at different mucosal sites, increasing the bioavailability of their cargo.

Lipid nanoparticles specifically targeting mucus are mainly designed to increase the systemic absorption of drugs through the oral or pulmonary routes.[74,84] Fan and co-workers grafted CSK on the surface of SLNs for oral delivery of salmon calcitonin.[74] CSK-modified salmon calcitonin-SLNs interacted with mucin and goblet cells, resulting in an increase in salmon calcitonin oral bioavailability by 2.45-fold compared to unmodified salmon calcitonin-SLNs.[74] Numerous mucosal-targeting lipid-based formulations have shown excellent results at the preclinical stage. However, more efforts should be made in the future regarding translation to the clinic. Indeed, a deeper comprehension of the delivery mechanisms of formulations and their toxicity at repeated doses as well as the transition from lab-scale to large-scale production are needed.

3.3. Mucus Penetration. Mucoadhesive polymers and muco-ligands could increase the retention and/or enhance the interaction between the mucus and delivery system. However, enhanced interactions with mucus are not the only parameters that affect nanomedicine efficiency. Indeed, a nanoparticle is specifically associated with the mucus, but it still has to penetrate and cross the mucus to reach cells, tissues, or organs underneath.[110] To improve mucus penetration, mucus-penetrating strategies have been developed that were initially inspired by the behavior of viruses. These mucus-penetrating strategies can lead to the effective penetration of the dense mucus when they are small enough or have a net-neutral highly hydrophilic surface.[110,111] Among all developed strategies, a dense coating of PEG on the surface of drug carriers has become the mainstream approach in the engineering of mucus-penetrating drug delivery systems. This approach has been applied to the eyes, nasal cavity, oral cavity, airways, gastrointestinal tract, and reproductive tract.[112−115] In addition to PEGylation, other surface-modifying polymers, such as poly(vinyl alcohol),[116] poly(2-alkyl-2-oxazoline),[117] and poly-(*N*-(2-hydroxypropyl)methacrylamide),[118] have been used to improve the mucus penetration of delivery systems. Another approach to increasing mucus penetration is the design of drug delivery systems decorated with mucolytic enzymes, such as *N*-acetyl-L-cysteine (NAC),[119] bromelain,[120] and papain.[121] The recent efforts, advantages, and limitations of different mucus-penetrating strategies have been well reviewed elsewhere.[122−124]

3.4. Future Perspectives in Mucosal Targeting. To further improve and increase lipid nanoparticle retention in the mucosae and thus their therapeutic efficiency, several strategies could be developed. One strategy would be to use the modified properties of the mucus in the pathological context (Table 3) to produce optimized and tailored lipid nanoparticle−mucus interactions.[125] Another is to adapt lipid delivery systems to the physicochemical properties of the mucus, such as pore size, pH, charge, viscoelasticity, and ionic strength. Bernkop-Schnürch's group developed phosphorylated material-decorated nanocarriers whose ζ potential could change following enzymatic degradation with alkaline phosphatase.[126−128] These nanoparticles contained phosphate functional groups, which had an overall negative charge that shifted to a positive/nearly neutral charge after enzymatic cleavage of the phosphate ester moiety.[126] Once the system crosses the mucus and reaches the epithelia, the surface charges are converted into positive charges/nearly neutral charges to facilitate cellular uptake.[127−129] Further research is needed to determine whether this strategy will be efficient and how it could eventually lead to particle aggregation. This strategy could be attractive to develop thiomer-based lipid nanoparticles for mucosal sites presenting a pH gradient (e.g., gastrointestinal or cervical mucus), which might be able to increase the permeation of nanoparticles into acidic luminal mucus without forming disulfide bonds.[129] Additionally, the same group found that phosphorylated SLNs aggregated (from ∼120 nm to 5−8 μm) when phosphates were removed by alkaline phosphatase.[130] After negative charges on the surface of nanoparticles were lost, a decrease in interparticle repulsion and an increase in interparticle electrostatic interactions led to particle aggregation.[130] The authors demonstrated that the size shift to a micron range (particle aggregation) after mucus penetration not only prevented a back-diffusion effect but also extended the drug release. These studies illustrate strategies to optimize drug delivery systems based on the physicochemical properties of the mucus and could be promising approaches to overcoming mucus barriers.

However, an important point is the balance between mucus adhesion and mucus diffusion. It is crucial to lengthen the retention time of the nanoparticles at the mucosal site, but for better therapeutic efficacy, enough nanoparticles should reach the underlying epithelium. PEG or Pluronic F127 surface modification has been successfully used to achieve this

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Table 4. Lipid Nanoparticle Targeting Ligands (A Nonexhaustive List)

ligand (example)[a]	size	advantages	disadvantages
Proteins			
antibody (trastuzumab, rituximab)	150 kDa	available for a large range of antigens, selectivity improvement by protein evolution,[150] good specificity	large, unstable, immunogenic, need of proper orientation
Fab[151]	50 kDa	versus antibodies: smaller, more stable, less immunogenic, same targeting capacity	
scFv[152]	25 kDa		
nanobody[153]	12−15 kDa		
affitin/affibody[154]	<10 kDa		
transferrin	80 kDa	−	
Peptides[148]			
RGD[155]	350 Da	versus proteins: smaller, more stable, modulable immunogenicity, easier production	limited colloidal stability
CPP (R6H4)[156]	1.5 kDa		
CPP (TAT)[157]	1.6 kDa		
Aptamers[148]			
AS1411,[158] transferrin receptor targeting	10 kDa	very high affinity and specificity heat-stable, nonimmunogenic, nucleic acid chemistry	faster biodegradation
Polymers			
HA[159]	5−1500 kDa	adjustable size and density	limited targets
Small molecules[148,160]			
mannose	180 Da	versus macromolecules: more stable	low choice, limited targets
folate	441 Da		

[a]CPP, cell-penetrating peptide; Fab, fragment antigen-binding; HA, hyaluronic acid; scFv: Single-chain variable fragment.

goal[131,132] in many nanocarriers, including some lipid-based formulation examples.[132−134]

4. MODIFICATION OF NANOPARTICLE SURFACE TO TARGET-SPECIFIC CELL TYPES

Targeted nanomedicine has been the "magic bullet" dream of nanoscale drug delivery systems[145,146] to actively recognize, bind to, and be taken up by the targeted cell population. Specific cell targeting is achieved by the attachment of a ligand to the surface of a nanoparticle. This ligand molecule should recognize another molecule, such as a receptor, present in the targeted cells in a selective or specific manner.

4.1. Ligands. The choice of the ligand nature is crucial. Antibodies are obvious ligands, as their function is to target a specific antigen. Alternatives include antibody fragments, nanobodies, other types of proteins, peptides, aptamers, oligonucleotides, and small molecules such as folic acid or carbohydrates.[147,148] Each ligand has its pros and cons (Table 4), and a particular ligand should be selected according to the targeted cell type, the lipid nanoparticle, and the range of molecules available.

In addition to antibodies, smaller fragments, peptides, and aptamers have been developed to replace full-size antibodies. Peptides can recognize specific receptors such as integrins with the arginine-glycine-aspartate (RGD) peptide or improve cell penetration when positively charged (cell-penetrating peptides). Aptamers are short single-stranded nucleic acid sequences with more affinity and selectivity for their designed antigen than antibodies that are heat-stable and nonimmunogenic. The main polymeric ligand is HA; it targets the CD44 receptor, which is overexpressed in many cancer cells, such as ovarian cancer cells.[149] Finally, small molecules include sugars, such as mannose against dendritic cells or folate used for cancer targeting.

4.2. Grafting of Ligands onto Lipid Nanoparticles. The grafting of ligands onto lipid nanoparticles must (i) maintain the integrity and functionality of the ligand as well as of the nanoparticle; (ii) expose the ligand at the surface of the particle; (iii) stabilize the ligand in the blood and/or biological medium; and (iv) preserve the affinity of the ligand for its target. Ideally, the grafting technique should have the following characteristics: (i) stereospecificity to control the localization of the conjugation on the ligand to obtain a homogeneous distribution of the ligand and to ensure that the ligand is oriented outward; (ii) simplicity and reproducibility; (iii) easy scale-up and characterization; and (iv) a high yield to limit costs.[151] The methods used to graft targeting ligands onto liposomes have been previously reviewed.[161] In this review, we will focus on the progress made over the last 10 years.

4.2.1. Noncovalent Surface Modifications. Lipid nanoparticles do not usually present reactive groups on their surface and do not tolerate organic solvents after being formulated. Moreover, chemical reactions add some complexity and the risk of destabilization of nanoparticles. Therefore, noncovalent techniques have been developed to decorate lipid nanoparticles with targeting ligands (Figure 3, Table 5).

