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Lipophilic polylysines mediate efficient DNA transfection in mammalian cells

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Low molecular weight ($M_r \approx 3000$) poly(L-lysine) (PLL) conjugated to *N*-glutarylphosphatidylethanolamine is an effective carrier to promote DNA-mediated transfection in cultured mammalian cells. The conjugates, named 'lipopolylysines', contained an average of two phospholipid groups per molecule of PLL. Similar conjugates of the non-degradable poly(D-lysine) also had a similar transfection activity, indicating that the degradation of the carrier is not required for the activity. Unconjugated polylysines had little activity. The transfection activity of the lipopolylysine has been optimized with respect to the DNA concentration, DNA/carrier ratio, incubation time and the presence of serum in the incubation medium. The binding of lipopolylysine with DNA was measured by the degree of retardation of DNA in agarose gel electrophoresis. It was found that at the optimal DNA/lipopolylysine ratio for transfection, all DNA were found in large complexes which did not enter the gel. The transfection activity of the lipopolylysine, under optimal conditions, was approximately 3-fold higher than that of lipofectin, a widely used commercial reagent. Moreover, lipopolylysine mediated transfection even in the presence of 10% calf serum; whereas the lipofectin lost about 70% of its activity under the same condition. However, unlike lipofectin the transfection activity of the lipopolylysine depended on scraping the treated cells. Furthermore, lipopolylysine only transfected attached monolayer cells, and not suspension cells.

Introduction

Delivery and the subsequent expression of exogenous DNA into living cells, i.e. DNA-mediated transfection, have become increasingly important for both basic research in cell biology and clinical studies of genetic therapy. Reagents which facilitate the transfection process have been developed, among which the cationic lipids or liposomes have received much attention [1]. These reagents readily complex with DNA and mediate the entry of DNA into cells. The protocol is relatively simple and non-toxic to the cells. For example, liposomes composed of mixtures of phospholipid and a cationic amphiphile such as DOTMA [2], cationic cholesterol derivatives [3], alkyl ammonium [4], and other double-chain amphiphiles [3], belong to this category of reagents. Particularly, liposomes containing DOTMA, i.e. 'lipofectin', have received a wide range of acceptance as a convenient and efficient reagent for transfection of mammalian cells [2,5]. Recently, it has been shown that lipofectin is also effective in the delivery of RNA [6], and proteins [7] into living cells.

Recently, another lipophilic molecule has been reported as an efficient transfection reagent, i.e. lipopolyamine [8]. This reagent mediates the transfection by itself; it does not require any phospholipid to form liposomes. In other words, a micellar complex of lipopolyamine and DNA is sufficient for transfection. The

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Abbreviations: CAT, chloramphenicol acetyl transferase; DCC, 1,3-dicyclohexylcarbodiimide; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOTMA, *N*[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; EDTA, ethylenediaminetetraacetic acid; LPDL, lipopoly(D-lysine); LPLL, lipopoly(L-lysine); NGPE, *N*-glutarylphosphatidylethanolamine; NHS, *N*-hydroxysuccinimide; PDL, poly(D-lysine); PLL, poly(L-lysine).

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cationic polyamine is an essential part of the reagent since it complexes with the anionic DNA. In this regard, polylysine molecules conjugated to the asialoorosomucoid [9] or transferrin [10] are also effective in the target-specific delivery of DNA to the cells which express the appropriate receptor. The role of the polylysine is again to serve as an efficient carrier for DNA.

It thus seems reasonable to assume that a target-specific, efficient DNA vehicle can be constructed with three components: a target-specific ligand, a polycation, and a lipophilic moiety. A relatively simple way to construct this carrier would be to conjugate the ligand to the lipopolyamine. However, there are only four primary amino groups on the lipopolyamine. Consumption of any one of these for ligand conjugation would probably reduce the DNA complexing activity of the molecule. We have therefore decided to use a larger polycation, i.e. the polylysine. As the first step of the project, we have prepared a polylysine-phospholipid conjugate, i.e. the lipopolylysine, and show in this report that it is an efficient transfection reagent. We have also optimized the transfection protocol and show that the transfection activity of the lipopolylysine is about 3-fold higher than that of the lipofectin.

