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Transfection by cationic gemini lipids and surfactants†

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Diseases that are linked to defective genes or mutations can in principle be cured by gene therapy, in which damaged or absent genes are either repaired or replaced by new DNA in the nucleus of the cell. Related to this, disorders associated with elevated protein expression levels can be treated by RNA interference via the delivery of siRNA to the cytoplasm of cells. Polynucleotides can be brought into cells by viruses, but this is not without risk for the patient. Alternatively, DNA and RNA can be delivered by transfection, i.e. by non-viral vector systems such as cationic surfactants, which are also referred to as cationic lipids. In this review, recent progress on cationic lipids as transfection vectors will be discussed, with special emphasis on geminis, surfactants with 2 head groups and 2 tails connected by a spacer.

1. Introduction

The technique of introducing nucleic acids into eukaryotic cells is called transduction when virus-mediated, and transfection when achieved by non-viral methods. Such techniques are not only important as research tools, but also find application in gene therapy. This has the potential to treat almost any hereditary disease because it aims to address a known defect: malfunctioning genes can be repaired directly by the delivery of functional DNA into the nucleus of a cell.^{1,2} Promising results have been obtained in recent gene therapy clinical trials in diverse areas like cancer³ and diseases of the blood,⁴ eye,⁵ and brain.⁶ Gene therapy can be implemented directly to cells in the body of the patient, or by way of stem cells that are removed from the patient and incorporated after transfection.^{7–9} Using the same or similar carriers^{10,11} cells can also be transfected by RNA instead of DNA, using *in vitro* transcribed mRNA to achieve protein expression.^{12–14} An even more powerful approach is the delivery of siRNA (small interfering RNA) as part of an RNAi (RNA interference)

strategy,^{15–18} in which the delivery of a short double stranded RNA segment (20 to 25 base pairs) can silence the matching gene, as schematically represented in Fig. 1. The short RNA is complementary to part of the mRNA of the gene and together they are bound in a RNA-induced silencing complex, in which the mRNA is degraded, thereby blocking its translation and shutting down the activity of the corresponding gene.¹⁹

Viral vectors are currently the most commonly used vectors in clinical trials, and viral gene therapy has been approved in China since 2003, and in Europe since 2013.¹ Lentiviral (LV) vectors capable of integration of a therapeutic gene stably into the host genome were successfully developed.²⁰ The 1st and 2nd generation LV vectors were incomplete retroviruses in which a therapeutic gene had been introduced, but they gave problems due to potential

Fig. 1 Schematic representation of the RNAi (RNA interference) strategy.¹⁹ Left, the DNA of a gene is transcribed in the nucleus to mRNA (messenger RNA) and then translated into a protein in the ribosome; right, antisense RNA derived from siRNA (small interfering RNA) is bound to the RNA-induced silencing complex together with the complementary mRNA, leading to degradation of the latter.

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† Electronic supplementary information (ESI) available: Abbreviations: CAT, chloramphenicol acetyltransferase; Chol-OH, cholesterol; DC-Chol, 3β-[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol; DOPE, dioleoyl-phosphatidylethanolamine; DOTAP, 1,2-di-(9Z-octadecenyl)-3-trimethyl-ammonium-propane; DOTMA, 1,2-dioleoyl-3-trimethylammonium-propane; GFP, green fluorescent protein; LNP, lipid nanoparticles; LV, lentiviral; PC, phosphatidyl choline; PEG, polyethylene glycol; PEI, polyethylenimine; siRNA, small interfering RNA; RNAi, RNA interference; SNALP, stabilized nucleic acid lipid particles. See DOI: 10.1039/c8md00249e

recombination with host viral sequences, replication, toxicity and immunogenicity.^{21,22} These problems are essentially solved in the 3rd generation LV vectors, which have led to many preclinical systems.²³ Clinical application is however still hampered by safety concerns caused by random integration in the host genome, lack of efficiency of the available pseudotyped vectors, and the cost of clinical grade production of large batches of vector.²⁴ The first viral systems developed for clinical use were adenoviral vectors, but they proved highly immunogenic and limited in tropism and efficacy. Vectors derived from adeno-associated viruses are considered less immunogenic, can be produced in sufficient quantities, albeit at considerable cost, and have reached the stage of clinical trial and FDA approval.^{25,26}

Because of the safety and cost problems of viral vectors there is an interest in non-viral vector systems. While such

systems are hindered by (intra)cellular barriers and immune defence mechanisms against which viruses have evolved means to overcome, they can be made biocompatible and produced at low cost, and are easy to use.²⁷ Non-viral delivery systems for DNA and RNA²⁸ include cationic surfactants, also known as cationic lipids,^{29–33} but also cyclodextrins,³⁴ and polymers such as polyethylenimine (PEI),³⁵ dendrimers such as G2-octaamine,³⁶ and materials related to carbon nanotubes.^{10,22} Non-viral carriers are now applied in a large number of clinical trials,³⁷ with a share for cationic surfactants^{30,38,39} of 6%.² Before addressing lipid carriers in detail, we will first discuss general properties of lipids and barriers in transfection.

Amphiphiles (molecules with one or more polar, hydrophilic head groups and apolar, hydrophobic tails) are only sparingly soluble in water, and above a certain concentration,

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Mark Damen (top left, Nijmegen, 1968) received his M.Sc at Radboud University, Nijmegen in 1993. He did his PhD with Dr. Feiters and Prof. Nolte on gemini and gemini-like alkylated peptides for gene transfection. Currently he is working as a chemistry teacher at Rivers International School Arnhem and Lorentz Lyceum Arnhem, the Netherlands.

Alexander J. J. Groenen (top middle, Veghel, 1988) received his M. Sc in Molecular Life Sciences in 2013 at the Radboud University, Nijmegen. He focused during his internships and thesis on (bio) organic chemistry. Afterwards, he has chosen another career direction and continued to work in the Supply Chain Management and Information Technology.

Stijn van Dongen (top right) has been in academia from 2001 through 2014 and involved in research at the ETH in Zürich, the ENS and Institut Curie in Paris, including his PhD (2010) with Profs. van Hest and Nolte at Radboud University, Nijmegen. The unifying theme of his projects has always been the use of chemistry to solve biological problems. He then moved into patent attorney practice, being admitted to the Dutch national order in early 2018, and qualifying as a European patent attorney later that year.

Roeland J. M. Nolte (bottom left) is Emeritus Professor of Organic Chemistry at Radboud University and former Director of its Institute for Molecules and Materials, and currently holds a special University Chair in Molecular Nanotechnology there. His research interests span a range of topics at the interfaces of Supramolecular Chemistry, Macromolecular Chemistry, and Biomimetic Chemistry, focussing on the design of catalysts and (macro)molecular materials. After his retirement in 2010 he received an ERC Advanced grant, allowing him to continue his work on processive catalytic systems, and another one in 2017, which is focused on the encoding of information into polymers.

Bob J. Scholte (bottom middle, Amsterdam 1953) received his PhD (1980), in biochemistry and microbiology, at the BCP Jansen Institute, University of Amsterdam, (Prof. Dr. K. van Dam and Dr. P. Postma). After postdoctoral work on coagulation factor VIII (Blood Transfusion Service Laboratory Amsterdam, Dr. D. Roos) and cytochrome P450 gene regulation (Biochemistry, Free University Amsterdam, Dr. G. Groot), he became research group leader and assistant professor at Erasmus MC Rotterdam (Pediatric Pulmonology, Cell Biology) in 1986. His team is active in gene therapy, in particular non-viral delivery systems, and experimental therapy of Cystic Fibrosis, using animal and cell culture models.

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the critical aggregate concentration (CAC), they will form aggregates, in which the polar head groups are in contact with the solvent, whereas the tails will interact with each other, due to the hydrophobic effect.⁴⁰ The packing parameter P relates the volume (V) of an amphiphilic molecule to its length (l) and head group area (a), via the equation $P = V/a \cdot l$, and determines what kind of aggregate ($P < 1/3$, micelle; $1/3 < P < 1.2$, cylindrical micelle; $1/2 < P < 1$, vesicle; $P = 1$, bilayers, or $P > 1$, inverted micelle) will be formed.^{41,42} Glycerol is the most important linker between polar head groups and hydrophobic tails in biological lipids, such as the phospholipids.⁴³ The phosphatidyl cholines (PC, Chart 1), the most important class of lipids (50%) in biological membranes, have a P value close to 1, and will therefore aggregate into a lamellar structure, such as a lipid bilayer. The phosphatidyl ethanolamine (PE, Chart 1) head group is just a little bit smaller than that of PC, resulting in $P > 1$, so that inverted micelles are the preferred aggregate of this amphiphile in water. Because of this tendency, phosphatidyl ethanolamines are examples of lyso-genic lipids, i.e. they disrupt bilayer structures, and find application as so-called helper lipids in transfection. Dispersion in water induces liquid-crystalline order in the bilayers; below the phase transition temperature T_c they are in a rigid state, above it in a fluid state. The introduction of tails with unsaturation, such as the oleoyl (C18:1) tails, also present in DOPE (see Chart 1), lowers T_c and increases the fluidity.

The DNA of eukaryotic cells, which has a length of the order of meters when fully stretched, is condensed to a size (μm range) compatible with that of the cell nucleus primarily by compaction in chromatin, which consists of nucleosomes.⁴⁴ These are formed by the electrostatic interaction of the negatively charged DNA with the histone proteins,⁴⁵ which are rich (30–40%) in the basic amino acids lysine and arginine. In specialized cells like the spermatozoa, the role of

the histones is taken over by smaller proteins, the so-called protamines, which are rich in arginine, and by polyamines like spermidine and spermine (see section 2.2).

Transfection can be regarded as a special problem in drug delivery. Mechanistically, the introduction of new genes or siRNA through non-viral vectors is not straightforward; multiple barriers need to be overcome (Fig. 2).^{46–50} Delivery starts with complexation and condensation of the polynucleic acid by the cationic lipid aggregate into a so-called lipoplex (step 1),^{30,31} which then interacts with the cell plasma membrane by electrostatic attraction to proteoglycans on the cell surface (step 2); we will encounter various examples where the efficacy and cell selectivity are enhanced by incorporating receptor binding components in the lipoplex. The lipoplex enters the cell by endocytosis, via an endosome (step 3). The nucleic acids then risk being degraded, by lysosomal DNA-se, when the pH is lowered by the action of ATP-ases which convert the endosome to a lysosome (step 4); in order to avoid this they should leave the endosome (step 5), an escape promoted by (pH-dependent) lysogenic (helper) lipids. DNA then needs to travel through the cytoplasm and enter the nucleus (step 6) so that it can reach the appropriate transcription machinery, which leads to its expression as a protein (step 7) and the therapeutic effect (step 8). In viral transfection, the capsid proteins facilitate transport of large viral DNA molecules from the cytosol through the nucleopores into the nucleus.⁵¹ Non-viral transfection of non-dividing cells is problematic, because plasmid DNA cannot pass from the cytosol through the nuclear membrane.⁵² This is briefly absent only during mitosis, so that plasmid DNA can be taken up in the nucleus of the two daughter cells. All non-viral transfection systems rely on this, and this is the most important reason why the extrapolation of results obtained *in vitro* with rapidly dividing cell cultures to *in vivo* applications with few dividing cells is often disappointing. The release of siRNA from the lipoplex is easier, since the molecule is much smaller than a DNA expression cassette gene;⁵³ furthermore it can perform its

A	R ² -O R ³ -O	R ¹ Head group	R ² , R ³	Example	Name
		H ₃ N ⁺ (CH ₂) ₂ O(PO ₂)O-	acyl	oleoyl	DOPE, 1a
		(phosphatidyl ethanolamine, PE)	acyl	oleoyl	DOPC, 1b
		Me ₃ N ⁺ (CH ₂) ₂ O(PO ₂)O-	palmitoyl + oleoyl		POPC, 1c
		(phosphatidyl choline, PC)	acyl	stearoyl	DSPC, 1d
		Me ₃ N ⁺ (Cl ⁻)	alkyl	oleyl	DOTMA, 2a
			acyl	oleoyl	DOTAP, 2b
		Me ₂ N-	acyl	oleoyl	DODAP, 3a
			alkyl	linoleyl	DLinDMA, 3b
			acyl	linoleoyl	DLinDAP, 3c
			acyl	palmitoyl	6
				DLin-KCn-MC3-DMA, 5a, R = linoleyl	
				L319, 5b	
				R = (CH ₂) ₆ O O (CH ₂) ₄ CH ₃	
B	R = linoleyl	Me ₂ N		Me ₂ N	5
					7c
					7b
					7a

Chart 1 (Pseudo)glyceryl (A) and related lipids (B and C).

Fig. 2 Steps and barriers to overcome in gene delivery with non-viral vectors:^{31,50} 1) complexation and condensation of the polynucleic acid by the cationic lipid into a lipoplex, 2) electrostatic attraction to the cell surface, 3) uptake by endocytosis, 4) degradation in a lysosome or 5) escape from the endosome, 6) entry into the nucleus, 7) expression as a protein, and 8) therapeutic effect.

therapeutic action in the cytosol, and therefore the nuclear entry does not present an additional hurdle.

It can be concluded that the following aspects are important: the carrier should i) compact the polynucleotide, reducing its size and sensitivity to mechanical and enzymatic attack, ii) allow effective uptake by endocytosis, iii) have a pH dependent lysogenic activity that allows escape from the endosomal compartment. Compaction of the DNA is promoted by multivalent cationic head groups. The emphasis in this overview will be on the development of multivalency, in which the so-called gemini surfactants, to be discussed in detail in section 3, play an important role. A complication in the comparison of the merits of the various transfection procedures are the variations in i) the standards used, ranging from the early lipid DOTMA 1, *via* LipofectinTM, TransfectamTM, LipofectamineTM, and Lipofectamine 2000TM,⁵⁴ to Lipofectamin PLUS 2000TM, ii) the use and choice of helper lipids, iii) the gene incorporated (often luciferase and/or β -galactosidase), or to be silenced, iv) the targeted cells (overview in ESI,[†] Table S1), and v) the cell viability measurements.

2. Lipid-based vectors

Lipid-mediated gene therapy was one of the earliest successful strategies used to introduce exogenous genetic material into host cells; it has received positive evaluations in clinical applications³⁹ and favourable reviews in comparative studies of available reagents.^{37,55,56} Structure–activity relationships of early cationic lipopolyamines showed two trends. First, the density and nature of the cationic head group both affect the transfection; generally, a reduced charge density results in the formation of larger cationic complexes and lower toxicity.⁵⁷ Second, for a given head group, the outcome of a change in the hydrophobic portion on transfection is difficult to predict;⁵⁸ most successful cationic lipids as vectors for transfection, however, contain at least one unsaturation.^{59,60} Unfortunately, increasing the cationic character of the lipids also increased their toxicity, a feature already known for PEI.⁶¹ Neither head nor tail is the sole determinant of transfection efficiency; both domains have to be taken into consideration. Unfortunately, the combination of a ‘best head group’ and a ‘best alkyl tail’ will not automatically result in the cationic lipid with the best general transfection activity.

