Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors

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Gene therapy research is still in trouble owing to a paucity of acceptable vector systems to deliver nucleic acids to patients for therapy. Viral vectors are efficient but may be too dangerous. Synthetic non-viral vectors are inherently safer but are currently not efficient enough to be clinically viable. The solution for gene therapy lies with improved synthetic non-viral vectors systems. This review is focused on synthetic cationic liposome/micelle-based non-viral vector systems and is a *critical review* written to illustrate the increasing importance of chemistry in gene therapy research. This review should be of primary interest to synthetic chemists and biomedical researchers keen to appreciate emerging technologies, but also to biological scientists who remain to be convinced about the relevance of chemistry to biology. (209 references.)

1. Introduction

Gene therapy may be described as the use of genes as medicines to treat disease, or more precisely as the delivery of nucleic acids by means of a vector to patients for some therapeutic purpose. Gene therapy is a therapeutic modality with enormous promise, but one that has to date failed regrettably to deliver much of therapeutic significance in spite of all the clinical interest.[1] The primary reason for current failure and the ensuing frustration in the field is the inadequacy of vectors used to deliver therapeutic nucleic acids to their desired site of action in cells of the target organs of choice. Researchers have been seduced by the apparent simplicity of gene therapy approaches to treatment leading to a drive for clinical applications before vector technologies have been adequately developed or understood. Predictably, there has been a dramatic loss of confidence in gene therapy research in recent times matched by a decline in scientific and public perceptions of gene therapy. This is unhelpful, gene therapy retains all future promise but there now needs to be a period of patient, logical technical and scientific development of new vector systems prior to any major second round of clinical trial activity.[1] This process is ongoing.

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1.1 Viral or non-viral?

Which type of new vector system should be most appropriate to develop, viral or non-viral, synthetic or physical? In our view, synthetic non-viral vector systems represent the only realistic choice for routine *in vivo* applications and gene therapy in the future. Synthetic non-viral vector systems have many potential advantages compared with viral systems, including significantly lower toxicity/immunogenicity and potential for oncogenicity, size independent delivery of nucleic acids (from oligonucleotides to artificial chromosomes), simpler quality control, and substantially easier pharmaceutical and regulatory requirements. Increasing public alarm particularly with viral vectors may also be strengthening these significant advantages. Ever present in the minds of the public and regulators is the potential for toxic side effects from the use of viral vectors. Therefore, basic clinical confidence in non-viral vectors is growing and the various advantages listed above inherent in synthetic non-viral vector systems should ensure substantial clinical uptake once the science and technology of these vector systems can be appropriately matured for routine clinical use. In our opinion, recent advances suggest that this process of maturation is now progressing with pace. Appropriate synthetic non-viral vector systems for *in vivo* applications and gene therapy should not now be far off in coming, and for reasons of lower toxicity if for nothing else, synthetic systems making use of cationic lipids (cytofectins) and liposomes should be paramount.

1.2 Barriers to effective nucleic acid delivery

1.2.1 Extracellular barriers. Much of our understanding of the extra- and intra-cellular barriers to nucleic acid delivery experienced by synthetic non-viral vectors has come from studying the behaviour of simple cationic liposome/micelle–DNA complexes (lipoplex, LD) *in vitro* and *in vivo*. Such simple systems have acted as trail-blazers, illuminating the potential problems and pitfalls that lie in the way of successful synthetic non-viral *in vivo* applications and gene therapy. Of primary significance, is the problem of instability in biological fluids with respect to aggregation and decomposition. LD particles are typically highly unstable in biological fluids (*e.g.*, high salt and serum) (Fig. 1). *In vivo* topical lung delivery is beset by problems from mucus,[2] intravenous (*i.v.*) and intra-arterial (*i.a.*) delivery by serum components such as acidic serum albumin proteins, low-density lipoprotein, macroglobulins and other small molecular weight components.[3–8] Hydrophobic, negatively charged proteins such as serum albumin associate with LD particles and inhibit direct cellular uptake, as well as opsonize complexes for reticulo-endothelial system (RES) scavenging.[8] Small molecular weight lipids like oleic acid and large glycosides like heparin also disrupt LD structural integrity by displacing nucleic acids and lipid components leading to heavily impaired transfection efficiency.[8] LD particles can even activate complement[9] and bacterially derived plasmid DNA (pDNA), most frequently used to prepare LD particles, also appears to be immunogenic eliciting immune responses from unmethylated-CpG islets.[10–13] Even cationic lipids themselves now appear vulnerable to innate immune system surveillance.[14]

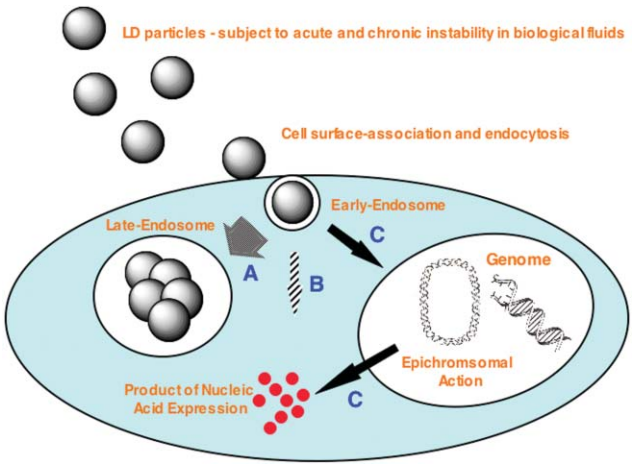


Fig. 1 Diagram to show process of LD particle cell entry. LD particles that have not succumbed to aggregation and/or serum-inactivation associate with the cell surface and enter usually by endocytosis. The majority in early endosomes become trapped in late endosomes (Path A) and the nucleic acids fail to reach the cytosol. A minority of are able to release their bound nucleic acids into the cytosol. Path B is followed by RNA that acts directly in the cytosol. Path C is followed by DNA that enters the nucleus in order to act. The diagram is drawn making the assumption that plasmid DNA has been delivered which is expressed in an epichromosomal manner. Reproduced with the permission of Bios Scientific Publications.[21]

These myriad problems ensure that LD particles, especially when delivered *i.v.*, have very short circulation times (<minutes) in biological fluids. A direct consequence of short circulation times is the classic first pass effect. After *i.v.*-injection of LD complexes, gene expression in the lung is typically 100-fold greater then in other organs such as the liver or spleen.[15] This is mainly due to the fact that the pulmonary circulation is the first capillary bed that LD complexes will encounter post-injection and enlarged serum-disrupted LD complexes will readily deposit in this lung microvasculature[15–17] possibly anchored by association with heparin proteoglycans on the pulmonary endothelial surface.[18] Cationic polymer–DNA (polyplex, PD) particles are also vulnerable in similar ways. Accordingly, any synthetic non-viral vector system that is intended to be viable for *in vivo* applications or gene therapy must at the very least be equipped with the capacity to evade these extracellular hazards and reach the desired cells in the target organ of choice.

These extracellular barriers discussed in this section of the review are far from exhaustive and are primarily valid as long as synthetic non-viral vector systems are involved in local delivery applications *in vivo* to lung, peritoneal cavity, vascular system or main filtration organs such as the liver. For systemic delivery to other organs including tumours, the circulatory barriers described above are just the beginning and significant issues concerning tissue penetration, cell organisation, and access to cells of interest through the extracellular matrix may become very significant.

1.2.2 Intracellular barriers. Assuming that a synthetic non-viral vectors system can survive to reach the target cells of interest, further intracellular barriers await. The mechanism of

cationic liposome/micelle-mediated nucleic acid delivery appears to be as follows; nanometric LD particles formed from the combination of cationic liposomes/micelles with nucleic acids in buffered, aqueous solution, enter cells by endocytosis triggered through non-specific interactions between complexes and the cell-surface proteoglycans of adherent cells (Fig. 1). Once inside, the pH of the endosome compartments drops from pH7 to 5.5 and a proportion of the bound nucleic acids escapes from early-endosomes into the cytosol to perform a therapeutic function there as in the case of RNA [Path B; (Fig. 1)], or else traffic from cytosol to the nucleus in order to perform a function there instead, as in the case of DNA [Path C; (Fig. 1)]. This process is surprisingly inefficient and every step of the delivery process is problematic.[19–21]

LD particles are not cell-type specific, they may be slow to enter cells (hours), are prone to endosome entrapment, and appear only to be weak facilitors of DNA entry into the cell nucleus. Tseng *et al.*[22] have provided convincing evidence that DNA entry within the nuclear envelope is impossible without the intervention of M-phase in the cell cycle when the nuclear membrane is partially dismantled to allow mitosis and cell division to take place. Nuclear-pore complexes appear unable to support facile entry of large pDNA into the nucleus. Indeed, the complexity of these pore complexes is only now being fully appreciated and access to the nuclear volume *via* these pore complexes should be regarded as one of the most severe barriers to effective DNA delivery to cells.[23–27] A further barrier to efficient DNA delivery appears to be the vulnerability of exogeneous DNA to digestion by cytosolic nucleases once DNA has escaped into the cytosol from early endosome compartments.[28,29] Studies involving fluorescence correlation spectroscopy have also revealed pDNA to bind extensively to immobile, cellular obstacles (cytoskeleton) in the cytosol thereby severely impeding intracellular migration of DNA towards the nucleus.[30,31]

Clearly, similar barriers are perceived for PD particles as well. Hence, any synthetic non-viral vector system that is intended to be viable for *in vivo* applications or gene therapy must at the very least be equipped with the capacity for rapid endosomal uptake followed by efficient endosmolysis, cytosolic trafficking and nuclear entry. Obviously, efficient nuclear entry is only required for DNA but not if RNA is involved. Conceivably, benefits could be had alternatively by avoiding endocytosis altogether and harnessing alternative cellular uptake mechanisms. However, this may well depend upon both vector characteristics and the nature of cells in the organs of choice that have been selected for nucleic acid delivery.

1.2.3 Formulation barriers. Curiously, one of the most important barriers to effective nucleic acid delivery is that of formulation. LD particles formed from cationic liposome/micelles and nucleic acids have been found typically difficult to formulate in a reproducible and scalable manner. Furthermore, they are susceptible to aggregation (in low ionic strength medium), are difficult to store long-term and do not as a consequence mediate reproducible nucleic acid delivery even *in vitro* and *ex vivo*. This formulation barrier cannot be underestimated. Any synthetic non-viral vector system that is intended for *in vivo* applications or gene therapy must be amenable to reproducible and scalable formulation with the nucleic acid of choice rendering particles that are both discrete, nanometric in dimension ($\leqslant$120 nm in diameter), and essentially single-size, mono-disperse in character. Furthermore, the capacity for long-term storage preferably without the requirement for refrigeration is indispensable as well. Should any of these characteristics be ignored or overlooked in the development of new synthetic, non-viral vector systems, then these systems are unlikely to satisfy increasingly stringent regulatory requirements for gene therapy clinical trials and cannot be expected to be of much use in other *in vivo* applications either. Recent research, our own included, has amply demonstrated that any attempts at systematic improvements of synthetic non-viral vector systems are destined to be fruitless unless the most fundamental problems associated with achieving reproducible and scalable formulations, resistance to aggregation, long term storage and properly reproducible transfection outcomes are convincingly solved prior to future attempts at systematic improvements.

2. ABCD nanoparticles

In the light of the foregoing discussion and given the numerous permutations of synthetic non-viral vector systems that have been developed over the last few years, there is a need to find a common language with which to discuss and appreciate these systems in a framework that allows us to relate different, individual systems and hence derive meaningful and realistic structure–activity correlations. Therefore, we would like to introduce the self-asscmbly **ABCD** nanoparticle concept as an appropriate structural paradigm for synthetic non-viral vector systems used for *in vitro, ex vivo* and/or *in vivo* applications (Fig. 2). In **ABCD** nanoparticles, nucleic acids (**A**) are condensed within functional concentric layers of chemical components designed for biological targeting (**D**), biological stability (**C**) and cellular entry/intracellular trafficking (**B**). For the purposes of this review, the **AB** core particle comprises nucleic acids (either DNA or RNA) (**A**) condensed and/or encapsulated by liposomes/micelles (**B**) in a non-covalent manner. Typically DNA may be in the form of pDNA or oligodeoxynucleotide (ODN) and RNA could be in the form

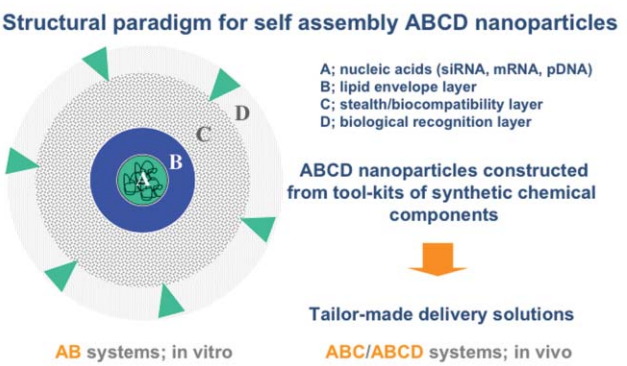


Fig. 2 ABCD nanoparticle concept. Graphic illustration of **ABCD** nanoparticle structure to show how nucleic acids (**A**) are condensed in functional concentric layers of chemical components purpose designed for biological targeting (**D**), biological stability (**C**), and cellular entry (**B**). Reproduced with the kind permission of Elsevier Academic Press.[209]

of messenger RNA (mRNA), oligonucleotide (ON) or small interference RNA (siRNA) for instance. **AB** core particles in our new nomenclature can be equivalent to LD (or even PD) particles and so should be expected to be appropriate for functional delivery of nucleic acids *in vitro*, perhaps *ex vivo* with limited applications *in vivo*.

For *in vivo* use, a stealth/biocompatibility polymer (**C**-layer) should be required that needs to be introduced by attachment to the surface of each **AB** core particle thereby conferring colloidal and structural integrity to **AB** core particles in biological fluids. Finally, biological-targeting ligands may be required as part of an optional exterior coating (**D**-layer) designed for the active targeting of nanoparticles firstly to the organ of choice *in vivo* but preferably to target cells of interest within the organ of choice. The requirement for biological-targeting ligands may not be obligatory. There are viable **ABC** nanoparticle systems that enter organs of choice *in vivo* by a process of passive targeting, namely organ/tissue accumulation through biophysical means without the need for active biological-targeting ligands. However, active biological-targeting processes are expected to subvert passive-targeting processes and ''reprogram'' nanoparticle systems to accumulate in alternative organs of choice with more precision, speed and efficiency than passive targeting processes will allow.

3. AB core particles

3.1 Cytofectins

AB core particles are equivalent to LD particles or other such particles generated by the condensation and/or encapsulation of nucleic acids. By far the majority of **AB** core particles have been generated by the combination of simple cationic liposome/micelle systems with pDNA. Simple cationic liposome/micelle systems are formed from either a single synthetic cationic amphiphile (known as a cytofectin; *cyto-* for cell and *-fectin* for transfection [*i.e.*, gene delivery and expression]) or more commonly from the combination of a cytofectin and a neutral lipid such as dioleoyl L-α-phosphatidylethanolamine (DOPE) **1** or cholesterol (Chol) **2** (Fig. 3). There are impressive numbers of cytofectins already described in the literature and available commercially[19,20,32–34] but all have in common a hydrophobic moiety covalently attached to a hydrophilic moiety through a polar linker (Fig. 3). Whilst hydrophobic regions are reasonably similar, polar linkers and cationic head groups vary quite substantially. The structures of a number of cytofectins are shown illustrating the structural diversity that is tolerated without necessarily impairing the efficiency of transfection (Fig. 3)! The cytofectin field now appears to be approaching saturation so that the creation of further novel structures is now much less likely to make a novel contribution unless their preparation is associated with the onward generation of **ABC** and **ABCD** nanoparticle systems for *in vivo* applications.

