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Synthetic mRNAs can become biopharmaceutics allowing vaccination against cancer, bacterial and virus infections. Clinical trials with direct administration of synthetic mRNAs encoding tumor antigens demonstrated safety and induction of tumor-specific immune responses. Although immune responses are generated by naked mRNAs, their formulations with chemical carriers are expected to provide more specificity and internalization in dendritic cells (DCs) for better immune responses and dose reduction. This review reports lipid-based formulations (LBFs) that have proved preclinical efficacy. The selective delivery of mRNA LBFs to favor intracellular accumulation in DCs and reduction of the effective doses is discussed, notably to decorate LBFs with carbohydrates or glycomimetics allowing endocytosis in DCs. We also report how smart intracellular delivery is achieved using pH-sensitive lipids or polymers for an efficient mRNA escape from endosomes and limitations regarding cytosolic mRNA location for translation.

KEYWORDS: dendritic cells • glycomimetics • glycotargeting • histidine • lipid-based formulations • lipoplexes • lipopolyplexes • mannose receptors • pH-sensitive delivery • RNA vaccines

Dendritic cells (DCs) play a critical role in antigen presentation to elicit a specific immune response against antigens which can be proteins, peptides and nucleic acids like plasmid DNA or mRNA encoding the antigens. In this context, the transfection of DCs with synthetic mRNA encoding antigen is becoming increasingly important for the design of innovative vaccines [1,2]. One major challenge to be tackled for their development is their targeting to DCs. It is crucial to develop delivery systems that *in vivo* protect mRNAs from degradation and help internalization in DCs and favor intracellular delivery in the right compartment. Since mRNA translation occurs in the cytosol, the transfection of DCs with synthetic mRNA avoids the necessity of mRNA to pass the selective barrier of the nuclear envelope. Therefore, the delivery of synthetic mRNA into the cytosol of non-dividing cells looks easier than that of plasmid DNA in the nucleus. When the synthetic mRNA encoding tumor antigen is expressed in DCs, it allows the antigen expression in its native form. When processed by the proteasome, all generated peptide epitopes of the antigen enter the endoplasmic reticulum (ER), bind to the major histocompatibility complex (MHC) class I molecules and then are expressed on the surface of DCs. The antigen presentation by the MHC class I complex leads to the induction of tumor-specific $CD8^+$ cytotoxic T lymphocytes that in turn kill tumor cells (FIGURE 1). The MHC class II antigen presentation is induced after the uptake of the antigen released in the extracellular medium or the transfection with mRNA encoding the antigen fused with the lysosomal sorting signal of LAMP1 protein targeting the MHC class II compartments (FIGURE 1). Natural mRNAs comprise a 5′ untranslated region, an open-reading frame consisting of the antigen nucleic sequence and a 3′ untranslated region. They are also ended by a cap structure at the 5′ terminus and a Poly(A) tail of at least 100–250 adenosine residues at the 3′ terminus. Synthetic mRNAs obtained by *in vitro* transcription have a similar structure (FIGURE 2). They are capped either with 3′-*O*-methyl-^{m7}5′ Gppp5′G (ARCA) or 2′-*O*-methyl-^{m7}5′ GppSp5′G (β-S-ARCA) anti-reverse cap analog allowing cap incorporation in the right orientation [3] and bear a Poly(A) tail comprising 64 or about 100 adenosine residues [4,5]. In contrast to plasmid DNAs, synthetic mRNAs cannot be integrated in the genome, do not bear promoter, terminator, enhancer and antibiotic-resistance nucleic sequences. There is

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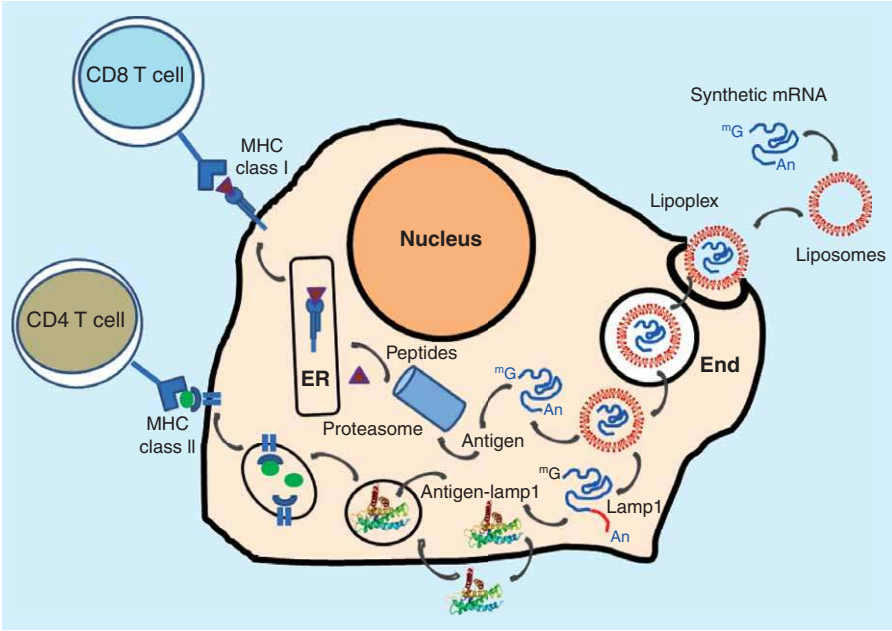

Figure 1. Loading of dendritic cells with synthetic mRNA, antigen processing and presentation.
mRNA formulations are internalized into intracellular vesicles by endocytosis. The escape of mRNA from acidic vesicles (endosomes, End) is generally carried out using pH-sensitive lipids. It is not yet known whether mRNA escapes from endosomes as free mRNA or still complexed with the delivery systems. Anyway once in the cytosol, mRNA reaches ribosomes for translation and antigen is expressed in its native form in the cytosol. After processing in the proteasome, all the peptide epitopes bind to MHC class I molecules in the endoplasmic reticulum and the complexes are then expressed on the DC cell surface, leading to the induction of antigen-specific cytotoxic T lymphocytes. The MHC class II antigen presentation is induced after the uptake of the antigen released in the extracellular medium or the transfection with mRNA encoding the antigen fused with the lysosomal sorting signal of LAMP1 protein targeting to the MHC class II compartments.

another emerging mRNA vaccine which is made of self-amplifying RNA or RNA replicon generated from single-stranded RNA viruses such as alphaviruses (Sindbis and Semliki Forest) and flaviviruses (Kunjin virus and Yellow Fever virus) (Figure 2). Replicon-based vaccines are longer RNA molecules (typically ~10 kb) than synthetic mRNAs. The mRNA encoding antigen of interest is inserted in the RNA virus in place of gene sequences encoding structural proteins. After transfection, the viral RNA polymerase replicates mRNA encoding antigen allowing expression of large amounts of antigens. Recently, non-viral formulations of synthetic self-amplifying RNA vaccines have been reported [6–8]. Most importantly is that synthetic mRNAs are not considered by the US FDA as genetically modified organism in contrast to plasmid [9].

