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(54) Titre : COMPLEXES STABLES D'ADMINISTRATION DE MEDICAMENTS COMPRENANT DES LIPIDES ET LEUR PROCEDE DE PRODUCTION

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(57) Abrégé/Abstract:
Novel stable, concentrated, biologically active and ready-to-use lipid- comprising drug delivery complexes and methods for their production are described. The biological activity of the complexes produced are comparable to the formulations prepared according to the prior art admixture method and upon purification, the complexes produced by the method of this invention are 50 to 500 fold more concentrated than the components of the complexes formed by admixture. The method described herein provides for the large scale production of lipid-comprising drug delivery systems useful for gene therapy and other applications.

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(54) Title: STABLE LIPID-COMPRISING DRUG DELIVERY COMPLEXES AND METHODS FOR THEIR PRODUCTION

(57) Abstract

Novel stable, concentrated, biologically active and ready-to-use lipid-comprising drug delivery complexes and methods for their production are described. The biological activity of the complexes produced are comparable to the formulations prepared according to the prior art admixture method and upon purification, the complexes produced by the method of this invention are 50 to 500 fold more concentrated than the components of the complexes formed by admixture. The method described herein provides for the large scale production of lipid-comprising drug delivery systems useful for gene therapy and other applications.

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Title of Invention

Stable Lipid-Comprising Drug Delivery Complexes And Methods For Their Production

Field of Invention

The present invention relates to cationic lipids and their use as vehicles for the transfer of nucleic acids or other macromolecules such as proteins into cells. More specifically, this invention relates to lipid-comprising drug delivery complexes which are stable, biologically active, capable of being concentrated, and to methods for their production.

BACKGROUND OF INVENTION

The development of new forms of therapeutics which use macromolecules such as proteins or nucleic acids as therapeutic agents has created a need to develop new and effective means of delivering such macromolecules to their appropriate cellular targets. Therapeutics based on either the use of specific polypeptide growth factors or specific genes to replace or supplement absent or defective genes are examples of therapeutics which may require such new delivery systems. Clinical application of such therapies depends not only on efficacy of new delivery systems but also on their safety and on the ease with which the technologies underlying these systems can be adapted for large scale pharmaceutical production, storage, and distribution of the therapeutic formulations.

Gene therapy has become an increasingly important mode of treating various genetic disorders. The potential for providing effective treatments, and even cures, has stimulated an intense effort to apply this technology to diseases for which there have been no effective

treatments. Recent progress in this area has indicated that gene therapy may have a significant impact not only on the treatment of single gene disorders, but also on other more complex diseases such as cancer. However, a significant obstacle in the attainment of efficient gene therapy has been the difficulty of designing new and effective means of delivering therapeutic nucleic acids to cell targets. Thus, an ideal vehicle for the delivery of exogenous genes into cells and tissues should be highly efficient in nucleic acid delivery, safe to use, easy to produce in large quantity and have sufficient stability to be practicable as a pharmaceutical.

Non-viral vehicles, which are represented mainly by the cationic liposomes, are one type of vehicle which have, for the following reasons, been considered for use in gene therapy. First, the plasmid DNA required for liposome-mediated gene therapy can be widely and routinely prepared on a large scale and is simpler and carries less risk than the use of viral vectors such as retroviruses. Second, liposome-mediated gene delivery, unlike retroviral-mediated gene delivery, can deliver either RNA or DNA. Thus, DNA, RNA, or an oligonucleotide can be introduced directly into the cell. Moreover, cationic liposomes are non-toxic, non-immunogenic and can therefore be used repeatedly in vivo as evidenced by the successful in vivo delivery of genes to catheterized blood vessels (Nabel, E.G., et al. (1990) Science, 249: 1285-1288), lung epithelial cells (Brigham, K.L., et al. (1989) Am. J. Respir. Cell Mol. Biol., 195-200, Stribling, R., et al. (1992) Proc. Natl. Acad. Sci. U.S.A., 89: 11277-11281), and other systemic uses (Zhu, N., et al. (1993) Science, 261: 209-211, Philip, R., et al. (1993) Science, 261: 209-211) of cationic liposomes.

Although a variety of cationic liposome formulations, including the commercially available

cationic liposome reagent DOTMA/DOPE (N-1,-(2, 3 - dioleoyloxy) propyl-N,N,N-trimethyl ammonium chloride/dioleoyl phosphatidylethanolamine), are known in the art (Felgner, P.L. et al. (1987) Proc. Natl. Acad. Sci. U.S.A., 84: 7413-7417), a cationic liposome formulation designated DC-Chol/DOPE (3βN-(N',N'-dimethylaminoethane)-carbamoyl cholesterol)/(dioleoyl phosphatidylethanolamine)) has been shown in in vitro studies (Gao, X., and Huang, L. (1991) Biochem. Biophys. Res. Commun., 179: 280-285) to be relatively non-toxic and more efficient than DOTMA/DOPE. Moreover, following extensive in vivo studies (Plautz, G.E., et al. (1993) Proc. Natl. Acad. Sci. U.S.A., 90: 4645-4649, Stewart, M.J., et al. (1992) Hum. Gene Ther., 3: 267-275) in which DC-Chol/DOPE was demonstrated to be both safe and efficacious as a nucleic acid delivery system, this formulation was approved by the U.S. Food and Drug Administration (FDA) and the U.K. Medicines Control Agency (MCA), and has been used in two separate gene therapy clinical trials (Nabel, G.J., et al. (1993) Proc. Natl. Acad. Sci. U.S.A., 90: 11307-11311, Caplen, N.J., et al. (1995) Nature Medicine, 1: 39-46).

However, the use of DC-Chol/DOPE and other currently existing cationic liposomes as vehicles for delivering nucleic acids to cellular targets are inconvenient for large scale therapeutic applications for a number of reasons. First, the ratios of liposome to nucleic acid utilized to form nucleic acid/liposome complex in the prior art admixture method results in the formation of complexes which are large in diameter and hence, relatively low in stability. Thus, none of the presently utilized cationic liposome formulations, including DC-Chol/DOPE, are designed as stable and ready-to-use pharmaceutical formulations of nucleic acid/liposome complex. This limitation of the admixture

method requires that the user prepare complex prior to each use, an inconvenience which requires special training of personnel. In addition, the preparation of complex by admixture prior to each use introduces a possible source of dosage variability which hinders evaluation of treatments utilizing these complexes due to possible over- or under-dosing of the recipient.

Second, the prior art admixture method of preparing nucleic acid/cationic liposome complexes prior to each use requires that a dilute nucleic acid solution (less than 4 μg/ml) and a dilute liposome dispersion (less than 50 μM) be used to prepare the nucleic acid/liposome complex in order to reduce the chance of forming large and less active aggregates. This limitation makes it difficult to make small biologically active complexes without using less than optimal conditions, such as reducing the amount of liposomes (which causes reduced nucleic acid transfer activity) or increasing the amount of liposome (which causes enhanced toxicity). Moreover, the fact that the complex must be made in dilute concentrations is a significant drawback to clinical applications, particularly in the case of intratumor injection of the complex, since only a small volume of the complex can be injected in each site (Nabel, G.J., et al. (1993) Proc. Natl. Acad. Sci. U.S.A., 90: 11307-11311).

Accordingly, an object of this invention is to provide stable, biologically active, lipid-comprising drug delivery complexes which are capable of being concentrated as well as methods of producing such complexes.

Summary of Invention

This invention provides methods for producing lipid-comprising drug delivery complexes having a net positive charge and/or a positively charged surface. By

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"drug" as used throughout the specification and claims is meant any molecular entity, which is either monomeric or oligomeric, and which, when complexed with lipid or with lipid and polycation, is being administered to an individual for the purpose of providing a therapeutic effect to the recipient. Thus, macromolecules having an overall net negative charge or regions of negativity would be expected to be capable of forming the delivery complexes of this invention. Macromolecules which are particularly suitable for use with the complexes of this invention are for example, DNA, RNA, oligonucleotides or negatively charged proteins. However, macromolecules having a positive charge (eq large cationic protein would also be expected to be capable of forming the complexes of this invention by sequentially complexing the cationic macromolecule with anionic lipid or polymer and then with cationic lipid.

The complexes of the present invention comprise a drug/lipid complex formed by mixing the drug to be delivered with cationic liposomes in a drug to lipid ratio such that the drug/lipid complex formed has a net positive charge and a drug/lipid/polycation complex formed by mixing drug with cationic liposomes and polycation in a drug to lipid to polycation ratio such that the drug/lipid/polycation complex formed has a net positive charge. By "net positive charge" as applied to the drug/lipid complex is meant a positive charge excess of lipid to drug. By "net positive charge" as applied to the drug/lipid/polycation complex is meant that the positive charges of the cationic lipid and the polycation exceed the negative charge of the drug. However, it is be understood that the present invention also encompasses drug/lipid and drug/lipid/polycation complexes having a positively charged surface irrespective of whether the net charge of the complex is positive, neutral or even

negative. A positively charged surface of a complex may be measured by the migration of the complex in an electric field by methods known to those in the art such as by measuring zeta potential (Martin, A., Swarick, J., and Cammarata, A., Physical Pharmacy & Physical Chemical Principles in the Pharmaceutical Sciences, 3rd ed. Lea and Febiger, Philadelphia, 1983), or by the binding affinity of the complex to cell surfaces. Complexes exhibiting a positively charged cell surface have a greater binding affinity to cell surfaces than complexes having a neutral or negatively charged surface.

The invention therefore relates to methods for producing these drug/lipid and drug/lipid/polycation complexes comprising mixing the drug to be delivered with cationic liposomes, and optionally polycation, in a ratio such that the complex formed has a net positive charge and/or a positively charged surface.

In another embodiment of this invention, the methods for producing drug lipid or drug/lipid/polycation complexes may further comprise the step of purifying said complexes from excess free components (drug, lipid, polycation) following their production.

The drug/lipid and drug/lipid/polycation complexes of this invention are generally stable, capable of being produced at relatively high concentration, and retain biological activity over time in storage. Such complexes are of utility in the delivery of nucleic acids, proteins and other macromolecules to cells and tissues.

According to one aspect of the present invention, there is provided a method for producing a nucleic acid/lipid/polycation complex having a net positive charge at pH 6.0-8.0 comprising a nucleic acid, at least one cationic lipid species, and at least one polycation, said method comprising mixing said nucleic acid with a solution comprising the at least one cationic lipid species and the at least one polycation in a ratio of nucleic acid to cationic lipid species to polycation such that said complex is formed, said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

According to another aspect of the present invention, there is provided a nucleic acid/lipid/polycation complex comprising at least one cationic lipid species, at least one polycation and nucleic acid; the ratio of said nucleic acid to said at least one cationic lipid species to said at least one polycation being such that said complex has a positive net charge at pH 6.0-8.0, wherein said complex is formed by adding the nucleic acid to a solution comprising the at least one cationic lipid species and the at least one polycation; said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40

large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

According to still another aspect of the present invention, there is provided a method for producing a noncovalent nucleic acid/lipid/polycation complex comprising a nucleic acid, at least one cationic lipid species and at least one polycation and having a net positive charge at pH 6.0-8.0, said method comprising mixing said nucleic acid with a solution comprising the at least one cationic lipid species and at least one polycation in a ratio of nucleic acid to cationic lipid species to polycation which results in the production of said complex, said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

According to yet another aspect of the present invention, there is provided a noncovalent nucleic acid/lipid/polycation complex having a net positive charge at pH 6.0-8.0 comprising a nucleic acid, at least one cationic lipid species and at least one polycation; said complex being produced by mixing a solution of the at least one cationic lipid species and the at least one polycation with the nucleic acid in a ratio of nucleic acid to cationic lipid species to polycation which results in the production of said complex, said at least one polycation being selected

from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

According to a further aspect of the present invention, there is provided a pharmaceutical composition comprising a complex as described herein and a pharmaceutically acceptable carrier.

According to yet a further aspect of the present invention, there is provided a use of the complex described herein as a vehicle for delivering nucleic acid to cells.

DESCRIPTION OF FIGURES

Fig. 1 shows a typical size distribution (mean diameter) of nucleic acid/liposome complexes prepared as an admixture from DC-Chol/DOPE (3:2) liposomes and pRSVL plasmid DNA (2 µg) at the indicated lipid to DNA ratios.

Figures 2A and 2B show the distribution of the liposome marker ^{3}H-cholesteryl hexadecyl ether (O) and the ^{32}P-DNA marker (■) among sucrose gradient fractions. The location of each fraction in the sucrose gradients of both Figures 2A and 2B is indicated at the top of Figure 2A.

Figure 2A shows the distribution of the ^{3}H and ^{32}P markers following ultracentrifugation of free liposomes (10 μmoles of DC-Chol/DOPE (2:3) in 2 ml volume) or free DNA (50 μg pRSVL DNA in a 2 ml volume) through a sucrose density gradient. Figure 2B shows the distribution of the ^{3}H and ^{32}P markers following ultracentrifugation of the DNA-lipid complex (formed via mixing of 20 μmoles DC-Chol/DOPE (2:3) liposomes and 0.4 mg pRSVL DNA in 2 ml volume) through a sucrose density gradient.

Figure 3 shows the transfection activities in CHO cells of admixture DNA/liposome complex (O), admixture DNA/liposome/poly-L-lysine(PLL) complex (□) DNA/lipid complex (●) and DNA/lipid/PLL complex (■). The DC-Chol/DOPE liposomes used to form the above complexes contained varying mole% of DC-Chol as indicated at the bottom of Figure 3. The DNA/lipid (●) and DNA/lipid/PLL (■) complexes were purified on a sucrose density gradient prior to being assayed for transfection activity. Transfection activity is indicated on the vertical axis as relative light units of luciferase activity.