Materials and Methods

Materials

N-Glutaryl-1,2-dioleoylphosphatidylethanolamine (NGPE), dioleoylphosphatidylethanolamine (DOPE), and dioloeylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids, Inc. 1,3-Dicyclohexylcarbodiimide (DCC) and triethylamine were purchased from Aldrich Chemical Company. *N*-Hydroxysuccinimide (NHS), poly(L-lysine) hydrobromide (PLL) (mol. wt. 3340), poly(D-lysine) hydrobromide (PDL) (mol. wt. 3800), dimethyl sulfoxide, and acetyl coenzyme A, lithium salt were obtained from Sigma Chemical Co. Lipofectin was purchased from Bethesda Research Laboratories Inc. D-*threo*[1,2-^{14}C]chloramphenicol (57 mCi/mmol) was purchased from ICN Biomedicals, Inc. pSV2CAT plasmid DNA was grown in *Escherichia coli* and purified by the CsCl gradient centrifugation method [11].

Synthesis of lipopolylysines

NGPE was dialyzed against 0.001 M HCl and then lyophilized and redissolved in chloroform. NGPE was activated as the *N*-hydroxysuccinimide ester by mixing with DCC at 1.2 equivalent in $CHCl_3$ and with NHS at 1 equivalent in dimethylformamide. The reaction proceeded at room temperature for 48 h. The crystal reaction byproduct, dicyclohexylisourea, was removed by filtration through glass wool. Lipopoly(L-lysine) (LPLL) or lipopoly(D-lysine) (LPDL) was prepared as follows: activated NGPE was added to PLL or PDL in dimethyl sulfoxide at a molar ratio of polylysine/NGPE = 1 : 2. Triethylamine at equal weight with PLL or PDL was added to the above mixture. The reaction was complete in a few min. Reaction products were analyzed on a silica gel plate using a solvent system of chloroform/methanol = 65 : 30. Polylysine and the phospholipid were stained by ninhydrin and Zinzade's solution, respectively [12]. Since all of the NHS ester of NGPE was consumed in the reaction, the stoichiometry of lipopolylysine was assumed to be polylysine/NGPE = 1 : 2. Lipopolylysines were used without any further purification.

Electrophoresis of DNA

LPLL suspension was prepared in 20 mM phosphate buffer (pH 7.4) at a concentration of 0.66 μg/ml. Various amounts of LPLL were mixed with 0.5 μg of DNA in serum free medium. The final volume was 20 μl. The mixture was incubated at room temperature for 15 min before loading onto a 1% agarose gel slab. Electrophoresis was carried out in TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer according to the standard technique [11].

Cell culture

L929 and Vero cells were grown in McCoy's medium containing 10% calf serum. RDM4 and CEM cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. HeLa cells were grown in Dulbecco's minimal essential medium supplemented with non-essential amino acids and 10% fetal bovine serum. For the attached cells, cultures of approximately 80% confluency were used for the transfection experiments.

Transfection

Polylysines, lipopolylysines or lipopolylysine/lipid mixtures were suspended in 200 μl phosphate buffer (20 mM, pH 7.4) and sonicated to clarity. The suspension and pSV2CAT DNA were diluted separately to 2.5 ml with serum-free medium and then mixed. The complexed DNA was then added to cells. After incubation at 37 °C, cells were washed three times with serum-free medium and then scraped and divided into two new plates. Serum containing growth medium was added back to the culture plate. Two days later, cells were harvested to assay for CAT activity. The transfection protocol for lipofectin was provided by the manufacturer. 40 μg of lipofectin was used to deliver 10 μg of DNA.

Assay of chloramphenicol acetyl transferase activity

CAT activity was determined by the method of Gorman et al. [13]. Cell extract was prepared by the freeze-thaw method. 0.5 to 1 mg of cellular protein was heat-treated at 65 °C for 10 min, and then added with 20 μl

of 4 mM acetyl-CoA and 0.2 μCi of [^{14}C]chloramphenicol. After 1.5 h incubation at 37°C, chloramphenicol and its derivatives were extracted with ethyl acetate and separated by thin-layer chromatography on a silica gel plate. The plate was autoradiographed and the appropriate spots were scraped for radioactivity counting to analyze the percentage of chloramphenicol acetylation. One unit of CAT activity is defined as conversion of one nanomole chloramphenicol into its acetylated derivatives per min.