Since the pioneering works of Sullenger and Gilboa [10], preclinical and clinical studies have been extensively tested for induction of antigen-specific immune responses against cancer cells or viruses by injection of autologous DCs transfected *ex vivo* with mRNA encoding specific antigens. Direct administration of synthetic mRNA encoding antigens is more attractive for vaccination of large population and clinical trials have been already initiated in patients with melanoma, renal cell carcinoma, prostate cancer and non-small-cell lung metastatic carcinoma. The anti-tumor mRNA vaccines are injected intradermal and comprise a cocktail of different mRNAs coding for the specific antigens to increase the extent of the immune response and are associated sometimes with an adjuvant. For example, mRNAs coding Melan-A, tyrosinase, gp100, melanoma-associated antigen (MAGE-A1), MAGE-A3, survivin and GM-CSF as adjuvant were injected for metastatic melanoma [11,12] and the tumor-associated antigens mucin 1, carcinoembryonic antigen, human epidermal growth factor receptor 2, telomerase, survivin and MAGE-A1 and GM-CSF as adjuvant were injected for renal cell carcinoma [13]. Phase IIa and IIb clinical trials initiated by CureVac (www.curevac.com) are running for prostate cancer [14] with mRNAs encoding four prostate antigens and for non-small-cell lung metastatic carcinoma cancer [15] with mRNAs encoding MAGE-C1, MAGE-C2, NY-ESO-1, survivin and 5T4 [16]. The first lesson learnt from these trials is about the safety of the strategy in humans and the specific and relevant immune responses that they were able to induce. Phase I clinical trials initiated by BioNTech (www.biontech.de) are also ongoing for melanoma (www.clinicaltrials.gov as #NCT01684241). Although it is reasonable to believe that the immune response is induced by DCs when DCs transfected with mRNA encoding antigens are injected, it is not excluded that the immune response might be in part obtained by cross-presentation of the antigen produced after mRNA transfection of other cells when mRNA encoding antigens are directly injected. Nevertheless, DCs remain the prime target.

In humans, myeloid DCs are derived from a hematopoietic progenitor expressing CD34 molecule in bone marrow. After activation, DC precursors circulate in the blood and lymph and then join the peripheral tissues where they reside as immature DCs. The Langerhans cells reside mainly in the skin tissue but also in lymph nodes and mucosa of the buccal, vaginal cavity and bladder. In the skin, Langerhans cells capture antigens before migration to secondary lymphoid tissues for presentation to lymphocytes with their MHC class I and class II. The interstitial DCs are located in the connective tissues of many organs and lymphoid tissues. In the presence of tissue damages or bacterial products, those immature DCs migrate to the secondary lymphoid organs via the lymphatic system, where they maturate and initiate the immune response. Conversely, DCs residing in

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lymphoid tissues directly capture antigens and present antigens to T lymphocytes. In humans, the other set of DC called plasmacytoid DCs circulate in the blood where they represent only 0.5% of the mononuclear cells. They are also observed in the lymphoid tissues. Circulating plasmacytoid DCs are preferentially recruited in the lymph nodes after exposition to an inflammatory stimulus. The locations of plasmacytoid DCs in inflammation sites and in secondary lymphoid organs suggest that they also play a major role in the initiation of the immune response.

mRNA is prone to degradation by RNAses which are present everywhere. Their anionic character does not facilitate penetration in DCs. It is thus crucial to develop delivery systems that protect mRNAs from degradation and help internalization in DCs and their intracellular delivery. This review aims to report advanced lipid-based formulations (LBFs) for direct administration of mRNA vaccines.

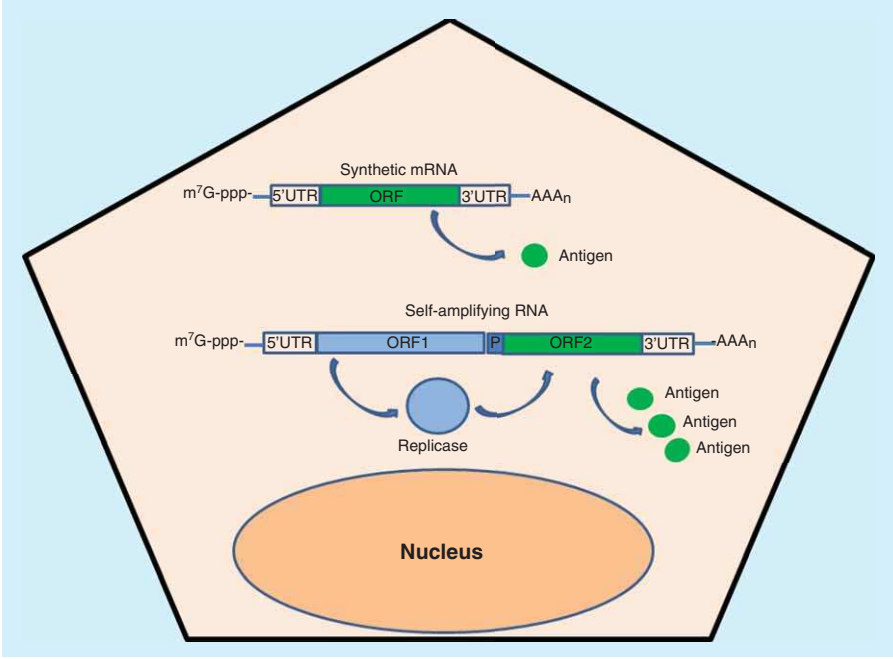

Figure 2. Synthetic mRNAs.
Synthetic mRNAs comprise tri moieties consisting of a capped (^{m}G) 5′ untranslated region, an open-reading frame made up of triplet codons series that code for each amino acid of antigen and a 3′ untranslated region ended with a Poly(A) tail (A_n). The mRNA 5′ and 3′ elements are recognized by initiation factors to allow ribosome binding and scanning along the 5′ untranslated region. The Poly(A) tail length is important for both mRNA translation and mRNA stability. The capping with the 3′-*O*-methyl-m75′Gppp5′G (ARCA) or 2′-*O*-methyl-m75′GppSp5′G (β-S-ARCA) anti-reverse cap analog avoiding cap incorporation in the reverse orientation increases translation efficiency. It is known that cytoplasmic mRNA of eukaryotic cells contains a tail of 100–250 adenosine residues (Poly(A) tail) at its 3′ terminus, which is involved in the mRNA translation and stability. Synthetic mRNA bear a Poly(A) tail of either 64 A or 100A. Self-amplifying RNAs or RNA replicons are generated from single-stranded RNA viruses. Replicon-based vaccines are longer RNA molecules (typically ~10 kb) than the conventional synthetic mRNA. The mRNA encoding antigen of interest is inserted in the RNA virus in place of gene sequences encoding structural proteins. The viral RNA polymerase replicates mRNA encoding antigen allowing expression of large amounts of antigens in dendritic cells.

Lipid-based formulations

There are different types of LBFs used to formulate synthetic mRNAs encoding antigens that have proved induction of specific immune responses in mice (Table 1). The chemical structures of lipids comprised in those LBFs are given in Table 2. Encapsulation of mRNA encoding influenza nucleoprotein in negatively charged PC/PS/Chol liposomes resulted in influenza vaccination despite the low encapsulation efficiency of negatively charged mRNA inside anionic or neutral liposomes [17]. To improve their interaction with liposomes, mRNAs can be formulated with cationic liposomes and the resulting LBFs are called lipoplexes (LP) (Figure 3). Despite the broad molecular diversity of cationic lipids designed for DNA transfection, few of them have been yet evaluated for mRNA transfection *in vitro* and *in vivo* [18,19].

A series of first papers demonstrated that cationic liposomes made with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or DOTAP:DOPE complexed with synthetic mRNA encoding antigen (ovalbumin [OVA], *Escherichia coli* β-galactosidase) enabled immunization of mice [20]. DOTAP:DOPE liposomes were also used to promote vaccination against HIV by using mRNA encoding HIV gag antigen [21]. These LBFs demonstrated efficiency to generate immune response after intravenous, subcutaneous and intradermal administrations. Most of physicochemical parameters of the above-mentioned mRNA LBFs are not reported, but with regards to the preparation and molecules length, we could assume that they formed cationic particles with size ranging from 100 to 200 nm. Ternary complexes comprising cationic liposomes – cationic peptide – pDNA or mRNA called lipopolyplexes (LPD and LPR, respectively) were initially developed by Huang and collaborators, for DNA transfection with DNA/polylysine/pH-sensitive anionic liposomes composed of DOPE:cholesteryl hemisuccinate:folate-polyethleneglycol-DOPE, DNA/protamine/DOTAP, DNA/protamine/DOTAP:DOPE or DNA/protamine/DOTAP:chol [22,23] (Figure 3). They have been also developed for siRNA transfection. When mRNA encoding *E. coli* β-galactosidase was mixed with protamine and unifectin, the resulting LPR demonstrated induction of immune responses after intravenous, subcutaneous and intradermal administrations in mice [24]. The delivery of mRNA vaccine against melanoma with other LPRs was reported by Mockey *et al.* [25]. Those LPRs were formed with PEGylated histidylated polylysine (PEG-HpK)/mRNA polyplexes and L-