A common form of noncovalent grafting is the integration of a lipid anchor into the lipid nanoparticle during formulation. This lipid anchor is often previously coupled with a polymer, such as PEG, as a spacer, with a terminal reactive group to react with the ligand.[162] Such lipid anchors include (i) phospholipids, with the most popular being 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE);[163,164] (ii) cholesterol;[162,164] or (iii) a single chain fatty acid, such as stearate.[163,165,166]

Alternatively, lipid anchors can be postinserted once nanoparticles have been formed. Postinsertion into lipid nanoparticles is time, temperature, and lipid concentration dependent.[167,168] This method is widely used because the insertion is stable and easy to perform. Conditions can be optimized by adjusting the temperature and the ligand/lipid ratio.[168] Postinsertion has been mainly used for liposomes[163,169,170] but has also been successfully adapted to phospholipid nanomicelles,[171] lipid-coated polystyrene nanoparticles,[8] LNCs,[172] and exosomes using sonication to increase the efficacy of postinsertion.[173]

Ionic interactions have been used to adsorb targeting ligands to the surface of lipid nanoparticles (i.e., electrostatic adsorption

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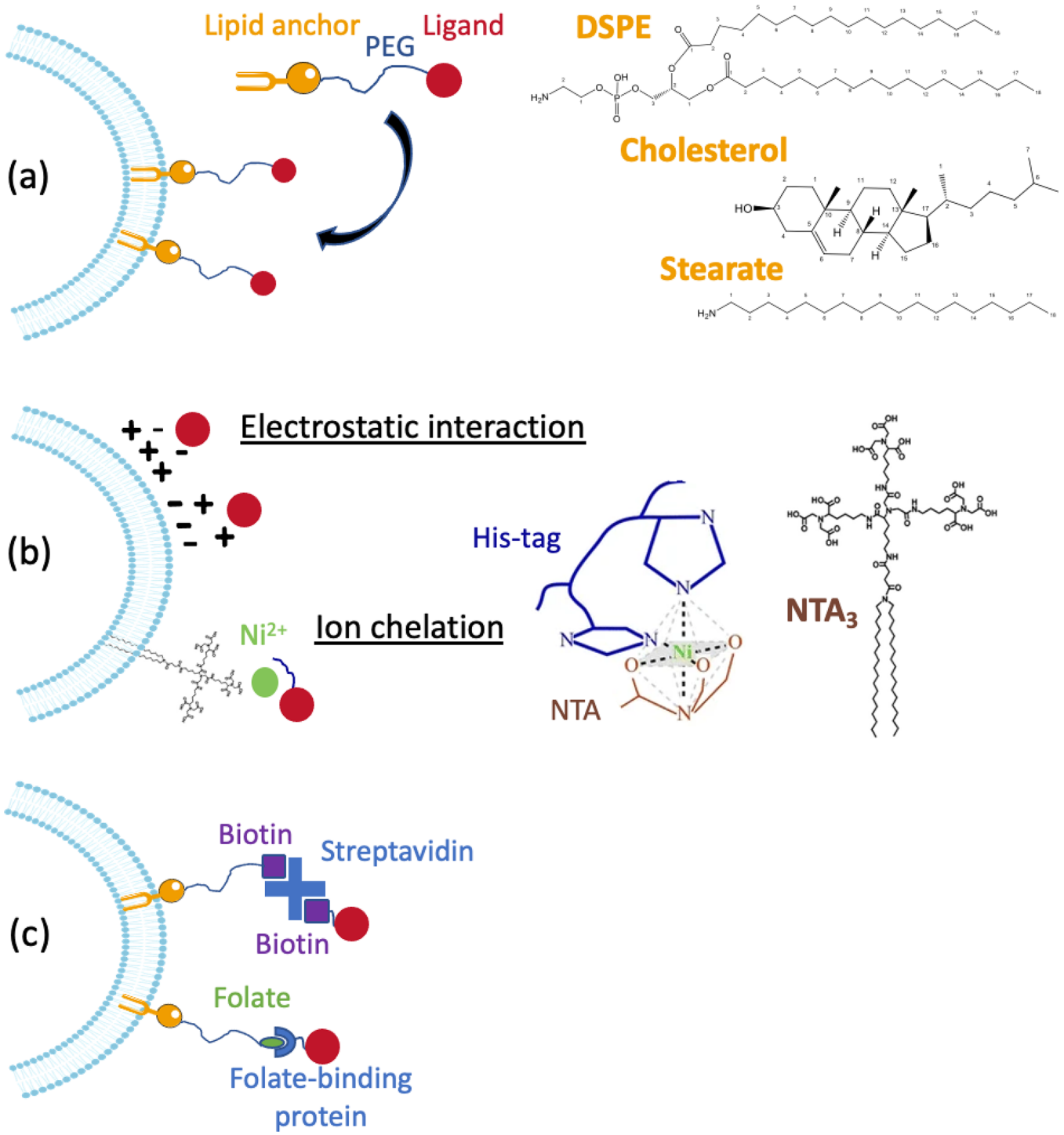

Figure 3. Noncovalent surface modification techniques. (a) Lipid anchor inserted in the membrane during formulation or by postinsertion and examples of lipid anchors. (b) Ionic interaction by electrostatic or NTA-nickel-His-tag chelation. (c) Biological interaction with streptavidin-biotin or folate-folate binding protein.

and chelation). Ionic interactions are quite weak, and biological media can induce their dissociation or ion exchange.[181] Therefore, multiple ionic interactions are needed. The objective is to pair up a charged nanoparticle with an oppositely charged ligand. This is the simplest method when feasible, as it does not need a chemical reaction. By default, lipid nanoparticles tend to have a slightly to a highly negative surface charge.[156,180] It is therefore possible to adsorb a positively charged ligand, such as a cell-penetrating peptide, to the surface of a lipid nanoparticle. Another strategy is to add a cationic lipid surfactant to the formulation to produce positively charged nanosystems that can then interact with a negatively charged ligand.[179,187] As a drawback, cationic nanoparticles can be toxic and are quickly cleared from the blood.[156,188] Therefore, ideally, the nanoparticle surface charge should be neutralized by the adsorption of the negative ligand before i.v. administration.

Ionic interactions can also be used for chelating strategies. It is indeed possible to obtain a stable noncovalent binding between

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Table 5. Noncovalent Surface Modification Techniques[a]

technique	ligands	advantages	disadvantages
Lipid anchor integration			
during nanoparticle formation	folate,[174] aptamer,[158] peptide,[175] mannose[176]	no additional step, stable	noncompatible with large ligands (proteins)
postinsertion of the ligand with the anchor	antibody,[163] scFv,[177,178] protein[170]	stable	additional step, heating
Ionic interaction			
electrostatic interaction	HA+CPP,[156] HA,[179] CPP,[180] antibody[181]	simplest	weak interaction, risk of dissociation, toxicity and clearance associated with a positive charge
NTA chelation	peptide,[182] protein[183]	spontaneous	unstable, His-tag ligand
Co^{II}-porphyrin chelation	protein[184]	more stable than NTA	His-tag ligand
Biological interaction			
folate binding	antibody[185]	specific and stable interaction, mild conditions	modified ligand
biotin-streptavidin	antibody[186]		

[a]His-tag, polyhistidine-tagged; NTA, nitrilotriacetic acid.

Table 6. Covalent Surface Modification Techniques, Examples, and Some Characteristics[193]

bond type	technique[a]	examples	advantages	disadvantages
cyanuric	cyanuric chloride	protein[200]	selectivity, no derivatization	potential toxicity, need basic pH ≥ 9
amide	EDC/sNHS	small molecule[201] peptide[202] antibody[203] HA[204]	common reactive groups: amine and carboxylate	two steps, no stereoselectivity, low yield, adjuvants
amide	pNP	antibody[205]	basic pH-specific, good yield, no adjuvant, hydrolysis of unreacted pNP	
cross-linker	DSC	antibody[206]		homobifunctional
isothiourea	isothiocyanate-amine	mannose[176]		
secondary amine	Schiff base + reductive amination	mannose[196] antibody[207]	common moieties: carbonyl and amine or 2 amines with glutaraldehyde[162]	no stereoselectivity, reduction step
cross-linker	SM(PEG)	peptides or antibody[7]	heterobifunctional	
thioether	thiol-maleimide	peptide[208] Fab′[209] antibody[195]	orthogonal, neutral pH-specific	need of a thiol group (can be added)
triazole	SPAAC	protein,[197] peptide[190]	orthogonal, high yield	specific moieties (cyclooctyne and azide)

[a]DSC, *N,N′*-disuccinimidyl carbonate; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; sNHS, sulfo-*N*-hydroxysuccinimide; pNP, *p*-nitrophenycarbonyl; SM(PEG), succinimidyl-[(*N*-maleimidopropionamido)-tetracosaethylene glycol] ester; SPAAC, copper-free strain-promoted cycloalkyne−azide cycloaddition.

nitrilotriacetic acid (NTA), nickel (Ni^{2+}), and a His-tag coupled to the targeting ligand upon aqueous incubation at room temperature.[189] However, Ni-based chelation is unstable after i.v. administration, and the associated ligand can be lost because of the competition with endogenous proteins or nickel can be removed by endogenous chelators.[184,189] Ni-NTA has thus been replaced by porphyrin-phospholipid to create a metallo-porphyrin-phospholipid bilayer in the presence of cobalt(II) ions, resulting in a more stable binding with His-tagged proteins[184,190] and improving the final coupling yield by spatial interaction.

Biological interactions can also be used for the noncovalent coupling of ligands. The advantages are that conjugation happens in physiological media with good specificity. The biological interaction with the highest affinity and most stability is between biotin and streptavidin.

The last technique, which does not require a chemical reaction, is gene engineering. Indeed, exosomes can be produced via previous transfection of the exosome-producing cells with a vector associating lamp2b, an exosomal membrane protein, with the desired targeting peptide.[191,192] The produced exosomes then have a targeting ligand at their surface without the need for further modifications.

4.2.2. Covalent Surface Modifications. Noncovalent grafting offers several advantages for the targeting of lipid nanoparticles, but the links it creates can be less stable and versatile than covalent coupling.[147,162] A covalent link is the result of a reaction between a ligand and a head reactive group at the end of a PEGylated lipid.[147] To be compatible with fragile ligands, the reaction should be performed in an aqueous solvent and should be free from side-product formation, such as a ligand−ligand or particle−particle association. These criteria significantly reduce the number of chemical reactions that can be used. We consider here the direct covalent coupling between a preformed lipid particle and a targeting ligand, but the same reactions can be used to link the ligand with a lipid polymer before formulation or postinsertion (see Section 4.2.1)[147] (Table 6 and Figure 4).