Results

Comparison of different transfection reagents

We have prepared several different transfection reagents and compared their transfection activities on L929 mouse fibroblast cells using a plasmid DNA (pSV2CAT) containing a standard marker gene coding for the *E. coli* CAT. These experiments were done by mixing the lipopolysine or other reagents with a constant amount (10 μg) of pSV2CAT DNA and added to cells in a serum-free medium. After 3 h of incubation at 37°C, cells were washed, scraped and recultured in fresh serum-containing medium for 2 days before the CAT activity of the cells was assayed. Table I shows the results of the experiment. Lipopoly(L-lysine) (LPLL) transfected the cells very efficiently; the expression of the CAT activity was approximately 6.4 mU/mg protein. Similarly efficient was the lipopoly(D-lysine) (LPDL), a non-biodegradable polymer conjugate. This result indicates that the degradation of the carrier is not essential for efficient transfection. The native poly(L-lysine) or poly(D-lysine) without the phospholipid conjugation was not active, indicating the importance of the lipophilic moiety for an efficient transfection reagent. It has been reported [2,4] that DOTMA and other cationic lipids exhibit their maximal transfection

TABLE I

Comparison of transfection efficiency for different transfection reagents

L929 cells were transfected with pSV2CAT (10 μg) alone or complexed with lipofectin (40 μg), PLL (44 μg), LPLL (66 μg), LPLL/DOPC (66 μg/138 μg), LPLL/DOPE (66 μg/130 μg), PDL (44 μg) or LPDL (66 μg). Cells were washed and scraped after a 3 h incubation with the mixtures and harvested 2 days later for CAT activity determination.

Transfection reagent	CAT activity (% of max)
E. coli CAT	50
lipofectin	31.7
PLL	1.3
LPLL	100
LPLL/DOPC	50
LPLL/DOPE	41.5
DNA	0.1
PDL	2.9
LPDL	87.8

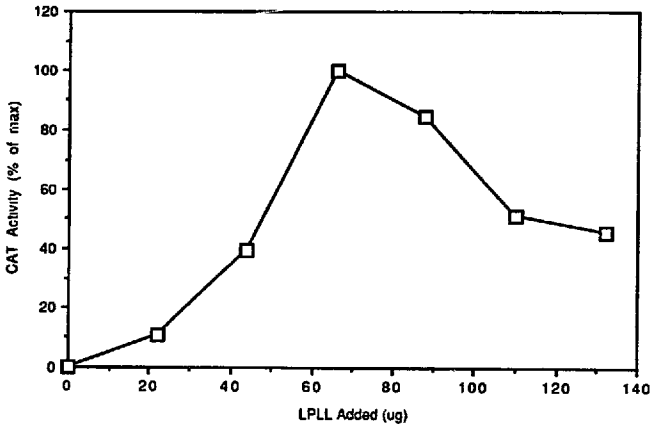

Fig. 1. The effect of LPLL dose on transfection efficiency. Various amounts of LPLL were complexed with 10 μg of pSV2CAT DNA. L929 cells were transfected with the mixtures and assayed for CAT activities following the standard procedures described in Materials and Methods.

activities when they are mixed with a phospholipid, particularly an unsaturated phosphatidylethanolamine, to form cationic liposomes. To test if mixing with phospholipids enhances the transfection activity, we have used DOPC and DOPE to mix with the LPLL (DOPC or DOPE/LPLL = 13:1, molar ratio) and tested the transfection activity. It was found that mixing with either DOPC or DOPE the activity of LPLL had decreased to about 50% of that shown by the LPLL alone (Table I). This result indicates that LPLL works more efficiently as a solo reagent: no liposomal formulation is needed. It is interesting to note that the transfection activity of LPLL was approximately 3-fold higher than that of the lipofectin, a cationic liposome formulation widely used for DNA transfection [2,14,15].

Effect of LPLL dose on the transfection efficiency

To test if the transfection activity of LPLL was dose-dependent, an experiment was done in which various amounts of LPLL were used with a constant amount (10 μg) of pSV2CAT DNA. Fig. 1 shows a bell-shaped dose-response curve with the optimal dose of LPLL occurring at approximately 60–70 μg. The decrease of CAT activity at higher doses was not due to the cytotoxicity of LPLL because cells treated with up to 132 μg LPLL per plate for 6 h still had more than 90% viability after 24-h post incubation.

