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Liposomes: from membrane models to gene therapy

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Abstract: Liposomes, which are self-closed vesicular structures composed of (phospho)lipid bilayers, have attracted considerable interest since their discovery in the 60's. Because of their organization and the versatility of their physicochemical properties these vesicles have been extensively studied as models for biological membranes. Since liposomes can sequester bioactive molecules, they are also widely used as drug delivery systems. The present report focuses on the renewed interest in the field with the advent of "sterically stabilized" (Stealth®) liposomes which, compared to "conventional" liposomes, have much longer circulation times *in vivo*, and on the use of cationic liposomes in non-viral gene delivery strategies. Liposomes are also very promising antigen carriers that can be used to design peptide-based synthetic vaccines.

INTRODUCTION

Liposomes are spherical structures composed of single or multiple concentric bilayers resulting from the self-assembly of amphiphilic molecules, such as phospholipids, in an aqueous medium. The polar head groups are located at the surface of the membranes, in contact with the medium, whereas the fatty acid chains form the hydrophobic core of the membranes, shielded from the water. These vesicles entrap in their interior volume part of the water phase and, consequently can capture and segregate polar molecules; moreover, because of the physicochemical properties of their constituents, they can also dissolve hydrophobic molecules in their bilayers. Liposomes can be prepared in many different sizes, ranging from small unilamellar vesicles (SUV's), whose smallest dia. are about 20 nm, to giant unilamellar vesicles (GUV's) up to tens of μm in dia. In between are the multilamellar vesicles (MLV's); i.e. the first generation of liposomes (1), of several hundreds of nm in dia., and the more recent large unilamellar vesicles (LUV's), characterized by high capture volumes, whose dia. can be adjusted (e.g. 100 or 200 nm) and size distribution narrowed-down by extrusion through specific membranes (2). In essence, liposomes are highly versatile structures whose properties can be modulated by changing number of parameters such as size, lamellarity, composition of the bilayers, surface charges and surface properties; for the chemist the (phospho)lipids which are the constituents of liposomes are also challenging molecules for designing analogs endowed with new properties and derivatives that are useful e.g. for coupling ligands to the surface of the vesicles. Consequently, liposomes have attracted an enormous interest (TABLE 1) : *i*) in basic research (chemistry and biology) overwhelmingly as membrane models, but they also offer attractive possibilities such as confining chemical reactions in very small volumes, *ii*) as vehicles for drug delivery and recently for gene transfer, *iii*) in biotechnology and pharmaceutical industry (development of antitumor drugs and liposome-based vaccines as well as cosmetics). The liposome community, which has generated a sizable amount of publications, is characterized by an unusual attraction for editing books in which the interested reader will find reviews on most aspects of this highly diversified field (3-8). In this presentation we will focus mainly on three domains in which liposomes have recently made a significant progress towards applications and/or provided important tools for new technologies :

- Stealth® (or sterically stabilized) liposomes
- Liposomes as carriers for antigens
- Cationic liposomes in gene transfer

Due to limited space we will not address the important issue of the mode of preparation and characterization of liposomes. Many techniques exist that are well validated, ranging from laboratory to industrial scale

(phospho)lipids derivatized with polyethylene glycol (Mr ~ 2 - 5 kDa). The major consequence is a profound change in the pharmacokinetic behavior of the SL. Whereas the half-life of conventional liposomes in blood, e.g. after i.v. injection, can be as short as 2 hours, the clearance rate of the corresponding SL is much reduced and some preparations have circulation $t_{1/2} > 40$ hours in humans. This corresponds to a *major breakthrough* in the application of particulate carriers for drug delivery. An unpredictable but far reaching discovery of the slower clearance of the SL by the RES *in vivo* is the so-called "enhanced permeability and retention" (EPR) effect. Long-circulating liposomes were found to accumulate in tumors and this can be used to increase dramatically the bioavailability of antitumor drugs in *e.g.* solid tumors. The EPR effect seems to be due to the prevalence of a "leaky" vasculature and to a limited lymphatic drainage in many tumors. This effect occurs also, to some extent, in areas of infections and inflammations. Consequently, SL which are captured more slowly by the liver and spleen, are passively targeted to tumor sites. For example, in tumor-bearing animals up to 25-fold higher liposomes can be found in the tumor several days after injection, i.e. up to 10% of the injected dose (10). Another consequence of this phenomenon is the use of SL for imaging. It should be mentioned that SL carrying doxorubicin (Doxil®), is presently developed by Sequus Inc..