Amide-bond coupling between an amine and a carboxylic group is one of the most popular techniques used to form a covalent link between a ligand and a lipid nanoparticle.[193] It is used for nanoparticles showing either carboxylate or amine moieties. *p*-Nitrophenylcarbonyl can also be added to the formulation to react with primary amines in an aqueous buffer at

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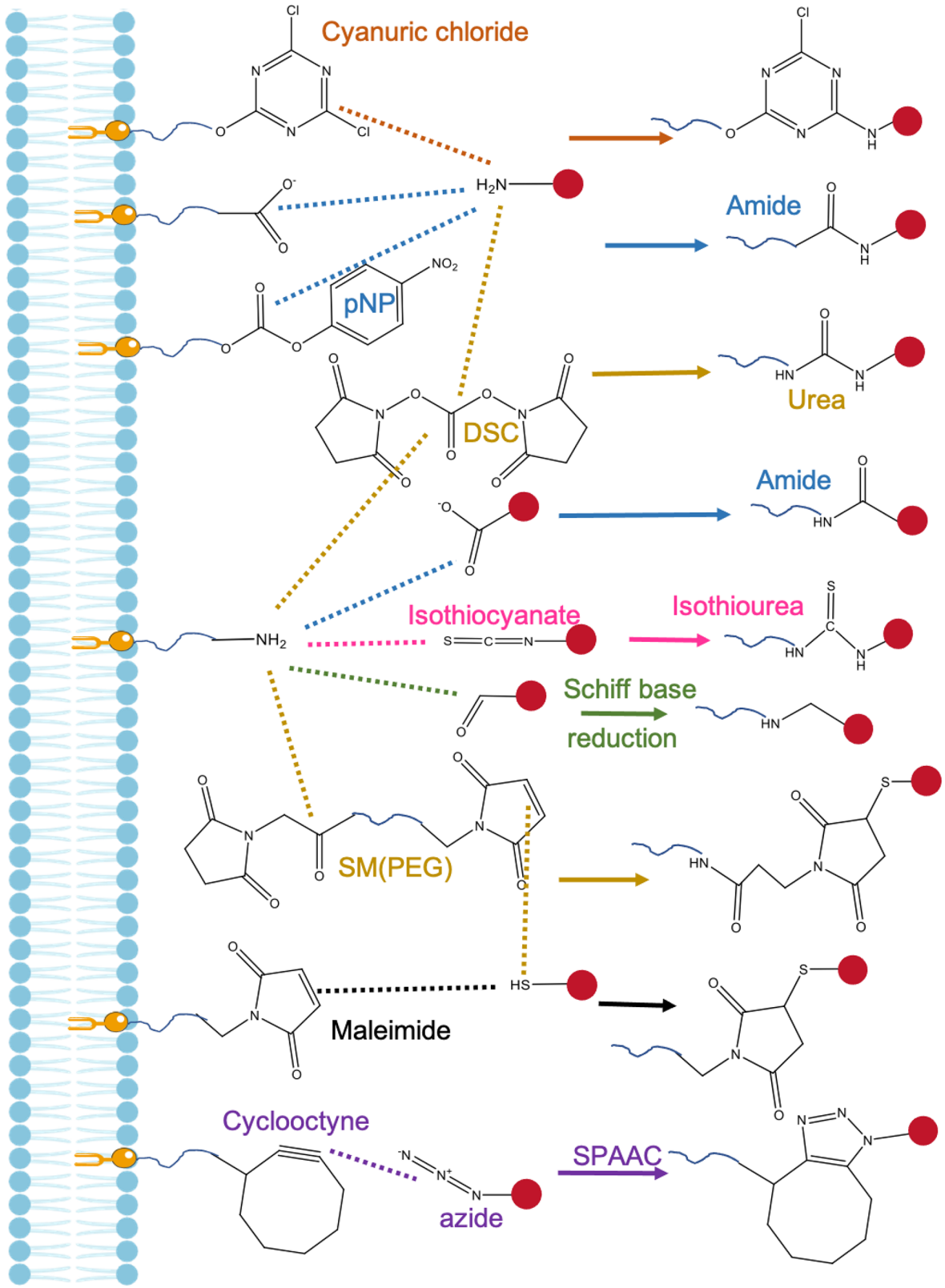

Figure 4. Covalent surface modification techniques. The available reactions are presented in the same order as in Table 12. pNP, *p*-nitrophenyl; DSC, *N*,*N*′-disuccinimidyl carbonate; SM(PEG), succinimidyl-[(*N*-maleimidopropionamido)-tetracosaethylene glycol] ester; SPAAC, copper-free strain-promoted cycloalkyne−azide cycloaddition.

pH between 8 and 9.5. This reaction does not need any adjuvant molecule or special conditions, except a pH change.[194]

Thioether-bond formation has also been used to attach a targeting ligand. Usually, a linker bearing a maleimide group specifically reacts with thiols in the pH range of 6.5−7.5 via a Michael addition reaction.[193] The ligand must contain a thiol group acquired from a cysteine or via thiolation using Traut's reagent (2-iminothiolane). This reagent is a cyclic imidothioest-

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er that can react with primary amines at pH 7−10 in a ring-opening reaction to exhibit a free sulfhydryl group.[151] *N*-Succinimidyl-3-(2-pyridyldithio)propionate can also be used to add a thiol group. The *N*-succinimidyl portion reacts with amine groups, whereas the pyridyldithiopropionate moiety can be reduced to a thiol by tris(2-carboxyethyl) phosphine hydrochloride or dithiothreitol. Once the ligand has reacted with the maleimide moiety, it is advised to block the remaining maleimide group by the addition of cysteine.[195]

Another strategy is to use a cross-linking agent that will successively bind to both lipid nanoparticles and ligands thanks to two adapted reactive groups. The cross-linking agent can be homobifunctional or heterobifunctional. The latter option has the advantage of preventing homologous ligations between two lipid nanoparticles or ligands. A very wide variety of commercial cross-linking agents is available.[151]

Two other bonds can also be created by the reaction with an amine group present on the surface of a lipid nanoparticle. The formation of a labile Schiff base with an aldehyde group can be further reduced to produce a stable secondary amine bond,[196] while the reaction with an isothiocyanate produces an isothiourea bond.[176]

Stable triazole bonding is obtained by click chemistry with copper-free strain-promoted cycloalkyne−azide cycloaddition (SPAAC) based on the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides to terminal alkynes. In an aqueous medium, this reaction is orthogonal against other biological functional groups and provides a good yield.[190,197] The click reaction with equivalent properties can also occur between a tetrazine and a *trans*-cyclooctene group.[198]

Finally, cyanuric chloride is a reagent with three aromatic chlorides that can react by nucleophilic substitution at basic pH. Each substitution requires specific temperature and time conditions, allowing selectivity. No derivatization is necessary, and unmodified proteins will be coupled by nucleophilic amino acids.[151]

Other techniques, such as disulfide-bond or hydrazone-bond formation, have been used in the past but not in the last 10 years. Disulfide bonds are unstable in reducing and acidic environments,[199] and hydrazone is also sensitive to hydrolysis.[151]

4.3. Current Limits and Challenges of Cell-Specific Targeting. Lipid nanomedicines presenting a modified surface (i.e., PEGylated) have reached the market (i.e., Caelyx), but despite 40 years of research on the active targeting of nanoparticles, no cell-specific targeted nanomedicine has yet been approved for clinical application, as recent improvements would not be able to translate as such in the clinic. It appears that the ligand targeting strategy faces many challenges. The whole paradigm of ligand targeting might be challenged, as comparative studies have shown that most targeted liposomes have a limited penetration efficiency when reaching the target site, with the liver still being the first organ where these materials accumulate.[148] A meta-analysis comparing active versus passive targeting showed a nonsignificant delivery efficiency improvement in a tumor model. Indeed, applying active targeting allowed an increase in tumor accumulation from 0.6% to 0.9% of the injected dose.[210]

Ligand targeting has been based on a simplified vision of targeted nanoparticles acting as targeted missiles. In the reality of living organisms, targeting nanoparticles, from the point of injection to the point of action, must cross several barriers of distinct scale: (i) systemic distribution through blood vessels; (ii) vascular extravasation to the targeted organs; (iii) tissue diffusion to the targeted cells;[211] and (iv) cell uptake and processing by cellular trafficking machinery. The rate-limiting steps for tumors seem to be vascular extravasation and the tissue microenvironment.[211,212] It has been shown that cell targeting mainly helps nanoparticle fate once the targeted tissue is reached. However, this targeting capacity can be lost during circulation in the blood due to protein corona adhesion.[211,213] In addition, once the tumor tissue is reached, a high-affinity ligand drives fast cell binding that prevents nanoparticle diffusion within the tissue.[214]

The future of nanomedicine targeting has to address simple and robust solutions to complex biological modeling. Physicochemical analysis of the targeted nanoparticle interactions with their biological environment should be studied more thoroughly. Innovative approaches such as computational modeling would help. The use of mathematical or computational multiscale modeling, considering the whole body, organ, tissue, and cell interactions, could facilitate the understanding and optimization of the entire process of targeted drug delivery.[214]

Nanomedicine design should be improved and move toward smart particles taking into account the different steps of the targeted nanoparticle journey; for instance, optimizing ligand localization and availability to interactions.[212] Nanomedicine design should improve the in vivo stability and pharmacokinetics, reduce circulation loss and off-target effects, and interact with cellular trafficking to direct efficient endosome release or escape depending on cargo sensitivity. Quantifying the nanotoxicity to target tissues must be routinely included within an investigational workflow. This will improve the translational potential of nanotechnology to a greater extent than adding new proofs of concept with complex nanomaterials or disease models with little to no therapeutic benefit.[148] Finally, the scale-up of such smart particles should be envisioned from the beginning of the material design.