Figure 4 shows the transfection activities of admixture DNA/liposome complex (O) and admixture DNA/liposome/PLL complex (□) compared to the transfection activities of DNA/lipid (●) and DNA/lipid/PLL complexes stored at 4°C for 130 days following their purification on a sucrose density gradient. The DC-Chol/DOPE liposomes used to form the above complexes contained varying mole% of DC-Chol as indicated at the bottom of Figure 4. Transfection activity is indicated on the vertical axis as relative light units of luciferase activity.

Figure 5 shows the concentration of extractable protein from CHO cells, 36 hours after the cells were treated with admixture DNA/liposome complex (O); admixture DNA/liposome/PLL complex (□); DNA/lipid complex (●); or DNA/lipid/PLL complex (■). The DNA/lipid and DNA/lipid/PLL complexes were purified on a sucrose density gradient prior to being assayed for transfection activity. The DC-Chol/DOPE liposomes used to form the above complexes contained varying mole% of DC-Chol as indicated at the bottom of Figure 5.

Figure 6 shows the results of CAT assays of tumor extracts prepared from mice having ovarian tumors. $2x10^6$ human ovarian carcinoma cells were subcutaneously injected into SCID mice at day 0. On day 14, 100 µl solutions containing pUCCMVCAT DNA (contains the chloramphenicol acetyl transferase gene of E. coli) 30 µg) complexed with DC-Chol liposomes (30 nmoles) in the form of admixture (lanes 1 and 2; duplicate samples) or the same amount of DNA in the form of purified complex (prepared from DNA:DC-chol liposome at ratio 1µg/25 nmoles, lanes 3 and 4; duplicate samples) were directly injected into tumors. 48 hours following transfection, the mice were sacrificed and tumor extract containing 100 µg protein was assayed for CAT activity. Lane 5 shows positive control CAT activity for standard E. coli CAT.

Figures 7A - 7C show the transfection activities of admixture DNA/lipid complex and purified and unpurified DNA/lipid/PLL complexes in 293 cells (Fig. 7A) C3 cells (Fig. 7B) and BL6 cells (Fig. 7C). Transfection activity is indicated on the vertical axis of Figures 7A-7C as relative light units of luciferase activity.

Description of Invention

This invention relates to lipid-comprising drug

delivery complexes having a net positive charge and/or a positively charged surface at pH 6.0-8.0. These complexes comprise cationic lipids, drugs, and optionally further comprise polycations. The invention further relates to a method for producing these complexes where the method may optionally include the step of purifying these formulations from excess individual components. For the production of the drug/lipid complexes of this invention, inclusion of the purification step is a preferred embodiment. It should be understood that where the purification step is applied to the drug/lipid/polycation complexes, the recovery of these complexes in a pure state free from excess components following purification is lower than the recovery of drug/lipid complexes following their purification since the peak containing the drug/lipid/polycation complex following sucrose purification via density centrifugation is broader than the peak containing drug/lipid complexes and hence, overlaps with the peaks of the free components.

The lipid-comprising drug delivery complexes of this invention are stable, capable of being produced at relatively high concentrations, and retain biological activity of the drug component over time in storage. The method of producing these complexes is based on a binding model between two oppositely charged polymers (e.g. negatively charged nucleic acid and positively charged lipids) in which the formation of large unstable aggregates is avoided by neutralizing the negative charge of the drug via the use of an excess amount of positive charge in the form of cationic liposomes or cationic liposomes and polycation. The complexes of this invention have been observed to retain their initial diameter and bioactivity over 4 months in storage in 10% sucrose buffer.

The "drug" which is contained in the lipid-

comprising drug delivery complexes of the present invention may be nucleic acids, polyanionic proteins, polysaccharides and other macromolecules which can be complexed directly with cationic lipids. However, cationic drugs (eg large cationic protein) can be directly complexed with an anionic lipid or sequentially complexed first with anionic lipid or polymer followed by cationic lipid. The use of this process permits delivery of positive or neutral charged drug to cells by the complexes of the present invention.

To produce drug/lipid and drug/lipid/polycation complexes with a net positive charge, the positive charge excess of lipid to drug or of lipid and polycation to drug may be up to about a 30-fold positive charge excess in the complex of total lipids to drug or of lipid and polycation to drug, preferably about a 2 to 10-fold charge excess and most preferably about a 2 to 6-fold charge excess. Complexes which posses a positive charge on their surface may have similar preferred ranges of surface charge excess to drug. To produce a nucleic acid/lipid complex having a positive charge excess of lipid to nucleic acid, mole amounts of cationic liposomal lipid to be mixed with 1 μg of nucleic acid to produce a nucleic acid/lipid complex which has positive charge excess of lipid to nucleic acid at pH 6.0-8.0 may range from about 0.1 nmol to about 200 nmol of lipid, preferably about 5 nmol to about 100 nmol lipid, depending on the positive charge content of the cationic liposome. Of course, if the drug were a protein, the amount of lipid to be mixed with 1 μg of negatively charged protein would be at least 10- fold less than the amount of lipid to be mixed with 1 μg of DNA as shown above since proteins are less charge dense than nucleic acids. Those of ordinary skill in the art would readily understand that depending upon the positive charge content of the cationic liposomes, different mole amounts of

different cationic liposomes would have to be mixed with an equivalent amount of drug to produce a positive charge excess of lipid to drug.

When a drug/lipid/polycation complex having a net positive charge and/or a positively charged surface is to be produced, the inclusion of the polycation reduces the amount of lipid which must be mixed with drug to the extent that the positive charge from the lipid may be less than the negative charge from the drug. This reduction in the amount of lipid reduces the toxicity of the polycation-containing formulations. Mole amounts of cationic liposomes to be used in formulating nucleic acid/lipid/polycation complexes may range from about 0.1 nmol to about 200 nmol lipid per 1 μg nucleic acid, more preferably from about 1 to about 25 nmoles lipid per 1 μg nucleic acid depending on the positive charge content of the cationic liposomes. It is to be generally understood that in producing the nucleic acid/lipid and nucleic acid/lipid/polycation complexes of the present invention, the mole amount of liposomes required to produce these complexes will increase as the concentration of nucleic acid mixed with the liposomes is increased.

Those of ordinary skill in the art would readily understand that when the complexes of the present invention are purified, the positive charge excess of cationic liposomes to drug or of cationic liposomes and polycation to drug immediately prior to mixing will be greater than the positive charge excess in the purified complexes of lipid to drug or of lipid and polycation since the purification step results in the removal of excess free lipids and/or free polycation.

In order to illustrate how the charges attributed to cationic lipid, drug and polycation may be determined at pH 6.0-8.0 the following example is provided. Assuming the drug to be delivered is DNA, one

determines the negative charge of the DNA to be delivered by dividing the amount of DNA to be mixed, or the amount of DNA in the complex, by 330, the molecular weight of a single nucleotide where one nucleotide equals one negative charge. Thus, the negative charge for 1 μg of DNA is 3.3 nmols.

For 10 nmol of DC-Chol/DOPE (2:3) liposomes one calculates the effective charge of the lipid by multiplying the amount of total liposomal lipid (10 nmol) by 0.4 (40% of the total liposomal lipid is the cationic lipid DC-Chol) to yield 4 nmol DC-Chol lipid in the liposomes. Since at pH 6-8, one molecule of DC-Chol has one positive charge, the effective positive charge of liposomal lipid at the time of mixing, or in the complex, is 4.0 nmol. Of course, those of skill in the art would readily understand that other cationic lipids may have a lesser or greater amount of positive charge per molecule of cationic lipid at pH 6-8.0 than DC-Chol.

Assuming the polycation to be mixed to form the complex is a bromine salt of poly-L-lysine (PLL), the positive charge of PLL at the time of mixing is obtained by dividing the amount of PLL to be mixed by 207, the molecular weight of one lysyl residue where one lysyl residue equals one positive charge. Thus, the positive charge for 1 μg of PLL is approximately 5.0 nmols. To calculate the positive charge contributed by lysyl residues in a formed complex, the amount of lysine present in the complex is divided by the molecular weight of one lysyl residue taking into account the weight of a counterion, if present.

Application of the above calculations to data presented in Table 1 herein (see Example 3) illustrates how a positive to negative charge ratio can be calculated both at the time of mixing of DNA and liposome and, after purification of the complex produced by the mixing of DNA

and liposome. In Table 1 of Example 3, 0.4 mg of DNA is mixed with 20 μmols of cationic DC-Chol/DOPE liposomes to produce DNA/lipid complex. For cationic liposomes having a DC-Chol/DOPE ratio of 4:6, the positive charge content of the liposomal lipid is calculated to be 8000 nmol and the negative charge content of the 0.4 mg DNA to be mixed with liposomes is calculated to be 1320 nmols based on the sample calculations presented in the above paragraphs. Therefore, the positive to negative charge ratio at the time of mixing is 6.06 (8000 divided by 1320). However, after the complex was purified, the lipid to DNA ratio of this purified complex was 12.7 nmol lipid/μg DNA as shown in Table 1 (see the "4:6 row"). This 12.7 ratio translates to a positive to negative charge ratio of 1.5 thus showing that purification removed excess positive charge of free liposomes.

Also in Table 1, where DNA/lipid/PLL complex was prepared by mixing 4 μmol of liposomes (4:6 DC-Chol/DOPE) and 1 mg PLL with 0.4 mg DNA, one can calculate the positive to negative charge ratio at the time of mixing as follows. Based on the sample calculations presented in the above paragraphs, the 4 μmol liposomal lipid contributes 1600 nmol of positive charge, the 1 mg of PLL contributes 5000 nmol of positive charge and the 0.4 mg DNA contributes 1,320 nmol of negative charge. Thus, the positive to negative charge ratio at the time of mixing liposomes, PLL and DNA is 5 $\left(\frac{1600 + 5000}{1320}\right)$.

It is further to be understood by those skilled in the art that the net charge of the complex may be determined by measuring the amount of DNA, lipid and when present, polycation in the complex by the use of an appropriate analytical technique such as the use of radioisotopic labelling of each component or by elemental analysis. Once the amounts of each component (DNA, lipid

and when present, polycation) in a complex at a given pH are known, one could then calculate the approximate net charge of that complex at the given pH taking into account the pK's of the components which may be known or determined analytically.

In a preferred embodiment, the drug is a nucleic acid sequence, preferably a nucleic acid sequence encoding a gene product having therapeutic utility.

In one embodiment of the invention, a method for producing nucleic acid/lipid complexes having a net positive charge and/or positively charged surface at pH 6-8.0, comprises, combining nucleic acids with cationic liposomes in a nucleic acid to lipid ratio such that the nucleic acid/lipid complex formed has a positive charge excess of lipid to nucleic acid.

In an alternative embodiment, nucleic acid and cationic liposome may be mixed with a polycation in a nucleic acid to lipid to polycation ratio such that the nucleic acid/lipid/polycation complexes formed have a positive charge excess of lipid and polycation to nucleic acid at pH 6-8.

In a preferred embodiment, the nucleic acid/lipid and nucleic acid/lipid/polycation complexes are produced by slowly adding nucleic acid to the solution of liposome or liposome plus polycation and mixing with a stirring bar where the mixing is allowed to proceed second. Alternatively, the liposome or liposome/ polycation mix can be added into a single chamber from a first inlet at the same time the nucleic acid is added to the chamber through a second inlet. The components are then simultaneously mixed by mechanical means in a common chamber.

The cationic liposomes mixed with drug or with drug and polycation to form the complexes of the present invention may contain a cationic lipid alone or a cationic

lipid in combination with a neutral phospholipid. Suitable cationic lipid species include, but are not limited to: 1, 2 bis(oleoyloxy)-3- (trimethylammonio) propane (DOTAP); N-1, -(2,3-dioleoyloxy) propyl -N, N, N-trimethyl ammonium chloride (DOTMA) or other N-(N, N-1-dialkoxy)-alklyl-N, N, N-trisubstituted ammonium surfactants; 1, 2 dioleoyl-3-(4'-trimethylammonio) butanoyl-sn-glycerol (DOBT) or cholesteryl (4' trimethylammonia) butanoate (ChOTB) where the trimethylammonium group is connected via a butanoyl spacer arm to either the double chain (for DOTB) or cholesteryl group (for ChOTB); DORI (DL-1, 2-dioleoyl-3-dimethylaminopropyl-B-hydroxyethylammonium) or DORIE (DL-1, 2-O-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium) (DORIE) or analogs thereof as disclosed in WO 93/03709; 1, 2-dioleoyl-3-succinyl-sn-glycerol choline ester (DOSC); cholesteryl hemisuccinate ester (ChOSC); lipopolyamines such as doctadecylamidoglycylspermine (DOGS) and dipalmitoyl phosphatidyesthanolamidospermine (DPPES) or the cationic lipids disclosed in US Patent Number 5,283,185, cholesteryl-3β-carboxyl-amido-ethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine, cholesteryl-3β-oxysuccinamido-ethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysuccinate iodide, 2-(2-trimethylammonio)-ethylmethylamino ethyl-cholesteryl-3β-oxysuccinate iodide, 3βN-(N', N'-dimethylaminoethane) carbamoyl cholesterol (DC-chol), and 3β-N-(polyethyleneimine)-carbamoylcholesterol.