Effect of DNA dose on the transfection efficiency

We then fixed the dose of LPLL at 66 μg and varied the DNA concentration. Again, a bell-shaped dose-response curve was obtained (Fig. 2). The optimal DNA concentration was 10 μg. For the subsequent experiments, we have used 66 μg LPLL and 10 μg DNA as a standard condition. The ratio of positive to negative charges of the LPLL-DNA complex under this optimal

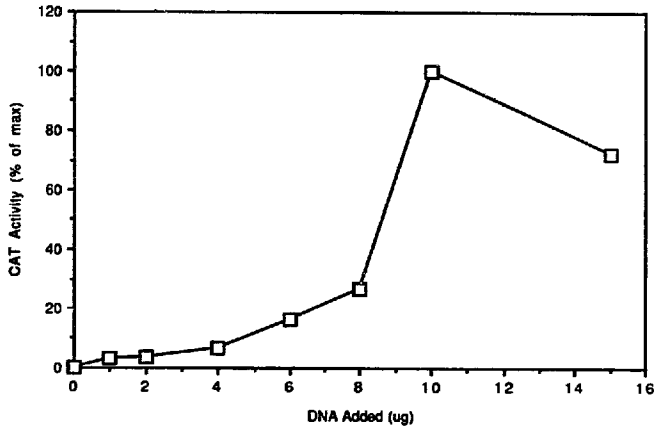

Fig. 2. The effect of DNA dose on transfection efficiency. Various amounts of pSV2CAT DNA were complexed with 66 μg of LPLL. L929 cells were transfected with the mixtures and assayed for CAT activities following the standard procedures described in Materials and Methods.

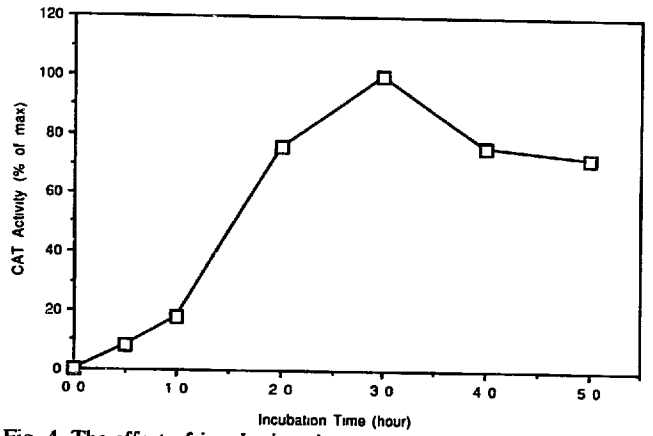

Fig. 4. The effect of incubation time on transfection efficiency. L929 cells were incubated with mixtures of 10 μg pSV2CAT DNA and 66 μg LPLL for various lengths of time. At the end of incubation, cells were washed and scraped and harvested 2 days later for CAT activity assay.

condition is approximately 6. At this ratio, slightly turbid complexes could be seen, but no precipitates were formed.

Electrophoresis of DNA-LPLL complexes

To examine the complex formation of DNA with LPLL, various amounts of LPLL were added to DNA and the complexes were electrophoresed in an agarose gel. The gel was stained with ethidium bromide to visualize DNA. As can be seen in Fig. 3, when 0.5 μg of DNA complexed with as little as 1.1 μg LPLL a retardation of the DNA bands in the gel (lane 2) had resulted. It indicates that the DNA-LPLL complex is larger in size and/or less negatively charged (hence less movement toward the anode) than the native DNA. At 3.3 μg LPPL, when the positive-to-negative charge ratio was 6, complete retardation of the DNA bands occurred (lane 4). Thus, the optimal DNA-LPLL complex of the charge ratio 6 was fairly large in size and/or not negatively charged.

Effect of incubation time on the transfection efficiency

The efficiency of the transfection was incubation time-dependent. Little transfection was detectable for incubation times shorter than 1 h. Maximal efficiency was observed at 3 h of incubation (Fig. 4). Although the cells had been incubated with the LPLL-DNA complex in a serum-free medium, no gross morphological changes or cell death was observed at the end of 6 h incubation.