To end this section on cell-specific targeting, it is worth noting that alternatives to ligand-associated targeting exist. This could be a short-term solution for the challenges associated with ligand coating. The main constituents of a lipid nanoparticle can help to selectively deliver its cargo to a targeted population. For instance, cholesterol-based NLCs show selectivity toward overexpressing low-density lipoprotein (LDL) receptor cells, such as some tumor cells.[215] In this case, cholesterol is not exposed at the surface but constitutes 60% of the NLC excipients. The same LDL receptor has been used recently in an elegant study where squalene-based nanoparticles were designed to interact with lipoproteins and be carried toward tumors by LDL.[216] Therefore, there is a place for promising strategies for tissue and cell targeting.

5. MODIFICATION OF NANOPARTICLE SURFACES TO CROSS BARRIERS

To improve the efficacy of lipid-based nanocarriers reaching a targeted tissue, one has to first identify the intrinsic features of the barriers to overcome. In the next section, the main characteristics of the most exploited routes of administration involving lipid-based nanocarriers will be briefly described. Additionally, the rational design of targeted lipid nanocarriers based on the target will be discussed, providing examples in each case.

5.1. Mucosal Barriers. The mucosal route has been used for both local and systemic drug administration.[217] Compared to other routes, mucosal delivery is a more patient friendly and

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Table 7. Examples of Active Targeting in Oral Drug Delivery

nanocarrier	surface modification	outcomes	refs
liposomes	biotin/biotin-DSPE	a 2-fold increase in insulin bioavailability with biotin-modified liposomes compared to plain liposomes	224, 225
		a 5.28-fold increase in insulin bioavailability with biotin-DSPE-modified liposomes compared to plain liposomes	
	folic acid	cefotaxime AUC (0-∞) and C_{max} were found to be 1.4−2-fold and 1.2−1.8-fold higher, respectively, for folic acid-coupled liposomes compared with folic acid-free liposomes	226, 227
		20% higher relative bioavailability than that of a subcutaneous insulin solution	
	tomato lectin	7.89% higher relative bioavailability than that of a subcutaneous insulin solution	228
	UEA-1	5.37% higher relative bioavailability than that of a subcutaneous insulin solution	236
SLN	WGA	in vitro, improved anticancer activity against A549 lung cancer cells compared with that of free PTX	230, 231
		in vivo, improved bioavailability and lung targeting of paclitaxel with WGA-SLNs	
		improved oral bioavailability of encapsulated insulin	
	HA2	in vitro, enhanced endosomal escape and improved permeability of insulin across Caco-2 cells	232
		in vivo, an improved hypoglycemic response with insulin-loaded HA2-SLNs via the oral route	
NLC	dextran/protamine	increased permeability of saquinavir across mucus-secreting cells in vitro	233
		a 9-fold increase in saquinavir permeability in vitro across Caco-2 cells	
LNC	DSPE-PEG	increased GLP-1 levels (up to 8-fold) in vivo in normoglycemic mice versus untargeted nanoparticles	238
		decreased administration frequency of exenatide from once daily to once every other day	
PLGA-lipid NPs	UEA-1	effective transport across M cells and further capture by mucosal dendritic cells	237

noninvasive alternative.[125,217] Bio/mucoadhesive drug delivery systems present several advantages: (i) by using mucoadhesive (bio)materials, the formulation remains for a longer period of time at the delivery site, which increases the bioavailability of the drug;[218] (ii) the formulation can target a particular site or tissue (e.g., the gastrointestinal tract) due to the grafting of some specific bioadhesive molecules;[219] (iii) an increased residence time, which when combined with controlled release, may result in reduced frequency of administration;[220,221] and (iv) drugs absorbed via a mucosal route other than the gastrointestinal route circumvent the hepatic first-pass effect and consequently have an increased half-life.[222,223]

5.1.1. The Gastrointestinal Tract. The gastrointestinal tract harbors a wide variety of cells that have become a target in the oral drug delivery field. Enterocytes have been targeted to increase the oral bioavailability of poorly water-soluble drugs; goblet cells have been targeted to overcome the mucus barrier; microfold (M) cells have been targeted as an approach to improve oral vaccination; and L cells have been targeted to increase endogenous GLP-1 secretion.[70] These are only a few examples (Table 7) highlighting approaches specific to lipid nanocarriers.

As the most abundant cell type in the intestinal epithelium, enterocytes have been the preferred target when considering oral drug delivery. Although not as exploited as their polymeric counterparts, lipid nanoparticles have been decorated with different ligands on their surface to increase their absorption and/or targeting across/to enterocytes. As an example, liposomes have been decorated with biotin,[224,225] folic acid,[226,227] or tomato lectin.[228] Biotin was added to the surface of liposomes by amidation between DSPE and biotin using *N*,*N*′-dimethylaminopyridine/*N*,*N*′-diisopropylcarbodiimide as the catalyst. DSPE-biotin was then added to the organic phase during the preparation of the liposomes and incorporated into them. A folic acid-poly(ethylene oxide)-cholesterol conjugate was used to incorporate folic acid onto the surface of liposomes toward increased folic acid receptor targeting.[229] Aggrawal et al. instead used a folic acid-polyethylene glycol-poly(allyl amine) hydrochloride construct to decorate the surface of the liposomes with folic acid.[227] Lectins were covalently linked to phosphatidylethanolamine (PE) via a two-stage carbodiimide method to synthesize lectin-lecithin conjugates,[228] which were ultimately incorporated onto the surface of the liposomes. SLNs have also been decorated for enterocyte targeting. Pooja et al. developed WGA-conjugated SLNs by a 1-ethyl-3-(-3-(dimethylamino)-propyl) carbodiimide-*N*-hydroxy succinimide (EDC-NHS) reaction.[230] Zhang et al. modified WGA via a WGA-*N*-glutaryl-phosphatidylethanolamine (WGA-*N*-glut-PE) conjugate.[231] Other than lectins, SLNs have been modified with an endosomal escape agent (GLFEAIEGFIENGWEGMIDGWYG, HA2) by incorporating HA2 in the aqueous solution during the formulation process.[232]

Dextran/protamine conjugates (with a positive charge) were incorporated on the surface of NLCs (with a negative charge) via electrostatic interactions to increase the oral bioavailability of lipophilic drugs, which increased their mucopenetration in vitro across mucus-secreting cell monolayers.[233]

To our knowledge, lipid-based nanocarriers failed to target M cells in an in vitro model of the follicle-associated epithelium and did not increase the transport of the encapsulated drugs via M cells.[234,235] We have found only a few examples regarding the surface modification of lipid nanocarriers toward M cell targeting compared to the large number of polymeric examples. Liposomes encapsulating the hepatitis B surface antigen (HBsAg) were developed and coupled with UEA-1 to increase transport across M cells.[236] UEA-1 was grafted onto the surface of liposomes via an EDC-NHS reaction. UEA-1 was also coupled to the surface of poly(lactic-*co*-glycolic acid) (PLGA)-lipid hybrid nanoparticles by carbodiimide chemistry.[237]

There is only one example of L cell-targeting LNCs with a modified surface.[238] DSPE-PEG_{2000} was postinserted within the PEG corona of LNCs to increase L cell stimulation. The same method was exploited previously to increase the oral bioavailability of paclitaxel.[239]

5.1.2. The Pulmonary Barrier. Pulmonary administration has been used not only for local treatment of lung diseases but also for systemic delivery.[240] What makes the lung an interesting site of administration is its large surface area (approximately 100 m^2), thin alveolar epithelium, and its vasculature, which allow high absorption of soluble and permeable active compounds.[240] However, molecules still have many obstacles and barriers to overcome.[241]

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Lipid nanoparticles can address some of these problems, as they can be easily formulated as an inhaled spray (as a powder or an aerosol[240]), have good tolerability, and possess mucoadhesive properties. Moreover, they have an impact on the pulmonary surfactant by creating a biophysical disruption due to protein and lipid (present on the surfactant) adsorption at their surface.[241]

The most common surface modification for the pulmonary delivery of lipid nanoparticles is the adsorption of a mucoadhesive polymer (see Part 3). For instance, Murata et al.[242] modified liposomes using oligosaccharide chitosan and poly(vinyl alcohol) with a hydrophobic anchor. These modifications allowed for a better pharmacological effect of calcitonin, a hormone that regulates calcemia, after pulmonary administration to rats.[242] Alternatively, an antibody against intercellular adhesion molecule-1 (ICAM-1), a glycoprotein overexpressed in the vasculature of acutely injured lungs, was grafted at the surface of NLCs.[243] When injected intravenously in a mouse acute lung injury model, ICAM-1-NLCs showed greater accumulation in the lungs than control NLCs (grafted with an isotype antibody). Additional examples can be found in Table 8.