Examples of preferred cationic lipids include cholesteryl-3β-carboxyamidoethylenetri-methylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-

carboxyamidoethyleneamine, cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysuccinate iodide, 2-(2-trimethylammonio)ethylmethylamino ethyl-cholesteryl-3β-oxysuccinateiodide, 3βN-(N', N'dimethyl-aminoethane)-carbamoyl-cholesterol (DC-chol), and 3βN-(polyethyleneimine)-carbamoyl cholesterol.

Since an attribute of the complexes of the invention is their stability during storage (i.e., their ability to maintain a small diameter and retain biological activity over time following their formation); it will be understood by those of ordinary skill in the art that preferred cationic lipids are those lipids in which bonds between the lipophilic group and the amino group are stable in aqueous solution. While such bonds found in cationic lipids include amide bonds, ester bonds, ether bonds and carbamoyl bonds, preferred cationic lipids are those having a carbamoyl bond. An example of a preferred cationic lipid having a carbamoyl bond is DC-Chol. Those of skill in the art would readily understand that liposomes containing more than one cationic lipid species may be used to produce the complexes of the present invention. For example, liposomes comprising two cationic lipid species, lysyl-phosphatidylethanolamine and β-alanyl cholesterol ester have been disclosed (Brunette, E. et al. (1992) Nucl. Acids Res., 20:1151).

It is to be further understood that in considering cationic liposomes suitable for use in mixing with drug and optionally with polycation, to form the complexes of this invention, the methods of the invention are not restricted only to the use of the lipids recited above but rather, any lipid composition may be used so long as a cationic liposome is produced.

Thus, in addition to cationic lipids, cationic liposomes used to form the complexes of the invention may

contain other lipids in addition to the cationic lipids. These lipids include, but are not limited to, lyso lipids of which lysophosphatidylcholine (1-oleoyllysophosphatidycholine) is an example, cholesterol, or neutral phospholipids including dioleoyl phosphatidyl ethanolamine (DOPE) or dioleoyl phosphatidylcholine (DOPC). The lipid complexes of the invention may also contain negatively charged lipids as well as cationic lipids so long as the net charge of the complexes formed is positive and/or the surface of the complex is positively charged. Negatively charged lipids of the invention are those comprising at least one lipid species having a net negative charge at or near physiological pH or combinations of these. Suitable negatively charged lipid species comprise phosphatidyl glycerol and phosphatidic acid or a similar phospholipid analog.

It is further contemplated that in the cationic liposomes utilized to form the complexes of the invention, the ratio of lipids may be varied to include a majority of cationic lipids in combination with cholesterol or with mixtures of lyso or neutral lipids. When the cationic lipid of choice is to be combined with another lipid, a preferred lipid is a neutral phospholipid, most preferably DOPE.

Methods for producing the liposomes to be used in the production of the lipid-comprising drug delivery complexes of the present invention are known to those of ordinary skill in the art. A review of methodologies of liposome preparation may be found in Liposome Technology (CFC Press NY 1984); Liposomes by Ortro (Marcel Schher, 1987); Methods Biochem Anol. 33:337-462 (1988) and U.S. Patent 5,283,185. Such methods include freeze-thaw extrusion and sonication. Both unilamellar liposomes (less than about 200 nm in average diameter) and multilamellar liposomes (greater than about 300 nm in

average diameter) may be used as starting components to produce the complexes of this invention.

In the cationic liposomes utilized to produce the drug/lipid complexes of this invention, the cationic lipid is present in the liposome at from about 10 to about 100 mole% of total liposomal lipid, preferably from about 20 to about 80 mole% and most preferably about 20 to about 60 mole%. The neutral lipid, when included in the liposome, may be present at a concentration of from about 0 to about 90 mole% of the total liposomal lipid, preferably from about 20 to about 80 mole%, and most preferably from 40 to 80 mole%. The negatively charged lipid, when included in the liposome, may be present at a concentration ranging from about 0 mole% to about 49 mole% of the total liposomal lipid, preferably from about 0 mole% to about 40 mole%. In a preferred embodiment, the liposomes contain a cationic and a neutral lipid, most preferably DC-Chol and DOPE in ratios between about 2:8 to about 6:4. It is further understood that the complexes of the present invention may contain modified lipids, protein, polycations or receptor ligands which function as a targeting factor directing the complex to a particular tissue or cell type. Examples of targeting factors include, but are not limited to, asialoglycoprotein, insulin, low density lipoprotein (LDL), folate and monoclonal and polyclonal antibodies directed against cell surface molecules. Potential targets include, but are not limited to, liver, blood cells, endothelial cells and tumor cells.

It is to be further understood that the positive charge of the complexes of this invention may be affected not only by the lipid composition of the complex but also by the pH of the solution in which the drug/lipid complexes are formed. For example, increasing pH (more basic) will gradually neutralize the positive charge of

the tertiary amine of the cationic lipid DC-Chol. In a preferred embodiment, the complexes of the present invention are produced, and stored, at a pH such that the complexes have a net positive charge and/or positively charged surface. A preferred pH range is pH 6.0 - 8.0, most preferably pH 7.0 - 7.8.

When a polycation is to be mixed with nucleic acid and cationic liposomes, the polycation may be selected from organic polycations having a molecular weight of between about 300 and about 200,000. These polycations also preferably have a valence of between about 3 and about 1000 at pH 7.0. The polycations may be natural or synthetic amino acids, peptides, proteins, polyamines, carbohydrates and any synthetic cationic polymers. Nonlimiting examples of polycations include polyarginine, polyornithine, protamines and polylysine, polybrene (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine and synthetic polypeptides derived from SV40 large T antigen which has excess positive charges and represents a nuclear localization signal. A preferred polycation is poly-L-lysine (PLL). In producing nucleic acid/lipid/polycation complexes of the present invention, the ratio of polycation to nucleic acid is kept fixed while varying the amount of liposome. However, those of skill in the art would recognize that the ratio of polycation to nucleic acid will be affected by the charge density of the liposome to be mixed with the nucleic acid and polycation. For example, if the charge density of liposomes is decreased as a result of changes in the lipid composition of the liposome (eg decreasing the ratio of cationic lipid: neutral lipid in the liposome), the amount of polycation to be mixed with nucleic acid and liposome may be increased to compensate for the decrease in positive charge contributed by the liposomes. However, when polycation is utilized, it is

preferred to use subsaturating amounts of polycation (ie amounts which will not saturate all the negative charge of the nucleic acid) in order to allow the cationic lipids to complex with the nucleic acid. Thus, in a preferred embodiment of the invention, a positive charge excess of lipid to nucleic acid is used even when polycation is mixed with lipid and nucleic acid. Amounts of polycation which may be mixed with 1 μg of nucleic acid and varying amounts of cationic liposomes in the present invention range from about 0.01 μg to about 100 μg of polycation per μg of nucleic acid, preferably from about 0.1 μg to about 10 μg of polycation per μg of nucleic acid.

Where purification of nucleic acid/lipid and nucleic acid/lipid/polycation complexes from excess free DNA, free liposomes and free polycation is desired, purification may be accomplished by centrifugation through a sucrose density gradient or other media which is suitable to form a density gradient. However, it is understood that other methods of purification such as chromatography, filtration, phase partition, precipitation or absorption may also be utilized. In a preferred method, purification via centrifugation through a sucrose density gradient is utilized. The sucrose gradient may range from about 0% sucrose to about 60% sucrose, preferably from about 5% sucrose to about 30% sucrose. The buffer in which the sucrose gradient is made can be any aqueous buffer suitable for storage of the fraction containing the complexes and preferably, a buffer suitable for administration of the complex to cells and tissues. A preferred buffer is pH 7.0-8.0 Hepes.

It is understood that in the present invention, preferred nucleic acid sequences are those capable of directing protein expression. Such sequences may be inserted by routine methodology into plasmid expression vectors known to those of skill in the art prior to mixing

with cationic liposomes or liposomes and polycation to form the lipid-comprising drug delivery complexes of the present invention. The amount of nucleic acid mixed together with cationic liposomes or with cationic liposomes and polycation may range from about 0.01 μg to about 10 mg, preferably from about 0.1 μg to about 1.0 mg. It is understood that where the nucleic acid of interest is contained in plasmid expression vectors, the amount of nucleic acid recited above refers to the plasmid containing the nucleic acid of interest.

The purification of the nucleic acid/lipid and nucleic acid/lipid/polycation complexes of the present invention serves to concentrate the nucleic acids and lipids contained in the resultant complexes from about 50 -fold to about 500 -fold such that the lipid content contained in the complexes may be as high as about 40 μmol/ml and the nucleic acid content may be as high as about 2 mg/ml.

The diameter of the complexes produced by the methods of the present invention is less than about 400 nm, preferably less than about 200 nm, and more preferably less than 150 nm.

The complexes formed by the methods of the present invention are stable for up to about one year when stored at 4° C. The complexes may be stored in 10% sucrose solution upon collection from the sucrose gradient or they may be lyophilized and then reconstituted in 10% sucrose solution prior to use. In a preferred embodiment, the complexes are stored in solution. The stability of the complexes of the present invention is measured by specific assays to determine the physical stability and biological activity of the complexes over time in storage. The physical stability of the complexes is measured by determining the diameter of the complexes by methods known to those of ordinary skill in the art, including for

example, electron microscopy, gel filtration chromatography or by means of quasi-elastic light scattering using a Coulter™ N4SD particle sizes as described in the Examples. The physical stability of the complex is "substantially unchanged" over storage when the diameter of the stored complexes is not increased by more than 100%, preferably by not more than 50%, and most preferably by not more than 30%, over the diameter of the complexes as determined at the time the complexes were purified.

Assays utilized in determining the biological activity of the complexes vary depending on what drug is contained in the complexes. For example, if the drug is nucleic acid encoding a gene product, the biological activity can be determined by treating cells in vitro under transfection conditions utilized by those of ordinary skill in the art for the transfection of cells with admixtures of DNA and cationic liposomes. Cells which may be transfected by the complexes includes those cells which may be transfected by admixture DNA/liposome complexes. The activity of the stored complexes is then compared to the transfection activity of complexes prepared by admixture. If the drug is a protein, then activity may be determined by a bioassay suitable for that protein.

It is further understood by those of skill in the art that the complexes of the present invention may be used in vivo as vectors in gene therapy.

Therapeutic formulations using the complexes of the invention preferably comprise the complexes in a physiologically compatible buffer such as, for example, phosphate buffered saline, isotonic saline or low ionic strength buffer such as 10% sucrose in H_2O (pH 7.4-7.6) or in Hepes (pH 7-8, a more preferred pH being 7.4-7.6). The complexes may be administered as aerosols or as liquid

solutions for intratumor, intravenous, intratracheal, intraperitoneal, and intramuscular administration.

The following examples illustrate various aspects of the invention but are intended in no way to limit the scope thereof.

EXAMPLES

Materials

DOPE was purchased from Avanti Polar Lipid, Inc.(Alabaster, AL). pRSVL, a plasmid which encodes the luciferase gene under the control of Rous sarcoma virus long terminal repeat, (De Wet, J.R. et al. (1987) Mol. Cell. Biol., 7: 725-737) was amplified in E. coli and purified using the standard CsCl-EtBr ultracentrifugation method (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual (2d ed) Cold Spring Harbor Laboratory Press: New York (1989)). All the tissue culture media were obtained from Gibco BRL (Gaithersburg, MD). Human embryonic kidney 293 cells, CHO (Chinese Hamster Ovary cells), BL6 and BHK (Baby Hamster Kidney cells) cells were from American Type Culture Collection (Rockville, MD). Mouse lung cells (MLC) are primary culture cells originally derived from the lung of a Balb/c mouse by Dr. S. Kenned (Oak Ridge National Laboratory, TN). 293, BL6, BHK and MLC cells were cultured with DMEM media, CHO cells were cultured with F12 media, and C3 Hela cells were cultured in RPMI-1640 medium. All media was supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and 100 unit/ml penicillin and 100 μg/ml streptomycin. Poly-L-lysine hydrobromide (MW 3000 and MW 25,600) and other chemicals were from Sigma (St Louis, MI).

DC-Chol was synthesized according to the method of Gao and Huang (1991) (Gao, X., and Huang, L. (1991) Biochem. Biophys. Res. Commun., 179: 280-285) with modifications in the purification steps as follows: after the reaction, 10 ml hexane was added and the mixture was extracted three times with 10 ml water. The organic phase was collected and dried under vacuum at 4°C. The resulting solid was dissolved in a minimal amount of absolute ethanol with heat, and recrystallized in acetonitrile at 0 °C. The purity of DC-Chol was at least > 95%, as analyzed by TLC method and ^{1}H-NMR and the yield of about 70% was a significant improvement over that of the previously reported method of Gao, X., and Huang, L. ((1991) Biochem. Biophys. Res. Commun., 179: 280-285).

Methods

Preparation and purification of complexes

Cationic liposomes at a 20 mM total lipid concentration were prepared from DC-Chol and DOPE at various ratios by a sonication method, according to a published procedure (Gao, X., and Huang, L. (1991) Biochem. Biophys. Res. Commun., 179: 280-285). Trace amount of ^{3}H cholesteryl hexadecyl ether (Amersham, Arlington Heights, IL) was included for quantitation purpose. The size of these liposomes was between 100 to 150 nm in diameter, as determined by quasi-elastic light scattering using a Coulter N4SD particle sizer (Coulter Electronics, Inc., Hialeah, FL). Unless indicated otherwise in the following Examples, DNA/lipid complexes were prepared at a typical laboratory scale by adding amounts of free DC-Chol/DOPE liposomes as indicated in each Example in a volume of 1 ml of a 2 mM Hepes buffer (pH 7.6) to a 15 x 7.5 polystyrene culture tube (Baxter,

McGraw Pare, IL), a micro-magnetic stirrer was placed in the tube, and the solution was well mixed. Amounts of pRSVL DNA as indicated in each Example were then added dropwise from a stock solution (0.2 mg/ml, in 2 mM Hepes buffer, pH 7.6) to the liposome solution over a period of 3 min. Trace amounts of pRSVL labeled with ^{32}P using a nick translation kit (Promega, Madison, WI) and ^{32}P dCTP (Amersham, Arlington Heights, IL) was included for the purpose of quantitation.