2.1. (Pseudo-)glyceryl lipids and terpenoids

Many lipid carriers for transfection take cationic analogues of phospholipids (1, Chart 1), the ‘pseudoglyceryl’ compounds⁶² 2, in which the phosphate ester in the 1-position is replaced by a nitrogen-based cationic group, as a starting point, because of the expected compatibility with the plasma membrane. The positively charged head group has a dual function: it compensates the negative charge of the phosphate groups of the polynucleotides and plays a role in the association between lipoplexes and cells. The term ‘lipofection’ was coined⁶³ to describe lipid-based gene transfection, *viz.* of

COS-7 (African green monkey kidney fibroblast) and CV-1 cells with chloramphenicol acetyl transferase (CAT), using 1,2-dioleoyl-3-trimethylammonium-propane (DOTMA, 2a; Chart 1) in combination with the so-called helper lipid DOPE (1a) in a weight ratio α (= cationic lipid/{cationic lipid + helper lipid}) of 0.5, a combination commercialized as LipofectinTM; 2a was also the first lipid to be used for RNA transfection, *viz.* of NIH 3T3 cells with luciferase mRNA.⁶⁴ Its observed toxicity was ascribed to the poor biodegradability of the lipid's ether bonds; replacement by ester bonds gave 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP, 2b),⁶⁵ which is more easily degraded *in vivo* and therefore less toxic, although it is not necessarily always the better transfection agent. It is worth noting that contrary to their biological analogues, the pseudoglyceryl molecules are racemic, and neither enantioselective synthesis nor separation of the enantiomers to study their biological activities separately have been attempted; because the lipids are adjuvants, this is considered to be less important than if they would be themselves the therapeutically active molecules.

It is not necessary that a permanent charge, as in a quaternary ammonium group, is present in the molecule; lipids with a procationic (protonable) group such as in a tertiary amine are also applicable. The lipoplex of DODAP 3a (apparent pK_a 5.8) with DNA could be formed at pH 4 and appeared in the cryo-electron microscope as multilamellar vesicles, probably with DNA between the lipid layers; it could be administered at physiological pH provided that a PEGylated lipid was added to prevent aggregation, and was eliminated from plasma significantly more slowly than free DNA or a lipoplex with a quaternary aminolipid.⁶⁶ Of a number of cationic lipids that were combined with fusogenic and PEG-lipids as well as siRNA to give so-called stabilized nucleic acid lipid particles (SNALP), the polyunsaturated acyl analogue of DODAP, DLinDMA 3b, was found to be the most active in silencing Neuro2A (mouse) cells transfected with luciferase with siRNA.⁶⁰ This was also the cationic component in the SNALP used to silence apolipoprotein B in non-human primates.⁶⁷ The activity could be further improved by installing a ketal group in the linker (DLin-K-DMA, 4a), and even more by incorporating additional methylene groups between ketal and ionisable head group (DLin-K2-DMA, 4b), resulting in effective suppression of the murine clotting factor VII in mouse hepatocytes by siRNA in the order 4b > 4a > 3a > DLinDAP 3b.⁶⁸ Even more active remote analogues are DLin-MC3-DMA 5a^{69,70} and its analogue L319 5b with a hydrolysable alkyl tail,⁷¹ which however are no longer (pseudo)glyceryl compounds (Chart 1B). Another interesting modification of phospholipids is that with nucleosides to give nucleolipids, which combine the molecular recognition principles seen in a DNA helix with the self-assembly characteristics of lipids, and feature hydrogen bonding and π -stacking forces as additional factors that promote lipoplex formation.⁷² Upon incorporation in DOPE liposomes (6/DOPE molar ratio 1/9) the anionic nucleolipid diC16-3'-T 6 (Chart 1) bound DNA at a Ca^{2+} concentration of 1 mM;⁷³ the low Ca^{2+} concentration in the cell

(1 μM) would therefore contribute to the release of DNA from the lipoplex. HEK293 cells could be transfected with pE-GFP (green fluorescent protein) at a relatively low toxicity. There appears to be no example of nucleosides attached to cholesterol or in a gemini, as discussed below for other head groups.⁷²

The combination of anionic nucleolipids with Ca^{2+} is a way to evade the toxicity associated with cationic lipids. Another approach in which the use of cations is minimized, at least for cell-lipoplex recognition, is cell surface engineering using the SnapFect system.⁷⁴ Here the cells to be transfected are immobilized and first exposed to mixed DOTAP (**2b**)/POPC (**1c**) liposomes containing the ketone-lipid **7a** (Chart 1C), which fuse with the cell membrane to give the so-called ketone-cell, with ketone functionalities at the surface. These can then react with the lipoplexes, based on **2b/1c** but also containing the oxamine-lipid **7b**, which reacts (clicks) with the ketone **7a** to give the oxime **7c**, promoting the interaction between target cell and lipoplex as well as subsequent transfection. The principle has been demonstrated for transfection of 3T3 fibroblast cells by GFP with high cell viability.

The steroid cholesterol (HO-Chol, shown in Chart 2 along with some other alternative hydrophobic tails) and its esters are the most important (30%) components of animal cell membranes, increasing the molecular packing by the hydrophobic interaction with the phospholipids' alkyl chains, while maintaining the membrane's fluidity and integrity. As expected on the basis of its compatibility with the cell membrane, it is possible to replace the two alkyl tails in a surfactant by a single Chol moiety, leading to vectors such as 3β -[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol, **8a**, Chart 2); with DOPE as a helper lipid, the CAT plasmid was incorporated and expressed in a variety of cells of mammalian origin.⁷⁵ Cationic derivatives of Chol proved, however, also to be protein kinase C (PKC) inhibitors,⁷⁶ which is detrimental to the physiological integrity of a cell; derivatives with a quaternary nitrogen head group were much stronger inhibitors than tertiary ones.⁷⁷

2.2. Multivalent cationic lipids

Most cationic lipids form polycationic aggregates (liposomes) of which the cationic groups can associate with the phosphate groups of the nucleic acids and thus neutralize their negative charges. In this reaction (eqn (1), step 1 in Fig. 2) the natural cationic counterions of the nucleic acid are

Chart 2 Alternative hydrophobic tails.

exchanged for the cationic groups of the lipid aggregate.⁷⁸ The lipoplex should preferentially be formed with an excess of positive charge, *i.e.* n for N^+ (cationic lipid) $> n$ in P^{n-} (polynucleotide), so that it is attracted to the negatively charged cell surface (Fig. 2, step 2); this means that the nominal charge ratio, indicated as N/P (because the positive charge in the lipid is typically on N, and the negative charge in the nucleotide on P), has to be >1 .

An important factor in the efficiency of the ion exchange reaction (eqn (1)) that leads to the lipoplex is the multivalency effect,⁷⁹ by which multiple binding interactions can give rise to binding constants much higher than the sum of individual binding interactions. A single polynucleotide molecule has multiple negative charges, which can interact with multiple cationic charges on the surface of a liposome; to incorporate multiple cationic charges into a lipid molecule would therefore greatly enhance its DNA condensation ability. Examples of linear and cyclic multicationic groups are shown in Chart 3. For example, the combination of the pseudoglycerol scaffold with carboxyspermine Sp(CO) gives 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanamine (DOSPA, **9**, Chart 3), an important component in the commercially available Lipofectamine.⁸⁰ 5-Carboxyspermylglycine dioctadecylamide (DOGS, **10**, Chart 3),⁸¹ which does not contain glycerol as a linker, is effective with saturated alkyl tails. The effect of (partial) replacement of the H atoms in the alkyl tails by fluorines has also been explored, as the resulting fluorocarbon tails are even more hydrophobic than their hydrocarbon analogues;⁸² indeed fluorinated analogues of **10**,^{83,84} but also of DOTMA **2a**⁸⁵ and DOSPA **9**⁸⁶ are also active in transfection, often with much reduced toxicity.

The multivalent lipid **11** (MVL5, Chart 3) contains an additional propane-ammonium moiety in its head group, and has its hydrophobic tails attached to a 3,4-dioleoyloxybenzoic acid scaffold; with DOPC **1b** (Chart 1) as a helper lipid, it was

Chart 3 Multivalent cationic lipids.

The conceptually simple bola **17** (Chart 4), with a single neutral hydroxyl group at one end and a trimethylammonium group on the other, did not form vesicles; combined with DOTAP (**17/2b** ratio 1 : 5), it was equally active in the transfection of HEK293 cells with GFP as **2b** alone, but with reduced cytotoxicity.¹⁰⁰ The symmetric multivalent bola **18** transfected HEK293, HepG2, NiH3T3, and HeLa, and 4T1 cells with luciferase (N/P 8, no helper lipid), with high cell viability (80–90%).¹⁰¹ Compound C₆C₂₂C₆ **19**, a gemini (see section 3) with

an extremely long spacer, had an activity comparable to that of Lipo2000* in transfection of COS-7 cells by GFP and luciferase with DOPE as the helper lipid ($0.2 < \alpha < 0.5$).¹⁰²

2.4. Bio-reducible and/or dimerizable lipids

Amphiphilic molecules that contain thiol groups in the hydrophobic part can be oxidatively linked by disulfide formation to give a bola as in the example of **16** in the previous section; this bolaamphiphile can then be decomposed into the 2 monomers by splitting of the disulfide bond in the more reductive environment in the cell. This principle can also be applied to thiol groups in the polar head groups of amphiphilic molecules, where oxidation (Scheme 1A) would lead to a disulfide-containing gemini (see next section 3.1), which could be split into thiol-containing monomers in the cell. In this review, most dimerizable surfactants will be treated as bio-reducible geminis in section 3 according to the type of head group and/or aliphatic tail involved; a few representative examples will be discussed here.

Investigations of a series of cationic Orn-Cys-alkylamine surfactants **20** (Scheme 1B)¹⁰³ revealed that their oxidative dimerization was accelerated in the presence of DNA; the DNA molecule thus served as a template, binding the cationic molecules by ion-exchange, which then leads to the formation of gemini surfactants, according to the reactions given in eqn (1) and Scheme 1A, respectively. The activity in transfection of luciferase in 3T3 murine fibroblasts, BNL CL2 murine hepatocytes, and HeLa cells increased in the order **20a** < **20b** < **20c**, but was less than that of Transfectam™ by an order of magnitude. The activity of transfection of mouse fibroblast L-cells with luciferase by disulfide analogues of the multivalent cationic lipid MVL5 **11**, the CMVL series **21**, were studied in mixed liposomes with DOPC.¹⁰⁴ With 40% DOPC, the best compounds **21d** and **21c** had activities comparable to that of Lipofectamine 2000™, but with much better cell viability; the activity decreased with the number of positive charges in the head group in the order **21d** > **21c** > **21b** > **21a**.

3. Gemini surfactants

3.1. Introduction

Gemini surfactants are a relatively young subfamily of amphiphilic molecules containing two head groups and two

aliphatic tails which are linked by a spacer between the head groups, or between the linker region connecting the heads and the tails (Chart 5).^{105–108} Their critical aggregate concentration is 10^3 -fold lower compared to the corresponding monovalent compounds (*i.e.* the classical surfactants with a single chain and a single head group), which reduces the amount of material required for *e.g.* solubilization and makes them interesting compounds for various applications,¹⁰⁹ in particular biomedical ones,^{110–113} including transfection.⁵⁰ The basic structure of geminis with their 2 head groups offers opportunities to take advantage of the multivalency effect (see section 2.2).

If the area of the combined head groups is not larger than the cross section of the two tails ($P \approx 1$), gemini surfactant molecules will aggregate into bilayers, and be compatible with the plasma membrane. An interesting difference in the behaviour of cationic surfactants in vesicle bilayers of anionic phospholipids is that all the anionic charge of the vesicle can be neutralized by addition of a classical cationic surfactant when the bilayer is in its 'liquid' state, and half of it when it is in the 'solid' state, whereas cationic gemini surfactants neutralize only half of the anionic charge, irrespective of the state of the bilayer.¹¹⁴ This difference is ascribed to the much lower propensity of gemini surfactants for 'flip-flop', which makes them much more similar to phospholipids than classical surfactants.¹¹¹ Progress in the understanding of the physical chemistry of gemini and multivalent lipids in transfection has recently been reviewed,¹¹³ and will not be discussed in detail here.

The spacer in gemini surfactants opens great possibilities for variation, in addition to variations of the head groups and tails in conventional surfactants.¹⁰⁷ It can be anything from a 2- to 10-carbon or longer aliphatic linker,⁹³ or a rather polar polyamine.¹¹⁵ In some gemini surfactants the spacer is so dominantly aliphatic that they should practically be considered bola-amphiphiles (*e.g.* compound **19** in section 2.3).^{92,93} The head groups can vary from anionic sulfate *via* non-ionic sugars to cationic ammonium moieties, either chiral or achiral.^{116–118} Geminis that are asymmetric due to the presence of a peptide spacer have been dubbed geminoid or gemini-like surfactants¹¹⁹ and will be discussed in section 4. Interestingly, a biological gemini phospholipid is known, cardiolipin (**22a**, Chart 5C), which is located in the inner mitochondrial membrane and binds protons as well as cationic

Scheme 1 A) Oxidative dimerization of an amphiphilic thiol into a disulfide gemini surfactant. B) Reducible transfection agents.

Chart 5 Gemini: A and B, general cationic gemini structure, connected at the head (A) or in the linker region (B); C, natural gemini (cardiolipin, **22a**) and cationic cardiolipin analogue **22b**.

Of a new set of derivatives of dimeric surfactants (Chart 7) having aza- (*m*-5N-*m*, 33a; *m*-7N-*m*, 33b; *m*-8N-*m*, 34) and imino- (*m*-7NH-*m*, 35) substituted spacer groups, the activities of the C12-analogues were studied, and 12-7NH-12 (35a) was found to be comparable to Lipofectamine Plus™ in the transfection of COS-7 cells with luciferase, albeit at the cost of a slightly higher toxicity.^{134,135} Compared to 23, the interest of 35 is in the protonable amine at the centre of the -7N- spacer, and 35a-d were therefore compared to analogues of 23 with a spacer of the same length, but without the amine (23a-d with *s* = 7).¹³⁶ Indeed the size of the aggregates of 35a-d was pH dependent, with smaller aggregates at higher pH, whereas those of 23a-c (*s* = 7) were pH-invariant. In the transfection of murine PAM 212 keratinocytes with luciferase, the activity increased going from 23 (*s* = 7) *via* 23 (*s* = 3) to 35, which came closest to Lipofectamine Plus™; within each group, the activity increased in the order *m* = 12 < 16 < 18.