Nevertheless, there are a small number of recent additions to the cytofectin-pantheon that are worth mentioning. N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) **9** (Fig. 3) syntheses and properties have been described a number of times.[35–37] However, unusually CDAN/DOPE cationic liposomes (1 : 1 m/m, Trojene™) have now been found to mediate very high efficiency pDNA delivery to cells *in vitro* in the presence of cell growth medium, hence allowing for mininum-handling transfection protocols to be developed.[38] Furthermore, CDAN/DOPE cationic liposomes (45 : 55 m/m, siFECTamine®) have also been shown to facilitate the delivery of siRNA *in vitro* to cells with even more effect, as shall also be described later in more detail.[39] For this reason, an attractive new solid phase methodology was very recently devised for the synthesis of CDAN in excellent yield that represents an effective synthesis of this increasingly critical cytofectin[40] (Scheme 1). Balaban and coworkers,[41,42] have reported functional solution-phase syntheses of novel pyridinium amphiphiles that enlarge on the contributions of others in this important area of cytofectin design,[43,44] that is also growing in importance (Scheme 2). Multipurpose Gemini surfactants have also found their way into cytofectins[45,46] (Scheme 3). Finally, one of the most innovative new cytofectins to emerge has been from Thompson and coworkers. They developed *O*-(2*R*-1,2-di-*O*-(1′*Z*,9′*Z*-octadecadienyl)-glycerol)-*N*-(bis-2-aminoethyl)-carbamate (BCAT) based on plasmenylcholine synthesis, whose enol ether linkages are primed for acid-catalysed hydrolysis in conditions of acid pH[47–49] (Scheme 4). The notional design objective was to ensure that BCAT should mediate DNA delivery to cells. Thereafter, acid-catalysed BCAT decomposition was expected to take place in acidic endosome compartments leading to enhanced endosmolysis thereby increasing the proportion of bound nucleic acids able to escape from early-endosomes into the cytosol. This approach to the endosomal barrier problem (outlined above) appears to have been less effective than expected owing to the unexpectedly slow rate of enol-ether hydrolysis at pH 5.5. This is unfortunate, but Thompson and coworkers are already at work innovating the next generation of acid-sensitive functional groups.

3.2 Characteristics of LD particles

Typically, cytofectin and neutral lipid components are mixed together in an appropriate mol ratio and then induced or formulated into unilammellar vesicles by any one of a number of methods including reverse phase evaporation (REV), dehydration–rehydration (DRV) and extrusion.[19,20,37] Alternatively, cytofectins may be assembled into micellar structures after being dispersed in water or aqueous organic solvents.[19,20] Unilamellar vesicles or micelles may then be combined with nucleic acids to form nanometric LD particles. Biophysical structure–activity studies designed to understand the structures of LD particles and their relationships to LD transfection efficiency have been numerous. Unfortunately, the diversity of cytofectin structures, LD particles and biological targets has resulted in considerable inconsistency in the results reported by the research groups concerned. For instance, LD mixtures with a positive/negative charge ratio higher than 1,[50,51] or close to 1[52–55] have been reported to be optimal for LD transfection *in vitro*. In direct contrast, LD mixtures with an overall positive/negative charge ratio of <1 appear to be optimal for LD transfections of COS-7 cells *in vitro* and even of Balb/c mice lungs *in vivo*.[36] These latter observations have been supported by the results of others too.[56]

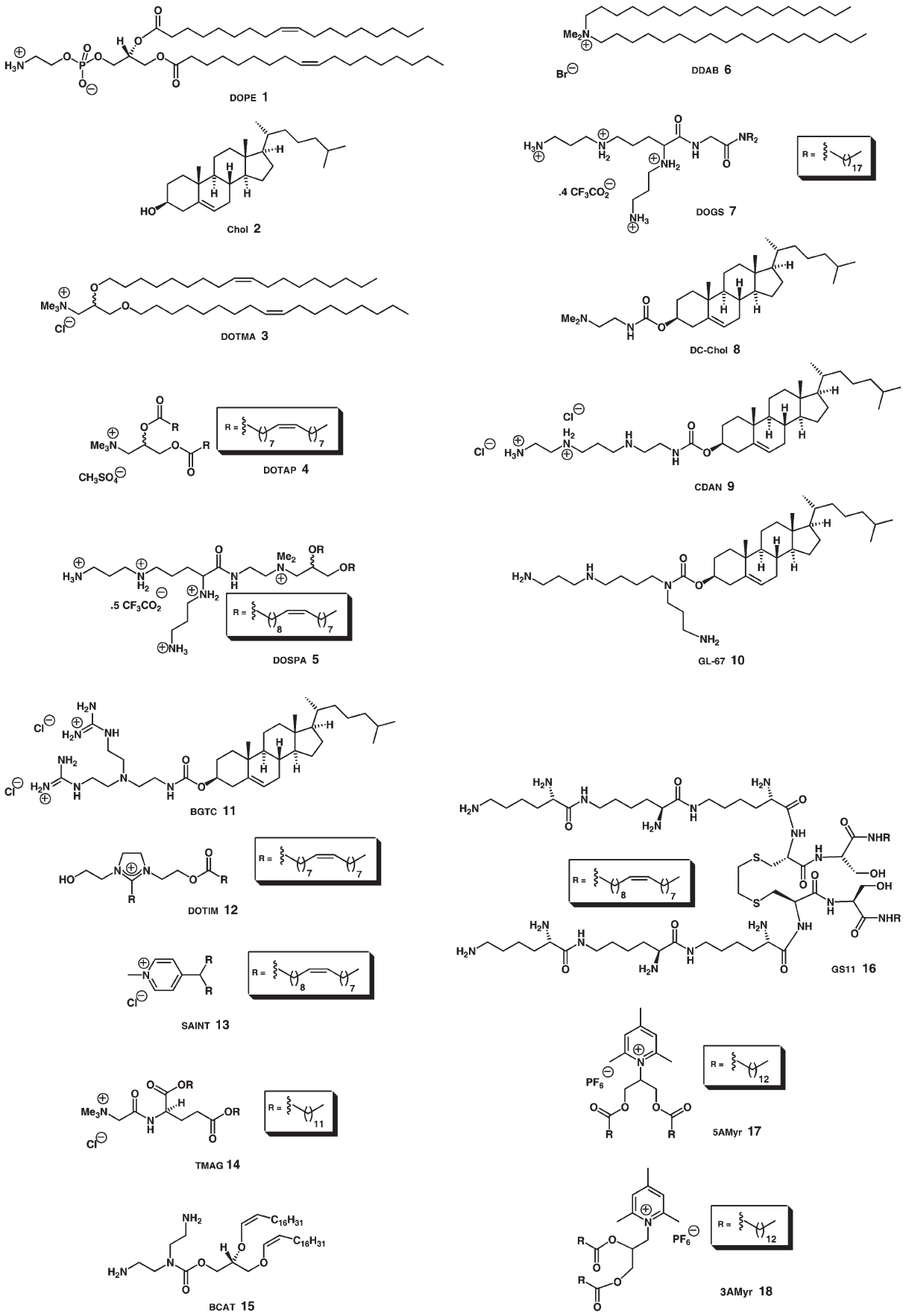
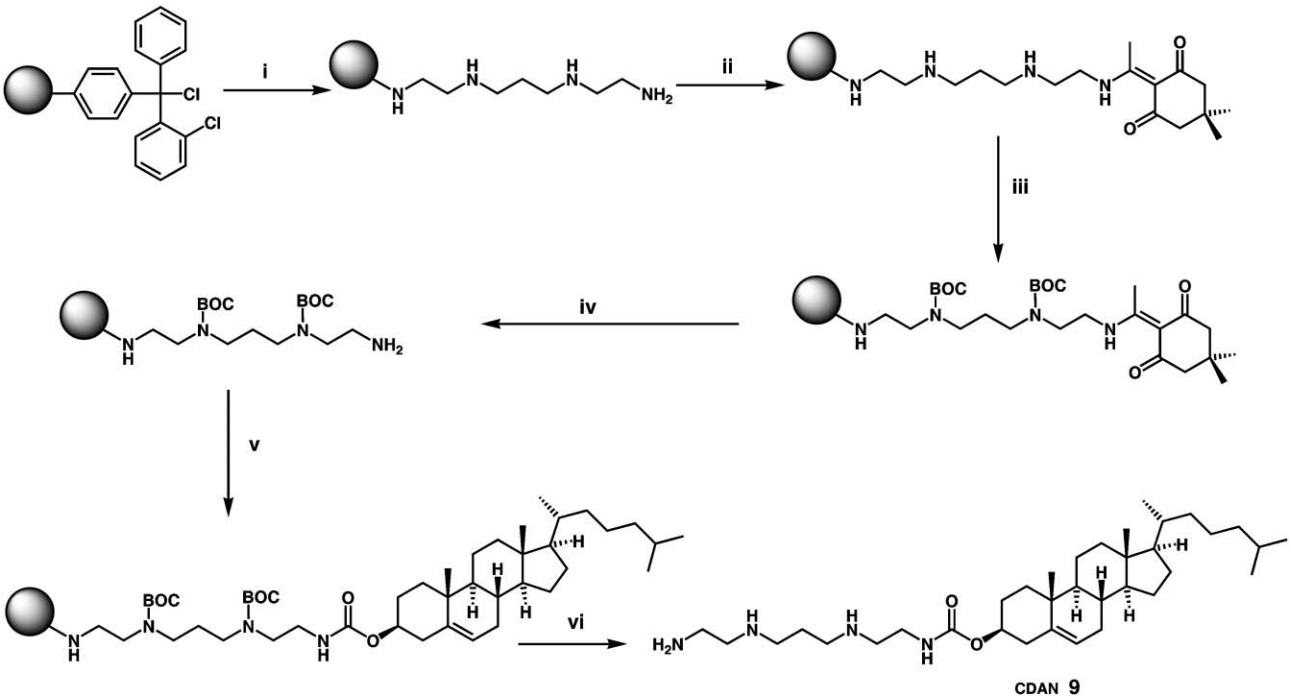
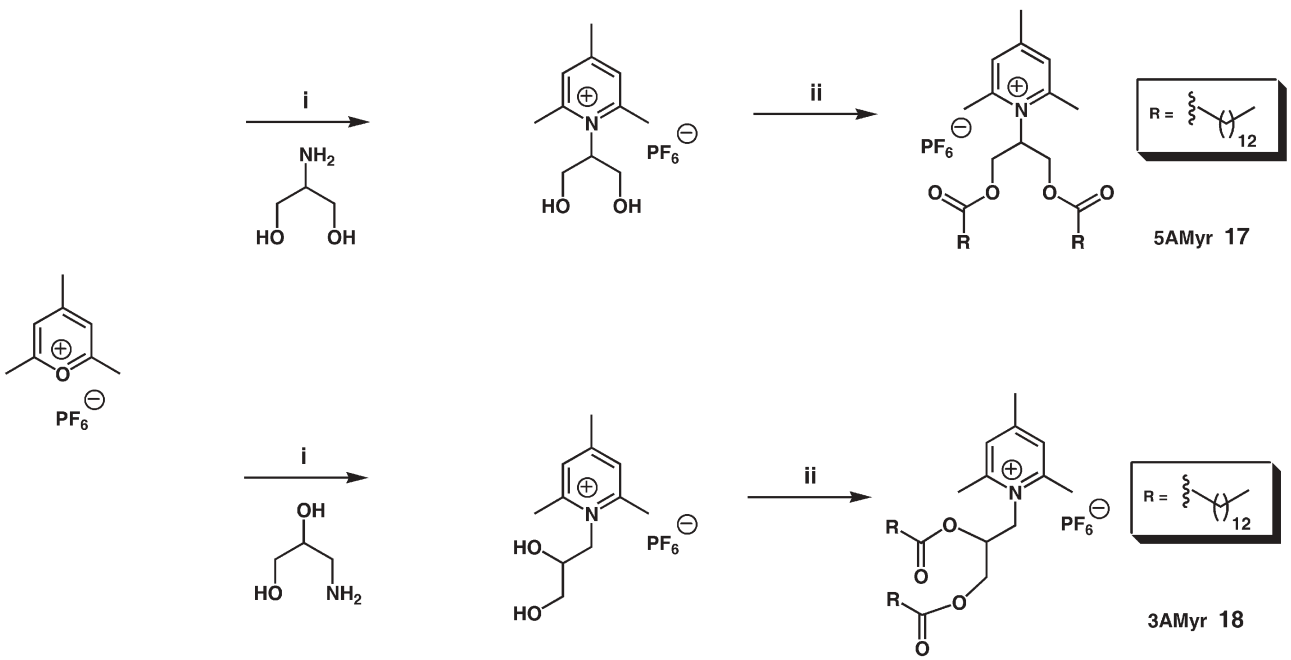


Fig. 3 Cytofectins and neutral lipids. Summary of important cytofectins and neutral lipids that are mentioned in the text.

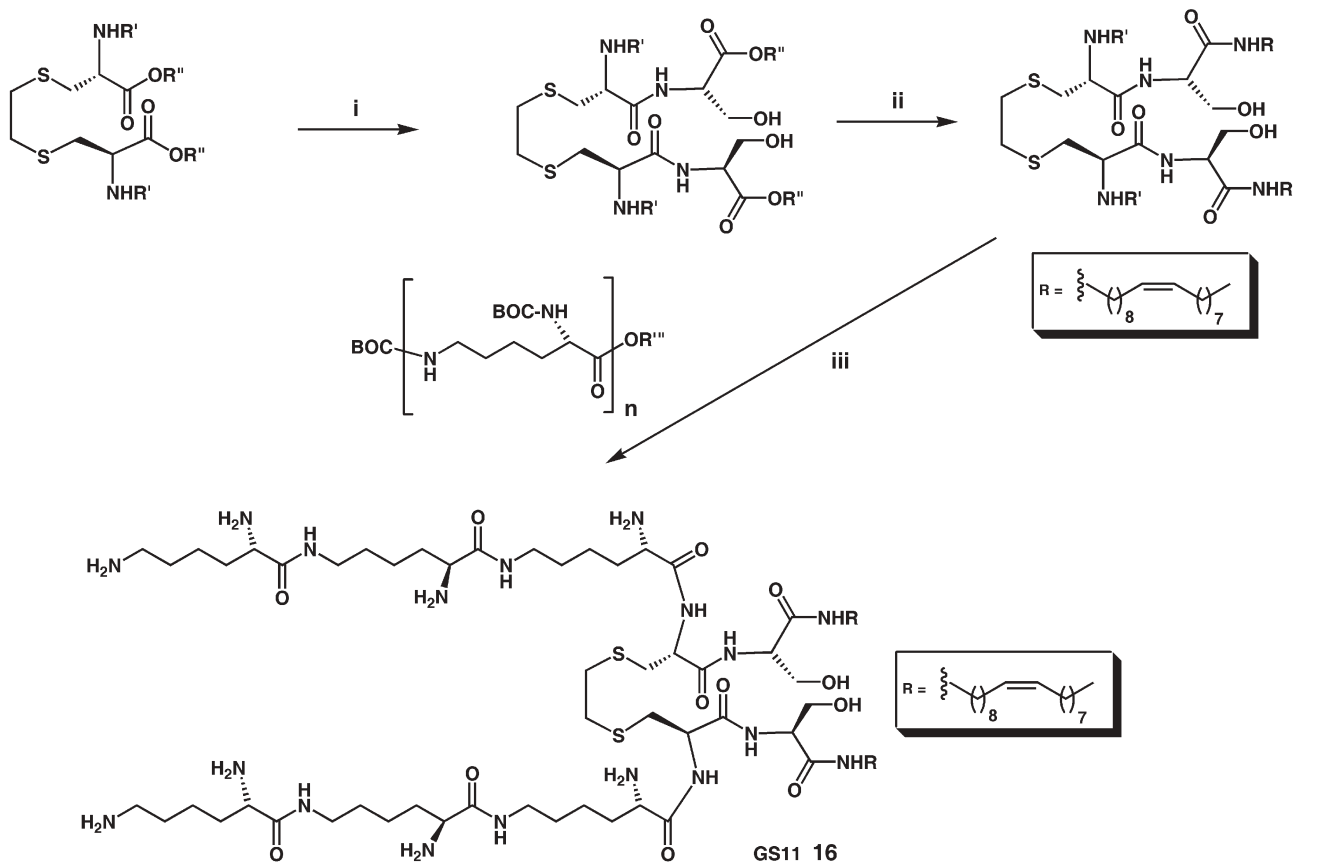


Scheme 1 *Reagents and conditions:* (i) polyamine (10 equiv.), CH_2Cl_2, rt, 2h, then MeOH (1000 equiv.), rt, 10 min; (ii) Dde-OH (10 equiv.), DMF, rt, 12 h; (iii) Boc_2O (5 equiv. per free amine), NEt_3 (2 equiv. per free amine), CH_2Cl_2, rt, 4 h; (iv) 2% hydrazine hydrate in DMF, rt, 10 min (rpt step × 2); (v) cholesterol chloroformate (10 equiv.), NEt_3 (3 equiv.), rt, 4 h; (vi) 50% TFA in CH_2Cl_2, rt, 1 h, 93%.[40]