5.1.3. The Vaginal Barrier. Topical vaginal delivery is hindered by mucosal vaginal fluids, presenting different viscosities and pH values that vary with menstrual cycle and age. In addition, the self-cleaning properties of the vaginal tract shorten the drug residence time, thus limiting its therapeutic effect.[246]

To overcome the vaginal mucus barrier, mucoadhesion is one of the most explored strategies to increase the residence time of nanomedicines. Mucoadhesive polymers such as chitosan derivatives have been used to decorate the surface of nanoparticles to increase nanoparticle−mucus interactions and then enable a higher penetration rate (see Section 4). However, the issue with this strategy is that nanoparticles are often trapped in the outer mucus layer and can be rapidly cleared. An alternative is to use PEG(2000)-liposomes to deliver interferon α-2b (IFN α-2b).[247] PEG endows the liposomes with a close-to-neutral charge, which results in weaker interactions than chitosan-coated liposomes. An ex vivo experiment on vaginal sheep epithelium showed that a PEGylated liposomal formulation increased IFN α-2b penetration through the vaginal mucus to a higher extent than the controls. Vaginal delivery using lipid nanoparticles has not been extensively explored, but a few examples are given in Table 9.

5.1.4. The Ocular Barrier. The eye is undoubtedly a challenging barrier to overcome. Most of the marketed formulations for eye-related diseases are based on eye drops. The residence time of this type of formulation is rather low and, consequently, so is the drug concentration. Multiple studies centered on lipid-based nanoparticles have evidenced the potential of these formulations to overcome ocular barriers. Different surface modifications have been exploited depending on the ocular barrier to overcome (Table 10).

To increase the retention time of the formulation on the surface of the eye, researchers have focused their attention on chitosan as a mucoadhesive. Sandri et al. coated SLNs with chitosan by postinserting chitosan onto the surface of these nanoparticles.[80,250] Eid et al. added PEG in addition to chitosan as a coating on the surface of SLNs encapsulating ofloxacin as a model drug.[251] The authors aimed to increase both the retention time and the transcorneal bioavailability of ofloxacin. In rabbits, no difference between ofloxacin-loaded chitosan-PEG-modified

Table 8. Strategies to Enhance Pulmonary Distribution

nanocarrier	target	surface modification	grafting technique	outcome	refs
NLCs	ICAM1	anti-ICAM1 antibody	amino groups of the antibody and amino groups of NH_2−PEG_{2000}-SA mediated by DSC	in vivo: increase of pulmonary distribution after i.v. injection	243
SLNs	lactoferrin receptor	lactoferrin	carbodiimide chemistry: the carboxylic group of lactoferrin with the amine group of the stearylamine of SLNs; mediated by EDC	in vivo: increase of pulmonary uptake compared to the control unconjugated SLNs at 24 h	244
liposome	–	DPPC-containing palmitoyl groups absorption enhancers	put directly in the liposome formulation	in vivo: DPPC liposomes loaded with insulin decreased glucose levels after pulmonary administration to rats compared to controls (free insulin and insulin-loaded liposomes)	245
liposome	–	polyvinyl alcohol with a hydrophobic anchor ($C_{16}H_{33}S$) and chitosan oligosaccharide	adsorption	in vitro: on a Calu-3 cell monolayer, oligoCS-modified liposomes increase epithelial permeability in vivo: after pulmonary administration, oligoCS-modified liposomes and polyvinyl alcohol-modified liposomes allowed a prolonged calcitonin effect	242
liposome	–	WGA to escape macrophage + CP for mucoadhesive properties	carbodiimide chemistry	in vitro: WGA-CP liposome uptake by A549 cells is higher than that of unmodified liposomes in vivo: Calcitonin-loaded CP-WGA liposomes highly decreased calcium blood levels compared with those in the control after pulmonary administration to rats	84

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Table 9. Examples of Surface Modification to Cross Vaginal Mucus Barrier

nanocarrier	surface modification	grafting technique	modification outcome	refs
liposomes	PEG2000	directly in the liposome formulation	in vitro: interaction with mucus is decreased ex vivo vaginal tract model: higher IFN α-2b release	247
liposomes	chitosan and HPMC	adsorption	ex vivo: coated liposomes with chitosan and HPMC have a higher permeation rate through the bovine vaginal mucosa than that of a free sildenafil solution	248
SLNs	heparin and polylysine	electrostatic layer-by-layer assembly method	no in vitro or in vivo test	249

Table 10. Examples of Active Targeting to the Eye

nanocarrier	surface modification	outcome	references
SLNs	SAP (electrostatic interaction)	significantly increased transfection compared with that of plain SLNs in HEK293 and ARPE-19 cells increased lysosomal escape	255
SLNs	Dex-Prot (electrostatic interaction)	specifically increased transfection of retinal cells in vitro compared to SLNs alone prolonged blood circulation in vivo compared to SLNs efficient in vivo transfection after retinal and topical administration successful transfection in retinal layers close to the administration site in an XLRS mouse model	256−259
SLNs	Dex-HA (electrostatic interaction)	a 7-fold increase of the transfection capacity of SLNs in vitro in ARPE-19 cells successful transfection in outer and inner retinal layers in an XLRS mouse model	258−260
SLNs	chitosan (electrostatic interaction)	increased in vitro mucoadhesion compared to plain SLNs	80, 250
SLNs	chitosan + PEG (electrostatic interaction)	increased ofloxacin concentration in the eye (two- to 3-fold increase compared to ofloxacin drops).	251
niosomes	protamine (electrostatic interaction)	improved cell transfection, DNA condensation and cell viability prolonged in vivo transfection up to one month upon administration	262
niosomes	HA	two times higher transfection efficiency specific retinal layer targeting in rats improved tacrolimus pharmacokinetics	263, 264
liposomes	PEG (covalent link)	good tolerability inhibition of *Acanthamoeba* encystment 60% regression of corneal damage when combined with chlorhexidine	267, 268
liposomes	PEG + APRPG (covalent link)	enhanced accumulation in CNV lesions compared to unmodified PEG-liposomes	269−271
liposomes	PAMAM G3.0 (electrostatic interaction)	good tolerability enhanced bioavailability increased cellular permeability	265
liposomes	HA (electrostatic interaction)	improved residence time compared to plain liposomes and the drug in solution 1.7-fold increase in the doxorubicin AUC	272
liposomes	chitosan (electrostatic interaction)	increased retention time prevention of burst drug release delay/prevention of cataracts	273
self-emulsifying drug delivery systems	Eudragit L100-55 (hydrophobic ion pairing)	increased ocular mucoadhesion sustained econazole nitrate release	274

SLNs and ofloxacin drops was observed in terms of tolerability, but the drug concentrations in the eye increased 2−3-fold with the nanoparticles.

Lipid nanoparticles are efficient drug/gene delivery systems to the eye.[252,253] del Pozo-Rodríguez et al. investigated the use of SLNs as nonviral vectors for gene therapy for retinal-related diseases.[254] Tailoring the SLN surface improved the transfection efficiency. The transfection capacity of plain SLNs in ARPE-19 cells (a retinal cell line) and HEK293 cells (epithelial kidney cells) led to a different nanoparticle trafficking profile depending on the cell type.[254] To increase the transfection capacity of the nanoparticles, they modified the surface of the SLNs by adding *sweet arrow peptide*.[255] The incorporation of this peptide on the surface of the nanoparticles by electrostatic interactions between the complexes and the SLN led to a greater transfection capacity of the nanoparticles in both cell lines. The *sweet arrow peptide* induced a shift in the mechanism of transport of the nanocarrier within the cells, favoring lysosomal escape and hence reducing degradation of the vector. This effect was dose dependent. As an alternative approach, the authors replaced sweet arrow peptide with dextran (Dex)-protamine (Prot) complexes on the SLN surface following the same procedure as that for the sweet arrow peptide.[256,257] Tailoring DNA-SLN with Dex-Prot greatly increased the transfection capacity of the nonviral vectors in ARPE-19 retinal cells. For X-linked juvenile retinoschisis (XLRS) treatment,[257] the formulation Dex-Prot containing the plasmid pCMS-EGFP or pCEP4-RS1 enhanced the expression of both GFP and retinoschisin in ARPE-19 cells. In vivo, after topical, subretinal, and intravitreal administration to rats, this formulation was able to transfect different cell types depending on the administration route. Hence, the authors demonstrated the potential of the formulation not only for retinal disorders but also for ocular surface-related diseases. To confirm the efficacy of the formulation in the pathological

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Table 11. Examples of Lipidic Nanoparticle Surface Modifications for BBB Targeting

nanocarrier	surface modification	grafting technique	modification outcome	refs
Target transferrin receptor				
Cationic SLNs	OX26	thiol-maleimide reaction	enhancement of the PK profile of baicalin in cerebrospinal fluid	280
LNCs	OX26	thiol-maleimide	in vitro: LNC-OX26 interacted with cells expressing TrF	278
liposomes	transferrin receptor DNA aptamer	thiol-maleimide reaction	better accumulation of DAL-TRAM in the brain parenchyma than with noncoated liposomes.	281
			better reduction of cocaine-induced hyperlocomotion than with noncoated liposomes in vivo	
liposomes	RI7217	thiol-maleimide or avidin-biotin	enhanced permeability in an in vitro BBB model hour after hour	282
			no control (hCMEC/D3 cell monolayers)	
			confocal microscopy after 2 h of incubation	
SLNs	OX26	thiol-maleimide	enhanced permeability in vitro (ECs derived from hematopoietic stem cells)	283
Tight junction opening				
SLNs	borneol	amide reaction with succinic anhydride as the linker.	enhanced permeability in an in vitro BBB model	275
			enhanced HBMEC cell uptake	
			higher and faster accumulation in the brain	
Target LDLR				
SLNs	apolipoprotein E (ApoE)	avidin-biotin	enhanced permeability in an in vitro BBB model (CMEC/D3 cell monolayers)	284
Combination strategy				
liposomes	ApoE, anti-TfR, and TREG	thiol-maleimide	in vivo: multifunctionalized liposomes have higher penetration in the brain than dual-labeled ApoE and anti-TrF liposomes	285

context of XLRS, Apaolaza et al. tested the nonviral vector in an Rs1h-deficient mouse model.[258,259] The authors compared the efficacy of Dex-Prot-DNA-SNA with the same vector containing HA instead of Dex.[260] After subretinal or intravitreal administration to Rs1h-deficient mice, the expression of retinoschisin was observed in all retinal layers except the outer nuclear layer after subretinal administration. This effect was maintained for 2 months postadministration, demonstrating the feasibility of the formulations for nonviral gene therapy treatment for retinal disorders. When comparing Dex-Prot-DNA-SLN with HA-Prot-DNA-SLN, the authors found that Dex-modified SLNs more efficiently transfected retinal layers closer to the administration site, whereas HA-modified SLNs diffused and transfected both the outer and inner retinal layers.

