Long-circulating vectors for the Systemic delivery of genes
David B Fenske*1, Ian MacLachlan2 & Pieter R Cullis1,3

Addresses
1Department of Biochemistry and Molecular Biology
University of British Columbia
2146 Health Sciences Mall
Vancouver
BC
Canada
Email: fenske@interchange.ubc.ca

2Provia Biotherapeutics
INEX Pharmaceuticals Corporation
100-8900 Glenlyon Parkway
Burnaby
BC
Canada
*To whom correspondence should be addressed

Keywords Gene therapy, lipoplexes, lipopolyplexes, liposomes, polyplexes, stabilized plasmid-lipid particles, systemic drug delivery

Introduction
Gene therapy was initially seen as a treatment for inherited genetic disorders by replacement of defective genes with functioning copies. This perspective has changed as current efforts focus on the development of genetic drugs capable of treating acquired diseases such as cancer and inflammation. The systemic nature of these diseases requires the development of gene delivery vehicles capable of accessing distal disease sites following systemic (intravenous) administration. Unfortunately, while numerous methods exist for effective in vitro gene delivery, current systems have limited utility for systemic applications. Viral vectors, for example, are rapidly cleared from the circulation, limiting transfection to 'first-pass' organs such as the lungs, liver and spleen. In addition, these systems induce immune responses that compromise transfection resulting from subsequent injections. In the case of non-viral vectors such as plasmid DNA-cationic lipid complexes (lipoplexes), the large size and positively charged character of these aggregates also results in rapid clearance. The highest expression levels observed with lipoplex systems are again in first-pass organs, particularly the lungs [1,2,3-6]. In addition, lipoplexes often give rise to significant toxicities both in vitro [7] and in vivo [8,9].

Progress towards achieving a gene delivery system for treatment of systemic disease has been slow: in this review we examine current approaches employing non-viral vectors, focusing on those systems with potential for systemic delivery. We report primarily on progress employing polymer and lipid-based systems over the past two years, as other reviews provide summaries of earlier material [10,11].

Properties required of a systemic gene therapy vector
The design features required of drug delivery systems that have systemic utility are becoming better defined. In particular, the carrier system must exhibit a long circulation time following intravenous injection. Unfortunately, most gene delivery systems, including viral vectors as well as non-viral vectors, eg, lipoplexes, polyplexes and lipopolyplexes, are rapidly cleared from the circulation and are preferentially taken up by the first-pass organs such as liver, lung and spleen. Here we review recent literature concerning the ability of non-viral vectors to act as systemic gene therapy agents. The most promising systemic vectors are liposomal systems in which plasmid DNA is encapsulated within a lipid bilayer. The stabilized plasmid-lipid particle (SPLP) system, for example, exhibits circulation half-lives of the order of 6 h following intravenous injection, and preferentially accumulates in distal tumors with gene expression primarily localized to the tumor site.

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Current vectors for in vivo gene delivery

Polymer-based delivery vehicles

Masmid DNA can be condensed into small particles (60 to 100 nm) by a variety of cationic polymer complexes, giving rise to polyplexes (plasmid DNA–cationic polymer complexes). Polyplexes formed from polylysine (PLL) and plasmid DNA are cleared from the circulation within 5 to 30 min, with the majority of DNA delivered to the liver. Rapid clearance of PLL polyplexes has been attributed to charge-mediated interactions with serum albumin and other proteins. One strategy to improve circulation times has been to coat the PLL/DNA complexes with a multivalent hydrophilic polymer (poly-N-(2-hydroxypropyl)methacrylamide) or pHPMA that prevents protein interaction, and reduces uptake by macrophages in vitro. Unfortunately, these systems have limited systemic utility since the circulation lifetimes of the pHPMA–PLL/DNA polyplexes are even shorter than observed for PLL/DNA. Both systems are taken up by the liver. Attachment of a targeting ligand (transferrin) to pHPMA–PLL/DNA polyplex increases transfection of K562 cells some 10-fold, but in vitro studies have not been performed.