To prepare purified lipid/PLL/DNA complexes, an amount of the above 0.2 mg/ml DNA solution as indicated in each Example was added to 1 ml PLL/liposome mixture containing amounts of liposomes and PLL as indicated in each Example. DNA/lipid complexes were loaded on the top of a sucrose step gradient composed of 0.5 ml each of 5%, 7.5%, 10% and 15% sucrose (w/w) and DNA/lipid/PLL complexes were loaded on top of a sucrose step gradient composed of 0.5 ml each of 5%, 10%, 15%, 20%, 25% and 30% sucrose (w/w). The DNA/lipid and DNA/lipid/PLL complexes were then purified from free lipid and PLL by ultracentrifugation at 100,000 g for 30 min at 4° C. After centrifugation, fractions of 200 µl were taken from the top to the bottom of the tube. Aliquots from each fraction were assayed for both ^{3}H and ^{32}P radioactivity using a scintillation counter. Fractions that contained peak value of the ^{32}P were collected and pooled. These pooled fractions were then assayed for particle size and for transfection activity.

<u>In vitro Transfection assay</u>

The biological activity of the above complexes were assayed by <u>in vitro</u> transfection of cells in the Examples as follows. Briefly, cells grown in 48 well plate were incubated with DNA/lipid complex diluted in 0.5

ml CHO-S-SFM (Gibco BRL) or with admixture DNA/liposome complex prepared according to Gao and Huang (1991) (Gao, X., and Huang, L. (1991) Biochem. Biophys. Res. Commun., 179: 280-285). For transfection of pRSVL DNA using DC-Chol liposomes in the presence of PLL, liposomes were first mixed with PLL, then complexed with DNA. All transfections were performed for 4 hours at 37°C. After transfection, cells were further cultured for 36 hours in the appropriate media containing 10% fetal bovine serum. Cells were then washed with PBS and lysed with 100 µl of 1X lysis buffer provided by a luciferase assay kit (Promega, Madison, WI). A 4 µl sample of the lysate was assayed for luciferase activity using 100 µl substrate solution from the reconstituted luciferase assay kit and an AutoLumat™ LB953 luminometer (Berthold, Germany). Protein concentration from each lysate was assayed by a Coomassie blue dye method according to the manufacturer's protocol (Pierce, Rockford, IL).

EXAMPLE 1

The Size Of The DNA/Lipid Complex Is Determined By The Ratio Of DNA To Lipid

This experiment was conducted to show that the size of the DNA-lipid complex formed by admixture changed as the ratio of DNA mixed with liposome varied. In brief, pRSVL plasmid DNA (2 µg) was mixed with varying amounts of DC-CHOL/DOPE (3:2) liposomes in 2 mM Hepes buffer at pH 7.6 in a final volume of 500 µL and after 7 minutes, the size of the complex was determined with a Coulter N4SD laser light scattering particle sizer operating in the unimodel mode.

As shown in Figure 1, large aggregates did not form when DNA was in excess (ratios of liposome to DNA less than 7) but at ratios of lipid to DNA that were

charge neutral (~10), the size of the complex reached a maximum. In addition, when the ratio of liposome-to-DNA was kept constant at 10 nmoles/μg, the size of the complex increased as both DNA and liposome concentration increased and eventually formed precipitates. However, when the ratio of liposomes to DNA was increased, the size of the complex was progressively reduced until the size of the complex became constant (250-300 nm) when the ratio of liposome-to-DNA exceeded 25 nmoles lipid/μg DNA. This result may be due to the fact that the DNA was perhaps coated by excess liposomes and therefore aggregation between the complexes did not occur.

Based on the data presented in Figure 1, lipid/DNA complexes were prepared using a liposome-to-DNA ratio of 50 nmoles/μg by slowly adding a DNA solution of 200 μg/ml to an excess amount (10 μmols) of liposome. The size of the complex formed was about 250 nm. When the ratio of liposome-to-DNA was changed to 25 nmoles/μg, the size of the complex increased to about 350 nm. The complexes formed using either the 25 nmole/μg or 50 nmole/μg ratios appeared to be physically stable since no precipitates formed during storage for four weeks at 4° C.

EXAMPLE 2

Purification Of DNA/lipid Complexes

When DNA was mixed with liposomes at a ratio of 1 μg/50 nmoles, an excess of free liposomal lipids was observed to co-exist with the DNA/lipid complex. Since excess free lipids are toxic to cells, an experiment was conducted to determine if free liposomal lipids could be separated from the DNA/lipid complex by a density gradient ultracentrifugation method. In brief, free liposomes (10 μmoles of DC-Chol/DOPE (2:3) in a volume of 2 ml); free DNA (50 μg pRSVL in a volume of 2 ml) and DNA/lipid

complex formed by mixing 20 μmoles DC-Chol/DOPE (2:3) and 0.4 mg pRSVL plasmid DNA (50 nmoles/μg) were each centrifuged for 30 minutes at 4° C at 100,000 g over a gradient consisting of 0.5 ml each of 5%, 7.5%, 10% and 15% sucrose (W/W). Fractions of 200 μl were then collected from the top to the bottom of the tube and assayed for the distribution of DNA marker (^{32}P, ■) and lipid marker (^{3}H, O). Figures 2A and 2B show the results of typical separations of free liposomal lipid, free DNA (Fig. 2A) and DNA/lipid complex (Fig. 2B) on the sucrose gradient. The results presented in Figure 2B show that after centrifugation, the complex formed a major band at the 10% sucrose layer. By comparison, Figure 2A shows that most of the radioactivity of the free DNA or free liposomal lipids distributed at the top half of the tube and did not enter the sucrose gradient. In addition, although the peak of ^{3}H and the peak of ^{32}P in Figure 2B co-existed at fraction number 16, there was a significant amount of the ^{3}H distributed in fractions 1 to 10 indicating that the excess free liposomal lipids were well separated from the DNA/lipid complex.

EXAMPLE 3

Physical Stability Of Purified Lipid/DNA And Lipid/PLL/DNA Complexes

DNA/lipid complexes were formed by mixing 20 μmoles of liposomes of various DC-Chol/DOPE compositions (see Table 1) with 0.4 mg pRSVL plasmid DNA at a ratio of 1 μg DNA/50 nmoles lipid. Lipid/PLL/DNA complexes were formed by mixing 4 μmoles of liposomes of various DC-Chol/DOPE compositions with 1 mg PLL (MW=3000) and 0.4 mg pRSVL plasmid DNA. Both complexes were then purified from free lipids, free DNA and free PLL by sucrose gradient centrifugation as described in the Methods section. The

peak fractions were collected and pooled. Pooled samples were then assayed for diameter immediately after collection (0 days) or after storage in 10% sucrose at 4°C for 120 days. Table 1 shows the results of these assays.

TABLE 1. Physical stability of purified lipid/DNA and lipid/PLL/DNA pre-complexes

Liposome composition (DC-chol/DOPE)	PLL (μg/μgDNA)	Size (nm) Day 0	Size (nm) Day 120	Purified complex Ratio of lipid/DNA (nmoles/μg)	Recovery of DNA (%Total)
2:8	0	168	280	23.2	51
3:7	0	187	252	14.0	66
4:6	0	175	195	12.7	73
5:5	0	174	210	13.2	70
6:4	0	198	232	10.1	69
2:8	2.5	165	287	20.8	17
3:7	2.5	99	101	19.2	22
4:6	2.5	138	132	38.3	29
5:5	2.5	184	178	22.4	27

The data presented in Table 1 shows that purified lipid/DNA and lipid/PLL/DNA complexes were small (under 200 nm) in size at day 0 and that their size did not increase dramatically with storage. Further, the ratios of DNA-to-lipid in the purified complexes was between 10 to 23 nmoles lipid/μg DNA depending on the composition of the liposomes used and this ratio did not change after storage for 120 days. A reciprocal relationship between the concentration of DC-Chol in the liposomes and the amount of the lipid in the complex was also observed indicating that liposomes enriched with

DC-Chol show stronger DNA binding or charge neutralizing activity than liposomes less enriched with DC-Chol. The far-right hand columns shows recovery of ^{32}P-labeled DNA in the DNA/lipid and DNA/lipid/PLL complexes following their purification on the sucrose density gradient. The results show that recovery of DNA in the non-PLL containing complexes was higher than that observed for the PLL-containing complexes.

EXAMPLE 4

Biological Activity Of The Purified Complexes In Various Cells

Since the DNA/lipid complexes formed by a mixture of liposomes to DNA having a high lipid to DNA ratio were both small and stable over time, experiments were conducted to compare the transfection activity of these complexes to the activity of DNA/liposome complex prepared by the admixture method.

In one experiment, CHO cells cultured in 48 well-plates were treated for 4 hours with an admixture of either 1 μg pRSVL and 10 nmoles DC-Chol/DOPE liposomes of different lipid compositions alone (O) or together with 1 μg PLL (MW=3,000) (□) or, the cells were treated with purified DNA/lipid complex (●) or purified DNA/lipid/PLL complex (■) formed by mixing 1 μg DNA with 50 nmoles DC-Chol/DOPE liposomes (DNA/lipid complex) or with 10 nmoles DC-Chol/DOPE liposomes and 1 μg PLL (DNA/lipid/PLL complex) followed by centrifugation through a sucrose density gradient as described in the Methods section. 36 hours after treatment, cells were lysed in 100 μl lysis buffer and 4 μl of the lysate was assayed for luciferase activity using 100 μl of luciferase substrate solution. Luciferase activity was then counted over a period of 20 seconds. The results presented in Figure 3 show that the most preferred liposome composition for transfecting CHO

cells was 40% DC-Chol and 60% DOPE. In addition, in the presence of additional 1 μg poly-L-lysine (PLL, MW=3,000), a 2-7 fold enhancement of the transfection activity was seen in most cases. Of particular interest, the activity of the purified DNA/lipid complex was similar to that of the admixture DNA/lipid complex when the same amount of DNA was added to cells. However, the transfection activity of the purified DNA/lipid/PLL complex was about 30% to 50% lower than that of the DNA/liposome/PLL complex prepared by the admixture procedure.

In order to determine that the results obtained in CHO cells were not cell-specific, the transfection activities of the purified DNA/lipid and DNA/lipid/PLL complexes in two other cells, BHK and mouse lung cells (MLC), were compared to that of DNA/liposome complexes formed by admixture.

In brief, cells (either BHK or MLC) grown in 48-well plates at 60% confluency were transfected with 1μg pRSVL complexed with 10 nmoles of DC-Chol liposomes (admixture complex), with the same amount of DNA mixed with liposomes at a DNA/liposome ratio of 1 μg/50 nmols to produce purified DNA/lipid complex or with purified DNA/lipid/PLL complex prepared at a DNA/liposome/PLL ratio of 1 μg/10 nmols/2 μg. Cells were then harvested at 36 hours post-transfection, and the luciferase activity of the transfected cell lysates was determined as described in the Methods section. The results of these experiments are shown in Tables 2 and 3.

TABLE 2. Expression of luciferase gene in BHK cells transfected with pRSVL

Liposome composition (DC-Chol/DOPE)	Luciferase Activity (Relative Light Units X 10^{-3})		
	Admixture DNA/liposome complex	Purified DNA/lipid complex	Purified DNA/lipid/PLL complex
2:8	91.8 ± 9.5	110.1 ± 5.2	214.6 ± 41.1
3:7	61.2 ± 19.9	1886.8 ± 266.7	151.7 ± 62.9
4:6	438.2 ± 14.4	1638.8 ± 63.9	446.3 ± 16.9
5:5	837.8 ± 8	1015.0 ± 41.2	234.2 ± 46.4

TABLE 3. Expression of luciferase gene in mouse lung cells transfected with pRSVL

Liposome composition (DC-Chol/DOPE)	Luciferase Activity (Relative Light Units X 10^{-3})		
	Admixture DNA/liposome complex	Purified DNA/lipid complex	Purified DNA/lipid/PLL complex
2:8	1.1 ± 0.7	0.4 ± 0.2	0.3 ± 0.1
3:7	1.5 ± 1.0	0.3 ± 0.0	4.1 ± 1.3
4:6	3.1 ± 0.2	2.0 ± 0.3	14.6 ± 3.1
5:5	0.1 ± 0.0	1.5 ± 1.2	10.1 ± 2.3

Interestingly, for the BHK cell line, the transfection activity of the purified DNA/lipid complex was significantly higher than that of the DNA/liposome complex formed by admixture. For cells such as MLC, which are difficult to transfect, purified complexes made from DNA/liposome/PLL mixtures were apparently superior to admixture complexes and to purified DNA/lipid complexes made without PLL.

In order to determine whether lipid/PLL/DNA complexes could be made using different ratios of lipid and nucleic acid and a different molecular weight PLL than

that used in the previous examples, the following experiment was conducted. Lipid/poly-L-lysine/DNA complex was prepared from 20 μg pRSVL plasmid DNA, 10 μg poly-L-lysine (MW 25,600), and DC-chol/DOPE liposomes (4.5/5.5 molar ratio) at the ratios of lipid to DNA shown in Table 4. The resulting complexes were then purified by sucrose gradient ultracentrifugation as described in the methods section. An aliquot of the purified complex containing 0.5 μg of DNA was used to transfect CHO cells, and luciferase activity was then measured. The results of this experiment are shown below in Table 4.