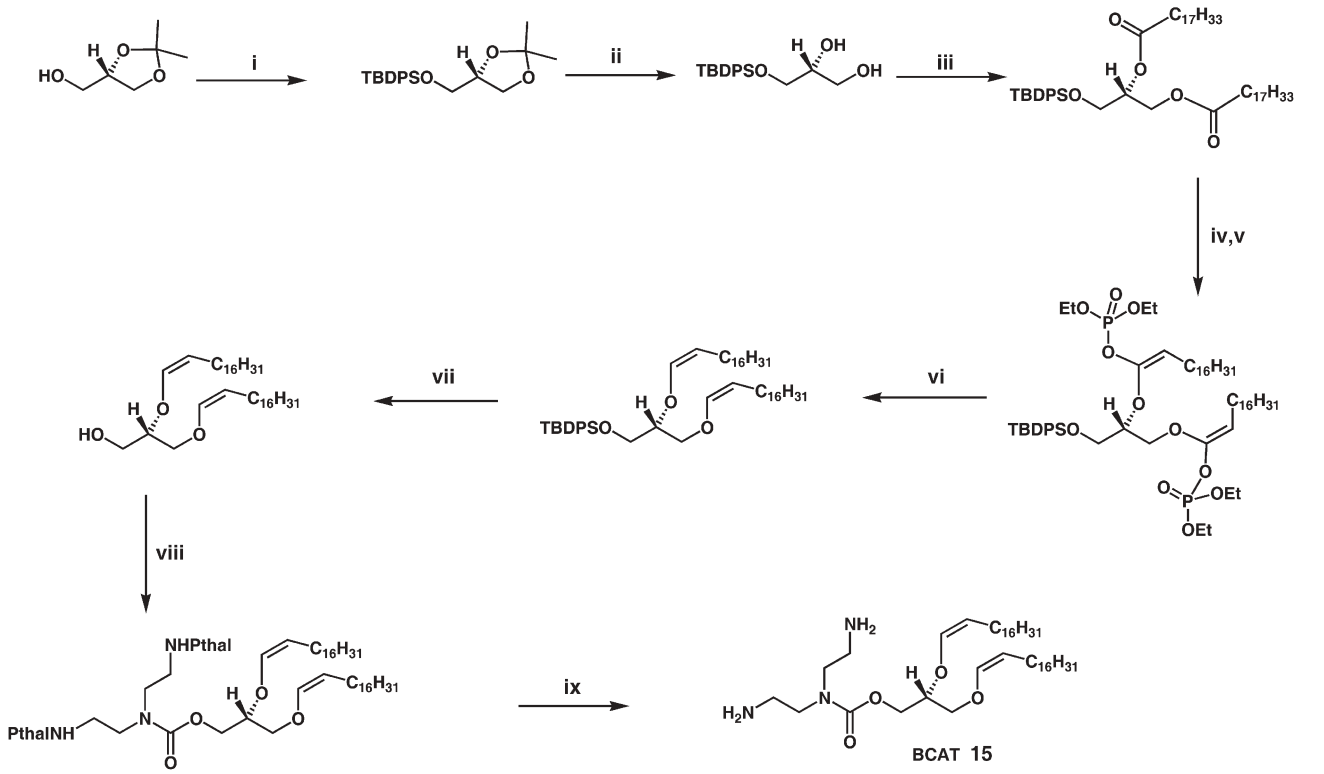
Similarly diverse views exist concerning the structures of LD mixtures optimal for transfection. In some circumstances, LD mixtures optimal for *in vitro* transfection appear to be heterogeneous and consist of a variety of particles and other structures all in dynamic equilibrium.[57,58] These particles and other structures have been variously identified and described by a number of researchers and they include multilamellar lipid/nucleic acid clusters (>100 nm in diameter)[59–63] perhaps with some surface associated nucleic acids,[64] or with thinly lipid-coated DNA nucleic acid strands[65] and even in the presence of free nucleic acids.[61] Such structural observations have led to a substantive debate concerning the relative



Scheme 2 *Reagents and conditions:* (i) NEt_3 (1.2 equiv.), EtOH/AcOH, 1–3 h, 50–85%; (ii) NEt_3 (2 equiv.), myristoyl-Cl (2.2 equiv.), AcCN, 3–5h, reflux.[42]



Scheme 3 *Reagents and conditions:* (i) L-serine (2 equiv.), K_2CO_3 (2 equiv.), THF/H_2O 1 : 1 v/v, rt, 72 h; (ii) (a) NHS (2 equiv.), DCC (2 equiv.), THF, rt, 24 h (b) NEt_3 (2 equiv.), oleylamine (2 equiv.), THF, rt, 48h; (iii) (a) BOC-protected L-K_3 (R‴ = *N*-succinimido) (>2 equiv.), H_2O/NaOH/THF, rt, 48 h (b) MeOH/HCl.[45]



Scheme 4 *Reagents and conditions:* (i) TBDPS-Cl, imidazole; (ii) HCl, MeOH; (iii) oleoyl-Cl, pyridine; (iv) LDA; (v) Et_2POCl, HMPA; (vi) $Pd(PPh_3)_4$, Et_3Al; (vii) TBAF, TBAH; (viii) (a) py_2CO, NEt_3 (b) *N*, *N*′-dipthalamidylethylenetriamine; (ix) N_2H_4–H_2O.[49]

importance of each of these structural entities for efficient *in vitro* transfection.

However, LD mixtures optimal for *in vitro* transfection do not necessarily have to be heterogeneous and substantially polydisperse. Our recent studies using cryo-electron microscopy have clearly demonstrated that LD mixtures optimal for *in vitro* and *in vivo* transfection may actually consist of discrete LD particles (size range; 60–250 nm in diameter) exhibiting bilamellar perimeters and striations with a periodicity of 4.2 ± 2 nm (Fig. 4).[36] Small-angle X-ray scattering (SAXS) and other cryo-electron microscopy studies of LD mixtures have revealed similar periodicities of approx. 6.5 and 3.5 nm that have been shown to result from the encapsulation of DNA molecules in regular periodic arrays within a multilamellar LD assembly[59,60,66–68] (Fig. 5). Therefore, the observed LD particles are most likely composed in a similar way. Hence in this case at least, optimal LD transfection *in vitro* and *in vivo* must be primarily mediated by these discrete, multilammellar LD particles.[36] The significance of discrete LD particles for optimal transfection has been supported by the results of at least one other published study comparing LD structure with *in vivo* transfection efficacy.[69] Evidence then suggests that the regular multilamellar bilayer structure ($L_{\alpha I}$) of LD particles should undergo a phase change in the endosome forming inverted hexagonal phase structures (H_{II}) that may disrupt endosome membranes and faciliate nucleic acid escape into the cytosol[70] (Fig. 5). Lipids like DOPE are well known to prefer H_{II} phases under physiological conditions of temperature and pH, and the $L_{\alpha I} \rightarrow H_{II}$ phase transition has been widely implicated as a key facilitator of membrane fusion and membrane disruption events. Hence the inclusion of DOPE in an LD system is likely to facilitate endosome escape of bound nucleic acids through induced $L_{\alpha I} \rightarrow H_{II}$ phase destabilisation.[71]

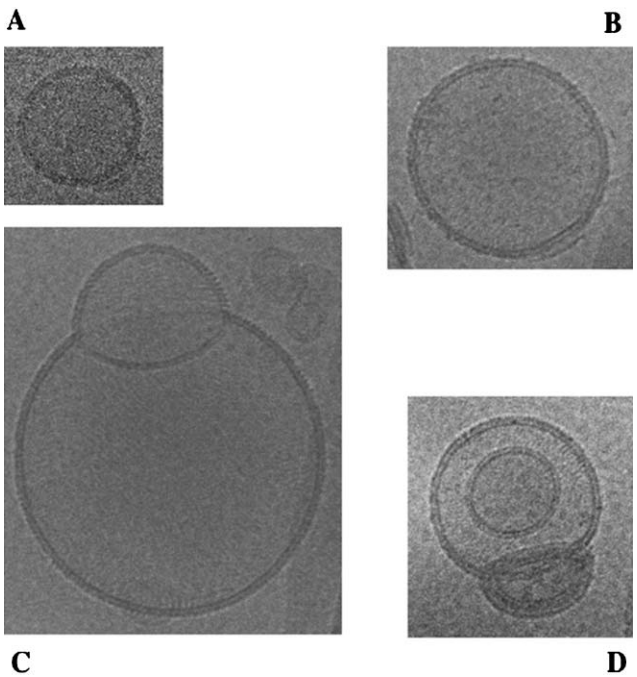


Fig. 4 Cryo-electron microscopy images of LD particles. These LD particles were formed after the combination of CDAN/DOPE cationic liposomes and pDNA in the [cytofectin]/[nucleotide] ([cyt]/[nt]) mol ratio of 0.6, optimal for *in vitro* and *in vivo* lung transfection. Final lipid concentration was 0.17 mM. Magnification is 150 000 × (1 cm = 67 nm).[36]

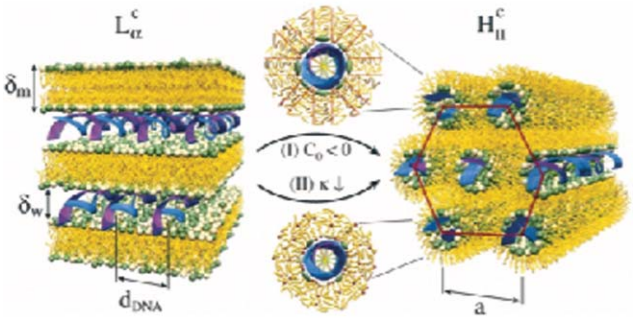


Fig. 5 LD particle internal structure and dynamics. *Left-hand side:* schematic of the lamellar $L_{\alpha I}$ phase of DNA molecules interacting with cationic bilayers forming a multilayered assembly typical of LD particle composition. DNA double helices are shown as ribbons (blue and purple), head groups of anionic/zwitterionic lipids are shown as white spheres while those of cytofectins are shown as grey spheres. The notation $\boldsymbol{\delta_m}$ refers to bilayer thickness, $\boldsymbol{\delta_w}$ to interbilayer separation and $\boldsymbol{d_{DNA}}$ to DNA interaxial spacing. *Right-hand side:* conversion from lamellar $L_{\alpha I}$ phase to the columnar, inverted hexagonal H_{II} phase thought to be typical of LD particle composition during the transfection process, takes place by two possible routes. The first involves pathway (**I**) typified by negative curvature $\boldsymbol{C_o}$ induced in each cationic monolayer due to the presence of DOPE **1**. The second involves pathway (**II**) typified by loss in membrane rigidity $\boldsymbol{\kappa}$ thereby encouraging phase inversion. Reproduced with permission from the American Association for the Advancement of Science.[70]

General biophysical structure–activity studies have generated few proper correlations between LD particles structure, physical attributes and transfection efficiency *in vitro*,[33] and in general there have been few attempts to derive unifying biophysical parameters able to account for differences in LD transfection efficiency *in vitro*, yet alone relate these parameters to *in vivo* transfection performance. One exception may be found in the work of Stewart *et al.*[36] wherein the physical properties of a systematic series of cationic liposomes and their corresponding LD mixtures were studied. Liposomes were formulated from DOPE **1** and cholesterol-based polyamine cytofectins such as CDAN **9** (Fig. 3). Successful *in vitro* transfection was linked to the ability of cationic liposome systems to (1) provide relatively inefficient neutralisation, condensation and encapsulation of nucleic acids into LD particles; and (2) present unprotonated amine functional groups ($pK_a < 8$) at neutral pH with the capacity for substantial endosome buffering, thereby enabling the osmotic shock mechanism to facilitate nucleic acid escape from endosome compartments as their internal pH is reduced from pH 7 to 5.5.[72,73] Critically, both main factors were observed to be under the control of the cytofectin polyamine head group structure. The inclusion of ''natural'' propylene and butylene spacings between the amine functional groups of head groups appeared to promote efficient neutralisation, condensation and encapsulation of nucleic acid. Inclusion of ''unnatural'' ethylene spacings appeared to promote the reverse effect although at the same time assisting the perturbation of amine pK_a values from 9–10 to below 7. The appearance of such

perturbed pK_a values now appears not only to be important to enable the osmotic shock mechanism for endosome escape but also to render LD particles metastable and prone to partial aggregation and sedimentation onto cell surfaces *in vitro*.[38] Such sedimentation is likely to be beneficial for transfection unless the given LD particles themselves induce cytotoxicity. Consistent with this observation, a correlation has also been established between transfection efficiency and enhanced membrane fluidity in both lipoplex and cellular membranes.[74]

3.3 Ternary LD particles

Ternary LD particles represent another form of **AB** nanoparticle system. In these systems the cationic character of the cationic liposome/micelle system used to condense and/or encapsulate nucleic acids is supplemented by an additional cationic entity. In the case of pDNA, this cationic entity is frequently used to precondense the pDNA prior to final condensation and encapsulation by cationic liposomes/micelles. This is particularly true of the lipid:protamine:DNA (LPD) system prepared using the salmon sperm-derived peptide protamine,[5,75–83] and the liposome:mu:DNA (LMD) system prepared from adenoviral derived peptide μ (mu).[37] Other cationic entities that have been used to condense nucleic acids prior to complexation with cationic liposomes/micelles include poly-L-lysine (pLL),[84,85] spermidine,[86] lipopolylysine,[87] histone proteins,[88] chromatin proteins,[89] human histone derived peptides,[90] L-lysine containing synthetic peptides,[91] not to mention a histidine/lysine (H-K) copolymer.[92] The formulation process of LMD is illustrated (Fig. 6). LPD particles may be prepared in a similar way.