The properties of SL, in terms of long circulation time and extravasation, have of course raised the possibility of the "active targeting" of these vesicles. This was done by coupling e.g. monoclonal antibodies at the extremity of long PEG spacer arms to avoid steric hindrance in the antigen/antibody recognition step. An interesting example of the coupling of a small molecular weight ligand to PEG was published by the group of Low (11,12). Because receptors of folate are frequently overexpressed at the surface of epithelial cancer cells this group has developed techniques that allow the conjugation of folic acid to SL (Fig. 2); they showed that only a long PEG spacer (250 Å) was able to mediate a ligand-specific endocytosis of the targeted liposomes by KB cells. Up to 50-fold greater quantities of doxorubicin could be delivered *in vitro* into cells by the targeted SL compared to non-targeted SL. Similar strategies have also been followed successfully with immunoglobulin fragments (13). Such experiments undoubtedly pave the way to an increased use of targeted SL *in vivo*.

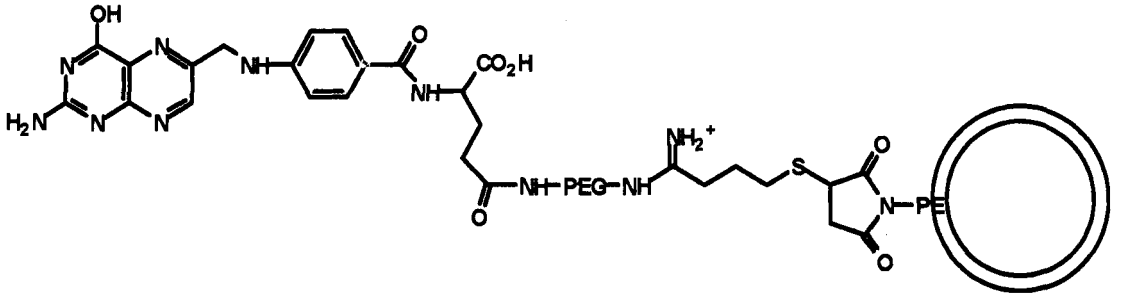

Fig. 2 Targeted liposome. Folic acid was conjugated to the surface of liposomes at the extremity of a PEG spacer-arm (length : 250 Å) to interact with tumor cells that overexpress the folate receptor (11,12).

LIPOSOMES AS CARRIERS OF PEPTIDE ANTIGENS - VACCINE DESIGN

The so-called "subunit vaccines" represent an important aspect of the modern approaches of vaccination, which include also recombinant viruses and microorganisms, genetic immunization, anti-idiotypic antibodies, etc. They are based on the utilization of isolated antigens or fragments of antigens (polysaccharides, (glyco)proteins, peptides, glycolipids). Among the many strategies that are available, the possibility to construct peptide-based vaccines is well acknowledged and is particularly appealing from an immunological viewpoint (14). Thus, peptides that mimic epitopes of pathogens, which can be synthesized with high reproducibility and purity (pathogen free), can be designed to induce well-defined monofunctional immune responses (i.e. B, Th, Tc epitope responses). The possibility to select the peptides allows the avoidance of unwanted immune responses and makes possible to orient the response toward an epitope that might otherwise be subdominant (e.g. in the natural protein). Multi-epitopic constructs can also be envisaged that contain for example protective and suppressive epitopes from the same and/or different pathogen(s).

The reader is again referred to references (3-8).

TABLE 1. Selected applications of liposomes

Biophysics : permeability, osmolarity, phase-transitions of membranes
Physical chemistry : colloid sciences, materials science (biocompatible surfaces),...
Chemistry : catalysis, compartmentization of reactions
Biochemistry/Biology : membrane fusion, reconstitution of membrane-associated proteins - functional studies,...
Pharmaceutics/Medicine : (targeted) drug delivery systems, vaccines, transfection vectors, medical diagnosis (imaging, immunoassays),...
Industrial : cosmetics, food industry, paints
Industrial products of pharmaceutical liposomes : AmBisome®(Amphotericin B; Nextar); Doxil® (Doxorubicin, SEQUUS),....