TABLE 4. Effect of lipid/DNA ratio on purified complex containing poly-L-lysine (MW 25,600)

Ratio (nmoles lipid/μg DNA)	Composition of purified complex (nmoles lipid/μg DNA)	Size of purified complex (nm)	Transfection activity[b] (counts (SD) X10^{-3})
3.3	1.1	89	108 (5)
6.6	2.5	98	6,065 (604)
12.5	4.3	101	5,846 (668)
20.0	9.6	35	7,633 (977)

The results show that in the presence of increased amounts of polycation, lower ratios of lipid to DNA may be used to produce DNA/lipid/polycation complexes having appreciable transfection activity.

EXAMPLE 5

Transfection Activity of Stored Complexes

CHO cells cultured in 48 well-plates were treated for 4 hours with admixture of 1 μg pRSVL and 10 nmoles of DC-Chol/DOPE liposomes of different DC-Chol/DOPE compositions alone (O) or together with 1 μg PLL (mw =

3,000) (□), or with purified DNA/lipid (●) or DNA/lipid/PLL (■) complexes stored at 4°C for 130 days in 10% sucrose. The purified complexes had been formed by mixing 1 μg pRSVL and 50 nmoles of DC-Chol/DOPE liposomes of different DC-Chol/DOPE compositions alone (DNA/lipid) or with 10 nmole of DC-Chol/DOPE liposomes and 1 μg PLL (DNA/lipid/PLL complex) followed by centrifugation through a sucrose density gradient as described in the Methods section. The results show that the luciferase activity of cell lysates prepared from cells transfected with the stored DNA/lipid and DNA/lipid/PLL complexes was comparable with the luciferase activity observed in cell lysates of cells transfected with the corresponding complexes prepared by admixture.

EXAMPLE 6

Comparative Cytotoxicity Of DNA/liposome Complexes Prepared By Admixture To That Of Purified DNA/Lipid Complexes

Cell toxicity of the different complexes was studied in CHO cells as follows. CHO cells were treated with admixture DNA/liposome complex (O), admixture/liposome/PLL complex (□); purified DNA/lipid complex (●); or purified DNA/lipid/PLL complex (■). The admixture complexes were formed by mixing 1 μg pRSVL DNA with 10 nmoles DC-Chol/DOPE liposomes of different DC-Chol/DOPE compositions alone or together with 1 μg PLL (mw = 3,000). The purified complexes were formed by mixing 1 μg pRSVL DNA with 50 nmoles DC-Chol/DOPE liposomes alone (DNA/lipid complex) or with 10 nmoles DC-Chol/DOPE liposomes and 1 μg PLL (DNA/lipid/PLL complex) followed by centrifugation through a sucrose density gradient as described in the Methods section. 36 hours after treatment, the cells were lysed, protein was extracted and then quantitated by a Coomassie blue dye method.

Fig. 5 shows the results of this experiment where the amount of total extractable protein recovered at the end of the experiment serves as an indicator of the portion of the cells which survived after the indicated treatment. The data presented shows that while the purified complex appeared to be slightly more toxic to the cells than the admixture DNA/liposome complex, morphologically, the transfection did not cause any serious cytotoxic effects in cells treated with either admixture complexes or the purified complexes, except that the cells treated with purified complexes containing high mole % DC-Chol were less confluent at the end of the experiment.

EXAMPLE 7

In Vivo Transfection of Tumors By Purified DNA/Lipid Complexes

3×10^6 human ovarian carcinoma cells were injected subcutaneously into SCID mice at day 0. 14 days later, 100 µl solutions containing pUCCMVCAT DNA (30 µg) complexed with DC-Chol (3:2 DC-Chol:DOPE) liposomes (30 nmoles) in the form of admixture (lanes 1 and 2) or the same amount of DNA in the form of purified DNA/lipid complex (prepared from DNA and DC-Chol liposomes at ratios of 1 ug DNA/25 nmoles lipid were directly injected into tumors. Animals were sacrificed 2 days later and tumor extracts containing 100 µg protein were assayed for CAT activity at 37°C according to Ausubel, et al. (1991) Current Protocols in Molecular Biology (Wiley, Boston), Vol. 1, pp. 9.6.2-9.6.5). The results show that purified complex, while prepared under non-optimal conditions, exhibited in vivo transfection activity.

EXAMPLE 8

Comparative Transfection Activity of Purified and Unpurified DNA/lipid/PLL Complexes With Admixture DNA/Lipid Complex

The transfection activities of purified and unpurified DNA/Lipid/PLL complexes and admixture DNA/lipid complex in three cell lines (293, BL6 and C3) were measured as follows:

Purified DNA/lipid/PLL complex was formed by mixing 1 μg PRSVL DNA with 10 nmoles DC-Chol/DOPE liposomes (2:3 mol/mol) and 1 μg PLL (MW = 25,600) followed by centrifugation through a sucrose density gradient as described in the Methods section.

Unpurified DNA/lipid/PLL complexes were formed by mixing 100 μg PRSVL DNA with 80 μg PLL (MW = 25,600) and 1702 nmol DC-Chol/DOPE (2:3 mol/mol) liposomes (i.e. a DNA/lipid/PLK ratio of 1 μg DNA/17 nmol lipid/0.8 μg PLL) in a final volume of 500 μl of water. 20 μl of the unpurified DNA/lipid/PLL complex (i.e. 4μg DNA, 3.2 μg PLL and 68.1 nmol lipid) was then added to 780 μl of serum free medium appropriate for the cell line to be transfected.

Admixture DNA/lipid complex was formed by mixing 1 μg PRSVL DNA with 10 nmols of DC-Chol/DOPE (3:2 mol/mol) liposomes.

293, C3 and BL6 cells grown to 80% confluence in 24-well plates were transfected for four hours at 37°C with 1 μg of DNA in the form of purified DNA/lipid/PLL complex, admixture DNA/lipid complex or unpurified DNA/lipid/PLL complex. After transfection, cells were were further cultured for 36-48 hours in the appropriate media containing 10% fetal calf serum. Luciferase activity was then measured as described in the Methods section.

The results presented in Figures 7A (293 cells)

unpurified DNA/lipid/PLL complex exhibited the highest transfection activity in all three cell lines tested.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that the basic constructions can be altered to provide other embodiments which utilize the methods and devices of this invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

CLAIMS:

1. A method for producing a nucleic acid/lipid/polycation complex having a net positive charge at pH 6.0-8.0 comprising a nucleic acid, at least one cationic lipid species, and at least one polycation, said method comprising mixing said nucleic acid with a solution comprising the at least one cationic lipid species and the at least one polycation in a ratio of nucleic acid to cationic lipid species to polycation such that said complex is formed, said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

2. The method of claim 1, wherein said ratio of nucleic acid to cationic lipid species is from 1 µg/0.1 nmol to 1 µg/200 nmol.

3. The method of claim 1 or 2, wherein said at least one polycation is present from 0.01 µg to 100 µg per 1 µg of nucleic acid.

4. The method of any one of claims 1 to 3, wherein the complex further comprises a neutral phospholipid.

5. The method of any one of claims 1 to 3, wherein the at least one cationic lipid species is 3βN-(N',N'-dimethylaminoethane)-carbamoyl cholesterol (DC-Chol).

6. The method of any one of claims 1 to 3, wherein the complex further comprises dioleoyl phosphatidylethanolamine (DOPE).

7. The method of any one of claims 1 to 6, wherein the at least one polycation is the peptide comprising polylysine.

8. The method of any one of claims 1 to 6, wherein the at least one polycation is the peptide comprising the nuclear localization sequence of SV40 large T antigen and which has excess positive charge.

9. The method of any one of claims 1 to 8, wherein the at least one polycation has a molecular weight of from 300 to 200,000 daltons.

10. The method of any one of claims 1 to 6, wherein the at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000 daltons.

11. A nucleic acid/lipid/polycation complex comprising at least one cationic lipid species, at least one polycation and nucleic acid; the ratio of said nucleic acid to said at least one cationic lipid species to said at least one polycation being such that said complex has a positive net charge at pH 6.0-8.0, wherein said complex is formed by adding the nucleic acid to a solution comprising the at least one cationic lipid species and the at least one polycation; said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic

lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

12. The complex of claim 11, wherein said ratio of the nucleic acid to the at least one cationic lipid species is from 1 µg/0.1 nmol to 1 µg/200 nmol.

13. The complex of claim 11 or 12, wherein said at least one polycation is present from 0.01 µg to 100 µg per 1 µg of nucleic acid.

14. The complex of any one of claims 11 to 13, wherein the at least one polycation is the peptide comprising polylysine.

15. The complex of any one of claims 11 to 13, wherein the at least one polycation is the peptide comprising the nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

16. The complex of any one of claims 11 to 15, wherein the at least one polycation has a molecular weight of from 300 to 200,000 daltons.

17. The complex of any one of claims 11 to 13, wherein the at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000 daltons.

18. The complex of any one of claims 11 to 17, wherein the at least one cationic lipid species comprises 3βN-(N',N'-dimethylaminoethane)-carbamoyl cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE).

19. A method for producing a noncovalent nucleic acid/lipid/polycation complex comprising a nucleic acid, at

least one cationic lipid species and at least one polycation and having a net positive charge at pH 6.0-8.0, said method comprising mixing said nucleic acid with a solution comprising the at least one cationic lipid species and at least one polycation in a ratio of nucleic acid to cationic lipid species to polycation which results in the production of said complex, said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

20. The method of claim 19, wherein the ratio of cationic lipid species to nucleic acid ranges from 0.1 nmol to 200 nmol lipid per 1 µg nucleic acid.

21. The method of claim 19, wherein the ratio of cationic lipid species to nucleic acid ranges from 1 nmol to 25 nmol lipid per 1 µg nucleic acid.

22. The method of any one of claims 19 to 21, wherein the polycation:nucleic acid ratio is 0.01 µg to 100 µg polycation per 1 µg nucleic acid.

23. The method of any one of claims 19 to 22, wherein the complex further comprises a neutral phospholipid.

24. The method of any one of claims 19 to 23, wherein the at least one polycation is the peptide comprising polylysine.

25. The method of any one of claims 19 to 23, wherein the at least one polycation is the peptide comprising the nuclear localization sequence of SV40 large T antigen and which has excess positive charge.

26. The method of any one of claims 19 to 25, wherein the at least one polycation has a molecular weight of from 300 to 200,000 daltons.

27. The method of any one of claims 19 to 23, wherein the at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000 daltons.

28. The method of claim 23, wherein said at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000, said at least one cationic lipid is 3βN-(N',N'-dimethylaminoethane)-carbamoyl cholesterol and the neutral phospholipid is dioleoylphosphatidlyethanolamine.

29. The method of any one of claims 19 to 28, wherein said complex has an average diameter of less than 400 nm.

30. The method of any one of claims 19 to 29, wherein said method further comprises the step of purifying said complex by a method selected from the group consisting of centrifugation, chromatography, filtration and phase partition.

31. A noncovalent nucleic acid/lipid/polycation complex having a net positive charge at pH 6.0-8.0 comprising a nucleic acid, at least one cationic lipid species and at least one polycation; said complex being produced by mixing a solution of the at least one cationic lipid species and the at least one polycation with the nucleic acid in a ratio of nucleic acid to cationic lipid species to polycation which results in the production of

said complex, said at least one polycation being selected from the group consisting of polyarginine, polyornithine, protamines, polylysine, polybrene™ (hexadimethrine bromide), histone, cationic dendrimer, spermine, spermidine, a peptide comprising polylysine and a peptide comprising a nuclear localization sequence of SV40 large T antigen and which has excess positive charge, wherein said at least one cationic lipid species is present in an amount of from about 10 to about 100 mole % of total liposomal lipid.

32. The complex of claim 31, wherein the ratio of cationic lipid species to nucleic acid ranges from 0.1 nmol to 200 nmol lipid per 1 µg nucleic acid.

33. The complex of claim 31, wherein the ratio of cationic lipid species to nucleic acid ranges from 1 nmol to 25 nmol lipid per 1 µg nucleic acid.

34. The complex of any one of claims 31 to 33, wherein the polycation:nucleic acid ratio is 0.01 µg to 100 µg polycation per 1 µg nucleic acid.

35. The complex of any one of claims 31 to 34, wherein the complex further comprises a neutral phospholipid.

36. The complex of any one of claims 31 to 35, wherein the at least one polycation is the peptide comprising polylysine.

37. The complex of any one of claims 31 to 35, wherein the at least one polycation is the peptide comprising the nuclear localization sequence of SV40 large T antigen and which has excess positive charge.

38. The complex of any one of claims 31 to 37, wherein the at least one polycation has a molecular weight of from 300 to 200,000 daltons.

39. The complex of any one of claims 31 to 35, wherein the at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000 daltons.

40. The complex of claim 35, wherein said at least one polycation is poly-L-lysine having a molecular weight of 300 to 200,000, said cationic lipid is 3βN-(N',N'-dimethylaminoethane)-carbamoyl cholesterol and said neutral phospholipid is dioleoylphosphatidlyethanolamine.

41. The complex of any one of claims 31 to 40, wherein the complex has an average diameter of less than 400 nm.

42. The complex of any one of claims 31 to 41, wherein said complex is substantially free of uncomplexed nucleic acid, lipid species and polycation.

