Chapter 1

Introduction

1.1 Nanomedicine and its history

Many drug candidates show potent biological activity but also exhibit poor water solubility, chemical instability, short half-lives in circulation, or inefficient cell uptake, and will therefore likely face significant delivery challenges.[1] Fortunately, a recent advance in nanotechnology for healthcare applications, named nanomedicine, could address those shortcomings and limitations, enhance the therapeutic efficacy of traditional drugs, and revolutionize the pharmaceutical industry landscape.[2] Nanoscale delivery vehicles are designed to aid the transport of diagnostic or therapeutic agents through biological barriers and to improve the physical, chemical, and biological properties (*e.g.* solubility, circulating half-life, less off-target side effects) of drug candidates.[3, 4] Within the field of nanomedicine a wide range of applications, such as drug delivery, vaccine development, antibacterials, diagnostics, imaging tools, wearable devices, implants, high-throughput screening platforms, and other healthcare-related areas are studied.[5]

The progress of nanomedicine has undergone different stages during the last 60 years (**Fig. 1**).[6-15] In 1964, researchers discovered the structure of liposomes and proposed them as carriers for drugs.[6] Thereafter a variety of other nanoscale biomaterials including dendrimers,[16] polymers,[7] targeted liposomes,[15] and PLGA nanoparticles[14] were explored for their potential to deliver drugs. With the FDA approval in 1995 of doxorubicin-loaded liposomes, marketed as Doxil®,[10] the field of nanomedicines entered a new era, and many other lipid-based pharmaceuticals entered the clinic to treat cancer, fungal infections, pain management, and to function as anti-viral therapies (**Table 1**).[17-30] To date, a variety of organic and inorganic nanomaterials have been applied as drug delivery vehicles.[9-13, 31, 32] In 2018 the first siRNA lipid nanoparticle (Onpattro) was approved and the successful authorization for two mRNA Covid-19 vaccines in 2020 kickstarted the era of gene therapy taking center stage in the field of nanomedicine.[33, 34]

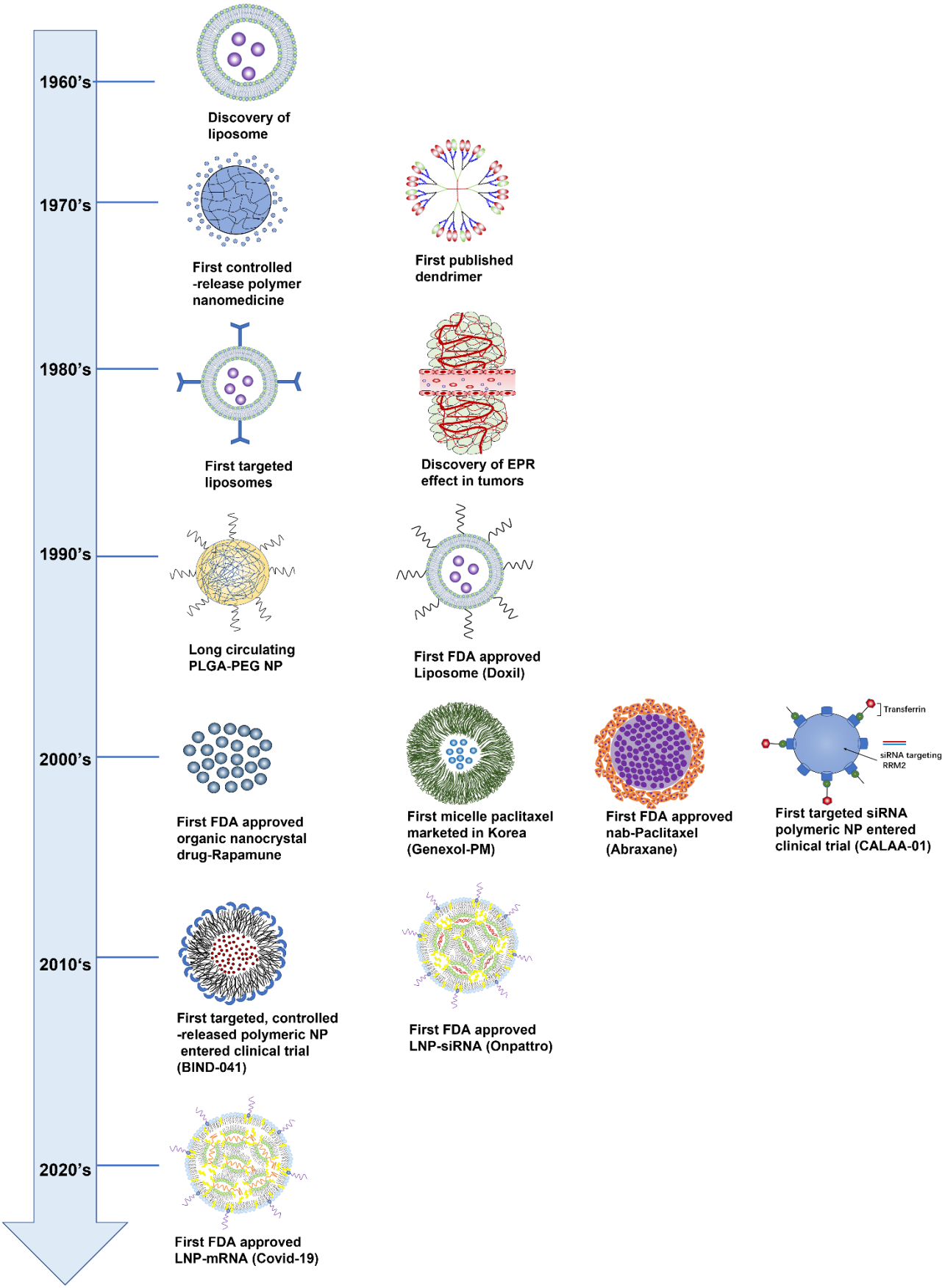

Figure 1. Important milestones in the field of nanomedicine.