The related tetraalkyl diammonium surfactants with spacer length $s = 3$ (**24a**) and $s = 6$ (**24b**) and Cl^- counterions, which can be considered to be gemini analogues of dioctadecyl-diammoniumchloride (DODAC), were tested for

$\begin{array}{c} \text{R}^1 \\ \\ \text{R}^2 - \text{N}^{\oplus}(\text{CH}_2)_s \\ \\ \ominus \\ \text{X} \end{array}$	$\begin{array}{c} \text{R}^1 \\ \\ \text{N}^{\oplus} - \text{R}^2 \\ \\ \text{R}^4 \\ \\ \text{X}^{\ominus} \end{array}$	a-d , R ³ =R ⁴ = a , lauryl; b , palmityl; c , stearyl; d , oleyl; e-g , s = 3, R ³ =phytanyl (Chart 2), R ⁴ = e , lauryl; f , palmityl; g , stearyl
23 , R ¹ =R ² = Me, X=Br		
24 , R ¹ =Me, X=Cl, R ² =R ³ =R ⁴ =oleyl;		a , n = 9; b , 11; c , 13
a , s = 3; b , s = 6
25 , R ¹ =R ² = Me, X=Cl, s = 2, R ³ =R ⁴ =		O(CH ₂) _n CH ₃
26 , R ¹ =R ² = Me, X=Br,		
s = 3, 4, 5, 6, 12, R ³ =R ⁴ =		a , n = 11; b , 13; c , 15
27 , (CH ₂) ₂ = 2 Me		O(CH ₂) _n CH ₃
28 , R ¹ =R ² =Me, X=Br, R ³ =R ⁴ =(CH ₂) ₂ OChol,		
s = 3, 4, 5, 6, 12;	29 , (CH ₂) _s = 2 Me	
30 , R ¹ = Me, R ² = CH ₂ CH ₂ OH, X = Br, R ³ =R ⁴ =(CH ₂) ₂ OChol,		
s = 3, 4, 5, 6, 12	31 , (CH ₂) _s = 2 Me	
32 , R ¹ =R ² =Me. X=Br. R ³ =R ⁴ =(CH ₂) ₇ OToc. s = 3, 4, 5, 6, 8, 12		

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Chart 7 Quaternary ammonium geminis with other spacers.

Because of the tendency of surfactants with phytanyl tails (Phy, Chart 2) to give fusogenic hexagonal lipid packings,¹³⁷ it was considered of interest to investigate gemini surfactants of type 23 with $n = 3$ and different alkyl tails, of which one is a phytanyl (23e–g, Chart 6).¹³⁸ In the synthesis of these compounds, the stereochemistry of one of the centres in the phytanyl bromide building block is not controlled, which implies that, due to the presence of other chiral centres in the phytanyl chain, 23e–g are prepared and studied as mixtures of diastereoisomers. Compared to the analogues 23a–c with $n = 3$, the hydrophobic volume in 23e–g was much increased, causing a shift in the packing parameter P from values around 0.35, giving cylindrical micelles, to approx. 0.55, more consistent with vesicles. The asymmetric geminis 23e–g were more efficient than 16-3-16 (23b, $n = 3$) in the transfection of ovarian cancer cells OVCAR-3 with GFP, but not as active as Lipofectamine 2000™. In a subsequent study, these results were compared to those for the phytanyl analogues of 35a, viz. 35d–f;¹³⁹ this series turned out not to transfect OVCAR-3 cells, in spite of the aforementioned activity of 35a in COS-7¹³⁴ and PAM 212¹³⁶ cells.

The 12-7N-12 cationic gemini 35a was appended at the central N with amino acids (Gly, Lys) and dipeptides (Lys-Gly, Lys-Lys), for which the structure of 12-7N(Lys-Gly)-12 is given as an example (36a(Lys-Gly), Chart 7).¹⁴⁰ In the transfection of COS-7 cells with interferon γ and DOPE as a helper lipid, optimum expression was reached after 72 h, with efficiencies decreasing in the order 36a(Gly) > 36a(Lys-Gly) > 36a(Lys) > Lipofectamin Plus™ > 36a(Lys-Lys) $\approx$ 35a. In the transfection of PAM 212 (murine keratinocytes) the activities of the 36a analogues were quite similar and much better than that of 35a although much worse than that of Lipofectamin Plus™; this standard performed very poorly with Sf 1Ep cottontail rabbit epithelial cells, where 36a(Gly) was the most efficient, followed by 36a(Lys-Gly) > 35a > 36a(Lys) > Lipofectamin Plus™ > 36a(Lys-Lys). The differences in efficiency of 35 and 36a for various epithelial cell types were ascribed to differences in the amount of proteoglycan on the cell surfaces. The presence of an amino acid/peptide in 36a is expected to soften the electrostatic interactions between lipoplex and proteoglycan by additional van der Waals and

H-bond interactions, as compared to 35a, and the larger hydrophilic head group results in higher biocompatibility and (although not investigated) reduced toxicity. The cellular uptake of lipoplexes of 35a and 36(Lys-Gly) was not affected by the attempted selective inhibition of clathrin-mediated and caveolae-mediated endocytosis with respectively genistein and chlorpromazine, but much reduced by methyl- β -cyclodextrin, which affects both pathways.¹⁴¹ The transfection with interferon γ /GFP was affected by inhibition of the caveolae-mediated pathway for 35a but not for 36a(Lys-Gly), whereas inhibition of the clathrin-mediated pathway enhanced the transfection by 35a while slightly reducing that by 36a(Lys-Gly). It is proposed that with both surfactants, endosomal escape from caveolae is possible, but that 36a(Lys-Gly) can escape from clathrin-coated endosomes by a proton-sponge effect, due to the buffering by the Lys residue, whereas 35a can not. More recently analogues of 36(Lys-Gly) a–c with varying tail lengths were compared, with the best result for the C16-derivative 36b with DOPE at N/P = 2.5.¹⁴²

The oxyethylene linker in 37¹⁴³ is reminiscent of the aforementioned (section 2.1) neutral but hydrophilic PEG moieties, which can be incorporated in cationic lipid aggregates to prevent their aggregation with anionic biomolecules. In the transfection of HEK293T cells by pEGFP-C3, 37c was most efficient (α 0.7), and better than the Lipofectamin 2000™ standard, both in the presence and absence of serum, followed by 37a and 37b; 37b also gave the poorest cell viability results, although better than the standard, while 37a was the best in this respect. In a number of other cell types, viz. HeLa, CaCo-2 (human epithelial colorectal adenocarcinoma), Hep3B and MDA-MB231 cells, 37a was always the best, and 37b the poorest transfection agent. The analogue of 23b (16-6-16) with 2 S atoms in the 6 atom-spacer, 38-S-S-38, is an example of the aforementioned (section 2.4) disulfide dimers, which can be reduced in the reductive environment of the cell by the thiol compound glutathione, according to the reaction in Scheme 1A, in this case giving the monomeric, non-gemini surfactant 38-SH (Chart 7).¹⁴⁴ It was demonstrated that glutathione can indeed split the S–S bond of 38-S-S-38 molecules in monolayers at the air–water surface, but reduction in the presence of DNA was much slower, resulting in a disappointing transfection activity. It is concluded that the S–S bond is not reduced effectively if it is in the hydrophobic part of the surfactant molecule. The transfection of COS-7 cells by GFP by the with *m*-phenylene gemini 39 with DOPE (α 0.2 or 0.5) was comparable to that of Lipo2000*; the corresponding monomer 40 was less effective at low α , and more toxic at low N/P.¹⁴⁵

The series 41a–d with oxyethylene spacers of varying length and the Chol linked by an O atom were also investigated (Chart 7).¹⁴⁶ All oxyethylene gemini surfactants were better than the monomer ‘half-gemini’ 31 in transfecting HeLa cells with GFP, with the best results for 41a ($n = 1$) and the worst for 41d ($n = 5$). A remarkable observation was that the transfection by 31 was strongly affected by the addition of 10% serum, whereas that by 41a–d was not. The analogues

42a–c (Chart 7) in which the Chol is not attached by an O but by a reducible disulfide bridge were tested for transfection of HeLa and HT1080 cells by GFP with DOPE, with practically no toxicity.¹⁴⁷ The aromatic linker in 42b proved to be a disadvantage in this case, since the order of transfection efficiency was $42a \approx 42c > 42b$ for HeLa cells, and $42a > 42c > 42b$ for HT1080 cells.

In compounds 43, the quaternary ammonium groups were linked by a redox-active ferrocenyl spacer. In the transfection of Caco-2, HEK293T, and HeLa cells with GFP, the compounds with the shorter ($n = 5$) were more active than those with the longer ($n = 10$) spacer, but less active than the analogues with just one quaternary ammonium/Chol group attached to the ferrocenyl.¹⁴⁸ As in earlier studies on ferrocenyl-containing cationic lipids, the activity was abolished when the ferrocenyl group was oxidized,¹⁴⁹ but for 43 it could be reinstated upon reduction of the lipoplex with ascorbic acid.

3.3. Carbohydrate geminis

Carbohydrates can be combined with lipids to give glycolipids, and given a positive charge to act as transfectants. This is of interest because many cell types have receptors for carbohydrate moieties,¹⁵⁰ and the high level of hydration of carbohydrates possibly weakens the binding of the cationic ammonium groups to the DNA, thus facilitating its release. The series 44 (Chart 8), with the open-chain galactose-derived carbohydrate directly linked to the ammonium, showed transfection in CHO, COS-1, MCF-7 and A549 (human lung adenocarcinoma epithelial) cells, in particular with the C14 (44a) and C16 (44b) tails, while the related compound 45 with a longer spacer between head group and alkyl tail was not biologically active.¹⁵¹

A series of geminis based on reduced glucose in which the spacer and alkyl tails were varied, was synthesized⁹³ and tested for biological activity both *in vitro* and *in vivo*.¹⁵² Of the series with saturated tails 46a–h, the surfactants with hexadecyl tails 46e–f appeared to have the highest transfection efficiencies, comparable to Lipofectamine Plus2000™, for β -galactosidase in CHO-cells without a helper lipid, and the activity was slightly higher for the derivatives with $s = 4$

Chart 8 Carbohydrate classical and gemini transfectants.

than for $s = 6$. The polar amine groups are procationic, and have an influence on each other, resulting in *e.g.* pK_a values of 8.3 and 5.8 for 46i¹⁵³ and pH-dependence of its aggregation behaviour and interaction with DNA.¹⁵⁴ In a subsequent study, the glucose and mannose oleyl surfactants with alkyl ($s = 6$) spacers (46i and 46j) were compared to their counterparts with oxyethylene spacers (47a and 47c) and their amide analogue 47b.¹⁵⁵ In the transfection of CHO-K1 cells with GFP, the activity decreased in the order $47a > 47c > 46j > 46i$, with activity of 46i comparable to that of the standard Lipofectamine 2000™, while the amide 47b showed no activity; only 47c was less toxic than the standard.

3.4. Geminis with multivalent head groups

The naturally occurring polyamine Sp (Chart 3) has been symmetrically appended with 2 Chol residues to give the compounds 48 (Chart 9).¹⁵⁶ Compound 48a and its analogue featuring S–S bonds between the Chol and Sp moieties, 48e, were more active than the standard Lipofectamine 2000™ in the transfection of HEK293 cells by pEGFP-C2 plasmid with DOPE ($\alpha = 0.5$) at N/P ratios > 4 .¹⁵⁷ The activity of 48e was slightly lower than that of 48a and was abolished by extracellular glutathione, presumably due to reduction of the S–S bond, which would result in detachment of the cationic Sp head group of 48e from the hydrophobic Chol part. Other variations in the connection between Sp and Chol had subtle effects, with the best results for 48b (N/P 6) and 48d (N/P 8) in transfection of HEK293 cells as measured by the percentage of fluorescent cells resp. mean fluorescence intensity, and for 48c (N/P 8) in GFP silencing in BHK IR-780 cells.¹⁵⁸

At N/P < 4 the cyclen-based gemini surfactants 49–50¹⁵⁹ (Chart 9) were less toxic than Lipofectamine 2000™ in

Chart 9 Geminis with multivalent head groups.

HEK293 cells. Their transfection activity was tested with luciferase (pGL-3) and eGFP (pEGFP-N1) plasmid DNA in HEK293 cells in the presence of the helper lipid DOPE. The amides **50** and the HO-Chol ester **49f** were relatively inactive, and although for the other esters **49** the unsaturated **49d** was slightly more active than its saturated analogue **49e**, the highest activities were observed for the relatively short-chain (C12 and C14) derivatives **49a** and **49b**; of the reverse esters **51**, the myristoyl analogue **51b** was the most active. Of the single-chain analogues **52**, **52a** was internalized in spite of the relatively large size of its lipoplex, but nonetheless resulted in much poorer transfection than displayed by its gemini analogue **49a**. Bio-reducible geminis with 2 cyclen head groups, an S-S linker, and hydrophobic tails connected as amides (**53**) or esters (**54**) were also investigated in combination with DOPE ($\alpha = 0.33$), and compared with a non-dimerizable single-chain analogue **55**.¹⁶⁰ All compounds **53–55** were less toxic than Lipofectamine 2000™ up to an N/P ratio of 8; the HO-Chol ester **54d** was least toxic but also had the weakest interaction with DNA and was not further tested for biological activity. In the transfection of HEK293 cells with luciferase, the amides **53** were more active than the esters **54**, with the activity decreasing in the order **53b** (oleyl) > **53a** (lauryl) > **53c** (linoleyl) and **54b** > **54a** ≈ **54c**. The activity of the best compound in this group, **53b**, was close to that of Lipofectamine 2000™. Because of the generally lower activities of these compounds, the activity of the non-gemini **55** was only approx. 1 order of magnitude lower than that of its closest gemini analogue **53a**. Quite recently, another cyclic multivalent head group, triazacyclododecane [12]ane-N₃, has been linked by a triazole to a tetraphenylethylene platform with varying alkyl tails to give **56**.¹⁶¹ The transfection of HepG2 cells by luciferase and DOPE decreased in the order **56b** > **56a** >> **56d** > **56c** and was in all cases less efficient than that by Lipo2000.

3.5. Geminis with delocalized charges

A strategy to overcome the toxicity problems of transfectants is to spread the positive charge of the cationic head. An early example is to use the amidine group like in diC14-amidine (Vectamidine, **57**, Chart 10),¹⁶² which with PE as a helper lipid was active in the transfection of CHO and K562 cells with CAT. The guanidinium group in the amino acid arginine is a biological cationic group with delocalized charge; it has been coupled to DOTMA **2a**, yielding Arg-DOTMA derivative **58**,¹⁶³ which gave good transfection of V79 (Chinese hamster lung) and HT29 cells. The positive charge can also be delocalized in a heterocyclic ring like pyridinium, as in the SAINT-2 (**59**, Chart 10, commercialized with DOPE as SAINT, Synthetic Amphiphile INTERaction)¹⁶⁴ and Sunfish **60**.¹⁶⁵ lipids.