Niosomes have emerged as a promising approach for gene delivery to the eye.[261] These nanocarriers, where phospholipids are replaced by nonionic surfactants, could be an alternative to liposomes. Similar to SLNs, niosomes have been modified to improve the transfection capacity of the formulation. Protamine/DNA complexes were mixed with niosomes forming protamine/DNA/niosome ternary vectors by electrostatic interactions.[262] Protamine improved the transfection efficiency of the niosomes as well as DNA condensation and cell viability while prolonging in vivo transfection for up to 1 month after administration. HA has also been used to modify the surface of niosomes.[263] The transfection capacity of the modified niosomes was significantly higher than that of unmodified niosomes (2-fold). Modified niosomes also exhibited selective targeting to the retinal layers and a 6-fold increase in transfection in vivo compared to the naked plasmid. Zeng et al. also observed an increased tacrolimus concentration when administered within HA-modified niosomes.[264] This concentration was 2.3-fold and 1.2-fold higher compared to a tacrolimus suspension and unmodified niosomes, respectively.

Liposomes have largely been exploited for ocular delivery.[265,266] As an example, PEGylated liposomes were prepared for the treatment of ocular keratitis caused by *Acanthamoeba*.[267,268] PEG was included in the formulation by replacing a conventional lipid (DSPE) with a PEG-modified lipid (DSPE-PEG) during the formulation. These PEGylated liposomes loaded with a therapeutic siRNA sequence were found to inhibit the encystment process of *Acanthamoeba*. Furthermore, a combination with chlorhexidine was able to reverse lesions associated with keratitis, reaching 60% corneal damage regression. For choroidal neovascularization treatment, PEGylated liposomes were decorated with the peptidic sequence Ala-Pro-Arg-Pro-Gly (APRPG) to target newly formed blood vessels[269−271] by replacing DSPE-PEG with DSPE-PEG-APRPG. Intravitreal administration of APRPG in a rat model of choroidal neovascularization allowed the accumulation of liposomes in choroidal neovascularization lesions lasting for at least 2 weeks postinjection. Lai et al. evaluated polyamidoamine dendrimer (PAMAM G3.0)-coated liposomes as an alternative drug delivery system to improve the stability of liposomes and the bioavailability of berberine and chrysophanol in age-related macular degeneration treatment.[265] The coated liposomes exhibited increased cellular permeability, enhanced bioadhesion to the cornea, and increased bioavailability while presenting good tolerability. HA-modified liposomes were tested as mucoadhesive carriers to improve the bioavailability of doxorubicin in the eye.[272] After their instillation into rabbits, the authors observed an increased residence time of the formulation compared to plain liposomes or the drug in solution and a 1.7-fold higher doxorubicin area under the curve (AUC). Liposomes have also been coated with chitosan to increase the retention time of the formulation in the eye. Huang et al. observed that chitosan coating indeed increased the retention time of the formulation while preventing burst release of the drug.[273] This formulation was effective in delaying or preventing the formation of cataracts in the eye.

Self-emulsifying drug delivery systems (SEDDS) have been less exploited in the eye than other lipid-based drug delivery systems and compared to other routes of administration. Elbahwy et al. developed mucoadhesive SEDDS using an entirely S-protected thiolated Eudragit L100-55.[274] The authors chose benzalkonium chloride to interact with the anionic

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Table 12. Surface-Modified Lipid-Based Nanoparticles for Topical Application to the Skin

nanocarrier	surface modification	outcome	refs
NLCs	polyarginine (electrostatic interactions)	increased cell penetration increased skin permeation alleviated inflammation compared to bare NLCs	298
NLCs	chitosan (electrostatic interactions)	decreased inflammation and psoriatic markers in vitro in a psoriatic model	299
NLCs	HA (covalent link)	increased drug penetration in vitro in Franz diffusion cells in the rat skin compared to drugs in solution prolonged and stronger anesthetic effect compared to drugs in solution highest anesthesia efficacy compared to other liposomes and EMLA-containing bupivacaine	300, 301
lipid nanoparticles	ASKAIQVFLLAG	selective uptake by keratinocytes improved skin barrier function and wound closure in burn wounds	302
lipid-polymer hybrid nanoparticles	chitosan	decreased inhibitory concentration of fusidic acid needed prolonged antibacterial activity	303

polymer via electrostatic interactions (hydrophobic ion pairing) to further lipidate thiolated Eudragit L100-55 for its incorporation within the SEDDS. Econazole nitrate was encapsulated into the mucoadhesive SEDDS, and this formulation exhibited higher ocular mucoadhesion and sustained drug release.

5.2. Nonmucosal Barriers. The surfaces of lipid nanoparticles have also been modified to cross nonmucosal barriers such as the blood−brain barrier (BBB), the inner ear, and the skin.

5.2.1. The BBB. The BBB is an anatomical barrier that protects the central nervous system (CNS) composed of endothelial cells, tight junctions, pericytes, astrocytes, and neurons.[275] Therapeutic compounds commonly administered intravenously or orally need to pass through the BBB to reach the CNS and generally display very low availability.[276] Nanomedicines can be used to improve drug availability, but even then, their crossing of the BBB is not very efficient. This is the reason why nanoparticle surface modification strategies have been proposed. The two major surface modifications that have been applied to lipid nanoparticles are based on either cationic polymer adsorption or grafting of a ligand that will interact with a receptor based on the endothelium to highjack receptor-mediated endocytosis. For the latter, transferrin, lactoferrin, insulin, and low-density lipoprotein receptors (LDLRs) have mainly been targeted.[277]

One of the most popular strategies is targeting the transferrin receptor (TrF), which is highly expressed on BBB endothelial cells,[278] or the LDLR. LDLs enter the CNS by binding to this receptor followed by transcytosis.[279] Examples of nanoparticle surface modification aiming for TrF or LDLR are collected in Table 11.

A less usual strategy was proposed by Song et al. They used borneol, a bicyclic monoterpene extracted from *Dryobalanops aromatica*, at the surface of SLNs to open the BBB tight junctions.[275]

5.2.2. The Inner Ear. The inner ear can be a reservoir of different pathologies, such as Meunier's disease, tinnitus, or sudden sensorineural hearing loss. To treat these diseases, therapeutics need to be delivered to the inner ear. Due to its anatomical position and complex geometry, the inner ear is difficult to reach.[286] Systemic administration has limited effects, as the inner ear is not very well vascularized,[286] and there is a blood−labyrinth barrier that is very similar to the BBB in terms of function. Hence, local delivery has been used; it can be extracochlear (i.e., intratympanic) or intracochlear.[287] Extracochlear delivery is considered the safest route of administration and is therefore the most commonly used. It does not require surgical intervention compared to intracochlear administration. Drugs can penetrate the inner ear through the round and ovale windows. The round window membrane (RWM) is a semipermeable membrane where small, lipophilic, and cationic molecules can penetrate easily to reach the stria tympani in the inner ear.[288] To date, nanoparticles have mostly been used to increase RWM permeability and overcome eustachian clearance (by mostly using nanohydrogels).[289]

Liposomes, LNCs, and SLNs have been used to deliver drugs to the inner ear with some success. For instance, SLNs increased the concentration of edaravone, an antioxidant neuroprotective molecule, in the cochlea.[290] LNCs administered to Sprague−Dawley rats at the RWM were able to cross this membrane and reach cells in the cochlea.[291]

Few studies have reported the use of targeted lipid nanoparticles for inner ear delivery, and when conducted, surface modification has mostly aimed to target spinal ganglion neurons.[292] Spinal ganglion neurons play a key role in signal transduction from hair cells to the brain. One of the specific biomarkers of these cells is TrkB. Additionally, these cells are affected during ear loss and are prone to cell death. Glueckert and colleagues[292] aimed to target these cells by decorating LNCs with A415 or A747, which are peptide ligands for TrkB. However, the targeted LNCs showed lower uptake than nonfunctionalized LNCs. The authors suggest that these results could be explained by the modification of the surface charge that is induced by the functionalization. Another possibility is that LNCs could mostly be taken up by micropinocytosis, a nonspecific internalization that overcomes TrkB receptor-mediated uptake.

5.2.3. The Skin. The skin has undoubtedly been one of the most exploited routes of administration when referring to lipid-based nanoparticles.[293,294] SLNs have been widely exploited for topical administration, mostly for cosmetic purposes. Since the commercialization of a lipid nanoparticle-based cream in 2005 (Cutanova Cream NanoRepair Q10 (Dr. Rimpler GmbH, Wedemark, Germany)), many others have followed. Additionally, the number of studies evaluating the potential of lipid nanoparticles on topical diseases has increased substantially, mostly for wound healing and skin regeneration.[295−297] Lipid nanoparticles are generally applied to the skin without any further surface modifications. The number of examples in the literature regarding surface-modified lipid-based nanoparticles for topical application to the skin are less abundant, and the most promising are described in Table 12.