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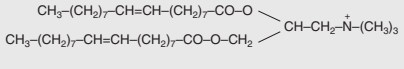
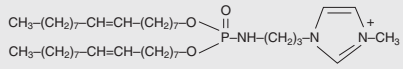
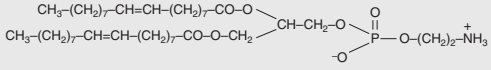
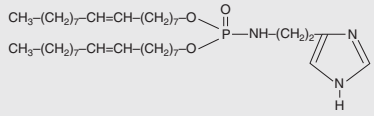
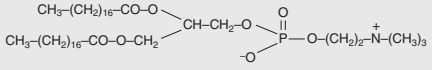
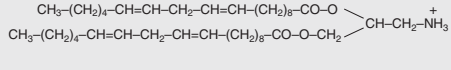
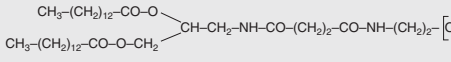
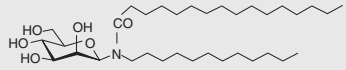
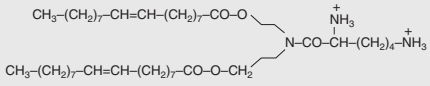
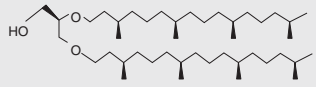
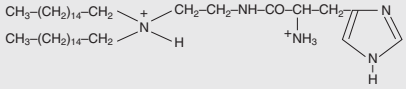
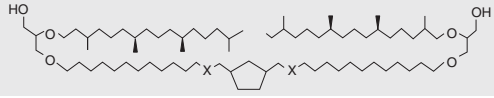
Table 1. A list of lipid-based mRNA vaccines delivery systems.

Vector	Targeted antigen	Size (nm)	ζ (mV)	Species	mRNA doses	Administration routes†						Ref.
						iv.	*sc.*	*IP*	*im.*	*ID*	*IN*	
L: PC/PS/Chol	NP			Mice		+	+	-	nr	nr	nr	[17]
LP: PC/Chol/DC-Chol	gp100	<200	nr	Mice	8 µg (× 2)	Intrasplenic injection						[83]
LP: DOTAP	OVA	nr	nr	Mice	5 µg (× 2)	+	-	nr	nr	+	nr	[20]
LP: DOTAP/DOPE	OVA	nr	nr	Mice	3 µg (× 2)	+	-	nr	nr	+	nr	[20]
LP: DOTAP/DOPE	HIV gag	nr	nr	Mice	20 µg (× 2)	nr	+	nr	nr	nr	nr	[21]
LPR: Unifectin + protamine	β-Gal	nr	nr	Mice	30 µg (× 1)	+	+	-	-	+	nr	[24]
LPR: HpK-PEG + HDHE/chol	MART-1	60–100	17	Mice	12.5 µg (× 2)	+	-	nr	-	nr	nr	[25]
LPR: HpK-PEG + KLN25/MM27	MART-1	100	14	Mice	25 µg (× 2)	+	-	nr	-	nr	nr	[26]
LPR: HpK-PEG + mannose KLN25/MM27	MART-1	140	18	Mice	25 µg (× 2)	+	-	nr	-	nr	nr	[26]
LNP: PBAE + DOTAP/DOPC lipid shell + DSPE-PEG2000	OVA	280	40	Mice	3 µg (× 2)	nr	nr	nr	nr	nr	+	[31]
LNP: DSPC: cholesterol: DMG-PEG2000: DLinDMA	RSV-F Replicon	79–121	nr	Mice	1 µg (× 2)	nr	nr	nr	+	nr	nr	[7]
CNE: Tween 80 with an oil phase containing Span 85, DOTAP and squalene	RSV-F replicon	86	nr	Mice	15 µg (× 2)	nr	nr	nr	+	nr	nr	[6]
CNE: Tween 80 with an oil phase containing Span 85, DOTAP and squalene	HIV gp140 replicon	86	nr	Rabbits	25 µg (× 2)	nr	nr	nr	+	nr	nr	[6]
CNE: Tween 80 with an oil phase containing Span 85, DOTAP and squalene	IE-1 hCMV replicon	86	nr	Macaques	75 µg (× 2)	nr	nr	nr	+	nr	nr	[6]

†Administration route: (+) the efficiency was demonstrated; (-) non-efficient when tested. nr: not reported.
Chol: Cholesterol; CNE: Cationic nanoemulsion; DC-Chol: 3ß-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride; DOTAP: 1,2-Dioleoyl-3-trimethylammonium-propane; DMG-PEG2000: 1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene Glycol-2000; DLinDMA: 1,2-Dilinoleyloxy-3-dimethylaminopropane; DOPC: L-α-Di-oleoyl phosphatidyl choline; DOPE: L-α-Di-oleoyl phosphatidyl ethanolamine; DOTMA: *N*-[1-(2,3-di-oleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride; Unifectin: L-Lysine-*bis*-(*O*,*O*′-9-octadecenoyl-β-hydroxyethyl)amide dihydroxychloride; DSPC: 1,2-Distearoyl-sn-glycero-3-phosphocholine; DSPE-PEG: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (ammonium salt); GFP: Green fluorescent protein; gp100: Glycoprotein 100 melanoma antigen; hCMV: Immediate early protein 1 from human cytomegalovirus; HDHE: L-Histidine-(*N*,*N*-di-*n*-hexadecylamine)ethylamide; HIV gag: gag protein of human immunodeficiency virus; HIV gp140: gp140 envelope glycoprotein of human immunodeficiency virus. HpK: Histidylated polylysine; ID: Intradermal injection; im.: Intramuscular injection; IN: Intranasal injection; IP: Intraperitoneal injection; iv.: Intravenous injection; KLN25: *O*,*O*-dioleyl-*N*-(3*N*-(*N*-methylimidazolium iodide)propylene) phosphoramidate; MM27: *O*,*O*-dioleyl-*N*-histamine phosphoramidate; L: Liposomes; LNP: Lipid nanoparticle; LP: Lipoplex; LPR: Lipopolyplex; MART-1: Melanoma antigen recognized by T cells 1; NP: Influenza virus nucleoprotein: OVA: Ovalbumin; PBAE: Poly(β-amino ester) poly-1; PC: Phosphatidylcholine; PEG: (Polyethylene glycol)-5000; PEG-HpK: Histidinylated polylysine methoxypolyethylene Glycol-5000; PS: Phosphatidylserine; RSV-F: Fusion glycoprotein of respiratory syncytial virus; sc.: Subcutaneous injection; β-Gal: *Escherichia coli* β-galactosidase.

histidine-(*N*,*N*-di-*n*-hexadecylamine) ethylamide (HDHE):cholesterol liposomes. Then, a second generation of LPRs has been designed by combining PEG-HpK/mRNA polyplexes with *N*-methyl imidazolium lipophosphoramidate (KLN25): histamine lipophosphoramidate (MM27) liposomes called Lip100 [26,27]. Lipophosphoramidates have a bio-inspired chemical structure from the natural phospholipids present in the cell membranes and they are less toxic than the other cationic lipids. To favor DCs targeting, mannosylated Lip100 liposomes were prepared to form mannose-LPR [26]. Those LPRs have a size range from 60 to 140 nm and a low global charge characterized by a ζ potential close to 15 mV.

Table 2. Chemical structures of lipids.