Mature adenovirus consists of an icosahedral, non-enveloped capsid particle (approx. 90 nm) enclosing a core complex that consists of a linear dsDNA viral genome (~36 kbp) non-covalently associated with two cationic proteins (proteins V [pV] and VII [pVII]) and the 19-residue mu peptide.[93,94] Mechanistic studies using the mu peptide have revealed how increasing pDNA–peptide interactions lead to progressive base-pair-tilting generating regions of high and low double helical stability, that in turn promote super-coiling followed by pDNA hydrophobic collapse.[95,96] In kinetic terms, the process of pDNA condensation and the reverse process of pDNA expansion appear to be equivalent to small single domain protein folding and unfolding respectively.[96] Chaotic behavior is also observed at low peptide/pDNA ratios (0.1–0.3 w/w) that becomes more uniform at higher ratios suggesting that with suboptimal ratios, pDNA is condensing in a multitude of conformations, each representing different stages of hydrophobic collapse in the search for the thermodynamically most stable (*i.e.*, the fully condensed pDNA molecule). This represents yet another analogy with protein folding. At higher ratios, peptide/pDNA complexes formed appear to be increasingly irreversible consistent with the formation of kinetically and/or thermodynamically stable, condensed pDNA molecules.[96] Such stable states could create problems for the successful transcription of DNA post delivery to cells, yet another barrier to successful delivery of DNA to cells that is yet to be understood!

Both LPD and LMD systems are able to form discrete, essentially mono-disperse (single-size) particles. DOTAP/Chol-based LPD systems were even more effective and were found to formulate into discrete, essentially single-size particles

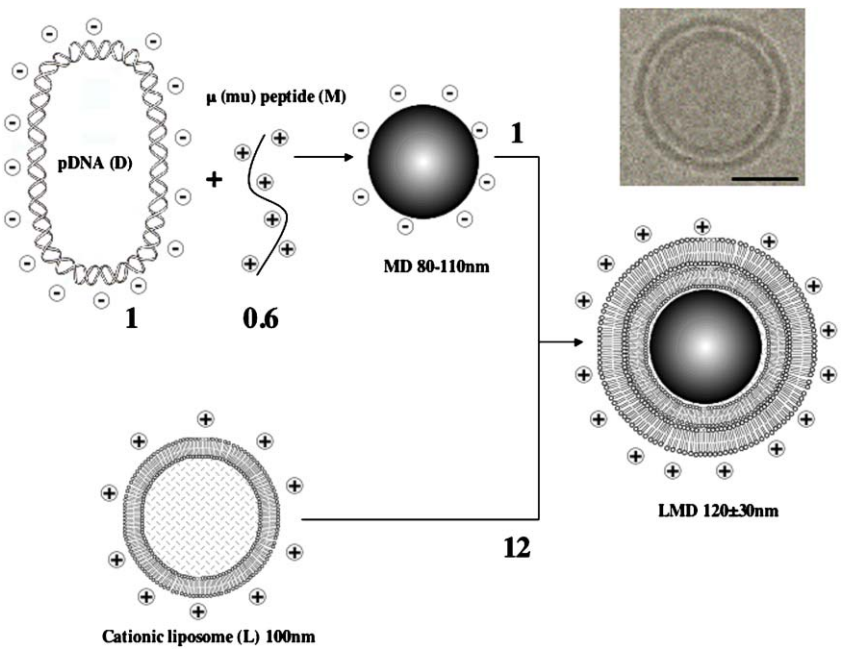


Fig. 6 LMD formulation. Schematic illustration of LMD particle formation. Initially, pDNA (D) is introduced under vortex mixing to mu peptide (M) in the ratio of 0.6:1 w/w forming MD particles. These themselves are then added under vortex mixing to cationic liposomes (L) in a ratio of 12:1 w/w with respect to pDNA, resulting in the formation of bilamellar LMD particles. *Inset:* cryo-electron microscopy image of LMD particle prepared with DC-Chol/DOPE cationic liposomes and pDNA (1 cm = 60 nm). Reproduced with the kind permission of Elsevier Academic Press.[37]

(approx 135 ± 42 nm).[77] DC-Chol/DOPE-based LMD systems were found to formulate into discrete single-size particles (approx. 120 ± 30 nm)[37] (Fig. 6). LMD particles can be formulated reproducibly that are amenable to long-term storage at −80 °C and stable up to a pDNA concentration of 5 mg ml^{-1} (nucleotide concentration 15 mM), a concentration appropriate for facile use *in vivo*.[37] Using LD systems, nucleotide concentrations >4 mM are difficult to achieve owing to ready LD particle aggregation above this concentration threshold.[35,36,97] Moreover, LMD transfections appear to be significantly more time and dose efficient *in vitro* than LD transfections. LMD transfection times as short as 10 min and DNA doses as low as 0.001 μg per well result in significant gene expression. Furthermore, LMD transfections will also take place in the presence of biological fluids (*e.g.*, up to 100% serum), conditions typically intractable to LD transfections, suggesting that LMD formulations exhibit an additional element of stability. In consequence, LMD transfection of murine lung *in vivo* was up to six-fold more dose efficient than transfection with GL-67/DOPE/DMPE-PEG^{5000} (1 : 2 : 0.05 m/m/m) (one of the best synthetic non-viral vector systems reported to date for lung transfection). LMD has been called an artificial virus-like nanoparticle (VNP) on the basis that cryo-electron microscopy shows LMD particles to consist of a mu:DNA (MD) particle encapsulated within a cationic bilamellar liposome (Fig. 6).

However, this additional element of LMD stability is unlikely to be adequate for general *in vivo* applications and gene therapy.[98] Indeed, corresponding LPD particles are readily modified by serum causing gradual vector disintegration, release of DNA and probable RES scavenging.[5,77] Released DNA is also noted to be susceptible to extracellular nuclease digestion. Furthermore, LPD particles have been found to promote a systemic, Th1-like innate immune response in mice, much more appropriate for a DNA vaccine than for gene therapy.[79] However, the general impression given is that LPD like LMD systems could have a role to play clinically for the passive delivery of genes to lung but are not appropriate for targeted gene delivery to other tissues.[5]

Studies carried out by confocal microscopy on dividing tracheal epithelial cells suggest that endocytosis is not a significant barrier to LMD transfection. However, the nuclear envelope remains a highly significant barrier. LMD particles were found to enter cells rapidly (minutes), and disintegrate almost immediately leaving mu peptide free to migrate to the nuclear zone (within 15 min) and pDNA to enter after a further 15–30 min. There is every possibility that both cytofectin and perhaps even mu peptide are exercising fusogenic behaviour with respect to early endosome membranes.[99–101] However, LMD does not appear to facilitate pDNA entry into the nucleus of growth arrested (aphidicolin-treated) cells suggesting that the nuclear pore complex remains a significant barrier to LMD transfection even though mu peptide has been shown to possess strong nuclear localisation sequence (NLS) characteristics.[102] The obvious solution is to ensure that mu peptide and pDNA remain in association for long enough within non-dividing (quiescent) cells for the DNA to utilise the NLS characteristics of the mu peptide to cross the nuclear membrane.[95,102] Evidence from DNA trafficking and expression studies using NLS peptides covalently or non-covalently associated with the pDNA appear to support this suggestion amply,[27,103] assisted by the presence of such elements as the SV40 enhancer in pDNA structure.[104]

Very recently, a new ternary LD system known as the multifunctional envelope-type nano-device (MEND) system was described.[105] The formulation process compares in an interesting way to the LMD and LPD systems involving a cationic DNA/polycation complex interacting with an anionic fusogenic lipid film prior to sonication into large but discrete particles (402 ± 73 nm) whose charge can be modified by the post-insertion of stearyl octa-arginine (STR-R_8) to give transfection competent particles (Fig. 7). Without doubt an imaginative, alternative way to arrive at condensed discrete particles. In the cases of LMD, LPD and perhaps MEND particles, these represent systems that can be formulated in a reproducible and scalable manner, that are resistant to aggregation in low ionic strength media, are amenable to long term storage and give properly reproducible transfection outcomes. Therefore, these are ideal platforms upon which to build viable lipid-based synthetic, non-viral vector systems for DNA delivery *in vivo* by a process of modular upgrading through systematic chemical adaptation with appropriate tool-kits of known or newly designed chemical components.

4 ABD particles

4.1 Synthetic ABD particles

Some fascinating examples of **ABD** particles have emerged in recent years notable for some *in vivo* viability although somewhat irregular in formulation. For instance, peptides consisting of an oligo-L-lysine moiety linked to a peptide moiety specific for cell surface integrin proteins have been combined with LD systems.[106–109] In the latter case, credible enhancements of at least an order of magnitude in *in vitro* transfection have been observed over and above the results of binary LD transfection owing to the involvement of integrin-mediated cell uptake.[107–109] Furthermore, enhancements to *in vivo* transfection have been reported as well, but the mechanism of these so-called lipid:integrin-targeting peptide:DNA (LID) systems does not actually appear to be integrin-receptor dependent in this case.[106]

Modular adaptation of LMD particles has arguably resulted in alternative **ABD** systems whose behaviour has given more clarity. A glyco-LMD variant was prepared by a *post-modification* strategy in which neoglycolipid micelles were combined with pre-formulated LMD particles (**AB** system) in order to encourage insertion of neoglycolipid molecules into the outer leaflet membranes of LMD particles using their hydrophobic lipid moieties. The syntheses of neoglycolipids is shown given the particular use of an aminoxy functional group to couple reducing sugars to the lipid moiety without the requirement for any protecting groups illustrating the high chemoselectivity of the coupling reaction (Scheme 5). This coupling reaction takes place in aqueous as well as organic solvents, ideal given the range of reducing sugars coupled.[98] The resulting glyo-LMD particles (**ABD** nanoparticles) were stable in high-salt medium (but not 100% serum) and mediated enhanced non-specific transfection of cells *in vitro*.[98]

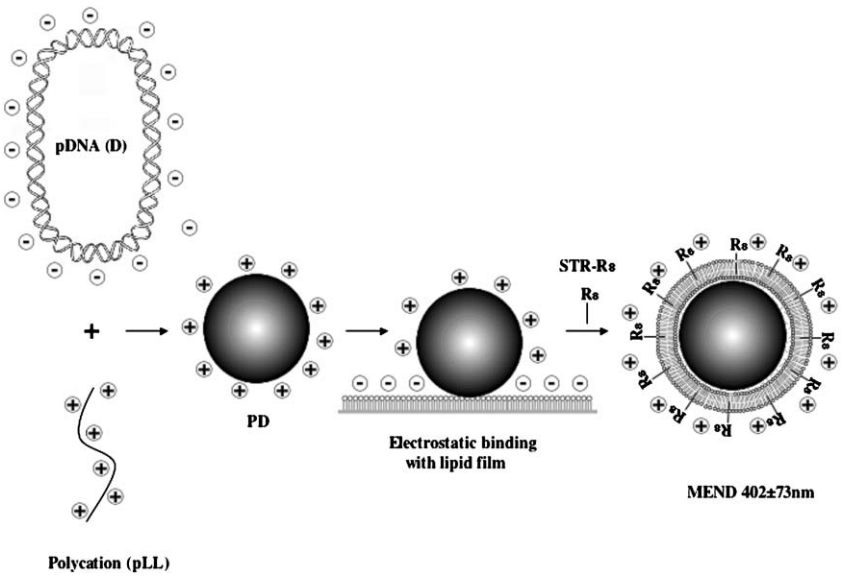
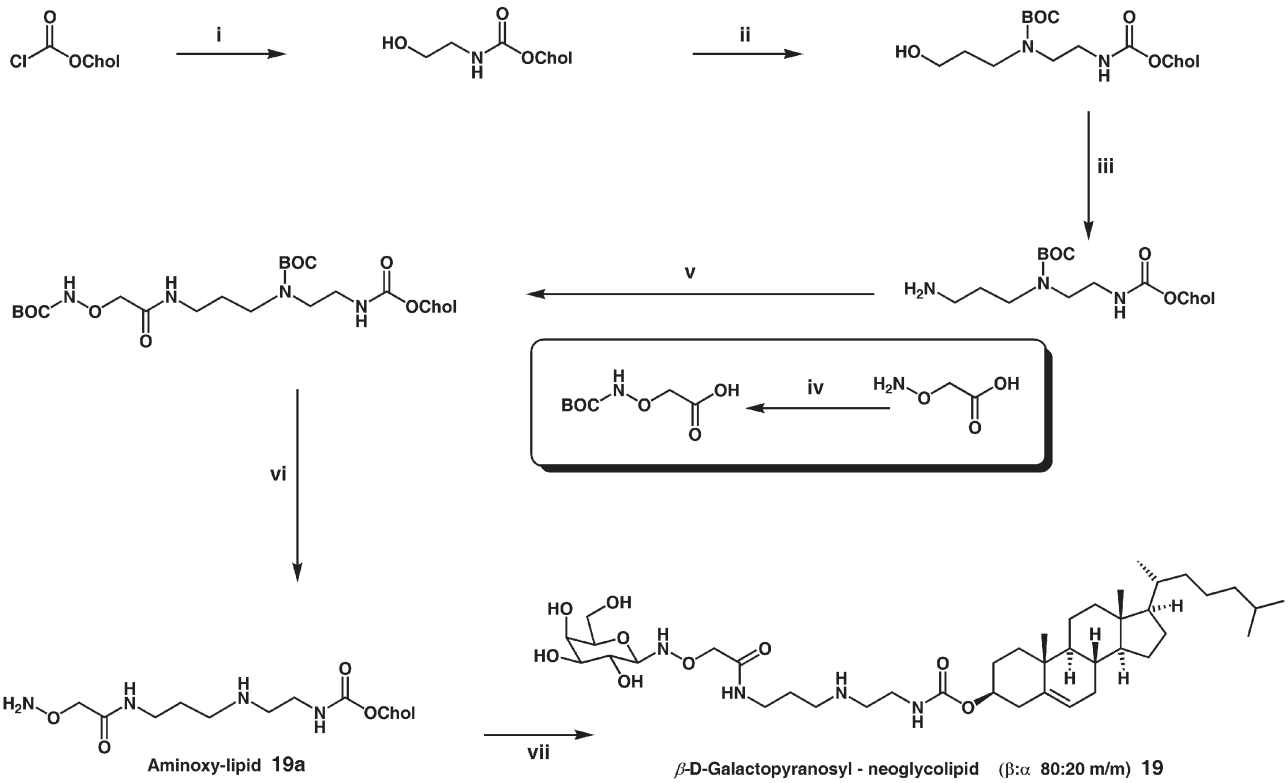
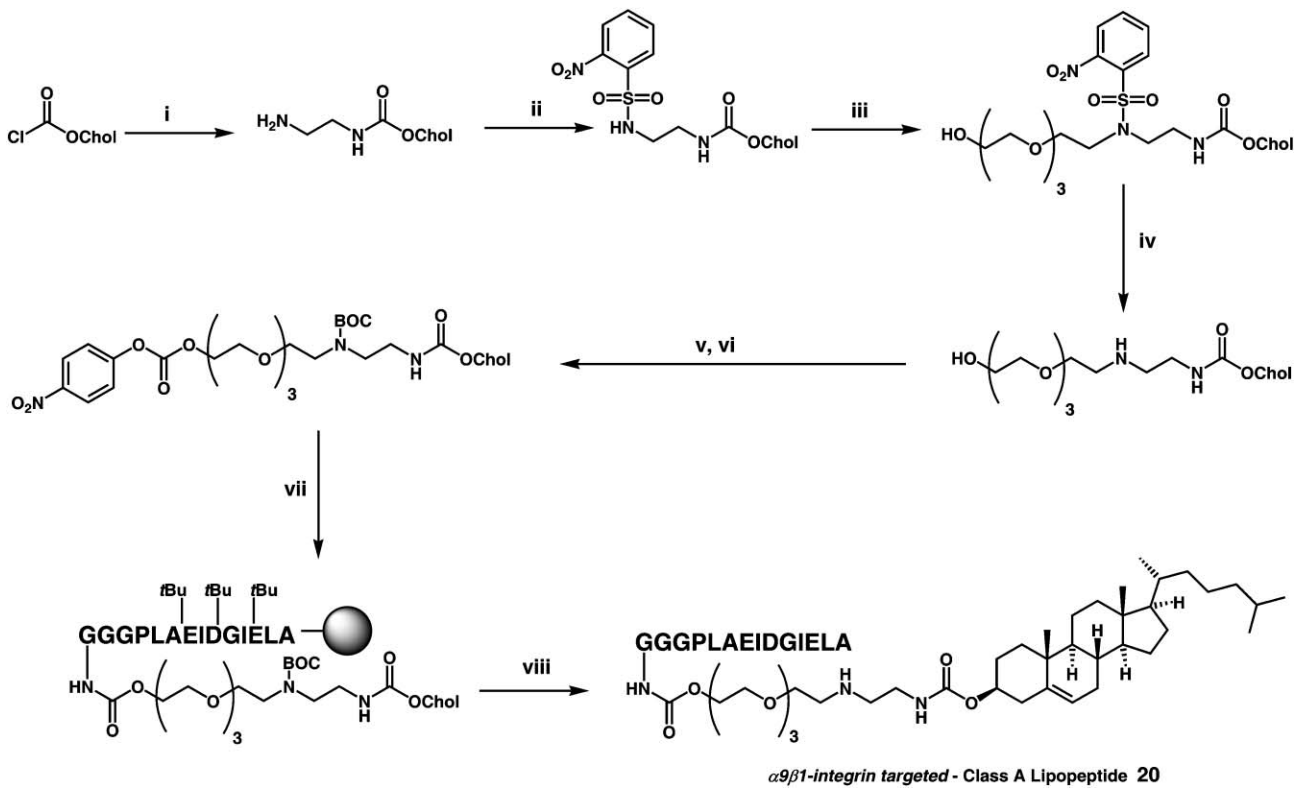