The pyridinium surfactants generally have lower cytotoxicity than lipids with quaternized amine head groups, as shown also in a direct comparison of the pseudoglycerol compound **3** (DOTAP) with its analogue with the quaternary

Chart 10 Transfectants with delocalized positive charge.

ammonium group substituted by 2,4,6-trimethylpyridinium **61**.¹⁶⁶ Imidazolium and imidazolium rings are other examples of heterocyclic rings in which positive charge can be delocalized. The latter was tested in the lipophosphoramidate framework (Chart 10),¹⁶⁷ where the methylimidazolium compounds **62c** and **62d** were found to be more efficient and less toxic than the guanidinium compounds **62a** and **62b**. The developments of the lipophosphonates and lipophosphoramidates have been reviewed elsewhere.¹⁶⁸

For pyridinium-containing geminis **63** (Chart 11A) related to **59**, with 4 alkyl tails, the highest transfection efficiencies were found for a pentamethylene spacer (**63**, $s = 3$).¹⁶⁴ In contrast to the transfection results of other gemini surfactants discussed above and below, pyridinium geminis were found to be less effective than their monomeric counterparts. For another group of geminis **64** the optimal spacer length for transfection of MCF-7 cells by luciferase with HO-Chol as helper lipid ($\alpha = 0.5$, N/P = 2) was found to be a dimethylene spacer ($s = 0$), which was comparable to DOTAP; remarkably the transfection increased again at spacer lengths $> s = 6$.¹⁶⁶ Insertion of nitrogens in the spacer of pyridinium geminis with varying nitrogen content (**65** vs. **66a**, which has Sp at the core of its spacer) and linker length (**65a–c**) did not improve the transfection of NCI-H23 (lung carcinoma) cells by luciferase with HO-Chol as helper lipid ($\alpha = 0.5$, N/P = 2) compared to **64** ($m = 0$ or 8), and showed a similar bimodal dependence on spacer length: **66a** > **65a** > **65b** > **65c** (virtually no activity).¹⁶⁹ Of derivatives of **66** where the nitrogens in the centre of the spacer had been amidated (**66b**, **c**) or alkylated (**66d**), only the latter was more active than Lipofectamine™, this time with a preference for DOPE ($\alpha = 0.5$) as a helper lipid. Appending the central N in **64b** with another amphiphilic pyridinium led to the unique ‘sesquigemini’ amphiphile **67**, which was less active than **66d** and Lipofectamine™, but was relatively indifferent to the presence or choice of helper lipid. Incorporation of a reducible disulfide in the spacer (**68**) resulted in loss of transfection activity, irrespective of the counterion (PF₆[−] or Cl[−]) used.

The ‘pseudogemini’ surfactants are called this way because they have 2 alkyl tails but only one pyridinium head

Chart 11 Headgroups with delocalized charge, A) and C) pyridinium resp. imidazolium geminis; B) pseudogemins.

group; the other polar element is an ester (**69**) or amide (**70**) in the linker (Chart 11B). With N/P = 2 and DOPE ($\alpha = 0.5$), these compounds gave transfection of NCI-H23 cells equivalent to that of Lipofectamine; the optimum alkyl chain length was the intermediate one (b) in both cases.¹⁷⁰ The observed lower (virtually absent) cytotoxicity of the ester **69** compared to the amide **70** was tentatively ascribed to the different products expected to be formed by enzymic hydrolysis. For the pseudogemini analogue **71** in which the amide bond is reversed HO-Chol was a better helper lipid than DOPE, and the transfection activity at N/P = 2 decreased in the order **71c** > **71a** > **71b**, with much reduced toxicity compared to **70**.¹⁷¹

Following the success of oxyethylene spacers in other gemini systems (**41**,¹⁴⁶ see section 3.2) the oxyethylene pyridinium geminis **72** (Chart 11A) with varying tail lengths were also prepared and tested.¹⁷¹ The transfection activity with DOPE ($\alpha = 0.33$) at N/P = 3 increased with *n*, to a value for *n* = 16 higher than that found for LipofectamineTM. It was then attempted to take advantage of the excellent transfection efficiency of gemini **72** (*n* = 16), due to its membrane destabilization and its high charge/mass ratio, with the good transfection activity and somewhat reduced cytotoxicity of the conventional pyridinium surfactant **61b** (a symmetric isomer of the pseudoglyceryl pyridinium compound **61a**, see Chart 10), by studying mixtures of these compounds.¹⁷² The addition of 5–10% gemini **72** to **61b** improved the transfection of NCI-H23 cells by GFP with DOPE **1a** as the helper lipid ($\alpha = 0.33$, N/P = 3). Moreover, there was evidence for a change in the endocytotic mechanism of uptake by the cell

(see section 2.2): transfection by **61b/1a** was inhibited by 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), which interferes with macropinocytosis, whereas transfection by **61b/72/1a** was also inhibited by genistein, which interferes with the caveolae pathway. Interestingly, with HO-Chol as the helper lipid ($0.33 < \alpha < 0.50$), the incorporation of **72** results in suppression of endocytosis involving clathrin, caveolae as well as macropinocytosis, which, in view of the improved transfection efficiency, suggest that yet another uptake mechanism operates in this case, possibly temporary poration of the cell membrane by **72**. The results for this class of pyridinium surfactants where the head groups are connected by spacers directly attached to the quaternary nitrogen atoms have been reviewed.¹⁷³

Another class of pyridinium geminis, **73a** (Chart 11C), with long alkyl tails connected to the N in the pyridinium groups which are linked by spacers connected to the *o*-position, has been known for some time,¹⁷⁴ but has only recently been used in transfection studies,¹⁷⁵ viz. of RD-4 cells by pEGFP-C1 with GenePORTERTM as a standard. The analogue of **73a** with *m* = 2 (spacer length 4 CH₂ groups) was approximately as active as the standard, and only marginally improved with DOPE as the helper lipid ($0.33 < \alpha < 0.67$); AFM revealed that this was the only analogue of **73a** that could condense DNA to particles with a diameter <100 nm. Of the other analogues of **73a** given in Chart 11, only the one with *p* = 6 had some activity, and required DOPE ($\alpha = 0.5$). The fluorinated analogues **73b** all required DOPE ($\alpha = 0.33$), and except for absence of any activity for *p* = 10, the dependence on the spacer length was completely different: the highest activity was observed for *p* = 6 (comparable to standard, lipoplex particles <100 nm), with much lower activities for *p* = 1 and 2.¹⁷⁶

The imidazolium gemini **74a** showed efficient transfection of HEK293 and HeLa cells with GFP with negligible cytotoxicity up to a concentration of 10⁻⁵ M (Chart 11D).¹⁷⁷ The dependence of the transfection of HEK293 cells by GFP with DOPE as a helper lipid ($\alpha = 0.2$) on the spacer length was investigated for the analogues **74b–e** with C16 instead of C12 tails,¹⁷⁸ and found to decrease in the order *m* = 0 (**74b**) > 3 (**74d**) > 1 (**74c**) > 10 (**74e**). In a comparison with the ammonium geminis **23b** and **41** and the alkyl-spaced imidazolium gemini **74c**, the oxyethylene analogue **75c** was biologically the most active; all geminis included in this comparison outperformed the standard Lipofectamine 2000TM. In a subsequent study¹⁷⁹ the knockdown of EGFP by the appropriate siRNA in EGFP-expressing HEK293T cells was achieved, this time with a different helper lipid, i.e. mono-oleoyl glycerol.¹⁸⁰

3.6. Amino acid and peptide geminis

Amino acids and peptides coupled to lipids are also used in transfection,¹⁸¹ for example in the aforementioned Arg-DOTMA derivative **58**.¹⁶³ Attachment of a stearyl group to the N-terminus of the cell-penetrating peptides TAT, REV, FHV and R₈ (stearyl-HN-peptide-CO-NH₂) improved the transfection of COS-7 cells with luciferase, with the best

results, comparable to Lipofectamine, for R_8 .¹⁸² Stearoyl-appended R_8 gave better condensation of the DNA, better adsorption to the cells, and better delivery to the nucleus than R_8 itself;¹⁸³ incorporation (10%) of the lipopeptide in liposomes led to uptake through macropinocytosis, resulting in less lysosomal degradation than in the case of clathrin-mediated endocytosis.¹⁸⁴

The aforementioned polyamine Sp (Chart 2) can be symmetrically connected by amide bonds to an amino acid/peptide and oleic acid in two different ways (Chart 12), *i.e.* with the peptide at both terminal N, and the oleoyl at the central N atoms (76), and the other way around (77).¹¹⁵ The transfection of a number of cells, CHO-DG44, C2C12 (mouse muscle), nonadherent MOPC315 (mouse tumor), and 1321N1 (neuroal) with luciferase by the Lys derivatives 76a and 77a and the peptides 76b and 77b was compared to that by Lipofectamine 2000TM. For all cell types, the geminis of type 77 were more active than 76. The peptide derivative 77b was more active than the Lys-derivative 77a except for the C2C12 cells where it was the other way around. The gemini surfactants were superior to Lipofectamine 2000TM except for the 1321N1 cells where they performed relatively poorly. In a subsequent study on analogues of 77a,¹⁸³ it was found that the Lys head group was superior to alternative amino acids with 2 amino groups, L-Orn, L-Dab (2,4-diaminobutyric acid), and L-Dap (2,3-diaminopropionic acid), in the transfection of HEK293 cells with luciferase; the D-Lys analogue appeared to be both slightly more active and less toxic than L-Lys. Of a series of analogues of 77a with an oleoyl and one other tail (C6, C10, C14, C18, C18:1, and C18:3) those with 2 tails with 18 C atoms were the most active, with activity decreasing in the order C18 > C18:1 > C18:3 for the other tail. The cyclosporin 78 showed a remarkable dependence on the nature of the links between the Lys residues in the peptide; compound 78a with an ϵ -link only between the 1st and 2nd Lys was one of the most active compounds, whereas the analogue 78b with ϵ -links between all Lys residues was not active at all.⁵⁰

Peptide geminis with Cys-Ser-alkylamine at the core linked by an ethylene bridge between the Cys-S, which are remotely related to the dimerizable cationic surfactant 20,¹⁰³ have also been investigated.^{50,184,185} Of a series of compounds with C16 tails 79, the ones with head groups consisting of a single (di) amino acid, Dab 79a and Lys 79c were not active in the transfection of CHO cells with luciferase;¹⁸⁴ for a series of peptide headgroups, the activity decreased in the order Lys $^{\epsilon}$ Lys 79d > (Lys)₂Lys 79e > Lys-Ser 79b. The transfection of CHO-K1 cells with β -galactosidase by 79d was improved by addition of either DOPE or Lipofectamine PlusTM, and even more by both. In a subsequent investigation, the C18:1 analogues 80 turned out to be even more active than the C16 compounds 79 and much more than the corresponding C12 compounds, and the effect of the linkage, α or ϵ , was systematically investigated for head groups consisting of 3 Lys residues.¹⁸⁵ The activity of these compounds in the transfection of CHO-DG44 cells without DOPE was compared to that of Lipofectamine PlusTM and found to decrease in the order 80d (ϵ,ϵ) > 80c (ϵ,α) > 80b (α,ϵ) > 80a (α,α) > 80e ((Lys)₂Lys);⁵⁰ compounds 80d and its C16 analogue as well as 80c were more active than Lipofectamine PlusTM.¹⁸⁵ These results can however not be generalized in the sense that for trilycine head groups all links should be ϵ , because of the aforementioned opposite result for the cyclosporin 78. The gemini character of compounds 80 is important for transfection, as demonstrated by the fact that compound 81, which is the 'half-gemini' analogue of 80b, is not active.⁵⁰

Another group of gemini surfactants based on lysine, *viz.* (R¹(CO)-Lys(H)-NH)₂(CH₂)_n 82 (Chart 12),¹⁸⁶ has a fatty acid (R¹(CO)) connected to the α -amino group, and the carboxylic groups linked by diamine spacers of length *n*. The first report⁵⁰ on 82 referred to products that may have contained up to 10 stereoisomers, due to the use of technical (*Z/E* mixture) oleic acid, combined with a racemization-prone synthetic protocol; this strategy was revised, starting from pure oleic acid.¹⁸⁶ For spacer length *n* = 6 the hydrophobic acyl tail was varied in length (R¹ = C8 to C18, 82d-i), and, for R¹ = C18, the degree of unsaturation (C18:1 *Z* 82a and *E* 82b, C18:2 (*Z,Z*) 82c). For R¹(CO) = oleoyl (C18:1 *Z*) the spacer length (*n* = 2–8, 82j–k, 82a) and the stereochemistry of the lysine building block (D-Lys, 82o) were varied; a 'half-gemini' derivative 83 with a single oleoyl tail and head group was also prepared. In the transfection of HeLa cells with β -galactosidase, with or without DOPE, the oleoyl derivative (C18:1 *Z*, 82a) was by far the best compound in this series. There was also a slight improvement with the change of Lys stereochemistry in 82o, such as also found for the aforementioned Sp derivative 78a. Without DOPE, the oleoyl derivative with *n* = 6 (82a) was far more active than those with *n* = 2–5 (82j–n) or *n* = 8 (82m), making this, along with the glycolipid 46i and the lipopeptide 80d, one of the most active compounds to come out of the ENGEMS project;⁵⁰ with DOPE, there was hardly an effect of spacer length, except a slightly lower activity for *n* = 3 (82k). The gemini structure of 82a was essential; its 'half-gemini' analogue 83 was less active by 2 orders of magnitude.

Chart 12 Amino acid-based gemini surfactants. Links between Lys in peptides are α unless otherwise indicated.

Related to **79** and **82** is the disulfide dimer **84** of a Lys-Cys peptide that is esterified at its C-terminus with oleyl-alcohol;¹⁸⁷ this showed no toxicity against HEK293 and HeLa cells like the standard Lipofectamine RNAi^{MAX}™, but it gave more efficient silencing of the protein kinase MEK1 with RNAi. The aggregates of **84** formed lipoplexes with siMEK1, in which the RNA was protected from degradation in serum, and from which it could be released by reduction of the S–S bond by dithiothreitol. In a subsequent report¹⁸⁸ the activity of **84** was compared to its non-reducible analogue with a single –CH₂– group in the place of the –S–S– functionality in **84**, and with its non-dimerizable ‘half-gemini’ analogue Lys-Ala-oleoyl in the silencing of EGFR in MCF-7 and HeLa cells. The half-gemini compound was both less active and more toxic. The lipoplexes of both geminis (N/P 10) were taken up in MCF-7 cells by clathrin-mediated endocytosis. Although the uptake of lipoplexes of **84** was less or equally efficient in respectively MCF-7 and HeLa cells than that of the –CH₂– analogue, the silencing was equally resp. more complete for **84**; this effect was ascribed to reduction of the S–S bond upon uptake, resulting in more efficient release of the siRNA from the lysosome. The efforts in this field have been reviewed at an early stage⁵⁰ as well as more recently.¹¹²

Of the multivalent protonable amphiphilic peptides represented by general structure **85** (Chart 12), EHCO (**85a**) stood out as particularly effective, at N/P values in the range 8–10 and without helper lipid, in the silencing of luciferase in U87-luc cells.¹⁸⁹ This was explained by its hemolytic activity at pH 5.4, but not at 6.5 and 7.4, which indicates that it will selectively disrupt endosomal membranes at the pH of the transition to lysosomes. In a study where EHCO (**85a**) was applied to silence the hypoxia-inducible factor (HIF-1 α) in U87 glioblastoma cells, it was shown by confocal laser microscopy that Alexa Fluor 488-labeled siRNA had indeed escaped from the lysosomes.¹⁹⁰ Hemolytic activity is not the only important factor for transfection activity, since CHO-GFP and HT29 were better transfected at N/P 12 by the histidine-free lipopeptides ECO (**85b**) and ECLn (**85c**), which is tentatively explained by an assumed tendency for the His-containing EHCO (**85a**) to take up a cylindrical shape, which would favour bilayer over non-bilayer structures.¹⁹¹ In this connection it was shown that oxidative polymerization of the 2 thiols in ECO (**85b**) upon lipoplex formation protects the siRNA in the lipoplex from degradation, and that it is released upon reduction by glutathione (see section 2.4).¹⁹² The cysteine thiol in EHCO (**85a**) can also be used to enhance selective delivery, for example by maleimide conjugation to a PEG-ylated peptide, to target cancer cells in which the bombesin receptor is overexpressed.¹⁹³ The results for these lipopeptides have recently been reviewed.¹⁹⁴