Lipid	Name	Lipid	Name
	DOTAP		KLN25
	DOPE (+)†		MM27 (+)
	DSPC		DLinDMA (+)
	DMG-PEG2000		Man-Lip
	Unifectin		Archaeol
	HDHE (+)		Tetraether diols

†(+) stand for lipids exhibiting acid-mediated membrane destabilization properties.
DMG-PEG2000: 1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene Glycol-2000; DLinDMA: 1,2-Dilinoleyloxy-3-dimethylaminopropane; DOPE: L-α-Di-oleoyl phosphatidyl ethanolamine; DOTAP: 1,2-Dioleoyl-3-trimethylammonium-propane; DSPC: 1,2-Distearoyl-sn-glycero-3-phosphocholine; HDHE: L-histidine-(*N*,*N*-di-*n*-hexadecylamine)ethylamide; KLN25: *O*,*O*-dioleyl-*N*-(3*N*-(*N*-methylimidazolium iodide)propylene) phosphoramidate; MM27: *O*,*O*-dioleyl-*N*-histamine phosphoramidate; Man-Lip: β-D-Mannopyranosyl-*N*-dodecylhexadecanamide; Tetraether diols: Synthetic hemimacrocyclic analogs of archaeal membrane bipolar lipids; Unifectin: L-Lysine-*bis*-(*O*,*O*′-9-octadecenoyl-β-hydroxyethyl)amide dihydroxychloride.

HDHE-, Lip100- and Man-Lip100 LPR containing 25 µg mRNA encoding MART-1 melanoma tumor antigen demonstrated efficient immunization against the murine B16F10 melanoma after only two intravenous injections. A MART-1-specific CTL immune response was generated as expected for cancer immunization. Subcutaneous and intramuscular administrations did not provide efficient immunization. Intradermal injection was not tested. The specificity of MM27, HDHE and PEG-HpK compounds lies in their imidazole moieties that when acquiring a cationic charge at pH <6.0 lead to an acid-mediated endosomes membrane destabilization favoring mRNA delivery in the cytosol.

Until now, there is no study reporting the structural organization between cationic lipids and mRNA. The required quantity of synthetic mRNA and their RNAse sensitivity actually limit physicochemical studies. In the case of pDNA, it is well demonstrated that when mixed together, lipids and DNA undergo topological transformation into compact quasi-spherical vesicles of 200–300 nm in diameter, in which DNA and lipids adopt an ordered multilamellar structure [28]. The structure of a supercoiled pDNA being different to that of a single-stranded mRNA, which often contains several hairpins, mRNA LP would likely exhibit another type of supramolecular organization. mRNA LPR are formed by combining cationic liposomes with preformed polyplexes. As for mRNA LP, the nature of the assembly of mRNA with a cationic polymer is not yet well known. Mockey *et al.* reported a transmission electronic microscopy analysis of MART-1 mRNA/PEG-HpK/HDHE:chol LPR [25]. It was shown that after mixing mRNA polyplexes of 50 nm in diameter with HDHE liposomes of 60–100 nm, vesicles of 60–100 nm were observed with a dense core in their middle presumably mRNA polyplexes entrapped inside liposomes. These transmission electronic microscopy observations in terms of morphology were in line with those reported for DNA/polylysine/DOPE:cholesteryl hemisuccinate: folate-polyethlene glycol-DOPE [22] and DNA/polylysine/DC-chol:DOPE [29]. CryoTEM observations of LPD100 of 136 nm made with pDNA indicated that interactions of KLN25/MM27 liposomes with DNA/PEG-HpK polyplexes did not lead to a complete reorganization of polyplexes [30]. The lamellar organization of DNA/liposome moieties observed at the periphery of the assembly, suggests that liposomes were associated with DNA/polymer complexes without a strong reorganization of the polyplex structure.

mRNA formulated with lipid nanoparticles (LNPs) was reported to induce an anti-tumor immune response after intranasal injection of 6 µg mRNA encoding OVA [31]. Those LNPs were composed of a core made with a poly(β-amino-ester) (PBAE) polymer exhibiting proton sponge property and a DSPE-PEG:DOTAP:DOPC lipid shell minimizing toxicity of the polycation core; DSPE-PEG being post-inserted into PBAE:DOTAP:DOPC particles (Figure 3) [32]. The supramolecular assembly is different to that of LPR. The preparation process leads to the formation of lipid-coated PBAE particles on which mRNA is adsorbed via electrostatic interactions onto the positively charged surface. The resulting LNP has a size of 280 nm in diameter and a high ζ potential of 40 mV that could favor interaction and adhesion on mucosal cells.

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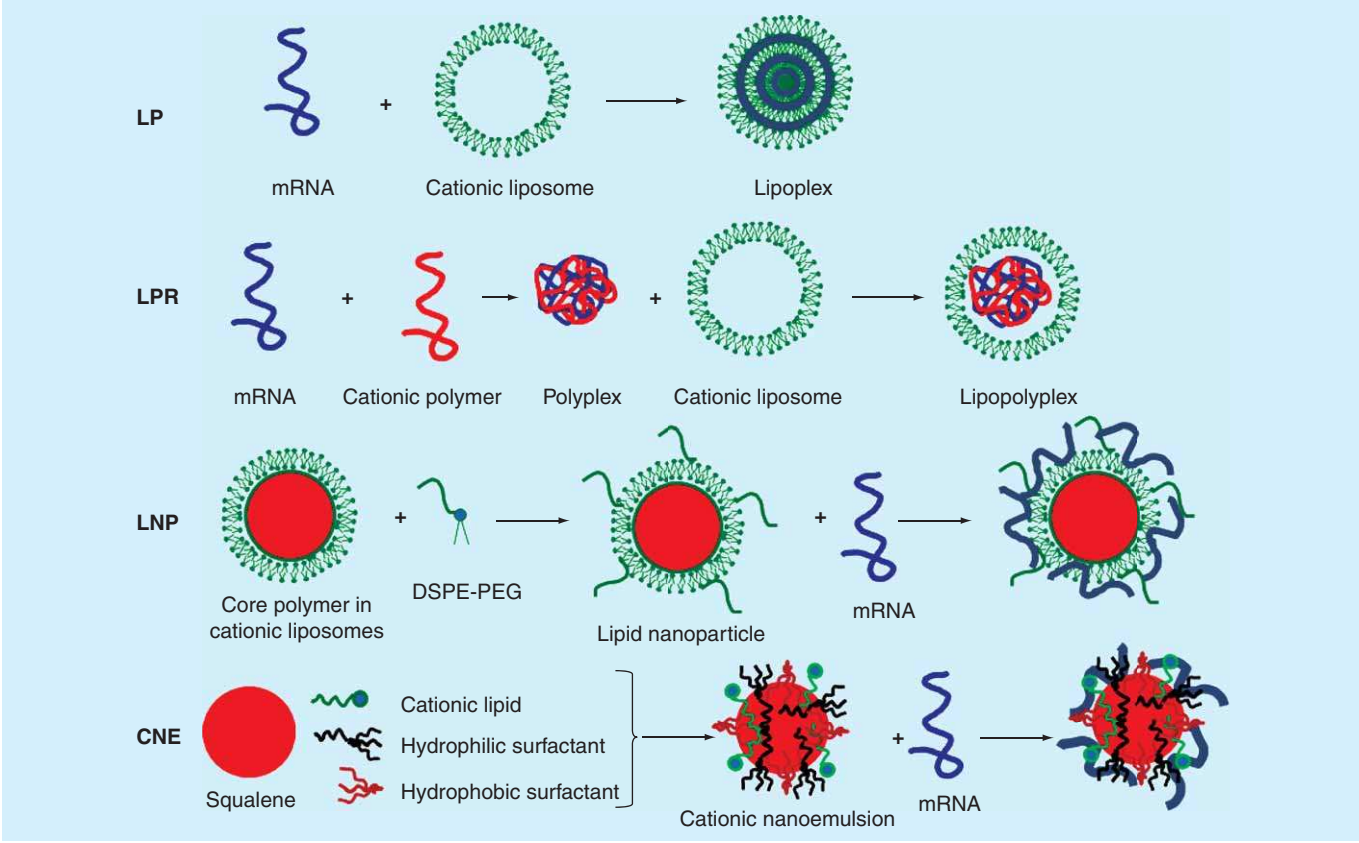

Figure 3. Schemes of lipid-based formulations.
CNE: Cationic nanoemulsion; LNP: Lipid nanoparticle; LP: Lipoplex; LPR: Lipopolyplex.