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Table 13. Targeted Lipid Nanoparticle Purification Techniques

technique[a]	advantages	disadvantages	ligands
SEC	fast, effective, robust	complexity, sample dilution (≥2), cost	postinsertion micelle,[163,168] protein,[172,312] peptide,[182,208] small molecule,[208] polymer[182]
dialysis	no dilution	slow (days), risk of drug release	postinsertion micelle,[313,314] protein,[203,205] peptide,[315] small molecule,[196] polymer[204]
ultracentrifugation	simple, nanoparticle concentration	not applicable to all lipid nanoparticles	postinsertion micelle,[8] protein,[181,201] peptide,[7,316] small molecule[201]
ultrafiltration	nanoparticle concentration, unbound ligand dosage allowed	risk of loss of nanoparticles	protein,[195,317] peptide,[180,316] aptamer[158]

[a]SEC, size-exclusion chromatography.

Table 14. Characterization Techniques of Targeted Lipid Nanoparticles

technique[a]	advantages	disadvantages	ligands
Physicochemical characterization			
dynamic light scattering	checking nanoparticle integrity	no or low impact of grafting	protein,[163,181] peptide,[180,316] small molecule,[196] polymer[159,173]
NP tracking analysis	Idem	Idem	peptide[192]
surface charge (ζ)	visible changes after grafting	salinity and pH artifacts	protein,[181,320] peptide,[175,180] aptamer,[158] small molecule,[176,196] polymer[159,173]
electron microscopy	direct visualization,	cost, no or low impact of grafting	protein,[195,203] small molecule,[196] polymer[159,204]
atomic force microscopy		no impact of grafting except for polymers	protein,[201] peptide,[321] small molecule[201]
Ligand detection and quantification			
absorbance/fluorescence spectroscopy	sensitivity (fluorescence), fluorescent dye	sensitivity (absorbance), limited applicability	peptide,[208,322] aptamer[158]
X-ray photon spectroscopy	high sensitivity	not quantitative, limited applicability	small molecule,[165] polymers[159]
infrared spectroscopy		not quantitative, limited applicability	small molecule[196]
FACS	quantitative, signal for each particle	not for particles <300 nm	peptide[316]
(micro)BCA		interference with lipids	protein,[205,317] peptide[180]
Bradford		interference with lipids	protein[201]
CBQCA	high sensitivity, no interference with lipids		protein[181]
fluorescamine	no interference with lipids	low sensitivity	peptide[323]
SDS gel electrophoresis	purification and characterization	semiquantitative,	protein,[163,209] peptide[7]
Western blot	purification and characterization	semiquantitative, limited applicability	protein,[197] peptide[191]
FRET-electrophoretic mobility shift assay	purification and characterization	low applicability	peptide[184]
(U/H)PLC		applicable only to small ligands, indirect measure	peptide,[190,208] small molecule[197,201]
microbead assay	direct information on ligand density	complex	protein[203]
^{3}H assay	quantitative	radioactivity	polymer[204]

[a]FACS, fluorescence-activated cell sorting; BCA, bicinchoninic acid; CBQCA, (3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde); SDS, sodium dodecyl sulfate; FRET, Förster resonance energy transfer; (U/H)PLC, (ultra) high-performance liquid chromatography.

Gao et al. modified the surface of NLCs encapsulating lornoxicam with polyarginine to increase the membrane translocation capability of nanoparticles.[298] NLCs were mixed with a solution of polyarginine (R11) using a 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl) imidodiacetic acid) succinyl nickel salt] spacer to increase the peptide binding affinity to NLCs. The decoration of the surface of the negatively charged NLCs with the positively charged R11 led to increased permeation of the NLCs into the skin in vitro compared with that of the unmodified NLCs. The anti-inflammatory effects of lornoxicam-NLCs-R11 were evaluated in vivo in a carrageenan-induced rat paw edema model. NLCs modified with R11 not only increased the skin penetration of the drug but also prolonged the anti-inflammatory effect, alleviating rat paw edema and the related inflammation.

Malgarim et al. evaluated chitosan as a coating agent for NLCs in vitro, in fibroblasts and psoriatic keratinocytes.[299] Chitosan-coated fucoxanthin-NLCs reduced the expression of β-defensin-2 (a psoriatic marker) by 40% and the expression of TNF-α compared to uncoated NLCs, demonstrating the potential of this formulation toward inflammation and hyperproliferation.

NLCs have been modified with HA for dermal purposes.[300,301] Yang et al. covalently linked HA to DSPE-PEG-NH_2. NLCs encapsulating ropivacaine and dexmedetomidine were modified with HA-PEG-DSPE. Modified NLCs significantly increased the penetration of the drugs between 2- and 4.7-fold compared to the drugs in solution. In vivo, NLCs prolonged and reinforced the antinociceptive effect of anesthesia compared to the drugs in solution. Yue et al. also modified the surface of NLCs with HA and linoleic acid to improve the local anesthetic properties of a formulation encapsulating bupivacaine.[300] Both

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HA and linoleic acid were covalently linked to PEG via the formation of amide bounds between their carboxyl groups and the amino groups of NH_2-PEG-NH_2. As per the previous example, the HA-modified NLCs increased in vitro penetration of bupivacaine compared to the unmodified nanoparticles or the drug in solution. In vivo, anesthesia antinociception was also evaluated via the tail-flick test. Bupivacaine-loaded HA-NLCs exhibited the most prolonged anesthesia efficacy compared to all of the controls and the highest maximum possible effect.

Li et al. prepared keratinocyte-targeted LNPs by an ethanol serial dilution method.[302] The nanoparticles were loaded with locked nucleic acid-modified anti-miR. To achieve selective delivery to keratinocytes, the LNPs were targeted with the peptide sequence ASKAIQVFLLAG (A5G33) (TLN_κ). A5G33 was conjugated to DSPE-PEG_{2000}-NH_2 and incorporated onto the surface of the LNPs. In vitro, the uptake of TLN_κ was significantly higher in keratinocytes than in untargeted LNPs and in keratinocytes than in endothelial cells or fibroblasts, displaying its selectivity. In vivo, the efficacy of the formulation was evaluated in burn wounds. TLN_κ encapsulating anti-miR-107 administered topically twice per week significantly restored skin barrier function and accelerated wound closure while upregulating the expression of junctional proteins (e.g., claudin-1 and ZO-1).

Thakur et al. evaluated chitosan-modified hybrids encapsulating fusidic acid as a strategy to treat wound infections.[303] Chitosan was added to the hybrids dropwise under magnetic stirring. The antibacterial activity of the formulation was evaluated by a broth microdilution assay using the methicillin-resistant *Staphylococcus aureus* (*MRSA 33591*), *Methicillin-sensitive Staphylococcus aureus* (*MSSA 25923*), and *Staphylococcus aureus* (*SA 22359*) strains. The inhibitory concentration of fusidic acid decreased from 5- to 4-fold compared to the drug alone, and the effect was prolonged over time.

6. HOW TO CHANGE THE LIPIDIC NANOPARTICLE SURFACE TO MODIFY RELEASE

Strategies related to surface modification of lipid nanoparticles to specifically modulate drug release are quite limited. These strategies mostly apply to SLNs and liposomes.

An SLN surface was coated with different types of chitosan to reduce the burst release occurring in acidic environments when the nanoparticles were administered via the oral route. Quarternized chitosan derivatives or *N*-carboxymethyl chitosan on curcumin-loaded SLNs stabilized the formulation and reduced the burst release.[304,305] *N*-Trimethyl chitosan-*g*-palmitic acid copolymer and *N*-carboxymethylchitosan have been used to minimize the release of resveratrol and carvedilol, respectively, in acidic environments.[306] A slower release of curcumin was also obtained by coating SLNs with a biopolymeric double layer using caseinate and pectin. The coating was chemically cross-linked by creating covalent bonds between caseinate and pectin.[307] Silica-coated liposomes loaded with curcumin displayed significantly higher stability against artificial gastric fluid and showed more sustained drug release in artificial intestinal fluid.[308]

Liposome surfaces have also been modified to trigger on-demand release after external stimuli application, such as heat, by, for example, incorporating temperature-sensitive molecules, such as *N*-isopropylacrylamide monomers.[309] Photosensitizers such as IR-780 and IR-700 have also been incorporated in the liposome membrane to trigger the release of lonidamine and calcein, respectively.[310,311]

7. PURIFICATION AND CHARACTERIZATION OF LIPID NANOPARTICLES

Different techniques have been used to purify the final lipid nanoparticles after surface modification and isolation from unbound ligands or coating agents (Table 13) and to validate and quantify bound surface modifiers (Table 14). However, this step is not always explained in detail in the literature. This is crucial for both reproducibility of the results and product characterization for translation to the clinic.

7.1. Removal of Unbound Ligands and Unbound Coating. Most of the coupling reactions leave unbound ligands, which are potentially responsible for competition with grafted ligands for the targeted sites or for increased immune reactions. This crucial step in the preparation of targeted nanoparticles can be challenging and has a considerable impact on the final product. Surprisingly, most articles reviewed for this work did not mention if, or how, they eliminated free ligands and purified the lipid nanoparticles.