STEALTH® LIPOSOMES AND (TARGETED) DRUG DELIVERY

The use of "conventional" liposomes, especially MLV's, as drug delivery systems is largely hampered by two main factors. The first one is the important tropism of these vesicles for the reticuloendothelial cell system (RES), i.e. mainly for macrophages which, in organs such as liver and spleen, clear the organism of circulating particles including, of course to our benefit, microorganisms. This efficient uptake of liposomes by phagocytosis, which seems largely attributable to the surface coating of the vesicles by serum factors (opsonization), is certainly at the origin of the common belief that liposomes are just good to be "gobbled-up by the liver". This tropism can somewhat be alleviated by use of smaller neutral vesicles, whose bilayers are in the gel phase at the body temperature. It should be noted, however, that the preferential uptake of large liposomes by macrophages can also be turned into a benefit ("passive targeting") in numerous cases where these cells are of importance; i.e. treatment of intracellular infectious diseases due to microorganisms or parasites, immunostimulation, etc...(3-8). Nevertheless, the limited *in vivo* circulation time of "conventional" liposomes severely restricts their use in more ambitious strategies such as targeted drug delivery where ligands, e.g. monoclonal antibodies, conjugated to the surface of the vesicles provide an interaction with specific target cells. The second factor which is potentially responsible for the limited extravasation of these vesicles is their size; for example, only liposomes of dia. $\leq$ 150 mm can escape the vascular system in organs where the endothelial lining of the blood vessels is fenestrated.

For these different reasons the advent of "sterically stabilized" liposomes (SL) raised renewed hopes for the feasibility of using liposomes as drug delivery systems (9). SL, also called Stealth® liposomes, are vesicles whose surface properties have been altered in such a way as to hinder opsonization phenomena (Fig. 1). This is reached by incorporating into the composition of the liposome small proportions ($\leq$ 5 mol%) of

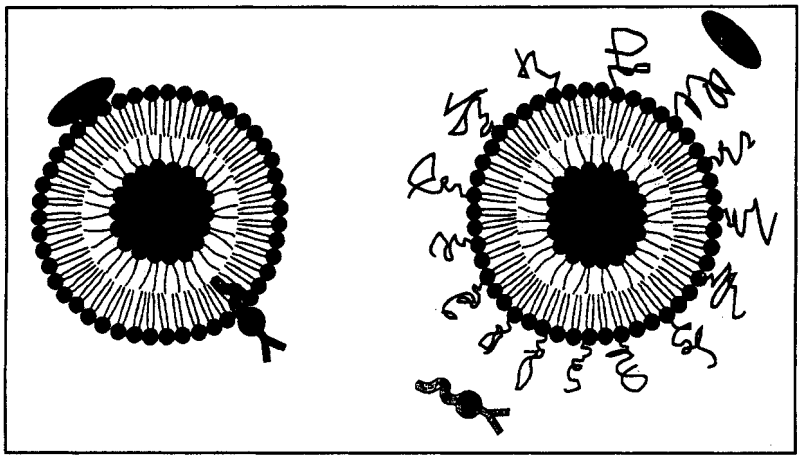

Fig. 1 Interaction between opsonization factors with conventional (left) and Stealth® liposomes (right). (From D.D. Lasic. *Am. Sci.* **80**, 20 (1992); with permission)

However, despite their interest, the impact of peptides in the development of effective means for the prevention of infectious diseases was, so far, somewhat limited. This is mainly due to the low immunogenicity of peptides and to the paucity of safe and powerful adjuvants, that are accepted for humans. The low immunogenicity of small peptides results from two main problems : i) they often act as haptens, i.e. as molecules that contain a B-lymphocyte epitope but lack a T-helper (Th) lymphocyte epitope; ii) their uptake and presentation by antigen presenting cells (APC) is often too limited to be efficacious. Classical solutions involve the coupling of the peptides to carrier proteins which offer Th-epitopes and an increased uptake by APC's. Very often this coupling step is poorly controlled from a chemical standpoint, and this of course offsets the benefits of using chemically well-defined peptides. Moreover, the carrier proteins introduce unrelated B-epitopes that might lead to carrier epitopic suppression. More interesting from a vaccination standpoint, some powerful peptide carriers have been developed such as the MAPs (15), ISCOMs (16) and hydrophobic anchors (17).