Another LNP was used for mRNA vaccination of mice with replicon or self-amplifying mRNA encoding the fusion (F) glycoprotein of respiratory syncytial virus (RSV) [7]. The lipid particles comprised 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, DMG-PEG2000 and 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA). Ethanol dilution was used to produce small uniform lipid particles of 79–121 nm size with a high degree of mRNA encapsulation. An F-specific IgG response was generated compared with naked replicon after two intramuscular injections of 1 µg mRNA LNP. Recently, the same team reported vaccination of different species with self-amplifying mRNA by using a cationic nanoemulsion (CNE) different from LNP (Figure 3) [6]. CNE comprised squalene, DOTAP and sorbitan trioleate mixed and heated to 37°C. The resulting oil phase was then combined with an aqueous phase consisting of Tween 80 in citrate buffer at pH 6.5. The resulting emulsion had a size of 86 nm. Protective RSV vaccination was obtained in mice after two intramuscular injections of 15 µg self-amplifying mRNA encoding the fusion (F) glycoprotein of RSV. High level of HIV neutralizing antibody titers was also generated in rabbits after two intramuscular injections of 25 µg self-amplifying mRNA encoding HIV gp140. Importantly, antibody and T-cell responses were induced in Rhesus macaques after two intramuscular injections of 75 µg self-amplifying mRNA encoding human cytomegalovirus envelope glycoprotein B (gB) and a fusion protein (pp65–IE-1) of phosphoprotein 65 (pp65) and immediate early protein 1 (IE-1). One advantage of CNE over LNP is that chemical products (DOTAP, squalene and sorbitan triolate) are already used in clinical trials.

It could be necessary to increase stability of liposomal formulations notably by enhancing their stability in the blood or for sterilization and preservation purposes. For instance, cholesterol known to stabilize the lipid bilayer of liposomes is used sometimes in place of DOPE in cationic liposomes. But in contrast to DOPE, cholesterol has no fusogenic properties to help intracellular delivery of cargo. Thus, a compromise should be found in the co-lipid choice to provide good extracellular stability of LBFs or good capacity at inducing endosome destabilization. In this context, *Archaea* that are non-pathogenic microorganisms could be of interest. The membrane of some extremophilic *Archaea* is composed of non-conventional lipids such as archaeol comprising two saturated isoprenoid chains attached to glycerol by ether linkages with a sn-2 stereochemistry (Table 2). In thermoacidophilic, methanogenic and some psychrophilic species, there are bipolar lipids consisting of tetraether 72-membered macrocycles formed by two biphytanyl chains ether linked at both ends to a glycerol unit. Those lipids are indeed involved in the maintenance of the microorganism membrane integrity in harsh environments. Synthetic archaeal tetraether lipids have

been synthesized [33,34]. For example, the hemimacrocyclic bipolar lipids made of an atom-long bridging chain containing a cyclopentane ring linked at both ends to two (S)-glycerol moieties via ether bonds, two phytanyl chains having a combined length equivalent to that of the bridging chain and hydroxyl groups (Table 2). Their insertion in liposomal formulations enhanced liposome stability. A tetraether macrocycle analog used as helper in lipophosphoramide cationic liposomes allowed efficient DNA transfection *in vivo* [35].

Influence of the administration routes

Synthetic mRNAs used in the above-mentioned clinical trials have been intradermally administrated. The administration route of mRNA LBFs might have a significant influence on both the quality of the induced protection, the frequency of the administration and intensity of side effects. All mRNA LP and lipopolyplexes listed in Table 1 have proved efficacy in mice after intravenous injection (iv.). The vaccine effect with LNPs and CNE listed in Table 1 was not evaluated via systemic injection. Cationic particles size higher than 100 nm are mostly retained in the liver Kupffer cells and are known also to be trapped in the lung. The presence of PEG moieties reducing the positive charge of the complexes, interaction with serum protein and aggregation limits the pulmonary capture. To induce an immune response, they should reach lymphoid organs. It has been shown that 24 h after iv. injection of LPR containing mRNA encoding EGFP, 3% of splenic DCs were EGFP-positive [26]. When LPR were made with mannosylated liposomes, the percentage reached 13% due to their targeting to DCs via the mannose receptor (MR). Remarkably, the vaccine effect was correlated with the number of transfected DCs suggesting that the immune response against cancer cells was mainly mediated by transfected DCs. After iv. injection, LPR and *a fortiori* mannosylated LPR could be also captured by mouse $CD11c^+$ $B220^+$ $Gr1^+$ plasmacytoid DCs present in the blood circulation, which could participate in the establishment of the immune response. Although here no toxic and/or side effects were observed in mice, transposition to human will require a more thorough analysis of the security.

When subcutaneous injection (sc.) in space between the skin and skeletal muscles was tested, some mRNA formulations listed in Table 1 induced the immune response. This administration route offers the possibility to transfect directly a population of DC present in the lymph node.

Intramuscular injection (im.) is easy to realize at large scale but when tested mRNA vaccines listed in Table 1 were not effective with LPRs. The mobility of LPR in muscle fibers depends on their sizes and their charges which likely impair their delivery in DCs. The mobility could be expected better with smaller particles (size <50 nm) and less positively charged particles. In contrast, replicon delivered with 1,2-distearoyl-sn-glycero-3-phosphocholine:cholesterol:DMG-PEG2000: DLinDMA LNP particles or CNE lipid emulsion induced antiviral immune response after im. injection. In the case of LPR vaccinations via im. injection, an immune response against cancer cells was sought. However, this route mobilizes circulating DCs which are from lymphoid origin turned toward a humoral immune response. In case of the above-mentioned LNP and CNE, an antiviral immune response was sought, so im. injections were more appropriate. The nasal route enables to reach local lymphoid tissues. One example of mRNA transfer after nasal administration was reported with LNP composed of PBAE polymer and DSPE-PEG:DOTAP:DOPC lipids (Table 1). A specific CTL immune response directed against E.G7-OVA tumor was obtained with mRNA encoding OVA [31]. Once in the nasal epithelium, particles can be transported via M cells to underlying nasal-associated lymphoid tissues rich in B, T cells and DCs. By this way, the transport via M cells of LBFs of several microns is expected. LBFs can be highly positively charged in order to increase adhesion on mucosal cells and chitosan can be added to increase adhesion. According to influenza vaccination expertise in the past, this route of administration requires precautions while the person is vaccinated. The intradermal injection (ID) is the administration of particles into the skin dermis, just below the epidermis. This is the route used for the clinical trials with anti-cancer mRNA vaccine. Compared with sc. and im. injection, ID injection permits to touch Langerhans cells which are from myeloid origin and induce a Th1 immune response with CTL induction. With LBFs, OVA and *E. coli* β-galactosidase mRNA vaccination was obtained with ID injection of DOTAP LP and protamine/Unifectin LPR (Table 1). ID reduces the volume of injection, for example, 100–300 µl or 10–20 µl are injected in humans and mice, respectively.

When tested, intraperitoneal injection did not provide significant immunization. Intratumoral injection would deliver mRNA encoding antigen in DCs present in tumors but it is not yet reporter for mRNA vaccine. Again small volume of vaccine will be injected in solid tumors. The injection of synthetic mRNA encoding tumor antigen (5–10 µl liquid) into inguinal lymph node (intranodal injection) has been found to elicit antitumoral immunity in mice [36,37]. Again small volumes of vaccine are injected. This type of administration is not yet reported with LBFs. The choice of the administration route of mRNA vaccines must be guided by the level and nature of the required vaccine effect. We must keep in mind that vaccination concerns large human population. Knowing that mRNA and LBFs will be produced at a large scale under good manufacturing practice (GMP) conditions at costs satisfying commodity low-cost vaccine markets, unsophisticated LBFs and small volumes for injection must be considered in strategic developments of mRNA vaccines.

mRNA internalization & intracellular trafficking

Clathrin-dependent endocytosis, caveolae-dependent endocytosis and macropinocytosis are the main mechanisms leading to cell internalization of extracellular molecules and particles. In few papers, synthetic mRNA is described to be taken up by DCs via the caveolae-dependent pathway involving lipid raft-rich membrane domains and scavenger-receptor(s), and macropinocytosis excluding any role of clathrin-coated pits [38].