The clinically approved liposome and albumin nanoparticles are defined as so-called first-generation nanomedicines, which overcome physicochemical barriers such as poor solubility or passive diffusion of drug molecules.[3, 35] Compared to conventional pharmaceuticals, nanomedicines generally have a large specific surface area and flexibility of surface functionalization enabling different drug loading, retention, and controlled release. As a result, these nanocarriers improve the solubility of poorly-soluble hydrophobic drugs, improve bioavailability, therapeutic effects, and/or release drugs in a sustained, controlled, or stimuli-triggered manner.[36] With these properties, systemic side effects and administration dosage and frequency could be substantially reduced.

More recently, nanomedicines have developed into more advanced nanosystems, so-called second-generation drug delivery systems. These formulations have increased circulation half-life and reduced immunogenicity, while targeting moieties have been introduced to promote cell-specific targeting. Typically these targeting moieties are designed to specifically bind to overexpressed receptors on the surface of cells to target high selectivity for a variety of applications.[37] As targeting moieties, a variety of molecules are being used including small peptides, natural proteins, monoclonal antibodies, aptamers, polymers, carbohydrates, and small targeting molecules (**Fig. 2**).

Table 1. Clinically approved liposome-based products

Clinical products (Approval year)	Administration	Active agent	Lipids/Drug ratio (molar ratio)	Indication	ompany
Doxil® (1995)	i.v.	Doxorubicin	HSPC:Cholesterol:PEG200-DSPE (56:39.5:5)	Ovarian, breast cancer, Kaposi's sarcoma	Sequus Pharmaceuticals
Abelcet® (1995)	i.v.	Amphotericin B	DMPC:DMPG (70:30)	Invasive severe fungal infections	Sigma-Tau Pharmaceuticals
Amphotec® (1996)	i.v.	Amphotericin B	Cholesteryl sulphate: Amphotericin B (1:1)	Severe fungal infections	Ben Venue Laboratories Inc.
DaunoXome® (1996)	i.v.	Daunorubicin	DSPC:Cholesterol (2:1)	AIDS-related Kaposi's sarcoma	NeXstar Pharmaceuticals
Ambisome® (1997)	i.v.	Amphotericin B	HSPC:DSPG:Cholesterol:Amphotericin B (2:0.8:1:0.4)	Presumed fungal infections	Astellas Pharma
Inflexal®V (1997)	i.m.	Inactivated hemaglutinine of Influenza virus strains A and B	DOPC:DOPE (75:25)	Influenza	Crucell, Berna Biotech
Depocyt® (1999)	Spinal	Cytarabine/Ara-C	DOPC, DPPG, Cholesterol and Triolein	Neoplastic meningitis	SkyPharma Inc.
Epaxal® (1999)	i.m.	Inactivated hepatitis A virus(strain RGSB)	DOPC:DOPE (75:25)	Hepatitis A	Crucell, Berna Biotech
Myocet® (2000)	i.v.	Doxorubicin	EPC:Cholesterol (55:45)	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan Pharmaceuticals
Visudyne® (2000)	i.v.	Verteporphin	Verteporphin,DMPC,EPG	Choroidal neovascularisation	Novartis
Mepact® (2001)	i.v.	Mifamurtide	DOPS:POPC (30:70)	High-grade, resectable, non-metastatic osteosarcoma	Takeda Pharmaceutical Limited
DepoDur™® (2004)	Epidural	Morphine sulfate	DOPC,DPPG,Cholesterol, and Triolein	Pain management	SkyPharma Inc.
Exparel® (2011)	i.v.	Bupivacaine	DEPC,DPPG,Cholesterol, and Tricaprylin	Pain management	Pacira Pharmaceuticals, Inc.
Marqibo® (2012)	i.v.	Vincristine	SM:Cholesterol (60:40)	Acute lymphoblastic leukaemia	Talon Pharmaceutics, Inc.
Onivyde™® (2015)	i.v.	Irinotecan	DSPC:MPEG-2000:DSPE (3:2:0.015)	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceutics, Inc.

i.v. (intravenous); i.m. (intramuscular); HSPC (hydrogenated soy phosphatidylcholine); PEG (polyethylene glycol); DSPE (distearoyl-sn-glycero-phosphoethanolamine); DSPC (distearoylphosphatidylcholine); DOPC (dioleoylphosphatidylcholine); DPPG (dipalmitoylphosphatidylglycerol); EPC (egg phosphatidylcholine); DOPS (dioleoylphosphatidylserine); POPC (palmitoyloleoylphosphatidylcholine); SM (sphingomyelin); MPEG (methoxy polyethylene glycol); DMPC (dimyristoyl phosphatidylcholine); DMPG (dimyristoyl phosphatidylglycerol); DSPG (distearoylphosphatidylglycerol); DEPC (dierucoylphosphatidylcholine); DOPE (dioleoly-sn-glycero-phophoethanolamine)

1.2 Lipid-based nanomedicines

Lipid-based nanoparticles are still the most widely employed nanocarrier in drug delivery and diagnostic applications.[30, 38, 39] Liposomes have been successful in delivering anti-cancer, anti-fungal, antibiotic, anesthetic, anti-inflammatory, and gene-based drugs. By careful design, long-circulating (*e.g.* by PEGylation), triggered release, and ligand-targeted delivery is obtained *in vivo*.[38]

As a drug delivery system, lipid-based nanomedicines such as liposomes possess multiple advantages: (i) liposomes can deliver both hydrophobic and hydrophilic molecules due to their amphiphilic lipid molecules that self-assemble into a hydrophilic core and a hydrophobic lipid layer; (ii) lipids are non-toxic and biodegradable; the large pool of lipid varieties enable us to manipulate the liposome structures and properties to achieve different goals by changing the lipid types and ratios; (iii) liposomes exhibit higher tissue accumulation through the enhanced permeability and retention (EPR) effect and better pharmacokinetics, which leads to enhanced therapeutic efficacy and reduced toxicity; (iv) liposomes protect the encapsulated drug, improving drug stability and prolonging its circulation half-life; (v) the large surface of liposomes could be further decorated with different functional moieties (polymers, ligands, and antibodies) to construct targeting and controlled-release drug delivery systems.[38-40] In summary, lipid-based nanomedicines have a proven track record in the successful delivery of a wide range of therapeutics for various diseases.