In such a context liposomes are of great interest. These phospholipid vesicles are characterized by a low toxicity and a low intrinsic immunogenicity (no carrier epitopic suppression). They can carry antigens (and adjuvants) either surface-bound, encapsulated or membrane-associated. Moreover, they physicochemical properties (size, pH-sensitivity, bilayer rigidity,..) can be manipulated to influence the mode of antigen presentation. Indeed, it was noted decades ago that these vesicles are able to increase the immune response against poorly immunogenic proteins associated to them (either entrapped or surface-bound); an important literature exists on that property which has been related to the passive targeting of liposomes to macrophages which act as APC's (18,19); protein-based liposomal vaccines have been developed against viral, bacterial and parasitic diseases. Comparatively, little had been done with small peptides antigens. Our aim was to reproduce, with simple liposomal constructs, the presentation by a pathogen of antigens to the immune system in order to trigger a functional immune response. Our first approach was very fundamental; we studied the different parameters affecting the immunogenicity of a liposome-associated model hexapeptide IRGERA (H_2N-Ile-Arg-Gly-Glu-Arg-Ala-COOH), i.e. the *C*-terminal peptide and major epitope of histone H3. This peptide is strictly a B-epitope and, unless conjugated to a carrier, is unable to induce an immune response. Our aim was twofold: i) to design a construct able to elicit anti-peptide antibodies that cross-react with histone H3, the parent protein, and ii) to find conditions that elicit the production of a long lasting IgG response. Many parameters have been studied and the model system that was successful is summarized in Fig. 3. The following parameters were of importance: i) the peptide must be surface-bound; ii) the vesicles must have a limited size (dia. $\leq$ 100 nm), and iii) the same liposomes that carry the peptide

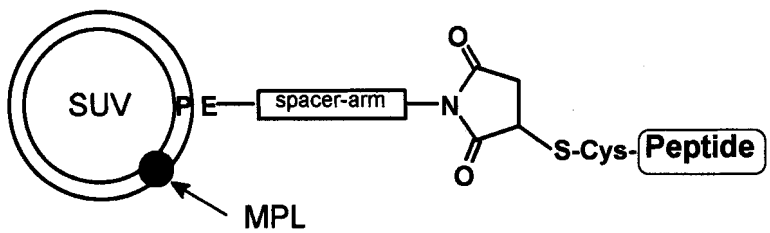

Fig. 3 Liposomal system able to trigger an immune response against an antigenic peptide. The peptide, bearing at the *N*-terminus an extra Cys or Cys-Gly spacer, was covalently coupled to the surface of preformed SUV containing a thiol-reactive phosphatidylethanolamine derivative (here a maleimide group) and MPL as adjuvant.

must contain an adjuvant such as monophosphoryl lipid A (MPL) (20,21). This latter amphipathic molecule, which is a non-toxic derivative of the lipopolysaccharide, is a macrophage activator and a B-lymphocyte mitogen that is currently tested in humans (22). With such a construct, using IRGERA as peptide, we obtained in BALB/c mice a potent and long-lasting immune response (production of IgGs) after boosting (21); importantly, the antibody titers was as high as those obtained with conventional immunization (i.e. peptide coupled to a carrier protein and injected with Freund's adjuvant) and the antibodies obtained cross-reacted with histone H3. The paramount influence of the liposome size on the immune response is in favor of a hypothesis involving the *targeting* of the constructs to specific B-lymphocytes that function as APCs, i.e. via the recognition of the peptide antigen by the sIg followed by the delivery of the mitogenic adjuvant (and isotype switching ?).