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Diken *et al.* showed a dominant role of macropinocytosis in the uptake of synthetic RNA by immature DCs *in vitro* and *in vivo* [39]. A recent paper reporting an endocytosis study of mRNA/polyethylenimine (PEI) polyplexes of 200 nm size in HeLa cells showed that mRNA colocalized with caveolin-1 after 30 min incubation, indicating the involvement of the caveolae pathway [40]. After 2 h, approximately 32% of mRNA polyplexes have left caveolae. Some mRNA polyplexes colocalized with clathrin vesicles suggesting that either the clathrin-mediated endocytosis occurred also or it was the result of the docking of caveolae onto endosomes allowing some mRNA complexes internalized *via* caveolae to join the clathrin-dependent pathway [41]. Although synthetic mRNA alone in DCs and complexed with PEI in HeLa cells were described to be taken up by clathrin-independent way, mRNA LP and mRNA LPR could be internalized in DCs by clathrin-dependent endocytosis in comparison with DNA LP and LPD. A confocal microscopy studies with DNA LPD100 and DNA mannosylated LPD100 revealed indeed that DNA uptake occurred through clathrin-mediated endocytosis in the DC2.4 murine DC line [42]. These studies were performed with genetically modified cells stably expressing fluorescent intracellular compartments such as Rab5-EGFP (early endosomes), Rab7-EGFP (late endosomes) or Cav-1-mRFP (caveolea), which are powerful cellular tools to delineate endocytosis mechanisms without the use of pharmacologic drugs [43]. Further works will be done to confirm uptake mechanism of mRNA LBFs.

Endosome escape

In both cases (clathrin-dependent and caveolae-dependent endocytosis), mRNA LBFs reach acidic vesicles (endosomes); the escape of mRNA can be achieved using pH-sensitive lipids becoming fusogenic in mildly acidic medium, that is, pH 6.5–5.0. Among the LBFs listed in Table 1, some of them comprise lipids that exhibit acid-mediated membrane destabilization properties (Table 2). DOPE and MM27 are neutral lipids used as helper with DOTAP and KLN25, respectively. Cationic lipids mediate mRNA condensation and protection against RNAses while DOPE and MM27 promote membrane destabilization. HDHE and DLinDMA are cationic lipids that combine both mRNA condensing and membrane destabilization activities.

DOPE has the propensity to form HII-phase structure that can induce supramolecular arrangements leading to the fusion of lipid bilayer [28]. DOPE indeed undergoes a lamellar-to-hexagonal phase transition in acidic medium, which likely facilitates fusion with or destabilization of the target membrane. The protonation of the polar head decreases the hydrophilic behavior of DOPE changing its shape for a conical one and a HII preference structure. DOPE incorporated in mRNA LP promotes the formation of HII phase structure in endosomes that likely destabilizes endosomal membrane and delivers mRNA in the cytosol.

HDHE and MM27 are lipids that have a polar head containing a histidine and an imidazole group, respectively. The protonation of the imidazole ring occurring around pH 6.0 greatly increases the fusogenic properties of liposomes with endosomal membrane causing destabilization and cytosolic delivery. Several other lipids containing histidine or imidazole have been synthesized and evaluated for gene transfer but today few of them were tested for mRNA transfection [44].

DLinDMA is an ionizable cationic lipid containing two double bonds *per* linoleyl hydrocarbon chain [45]. Further developments of this lipid family have identified DLin-KC2-DMA (with methylene groups added between the DMA head group and the ketal ring linker) exhibiting better activity. The proposed mechanism of action for membrane disruptive effects of DLinDMA involves the formation of inverted, non-bilayer phases such as the hexagonal HII phase (like DOPE) occurring when the protonated DLinDMA in endosome and an anionic lipid (i.e., phosphatidylserine of endosomal membrane) mixed together adopt a molecular 'cone' shape. The association of HpK-PEG – a protonable histidine-rich polymer – with HDHE or KLN25/MM27 liposomes in mRNA LPR further enhances the endosome destabilization capacity. Indeed, poly (L-histidine) is known to promote membrane fusion in acid medium. In addition, imidazole is a weak base (pKa of 6.0) that is suitable to induce a proton sponge effect inside endosomes containing mRNA. The capture of protons generated by the proton ATPase increases the lumen osmolarity and endosome swelling, which likely facilitates mRNA delivery in the cytosol.

PBAE is a pH-sensitive polymer promoting also endosome disruption by a proton sponge effect (Table 1). Other polymers could be exploited as endosomolytic agent. For instance, poly(amidoamine) polymers undergo a marked conformational change from a relatively coiled hydrophobic structure at neutral pH to a relaxed hydrophilic structure at acidic pH. Some polyanions bearing carboxyl groups such as poly(acrylic acid) derivatives and succinylated poly(glycidol)s exhibit pKa values close to 6 and acquire hydrophobic character at low pH [46]. They can promote destabilization and fusion of negatively charged or uncharged membranes.

Cytosolic release

It is not yet known whether synthetic mRNA escapes from endosomes as free mRNA or is still associated with LBFs. Anyway once in the cytosol, mRNA must reach ribosomes for translation. No data have been reported yet on the cytosolic mobility of an exogenous synthetic mRNA. Studies have shown that the cytosol mobility of a plasmid DNA is drastically reduced soon to 500 bp and that above 2000 bp does not diffuse [47]. The size of MART-1, TRP2, MAGE1, GFP, luciferase synthetic mRNA including the 5′ untranslated region (60 nucleotides) and the 100 adenosine poly(A) stretch nucleotides are 514 b, 1816 b, 1090 b, 875 b and 1810 b, respectively. Thus, their cytosol diffusion would be very low or not even if their sizes are smaller than plasmid DNA. Moreover, single-stranded mRNAs contain several hairpins that likely impair their mobility. Anyway, synthetic self-amplifying

mRNAs of 10 kb do not diffuse. If synthetic mRNA escapes from endosomes associated with LBFs, they should be dissociated for translation by ribosomes. Indeed, *in vitro* translation of synthetic mRNA is drastically abolished after complexation with cationic liposomes or cationic polymers. For example, the luciferase activity produced by synthetic mRNA encoding luciferase after incubation with reticulocyte extracts is totally inhibited after complexation with a cationic polymer such as PEI or cationic liposomes such as KLN25/MM27 (personal unpublished data). The translatability of mRNA encoding luciferase complexed with protamine is also strongly inhibited *in vivo* after intradermal injection while luciferase expression is obtained after injection of naked mRNA [48]. Thus, mRNA extraction from mRNA LBFs by intracellular biomolecules should occur in the cytosol or before endosome escape. The intracellular location of synthetic mRNA after endocytosis is not yet known. A recent study indicates that 3 h after transfection of HeLa cells with a synthetic mRNA complexed with PEI, a small part of the mRNA colocalized with stress granules but not in P bodies involved in mRNA degradation even after 18 and 48 h post-transfection [40]. The presence of synthetic mRNA in stress granules would be in agreement with accumulation of endogenous mRNA observed under stress conditions such as those induced by transfection with cationic polymers. Such storage could hamper the mRNA expression and studies deserve to be performed to unravel the trafficking and mobilization of stored mRNA. Some mRNAs dissociated from PEI were nevertheless observed.

Table 3. Dendritic cell sugar receptors.