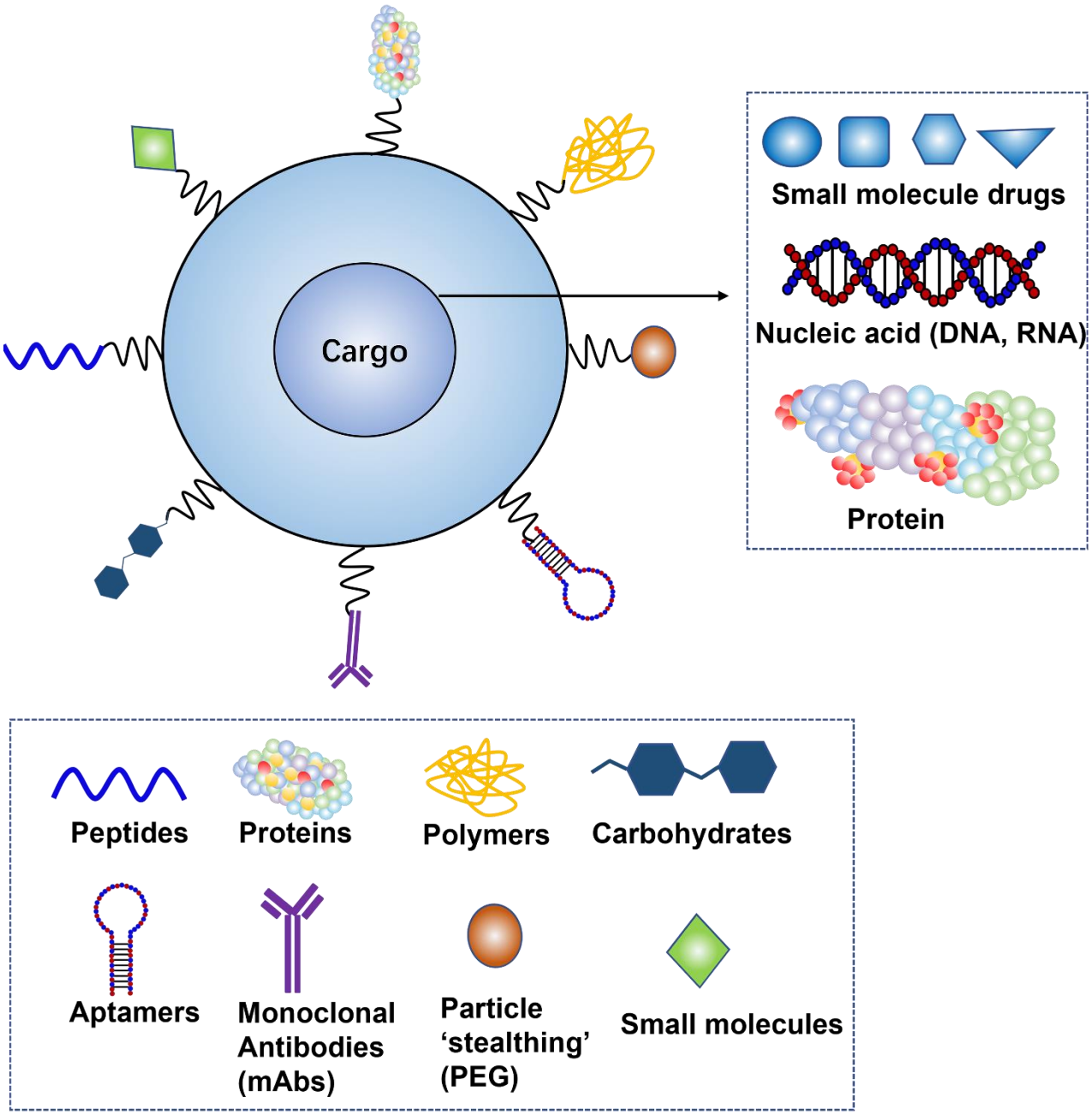

Figure 2. The schematic representation of engineered nanomedicines. The various surface modifications that are commonly pre-engineered could specifically target cells, and the types of encapsulated cargos are highlighted.

1.3 RNA therapy

Gene therapy has attracted attention over the last decades, as it possesses the potential to treat a genetic disorder from its origins by counteracting or replacing a malfunctioning gene within the cells adversely affected by the condition.[41] Genetic cargo containing DNA, mRNA, small interfering RNA (siRNA), and microRNA (miRNA) mimics, can either express specific genes, knockdown gene expression, or upregulate target genes via several mechanisms.[42] For DNA therapy, once the DNA cargo is internalized into target cells and released into the cytoplasm, it still needs to undergo nuclear trafficking and transcription into RNA, and its functionality depends on the nuclear envelope breakdown during cell division; this represents a major hurdle to DNA delivery efficacy.[43, 44] In contrast, RNA delivery is relatively simple as it only needs to reach the cytoplasm of cells to be functional and typically (m)RNA is less immunogenic compared to DNA.

Messenger RNA (mRNA) has recently come into focus as a potential new drug class to deliver genetic information, which provides tremendous flexibility and a broad therapeutic utility.[45] Potential applications include protein replacement therapies, vaccines, and gene editing (**Fig. 3**).