To validate this approach, we have tested if such liposomal constructs could protect mice against an influenza virus infection. OF1 mice (for which the infection by the virus is lethal) were immunized with liposomes to which cyclic peptides were coupled. These two peptides (D-loop and K-loop; Fig. 4) were designed to mimic the site-A (i.e. a major epitope that forms a loop between residues 139-146) of influenza haemagglutinin. Loop-D, i.e. the smallest and structurally the most constrained one, was found by modeling (NMR) to fit best the conformation of site-A; in contrast, loop-K, which is larger and more flexible, shows less overlap with site-A. Both peptides, when classically coupled to carrier proteins, were found earlier to afford a protection to about 80% of the animals against a nasal challenge with a LD_{50} dose of A/NT/60/68 influenza virus (23). To our great satisfaction, our liposomal constructs were almost as potent in immunizing the animals since they afforded about 75% protection which, however, was restricted to the only loop-D (24). Importantly, the animals that were immunized with the liposomes, and escaped influenza, all remained perfectly healthy. These results pave the way to the design of liposome-based synthetic peptide vaccines.

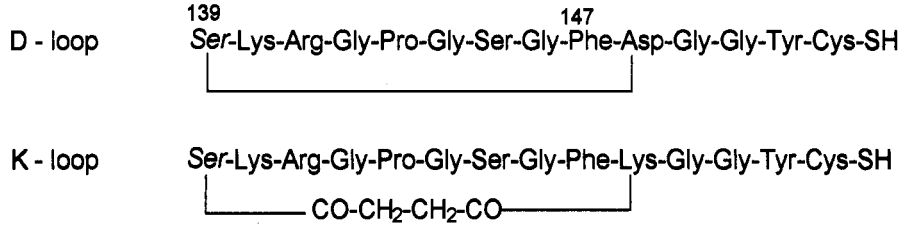

Fig. 4 Cyclic peptides, that mimic the site-A (139-146) of influenza A virus haemagglutinin, that were conjugated to SUV used in the immunization experiments (24).

Recently we have synthesized new polyoxyethylene-based spacer-arms (cf. Fig. 3), that are functionalized with other thiol-reactive functions than maleimide (25), and studied their immunogenicity; we thus found ways to reduce to a minimum the intrinsic immunogenicity of the carrier (26). At present we have started the exploitation of our "second generation" liposomal constructs : we have devised a strategy that allows a chemically controlled coupling of two different peptides, such as B and Th-epitopes, to the same preformed liposome. These vesicles induce particularly potent and long lasting immune responses even in the absence of MPL, i.e. with a completely synthetic system (Boeckler *et al.*, to be published).

CATIONIC LIPOSOMES AND GENE DELIVERY

Introduction of genes into cells is a major technique in cell biology. It has been used for genetic engineering of microorganisms, plants and animals. The interest in this research has dramatically increased with the advent of gene therapy. This therapeutic approach is based on systems that provide *in vivo* efficient transfer and expression of genetic information into target cells. At present, this challenging feat is best achieved with viruses (e.g. recombinant retroviruses, adenoviruses and adeno-associated viruses); however the genes that can thus be transferred are of limited size (< 7 kbases) and the modified viruses used in such experiments are not without drawbacks (some viruses transfect only dividing cells, the transgene expression is limited in time, transfected cells trigger immune responses, etc..).

Many other techniques, including conventional and, more recently, cationic liposomes, have been developed as alternate means to transfer nucleic acids into cells both *in vitro* and *in vivo*; they form the basis of the *non-viral gene delivery systems*. Thus recently, synthetic vectors have been very actively developed among which (poly)cationic amphipathic molecules, able to complex and compact DNA, proved the most attractive (Fig. 5) (reviewed in 27,28). The application of these agents is however hampered by their relatively low efficiency, compared to viruses, and by the necessity of an overall net positive charge of the transfecting particles (i.e. excess of cationic charges with respect to DNA phosphates). This latter property results, *in vivo*, in a low bioavailability and lack of cell specificity, possibly due to interaction with cell surface proteoglycans. The challenges faced by the non-viral gene delivery systems are quite formidable; but they are those faced by all particulate drug delivery systems. These include stability of the particles in serum when administered systemically, extravasation to reach extravascular cellular targets, efficient uptake by cells via endocytosis, exit of the transgene from the endosomes, plus additional hurdles such as