Receptors	Ligands	Receptor type
MR (CD206)	Mannose-terminated glycans, fucose-terminated glycans, 3-sulfo-LewisA, Tri-GlcNAc, $Man_9GlcNAc_2$-, PIMs	Type I transmembrane
DC-SIGN (CD209)	$Man_9GlcNAc_2$-, high mannose *N*-glycan, Le^b or Le^x, α-fucose-1–4GlcNAc, lacto-*N*-fucopentaose III containing Le^x, $GlcNAc_2$ Man_3, Man_4 oligossacharides, Manα1–3(Manα 1–6)Manα1-, PIMs, LAM, ManLAM	Type II transmembrane
DEC-205		Type I cell surface protein
Dectin-2	High-mannose carbohydrates with relatively low affinity	Type II transmembrane
Dectin-1	β-Glucan	Type II transmembrane
Langerin (CD 207)	Mannose, fucose, *N*-acetylglucosamine, mannan	Type II transmembrane

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DEC-205: Dendritic cell-specific 205 kDa molecular weight receptor; GlcNac: *N*-acetyl-β-D-glucopyranosylamine; LAM: Lipoarabinomannan; Le^b: $Lewis^b$; Le^x: $Lewis^x$; Man: α-D-mannopyranosyl; ManLAM: Mannosylated LAM; MR: Mannose receptor; PIMs: Palmitoyl-phosphatidylinositol dimannosides.

DCs specific delivery

The selective delivery of mRNA LBFs to DCs will favor mRNA accumulation in DCs and could reduce the effective mRNA dose. The targeting of these cells can be achieved by decoration of mRNA LBFs with ligands that are selectively recognized by receptors on the surface of DCs and permit their endocytosis. DCs express several sugar receptors called membrane lectins and most of them are involved in antigen capture and presentation. Thus, carbohydrate-based targeting is an obvious manner to enhance mRNA LBFs uptake, antigen presentation and immune response. The DC sugar receptors and their ligands are listed in Table 3.

Mannose receptor

The MR expressed by macrophages is also expressed by DCs [49,50]. This is a C-type I transmembrane lectin. Its three distinct extracellular binding sites recognize a wide range of endogenous and exogenous ligands (for review see [51]). MR constitutively recycles between the plasma membrane and early endosomes, even in the absence of any ligand. At the steady state, 10–30% of the receptor is expressed on the cell surface. MR recognizes mannose- and fucose-terminated glycans, Man_9-, 3-sulfo-LewisA, tri-GlcNAc and palmitoyl-phosphatidylinositol dimannoside. MR induces clathrin-dependent endocytosis.

DC-specific intercellular adhesion molecule-3-grabbing non-integrin

DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a type II transmembrane receptor which is mainly expressed by myeloid, dermal, interstitial DCs and by monocyte-derived DCs [52]. DC-SIGN contains a carbohydrate recognition domain that binds high-mannose- ($Man_9GlcNAc_2$-, Man_4-, Manα1–3(Manα1-6)$Man\alpha_1$-), fucose- (αfucose-1-4GlcNAc-, lacto-*N*-fucopentaose III-) and *N*-acetylglucosamine ($GlcNAc_2Man_3$-)-terminated glycans as well as Lewis-type blood antigens (Le^b or Le^x trisaccharide) [53,54]. Moreover, palmitoyl-phosphatidylinositol dimannoside that are present in the cell wall of *Mycobacterium tuberculosis* as part of the lipoarabinomannans or mannan-capped lipoarabinomannans bind to DC-SIGN [55]. After binding to DC-SIGN, glycosylated antigens are internalized, processed and epitopes presented. The fact that DC-SIGN is not expressed by murine DCs renders difficult investigations in relevant mouse models.

DEC-205

DEC-205 is a type I cell surface protein expressed primarily by DCs, an integral membrane protein homologous to the macrophage MR and related receptors, which are able to bind carbohydrates and mediate endocytosis [56]. The associated antigen presentation requires high-level expression of this receptor.

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DEC-205 is rapidly internalized via clathrin-mediated endocytosis, and is delivered to a multi-vesicular endosomal compartment that resembles the MHC class II-containing vesicles implicated in antigen presentation [57]. Antibody directed against DEC-205 provided *in vivo* targeting of antigens to DCs leading to improved T-cell vaccination and recent clinical applications were reported [58].

Dectin-1

Dectin-1 is a type II transmembrane protein receptor expressed on DCs that binds β-1,3 and β-1,6 glucans [59]. β-Glucans are polysaccharides of β-D-glucose extracted from the cell walls of different species of mushrooms, yeasts, oat, barley, seaweeds, algae and bacteria. However, β-glucans target other immune cells such as macrophages, neutrophils, monocytes, NK cells. The activation of dectin-1 by β-glucans leads to the upregulation of surface molecules on DCs and cytokine secretion. Dectin-1 mediates activation of monocyte-derived human DCs.

Dectin-2

Dectin-2 is a C-type lectin expressed by DCs and macrophages [60]. This receptor is important for host defense against *Candida albicans*. Dectin-2 binds high mannose-type carbohydrates including those exposed on the surface of *C. albicans* [61].

Langerin

Langerin is a C-type II transmembrane sugar receptor expressed on the surface of Langerhans cells allowing recognition of pathogens and their phagocytosis into Birbeck granules that are subdomains of the endosomal compartment [62]. Langerin recognizes high mannose structures, Manα1-2Man containing oligosaccharides, β-glucans, fucosylated blood group antigens and glycans with terminal 6-sulfated galactose [63–65].

Glycotargeting

Carbohydrate-based targeting is an obvious manner to enhance mRNA LBFs uptake, antigen presentation and immune response. For this purpose, mono- or disaccharides are usually used. Indeed, the synthesis of high mannose structures and complex oligosaccharides exhibiting high affinity for membrane lectins is difficult, expensive and yields are weak. The binding affinities of monovalent carbohydrate ligands such as mannose typically are weak ($K \approx 10^{-3}$ to 10^{-4} M). The affinity is increased by 1 or 2 orders of magnitude (10–100 µm) when several monosaccharide units are coupled on a scaffold such as peptide, protein or dendrimer. The multivalent decoration with monosaccharide units of low affinity forms clusters that increase the apparent affinity of the monosaccharide for its receptor. This method has been used in the past to generate neoglycoproteins [66]. Bovine serum albumin substituted with at least 25 ± 5 phenylthiocarbamyl-glycoside residues exhibits good affinity and is selectivity recognized by membrane lectins via an avidity phenomenon. The targeting of human peripheral blood monocytes-derived DCs was achieved with bovine serum albumin bearing 25 α-mannose or 25 α-fucose units [49]. Higher substitution increased non-specific binding, and lower substitution led to low binding and uptake. DC-SIGN glycotargeting was achieved with dendrimers substituted with 32 mannose residues [67]. The density of sugar moieties on the surface of liposomes or particles is an important parameter for efficient targeting of these receptors and receptor-mediated endocytosis. For instance, the effect of mannose density on the biodistribution of mannosylated lipid emulsions upon systemic injection in mice showed that emulsions with 5.0 and 7.5% of mannose-cholesterol were rapidly eliminated from the blood circulation and preferentially accumulated in the liver compared with those containing less mannose [68]. A selective gene delivery in DCs was achieved with mannosylated LPD100 prepared with PEG-HpK polymer (Table 1) and mannosylated liposomes (Man-Lip100) comprising KLN25/MM27 (Table 1) and β-D-mannopyranosyl-*N*-dodecylhexadecanamide (Man-Lip) (Table 2) [42]. DC selectivity was observed when liposomes contained 11% Man-Lip. Conversely, those containing 3% Man-Lip did not show specificity. The transfection of DC2.4 cells with Man_{11}-LPD100 containing DNA encoding luciferase gene gave luciferase activity two- to three-times higher than with non-mannosylated LPD100. A higher mRNA targeting of DCs was achieved *in vivo* with Man_{11}-LPR100 containing mRNA encoding EGFP and a higher vaccination was obtained with Man_{11}-LPR100 containing mRNA encoding MART-1 [26]. Besides clustering, antennary structuration of sugar moiety can enhance the monosaccharide affinity for its receptor. Mono-, di- and tetra-antennary mannosylated lipid derivatives revealed that liposomes containing multi-branched mannosylated lipids displayed higher binding affinity for MR compared with mono-mannosylated analogs [69]. Di-antennary mannosylated lipids were as efficient as the tetra-antennary lipids indicating that the di-antennary structure was sufficient. Glycosylated archaeal tetraether macrocycles bearing mono- or tri-antennary lactose or mannose at one or the two terminal ends were synthesized [33,34,70]. Such glycolipids incorporated into liposomes allowed specific interactions with their respective cell membrane receptors. Moreover, archaeal lipids exhibit adjuvant properties independent of Toll-like receptors activation [71]. Thus, mannosylated liposomes containing archaeal lipids that combine DC targeting and adjuvant properties would be useful to improve efficacy of mRNA vaccines.