43. A pharmaceutical composition comprising the complex of any one of claims 31 to 42, and a pharmaceutically acceptable carrier.

44. A use of the complex of any one of claims 31 to 42 as a vehicle for delivery of the nucleic acid to cells.

45. The use of claim 44 wherein the cells are cells *in vivo*.

SMART & BIGGAR

OTTAWA, CANADA

PATENT AGENTS

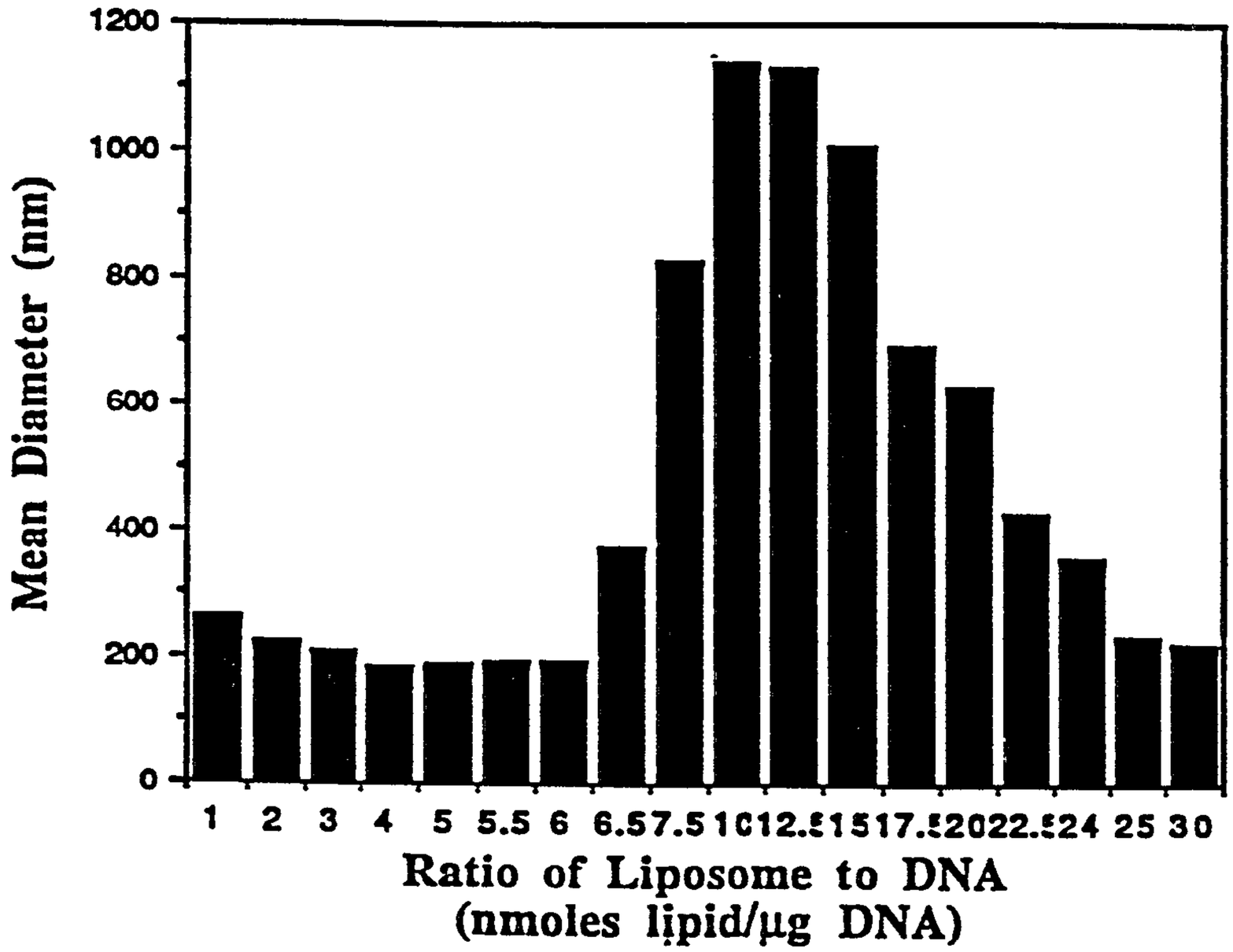
1200
1000
800
600
400
200
0
Mean Diameter (nm)
1 2 3 4 5 5.5 6 6.5 7.5 10 12.5 15 17.5 20 22.5 24 25 30
Ratio of Liposome to DNA
(nmoles lipid/µg DNA)

FIG. 1

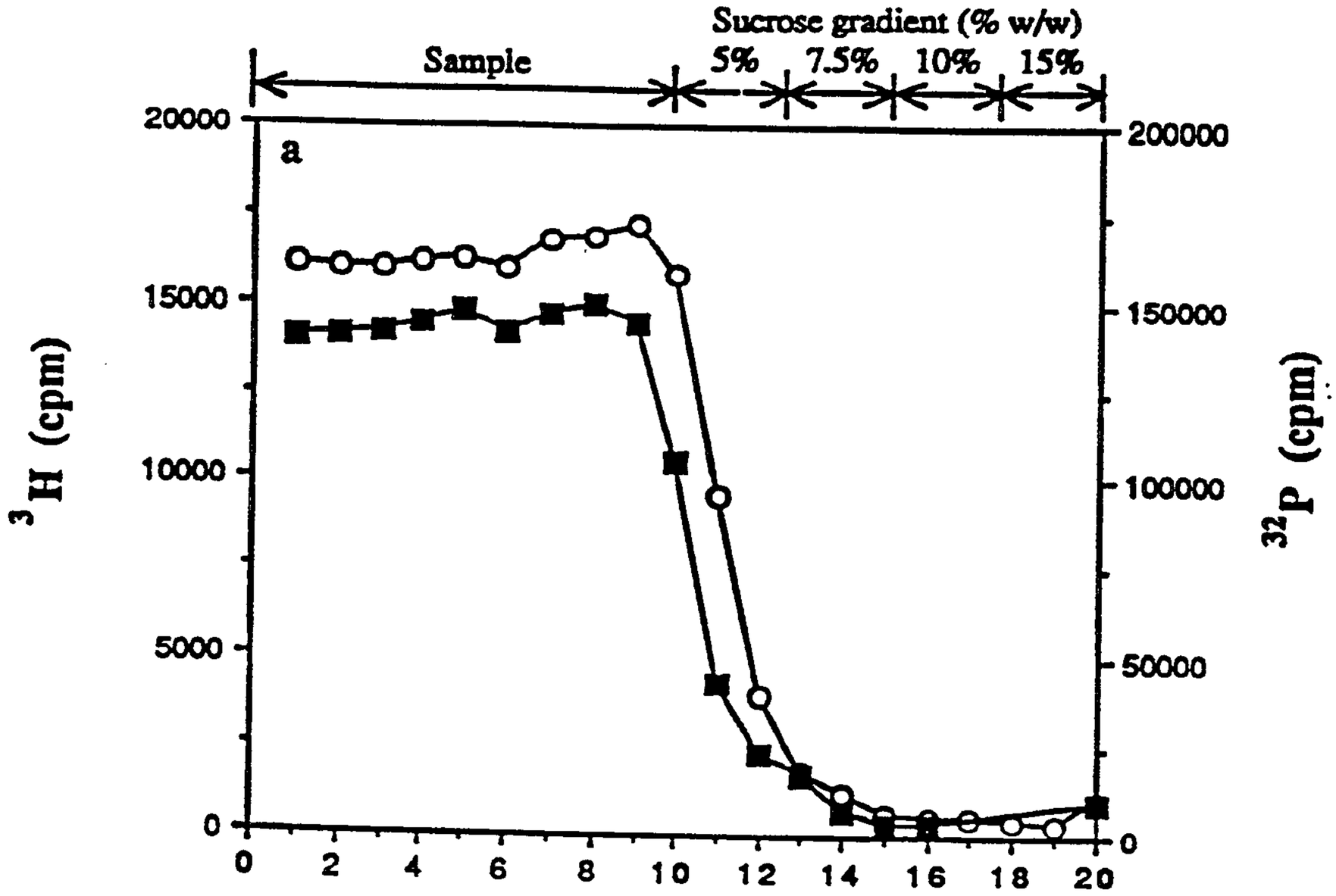
Sucrose gradient (% w/w)
Sample
5%
7.5%
10%
15%
a
20000
15000
10000
5000
0
200000
150000
100000
50000
0
^{3}H (cpm)
^{32}P (cpm)
0 2 4 6 8 10 12 14 16 18 20

FIG. 2A

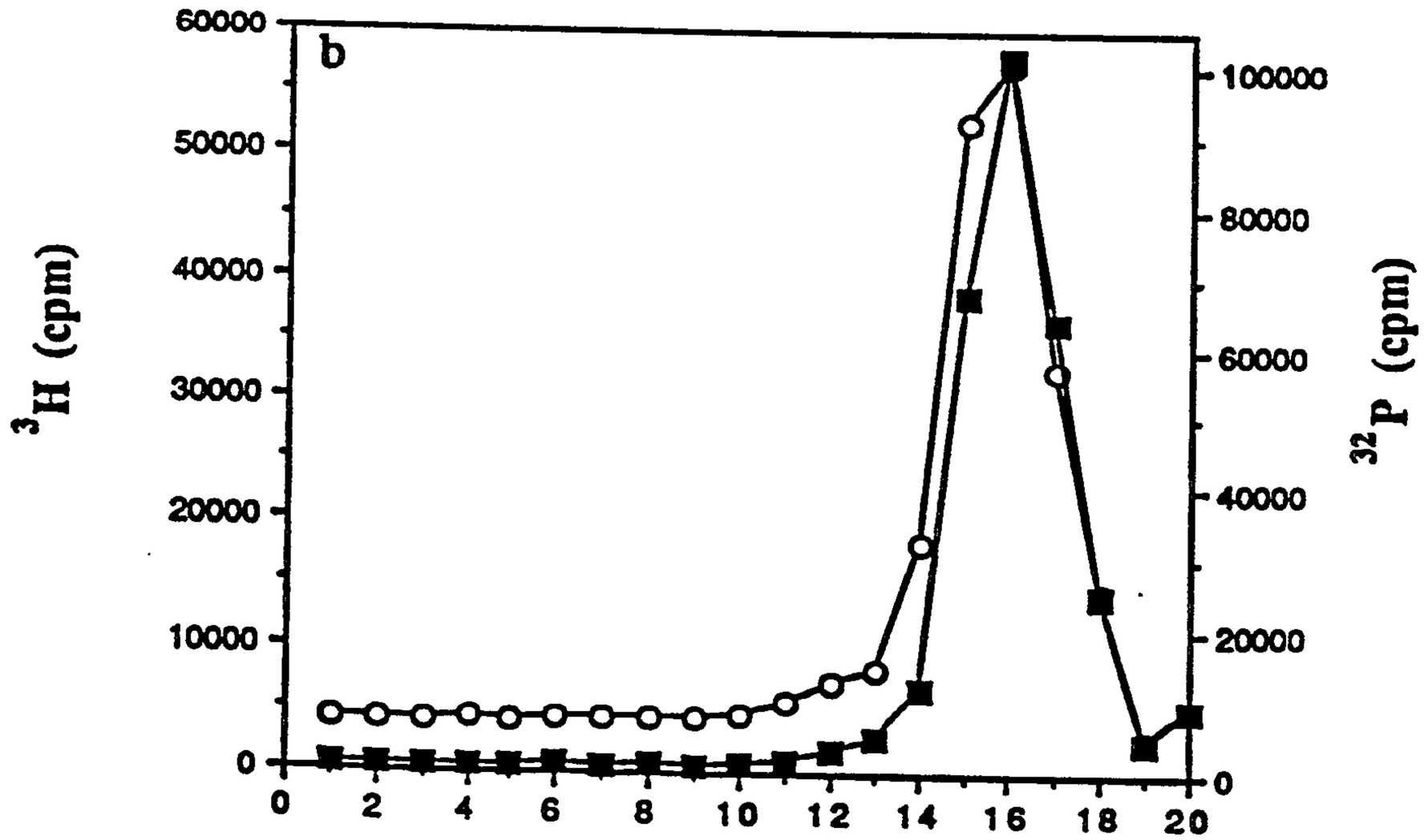
b
60000
50000
40000
30000
20000
10000
0
100000
80000
60000
40000
20000
0
^{3}H (cpm)
^{32}P (cpm)
0 2 4 6 8 10 12 14 16 18 20
Fraction number