4. Asymmetric geminis and beyond

4.1. Gemini surfactants with chiral spacers

Some cationic gemini surfactants bear one or more chiral centers in the spacer group, which can lead to asymmetry in

the molecular structure. Tartaric acid has a 4C-skeleton with 2 chiral centres. In an early example (Chart 13),¹⁹⁵ the hydroxy groups of the *L*- or (*R,R*)-enantiomer were esterified with palmitic acid, while the carboxylic acid groups were linked by an amide bond to one of the amine groups of 1,2-diaminoethane (**86a**), the α -amino group of the amino acid lysine (**86b**), or a combination of both (**86c**). Compared to the other gemini surfactants studied in the European Network on Gemini Surfactants (ENGEMS) project (see below), the activity of the compounds **86b** and **86c** in the transfection of CHO-K1 cells with luciferase plasmid DNA was moderate, while the toxicity, disappointingly, was more than average; compound **86a** was not active at all. More recently, a set of novel geminis based on tartaric acid, with the carboxylic acid groups esterified with alkyl (C12, **87a**; C16, **87b**) alcohols, and the hydroxyl groups with aminocaproic acids was investigated in Hek293T and HeLa cells,¹⁹⁶ and found to give GFP expression comparable to or better than DC-Chol **8a** and DOTAP **2b**, with significantly lower toxicity. The efficiencies of the mixed aggregates composed of the neutral helper lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and the stereoisomers of the cationic dimeric surfactant with 2 chiral centres in the spacer, 2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonium) butane dibromide **88** (**88a**, (2*S*,3*S*); **88b** (2*R*,3*R*); **88c** (2*S*,3*R*)), as transfection vectors were tested using the eGFP coding plasmid and COS-1 cells.¹⁹⁷ In these systems, only **88c** was more efficient than Fugene, and it also showed the lowest toxicity, whereas the half-gemini analogue of **88**, consisting of one of the surfactant tails and head groups with a 2-methoxyethyl group instead of the spacer, had no transfection activity at all.

Gemini-like amphiphilic peptides consisting of a peptide spacer with the N- and C-termini appended with hydrophobic groups are also to be considered ‘geminoids’.¹¹⁹ As an example, the SPKR peptide (**89**-SPKR, Chart 13, with R¹, R², R³, R⁴ representing the Ser, Pro, Lys and Arg side chains, respectively) inspired by biological nucleic acid binding motifs,^{52,198} was appended with unsaturated (oleoyl/oleyl) alkyl tails. The versatility of the chemical approach allowed peptides related to SPKR to be studied, and it was found that the Pro and at least one of the cationic (Lys, Arg) residues are essential for

Chart 13 Geminis with chiral spacers.

the biological activity. 89-SPKR and the analogue 89-APKR showed excellent plasmid DNA transfection of HeLa and Hek293T cells with GFP, and quenching of GFP by siRNA in 1207 human bladder carcinoma cells transfected with GFP, with only a moderate cytotoxic effect. Importantly, this can be achieved without the addition of a lysogenic helper lipid, provided that the lipid tails contain unsaturations.

Analogues of the gemini amphiphilic peptides 85¹⁹⁴ in which a lysine residue (K) is connected to other amino acids at both (α and ϵ) amino groups as in 90 and 91 are also asymmetric and fall under the geminoid definition. Of the 2 ethylenediamine derivatives EKHCO (90a) and EHHKCO (90b), the hemolytic activity of the former showed the largest increase at lower pH (5.5) and displayed the larger transfection activity, at N/P = 12 for U87 glioblastoma cells with pCMV-luciferase.¹⁹⁹ The derivatives 91 with the Sp headgroup (Chart 3) were studied in the silencing of the U87-Luc cells with N/P = 12.²⁰⁰ For the compounds without β -Ala ($z = 0$), the activity increased in the series 91c < 91d < 91a < Lipofectamine 2000 < 91c; the activities of the β -Ala ($z = 1$) containing analogues 91d–h were slightly lower and followed the same order, except for 91h which was the most active of all compounds 91.

4.2. Lipidoids

New cationic lipids for transfection have not only been explored by design but also by combinatorial approaches. In one such approach, a number of polyamines have been reacted with acrylamides with amine residues of varying chain length (Scheme 2A), and the crude products, coined 'lipidoids', tested for their activity in the silencing of HeLa cells transfected with luciferase by siRNA.²⁰¹ Not surprisingly, some gemini products, such as 92a (64N-16) and its C15-analogue, were found to be very effective. Interestingly, some of the products of more complex amines and acrylamides with shorter (C12) tails, such as 98N-12, turned out to be even more active, in particular with 5 tails attached (92b). In combination with HO-Chol and PEG-lipid, this compound was particularly effective in silencing the expression of the blood-clotting Factor VII, which can be detected in serum, by mouse hepatocytes. In a formulation with HO-Chol and PEG-lipid, 92b could also silence the expression of apolipoprotein by hepatocytes in cynomolgus monkeys; the mass of material relative to the siRNA in this experiment was approx. one third, with fewer components, of that in the aforementioned experiments⁶⁷ with SNALP with 3b.

In another combinatorial study involving acrylate esters in addition to acrylamides, the methoxy compound 93a was found to be most active in luciferase silencing in HeLa cells, with however low cell viability.²⁰² The result of factor VII silencing in mice was better for the alcohol 93b, with >80% knockdown at 12:1 lipid/siRNA weight ratio. In another study, the combination of the amide 93c with the C13-acrylate analogue of 93b (98O-13), which are each individually not active, was found to be synergistic in this factor VII

Scheme 2 Lipidoids from A) acrylates, B) epoxides, C) rings.

silencing.²⁰³ The bioreducible analogue 93e with an S–S bond in the acrylate was found to be very effective in silencing GFP in MDA-MB-231 cells expressing this protein.²⁰⁴

In the exploration of more combinations of amines with acrylate esters, it was found that lipid nanoparticles (LNPs) of DSPC (1d), HO-Chol, PEG-lipid and any of the lipidoids 94a, 94c, and 94d were also very active.²⁰⁵ Based on the result that the optimum alkyl tail length and substitution number were 12–13 and 4, respectively, the next generation of lipidoids was developed within the same study, which yielded 94e as the most effective compound. The most active lipidoids in the silencing of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in human colorectal adenocarcinoma (Caco-2) cells were 94b, 94d and 94f.^{206,207} The 3-tailed version of 94b was also applied in the silencing of the anti-apoptotic protein Mcl1, which resulted in apoptosis of mantle cell lymphoma cells,²⁰⁸ and of tumor necrosis factor α in a macrophage-fibroblast model for diabetic foot ulcers.²⁰⁹

In another combinatorial approach, amines were reacted with racemic alkyl epoxides of varying tail lengths (Scheme 2B) to give the substituted aminoalcohol products 95 as mixtures of diastereomers.²¹⁰ Following initial screening by silencing of luciferase in HeLa cells, the compounds 95a–c were identified as being most effective in factor VII silencing in mouse hepatocytes, with no evidence of adverse effects. In a formulation with DSPC, HO-Chol, and PEG, compound 95c was found to be more potent by two orders-of-

magnitude than the best acrylamide lipidoid **92b**. For the correlation between activity *in vitro* and *in vivo*, the RNA entrapment efficiency had a higher predictive value than the zeta potential and the particle size, provided that the latter was <200 nm.²¹¹ In a microscopic study of the transfection by LNPs based on **95c** of mouse embryonic fibroblasts with or without NPC1 (Niemann-Pick type C1 glycoprotein), it was found that transfection was more efficient without NPC1, implying that this protein is responsible for exocytosis of 70% of the nucleotide.²¹² In a study where **95c**-LNP loaded with m-RNA for erythropoietin (EPO) were intravenously injected in mice, the EPO level in serum was raised by including the classical helper lipid DOPE (**1a**) instead of DSPC (**1d**) in the formulation.²¹³ LNPs with lipopeptide lipidoids are called lipopeptide nanoparticles (LPNs). In a study where amine groups incorporated in amino acid derivatives were reacted by reductive amination, Michael addition to acrylate esters, and epoxide opening, the particularly effective compound cKK-E12 (**95d**), derived from the reaction of diketopiperazine of the amino acid lysine with the C12-epoxide, was found to be particularly effective and selective in the silencing of factor VII in mouse hepatocytes, also when compared to **92b** and **95c**.²¹⁴ LPNs based on **95d** were also successfully applied to deliver mRNA vaccines to B lymphocytes of the immune system in the spleen of mice to enhance their cytotoxic response to a melanoma tumor.²¹⁵ In a study on the transfection of mice with the mRNA for EPO, the activity of the linoleyl analogue OF-02 (**95e**) was even higher.²¹⁶

Lipidoids have also been used with DNA, for example 14N-87 (**92d**), which was more active than the standard Lipofectamine 2000 in the transfection of HeLa cells with β -galactosidase.²¹⁷ Related to the diketopiperazine platform of **95d** is the triazine in **96**, which was applied in simultaneous transfection of pDNA and siRNA, *viz.* expression of GFP and silencing of luciferase in HeLa cells.²¹⁸

5. Conclusions and outlook

The past decade has seen tremendous progress in the development, by design and synthesis as well as combinatorial approaches, of lipid-based vectors for DNA and RNA delivery. In this review, the emphasis was on gemini and gemini-like surfactants, which proved to be more effective than their single head group/single tail counterparts in many (but not all) cases where a comparison could be made. The spacer/linker region offers an important additional source of variation/optimization in geminis that is not available to classical surfactants.

In the design of vectors, many aspects of molecular and aggregate structure must be considered. Electrostatics must be balanced with respect to the interaction with the polynucleotide and the target cell; the lipid should preferably be biodegradable and its positive charge delocalized to minimize cytotoxicity. Advantage can be taken of concepts from biology, such as recognition of carbohydrates and peptides by cellular receptors, as well as specific peptide sequences

which are used for DNA binding and cell-penetration, and are also being explored for endosomal escape²¹⁹ and nuclear localization.²²⁰ In our review, the emphasis has been more on the exploration of chemical space, but it should be noted that insights in physicochemical concepts, such as surface charge density, effective charge density, lipoplex polymorphism and fusogenicity have also been developed; their impact on nucleic acid complexation, cellular uptake, endosomal escape, cytoplasmic mobility, and nuclear entry has been reviewed elsewhere.^{39,113,221,222} Cationic lipids already are used in laboratories for biological research purposes on a large scale, and recent surveys^{37,223} mention clinical trials with liposomes or LNPs for various therapies, *viz.* with plasmid DNA for (among others) cystic fibrosis, various lung cancers, various squamous cell carcinomas, glioblastoma, and with siRNA for amyloidosis, muscular dystrophy, neuroendocrine tumor and adrenocortical carcinoma, liver and pancreatic cancer, and hepatic fibrosis.

Cystic fibrosis is of special interest since it is the most frequent lethal recessive congenital disease in Caucasians, with a carrier frequency of 1/30.²²⁴ The genetic defect, *viz.* the Cystic fibrosis transmembrane conductance regulator (CFTR), has been known for almost 30 years, it is monogenic, and the lungs are easily accessible; nonetheless, no gene therapy is available to date. This has a number of causes: i) the thickened mucus, which is an important feature of this condition, presents an additional barrier to transfection, ii) the airways are heavily defended by the innate and cellular immune system, and iii) the target cells have low mitotic activity, which dramatically reduces the efficacy of non-viral DNA delivery systems.²²⁵ The lipid used in the most recent clinical trial,²²⁶ GL-67 **8b**, resulting from modification of DC-Chol **8a** with Sp,²²⁷ and its formulation with DOPE for the aerosol delivery of functional were also developed some time ago.²²⁸

Quite recently, gene editing using the CRISPR-(clustered regularly interspaced short palindromic repeats) associated (Cas) proteins has emerged as a promising technique to proceed beyond the addition of genes as in gene therapy described above, *viz.* to repair genes in the cell nucleus.²²⁹ It is based on delivery of a CRISPR dependent gene editing nuclease – Cas9, and guide RNA molecules, which allow efficient editing of specific gene sequences, and can be applied to stem/progenitor cells, in experimental and potentially in clinical applications. Like conventional gene therapy, this requires that a carrier complexed to polynucleotides is brought into the cell.²³⁰ Cas9 expression can be achieved with a viral vector,^{231,232} but also by non-viral vectors²³³ such as polymeric and hybrid microcarriers, made of degradable polymers such as polypeptides and polysaccharides and modified by silica shell,²³⁴ and by electroporation as in the example of a human embryo.²³⁵ However, since only short term expression of Cas9 is required to allow gene editing, mRNA or CAS9 protein delivery can also be applied, which is quite effective in non-dividing cells, and avoids the safety issues involved with viral vectors. This notion has opened a whole new field of applications for non-viral delivery systems, although many

of the delivery issues have remained. Cationic lipids have also been used for this purpose, such as the aforementioned bioreducible acrylate-derived lipid 92e²⁰⁴ in the case of the delivery of a protein-RNA adduct in HeLa-DsRed cells,²³⁶ and the lipid with C3 symmetry 97 in the delivery of Cas9 mRNA and single-guide RNA in C57BL/6 mice.²³⁷

Conflicts of interest

The authors declare no competing interest.

