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Synthethic gene transfer vectors

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Abstract. Gene transfer into mammalian cells is a prerequisite to gene therapy. Designed synthetic DNA carriers could be attractive alternatives to presently used viral vectors. Toward this end, lipopolyamines have been developed, which spontaneously condense DNA and coat it with a cationic lipid layer. The resulting nucleolipidic particles transfect efficiently various eukaryotic cells.

Gene therapy is "à la mode" and extends well beyond its own research fields, as is evidenced by special issues of multidisciplinary journals and frequent headlines in the media. This widespread interest has a number of origins. Most significantly, gene therapy is a conceptual revolution: for the first time DNA is considered as a drug, providing a general framework for curing (and not only treating) thousands of hereditary diseases. But gene therapy is also a new type of weapon in the fight against acquired diseases of larger social incidence, either multigenetic disorders such as cancer, or those resulting from foreign viral genes. Besides these concrete reasons, it also satisfies one of the medicine's greatest dreams: molecular surgery at the root of a disease. Clinical attempts to transfer genes into humans have already begun ("Immunotherapy of patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction"[1]).

Broadly speaking, gene therapy includes several approaches: molecular replacement of a mutated gene (by homologous recombination), addition of an extra gene resulting in the synthesis of a therapeutic protein, and transcriptional modulation of endogeneous cellular (or possibly viral) gene expression by drugs. In this last approach, it has been amply demonstrated since 1987 that some double stranded DNA sequences can be recognized through triple strand formation with synthetic oligonucleotides[2], or through strand replacement with entirely non-natural compounds[3]. While chemistry leads in this domain, it is almost absent from gene replacement and addition therapies, where the "drug" is DNA; yet here the power of organic synthesis could help creating artificial drug carriers. Indeed, these therapies rely on gene transfer (transfection), i.e. harmless introduction of the gene of interest into cells (figure 1). Most current vectors are engineered (recombinant) viruses[1] that involve complex technologies and suffer from intrinsic limitations (see below). Synthetic gene transfer vectors, although probably less efficient than viruses, do not raise the problems of working with biological and potentially infectious vectors.

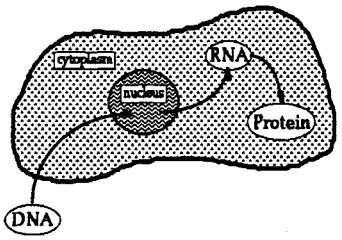


Figure 1. Gene transfer into an eukaryotic cell: a polyanionic macromolecule (DNA) is carried across the cytoplasmic lipid membrane, the nuclear membrane and finally is expressed into the corresponding protein.

Gene therapy is recent and is just coming out of age. However gene transfer techniques are also among the most powerful tools of cell biology research (ranging from the study of intracellular gene/protein function and regulation to that of complex processes such as embryogenesis). Moreover, gene transfer has profound economic implications as a prerequisite to genetic engineering of microorganisms, plants and animals (for crop and livestock improvement)[4]. It is therefore not surprising that many gene transfer techniques have been devised since the early seventies, when gene manipulation emerged from research laboratories. Before reviewing the most common techniques, it is worthwile considering how this problem has been solved in evolution.

Natural gene transfer is a highly complex process

Human fertilization has been studied in much detail for obvious reasons. Encounter of the parental genomes is more than pleasant, it is also a multistage, extremely complex process which prevents interspecies fertilization and polyspermy. Unfortunately, the key gene transfer step is the least well understood: after receptor-mediated lateral binding of the sperm head to the egg cytoplasmic membrane, a fusogenic sperm protein (and possibly acrosomal enzymes) help the membranes to merge. The sperm genome, which is highly compacted by polycationic proteins (protamins), can then penetrate into the egg.

Viral infection is another example: viruses are small (ca. 1000 Å) nucleic acid-containing particles, which need the cellular machinery for multiplication (figure 2). Infection begins by multiple binding to a cell surface receptor (protein or glycolipid) which triggers entry by direct membrane fusion, or alternatively by endocytosis and subsequent fusion-mediated escape. Although the structures of several fusogenic proteins are known, the molecular mechanism of fusion is poorly understood, mainly because it is a very short-lived event. Viral genomes, like sperm genomes, are compacted by cationic proteins and by polyamines such as spermine, in order to fit into the nucleocapsid.