the intracellular targeting of the transgene to the nuclei followed by its stable expression. Needless to say that all these requirements cannot be met easily. At present, although the search for new transfecting molecules remains a hot field, the focus has moved towards a better understanding and control of the different parameters that condition a successful outcome of this approach. The first step, involving the formation of the transfection complexes between the plasmids and the (poly)cationic amphiphiles, remains presently a very subtle formulation problem, especially for making particles that are stable under *in vivo* conditions. This is now studied at (ultra)structural levels (e.g. electron microscopy, X-rays) to better understand the phases formed after mixing DNA with preformed cationic liposomes or by direct addition of the cationic transfection agents (29,30). Recently also, an interesting hypothesis has emerged that rationalizes the mechanism of nucleic acid release from the cationic liposome/DNA complexes in the endosomal compartment (i.e. after cellular uptake) and its delivery into the cytoplasm (31).

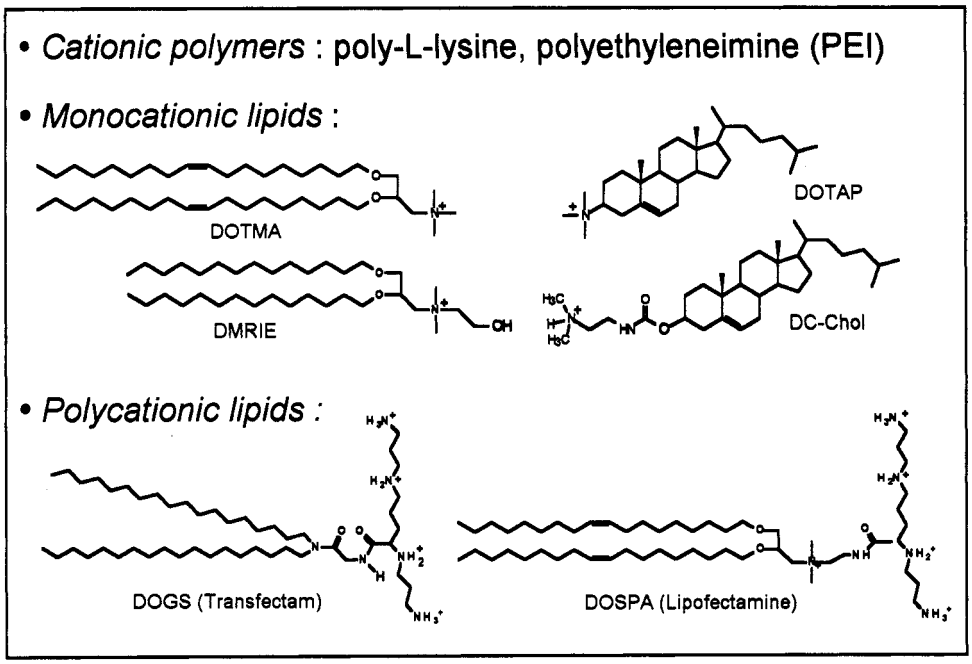

Fig. 5 Selected transfection agents

Because of the lack of cell specificity of the positively charged transfection particles, *targeted transfection* represents a second generation approach where the target cells can, in principle, be selected on the basis of specific interactions, e.g. ligand/receptor (reviewed in 28). Within this context, the presence of receptors specific for Gal/GalNAc ligands at the surface of cells such as hepatocytes (i.e. asialoglycoprotein receptor), macrophages and some metastases which has been exploited to target bioactive molecules and vectors (32) is of particular interest. Thus, targeted poly(L-lysine)-based gene delivery systems have been designed in which asialoglycoproteins, lactose (reviewed in 32) and synthetic multiantennary galactose ligands (33) were conjugated to the polycationic polymer. We have explored the possibility to transfect, *in vitro*, human hepatoma HepG2 cells with *electrically neutral* lipospermine/DNA transfection particles, to which synthetic tri-antennary galactose ligands were added to provide an interaction with these cells that express Gal/GalNAc receptors at their surface. Our strategy consists in replacing the non-specific electrostatic interaction by a specific recognition that triggers a receptor-mediated endocytosis of the transfection complex. Lipospermine (DOGS,Transfectam®, see Fig. 5) was chosen as transfection agent because of its efficiency with many cells (34).