FIG. 2B

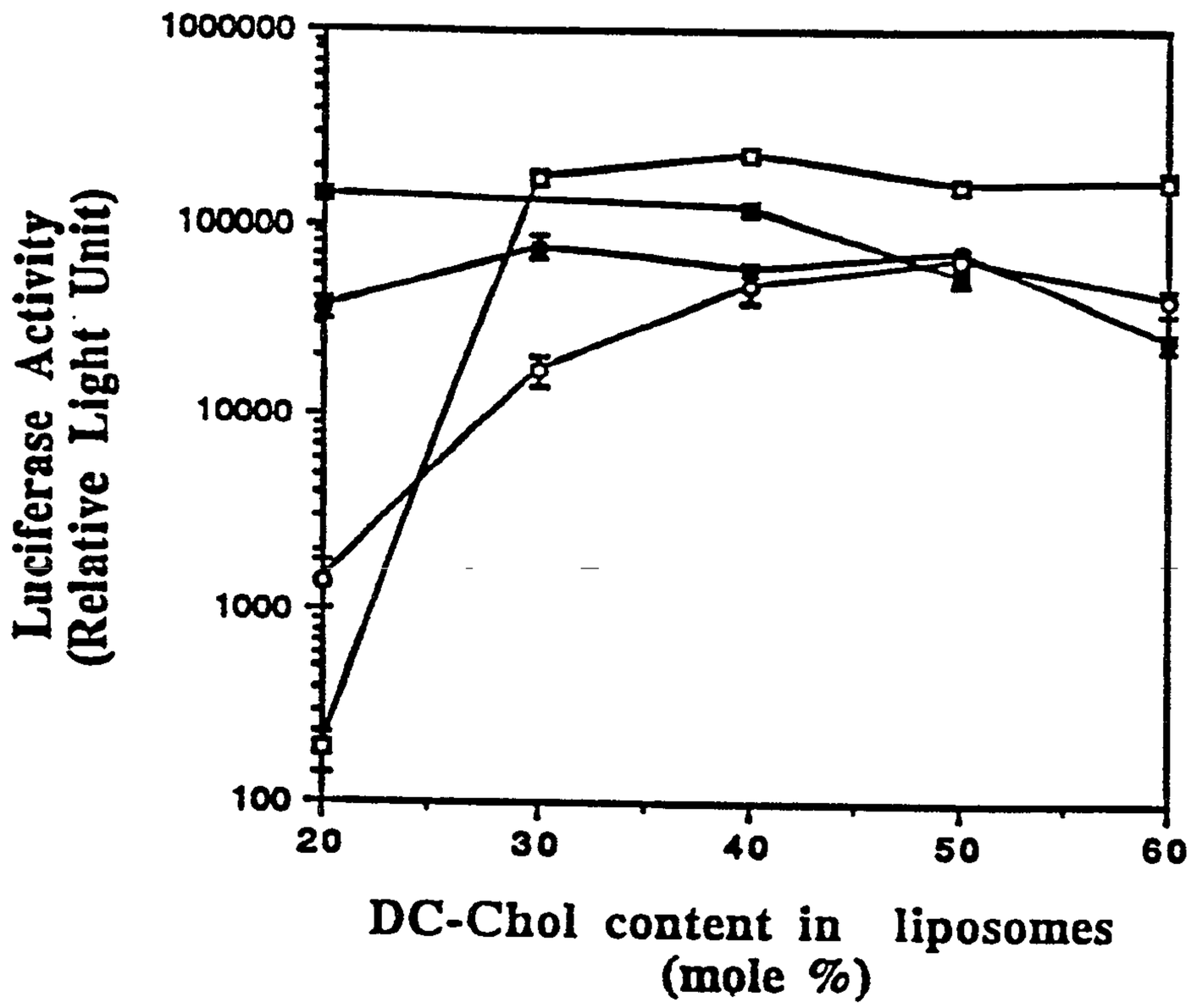
1000000
100000
10000
1000
100
Luciferase Activity
(Relative Light Unit)
20
30
40
50
60
DC-Chol content in liposomes
(mole %)

FIG. 3

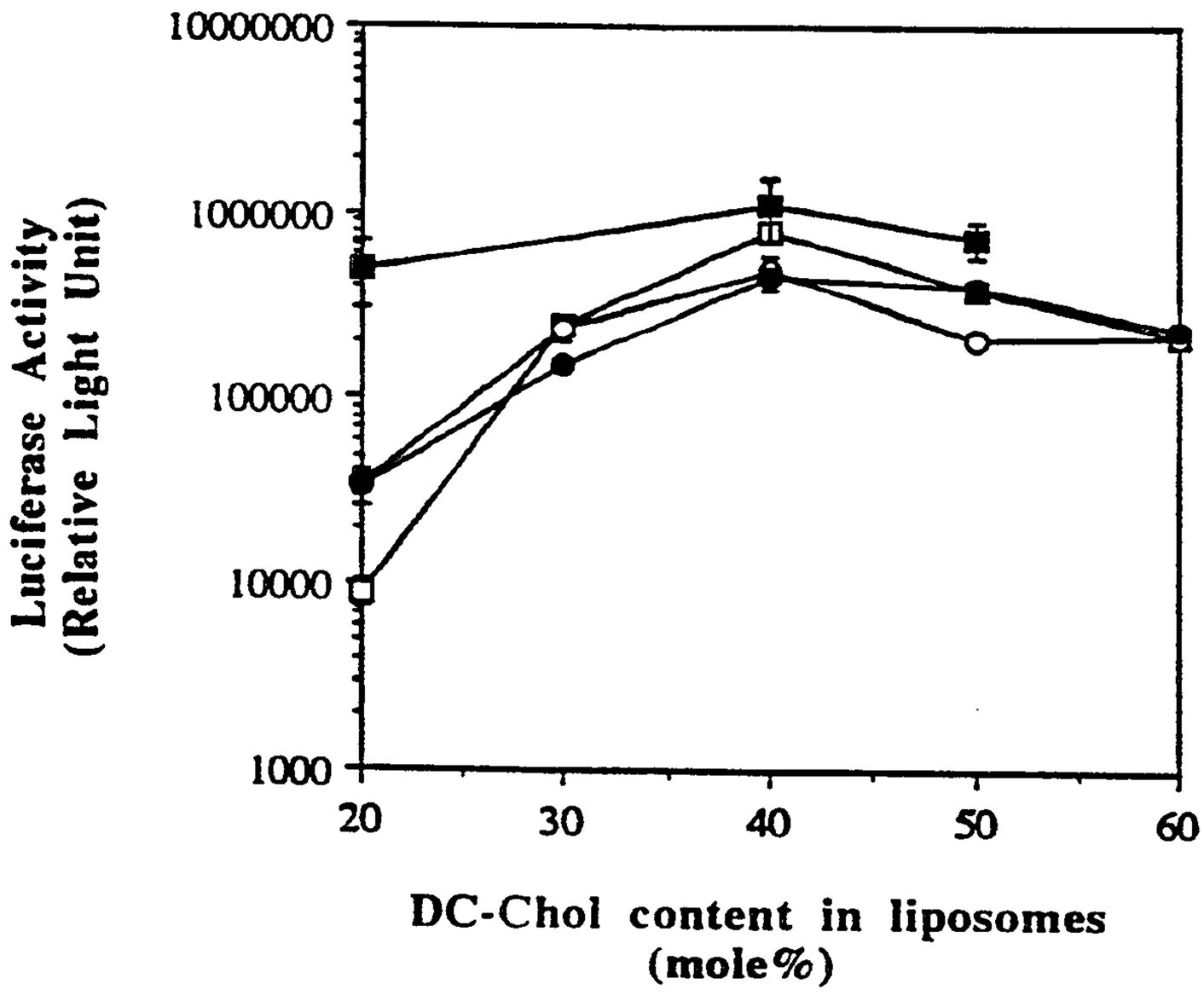
10000000
1000000
100000
10000
1000
Luciferase Activity
(Relative Light Unit)
20
30
40
50
60
DC-Chol content in liposomes
(mole%)

FIG. 4

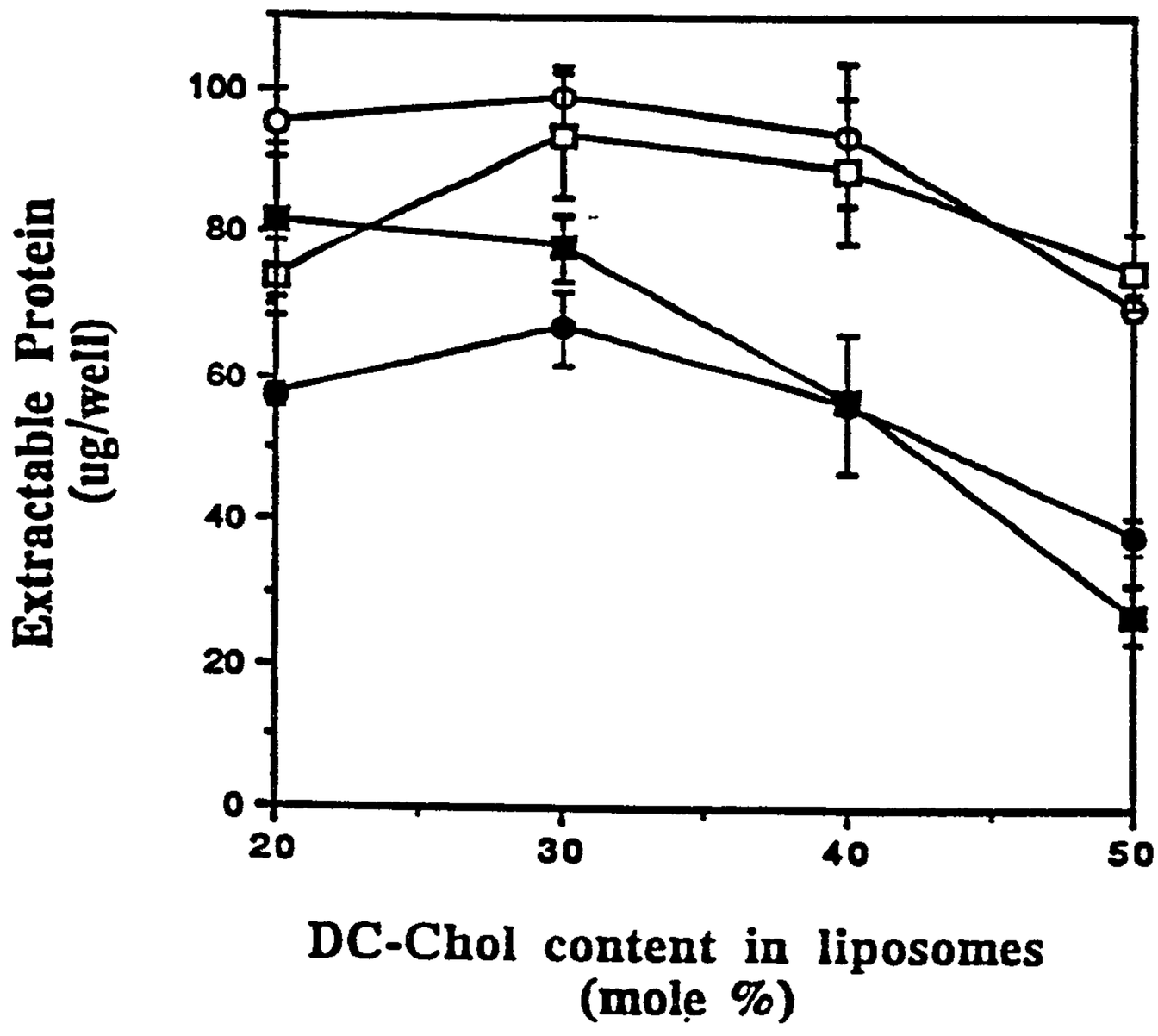
Extractable Protein
(ug/well)
100
80
60
40
20
0
20
30
40
50
DC-Chol content in liposomes
(mole %)