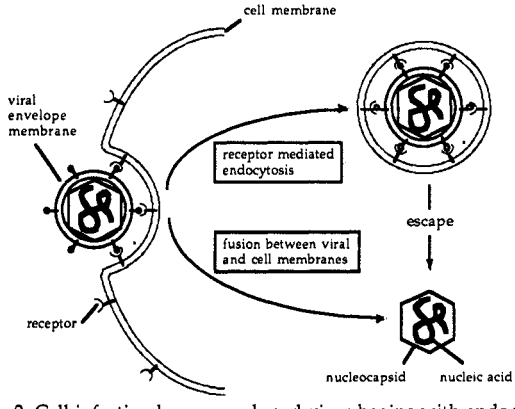


Figure 2. Cell infection by an enveloped virus begins with endocytosis or direct membrane fusion.

Thus, natural systems devoted to gene transfer have highly compacted silent genomes, and show specific cell surface binding that triggers penetration through several possible pathways, all of which involving the rupture of a lipid membrane.

Gene transfer techniques

The simplest method to introduce DNA into a cell is direct mechanical microinjection. However, for practical reasons, injection is only used to transfect eukaryotic germline cells for production of transgenic species. Other gene transfer techniques are indirect and variously employ biological vectors (recombinant viruses) or model liposomes[5], DNA coprecipitation with polycations, cell membrane perturbation by chemical (solvents, detergents, polymers, enzymes) or physical means (mechanic, osmotic, thermic, electric shocks). These techniques are of variable efficiency and complexity, and none could claim to cover the large range of cell types and environmental conditions encountered. However, for a given task, some techniques have become more popular than others .

Gene therapy relies almost exclusively on *recombinant viruses* (essentially retroviruses) which carry the gene of therapeutic interest into cells by the mechanisms shown above (figure 2); they are far more efficient than artificial vectors. The use of a biological carrier however raises two problems: to encapsulate the newly engineered genome into an empty viral particle and to prevent the new viruses from becoming infectious. Encapsidation is performed in genetically transformed animal cells maintained in culture and is complex and costly. Apart from the long term safety risks already mentioned, retroviral vectors also have other limitations: virus concentrations are less than ca. one million particles per ml (by comparison, one microgram of plasmid DNA/ml is a millionfold more concentrated); the retroviral genome is small (less than 10^4 nucleic base pairs = 10 kbp), hence not more than 5 kbp of foreign information can be added. A search for larger viral vehicles is ongoing[6].

In comparison with biological vectors, transfection by *DNA coprecipitation with calcium phosphate or cationic polymers*[7] looks amazingly simple. Although historically independent, these techniques share similar mechanisms and advantages, and have quickly become the most popular ways of introducing DNA into cells of various origins. They consist of the formation of a finely divided precipitate of polyanionic nucleic acid with calcium phosphate or with a commercial high molecular weight cationic polymer (e.g. diethylaminoethyl-dextran or polybrene, a linear polymeric quaternary ammonium salt). The cationic precipitate is "eaten" by the cells (phagocytosis). The success of these techniques comes from simplicity and low cost, although they are neither very efficient nor reproducible, and often toxic to the cells. Efficiency is improved when cells are incubated in presence of glycerol or DMSO. The coprecipitation method is essentially restricted to the *ex vivo* transfection of phagocytic cells.

Electroporation (figure 3) is based on the finding that when a strong electric field (typically kilovolts/cm) is applied to a cell suspension for a few microseconds, some regions of the cytoplasmic membrane undergo a slowly reversible breakdown, transiently allowing DNA to enter the cells[8]. Electroporation has become the method of choice *in vitro*, especially for cells which are resistant to the calcium phosphate technique. Many apparatuses have been designed, but the technique remains tricky to optimize (figure 3, right) as the electric shock must be strong enough to perturb most of the cells, yet leave them viable.

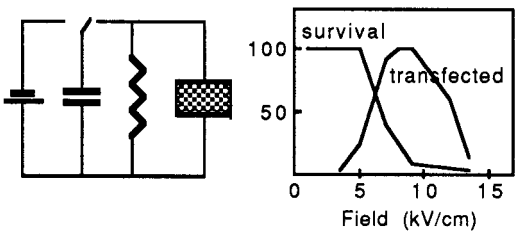


Figure 3. Left: Principle of electroporation of cells in suspension. Right: typical profiles of percent cell survival and cells transfected (among the surviving), as functions of field strength.

The *particle gun* represents the most recent physical transfection technology[9]: micronsize tungsten or gold particles are coated with DNA and propelled onto cells (figure 4). High velocity microprojectiles reach tissues such as liver *in vivo* , and also seem very promising for use on eukaryotic cells that possess walls such as plant cells.