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References

- 1 T. Wirth, N. Parker and S. Ylä-Herttua, *Gene*, 2013, **525**, 162–169.
- 2 S. L. Ginn, I. E. Alexander, M. L. Edelstein, M. R. Abedi and J. Wixon, *J. Gene Med.*, 2013, **15**, 65–77.
- 3 Y.-J. Ren and Y. Zhang, *Expert Opin. Biol. Ther.*, 2014, **14**, 1581–1592.
- 4 A. W. Nienhuis, *Blood*, 2013, **122**, 1556–1565.
- 5 J. D. Sengillo, S. Justus, Y.-T. Tsai, T. Cabral and S. Tsang, *Am. J. Med. Genet., Part C*, 2016, **172**, 349–366.
- 6 F. Piguet, S. Alves and N. Cartier, *Hum. Gene Ther.*, 2017, **28**, 988–1003.
- 7 A. Aiuti, L. Biasco, S. Scaramuzza, F. Ferrua, M. P. Cicalese, C. Baricordi, F. Dionisio, A. Calabria, S. Giannelli, M. C. Castiello, M. Bosticardo, C. Evangelio, A. Assanelli, M. Casiraghi, S. di Nunzio, L. Callegaro, C. Benati, P. Rizzardi, D. Pellin, C. di Serio, M. Schmidt, C. von Kalle, J. Gardner, N. Mehta, V. Neduva, D. J. Dow, A. Galy, R. Miniero, A. Finocchi, A. Metin, P. P. Banerjee, J. S. Orange, S. Galimberti, M. G. Valsecchi, A. Biffi, E. Montini, A. Villa, F. Ciceri, M. G. Roncarolo and L. Naldini, *Science*, 2013, **341**, 1233151.
- 8 A. Biffi, E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, C. Baldoli, S. Martino, A. Calabria, S. Canale, F. Benedicenti, G. Vallanti, L. Biasco, S. Leo, N. Kabbara, G. Zanetti, W. B. Rizzo, N. A. L. Mehta, M. P. Cicalese, M. Casiraghi, J. J. Boelens, U. del Carro, D. J. Dow, M. Schmidt, A. Assanelli, V. Neduva, C. di Serio, E. Stupka, J. Gardner, C. von Kalle, C. Bordignon, F. Ciceri, A. Rovelli, M. G. Roncarolo, A. Aiuti, M. Sessa and L. Naldini, *Science*, 2013, **341**, 1233158.
- 9 L. Naldini, *Nature*, 2015, **526**, 351–360.
- 10 P. Kesharwani, V. Gajbhiye and N. K. Jain, *Biomaterials*, 2012, **33**, 7138–7150.
- 11 V. Campani, G. Salzano, S. Lusa and G. De Rosa, *Nanomaterials*, 2016, **6**, 131.
- 12 G. J. Dimitriadis, *Nature*, 1978, **274**, 923–924.
- 13 U. Sahin, K. Karikó and Ö. Türeci, *Nat. Rev. Drug Discovery*, 2014, **13**, 759–780.
- 14 M. A. Islam, E. K. G. Reesor, Y. Xu, H. R. Zope, B. R. Zetter and J. Shi, *Biomater. Sci.*, 2015, **3**, 1519–1533.
- 15 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806–811.
- 16 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494–498.
- 17 R. Kanasty, J. R. Dorkin, A. Vegas and D. Anderson, *Nat. Mater.*, 2013, **12**, 967–977.
- 18 C. Wan, T. M. Allen and P. R. Cullis, *Drug Delivery Transl. Res.*, 2014, **4**, 74–83.
- 19 R. Agami, *Curr. Opin. Chem. Biol.*, 2002, **6**, 829–834.
- 20 L. Naldini and I. M. Verma, *Adv. Virus Res.*, 2000, **55**, 599–609.
- 21 A. Fischer and M. Cavazzana-Calvo, *Lancet*, 2008, **371**, 2044–2047.
- 22 M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, 2009, **109**, 259–302.
- 23 C. Gándara, V. Affleck and E. A. Stoll, *Hum. Gene Ther. Methods.*, 2018, **29**, 1–15.
- 24 J. C. M. van der Loo, W. P. Swaney, E. Grassman, A. Terwilliger, T. Higashimoto, A. Schambach, C. Baum, A. J. Thrasher, D. A. Williams, D. L. Nordling, L. Reeves and P. Malik, *Gene Ther.*, 2012, **19**, 246–254.
- 25 J. Baruteau, S. N. Waddington, I. E. Alexander and P. Gissen, *J. Inherited Metab. Dis.*, 2017, **40**, 497–517.
- 26 S. Russell, J. Bennett, J. A. Wellman, D. C. Chung, Z.-F. Yu, A. Tillman, J. Wittes, J. Pappas, O. Elci, S. McCague, D. Cross, K. A. Marshall, J. Walshire, T. L. Kehoe, H. Reichert, M. Davis, L. Raffini, L. A. George, F. P. Hudson, L. Dingfield, X. Zhu, J. A. Haller, E. H. Sohn, V. B. Mahajan, W. Pfeifer, M. Weckmann, C. Johnson, D. Gewaily, A. Drack, E. Stone, K. Wachtel, F. Simonelli, B. P. Leroy, J. F. Wright, K. A. High and A. M. Maguire, *Lancet*, 2017, **390**, 849–860.
- 27 B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J.-P. Vigneron, J.-M. Lehn and P. Lehn, *Curr. Pharm. Des.*, 2005, **11**, 375–394.
- 28 H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, *Nat. Rev. Genet.*, 2014, **15**, 541–555.
- 29 P. P. Karmali and A. Chaudhuri, *Med. Res. Rev.*, 2007, **27**, 696–722.
- 30 S. Bhattacharya and A. Bajaj, *Chem. Commun.*, 2009, 4632–4656.
- 31 X. X. Zhang, T. J. McIntosh and M. W. Grinstaff, *Biochimie*, 2012, **94**, 42–58.
- 32 D. F. Zhi, S. B. Zhang, S. H. Cui, Y. A. Zhao, Y. H. Wang and D. F. Zhao, *Bioconjugate Chem.*, 2013, **24**, 487–519.
- 33 B. Draghici and M. A. Ilies, *J. Med. Chem.*, 2015, **58**, 4091–4130.
- 34 C. Ortiz Mellet, J. M. García Fernández and J. M. Benito, *Chem. Soc. Rev.*, 2011, **40**, 1586–1608.

- 35 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297–7301.
- 36 S. Malhotra, H. Bauer, A. Tsische, A. M. Staedtler, A. Mohr, M. Calderón, V. S. Parmar, L. Hoeke, S. Sharbati, R. Einspanier and R. Haag, *Biomacromolecules*, 2012, **13**, 3087–3098.
- 37 M. Foldvari, D. W. Chen, N. Nafissi, D. Calderon, L. Narsineni and A. Rafiee, *J. Controlled Release*, 2016, **240**, 165–190.
- 38 R. Srinivas, S. Samanta and A. Chaudhuri, *Chem. Soc. Rev.*, 2009, **38**, 3326–3338.
- 39 E. Junquera and E. Aicart, *Curr. Top. Med. Chem.*, 2014, **14**, 649–663.
- 40 C. Tanford, *The Hydrophobic Effect, Formation of Micelles and Biological Membranes*, Wiley Interscience, New York, 1973.
- 41 J. N. Israelachvili, D. J. Mitchell and B. W. Ninham, *J. Chem. Soc., Faraday Trans. 2*, 1976, 1525–1568.
- 42 J. N. Israelachvili, S. Marcelja and R. G. Horn, *Q. Rev. Biophys.*, 1980, **13**, 121–200.
- 43 H. Brockerhoff, in *Bio-Organic Chemistry*, ed. E. E. van Tamelen, Academic Press, New York, 1977, pp. 1–20.
- 44 P. J. Horn and C. L. Peterson, *Science*, 2002, **297**, 1824–1827.
- 45 K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond, *Nature*, 1997, **389**, 251–260.
- 46 M. Belting, S. Sandgren and A. Wittrup, *Adv. Drug Delivery Rev.*, 2005, **57**, 505–527.
- 47 J. Nguyen and F. C. Szoka, *Acc. Chem. Res.*, 2012, **45**, 1153–1162.
- 48 N. Belmadi, P. Midoux, P. Loyer, C. Passirani, C. Pichon, T. Le Gall, P.-A. Jaffres, P. Lehn and T. Montier, *Biotechnol. J.*, 2015, **10**, 1370–1389.
- 49 K. M. Stewart, K. L. Horton and S. O. Kelley, *Org. Biomol. Chem.*, 2008, **6**, 2242–2255.
- 50 A. J. Kirby, P. Camilleri, J. Engberts, M. C. Feiters, R. J. M. Nolte, O. Söderman, M. Bergsma, P. C. Bell, M. L. Fielden, C. L. García Rodríguez, P. Guédât, A. Kremer, C. McGregor, C. Perrin, G. Ronsin and M. C. P. van Eijk, *Angew. Chem., Int. Ed.*, 2003, **42**, 1448–1457.
- 51 N.-D. van Loo, E. Fortunati, E. Ehlert, M. Rabelink, F. Grosveld and B. J. Scholte, *J. Virol.*, 2001, **75**, 961–970.
- 52 M. Wilke, E. Fortunati, M. van den Broek, A. T. Hoogveen and B. J. Scholte, *Gene Ther.*, 1996, **3**, 1133–1142.
- 53 W. Li and F. C. Szoka Jr, *Pharm. Res.*, 2007, **24**, 438–449.
- 54 B. Dalby, S. Cates, A. Harris, E. C. Ohki, M. L. Tilkins, P. J. Price and V. C. Ciccarone, *Methods*, 2004, **33**, 95–103.
- 55 A. S. Arnold, V. Laporte, S. Dumont, A. Appert-Collin, P. Erbacher, G. Coupin, R. Levy, P. Poindrona and J.-P. Gies, *Fundam. Clin. Pharmacol.*, 2006, **20**, 81–89.
- 56 S. Yamano, J. Dai and A. M. Moursi, *Mol. Biotechnol.*, 2010, **46**, 287–300.
- 57 E. Guénin, A.-C. Hervé, V. Floch, S. Loisel, J.-J. Yaouanc, J.-C. Clément, C. Férec and H. Des Abbayes, *Angew. Chem., Int. Ed.*, 2000, **39**, 643–645.
- 58 R. P. Balasubramaniam, M. J. Bennett, A. M. Aberle, J. G. Malone, M. H. Nantz and R. W. Malone, *Gene Ther.*, 1996, **3**, 163–172.
- 59 J.-S. Remy, C. Sirlin, P. Vierling and J.-P. Behr, *Bioconjugate Chem.*, 1994, **5**, 647–654.
- 60 J. Heyes, L. Palmer, K. Bremner and I. MacLachlan, *J. Controlled Release*, 2005, **107**, 276–287.
- 61 Y. H. Kim, J. H. Park, M. Lee, Y. H. Kim, T. G. Park and S. W. Kim, *J. Controlled Release*, 2005, **103**, 209–219.
- 62 R. A. Moss and S. Bhattacharya, *J. Phys. Org. Chem.*, 1992, **5**, 467–470.
- 63 P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413–7417.
- 64 R. W. Malone, P. L. Felgner and I. M. Verma, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 6077–6081.
- 65 R. Leventis and J. R. Silvius, *Biochim. Biophys. Acta*, 1990, **1023**, 124–132.
- 66 S. C. Semple, S. K. Klimuk, T. O. Harasym, N. Dos Santos, S. M. Ansell, K. F. Wong, N. Maurer, H. Stark, P. R. Cullis, M. J. Hope and P. Scherrer, *Biochim. Biophys. Acta*, 2001, **1510**, 152–166.
- 67 T. S. Zimmermann, A. C. H. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L. V. Nechev, L. R. Palmer, T. Racie, I. Röhl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.-P. Vornlocher and I. MacLachlan, *Nature*, 2006, **441**, 111–114.
- 68 S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougères, I. MacLachlan, P. R. Cullis, T. D. Madden and M. J. Hope, *Nat. Biotechnol.*, 2010, **28**, 172–176.
- 69 M. Jayaraman, M. Steven, B. L. Ansell, Y. K. Mui, J. Tam, X. Chen, D. Butler, L. Eltepu, S. Matsuda, J. K. Narayanannair, K. G. Rajeev, I. M. Hafez, A. Akinc, M. A. Maier, M. A. Tracy, P. R. Cullis, T. D. Madden, M. Manoharan and M. J. Hope, *Angew. Chem., Int. Ed.*, 2012, **51**, 8529–8533.
- 70 J. Gilleron, W. Queres, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier and M. Zerial, *Nat. Biotechnol.*, 2013, **31**, 638–646.
- 71 M. A. Maier, M. Jayaraman, S. Matsuda, J. Liu, S. Barros, W. Queres, Y. K. Tam, S. M. Ansell, V. Kumar, J. Qin, X. Zhang, Q. Wang, S. Panesar, R. Hutabarat, M. Carioto, J. Hettinger, P. Kandasamy, D. Butler, K. G. Rajeev, B. Pang,