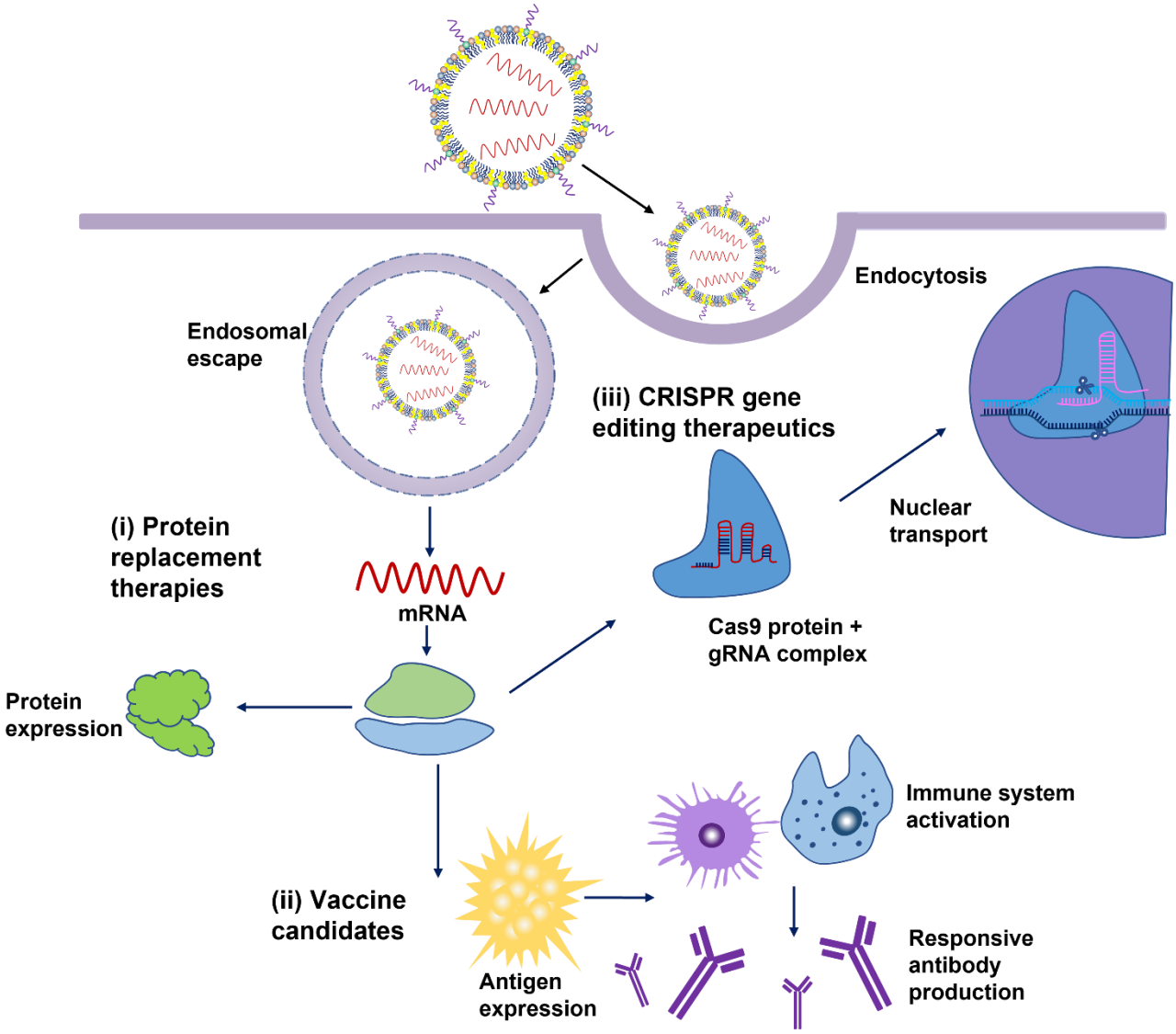

Figure 3. Therapeutic applications of mRNA-nanomedicines include: (i) protein replacement therapies, (ii) vaccine candidates, and (iii) CRISPR gene editing therapeutics.

The development of *in vitro*-transcribed (IVT)-mRNA-based therapeutics has the following advantages: (i) mRNA has no potential risk of insertional mutagenesis since it do not integrate into the genome; (ii) mRNA degradation can be achieved by physiological metabolic pathways; (iii) industrial production of IVT mRNA is relatively simple and inexpensive.[46] The IVT-mRNA produced from a plasmid DNA backbone contains a 5' cap, a 5' untranslated sequence (UTR), an open reading frame coding for the protein of interest, a 3' UTR, and a poly(A) tail (**Fig. 4**), all of these fragments can influence mRNA stability and translation. [46, 47]

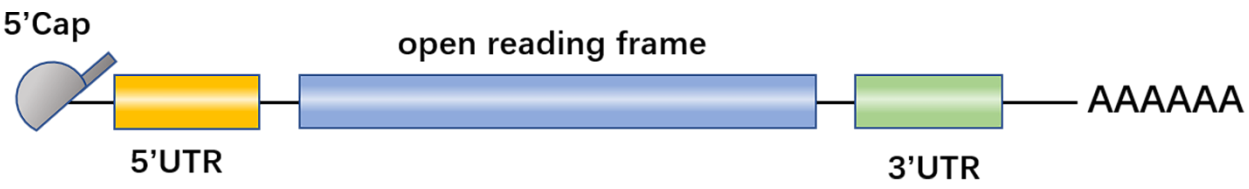

Figure 4. The key structural elements of *in vitro*-transcribed (IVT) mRNA.

1.3.1 mRNA as a protein replacement therapy

Genetic disorders originate from inherited or acquired gene mutations, resulting in abnormal protein expression.[48] Using IVT mRNA as a therapeutic drug to express a desired protein is the most straightforward application. The therapeutic proteins translated by mRNA are generally engineered to display low immunogenicity, prolonged stability, and efficient expression.[44] mRNA exerts its effect at the cytoplasm and expresses protein transiently and degrades via extracellular ribonucleases easily, which avoids the adverse effect of permanent expression. Therapeutic mRNA can be applied to restore the malfunction of a single defined protein caused by rare monogenic diseases, and it can be translated to modulate cellular behavior by expressing transcription of growth factors like vascular endothelial growth factor (VEGF) and cystic fibrosis transmembrane conductance regulator (CFTR).[49]

mRNA as a therapeutic has been studied to treat a range of hereditary or acquired metabolic diseases and regenerative medicine.[50] For example, in a mouse model of a lethal congenital lung disease caused by surfactant protein B (SP-B) deficiency, local delivery of modified SP-B mRNA to the lung greatly restored wild-type SP-B expression, and treated mice survived.[51] In another study intramyocardial injection of modified mRNA encoding human vascular endothelial growth factor A (VEGF-A) improved heart regeneration in the myocardial infarction mice model.[52] Finally, sustained mRNA delivery expressing therapeutic human a-galactosidase protein resulted in clinically relevant biomarker reduction in a mouse Fabry disease model.[53]

1.3.2 mRNA vaccines

Vaccines play a critical role in maintaining global health by preventing infection and transmission of multiple diseases worldwide. Vaccines work by exposing a patient to a part or whole pathogen, thus activating the immune system of the subject.[54] Traditional vaccines include live-attenuated, inactivated, and replication-defective pathogens as well as subunit and conjugate vaccines.[55] Traditional vaccine technologies have been used across a wide range of bacterial and viral pathogens, and the widespread utilization of clinically approved live-attenuated vaccines completely eradicated the smallpox virus and greatly reduced the incidences of polio, measles-mumps-rubella (MMR), yellow fever, and other childhood diseases.[56] However, they have not been successful in some diseases such as persistent infections, rapidly evolving pathogens with high sequence variability, complex viral antigens, and emerging pathogens.[54] The newly emerging infectious virus outbreaks require rapid vaccine technology development and large-scale production, and non-infectious disease like cancer also demand novel vaccine technology since conventional approaches are not applicable. Thus, novel vaccine platforms are highly needed.