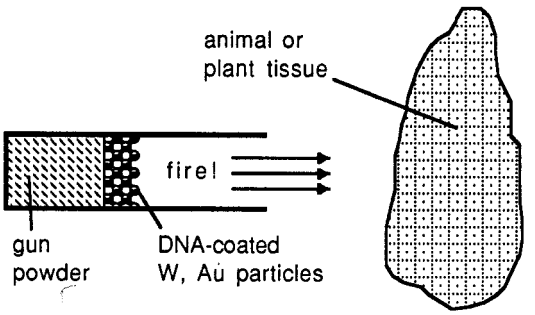
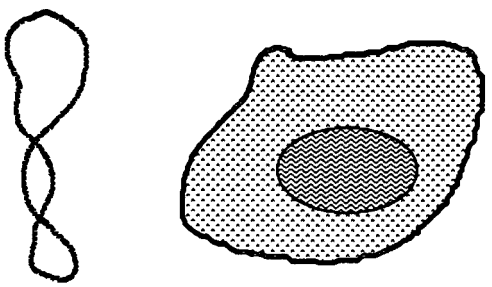


Figure 4. The particle gun technology.

Synthetic gene transfer vectors

In the the numerous gene transfer techniques mentioned previously, the most elaborate compounds which appear are commercial solvents (DMSO, glycerol) and polymers (polyethyleneglycol, polybrene). Surprisingly, despite the creative power of chemistry, there has been no rational design of artificial gene transfer vectors until recently. Within a few years, several groups reported the synthesis of molecules capable of compacting DNA and transfering it into cells[10-21]. Indeed, juxtaposition of a DNA molecule with an eukaryotic cell leads to the following common sense remarks with regard to transfection:



-plasmid DNA and the potential recipient cell are of similar size. Nature (see above) and spaghetti lovers both solved this contents/container puzzle in a similar fashion, namely compaction prior to ingestion.

-a macromolecular polyanion (typically 10^4 charges) will not spontaneously cross an intact lipid membrane, nor will it even bind to the negatively charged cell surface. Therefore a synthetic vector should not only condense DNA, but also mask its anionic nature. Furthermore, it should bind it to the cell surface in such a way as to trigger membrane destabilization or endocytosis.

Two classes of gene transfer agents have been designed along these lines (figure 5): hemisynthetic polypeptides[12,15,17], where a DNA-binding polycationic peptide (protamin, polylysine) is chemically linked to another protein (asialoorosomucoid, ferritin, insulin) whose recognition by a given cell surface receptor leads to active endocytosis; synthetic cationic lipids[10,11,13,14,16,18,19,21], where hydrophobic effects provide a kind of cationic double-faced sticky tape to link the anionic DNA and cell surface together.

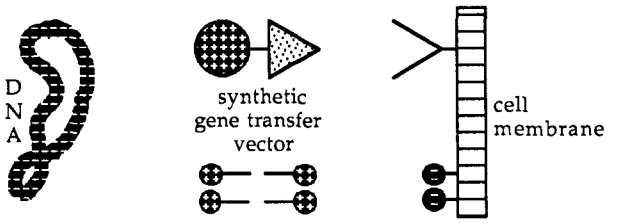


Figure 5. Current synthetic gene carriers are chimeric bifunctional proteins (upper) or cationic lipids (lower) able to compact DNA and bind it to the cell surface.

Polylysine-linked proteins carry DNA into cells by receptor mediated endocytosis

Oppositely charged polymers such as DNA and polylysine condense each other into neutral soluble particles which should not bind nor enter efficiently into cells. However, the cationic polypeptide can be linked (usually through a sulfur bridge) to a protein which naturally enters a cell by endocytosis, and thus compacted DNA may be cotransfered with the protein. The corresponding receptor may be shared by many cell types as part of their general metabolism (e.g. ferritin[15] which is an iron carrier), or alternatively DNA may be targeted to special cells which degrade asialoglycoproteins[12] or respond to insulin[17]. In any case, the nucleic acid must escape the degradative pathway of endosomes, as in the coprecipitation method discussed above. This has been shown for transferrin-polylysine gene delivery which is only efficient in presence of lysosomatropic agents[22]. However, when the complexes are linked to inactivated adenoviral particles which help them to escape from endosomes, transfection is enhanced by orders of magnitude[23]. This latter technique, although not straightforward, is among the most efficient to date.