FIG. 5

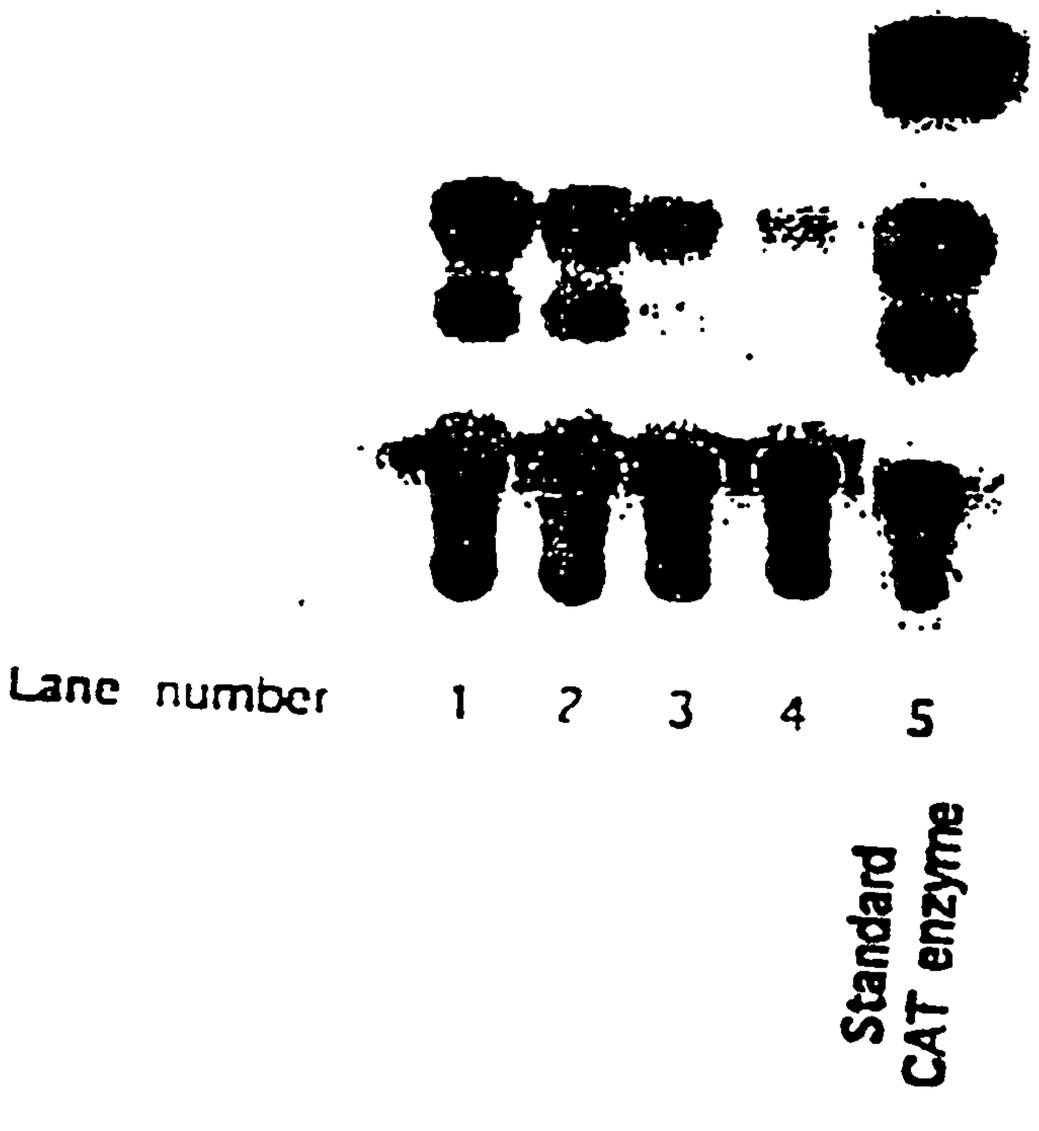

FIG. 6

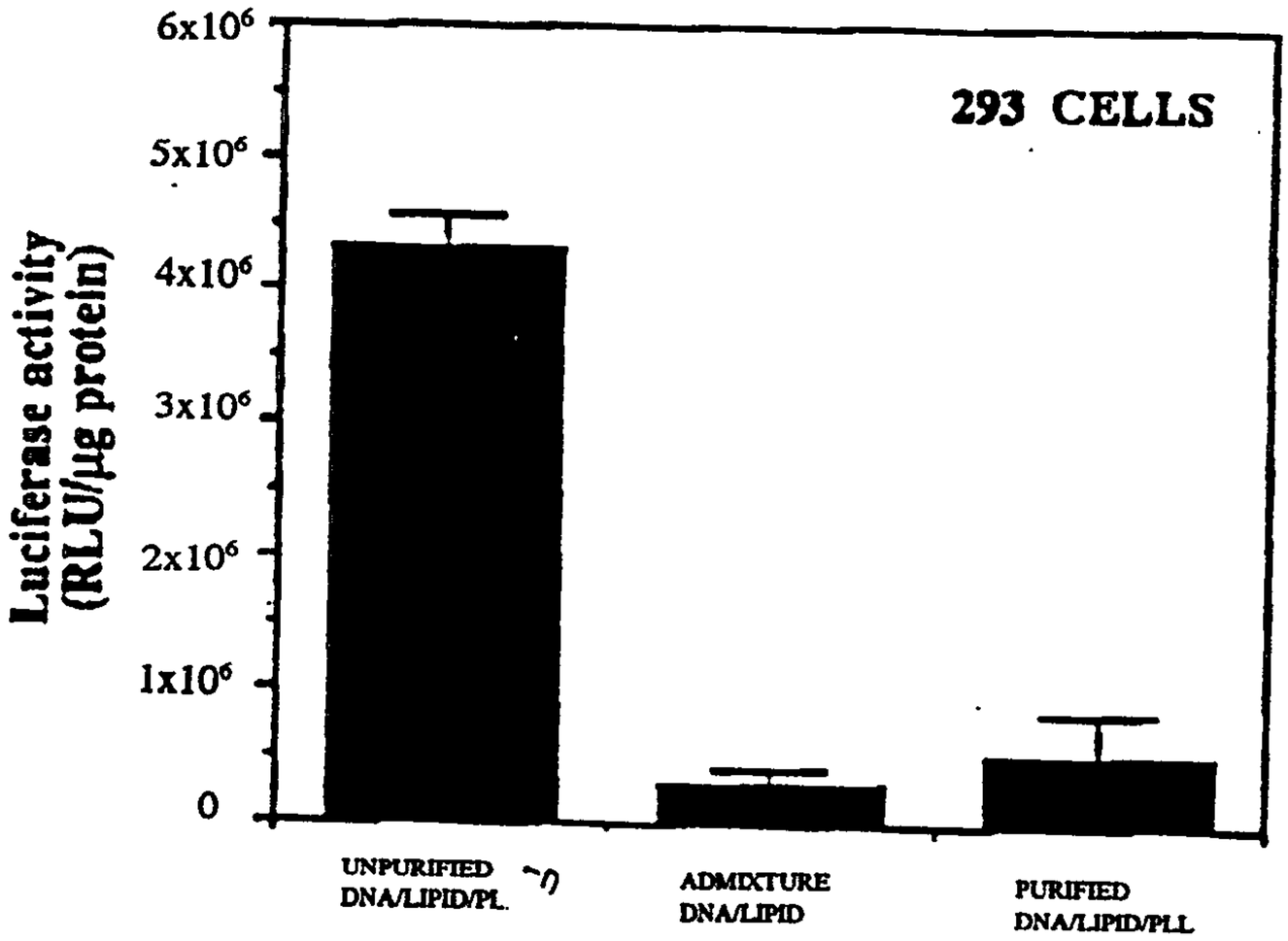
293 CELLS
Luciferase activity (RLU/µg protein)
6x10⁶
5x10⁶
4x10⁶
3x10⁶
2x10⁶
1x10⁶
0
UNPURIFIED DNA/LIPID/PL
ADMIXTURE DNA/LIPID
PURIFIED DNA/LIPID/PLL

FIG. 7A

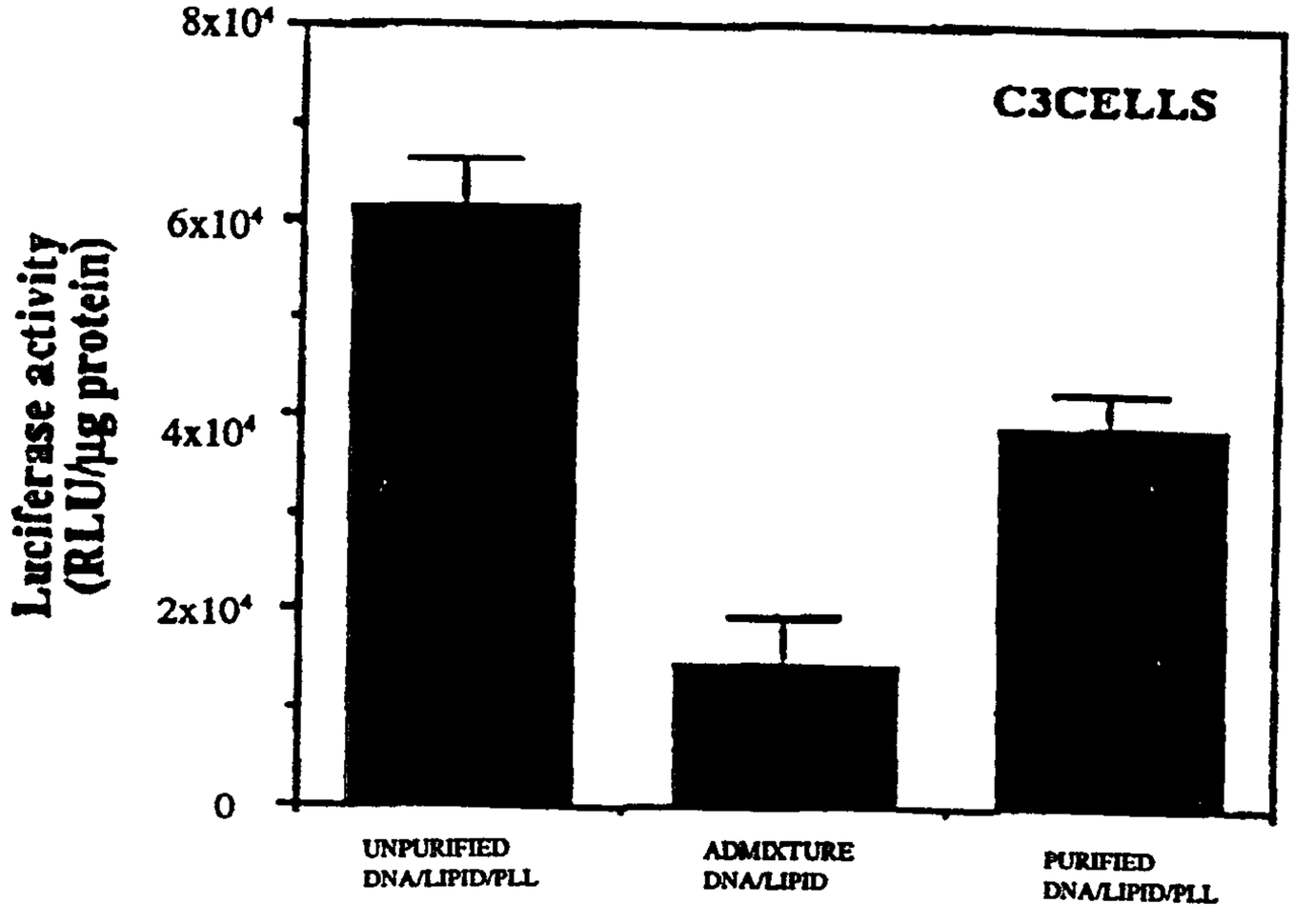
C3CELLS
Luciferase activity
(RLU/µg protein)
8x10⁴
6x10⁴
4x10⁴
2x10⁴
0
UNPURIFIED
DNA/LIPID/PLL
ADMIXTURE
DNA/LIPID
PURIFIED
DNA/LIPID/PLL

FIG. 7B

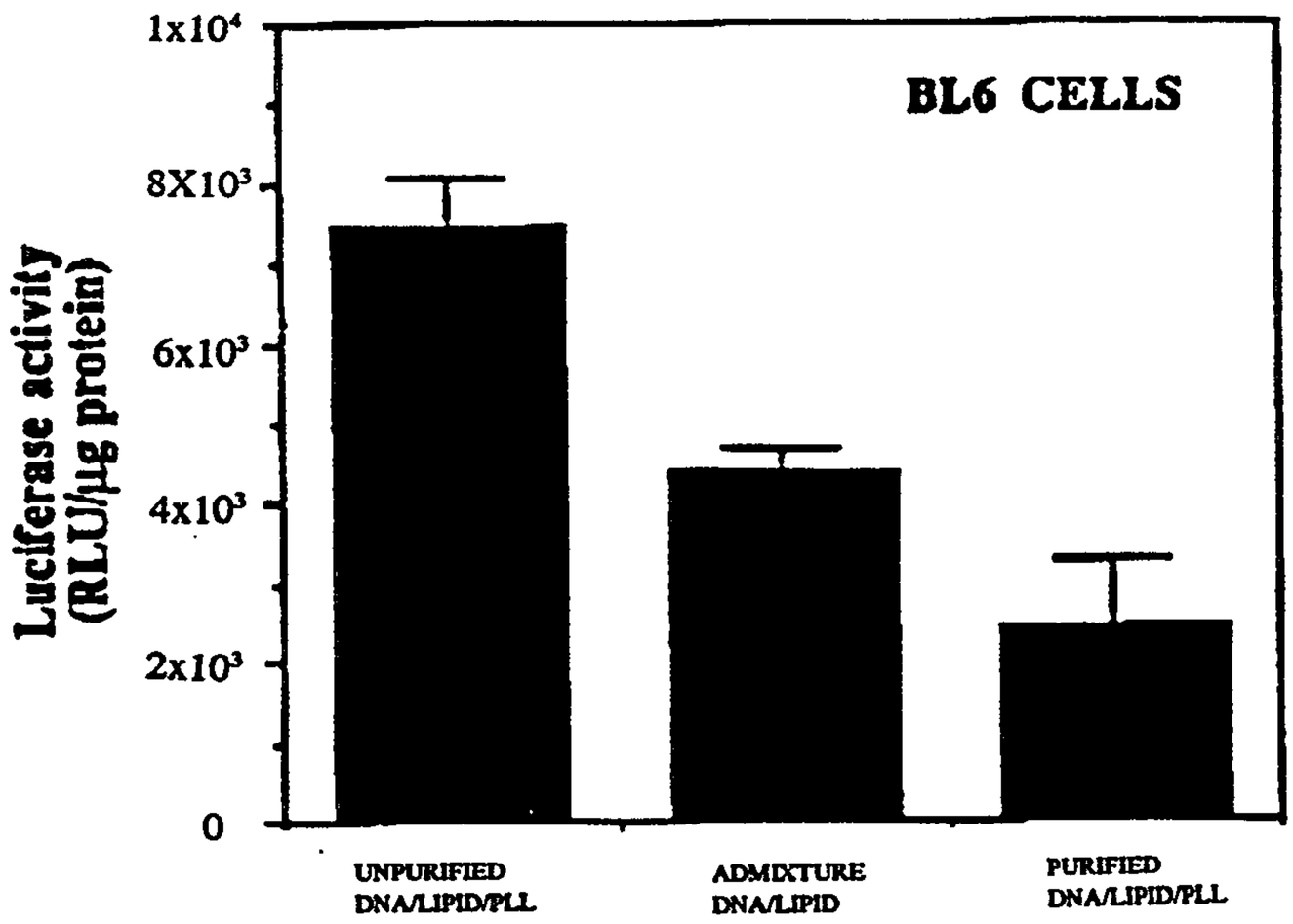
BL6 CELLS
Luciferase activity (RLU/µg protein)
1x10⁴
8X10³
6x10³
4x10³
2x10³
0
UNPURIFIED DNA/LIPID/PLL
ADMIXTURE DNA/LIPID
PURIFIED DNA/LIPID/PLL

FIG. 7C