Fig. 7 MEND formulation. Schematic illustration of MEND particle formation. Initially, cationic PD particles are formed from pDNA (D) and cationic polymer (P) (usually pLL). These associate electrostatically with a negatively charged mono-layer lipid film and are then encouraged to form particles by a process of hydration and sonication. Final post-modification with STR-R_8 results in the formulation of cationic MEND particles (see text for references). Reproduced with the kind permission of Elsevier Academic Press.[209]



Scheme 5 *Reagents and conditions:* (i) CH_2Cl_2, $HO(CH_2)_2NH_2$ (2.2 equiv.), 10 h, 97%; (ii) (a) CH_2Cl_2, 0 °C, NEt_3 (3 equiv.), MsCl (2.5 equiv.), 10 min; then 1 h at rt, 98% (b) THF, $HO(CH_2)_3NH_2$ (10 equiv.), 6 h, 96% (c) CH_2Cl_2, NEt_3, Boc_2O, rt, 5 h, 90%; (iii) (a) CH_2Cl_2, 0 °C, NEt_3 (3 equiv.), MsCl (2.5 equiv.), 10 min; then 2 h at rt, 90% (b) DMF, 80 °C, NaN_3 (5 equiv.), NaI (1 equiv.), 3 h, 95% (c) THF, PMe_3 (1.15 equiv.), rt, 3h (d) H_2O/NH_3, 88%; (iv) CH_2Cl_2, NEt_3, Boc_2O, rt, 5 h, 98%; (v) (a) EtOAc, NHS (1 equiv.), DCC (1 equiv.), 10 h, rt (b), EtOAc/THF 95/5 v/v, NEt_3 (pH8), 2 h, rt, 90%; (vi) CH_2Cl_2, TFA (15 equiv.), 0 °C, N_2, 5 h, 86%; (vii) saccharide, AcOH/DMF 1/1 v/v, rt.[98]

A peptido-LMD variant was also prepared very recently by a *pre-modification* strategy in which lipopeptides of two classes were formulated into cationic liposomes prior to LMD formulation. LMD formulations were prepared using both CDAN **9** and DC-Chol **8** cytofectins, The synthesis of one lipopeptide is shown, notable for the application of a novel



Scheme 6 *Reagents and conditions:* (i) $H_2NCH_2CH_2NH_2$ (200 equiv.), 2 days, 65%; (ii) (a) 2-NsCl (1.3 equiv.), NEt_3 (1.5 equiv.), CH_2Cl_2, 14 h, 87% (b) BnBr (1.1 equiv.), Ag_2O (1.5 equiv.), 20 h, generating 55%; (iii) tetraethyleneglycol (TEG) (1.3 equiv.), DTBAD (1.5 equiv.) slow addition over 1 h in CH_2Cl_2, PPh_2py (1.5 equiv.), CH_2Cl_2, 3 h, 71%; (iv) Na (10 equiv.), $C_{10}H_8$ (10 equiv.), −30 °C, 45 min, 74%; (v) Boc_2O (1 equiv.), NEt_3 (1.1 equiv.), CH_2Cl_2, 10 h, 84%; (vi) NEt_3 (2 equiv.), DMAP (2 equiv.), *p*-nitrophenyl chloroformate (3 equiv.), CH_2Cl_2, 10 h, 92%; (vii) fully protected peptide (Fmoc deprotected on *N*-terminus) (0.5 equiv.), NEt_3 (2.5 equiv.), DMF, 18 h; (viii) 95% v/v TFA/H_2O, 90 min, 10% over two steps.[110]

variation of the Fukuyama–Mitsunobu reaction (Scheme 6), that now appears to have general applications in the synthesis of complex secondary amines.[110] The peptide sequence used (tenascin peptide sequence: PLAEIDGIELA) was previously shown to target $\alpha_9\beta_1$-integrin proteins predominant on upper airway epithelial cells in mammals. When peptido-LMD systems were prepared using CDAN **9** cytofectin, no evidence of receptor-mediated enhancement of transfection was observed. Instead, even with as little as 0.05 mol% of lipopeptide present in each peptido-LMD particle, transfection was at least 10-fold more effective than found for corresponding LMD systems without peptide present, irrespective of whether the cells under investigation expressed $\alpha_9\beta_1$-integrin proteins or not! Such non-specific peptide enhancement may be interesting but is not necessarily desirable. When peptido-LMD systems were prepared using DC-Chol **8** cytofectin, thereby reducing the overall positive charge of each particle, a modest element of specific enhancement was observed (2-fold) over a general background enhancement that was otherwise non-specific.[110]

Non-specific enhancements appear to be a hazard (or perhaps even an advantage under some circumstances) of using **ABD** systems. There has been some apparent success in using neoglycolipids as **D**-layer targeting agents inserted into LD complexes. For instance, Behr and coworkers reported *in vitro* galactose-receptor mediated uptake into hepatoma cells of LD complexes formulated with a triantennary galactolipid.[50] By contrast, Kawakami *et al.* have suggested liver targeting *in vivo* using LD complexes formulated with a mannosyl neoglycolipid as targeting agent. However, non-specific mannosyl induced LD stabilisation leading to longer circulation times seems to be a sufficient explanation to account for these results too.[111,112] An additional example of non-specific enhancements in **ABD** systems has been provided from experiments with the Transferrin (Tf) protein. Tf has been used quite frequently for **D**-layer biological targeting on the basis that transferrin receptors (TfR) are found routinely at the surface of vascular endothelial cells associated with tumours or the blood brain barrier and are rapidly internalised upon binding to Tf.[113–118] However, whilst *in vitro* and *ex vivo* transfections with a Tf-LD **ABD** system are enhanced relative to binary LD transfection, the mechanism is quite clearly TfR independent, the protein instead acting primarily to promote endosome disruption and subsequent escape of complexed DNA into the cytosol,[113,114] and even entry into the nucleus.[119] In addition, Tf is an acidic protein, negatively charged at neutral pH. Accordingly, the association of Tf with binary LD systems seems to reduce the overall positive charge and provides simultaneously a combined stereo-electronic barrier to biological fluid components allowing *in vitro* transfection to take place, even in 60% serum. In the latter context, human serum albumin (HSA) has been deliberately combined with

binary LD systems in order to create negatively charged, sterically protected complexes appropriate for *in vitro* transfection in the presence of up to 30% serum and even for lung or spleen transfection *in vivo*.[120] Lectin proteins also promote *in vitro* transfection in the same context.[121]

Chang and coworkers have suggested that a simple reformulation of Tf-LD **ABD** systems is sufficient to give highly compact particles with a relatively uniform size (50–90 nm) comprising a dense Tf-DNA core enveloped by a membrane coated with Tf molecules spiking the surface. This system appears to render enhanced stability, improved *in vivo* gene transfer efficiency, and long-term efficacy for systemic p53 gene therapy of human prostate cancer when used in combination with conventional radiotherapy.[122] Others have reported the need to introduce protamine giving the equivalent of a Tf-LPD **ABD** system that is able to transfect cells in a number of tissues post *i.v.* injection,[123] otherwise alternative Tf-LD **ABD** systems have been administered directly (intra-tumoural injection) to subcutaneous mouse xenograft models of human prostate cancer,[124] or else by *i.a.* administration into hepatocellular carcinoma.[125] Such data is certainly consistent with the possibility of some TfR-mediated uptake of Tf-LD particles by some tissues.[126]

Chang and coworkers have also developed alternatives to the Tf-LD **ABD** systems. For instance, using an anti-TfR antibody variable region fragment (anti-TfR scFv), they have produced an anti-TfR scFv-LD system with the scFv covalently attached to a number of cytofectins that appears to show some promise for systemic p53 gene therapy in a number of human tumour models including human breast cancer metastasis.[127,128] This anti-TfR scFv-LD system has been further improved by the inclusion of a cationic peptide (HoKC) to precondense pDNA during complex formation (*i.e.*, in a similar way to LMD and LPD systems).[129] However, the final *coup-de-grâce* has been to demonstrate that anti-TfR scFv-containing **ABD** systems are in fact inferior to a complete **ABCD** system in which a post-coating strategy was employed taking pre-formed LD particles (**AB** core particles) that were sequentially conjugated with PEG polymer (**C**-layer) and then anti-TfR scFv (**D**-layer).[130] However, there may yet be a future for simple monoclonal antibody (MAb)-LD **ABD** systems with or without covalent coupling of the antibody to cytofectins,[131,132] and even for **ABD** systems with associated lectins.[121]

4.2 Semi-synthetic ABD particles

Difficulties experienced in working with fully synthetic **ABD** particles have also resulted in the development of some semi-synthetic virosome systems. The term virosome was originally coined in reference to combinations of liposomes and various virus glycoproteins but is now more generally used to refer to various types of viral/non-viral hybrid vector systems. Of these the HVJ-liposome system is instructive. This semi-synthetic system is prepared from a combination of UV-irradiated virions of the Hemagglutinating Virus of Japan (HVJ; Sendai virus) and liposomes in which are encapsulated nucleic acids complexed with the High Mobility Group 1 (HMG-1) protein.[133,134] The HMG-1 protein is there to assist nuclear access and localisation of delivered nucleic acids as well as promoting gene stabilisation within the nuclear envelope.[133,135] Conventional HVJ-liposomes are negatively charged,[133,134,136,137] however an HVJ-cationic liposome system has recently been developed, based on the cytofectin DC-Chol **8**, that appears to transfect various mammalian cell types *in vitro* 100–800 fold more effectively than conventional HVJ liposomes.[138] In addition, HVJ-cationic liposomes prepared with the cytofectin *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) **14** (Fig. 3), have also proved able to mediate delivery of nucleic acids to tracheal and bronchiolar epithelial cells *in vivo* with reasonable efficiency.[139] One major reason for the success of HVJ-liposome systems is the presence of the hemagglutinin-neuraminidase (H_N) and fusion (F) glycoproteins in the liposome bilayer (Fig. 8). These are fusogenic proteins that allow HVJ-liposomes to interact with cell surface sialic residues, fuse with the cell membrane and then release encapsulated nucleic acids directly into the cytosol, bypassing endocytosis altogether.[134] For this reason, HVJ liposomes have also been called fusogenic liposomes.[140] The clear success of HVJ-cationic liposomes has resulted in the development of a number of other cationic virosome systems including systems prepared with the influenza membrane fusion protein hemagglutinin that were used to deliver genes

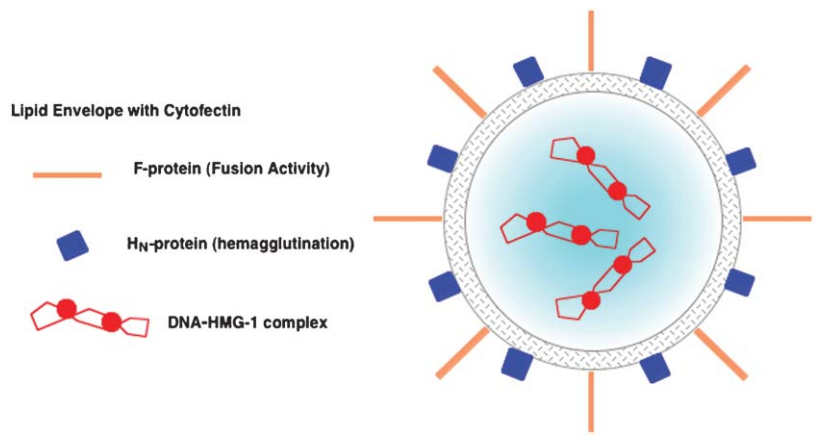


Fig. 8 HVJ-cationic liposome system. Cytofectins are incorporated in the lipid envelope. Reproduced with the kind permission of Bios Scientific Publishers.[21]

to cells *in vitro*,[141] cationic lipid-reconstituted influenza-virus envelopes used to deliver an ODN to cells *in vitro*,[142] and LD complexes prepared from DOTMA/DOPE cationic liposomes and pDNA doped with the partially purified G glycoprotein of the vesicular stomatitis virus envelope (VsV-G).[143] These semi-synthetic **ABD** nanoparticle systems are sure to remain of great interest going forward with one caveat. Virosomes contain virus proteins that could be very immunogenic *in vivo*, thereby creating potentially serious problems for repeated use of virosome vectors as for complete viral vectors.

5 ABC particles

Particles of this type appear to be altogether more promising for *in vivo* applications and gene therapy owing to passive targeting of particles stabilised with the aid of **C**-layer stealth/biocompatibility polymers. Passive targeting is the process by which stabilised nanoparticles accumulate with time into organs possessing enhanced microvasculature (such as solid tumours, infection, and inflammation sites) by means of long-term circulation in biological fluids, without the requirement for **D**-layer active targeting agents. By far the most popular **C**-layer molecule is polyethyleneglycol (PEG). PEG provides a steric barrier to interaction with biological fluid components and prevents uptake of liposomal vesicles by cells of the RES.[19,144] Safinya and coworkers have recently demonstrated that only PEG with a molecular weight of 2000 Da and above gives adequate stealth characteristics.[145] Hong *et al.* reported one of the first attempts to generate a self assembly **ABC** complex.[86] In this instance, DDAB/Chol cationic liposome-based LD particles were stabilised for storage by inclusion of *N*-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl]-DSPE (PEG-PE) and partially stabilised in the circulation *in vivo*. However, >1 mol% of PEG-PE proved sufficient to reduce lung *in vivo* transfection efficacy to a fraction of the transfection level mediated by DDAB/Chol cationic liposomes alone, indicative of a necessary compromise between the requirement to include PEG-PE for stabilisation purposes countered by the requirement to keep levels modest in order prevent "steric blocking" of LD transfection.