Glycomimetics

In order to deal with the complexity of complex oligosaccharide synthesis, new synthetic molecules mimicking natural sugar properties have emerged. For example, a Manα1-2Man disaccharide and a Lewis X trisaccharide glycomimetic compounds with high affinity for DC-SIGN and low structural complexity have been synthesized [72]. Dendrimers bearing multiple copies of pseudo-1,2-mannobioside were able to block DC-SIGN-dependent HIV trans-infection of T cells [73]. A class of Lewis X mimic containing a fucosylamide anchor has demonstrated better specificity toward DC-SIGN relative to langerin [74]. Shikimic acid, a natural compound important in plants and microorganisms, can be

transformed into monovalent and multivalent glycomimetics recognized by C-type lectins. It has been demonstrated that lysine-based clusters of carboxylic acids such as quinic and shikimic acid mimicking mannose are effective ligands for the MR of DCs. Shikimic acid can be used as scaffold to mimic α-D-mannose [75] and α-L-fucose can be mimicked by one epimer of shikimic acid (-)-4-epi-shikimic acid) [76]. A class of non-carbohydrate ligands containing a quinoxalinone core has been found to be effective at blocking DC-SIGN interaction with carbohydrates [77]. Recently, cationic mannose-mimicking amphiphilic molecule with quinic and shikimic acid head groups were synthesized and found to target plasmid DNA to antigen-presenting cells via MRs [78]. A cationic amphiphilic molecule containing mannose-mimicking shikimoyl head group holds promise as MR selective *ex vivo* DC-transfection vector for use in DC-based DNA vaccination [79].

Expert commentary

Recent advances have demonstrated promising therapeutic applications of mRNA vaccines to fight against cancers. Further trials will surely demonstrate their efficiency against viral and bacterial infections. However, it is mandatory to develop robust and smart delivery systems capable to deliver *in vivo* synthetic mRNAs encoding antigens specifically into DCs in order to reduce mRNA doses and/or side effects. Different types of LBFs including LP, LPR, LNPs and CNE for mRNA deliver are proposed and preclinical studies demonstrated their potentiality to induce antigen-specific immune response. No clinical trial is yet reported with one of LBFs listed in Table 1. According to a wide range of available lipid molecules, the composition of liposomes which comprise at least two types of lipids and the possibility of adding a variety of polymers, a lot of combinations could be prepared to form different types of LBFs. It is known that there is a gap to realize their technical transfer to clinical trials.

First, it is important to compare the performance of each delivery system in order to determine those that will be pushed for clinical trials. This comparative evaluation should include the determination of the best administration routes for the required vaccination. Intradermal injection seems promising for anti-tumor vaccination and intramuscular for anti-viral immunization. Second, the exploitation of ligands recognizing sugar receptors such as the mannose and DC-SIGN receptors have to be pursued to increase selectivity of mRNA vaccines and reduce doses. For this purpose, it is crucial to improve the synthesis in high quantity of high-affinity oligomannose moieties as well as powerful coupling reactions for the synthesis of mannosylated lipids or LBFs under GMP conditions. The use of glycomimetics is an interesting alternative to arm mRNA LBFs with DC-targeting molecules. One has to keep an open mind for a possible cross-presentation after transfection of other cells with mRNA encoding antigens even if DCs are transfected. Regarding clinical trials, cocktails of mRNAs encoding different tumor antigens and other proteins were injected. The reduction of mRNA doses will contribute to inject either a cocktail of mRNA LBFs or LBFs containing a cocktail of mRNA. At the cellular level, certain LBFs contain pH-sensitive devices either fusiogenic lipids such as DOPE, DLinDMA and histidylated lipids or protonable polymers such as histidinylated polylysine and PBAE to promote endosome escape. Third, it is important to engage studies to determine qualitatively and quantitatively the escape of mRNA from endosomes and its intracellular pathway to reach ribosomes in order to increase antigens production at low mRNA doses. The absence of systems allowing the production of large quantity of synthetic mRNAs slows down characterizations of mRNA LBFs, cell investigations and preclinical evaluations. Moreover, the availability of large quantity of GMP mRNA LBFs will be essential for clinical trials. It is expected that methods for the bioproduction of synthetic mRNAs will be developed. Lastly, LBFs could include gas microbubbles to enable local (sc., ID, intratumoral, im.) delivery of mRNA with ultrasounds. This method called sonoporation induces plasma membrane destabilization with gas microbubbles under ultrasounds [80]. During this process, extracellular molecules in the vicinity of the plasma membrane can penetrate in the cells. For instance, perfluorobutane lipid (DPPC, DSPE-PEG-biotin) microbubbles were used to sonoporate DCs *ex vivo* with mRNA/DOTAP:DOPE:DSPE-PEG-2000-biotin LP [81].

Five-year view

Within the next 5 years, synthetic mRNAs encoding antigens can become biopharmaceutics allowing vaccination against cancer, bacterial and virus infections. Moreover, synthetic mRNA will be used for regenerative medicine with the reprogramming of somatic cells from different tissues and species to obtain pluripotent stem cell lines [82]. Acquiring more knowledge on the complex life of endogenous mRNA (mRNA surveillance, trafficking and interplay with translation control mechanism) is crucial for a better optimization of synthetic mRNA expression under a specific condition. The current LBFs are expected to demonstrate clinical efficacy and be ready to become marketed products. More knowledge is expected to be gained to design, characterize and demonstrate preclinical efficiency of one or two smart mRNA LBFs that will enable an efficient targeting and delivery of synthetic mRNA or self-amplifying RNA into DCs. Moreover, they will meet the criteria requested for the vaccination of large human population. This includes the determination of the best administration route, the GMP production of synthetic mRNAs, lipids, polymers and LBFs.

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Key issues

- Cancer clinical trials with direct administration of synthetic mRNAs encoding tumor antigens demonstrated safety and induction of specific and relevant immune responses.
- It is desirable to develop smart delivery systems suitable to protect synthetic mRNA from RNAse degradation and help it to penetrate in dendritic cells (DCs) to improve vaccination and reduce mRNA doses.
- Lipoplexes, lipopolyplexes, lipid nanoparticles and lipid emulsions are suitable lipid-based formulations (LBFs) for mRNA delivery. Some of them have proved preclinical efficacy but till now clinical efficacy is missing.
- When LBFs are internalized in DCs by clathrin- or caveolae-dependent endocytosis, the escape of mRNA from acidic vesicles (endosomes) can be promoted by using pH-sensitive lipids and polymers that induce membrane fusion and proton sponge effect in mildly acidic medium, respectively.
- It is not yet known whether mRNA is delivered in the cytosol free or associated with LBFs. No data are yet reported on the intracellular trafficking, location and cytosolic mobility allowing synthetic mRNAs to reach ribosomes for translation.
- The selective delivery of mRNA vaccines to DCs favoring its intracellular accumulation aims at reducing the effective dose. DC-targeting can be achieved by decoration of LBFs with selective ligands recognized by surface receptors of DCs allowing endocytosis. For this purpose, glycotargeting with carbohydrates or glycomimetics recognized by sugar receptors expressed by DCs notably the mannose and DC-SIGN receptors is a good strategy to enhance antigen uptake, presentation and immune response.

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