In our study we have asked the following two questions: i) will the conjugation of galactose ligands to the surface of 1 charge eq. particles (i.e. where the number of spermine positively charged headgroups were calculated to exactly neutralize the negative charges of DNA) provide a specific interaction with HepG2 cells and an effective uptake via a receptor-mediated endocytosis ? ii) is the quantity of lipospermine present in 1 charge eq. complexes sufficient to provide an efficient transfection ? To answer these ques-

tions, targeted transfecting particles carrying at their surface synthetic triantennary galactose ligands (35,36) were prepared (Fig. 6) and then tested for their ability to transfect HepG2 cells (37).

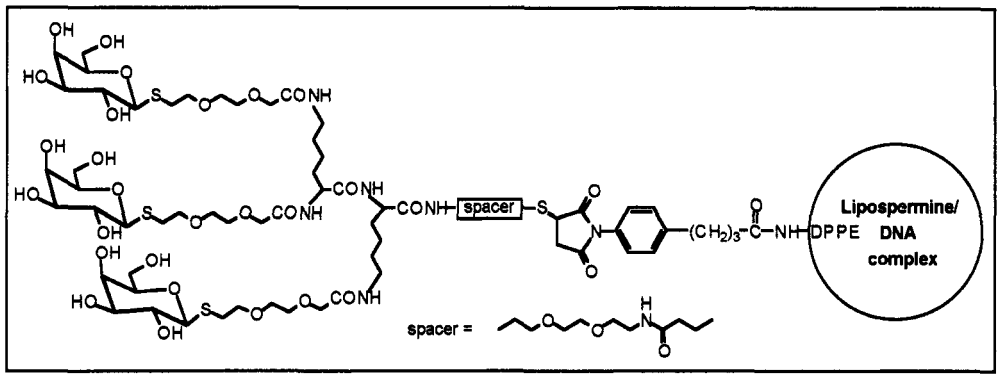

Fig. 6 Targeted transfection complex obtained by condensation of pCMV-Luc plasmid with lipospermine in the presence of a triantennary neo-galactolipid.

Transfection with neutral lipospermine/DNA particles, containing various proportions of the galactose ligand (Fig. 6), resulted in a considerably increased yield when compared to the same particles lacking the ligand. The presence of the galactose ligand, up to 25 mol% compared to lipospermine, increased the transfection efficacy > 1 000-fold, approaching the value observed with optimized positively charged (6 charge eq.) complexes. A dependence on the ligand concentration could be observed, i.e. at 5 mol% the transfection yield was about 15-fold lower than at 25 mol% where a plateau was reached. The transfection efficacy of the targeted complexes was also found to be dependent on the structure of the ligand; thus, a biantennary neo-galactolipid known to have a lesser affinity for the Gal/GalNAc receptor (36) was 2 orders of magnitude less efficient than the tri-antennary whereas mono-galactosyl ligands gave results next to the control levels (37). To demonstrate that the observed enhancement in transfection resulted indeed from a recognition of the neutral targeted particles by the galactose receptor of HepG2 cells, we have performed several controls (37): e.g. experiments similar to the ones described above were accomplished with 3T3 fibroblasts, which do not express the galactose receptor at their surface, and no transfection above control could be observed. In conclusion, *selective targeted transfection can be achieved in vitro with neutral particles composed of lipospermine/DNA/neo-galactolipids*. It remains to be shown whether these properties can be exploited for targeted *in vivo* gene delivery after systemic administration. Although lipospermine-based complexes have not been developed as yet towards that aim, some formulations have recently been devised that may be suitable for such purposes (38) and our synthetic ligands for the Gal/GalNAc receptor might represent attractive homing devices.

Active research in the field of non-viral gene delivery is still very much needed to render this approach useful *in vivo* and to fulfill the enormous hopes raised by the prospect of gene therapy. Recent progress indicates that in some cases this mode of gene delivery slowly approaches the efficiency of viral vectors, possibly without their drawbacks.

CONCLUSION

Liposomes constitute a world in themselves as study objects in fundamental sciences and also as sophisticated tools in biotechnology. We are certainly far from having exhausted all the possibilities of these "bags of tricks" and the last decade, e.g. with the advent of the SL and cationic liposomes, has taught us that new, and unpredictable, directions may still be ahead of us.

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