There are essentially three different ways in which **ABC** nanoparticles may be formulated with an exterior PEG **C**-layer (Fig. 9). These are:

1) *Pre-modification:* where a PEG-lipid is formulated into cationic liposomes prior to the addition of nucleic acids.[86]

2) *Post-modification:* where PEG-lipids in the form of micelles are combined with preformulated **AB** nanoparticle systems in the expectation that free and micellar PEG-lipids will transfer from free solution or micellar state and insert their

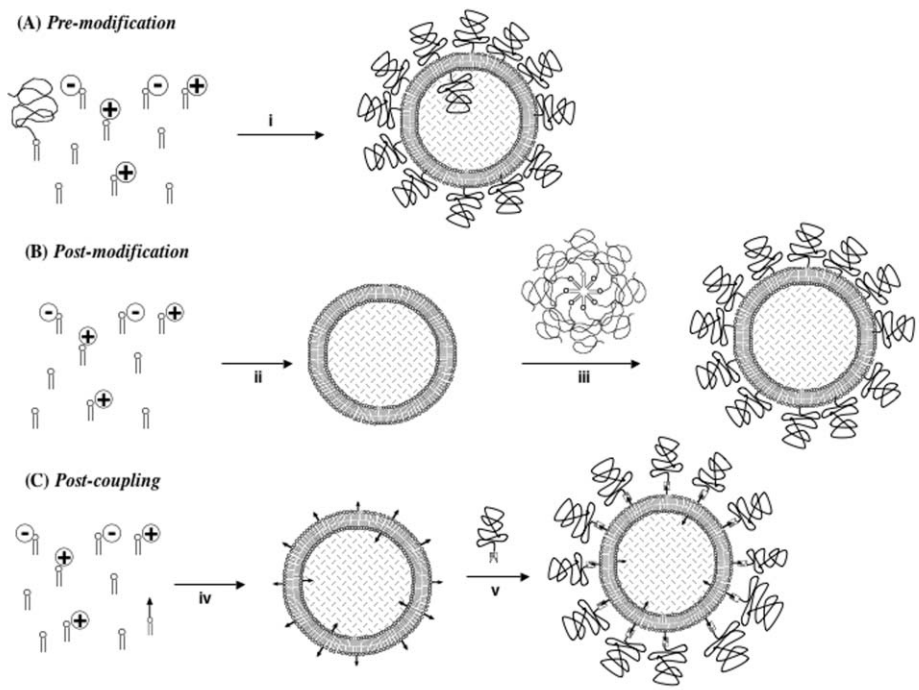


Fig. 9 C-layer stealth molecule incorporation strategies. **Top:** *Pre-modification* implies that PEG lipids are incorporated into cationic liposomes directly (step i) prior to the addition of any given nucleic acid; **Middle:** *Post-modification* strategy implies that simple cationic liposome (or LD, LsiR, LMD or LPD-like) systems are prepared in advance (step ii) and then PEG lipid micelles are incubated (step iii) with the liposome (or LD, LsiR, LMD or LPD-like) particles to encourage micelle breakdown and insertion of PEG lipids *via* their hydrophobic moieties into the outer leaflet membrane of whichever type of particle is being prepared; **Bottom:** *Post-coupling* strategy implies that simple cationic liposome (or LD, LsiR, LMD or LPD-like) systems are formulated with a coupling-lipid that enters lipid membranes (step iv). This coupling-lipid comprises a polar, functional group (black arrow) with very high chemoselectivity for certain complementary functional groups introduced into the termini (checked complementary-arrow shape) of modified PEG-molecules, allowing for highly efficient coupling in the subsequent conjugation step (step v). Post-coupling is flexible enough to allow for the introduction of other biological compatibility/stealth molecules and/or biological targeting molecules (see text for references). (Cationic liposome/micelle–siRNA complex = LsiR.) Reproduced with the kind permission of Elsevier Academic Press.[209]

hydrophobic lipid moieties into the outer leaflet membranes of **AB** nanoparticles.[98]

3) *Post-coupling:* where PEG-polymers are equipped with reactive functional groups that bioconjugate in aqueous conditions with complementary functional groups presented on the outside surface of the **AB** nanoparticle.[98,102]

Free amino functional groups on the surface of LMD particles can be modified easily by post-coupling with a PEG-succinimide activated-ester, giving a simple LMD-based **ABC** nanoparticle.[102] Perhaps surprisingly, these particles were observed to enter cells with ease even though prevailing opinion would have suggested that the PEG stealth layer might provide a steric barrier to cellular uptake. Instead, cellular uptake was found to be rapid (minutes) and substantial, but particles appeared to be entrapped in endosome compartments post cell entry and no measurable transfection was observed.[98,102] PEG has undeniable stealth/biocompatibility properties, and clearly facilitates the cellular uptake of attached nanoparticles in spite of these effects. Unfortunately, PEG also appears to block subsequent endosome escape completely. The obvious solution appears to be some form of triggered release of attached PEG once nanoparticles become trapped in endosome compartments revealing naked LMD particles (**AB** core nanoparticles) that are able to effect endosmolysis and onwards transfection.[102] Other polymers such as pluronic acid (a propylene oxide containing block co-polymer) and oligosaccharides also promote DNA uptake into cells.[98,146] This appears fundamental. Any given **ABC** (or even **ABCD**) system delivering DNA is only likely to be properly clinically viable once triggerable, meaning that they should be completely stable and non-reactive in extracellular fluids but unstable once recognised and/or internalised by target cells in the organ of choice. This paradox goes to the heart of the matter. **ABC** (or even **ABCD**) nanoparticles that are not triggerable are unlikely to be effective particles for DNA delivery by their very nature.

In the absence of triggered release, time-dependent release of PEG-lipids has turned out to be a reliable if not entirely effective alternative. According to this approach, PEG-lipids with hydrophobic moieties of variable chain-length will have variable affinities for the outer leaflet membrane of the **AB** core particle into which they are inserted. The shorter the alkyl chain, the lower will be the affinity and the lower will be the PEG-lipid residence time in the membrane (usually referred to in terms of residence half-life $t_{1/2}$). Ideally, PEG-lipids should be retained as far as the organ of choice *in vivo* and even up until the target cells, before dissociation and exposure of naked **AB** core nanoparticles to enter cells. This feature is characteristic of the rationale leading to the stabilized plasmid-lipid particle (SPLP) systems, the most developed of **ABC** nanoparticle systems to date. First generation SPLP system contained DOPE **1** (84 mol%), low levels (6 mol%) of cationic lipid dioleyldimethylammonium chloride (DODAC) and quite high levels of a PEG-Ceramide with an arachidoyl acyl group (PEG-CerC_{20}) (10 mol%).[147] The surface tenacity of PEG-CerC_{20} ($t_{1/2} > 13$ days) proved such an intractable steric barrier to transfection *in vitro* that PEG-CerC_{20} was replaced by PEG-CerC_8 ($t_{1/2} < 1.2$ min) with an octanoyl acyl group. Entrapment of pDNA was then accomplished by a detergent dialysis procedure (55–70% efficient), giving second-generation DOPE/DODAC/PEG-CerC_8 SPLP particles containing DODAC (24–30 mol%) and PEG-CerC_8 (15 mol%) (diameter approx. 100 ± 40 nm),[148] that were able to effect transfection of cells *in vitro* and regional delivery of pDNA *in vivo*. The formulation procedure is illustrated diagrammatically (Fig. 10). One of the most important aspects about SPLP particles is their very structural integrity (no changes in size or DNA encapsulation at 4 °C for 5 months).

SPLP particles were designed for passive targeting. That is to say, particles were designed for long term circulation *in vivo* enabling the gradual partition of particles into interstitial spaces in diseased tissue (such as tumour) by extravasation

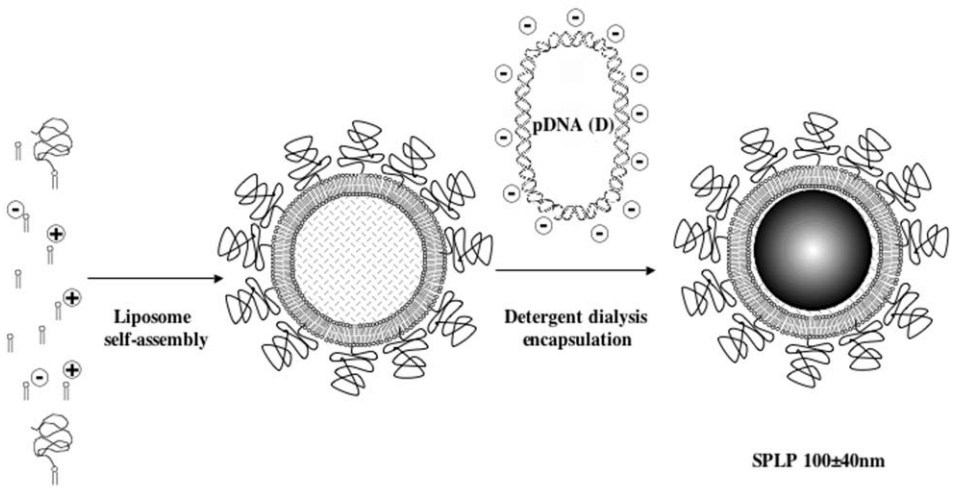
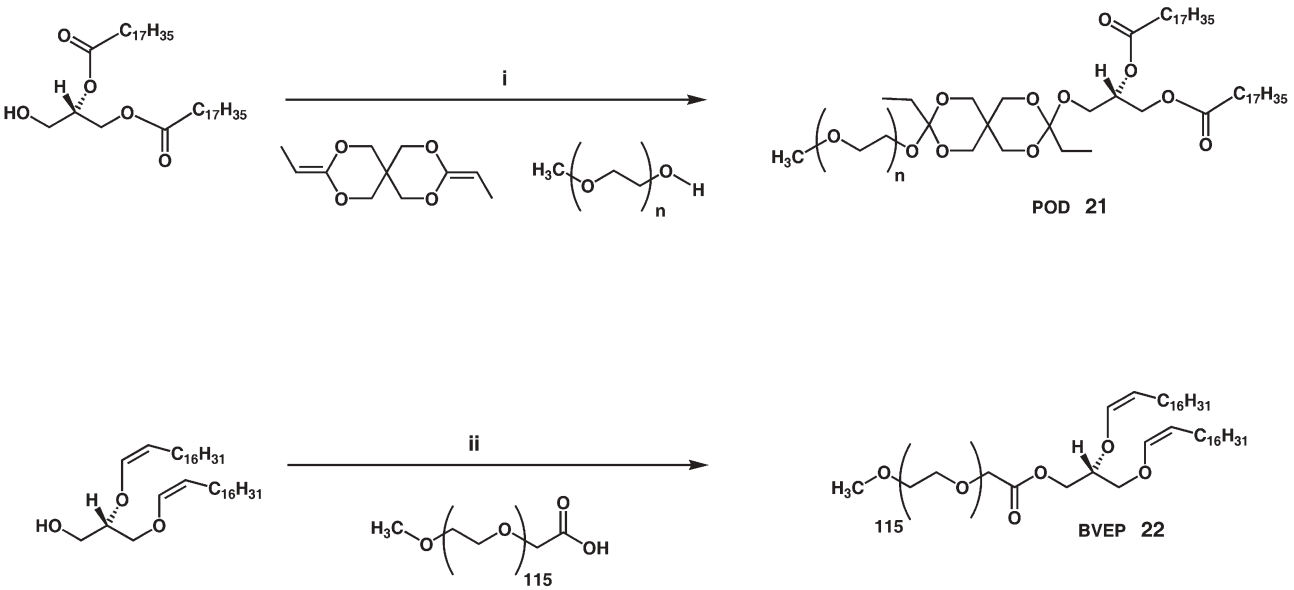


Fig. 10 SPLP formulation. Formation of low charge cationic PEG-liposomes by pre-modification with appropriate PEG lipids. The subsequent introduction of condensed pDNA requires some form of detergent analysis (encapsulation efficiency approx. 60%). Excess pDNA must be removed afterwards by column chromatography in order to yield stabilised plasmid-lipid (SPLP) particles ready for use (see text for references). Reproduced with the kind permission of Elsevier Academic Press.[209]

from blood *via* the enlarged sinusoidal gaps that typically exist between the endothelial cells which line the vasculature in diseased and inflamed organs. Given the severity of the extracellular *in vivo* environment only PEG-CerC_{20} SPLP particles were found to give modest, detectable transfection in animals post *i.v.*-administration.[149] Other systems such as the PEG-CerC_8 SPLP particles were not sufficiently robust to effect passive targeting even though their transfection properties were in principle superior. In other words, in seeking to find the balance between very high extracellular structural integrity (stability) and the very low intracellular or local structural integrity (instability) necessary to promote transfection, the requirement for extracellular stability was too overwhelming with the result that SPLP particles were developed with appropriate extracellular stability but insufficient instability for effective transfection once at the target cells of interest. Improvements continue to be made in order to increase DNA encapsulation efficiency and to improve transfection efficiency.[150–153] SPLP systems are now under evaluation in Phase I clinical trials (Protiva, unpublished data), the first **ABC** nanoparticle to be so evaluated. Data is awaited eagerly. Undoubtedly, SPLP systems represent a key synthetic, non-viral platform technology, and one that others are aiming to emulate increasingly and improve upon.[154]

The absence of triggered release is still perceived to be the main limitation of SPLP systems. Accordingly, Szoka and coworkers have assembled their own SPLP system taking advantage of a designed ortho ester PEG-lipid known as polyethyleneglycol-diorthoester-distearoyl glycerol conjugate lipid (POD) **21**. POD **21** is one of the best bespoke-pH-triggered PEG-lipids to date. The structure and synthesis of POD **21** are shown[155,156] (Scheme 7). The key design feature of such a pH-triggered PEG-lipid is that the triggerable linker should be completely stable at pH 7 and sufficiently destabilised at pH 5.5 to completely and irreversibly dissociate within 1 hour at the very least. Such a requirement is severe but is essential to ensure quantitative release of nucleic acid from endosomes! Biophysical release studies were performed with POD-loaded liposomes suggesting that the release half-life, $t_{1/2}$, at pH 5.5 was approx. 10 min.[157] Hence, proof of concept studies were carried out with a POD-SPLP formulation (DOPE/DOTAP/POD 68 : 12 : 20 m/m/m) into which pDNA was encapsulated (40–45% efficiency) by detergent dialysis (as above) giving 60 nm particles. These were found to mediate transfection *in vitro* much less effectively than simple DOTAP/DOPE cationic liposomes mixed with pDNA. However, POD-SPLP systems were up to three orders of magnitude more effective at transfection than equivalent pH-insensitive nanoparticle systems formulated with PEG-DSG rather than POD **21**.[158] Both POD-SPLP particles and particles of the equivalent pH-insensitive systems were found to enter cells in line with data obtained with LMD-based **ABC** nanoparticles.[102] Therefore, the clear implication is that the enhanced transfection efficiency of the POD-SPLP system was the result of the triggered release of PEG in the endosome leading to considerably enhanced endosmolysis and pDNA escape to the nucleus. Proof of concept studies *in vivo* are now awaited, so too are alternative next generation triggerable **ABC** nanoparticle systems for DNA delivery.