Polyplexes have been formed from a variety of other polymers, examples of which include polyethylenimines (PEI), galactosyl-PLSP (a lysine-serine copolymer with specific properties required for systemic delivery). Lipopolyplexes are formed from polyplexes and preformed liposomes. As with polyplexes and lipoplexes, the lipopolyplexes have potential for in vivo gene delivery to specific tissues, but do not possess the pharmacokinetic properties required for systemic delivery. Lipopolyplexes, which exhibit diameters in the range of 50 to 300 nm, are often smaller than lipoplexes, but it is difficult to assess whether this impacts circulation lifetimes as this parameter is rarely reported. One system composed of (PC/PE/Chol)/PlA/DNA exhibited a circulation half-life of 16 min. In terms of biodistribution, lipopolyplexes accumulate in lung, heart, spleen, liver and kidneys. Gene expression has been observed in those tissues for a lipopolyplex vector made from DOTAP/protein/DNA. Interestingly, lipopolyplexes containing the fusogenic ‘helper’ lipid DOPE yielded less gene expression than comparable systems containing cholesterol. GFP gene expression in liver was observed 24 h after injection of a lipopolyplex formed from acetylated PEI/DOPE liposomes and plasmid DNA.

Plasmid DNA encapsulated in liposomes

A variety of techniques have been described for encapsulating DNA within liposomes, but most of these give rise to systems with low DNA-to-lipid ratios and sizes in the range of 100 to 500 nm. The major challenge is to efficiently encapsulate plasmid in small liposomal systems. Efficient encapsulation of small molecules is increasingly straightforward, however, encapsulation of macromolecules such as plasmids presents a much more difficult challenge. Recently, Shangguan et al. described a method for condensing plasmid with spermine, which was then encapsulated within a novel N-acyl phosphatidylethanolamine-containing delivery vehicle (N-C12-DOPE/DOPE liposomes). The resulting particle diameter was approximately 200 nm.
These systems, transfected OVCAR-3 cells in vitro at levels comparable to lipoplexes. Unfortunately, in vivo biodistribution or transfection data were not reported. Bailey and Sullivan [34**] have achieved efficient encapsulation of plasmid DNA in small neutral liposomes, employing a protocol utilizing ethanol and calcium with subsequent removal of ethanol by dialysis.

These neutral lipid complexes (NLCs), composed of plasmid DNA encapsulated in vesicles of DOPE, DOPE:DOPE and DOPE:DOPE:Chol, have circulation lifetimes > 1 h. To date, neither in vitro nor in vivo transfection potencies have been reported.

Targeted transfection in the brain has been achieved using neutral PEGylated immunoliposomes containing plasmid [35**]. The plasmid was encapsulated within vesicles prepared from POPC, DDAB and DSPE-PEG, by a procedure in which a mixture of plasmid and sonicated vesicles were freeze-thawed and extruded, with subsequent removal of external DNA by nuclease digestion. A monoclonal antibody directed against the transferrin receptor was then attached to the liposome surface. The resulting particles have a diameter of the order of 70 nm, but a circulation lifetime of only 5 min. Nevertheless, the presence of the transferrin mAb increased uptake in the liver and brain, with gene expression observed in the liver, lung, spleen and brain that peaked at 48 h.

Liposomal DNA carriers known as stabilized plasmid-lipid particles (SFLP) have been developed and characterized in the last two years. SFLP are small (70 nm) and consist of plasmid DNA encapsulated within a lipid bilayer composed of DOPE, a cationic lipid (usually DODAC) and PEG-ceramide (PEG-Cer) [31**]. SFLP are formed by a procedure in which mixtures of plasmid and lipid are co-solubilized in the detergent octyl-glucopyranoside (OGP), which is then removed by dialysis. When conditions are optimized, high plasmid encapsulation efficiencies can be achieved (50 to 70%). Unencapsulated DNA is removed by DEAE-Sepharose chromatography, and empty vesicles are removed by sucrose density gradient centrifugation. The resulting SFLP are small, monodisperse particles of ~ 70 nm diameter that consist of plasmid encapsulated in a unilamellar lipid vesicle [9**]. The plasmid:lipid ratio of the purified SFLP is 62.5 μg/μmol [31**], corresponding to one plasmid per SFLP [9**,31**]. SFLP provide protection of plasmid DNA from DNase I and serum nucleases [31**], and are highly stable in serum.