Lipospermine-mediated gene transfer is very efficient when the nucleolipid-particles bear a net positive charge

The smallest natural polycations able to compact DNA are the polyamines spermidine and spermine; this interaction is however quickly reversible in physiological conditions. *Lipopolyamines*, i.e. amphiphiles with a self aggregating hydrocarbon tail linked to a cationic DNA-binding headgroup, have been shown to stably condense nucleic acids into discrete nucleolipidic particles[10] which may be further coated with an excess lipid layer (figure 6).

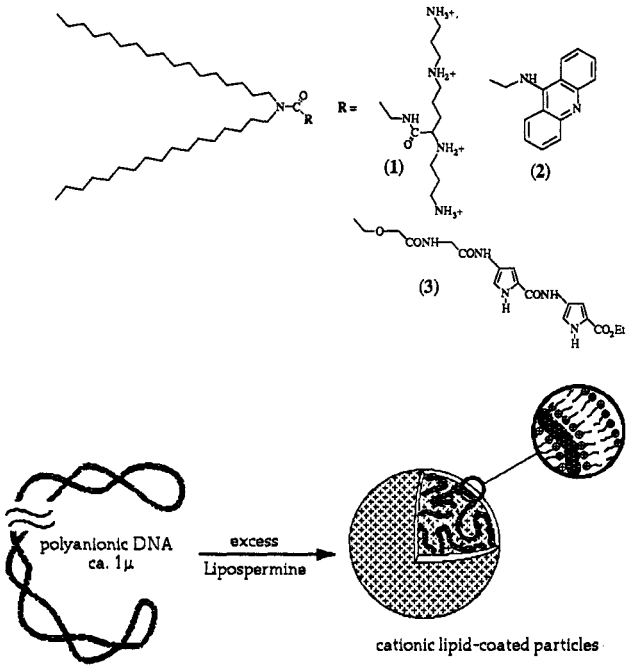


Figure 6. Upper: structure of a lipospermine (1), and related acridine (2) and netropsin (3) analogs; lower: lipospermine induced condensation of plasmid DNA.

Such polycationic particles, where the DNA charge has been reversed, bind cooperatively to anionic residues on the cell surface (figure 7)[13]. Lateral diffusion and cell deformability result eventually in their zipper-like engulfment. Such a spontaneous process may lead to endocytosis or direct membrane fusion at the most curved edges, both mechanisms formally reminiscent of viral entry[24]. The intracellular fate of the nucleolipidic particles is unknown, but some of them must end up in the cytoplasm, reach the nucleus and become at some stage uncoated, since active exogene transcription is observed.

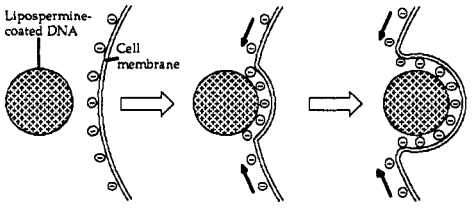


Figure 7. Spontaneous 'zipper' engulfment of a rigid cationic particle.

This view is supported by recent electron microscopy work and is consistent with the following indirect observations:

- Cationic lipid-mediated gene transfer is efficient only when the particles bear a strong positive charge (figure 8), irrespective of the individual charge of the lipid[13].
- Lipospermines with a single hydrocarbon chain have been synthesized. They form micelles instead of bilayers and still condense DNA. However they do not provide a cationic surface for interaction with the cells and as a result no transfection is observed.

- Lipids with other strong DNA-binding headgroups (**2** intercalates between base pairs, **3** binds into the DNA minor groove; see figure 6) are unable to transfect cells.

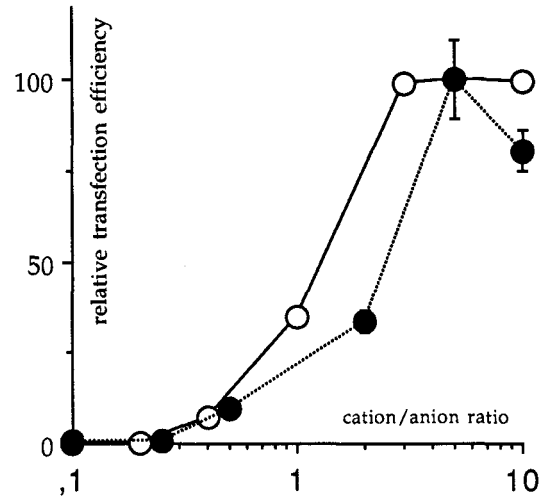


Figure 8. Gene transfer efficiency as a function of the mean ratio of cationic lipospermine over anionic DNA charges. 3T3 rat fibroblasts (open circles) or rat cerebellar neurons (filled circles) were transfected with a plasmid containing the bacterial chloramphenicolacetyl-transferase (CAT) gene. This gene is absent from mammalian cells, so determination of CAT activity in cell extracts 48h post transfection provides a convenient measure of gene transfer level.