Other **ABC** nanoparticles have been described mostly with regard to triggered or time dependent release properties and provide useful supporting studies. For instance, **ABC** nanoparticle systems have recently been constructed using short and long-chain SAINT-PEG lipids constructed from pyridinium cytofectins that have variable length residence times in the same way that PEG-ceramide lipids do.[159] Alternatively, Thompson and coworkers have adapted their chemical routes to cytofectin BCAT and diplasmenylcholine in order to prepare a novel acid labile PEG-lipid (*R*)-1,2-di-*O*-(1′*Z*, 9′*Z*-octadecadienyl)-glyceryl-3-(ω-methoxy-poly(ethylene) glycolate 5000) (BVEP) **22**[49,160,161] (Scheme 7). Elegant though the idea is, biophysical release studies were performed with BVEP -loaded liposomes suggesting that the release half-life, $t_{1/2}$, at pH 4.5 was approx. 4 hours.[160] In this respect, the vinyl



Scheme 7 *Reagents and conditions:* (i) pTSA, THF, 40 °C; (ii) DCC, DMAP, methoxy-poly(ethyleneglycol)-carboxymethyl (MPEGA).[49,155]

ether functional group is probably just too stable to provide for rapid and effective enough triggered release in DNA delivery involving **ABC** nanoparticles. Otherwise, two other studies are worthy of note. PEG-lipids appear to stabilize LD particles prepared from bis(guanidinium)-tren-cholesterol (BGTC)/DOPE liposomes. Subsequent **ABC** nanoparticles will transfect murine lung *in vivo* even without triggered release suggesting that topical lung administration may be exceptional.[162] Finally, Scherman and coworkers have been demonstrating how the judicious introduction of anionic PEG moieties can tune the systemic circulation lifetimes of **ABC** nanoparticles.[163,164]

6 ABCD particles

For most *in vivo* applications and gene therapies, true **ABCD** nanoparticle systems are perhaps the best proposition. The number of these is growing in spite of the obvious technical problems surrounding reproducible and scalable formulation of an **AB** core particle alongside controlled and reliable association of **C** and **D** layer molecules. This remains an ongoing problem. Pardridge and coworkers have reported really impressive results using an **ABCD** system comprising an LD core particle prepared from cationic liposomes with minimal cytofectin. This LD core is doped with PEG-PE variants, one for stabilisation and one for the covalent attachment of an anti-TfR monoclonal antibody (OX26) specific for TfR that is overexpressed by cells at the blood brain barrier (BBB) and also in peripheral organs such as liver and spleen.[165,166] Their main **ABCD** system is comprised of POPC/DDAB/DSPE-PEG^{2000} (19.2 : 0.2 : 0.6 m/m/m) liposomes where the DSPE-PEG^{2000} is distributed DSPE-PEG^{2000}/DSPE-PEG^{2000}-Maleimide (95 : 5 m/m). Particles were prepared by initial mixing of all of the lipids together in chloroform solution followed by solvent evaporation, rehydration, sonication, pDNA addition, concluding with multiple freeze-thaw cycles and extrusion. This arduous process (20% efficient) yielded **ABC** nanoparticles that were coupled to OX26 antibody overnight, yielding complete **ABCD** particles (35–50 OX26 MAb/particle; 45–114 nm) after final Sepharose Cl4B gel filtration to remove excess unreacted antibody[166] (Fig. 11).

These **ABCD** nanoparticles are almost completely neutral in charge and do not possess any triggered release system. Therefore, the impressive biological data showing transfection in liver, spleen and brain must be a consequence of active targeting mechanisms involving TfR interactions. From these beginnings, Pardridge and coworkers have developed further the formulation protocols for these pegylated immunoliposome (PIL) systems and demonstrated that PILs have impressively low levels of associated systemic toxicity.[167] Additional applications of PILs include the delivery of pDNA-directed epidermal growth factor receptor (EGFR) antisense mRNA using particles "armed" with both an anti-TfR MAb and an anti-insulin receptor (INSR) MAb, the first to promote crossing of the BBB and the second to promote transport of pDNA to the nucleus across plasma and nuclear membranes in the target brain tumour.[168,169] Dual targeting was deemed essential to ensure targeting across both the tumour cell membrane and microvasculature barrier to reach

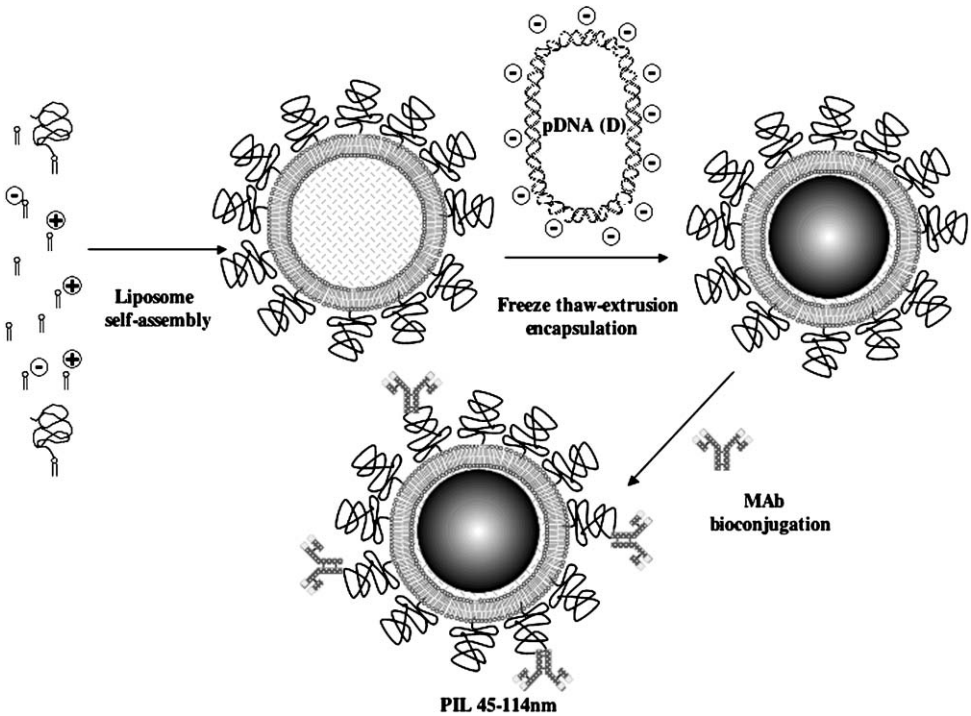


Fig. 11 PIL formulation. Formation of low charge cationic PEG-liposomes by pre-modification with appropriate PEG lipids. The subsequent introduction of condensed pDNA requires extensive freeze–thaw and extrusion steps (encapsulation efficiency approx. 20%). Excess pDNA must be removed afterwards. Final monoclonal antibody (MAb) coupling then takes place to yield pegylated immunoliposomes (PIL) particles ready for use (see text for references). Reproduced with the kind permission of Elsevier Academic Press.[209]

cells deep within the cancer tissue. Furthermore, TfR MAb-targeted PILs were used to mediate pDNA-directed short hairpin interference RNA (shRNAi) downregulation of transgenic luciferase activity *in vivo* in rat cranial brain tumours by up to 90%.[170]

Others are also moving towards success with the active targeting of tumours after *i.v.* injection of alternative **ABCD** systems prepared using MAbs or folate as the targeting ligand.[159,171,172] One of the most interesting variations on this theme has been the creation of folate receptor (FR)-targeted DDAB/CHEMS/f-PEG-PE liposomes comprising folate (f) conjugated PEG-PE (f-PEG-PE), cholesteryl hemisuccinate (CHEMS) and the cytofectin dimethyldioctadecylammonium bromide (DDAB) **6**, that are combined with poly-L-lysine (pLL) condensed pDNA to give **ABCD** nanoparticles competent to mediate FR-specific delivery of pDNA to cells *in vitro*.[173] However, whether or not this system will operate effectively *in vivo* remains to be seen.

7. The future role of biology; A layer innovations

This section is included owing to the fact that most chemists and chemical biologists interested in gene therapy related problems do not pay adequate attention to the nature of the encapsulated nucleic acid (**A**) that is to be delivered. Normally, chemists and many others assume that once the nucleic acid is "delivered to the nucleus", then their role is over. However, this is too simplistic. At the very least, the chemist should be aware of the different nucleic acid "payload" possibilities that are available for delivery. At the very best, the chemist should not only have a feel for the differences in molecular biology that govern their mechanisms of action of each alternative payload, but appreciate how synthetic non-viral vector systems could be adapted or fitted out for different nucleic acid payloads according to those mechanisms of action. Indeed, some synthetic non-viral vectors will also be better adapted naturally for the delivery of some nucleic acid payloads compared with others. For instance, while LD systems that tend to form metastable, large particles (>150 nm) appear to be well adapted for pDNA delivery *in vitro*,[38] LD systems with a membrane active component that generate small, stable particles (<150 nm) appear to be better adapted for the efficient delivery of anti-sense phosphorothiolate oligonucleotides *in vitro*.[174]

7.1 DNA constructs

Broadly speaking, payloads will either be DNA or RNA in character. In the case of DNA, pDNA has been the most commonly used form of DNA and has also been the most commonly used form of nucleic acid. Much of the data described in the preceding chapters has been acquired using pDNA. DNA must always be delivered to the nucleus in order to demonstrate a function, as described previously, but problems are far from over once there. Typically, gene expression takes place post-nuclear delivery and declines to background levels between 7 and 14 days post-transfection. This is known as plasmid silencing. Plasmid silencing is unhelpful for most projected *in vivo* applications or gene therapy. Curiously, the reasons for plasmid silencing do not appear to be plasmid shedding (loss of pDNA from cells) or plasmid CpG methylation as might be expected,[175] suggesting that other mechanisms are involved such as chromatin remodelling (nuclear protein condensation of pDNA leading to inactivation of gene expression). While research into plasmid silencing would be of undoubted use in the design of long-term expression plasmids such as plasmid minicircles,[176] molecular biology has not stood still and a number of alternatives to simple epichromosomal pDNA now exist that could be used in place.

A potential way to enhance long term expression in pDNA may be to introduce elements of DNA structure involved in the control of gene expression. Obviously, just as open-reading frames (ORFs) (genes or sections of genes) only comprise a fraction of chromosomal DNA in any one cell so open-reading frames do not comprise the entirety of any one plasmid. There are now known to be elements such as Locus Control Regions (LCRs) and Ubiquitous Chromatin Opening Elements (UCOEs) that act to sustain associated ORFs in states appropriate for transcription (into mRNA) and promote long term expression.[177] These have proven themselves in viral vector systems and now await analysis in synthetic non-viral vector systems.

Otherwise, the arrival of mammalian transposons (transposable elements) now looks remarkably promising. Transposons are stretches of DNA (either linear or circular) that are capable of insertion into chromosomal DNA at defined sites with the assistance of a transposase enzyme.[178] Of particular significance is the Sleeping Beauty transposon element originally identified in the Zebra fish genome through sequence similarity with active transposons (Tc1/mariner transposable elements) found in *Drosophila* and *Caenorhabditis elegans* but rendered inactive in Zebra fish through deleterious mutations. Reversal of these mutations has created a transposon active in mammalian cells and able to insert a transgene embedded in the transposon sequence into mammalian chromosomal DNA leading to long term transgene expression (months)[179–181] (Fig. 12). Proof of principle studies with non-viral delivery of Sleeping Beauty transposon to cells have been accomplished,[182] although there are concerns that this transposon integrates into too many sites in chromosomal DNA and therefore may be cancer-inducing (oncogenic) in the same way that retroviridae are. The physicochemical properties of Sleeping Beauty transposon integration sites in chromosomal DNA are known (palindromic AT repeat) and are theoretically numerous.[183] However, transposase enzymes do not integrate transposons into genes under active transcription unlike retroviridae. Therefore, the risks of oncogenicity are much reduced but remain realistic at this stage. Accordingly, there have been proposals for the construction of chimeric-transposase enzymes engineered with binding domains (such as zinc-finger domain proteins) with high affinity for select DNA sequences that could guide the transposon to site specific integration.[184] This idea is seductive but requires complete validation. An alternative approach has been suggested and validated using an integrase enzyme ϕC31 from bacteriophage. In this instance, the enzyme integrates an alternative transposable element that interacts with chromosomal DNA at binding sites less prevalent than the Sleeping Beauty integration sites and consequently is perceived to

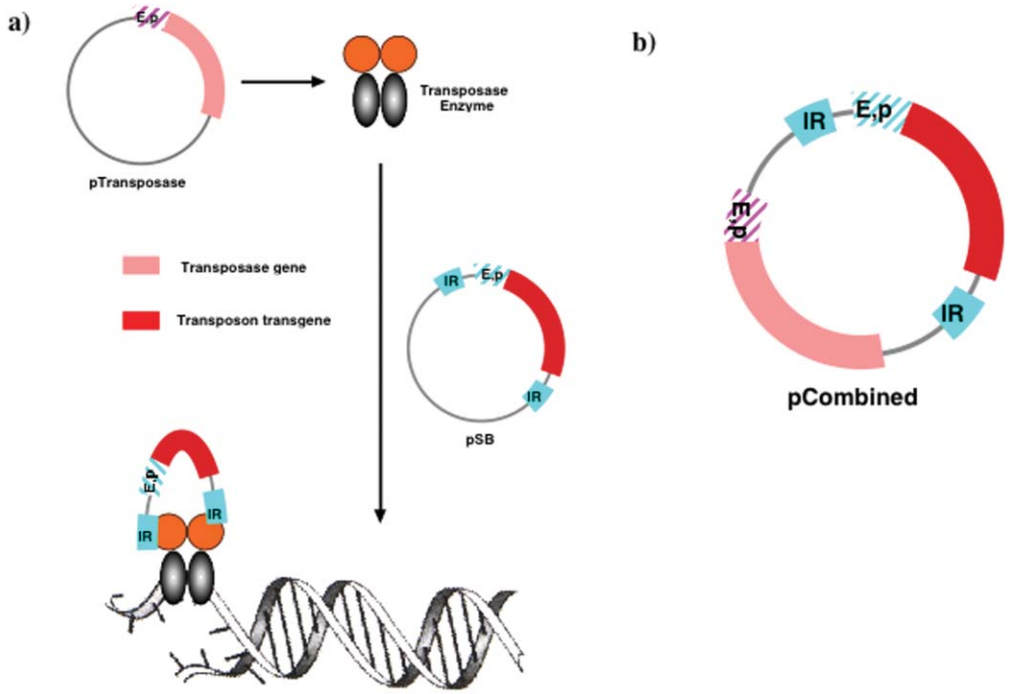


Fig. 12 Outline mechanism of Sleeping Beauty transposon mechanism. (**a**) Schematic to show how dimeric sleeping beauty transposase is coded for by one pDNA (pTransposase) and how this expressed enzyme then captures the inverted repeats (IRs) found in the pDNA (pSB) harbouring the transposon. Dimeric transposase then excises the complete transposon and relocates to defined chromosomal DNA binding sites where insertion of the complete transposon is finally assisted (see text for references); (**b**) Schematic to show how transposon and transposase gene could be integrated together on the same pDNA.

minimise the risks of oncogenicity in comparison[185–188] (Fig. 13). This approach is still in the early stages of technical development but appears potentially useful.

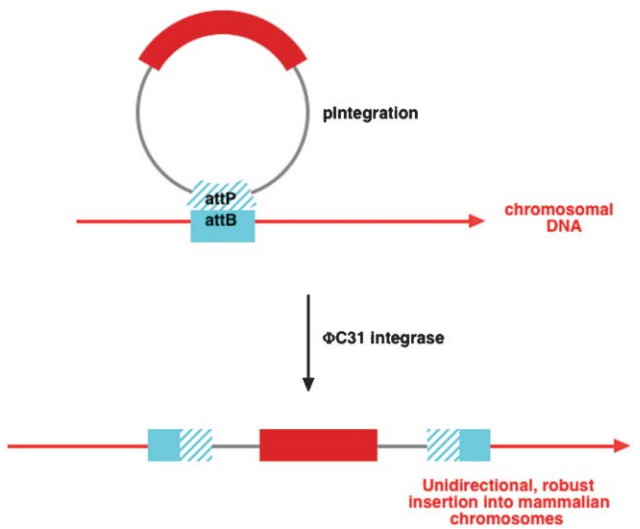


Fig. 13 Outline mechanism of phage integrase mechanism. Schematic to show how **attP** DNA sites in a given pDNA recognise and interact with complementary **attB** sites in chromosomal DNA. Thereafter, phage integrase enzyme **ϕC31** performs insertion of the entire pDNA into the **attB** sites in a unidirectional and robust manner without the need for cofactors (see text for references). The same will be true when **attB** sites are located in pDNA and interact with **attP**-like sites in chromosomal DNA.