- K. Charisse, K. Fitzgerald, B. L. Mui, X. Du, P. Cullis, T. D. Madden, M. J. Hope, M. Manoharan and A. Akinc, *Mol. Ther.*, 2013, **21**, 1570–1578.
- 72 C. M. LaManna, H. Lusic, M. Camplo, T. J. McIntosh, P. Barthélémy and M. W. Grinstaff, *Acc. Chem. Res.*, 2012, **45**, 1026–1038.
- 73 S. Khiati, N. Pierre, S. Andriamanarivo, M. W. Grinstaff, N. Arazam, F. Nallet, L. Navailles and P. Barthélémy, *Bioconjugate Chem.*, 2009, **20**, 1765–1772.
- 74 P. J. O'Brien, S. Elahipanah, D. Rogozhnikov and M. N. Yousaf, *ACS Cent. Sci.*, 2017, **3**, 489–500.
- 75 X. Gao and L. Huang, *Biochem. Biophys. Res. Commun.*, 1991, **179**, 280–285.
- 76 R. Bottega and R. M. Epand, *Biochemistry*, 1992, **31**, 9025–9030.
- 77 H. Farhood, R. Bottega, R. M. Epand and L. Huang, *Biochim. Biophys. Acta*, 1992, **1111**, 239–246.
- 78 G. S. Manning, *Q. Rev. Biophys.*, 1978, **11**, 179–246.
- 79 M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.
- 80 G. Gebeyehu, J. A. Jessee, V. C. Ciccarone, P. Hawley-Nelson and A. Chytil, *U.S. Pat.*, US5334761A, 1994.
- 81 J.-P. Behr, B. Demeneix, J.-P. Loeffler and J. Perez-Mutul, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 6982–6986.
- 82 J. G. Riess, *Tetrahedron*, 2002, **58**, 4113–4131.
- 83 J. Gaucheron, C. Santaella and P. Vierling, *J. Gene Med.*, 2001, **3**, 338–344.
- 84 J. Gaucheron, C. Santaella and P. Vierling, *Bioconjugate Chem.*, 2001, **12**, 114–128.
- 85 J. Gaucheron, C. Santaella and P. Vierling, *Biochim. Biophys. Acta*, 2002, **1564**, 349–358.
- 86 C. Boulanger, C. Di Giorgio, J. Gaucheron and P. Vierling, *Bioconjugate Chem.*, 2004, **15**, 901–908.
- 87 K. Ewert, A. Ahmad, H. M. Evans, H.-W. Schmidt and C. R. Safinya, *J. Med. Chem.*, 2002, **45**, 5023–5029.
- 88 N. F. Boussein, C. S. McAllister, K. K. Ewert, C. E. Samuel and C. R. Safinya, *Biochemistry*, 2007, **46**, 4785–4792.
- 89 T. Fujiwara, S. Hasegawa, N. Hirashima, M. Nakanishi and T. Ohwada, *Biochim. Biophys. Acta*, 2000, **1468**, 396–402.
- 90 Q.-D. Huang, H. Chen, L.-H. Zhou, J. Huang, J. Wu and X.-Q. Yu, *Chem. Biol. Drug Des.*, 2008, **71**, 224–229.
- 91 H.-J. Wang, X. He, Y. Zhang, J. Zhang, Y.-H. Liu and X.-Q. Yu, *RSC Adv.*, 2015, **5**, 59417–59427.
- 92 J.-H. Fuhrhop and J. Mathieu, *Angew. Chem., Int. Ed. Engl.*, 1984, **23**, 100–113.
- 93 J. M. Pestman, K. R. Terpstra, M. C. Stuart, H. A. van Doren, A. Brisson, R. M. Kellogg and J. B. F. N. Engberts, *Langmuir*, 1997, **13**, 6857–6860.
- 94 J. Gaucheron, C. Santaella and P. Vierling, *Bioconjugate Chem.*, 2001, **12**, 569–575.
- 95 N. Jain, V. Goldschmidt, S. Oncul, Y. Arntz, G. Duportail, Y. Mely and A. S. Klymchenko, *Int. J. Pharm.*, 2012, **423**, 392–400.
- 96 S. Denoyelle, A. Polidori, M. Brunelle, P. Y. Vuillaume, S. Laurent, Y. ElAzhary and B. Pucci, *New J. Chem.*, 2006, **30**, 629–646.
- 97 M. Brunelle, A. Polidori, S. Denoyelle, A.-S. Fabiano, P. Y. Vuillaume, S. Laurent-Lewandowski and B. Pucci, *C. R. Chim.*, 2009, **12**, 188–208.
- 98 J.-X. Chen, X.-D. Xu, S. Yang, J. Yang, R.-X. Zhuo and X.-Z. Zhang, *Macromol. Biosci.*, 2013, **13**, 84–92.
- 99 H. Zeng, M. E. Johnson, N. J. Oldenhuis, T. N. Tiambeng and Z. Guan, *ACS Cent. Sci.*, 2015, **1**, 303–312.
- 100 M. Khan, C. Y. Ang, N. Wiradharma, L. K. Yong, S. Liu, L. Liu, S. Gao and Y.-Y. Yang, *Biomaterials*, 2012, **33**, 4673–4680.
- 101 D. Paiva, T. Markowski, B. Dobner, G. Brezesinski, H. Möhwald, M. do Carmo Pereira and S. Rocha, *Colloid Polym. Sci.*, 2015, **293**, 3167–3175.
- 102 M. Martínez-Negro, A. Guerrero-Martínez, L. García-Río, Ò. Domènech, E. Aicart, C. Tros de Ilarduya and E. Junquera, *ACS Omega*, 2018, **3**, 208–217.
- 103 E. Dauty, J.-S. Remy, T. Blessing and J.-P. Behr, *J. Am. Chem. Soc.*, 2001, **123**, 9227–9234.
- 104 R. S. Shirazi, K. K. Ewert, C. Leal, R. N. Majzoub, N. F. Boussein and C. R. Safinya, *Biochim. Biophys. Acta*, 2011, **1808**, 2156–2166.
- 105 F. M. Menger and C. A. Littau, *J. Am. Chem. Soc.*, 1991, **113**, 1451–1452.
- 106 R. Zana and Y. Talmon, *Nature*, 1993, **362**, 228–230.
- 107 R. Zana, *J. Colloid Interface Sci.*, 2002, **248**, 203–220.
- 108 F. M. Menger and J. S. Keiper, *Angew. Chem., Int. Ed.*, 2000, **39**, 1907–1920.
- 109 H. C. Rath, DE19622612, 1997.
- 110 S. D. Wettig, R. E. Verrall and M. Foldvari, *Curr. Gene Ther.*, 2008, **8**, 9–23.
- 111 C. Bombelli, L. Giansanti, P. Luciani and G. Mancini, *Curr. Med. Chem.*, 2009, **16**, 171–183.
- 112 L. Pérez, A. Pinazo, R. Pons and M. Infante, *Adv. Colloid Interface Sci.*, 2014, **205**, 134–155.
- 113 E. Junquera and E. Aicart, *Adv. Colloid Interface Sci.*, 2016, **233**, 161–175.
- 114 A. A. Yaroslavov, O. Y. Udalykh, N. S. Melik-Nubarov, V. A. Kabanov, Y. A. Ermakov, V. A. Azov and F. M. Menger, *Chem. – Eur. J.*, 2001, **7**, 4835–4843.
- 115 G. Ronsin, C. Perrin, P. Guédat, A. Kremer, P. Camilleri and A. J. Kirby, *Chem. Commun.*, 2001, 2234–2235.
- 116 R. Oda, I. Huc and S. J. Candau, *Angew. Chem., Int. Ed.*, 1998, **37**, 689–691.
- 117 K. Jennings, I. Marshall, H. Birrell, A. Edwards, O. Söderman, A. J. Kirby and P. Camilleri, *Chem. Commun.*, 1998, 1951–1952.
- 118 S. Aleandri, M. G. Bonicelli, F. Bordini, S. Casciardi, M. Diociaiuti, L. Giansanti, F. Leonelli, G. Mancini, G. Perrone and S. Sennato, *Soft Matter*, 2012, **8**, 5904–5915.
- 119 M. Damen, J. Aarbiou, S. F. M. van Dongen, R. M. Buijs-Offerman, P. P. Spijkers, M. van den Heuvel, K. Kvashnina, R. J. M. Nolte, B. J. Scholte and M. C. Feiters, *J. Controlled Release*, 2010, **145**, 33–39.
- 120 A. J. Chicco and G. C. Sparagna, *Am. J. Physiol.*, 2007, **292**, C33–C44.

- 121 K. Kasireddy, S. M. Ali, M. U. Ahmad, S. Choudhury, P.-Y. Chien, S. Sheikh and I. Ahmad, *Bioorg. Chem.*, 2005, **33**, 345–362.
- 122 P.-Y. Chien, J. Wang, D. Carbonaro, S. Lei, B. Miller, S. Sheikh, S. M. Ali, M. U. Ahmad and I. Ahmad, *Cancer Gene Ther.*, 2005, **12**, 321–328.
- 123 H. S. Rosenzweig, V. A. Rakhmanova and R. C. MacDonald, *Bioconjugate Chem.*, 2001, **12**, 258–263.
- 124 I. Badea, R. Verrall, M. Baca-Estrada, S. Tikoo, A. Rosenberg, P. Kumar and M. Foldvari, *J. Gene Med.*, 2005, **7**, 1200–1214.
- 125 P. Pinnaduwaage, L. Schmitt and L. Huang, *Biochim. Biophys. Acta*, 1989, **985**, 33–37.
- 126 M. Muñoz-Úbeda, S. K. Misra, A. L. Barrán-Berdón, S. Datta, C. Aicart-Ramos, P. Castro-Hartmann, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *Biomacromolecules*, 2012, **13**, 3926–3937.
- 127 J. Gaucheron, T. Wong, K. F. Wong, N. Maurer and P. R. Cullis, *Bioconjugate Chem.*, 2002, **13**, 671–675.
- 128 E. Fiscaro, C. Compari, E. Duce, G. Donofrio, B. Rózycka-Roszak and E. Woźniak, *Biochim. Biophys. Acta*, 2005, **1722**, 224–233.
- 129 A. Bajaj, B. Paul, S. S. Indi, P. Kondaiah and S. Bhattacharya, *Bioconjugate Chem.*, 2007, **18**, 2144–2158.
- 130 A. Bajaj, P. Kondiah and S. Bhattacharya, *J. Med. Chem.*, 2007, **50**, 2432–2442.
- 131 J. Biswas, S. K. Mishra, P. Kondaiah and S. Bhattacharya, *Org. Biomol. Chem.*, 2011, **9**, 4600–4613.
- 132 K. Kumar, B. Maiti, P. Kondaiah and S. Bhattacharya, *Mol. Pharmaceutics*, 2015, **12**, 351–361.
- 133 K. Kumar, B. Maiti, P. Kondaiah and S. Bhattacharya, *Org. Biomol. Chem.*, 2015, **13**, 2444–2452.
- 134 S. D. Wettig, C. Wang, R. E. Verrall and M. Foldvari, *Phys. Chem. Chem. Phys.*, 2007, **9**, 871–877.
- 135 S. D. Wettig, I. Badea, M. Donkuru, R. E. Verrall and M. Foldvari, *J. Gene Med.*, 2007, **9**, 649–658.
- 136 M. Donkuru, S. D. Wettig, R. E. Verrall, I. Badea and M. Foldvari, *J. Mater. Chem.*, 2012, **22**, 6232–6244.
- 137 M. Hato, H. Minamikawa, K. Tamada, T. Baba and Y. Tanabe, *Adv. Colloid Interface Sci.*, 1999, **80**, 233–270.
- 138 H. Wang and S. D. Wettig, *Phys. Chem. Chem. Phys.*, 2011, **13**, 637–642.
- 139 H. Wang, T. Kaur, N. Tavakoli, J. Joseph and S. Wettig, *Phys. Chem. Chem. Phys.*, 2013, **15**, 20510–20516.
- 140 P. Yang, J. Singh, S. Wettig, M. Foldvari, R. E. Verrall and I. Badea, *Eur. J. Pharm. Biopharm.*, 2010, **75**, 311–320.
- 141 J. Singh, D. Michel, J. M. Chitanda, R. E. Verrall and I. Badea, *J. Nanobiotechnol.*, 2012, **10**, 7.
- 142 M. A. Al-Dulaymi, J. M. Chitanda, W. Mohammed-Saeid, H. Y. Araghi, R. E. Verrall, P. Grochulski and I. Badea, *AAPS J.*, 2016, **18**, 1168–1181.
- 143 A. L. Barrán-Berdón, S. K. Misra, S. Datta, M. Muñoz-Úbeda, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *J. Mater. Chem. B*, 2014, **2**, 4640–4652.
- 144 V. M. J. Säily, S. J. Ryhänen, H. Lankinen, P. Luciani, G. Mancini, M. J. Parry and P. K. J. Kinnunen, *Langmuir*, 2006, **22**, 956–962.
- 145 M. Martínez-Negro, A. L. Barrán-Berdón, C. Aicart-Ramos, M. L. Moyá, C. Tros de Ilarduya, E. Aicart and E. Junquera, *Colloids Surf., B*, 2018, **161**, 519–527.
- 146 A. Bajaj, P. Kondaiah and S. Bhattacharya, *Bioconjugate Chem.*, 2007, **18**, 1537–1546.
- 147 A. Bajaj, P. Kondaiah and S. Bhattacharya, *J. Med. Chem.*, 2008, **51**, 2533–2540.
- 148 G. Vulugundam, K. Kumar, P. Kondaiah and S. Bhattacharya, *Org. Biomol. Chem.*, 2015, **13**, 4310–4320.
- 149 N. L. Abbott, C. M. Jewell, M. E. Hays, Y. Kondo and D. M. Lynn, *J. Am. Chem. Soc.*, 2005, **127**, 11576–11577.
- 150 M. Hashida, M. Nikshikawa, F. Yamashita and Y. Takakura, *Adv. Drug Delivery Rev.*, 2001, **52**, 187–196.
- 151 Y. V. Mahidhar, M. Rajesh and A. Chaudhuri, *J. Med. Chem.*, 2004, **47**, 3938–3948.
- 152 M. L. Fielden, C. Perrin, A. Kremer, M. Bergsma, M. C. Stuart, P. Camilleri and J. B. F. N. Engberts, *Eur. J. Biochem.*, 2001, **268**, 1269–1279.
- 153 M. Johnsson, A. Wagenaar and J. B. Engberts, *J. Am. Chem. Soc.*, 2003, **125**, 757–760.
- 154 P. C. Bell, M. Bergsma, I. P. Dolbnya, W. Bras, M. C. A. Stuart, A. E. Rowan, M. C. Feiters and J. B. F. N. Engberts, *J. Am. Chem. Soc.*, 2003, **125**, 1551–1558.
- 155 L. Wasungu, M. Scarzello, G. Molema, G. van Dam, J. B. F. N. Engberts and D. Hoekstra, *J. Mol. Med.*, 2006, **84**, 774–784.
- 156 M. A. Maslov, T. O. Kabilova, I. A. Petukhov, N. G. Morozova, G. A. Serebrennikova, V. V. Vlassov and M. A. Zenkova, *J. Controlled Release*, 2012, **160**, 182–193.
- 157 P. A. Puchkov, E. V. Shmendel, A. S. Luneva, N. G. Morozova, M. A. Zenkova and M. A. Maslov, *Bioorg. Med. Chem. Lett.*, 2016, **26**, 5911–5915.
- 158 P. A. Puchkov, I. A. Kartashova, E. V. Shmendel, A. S. Luneva, N. G. Morozova, M. A. Zenkova and M. A. Maslov, *Bioorg. Med. Chem. Lett.*, 2017, **27**, 3284–3288.
- 159 H.-J. Wang, Y.-H. Liu, J. Zhang, Y. Zhang, Y. Xia and X.-Q. Yu, *Biomater. Sci.*, 2014, **2**, 1460–1470.
- 160 Y.-M. Zhang, Y.-H. Liu, J. Zhang, Q. Liu, Z. Huang and X.-Q. Yu, *RSC Adv.*, 2014, **4**, 44261–44268.
- 161 A.-X. Ding, Z.-L. Tan, Y.-D. Shi, L. Song, B. Gong and Z.-L. Lu, *ACS Appl. Mater. Interfaces*, 2017, **9**, 11546–11556.
- 162 J.-M. Ruysschaert, A. El Ouahabi, V. Willeaume, G. Huez, R. Fuks, M. Vandenbranden and P. Di Stefano, *Biochem. Biophys. Res. Commun.*, 1994, **203**, 1622–1628.
- 163 J. A. Heyes, D. Niculescu-Duvaz, R. G. Cooper and C. J. Springer, *J. Med. Chem.*, 2002, **45**, 99–114.
- 164 I. van der Woude, A. Wagenaar, A. A. Meekel, M. B. ter Beest, M. H. J. Ruiters, J. B. F. N. Engberts and D. Hoekstra, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 1160–1165.
- 165 R. Hulst, I. Muizebelt, P. Oosting, C. Van der Pol, A. Wagenaar, J. Šmisterová, E. Bulten, C. Driessen, D. Hoekstra and J. B. F. N. Engberts, *Eur. J. Org. Chem.*, 2004, **4**, 835–849.