Recently, novel *in vitro*-transcribed (IVT) mRNA vaccine technology offers the potential to revolutionize vaccine development as they are well-suited to address the limitations of existing conventional vaccine technology, especially as vaccine platforms against infectious diseases and several types of cancer.[46] mRNA-based vaccines possess multiple advantages over conventional vaccines. First, multiple proteins can be translated: mRNA can be engineered to translate into different

types of proteins to act as antigens to stimulate immune responses.[57, 58] Second, safety: mRNA translation is achieved by the ribosomes in the cytoplasm, requiring no need to enter the nucleus, thus efficacy can be greatly enhanced compared to DNA-based vaccines, which also rule out the potential risk to integrate into genomes.[57, 58] Third, efficacy: diversified modification and delivery vectors can enhance the stability and translation efficiency of mRNA.[57, 58] The functional carriers enable the rapid uptake and efficient expression of mRNA in the cytoplasm and can be administered repeatedly. Finally, production: mRNA vaccines are capable of rapid and large-scale manufacturing with the *in vitro* transcription technology advances greatly boosting the process of vaccine development.[57, 58] Researchers have successfully adopted mRNA vaccines to elicit protective immunity against many infectious diseases (*e.g.* Zika virus, powassan virus, HIV-1 virus, influenza virus) in animal models with the technology of LNP-mRNA delivery tools.[59-62]

Table 2. Representative clinical trials of lipid nanoparticle-mRNA therapeutics against infections, cancer, and genetic disorders

Name	Disease	Encoded protein	Administration route	ClinicalTrials.gov identifier	Phase
Infections					
CVnCoV	SARS-CoV-2	Spike	i.m.	NCT04652102	III
LNP-nCoVsaRNA	SARS-CoV-2	Spike	i.m.	ISRCTN17072692	I
ARCT-021	SARS-CoV-2	Spike	i.m.	NCT04728347	II
ARCoV	SARS-CoV-2	Receptors-binding domain	i.m.	ChiCTR2000034112	I
mRNA-1440	Influenza H10N8	Haemagglutinin	i.m.	NCT03076385	I
mRNA-1851	Influenza H7N9	Haemagglutinin	i.m.	NCT03345043	I
mRNA-1893	Zika virus	Pre-membrane and envelope glycoproteins	i.m.	NCT040649-5	I
mRNA-1345	Respiratory syncytial virus	F glycoproteins	i.m.	NCT04528719	I
mRNA-1653	Metapneumovirus and parainfluenza virus type 3 (MPV/PIV3)	MPV and PIV3 F glycoproteins	i.m.	NCT03392389	I
mRNA-1647	Cytomegalovirus	Pentameric complex and B glycoproteins	i.m.	NCT04232280	II
mRNA-1388	Chikungunya virus	Chikungunya virus antigens	i.m.	NCT03325075	I
CV7202	Rabies virus	G glycoproteins	i.m.	NCT03713086	I
mRNA-1944	Chikungunya virus	Antibody against chikungunya virus	i.v.	NCT03829348	I
Cancer					
mRNA-5671/V941	Non-small-cell lung cancer, colorectal cancer, pancreatic adenocarcinoma	KRAS antigens	i.m.	NCT03948763	I
mRNA-4157	Melanoma	Personalized neoantigens	i.m.	NCT03897881	II
mRNA-4650	Gastrointestinal cancer	Personalized neoantigens	i.m.	NCT03480152	I/II
FixVac	Melanoma	NY-ESO-1, tyrosinase, MAGE-A3, TPTE	i.v.	NCT02410733	I
TNBC-MERIT	Triple-negative breast cancer	Personalized neoantigens	i.v.	NCT02316457	I
HARE-40	HPV-positive cancers	HPV oncoproteins E6 and E7	i.d.	NCT03418480	I/II
RO7198457	Melanoma	Personalized neoantigens	i.v.	NCT03815058	II
W_ova1	Ovarian cancer	Ovarian cancer antigens	i.v.	NCT04163094	I
Genetic disorders					
mRNA-3704	Methylmalonic acidaemia	Methylmalonyl-CoA mutase	i.v.	NCT03810690	I/II
mRNA-3927	Propionic acidaemia	Propionyl-CoA carboxylase	i.v.	NCT04159103	I/II
MRT5201	Ornithine transcarbamylase deficiency	Ornithine transcarbamylase	i.v.	NCT03767270	I/II
MRT5005	Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator	Inhalation	NCT03375047	I/II
NTLA-2001	Transthyretin amyloidosis with polyneuropathy	CRIPR-Cas9 gene editing system	i.v.	NCT04601051	I

HPV, human papillomavirus; i.m., intramuscular; i.v., intravenous; KRAS, Kirsten rat sarcoma 2 viral oncogene homologue; MAGE-A3, melanoma antigen family A; NY-ESO-1, New York esophageal squamous cell carcinoma; SARS-CoV-2, severe acute syndrome coronavirus 2; TPTE, putative tyrosine-protein phosphatase; CoA, coenzyme A; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9.