Several techniques can be used to separate bound and unbound ligands from lipid nanoparticles, including size exclusion chromatography (SEC)[318] and gel filtration (Table 13). These techniques allow the quick and efficient separation and recovery of nanoparticles. Sephadex G-50 has been used to remove small ligands from liposomes,[182,208] while Sepharose CL-4B has been used to remove larger ligands, such as antibodies, from LNCs or liposomes.[172,312]

Dialysis is also commonly used to remove unbound ligands. The molecular weight cutoff can be adapted to the size of the ligand. A small cutoff has been used to remove free HA[204] or peptides[162,315] from liposomes and SLNs, while for antibodies, higher cutoffs of 100, 300, or 1000 kDa have been used.[203,319]

Ultracentrifugation is a very simple technique for the purification and concentration of nanoparticles. Unfortunately, not all lipid nanoparticles tolerate ultracentrifugation. LNCs, for instance, are destroyed by ultracentrifugation. Fortunately, most liposomes tolerate ultracentrifugation, with described cycles of 5900*g*, 5 min to 100,000*g*, 45 min.[181,197,201] Hybrid nanoparticles with a lipidic membrane handle centrifugation well.[7,8]

With an adapted molecular weight cutoff, ultrafiltration is efficient and compatible with all nanoparticles, but nanoparticle recovery may be impaired by particle adhesion to the ultrafiltration membrane.

7.2. Characterization of Targeted Lipid Nanoparticles. The final step of targeted lipid nanoparticle formulation is the characterization of their physicochemical properties, the validation of the presence of the ligand or coating agent at the nanoparticle surface, and, if possible, its quantification. This last step is not always described but is critical for therapeutic activity.

Several complementary methods are often used to characterize targeted lipid nanoparticles (Table 14).

7.2.1. Detection and Quantification of Ligands on the Nanoparticle Surface. Absorbance and fluorescence spectroscopy are commonly used to quantify ligands, as many biological molecules absorb ultraviolet (UV) light (i.e., peptides via tryptophan[208] or aptamers). Unfortunately, antibody concentrations are often too low to allow their detection or adsorption. Spectroscopy based on X-ray photons allows the detection of specific atoms such as nitrogen. It can be used if nitrogen is specifically present in the ligand and not on the nanoparticle surface.[324] Fourier transform infrared (FTIR) spectroscopy can also be used to detect additional chemical groups, such as mannose coating,[196] but the information is qualitative and not

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quantitative. If the ligand is labeled with a fluorescent dye and the nanoparticle is larger than 300 nm, flow cytometry (FC) can be used and comes with the advantage of allowing quantification of the proportion of nanoparticles bearing the ligand.[316]

Amine quantification by colorimetric assays has been used to quantify the percentage of ligand bound or unbound to the nanoparticles, providing that the nanoparticle itself does not react with the test reagent. Indeed, bicinchoninic acid (BCA)[205,325] and Bradford assays[181] have been used to quantify proteins grafted onto lipid nanoparticle surfaces but cannot be used with LNCs owing to their strong interaction with the assay reagents. Alternatively, (3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde) (CBQCA) and fluorescamine assays can be used. The BCA assay is very sensitive to lipids and surfactants; CBQCA is very sensitive (10 ng/mL);[326] and micro-BCA is limited to 500 ng/mL.[201] The fluorescamine fluorometric assay does not interfere with other components, such as lipids, but it lacks precision.[323]

Peptides and protein ligands can also be detected and even quantified by electrophoresis. Lipid nanoparticle electrophoresis allows the separation and detection of any remaining unbound ligand.[209] In association with standards and image analysis, quantification is also possible.[7,163] Western blotting can also be used to detect specific proteins on the nanoparticle surface.[197] Alvarez-Erviti et al. separated targeted exosomes from their unbound ligand using bead-grafted antibodies against the ligand and then performed a Western blot assay for semiquantification.[191] Finally, the association of the electrophoretic mobility shift assay with Förster resonance energy transfer (FRET) has been used for a fusion peptide inserted on a liposome by metal complexation with its His-tag tail.[184] For smaller ligands, such as peptides and small molecules, high-performance liquid chromatography (HPLC) or ultraperformance liquid chromatography can be used to quantify unbound ligands.[190,197,201,208] Large proteins such as antibodies are not suitable for UV-associated chromatography owing to a lack of sensitivity.

A microbead assay was used to determine the surface density of antibody-grafted liposomes.[203] This measurement was obtained by comparison between reference microbeads containing a defined antibody density and liposomes encapsulated by microbeads that reacted with the ligand. Given the importance of the ligand density on the targeting ability,[327] more techniques should follow this example. The impact of post-insertion on lipid nanoparticle membrane fluidity can be studied using the BODIPY-PC compound, followed by fluorescence polarization measurements.[173] Finally, tritium (^{3}H)-labeled molecules can also be used to quantify the number of ligands in a formulation.[204]

7.2.2. Evaluation of Nanoparticle Targeting Ability. Once the presence of the ligand on the nanoparticle surface has been confirmed, its targeting activity needs to be validated in a biological model (mainly in vitro but also in vivo). Most techniques require fluorescently labeled lipid nanoparticles incorporating a lipophilic tracer or fluorescent cargo in the formulation. The first step is almost always incubation of the nanomedicine with the targeted cell or receptor, in general for a duration of 1−24 h. The techniques used to quantify the percentage of nanoparticles associated with the cells vary and are summarized below.

FC provides relatively accurate cell numbers but requires detachment of adherent cells.[176,205,325] Confocal microscopy allows the localization of nanoparticles in the cells and can be associated with endosome and Golgi markers to study lipidic nanoparticle trafficking.[173,320] However, quantification by confocal microscopy is trickier than fluorescence-activated cell sorting (FACS), but the two techniques can provide complementary information. It is also possible to quantify cell uptake in a 3D spheroid model by FC and confocal microscopy.[174] Direct plate absorbance or fluorescence reading is feasible with either cell culture[173,177] or immobilized targeted receptors in the wells.[192]

Alternatively, the targeting activity of nanoparticles can be measured without the use of fluorescent dyes. Surface plasmon resonance analysis measures the binding kinetics of targeted nanoparticles to receptors coated on a Biacore sensor chip.[177,205] Finally, cell uptake has been investigated by HPLC by quantifying the drug from the cell lysate.[317]

In vivo targeting analysis provides high-value information about the real comportment of targeted lipid nanoparticles in a living organism. It is possible to follow the nanoparticle fate using an in vivo fluorescent imaging system (e.g., IVIS). Longitudinal quantification can be performed on whole animals or separated organs.[181,206] Blood samples can also be analyzed for pharmacokinetic modeling.[205] Targeted nanoparticle localization and association with the targeted cell type can also be assessed by immunofluorescence.[173]

8. CHALLENGES AND FUTURE PERSPECTIVES

The formulation of drugs in lipid nanocarriers has attracted attention for different routes of administration and different biomedical applications. Lipid nanocarriers are an important field of research in both academia and industry. A diverse set of marketed products illustrates their high potential in drug delivery, for example, Doxyl, a PEGylated liposome for doxorubicin delivery, or Comirnaty for COVID-19 mRNA vaccine delivery.

In this review, we have illustrated that the pharmaceutical and therapeutic properties of lipid nanoparticles can be significantly improved by modifying their surface (i) to interact with mucus, (ii) to target specific cells, (iii) to cross barriers, and (iv) to modify drug release. These surface-modified lipid nanoparticles have mainly been tested in preclinical models with some promising results.

For clinical translation and commercial development, surface modification of lipid nanoparticles is even more challenging than that for plain nanoparticles and must address regulatory hurdles. It requires robust manufacturing of the particles and pharmaceutical dosage forms. Adapted analytical tools to characterize drug-loaded nanoparticles and their surfaces must be developed and validated. Identifying the critical physicochemical attributes and therefore the critical quality attributes (CQAs) that are responsible for their therapeutic performance is essential. The type of formulation should not affect the design of clinical trials.[328,329] Manufacturing surface-modified lipid nanoparticles presents several challenges. The shift from small-scale laboratory production to large-scale manufacturing under good manufacturing practice (GMP) conditions is a bottleneck, particularly for more sophisticated nanoparticles. Multistep production must be robust and reproducible. The use of a quality by design (QbD) approach and the identification of the CQAs that affect the product characteristics and therapeutic performance helps to limit the batch-to-batch variability and warrant the production of purified and stable particles.[328] Microfluidics are increasingly used to formulate lipid nanoparticles[330,331] and also contribute to improving robustness.[332]

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In addition to the usual physicochemical characterization of nanomedicines, for example, drug loading or size, surface modification of lipid nanoparticles implies that the surface must be well characterized. Developing robust analytical techniques to characterize the particulate system and its CQAs is mandatory for translation to the clinic.[328,333] Several guidelines have been issued by regulatory agencies, and reference laboratories have been created.

Based on the pharmacopeia and generally recognized as safe status of most lipid excipients available at GMP quality, the QbD approach and the identification of CQAs to enable the development of a formulation that maximizes the possibility of success, the existing manufacture and characterization of lipid-based nanomedicines that are marketed, and the promising preclinical studies of surface-modified lipid-based nanoparticles, it is likely that more advanced lipid nanoparticles will enter clinical trials in the near future and ultimately will be marketed.

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VOCABULARY

lipid nanoparticles, nanocarriers composed mainly by lipids; **targeted nanocarriers**, surface-modified nanocarriers devoted to actively deliver their cargo to a specific site of action; **ligand**, usually considered a moiety used to bind a drug with specificity to a receptor; **nanoparticle PEGylation**, a strategy that provides the nanoparticle with stealth properties to improve drug delivery efficiency; **nanoparticle grafting**, to modify the nanoparticle with a ligand/moiety toward increased drug delivery efficiency

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