- 166 M. A. Ilies, B. H. Johnson, F. Makori, A. Miller, W. A. Seitz, E. B. Thompson and A. T. Balaban, *Arch. Biochem. Biophys.*, 2005, **435**, 217–226.
- 167 M. Mével, G. Breuzard, J.-J. Yaouanc, J.-C. Clément, P. Lehn, C. Pichon, P.-A. Jaffrès and P. Midoux, *ChemBioChem*, 2008, **9**, 1462–1471.
- 168 M. Berchel, T. Le Gall, H. Couthon-Gourvès, J.-P. Haelters, T. Montier, P. Midoux, P. Lehn and P.-A. Jaffrès, *Biochimie*, 2012, **94**, 33–41.
- 169 M. Ilies, W. A. Seitz, B. H. Johnson, E. L. Ezell, A. L. Miller, E. B. Thompson and A. T. Balaban, *J. Med. Chem.*, 2006, **49**, 3872–3887.
- 170 U. Satyal, B. Draghici, L. L. Dragic, Q. Zhang, K. W. Norris, M. Madesh, E. Brailoiu and M. A. Ilies, *ACS Appl. Mater. Interfaces*, 2017, **9**, 29481–29495.
- 171 V. D. Sharma, E. O. Aifuwa, P. A. Heiney and M. A. Ilies, *Biomaterials*, 2013, **34**, 6906–6921.
- 172 V. D. Sharma, J. Lees, N. E. Hoffman, E. Brailoiu, M. Madesh, S. L. Wunder and M. A. Ilies, *Mol. Pharmaceutics*, 2014, **11**, 545–559.
- 173 V. D. Sharma and M. A. Ilies, *Med. Res. Rev.*, 2014, **34**, 1–44.
- 174 P. Quagliotto, G. Viscardi, C. Barolo, E. Barni, S. Bellinva, E. Fisicaro and C. Compari, *J. Org. Chem.*, 2003, **68**, 7651–7660.
- 175 E. Fisicaro, C. Compari, F. Bacciottini, L. Contardi, N. Barbero, G. Viscardi, P. Quagliotto, G. Donofrio, B. Różycka-Roszak, P. Misiak, E. Woźniak and F. Sansone, *J. Phys. Chem. B*, 2014, **118**, 13183–13191.
- 176 E. Fisicaro, C. Compari, F. Bacciottini, L. Contardi, E. Pongiluppi, N. Barbero, G. Viscardi, P. Quagliotto, G. Donofrio and M. P. Krafft, *J. Colloid Interface Sci.*, 2017, **487**, 182–191.
- 177 T. Zhou, A. Llizo, P. Li, C. Wang, Y. Guo, M. Ao, L. Bai, C. Wang, Y. Yang and G. Xu, *J. Phys. Chem. C*, 2013, **117**, 26573–26581.
- 178 S. K. Misra, M. Muñoz-Úbeda, S. Datta, A. L. Barrán-Berdón, C. Aicart-Ramos, P. Castro-Hartmann, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *Biomacromolecules*, 2013, **14**, 3951–3963.
- 179 M. Martínez-Negro, K. Kumar, A. L. Barrán-Berdón, S. Datta, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *ACS Appl. Mater. Interfaces*, 2016, **8**, 22113–22126.
- 180 C. Leal, N. F. Boussein, K. K. Ewert and C. R. Safinya, *J. Am. Chem. Soc.*, 2010, **132**, 16841–16847.
- 181 Y. Zhao, S. Zhang, S. Cui, B. Wang and S. Zhang, *Expert Opin. Drug Delivery*, 2012, **9**, 125–139.
- 182 S. Futaki, W. Ohashi, T. Suzuki, M. Niwa, S. Tanaka, K. Ueda, H. Harashima and Y. Sugiura, *Bioconjugate Chem.*, 2001, **12**, 1005–1011.
- 183 M. Castro, D. Griffiths, A. Patel, N. Patrick, C. Kitson and M. Ladlow, *Org. Biomol. Chem.*, 2004, **2**, 2814–2820.
- 184 P. Camilleri, A. Kremer, A. J. Edwards, K. H. Jennings, O. Jenkins, I. Marshall, C. McGregor, W. Neville, S. Q. Rice, R. J. Smith, M. J. Wilkinson and A. J. Kirby, *Chem. Commun.*, 2000, 1253–1254.
- 185 C. McGregor, C. Perrin, M. Monck, P. Camilleri and A. J. Kirby, *J. Am. Chem. Soc.*, 2001, **123**, 6215–6220.
- 186 M. Damen, E. Cristóbal-Lecina, G. Colom Sanmartí, S. F. M. van Dongen, C. L. García Rodríguez, I. P. Dolbnya, R. J. M. Nolte and M. C. Feiters, *Soft Matter*, 2014, **10**, 5702–5714.
- 187 Y. Zheng, Y. Guo, Y. Li, Y. Wu, L. Zhang and Z. Yang, *New J. Chem.*, 2014, **38**, 4952–4962.
- 188 X.-F. Ma, J. Sun, C. Qiu, Y.-F. Wu, Y. Zheng, M.-Z. Yu, X.-W. Pei, L. Wei, Y.-J. Niu, W.-H. Pang, Z.-J. Yang, J.-C. Wang and Q. Zhang, *J. Controlled Release*, 2016, **235**, 99–111.
- 189 X.-L. Wang, S. Ramusovic, T. Nguyen and Z.-R. Lu, *Bioconjugate Chem.*, 2007, **18**, 2169–2177.
- 190 D. L. Gillespie, M. T. Aguirre, S. Ravichandran, L. L. Leishman, C. Berrondo, J. T. Gamboa, L. Wang, R. King, X. Wang, M. Tan, A. Malamas, Z.-R. Lu and R. L. Jensen, *J. Neurosurg.*, 2015, **122**, 331–341.
- 191 A. S. Malamas, M. Gujrati, C. M. Kummitha, R. Xu and Z.-R. Lu, *J. Controlled Release*, 2013, **171**, 296–307.
- 192 X.-L. Wang, R. Xu and Z.-R. Lu, *J. Controlled Release*, 2009, **134**, 207–213.
- 193 M. Gujrati, A. Malamas, T. Shin, E. Jin, Y. Sun and Z.-R. Lu, *Mol. Pharmaceutics*, 2014, **11**, 2734–2744.
- 194 M. Gujrati, A. Vaidya and Z.-R. Lu, *Bioconjugate Chem.*, 2016, **27**, 19–35.
- 195 P. J. J. A. Buijnsters, C. L. García Rodríguez, E. L. Willighagen, N. A. J. M. Sommerdijk, A. Kremer, P. Camilleri, M. C. Feiters, R. J. M. Nolte and B. Zwanenburg, *Eur. J. Org. Chem.*, 2002, 1397–1406.
- 196 N. Wan, Y.-Y. Jia, Y.-L. Hou, X.-X. Ma, Y.-S. He, C. Li, S.-Y. Zhou and B.-L. Zhang, *Biol. Pharm. Bull.*, 2016, **39**, 1112–1120.
- 197 C. Bombelli, F. Faggioli, P. Luciani, G. Mancini and M. G. Sacco, *J. Med. Chem.*, 2005, **48**, 5378–5382.
- 198 E. Fortunati, E. Ehlert, N.-D. van Loo, C. Wyman, J. A. Eble, F. Grosveld and B. J. Scholte, *Gene Ther.*, 2000, **7**, 1505–1515.
- 199 R. Xu, X.-L. Wang and Z.-R. Lu, *Langmuir*, 2010, **26**, 13874–13882.
- 200 R. Xu, X.-L. Wang and Z.-R. Lu, *Huaxue Tongbao*, 2012, **57**, 3979–3984.
- 201 A. Akinc, A. Zumbuehl, M. Goldberg, El. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougères, J. R. Dorkin, K. N. Jayaprakash, M. Jayaraman, M. John, V. Kotliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Y. Sah, J. Soutschek, I. Toudjarska, H.-P. Vornlocher, T. S. Zimmermann, R. Langer and D. G. Anderson, *Nat. Biotechnol.*, 2008, **26**, 561–569.
- 202 K. P. Mahon, K. T. Love, K. A. Whitehead, J. Qin, A. Akinc, E. Leshchiner, I. Leshchiner, R. Langer and D. G. Anderson, *Bioconjugate Chem.*, 2010, **21**, 1448–1454.
- 203 K. A. Whitehead, G. Sahay, G. Z. Li, K. T. Love, C. A. Alabi, M. Ma, C. Zurenko, W. Querbes, R. S. Langer and D. G. Anderson, *Mol. Ther.*, 2011, **19**, 1688–1694.

- 204 M. Wang, K. Alberti, A. Varone, D. Pouli, I. Georgakoudi and Q. Xu, *Adv. Healthcare Mater.*, 2014, 3, 1398–1403.
- 205 K. A. Whitehead, J. R. Dorkin, A. J. Vegas, P. H. Chang, O. Veiseh, J. Matthews, O. S. Fenton, Y. Zhang, K. T. Olejnik, V. Yesilyurt, D. Chen, S. Barros, B. Klebanov, T. Novobrantseva, R. Langer and D. G. Anderson, *Nat. Commun.*, 2014, 5, 5277–5286.
- 206 R. L. Ball, C. M. Knapp and K. A. Whitehead, *PLoS One*, 2015, 10, e0133154.
- 207 R. L. Ball, P. Bajaj and K. A. Whitehead, *Sci. Rep.*, 2018, 8, 2178.
- 208 C. M. Knapp, J. He, J. Lister and K. A. Whitehead, *Exp. Biol. Med.*, 2016, 241, 1007–1013.
- 209 L. N. Kasiewicz and K. A. Whitehead, *Acta Biomater.*, 2016, 32, 120–128.
- 210 K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Y. Sah, A. de Fougères, K. Fitzgerald, V. Kotliansky, A. Akinc, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, 107, 1864–1869.
- 211 K. A. Whitehead, J. Matthews, P. H. Chang, F. Niroui, J. R. Dorkin, M. Severgnini and D. G. Anderson, *ACS Nano*, 2012, 6, 6922–6929.
- 212 G. Sahay, W. Querbes, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer and D. G. Anderson, *Nat. Biotechnol.*, 2013, 31, 653–658.
- 213 K. J. Kauffman, J. R. Dorkin, J. H. Yang, M. W. Heartlein, F. DeRosa, F. F. Mir, O. S. Fenton and D. G. Anderson, *Nano Lett.*, 2015, 15, 7300–7306.
- 214 Y. Dong, K. T. Love, J. R. Dorkin, S. Sirirungruang, Y. Zhang, D. Chen, R. L. Bogorad, H. Yin, Y. Chen, A. J. Vegas, C. A. Alabi, G. Shay, K. T. Olejnik, W. Wang, A. Schroeder, A. K. R. Lytton-Jean, D. J. Siegwart, A. Akinc, C. Barnes, S. A. Barros, M. Carioto, K. Fitzgerald, J. Hettinger, V. Kumar, T. I. Novobrantseva, J. Qin, W. Querbes, V. Kotlianski, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, 111, 3955–3960.
- 215 M. A. Oberli, A. M. Reichmuth, J. R. Dorkin, M. J. Mitchell, O. S. Fenton, A. Jaklenec, D. G. Anderson and R. Langer, *Nano Lett.*, 2017, 17, 1326–1335.
- 216 O. S. Fenton, K. J. Kauffman, R. L. McClellan, E. A. Appel, J. R. Dorkin, M. W. Tibbitt, M. W. Heartlein, F. DeRosa, R. Langer and D. G. Anderson, *Adv. Mater.*, 2016, 28, 2939–2943.
- 217 S. Sun, M. Wang, S. A. Knupp, Y. Soto-Feliciano, X. Hu, D. L. Kaplan, R. Langer, D. G. Anderson and Q. Xu, *Bioconjugate Chem.*, 2012, 23, 135–140.
- 218 Y. Dong, A. A. Eltoukhy, C. A. Alabi, O. F. Khan, O. Veiseh, J. R. Dorkin, S. Sirirungruang, H. Yin, B. C. Tang, J. M. Pelet, D. Chen, Z. Gu, Y. Xue, R. Langer and D. G. Anderson, *Adv. Healthcare Mater.*, 2014, 3, 1392–1397.
- 219 A. El-Sayed, S. Futaki and H. Harashima, *AAPS J.*, 2009, 11, 13–22.
- 220 A. P. Lam and D. A. Dean, *Gene Ther.*, 2010, 17, 439–447.
- 221 C. R. Safinya, K. K. Ewert, R. N. Majzoub and C. Leal, *New J. Chem.*, 2014, 38, 5164–5172.
- 222 M. Muñoz-Úbeda, S. K. Misra, A. L. Barrán-Berdón, C. Aicart-Ramos, M. B. Sierra, J. Biswas, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *J. Am. Chem. Soc.*, 2011, 133, 18014–18018.
- 223 H. Ragelle, F. Danhier, V. Préat, R. Langer and D. G. Anderson, *Expert Opin. Drug Delivery*, 2017, 14, 851–864.
- 224 J. S. Elborn, *Lancet*, 2016, 388, 2519–2531.
- 225 M. Conese, F. Ascenzioni, A. C. Boyd, C. Coutelle, I. De Fino, S. De Smedt, J. Rejman, J. Rosenecker, D. Schindelhauer and B. J. Scholte, *J. Cystic Fibrosis*, 2011, 10(Suppl. 2), S114–S128.
- 226 E. W. F. W. Alton, *et al.*, *Lancet Respir. Med.*, 2015, 3, 684–691.
- 227 E. R. Lee, J. Marshall, C. S. Siegel, C. W. Jiang, N. S. Yew, M. R. Nichols, J. B. Nietupski, R. J. Ziegler, M. B. Lane, K. X. Wang, N. C. Wan, R. K. Scheule, D. J. Harris, A. E. Smith and S. H. Cheng, *Hum. Gene Ther.*, 1996, 14, 1701–1717.
- 228 S. J. Eastman, J. D. Tousignant, M. J. Lukason, H. Murray, C. S. Siegel, P. Constantino, D. J. Harris, S. H. Cheng and R. K. Scheule, *Hum. Gene Ther.*, 1997, 8, 313–322.
- 229 K. S. Bosley, M. Botchan, A. L. Bredenoord, D. Carroll, R. A. Charo, E. Charpentier, R. Cohen, J. Corn, J. Doudna, G. Feng, H. T. Greely, R. Isasi, W. Ji, J.-S. Kim, B. Knoppers, E. Lanphier, J. Li, R. Lovell-Badge, G. S. Martin, J. Moreno, L. Naldini, M. Pera, A. C. F. Perry, J. C. Venter, F. Zhang and Q. Zhou, *Nat. Biotechnol.*, 2015, 33, 478–487.
- 230 Z. Glass, M. Lee, Y. Li and Q. Xu, *Trends Biotechnol.*, 2018, 36, 173–185.
- 231 H. Ma, N. Marti-Gutierrez, S.-W. Park, J. Wu, Y. Lee, K. Suzuki, A. Koski, D. Ji, T. Hayama, R. Ahmed, H. Darby, C. Van Dyken, Y. Li, E. Kang, A.-R. Park, D. Kim, S.-T. Kim, J. Gong, Y. Gu, X. Xu, D. Battaglia, S. A. Krieg, D. M. Lee, D. H. Wu, D. P. Wolf, S. B. Heitner, J. C. Izpisua Belmonte, P. Amato, J.-S. Kim, S. Kaul and S. Mitalipov, *Nature*, 2017, 548, 413–419.
- 232 C. Liu, L. Zhang, H. Liu and K. Cheng, *J. Controlled Release*, 2017, 266, 17–26.
- 233 M. Wang, Z. A. Glass and Q. Xu, *Gene Ther.*, 2017, 24, 144–150.
- 234 A. S. Timin, A. R. Muslimov, K. V. Lepik, O. S. Epifanovskaya, A. I. Shakirova, U. Mock, K. Riecken, M. V. Okilova, V. S. Sergeev, B. V. Afanasyev, B. Fehse and G. B. Sukhorukov, *Nanomed.: Nanotechnol., Biol. Med.*, 2018, 14, 97–108.
- 235 F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott and F. Zhang, *Nat. Protoc.*, 2013, 8, 2281–2308.
- 236 M. Wang, J. A. Zuris, F. Meng, H. Rees, S. Sun, P. Deng, Y. Han, X. Gao, D. Pouli, Q. Wu, I. Georgakoudi, D. R. Liu and Q. Xu, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, 113, 2868–2873.
- 237 C. Jiang, M. Mei, B. Li, X. Zhu, W. Zu, Y. Tian, Q. Wang, Y. Guo, Y. Dong and X. Tan, *Cell Res.*, 2017, 27, 440–443.