1.4 mRNA delivery with lipid nanoparticles

mRNA is very large (300–5,000 kDa, ~1–15 kb), hydrophilic, and membrane impermeable due to its negative charges. Furthermore, mRNA is inherently unstable and susceptible to endonuclease degradation with an intracellular half-life < 7 hours.[63] RNA delivery needs to overcome multiple barriers, such as enzymatic degradation, uptake by the reticuloendothelial system, lack of selective tissue accumulation, kidney filtration, and limited intracellular entry and endosomal escape.[64] An ideal mRNA delivery vector must therefore protect against serum endonucleases, evade immune detection, prevent nonspecific interactions, avoid renal clearance, and promote cell entry.[42]

Developing safe and effective gene vectors has been the main focus. Gene vectors can be categorized into two major classes: viral and nonviral vectors. In fact, ~70% of gene therapy clinical trials carried out so far have used modified viruses such as retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAVs).[42] Viral vectors can efficiently transduce mammalian cells, however, the potential carcinogenesis,[65] immunogenicities,[66] broad tropisms,[67] limited packaging capacity, and difficult industrial manufacturing limited their wide applications.[68] Non-viral vectors could overcome those limitations with different biomaterial designs and modifications, including lipids, lipid-like materials, polymers, and inorganic nanoparticles.[45]

Lipid-based nanocarriers are the oldest and most commonly used vector for nucleic acid delivery.[42, 69-71] Initially, permanent cationic lipids were used to encapsulate and transfect RNA to target cells. The positively charged (cationic) amine head groups form an electrostatic complex with the negatively charged RNA, permitting compaction of the RNA in the core of the lipid-based nanoparticles.[72] However, these cationic liposomes often suffered from poor pharmaceutics, cellular toxicity, aggregation with erythrocytes, recruitment of the immune response (interaction with Toll-like receptor or other intracellular proteins), and rapid plasma clearance.[71, 73, 74] Thus, lipid nanoparticles composing ionizable lipids with less toxicity were designed to encapsulate RNA. These lipids are charged at mildly acidic pH and form a complex with RNA to assemble into stable lipid-nanoparticles (LNPs) that are neutral under physiological pH conditions.[71] These LNPs consist of both amorphous and lamellar core structures, whereas the core structure contains a mixture of amorphous, unilamellar, and polymorphic structures.[75, 76] They possess no extra charges, therefore they are exempt from maintaining the balance of the charges and transfection efficacy. They have been considered the most advanced methods for RNA-based therapeutics, as evidenced by the clinical approvals of three LNP formulations, Alnylam's Patisiran (ONPATTRO™), Pfizer's BNT162b2 and Moderna's mRNA-1273.[77] Many other lipid nanoparticle-mRNA formulations have been developed and are under clinical evaluation for the prevention and treatment of virus infections, cancer, and genetic diseases (**Table 2**).[42, 47, 48, 50, 54, 78-84]

LNPs are composed of (i) an ionizable lipid or cationic lipid or polymeric biomaterials bearing tertiary or quaternary amines that can efficiently condense mRNA; (ii) a zwitterionic lipid that serves as a helper lipid to enhance stability and fusogenicity, such as DSPC, DOPE; (iii) cholesterol or cholesterol analogs to stabilize the formulation by modulating membrane integrity and rigidity; (iv) a polyethylene glycol (PEG)-lipid to enhance the stability by decreasing particle aggregation, and to prolong blood circulation time.[80, 85]

After administration, there are multiple extracellular and intracellular barriers awaiting LNP-mRNA formulations to overcome in order to function *in vivo* (**Fig. 5**).[45, 50, 80] First, mRNA needs to be protected from extracellular ribonucleases abundantly present in blood and skin after systemic or local delivery.[45, 50, 80] Second, LNPs should avoid clearance by renal glomerular filtration and the mononuclear phagocyte system (MPS).[45, 50, 80] Third, LNPs need to reach the target tissue and organs, cross the cell membrane, and be internalized by the target cells.[45, 50, 80] Finally, the mRNA must escape from endo/lysosomes, and be transported into the cytoplasm.[45, 50, 80]

Lipid nanoparticle-mRNA formulations are usually manufactured by rapid microfluidic mixing where mRNA is encapsulated in the interior core through electrostatic interactions with the ionizable lipids.[47, 86] This stable nanostructure protects mRNA molecules from nuclease degradation in physiological fluids.[47, 86] The PEG-lipids reduce recognition by the MPS and clearance by renal filtration, improving the stability and circulation lifetime of LNPs.[47, 87, 88] The targeted delivery of LNP-mRNA can be improved by modifying and optimizing the nanoparticles, for example, selective organ targeting (lung, spleen, and liver, respectively) can be achieved by the addition of supplemental molecules.[89] Moreover, surface modification of nanoparticles with targeting moieties (*e.g.* antibodies) can also be engineered to deliver mRNA into inflammatory leukocytes for treating inflammatory bowel disease,[90] and targeting epidermal growth factor receptor (EGFR)-positive tumor cells for cancer treatments.[91] Once LNP-mRNA reaches target cells, they are usually internalized by cells through multiple endocytosis mechanisms depending on nanoparticles' properties and cell types, including macropinocytosis and clathrin-mediated and caveolae-mediated endocytosis.[47, 92-94] After cellular internalization, LNP-mRNA needs to escape from the endosome into the cytoplasm, which is crucial for effective mRNA delivery and translation into the corresponding protein.[95-97]