More speculative arguments go along the same lines:

- Lipospermines, as well as other cationic lipids able to transfect cells, do not form stable bilayer structures. They may therefore locally destabilize cellular membranes and help the particles to reach the cytoplasm.

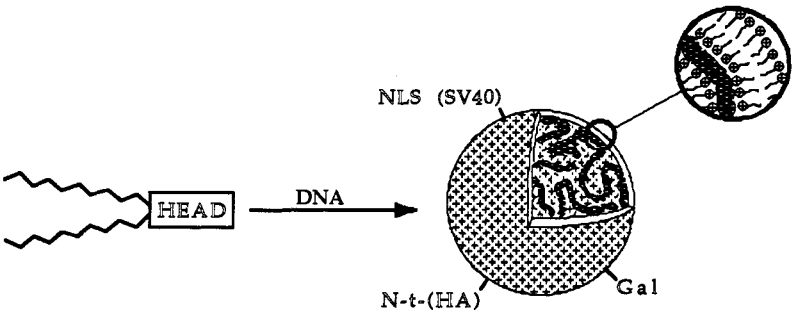
- Nuclear localization signals borne by endogeneous nuclear proteins and viral capsids contain an exposed stretch of at least five cationic aminoacids which could make them to accumulate in the nucleus after nuclear pore crossing and ionic binding to genomic DNA until free concentrations are equal. Positively charged nucleolipidic particles could be caryophilic for similar reasons, provided their size is compatible with nuclear pore crossing.

Cationic lipids are attractive alternatives to classical *in vitro* techniques

When compared to the popular calcium phosphate method, transfection with cationic lipids is as straightforward (requiring only mixing of components), yet it is far more efficient. This new technique has a major advantage of being applicable to almost all animal cell types, as it is based on non-specific ionic interaction (figure 7). It is also of low toxicity, provided the chemical carrier is designed to be biodegradable. Therefore cells resistant to classical techniques, as well as fragile cells of various origins, can be efficiently transfected *in vitro*[25] (neurons, keratinocytes, lymphoïd cells). Recently several synthetic cationic lipids have been commercialized for such gene transfer purposes.

Future improvements with cationic lipids could come from a *modular transfection system* where each molecular component would be responsible for a key step of viral infection. Such a system would still be based on a neutral lipopolyamine-DNA core particle to which other synthetic lipids could add new properties *via* their headgroups:

Lipid Headgroup	Property	Function
$Sper^{4+}$	DNA condensation	core particle
$(Gal)_n$	receptor binding	targeting
(N-t-HA) peptide	fusogenic	cytoplasm entry
(NLS-SV40) peptide	nuclear localization	caryophily



A lipid with a given oligo-peptide (or saccharide) headgroup should direct the nucleolipidic particle to the desired cell surface receptor; a lipid with a virus derived fusogenic peptide headgroup could help DNA to enter the cytoplasm; similarly, a lipid bearing a nuclear localization signal would provide nuclear tropism to the core particle. Thus, upon adding such a lipid mixture to DNA, the lipopolyamine component would bind to and condense it, leaving at the surface of the particle various signals for its trafficking toward the cell nucleus: a multi-ingredient soup which self-organizes into a programmed supramolecular device. Such improvements await further experimental work, but it may well be that the high transfection efficiency already observed with lipopolyamines alone is due to a unique (and fortunate) combination of properties such as DNA protection against nucleases, cellular membrane destabilization and caryophily.

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(6) Currently, adeno and herpes viral vectors are being developped for this purpose. Why is there such pronounced interest towards the introduction of large pieces of genetic information into cells? Eukaryotic genes have a mosaic structure where the protein-coding information (exons) is interrupted by long stretches of non-coding nucleotides (introns). On the other hand, gene transcription is controlled by protein binding to defined nucleotide sequences, some of which may be very distant from the coding region. Regulation and stability of an exogene in a living context requires the entire gene, typically over 10^5 base pairs long.

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