Artificial chromosomes represent the main alternative to pDNA and may be delivered with synthetic non-viral vectors to cells owing to the fact that these vector systems are able to deliver any nucleic acid construct irrespective of size. Clearly artificial chromosomes (50 kbp–1 Mbp) are much larger than pDNA (typically 4–7 kbp) but have been constructed to express genes in an epichomosomal manner supported by all the main features of a chromosome (such as the centromere) so that they operate as pseudo-chromosomes. Depending upon source of sequences and genes, there are bacterial artificial chromosomes (BACs),[189–191] P1-derived artificial chromosomes (PACs),[189,192,193] yeast artificial chromosomes (YACs),[194,195] mammalian artificial chromosomes (MACs),[196–198] and human artificial chromosomes (huACs).[199,200] Proof of concept has been demonstrated using synthetic non-viral vector systems and artificial chromosomes resulting in long term expression in cells *in vitro*,[189,192,198] and even *in vivo*.[190,191] At the other end of the spectrum, DNA aptamers and ribozyme DNAs have also been delivered successfully to cells *in vitro*. HIV-1 gene expression was successfully inhibited by the intervention of anti-HIV Rev-binding aptamer [RBE(apt)], and a ribozyme directed against the HIV-1 env gene,[201] both delivered by an **ABD** nanoparticle.

7.2 RNA constructs

In the case of RNA, one might consider the delivery of mRNA but the complex, heterogeneous secondary structure of such molecules and perceived vulnerability to hydrolysis have

ensured that mRNA has seen little application. However, the delivery of siRNA is set to transform the use of RNA and potentially even the face of therapeutic medicine itself. The concept of siRNA has risen with incredible speed within the past two years.[202] The phenomenon of RNA interference (RNAi) has a provenance stretching back to at least 1995 when large double stranded RNAs (dsRNA) were found to silence genes in nematodes by a mechanism that is only now being properly appreciated (Fig. 14). According to this mechanism, dsRNA is broken down into siRNA duplexes (typically comprising 2 base overhangs at each 3′-end and a central antiparallel 19bp double helical region) by an enzyme system known as DICER. The siRNAs then associate with a protein complex (RISC) that interacts in an asymmetric manner with each siRNA, separating sense (S) and antisense (AS) strands from each other and preferentially adopting the AS over the S strand as a "template" to bind target mRNA. Target mRNA is singled out for destruction by this activated RISC through

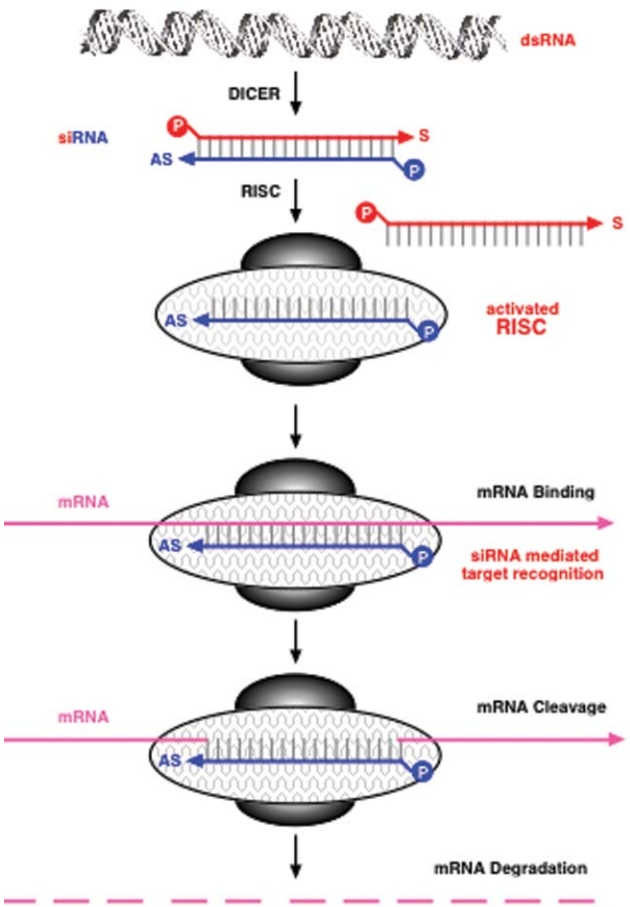


Fig. 14 Outline of siRNA mechanism of action. Small interference RNA (siRNA) is derived from long double-stranded RNA (dsRNA) through the action of the DICER enzyme system. Interaction of siRNA molecules with the RISC enzyme system results in sense (**S**)/anti-sense (**AS**) strand separation and the probable capture of individual **AS** strands by RISC. So activated, RISC recognises mRNA molecules with Watson–Crick base-pair complementary to bound AS strand. Once recognised and bound, mRNA is cleaved and then degraded.[202]

siRNA-mediated target recognition apparently provided by the AS strand of siRNA bound to RISC that presumably makes complementary Watson–Crick base pair interactions with a corresponding region in target mRNA.[203,204]

What makes siRNA so potent is that numbers of appropriate siRNAs may be identified with the capacity to complement corresponding regions in a target mRNA of interest, after which these siRNAs may be sifted with defined rule sets so as to identify those siRNAs (approx. 1–3% per gene) with S and AS strand base sequences that are optimal for RISC-mediated destruction of the target mRNA of interest.[205] Furthermore, these sequences may be screened at one higher level by means of high-end bioinformatics analyses (such as the siDIRECT analysis) ensuring that they have no likelihood of cross-reactivity with other off-target mRNA sequences and hence little likelihood of eliciting undesirable cellular toxicities.[206] The role of siRNA in genomics and target validation through specific gene knockdown and phenotypic characterisation is now beyond question. Moreover, with such apparent precision, a role of siRNA therapeutics now seems very credible. Gratifyingly, cationic liposome mediated siRNA delivery to cells (siFection) is particularly suitable for siRNA applications, not the least because cationic liposome systems such as CDAN/DOPE (45 : 55 m/m, siFECTamine®) have been specially formulated and adapted for *in vitro* siFection of cells resulting in maximum gene knockdown efficacy (>90%) with absolutely minimal toxicity.[39] The siFECTamine® cationic liposome system can be upgraded in a modular fashion for *in vivo* siFection (see below). Clearly, successful siFection does not require siRNA delivery to cell nuclei and also there is no real equivalent to the "long term expression" problem with pDNA. Therefore effective delivery of siRNA *in vivo* looks to be much more straightforward to achieve than effective delivery of pDNA. Hence the future for *in vivo* applications of siRNA looks bright not to mention the possibility of siRNA therapeutics as well.

8. The future role of chemistry; B, C and D layer innovations

Previously, we have noted that platform technologies like LMD and SPLP systems should be the only meaningful way forward for cationic liposome/micelle-based systems for *in vivo* applications and gene therapy. Our reasons for stating this were that these systems represent well-characterised transfection vehicles constructed from tool-kits of well-defined chemical components, that can be formulated in a reproducible and scalable manner, giving rise to reproducible transfection outcomes. We would now like to take the opportunity to update our comments in the light of the self-assembly **ABCD** nanoparticle concept presented in this review. Hence, in our revised view the most appropriate way forward for cationic liposome/micelle-based synthetic non-viral vector systems, is now the creation of **ABC** and **ABCD** nanoparticle systems that have been self-assembled in a modular and sequential fashion from tool-kits of well-defined chemical components. These systems must formulate with nucleic acids of choice in a reproducible and scalable manner giving discrete and well-defined particles with a narrow particle

size-distribution in solution (*i.e.*, narrow polydispersity) centred around 70–100 nm diameter, thereby being too large for rapid excretion and too small for rapid RES clearance. Furthermore, these particles should be able to give reproducible transfection outcomes (or siFection outcomes, as appropriate) *in vivo* with minimal toxicity. The combination of all these features should ensure regulatory confidence in the therapeutic applications of such **ABC** and **ABCD** nanoparticle systems. Furthermore, the very self-assembly and modular build characteristics of such nanoparticle systems should ensure that nanoparticles can be tailor-made for individual nucleic acid delivery requirements by using a range of different tool-kits of well-defined chemical components. It is in the synthesis and integration of these tool-kits of chemical components that the future opportunity for chemistry now lies.

As stated above, the cytofectin (**B**-layer components) field appears to be approaching saturation. There are numerous examples of cytofectins in the literature and the field of non-viral gene therapy is now less unlikely to benefit from further additions. However, there is real benefit in the synthesis of new lipids to facilitate the attachment and function of the stealth/biocompatibility **C**-layer. For instance, lipids containing the aminoxy functional group shown above (Scheme 5) can be synthesised, formulated into complexes with nucleic acids and then used to effect the post-coupling of PEG-aldehydes in aqueous medium. The result of this aqueous post-coupling procedure appears to be the highly efficient, reliable and non-disruptive introduction of a biocompatible/stealth **C**-layer resulting in robust **ABC**/**ABCD** nanoparticles, such as siFECTplus™ nanoparticles for the functional delivery of siRNA to cells in organs *in vivo* (see **CONZENTRx**™ systems of IC-Vec Ltd, unpublished results). Post coupling through aminoxy functional groups is clearly potentially effective, but what of other functional groups? Alternatively, requirements for **B** and **C** layer innovation could be combined in the quest for alternatives to the pH-triggerable PEG-lipids of Szoka or Thompson and coworkers (Scheme 7). The recent review of Guo and Szoka[207] was compiled not only to illustrate pH triggering but also redox potential, temperature and even enzymatic triggering processes. There is plenty of room for chemical innovation here!

Then there is the question of PEG itself. This remains the mainstay for most *in vivo* applications involving viable **ABC** and **ABCD** nanoparticles. However, this large and unwieldy ''stealth/biocompatibility'' molecule has already been shown to be refractory for transfection with pDNA.[98,102] Triggered release of PEG from the **AB** core once nanoparticles have entered cells, seems imperative in order for effective pDNA transfection to take place,[158] although the presence of attached PEG may in fact be much less a problem for siFection (delivery of siRNA). Nevertheless, efforts should be put into finding alternative hydrophilic polymers that can mimic the biocompatibility and stealth properties of PEG without the refractory characteristics and lack of biodegradability. Some alternative hydrophilic polymers have already been described by Seymour and coworkers, including poly-[*N*-(2-hydroxypropyl)-methacrylamide] (pHMPA).[208] Once again, there should be a host of other possibilities once the enthusiasm of polymer chemists can be engaged on this problem. Once prepared, each prospective surrogate of PEG will have to be rigorously evaluated for biocompatibility and stealth properties coupled with low toxicity and adequate biodegradability. However, this process should be encouraged at the earliest opportunity.

In general, there is a real requirement for more and better bioconjugation methodologies for the coupling of biological targeting moieties to core **AB** or **ABC** nanoparticles. Thus far, bioconjugation methodologies have been few and rather inefficient, including the aqueous coupling between free thiol groups and maleimide functional groups, or free amino groups and succinimide-activated esters. Moreover, there is usually little effort to characterise and confirm the results of most bioconjugation reactions that have been described in the literature, and correspondingly little real effort to separate bioconjugation products from reactants! This is woeful and also needs addressing at the earliest opportunity. Aminoxy-aldehyde aqueous functional group coupling and robust high performance liquid chromatography (HPLC) analyses have recently been developed (IC-Vec Ltd, unpublished results) and appear to represent an effective means of **D**-layer bioconjugation. However, once again much more chemical diversity is required for reproducible and scalable aqueous coupling of peptides, proteins and/or oligosaccharide targeting moieties to core **AB** or **ABC** nanoparticles.

By way of final comment, the circulatory extracellular barriers discussed in *Section 1.2.1* of this review were said to be far from exhaustive and are primarily valid as long as synthetic non-viral vector systems are involved in local delivery applications *in vivo* to lung, peritoneal cavity, vascular system or main filtration organs such as the liver. For systemic delivery to other organs including tumours, there are potentially other significant issues concerning tissue penetration, cell organisation, and access to cells of interest through the extracellular matrix that have not been addressed substantially in this review. These additional barriers are only just beginning to be thought about for synthetic non-viral vector systems and may be insurmountable. However, this will not become clear until extensive pharmacokinetic studies can be carried out with radioactive or specifically fluorescent-labelled **ABC** or **ABCD** nanoparticles. Therefore, chemical synthesis of bespoke probes for multiply-labelled nanoparticles,[102] is yet another area of chemical synthesis and innovation that could contribute significantly to synthetic non-viral vector gene therapy going forward, used in combination with *in vivo* studies and increasingly sophisticated *in vitro*/*ex vivo* cell model systems (spheroids and 3D cellular multilayers) designed to study these additional barriers to successful transfection in isolation.

Main abbreviations

DOPE	dioleoyl-L-α-phosphatidylethanolamine
Chol	cholesterol
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)-propane

DOSPA	2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate
DDAB	dimethyldioctadecylammonium bromide
DOGS	dioctadecylamidoglycylspermine
DC-Chol	3β-[*N*-(*N′*,*N′*-dimethylaminoethane)carbamoyl]cholesterol
CDAN	N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine
BGTC	bis-guanidinium-tren-cholesterol
DOTIM	1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride
SAINT	Synthetic Amphiphiles INTerdisciplinary
TMAG	*N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride
BCAT	*O*-(2R-1,2-di-*O*-(1′*Z*,9′Z-octadecadienyl)-glycerol)-*N*-(bis-2-aminoethyl)carbamate
GS11	Gemini Surfactant 11
5AMyr	1-(1,3-dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate
3AMyr	1-(2,3-dimyristoyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate
DODAC	dioleyldimethylammonium chloride
PEG-PE	*N*-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl]-DSPE
PEG	polyethylene glycol
DSPE	distearoyl-L-α-phosphatidylethanolamine
PEG-CerC_{20}	PEG-Ceramide bioconjugate with an arachidoyl acyl group
PEG-CerC_8	PEG-Ceramide bioconjugate with an octanoyl acyl group
POD	polyethyleneglycol-diorthoester-distearoyl glycerol conjugate
DSG	distearoyl glycerol
BVEP	(*R*)-1,2-di-*O*-(1′*Z*,9′*Z*-octadecadienyl)-glyceryl-3-(ω-methoxy-poly(ethylene) glycolate 5000)
pHMPA	poly-[*N*-(2-hydroxypropyl)methacrylamide]
CHEMS	cholesteryl hemisuccinate
pLL	poly-L-lysine
LD	lipoplex (cationic liposome/micelle–DNA complex)
LsiR	cationic liposome/micelle–siRNA complex
LPD	liposome:polycation:DNA (lipid:protamine:DNA)
LMD	liposome:mu:DNA
mu	μ peptide (of adenovirus)
MEND	multifunctional envelope-type nano device
STR-R_8	stearyl octaarginine
SPLP	stabilized plasmid-lipid particles
HVJ	hemagglutinating virus of Japan (Sendai virus)
PIL	pegylated immunoliposome
ODN	oligodeoxynucleotide
ON	oligonucleotide
pDNA	plasmid DNA
siRNA	small interference RNA
shRNAi	short hairpin interference RNA
BAC	bacterial artificial chromosome
YAC	yeast artificial chromosome
MAC	mammalian artificial chromosome
PAC	P1-derived artificial chromosome
huAC	human artificial chromosome
RES	reticulo-endothelial system
Tf	transferrin
TfR	transferrin receptor
EGFR	epidermal growth factor receptor
INSR	insulin receptor
f	folate
FR	folate receptor

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