The pharmacokinetics, tumor accumulation and transfection properties of SFLP composed of DOPE/DODAC/PEG-Cer (83:7:10)[9**,31**], have been extensively characterized in several mouse tumor models. Following intravenous injection into mice bearing subcutaneous Lewis lung carcinomas, the circulation half-life of SFLP was 6.1 ± 1.1 h and 7.2 ± 1.6 h, as assessed by lipid and DNA markers, respectively [9**]. Lipoplexes examined under identical conditions had circulation half-lives < 15 min [9**]. A representative example of the clearance of SFLP from the circulation of neuro-2a tumor-bearing mice following a single intravenous dose of SFLP is shown in Figure 1 [36]. Note that 10% of the injected dose is still present in the circulation 24 h after injection.

Accumulation of SFLP at distal tumor sites and gene expression at those sites has been observed in mouse tumor models following intravenous injection. Studies employing the subcutaneous Lewis lung carcinoma revealed the presence of SFLP in liver, plasma and tumor at 24 h after injection, with little found in the lung and spleen [9**]. In contrast, lipoplexes were located almost exclusively in the liver. Approximately 3% of the SFLP dose (~ 1000 copies per cell) was found at the tumor at 24 h, as contrasted with < 0.2% of the lipoplexes dose. Naked DNA and lipoplexes gave rise to no gene expression at the tumor, whereas SFLP gave significant gene expression peaking at 48 h. Further, SFLP elicited no toxic side effects at dose levels as high as 100 μg DNA per mouse, whereas lipoplexes resulted in raised liver enzyme levels at doses > 20 μg per mouse.

The accumulation of SFLP-associated plasmid DNA at a distal neuro-2a tumor site following intravenous injection is shown in Figure 2 [36]. The amount of intact plasmid delivered to the tumor is substantial, corresponding to > 10% of the total injected dose per gram of tumor at 24 h. This leads to significant levels of gene expression at the tumor at 24 h, which reaches a maximum 72 h after injection (Figure 3) [36]. It is striking that the highest luciferase activity is located in the tumor, with other tissues giving only low levels of transfection. At the later time points, the transfection levels in the tumor are two orders of magnitude greater than in other tissues. These results confirm that long-circulating liposomal vectors are capable of preferential disease-site targeting and gene transfer.
The levels of gene expression achieved at a distal tumor site following intravenous injection of SPLP are encouraging. However, some therapeutic proteins may require higher levels of expression to achieve therapeutic benefits. In this regard, the transfection potency of SPLP systems can be altered by employing different cationic lipids [37]. Transfection potencies can also be enhanced by raising the cationic lipid content of the SPLP, which can be achieved by inclusion of citrate [38] or phosphate [39] buffer in the dialysis medium. Further enhancements can be achieved by using PEG coatings that dissociate from the SPLP over time. The presence of the PEG coating which is required for SPLP formation, also inhibits interactions with cells, and thus uptake into cells is enhanced if the PEG molecules dissociate over time. The dissociation rates of PEG-Cer molecules from lipid vesicles can be adjusted by changing the length of the N-acyl chain on the ceramide anchor. For example, PEG-CerC, will dissociate with a half-life of < 1.2 min [40], whereas for PEG-CerC and PEG-CerC, the dissociation half-lives are 1.2 h and > 13 days, respectively [31]. It was found that SPLP containing 24% DODAC and PEG-CerC gave optimized levels of transfection in COS-7 and HepG2 cells in vitro [38]. The transfection properties of these systems following intravenous injection have not yet been reported.

Future directions

In summary, most gene delivery vectors do not possess the basic pharmacokinetic properties required for systemic applications. Polyplexes, lipoplexes and lipopolyplexes all have potential for gene delivery to organs such as lung, liver and spleen, but their application to systemic diseases requires modifications, such as PEGylation, to enhance the circulation lifetimes of these particles. The most promising systems are those where plasmid DNA is encapsulated within a lipid bilayer. The best characterized of these is the SPLP, which possesses long circulation lifetimes and which preferentially delivers plasmid to distal tumor sites following intravenous injection, with associated gene expression. Furthermore, this system is flexible, and enhanced levels of expression may be achieved by modifying the lipid composition. Alternatively, the use of PEG-Cer molecules with optimized dissociation rates may result in enhanced in vivo activity. The PEG-Cer needs to remain with the SPLP long enough to allow accumulation at a site of disease, and then dissociate, leading to particle destabilization and enhanced uptake into cells. Finally, the inclusion of targeting ligands that enhance uptake into target cells may also be expected to enhance gene transfer.

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References

- of outstanding interest
- of special interest


* Describes formation and characterization of SPLP.