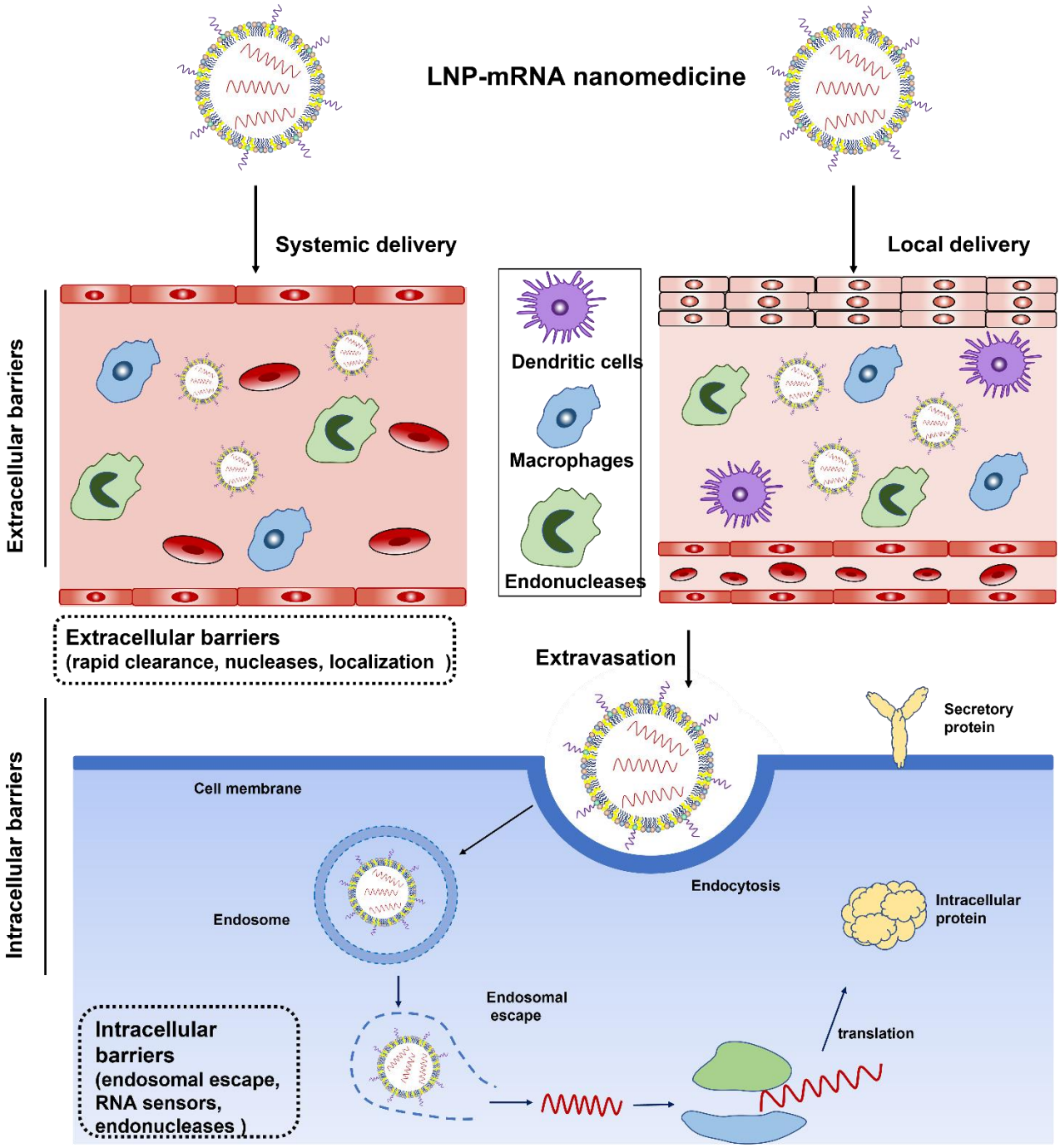

Figure 5. Physiological barriers (extracellular and intracellular) for lipid nanoparticle–mRNA (LNP–mRNA) nanomedicine after systemic and local delivery.

1.5 Endosomal escape

LNPs gain entry into the cells by exploiting membrane-derived endocytic pathways, the genetic cargo accumulates in the early endosome, which acts as a sorting and recycling organelle from which genetic cargo should rapidly escape into the cytosol to avoid progressive and fatal degradation.[98] Their transfection performance depends on this endo/lysosomal escape efficiency. Studies showed that only <2% of siRNA delivered by LNPs was able to escape endosomal compartments into the

cytoplasm.[95] For mRNA it was found that less than 5% delivered by LNPs was able to reach the cytoplasm.[99] In general, LNP enter cells via the formation of early endosomes (EE), where the pH gradually lowers from 6.5 to 5.5. Maturation into late endosomes further lowers the pH 5.5-5.0. Finally, LNPs fuse with lysosomes with pH down to 4.5-5.5, where multiple enzymes (lipases, nucleases, glycosidase, proteases, phosphatases, sulfatases) dismantle the LNP assembly.[100]

The delivery vectors are considered able to escape from endosomes through the proton sponge effect, the buffering capacity attenuates the decline of acidic endosomal pH, thus driving the osmotic pressure increase and ultimate endosomal rupture.[64, 101, 102] Current research is focused on amplifying endosomal escape and minimizing the toxicity of delivery vectors, therefore achieving satisfactory transfection performances. To accomplish these challenging goals, new materials have been designed, which are responsive to external stimuli, such as light, redox state, enzymes, and pH.[103] For LNPs, optimizing the pKa of ionizable lipids, using branched tails and biodegradable lipids, modulating the type (*e.g.* cholesterol, helper lipids [DSPC, DOPE]) and the ratio of lipids have been reported to increase the endosomal escape.[76, 93, 104-113]

1.6 Membrane fusion

Membrane fusion, one of the most fundamental processes in life, mediates housekeeping functions-endocytosis, constitutive secretion, and recycling of membrane components.[114] It underlies many cellular activities, such as viral infection, fertilization, and neurotransmitter release, and usually occurs when two separate lipid membranes merge into a single continuous bilayer.[115] The most typical membrane fusion is exocytosis, whereby an incoming vesicle docks to the membrane, opposing membranes are connected forming a hemifusion stalk, then fusion pores expand to release their contents (*e.g.* hormones or neurotransmitters) into the extracellular milieu, or to deposit receptors, transporters, channels or adhesion molecules into the limiting membrane.[116, 117]

Unlike typical endocytosis which needs to undergo endosome/lysosome degradation after internalization, membrane fusion has been recognized as able to overcome endosomal entrapment by driving direct fusion with the plasma membrane and subsequent delivery into the cytosol (**Fig. 6**).[118] With membrane fusion, the delivery efficiency of nanomedicine could be greatly improved. Among membrane fusion machinery components, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins have been well-characterierized and identified as critical components for multiple processes.[119] Inspired by SNARE proteins, our lab has innovated artificial coiled-coil peptides that could mediate efficient liposomal delivery with enhanced therapeutic effect.[120-122]

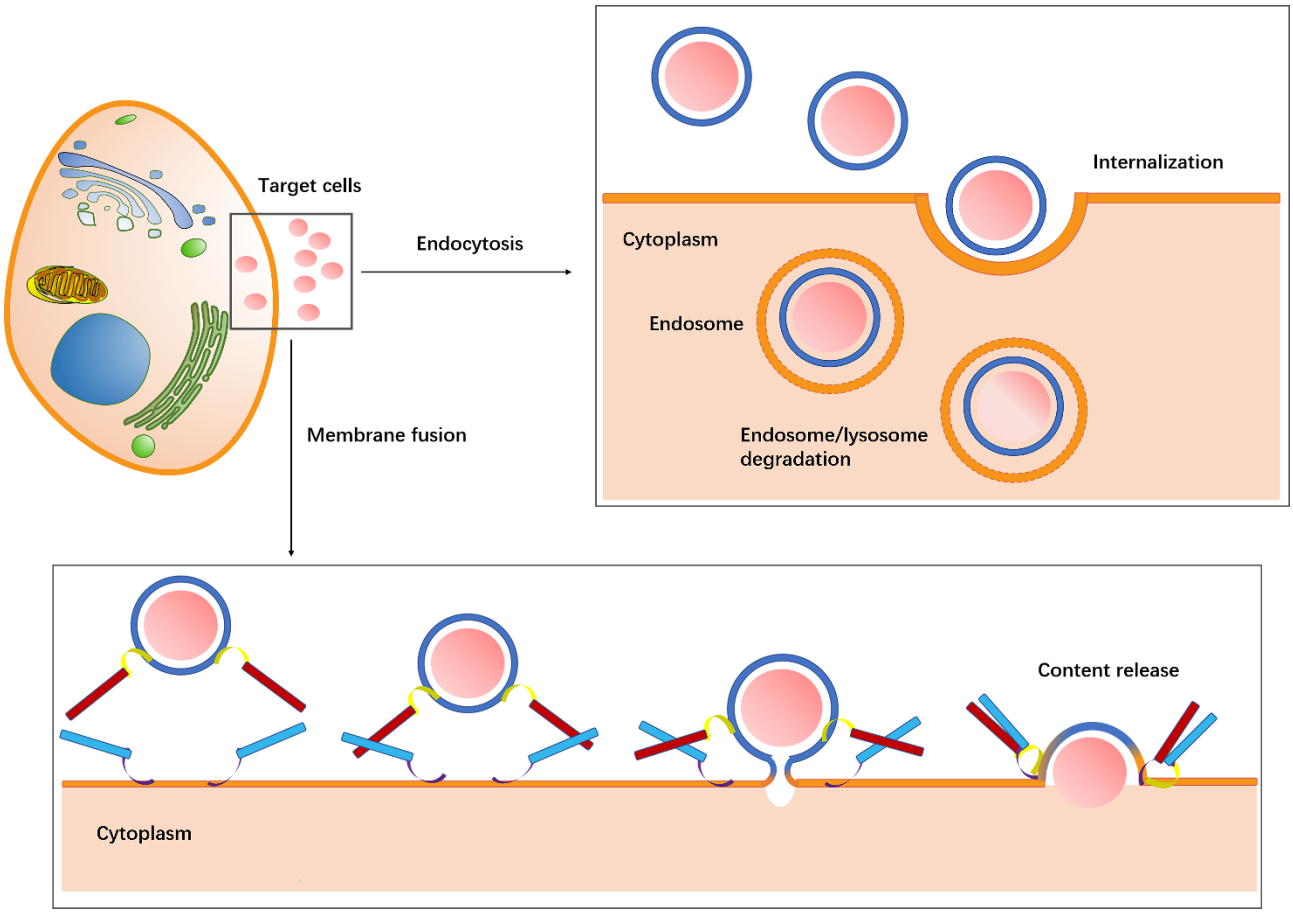

Figure 6. Cellular uptake differences between endocytosis and membrane fusion.

1.7 Aim and outline of this thesis

This thesis focuses on the application of lipid-based nanomedicine in drug delivery, including small molecular antitumor drugs and biomacromolecules including mRNA, and evaluates their biological performance. We have modified liposomes and LNPs with fusogenic coiled-coil peptides to enhance the drug/mRNA delivery efficiency (**Chapter 2-4**), and also investigated how the lipid composition of LNPs influences the immune response (**Chapter 5**).

In **Chapter 2** fusogenic coiled-coil peptides are used to facilitate mRNA delivery *in vitro*. By modifying LNPs with coiled-coil peptides, we show that enhanced transfection efficiency can be achieved independent of cell type. This study shows that the fusogenic coiled-coil LNP system enhances mRNA transfection and holds great promise for future mRNA-based therapies.

In **Chapter 3** we will demonstrate that a coiled-coil peptide dimer facilitates drug delivery within cells and is mainly driven by membrane fusion. By careful peptide dimer design, we investigated their structural differences, membrane binding affinity, cellular uptake efficiency, and pharmacological effects after encapsulating antitumor drugs. It was shown that the parallel PK4 dimer induces the highest cellular uptake, and superior antitumor efficacy compared to the other designs. This study offers important mechanistic insights into the design of coiled-coil driven membrane

fusion systems and also provided novel strategies to develop peptide-based biomaterials to induce improved drug delivery efficiency.

In **Chapter 4**, we further applied the coiled-coil peptide modified LNP to transfect the human-induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Different incubation methods of coiled-coil peptide modified LNPs are compared and a novel 1-step incubation protocol is developed resulting in a high mRNA transfection efficiency. Furthermore, the enhanced mRNA transfection was independent of LNP lipid composition when following the 1-step incubation protocol. This study forms the basis of future *in vivo* research towards the development of efficient cardiomyocyte transfection and stimulation of cardiac repair and ultimately regeneration to rescue the ischemic myocardium.

In **Chapter 5**, we evaluated the influence of lipid compositions of LNPs on immune responses by studying a panel of LNP formulations. This was done by keeping the ionizable lipids constant, replacing cholesterol with β-sitosterol, and changing the fusogenic helper lipid DOPE content. We studied the ability of this LNP library to induce antigen presentation and T cell proliferation, and identified four leading LNP formulations (C12-200-cho-10%DOPE, C12-200-sito-10%DOPE, cKK-E12-cho-10%DOPE and cKK-E12-sito-30%DOPE) that induced robust T cell proliferation and enhanced IFN-γ, TNF-α, IL-2 expression. This study proved that T cell proliferation is strongly dependent on LNP composition.

Chapter 6 summarizes the main finding of this thesis and discusses the future perspectives about the coiled-coil peptides modified nanomedicines and their use in mRNA-based therapies.

1.8 References

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