Effect of serum concentration on the transfection efficiency

Although the transfection incubation was routinely done in a serum-free medium, we have tested the effect of serum addition to the incubation medium on the efficiency of transfection. It is clear from the data in

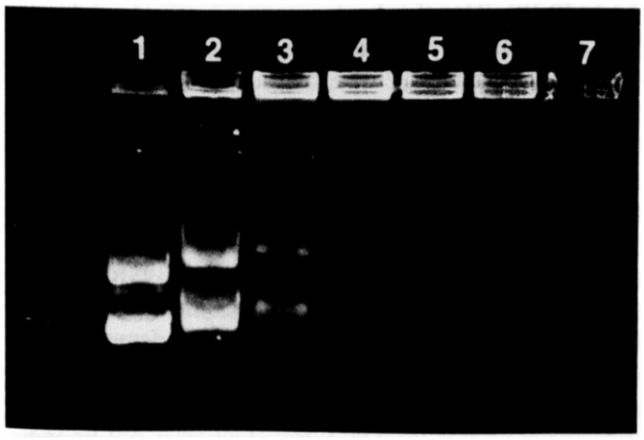

Fig. 3. The effect of LPLL dose on the electrophoretic mobility of plasmid DNA. 0.5 μg of pSV2CAT was complexed with 0, 1.1, 2.2, 3.3, 4.4, 5.5 and 6.6 μg (lane 1 to 7, respectively) of LPLL in serum free medium. The complexes were electrophoresed on 1% agarose gel. DNA was visualized with ethidium bromide.

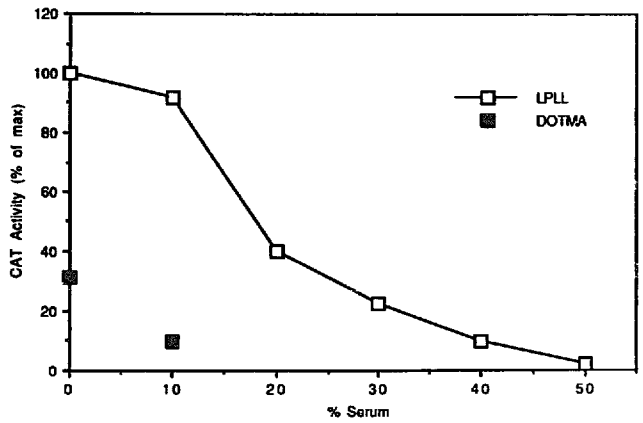

Fig. 5. The effect of serum concentration on transfection efficiency. 10 μg pSV2CAT DNA and 66 μg of LPLL were complexed and then mixed with various amounts of serum. The mixtures were added to L929 cell monolayers. After a 3 h incubation, cells were washed, scraped and harvested 2 days later for CAT activity assay.

Fig. 5 that the calf serum produced a concentration-dependent inhibition effect on the transfection efficiency. Almost no transfection was detectable at 40–50% serum concentration. however, considerable (90% or greater) amount of the transfection activity was still observed at 10% serum concentration. In contrast, little transfection activity was observable if 10% serum was added to the lipofectin reagent (Fig. 5). This result indicates that LPLL can tolerate the inhibitory effect of the serum better than the lipofectin. Most mammalian cells are healthy in a medium containing 10% serum. Thus, the lipopolylysine reagent may be more useful than the lipofectin for those cells which require serum for viability.

Effect of cell scraping on the transfection efficiency

We routinely transfer the transfected cells into new plates at a lower cell density to avoid cell overgrowth during the 2-day postincubation period. The transfer was done by mechanically scraping the cells off the plate and reculturing them in new plates. It turns out that the scraping step is important for the maximal transfection activity, because cells transferred by using either trypsin or EDTA showed only low levels of activity (Table II). Cells not transferred to the new plates also showed a low activity. Interestingly, the transfection activity of lipofectin was also enhanced by the scraping procedure by approximately 3-fold (Table II).

Effect of cell type on the transfection activity of LPLL

L929 cells are monolayer cells. It was interesting to see if LPLL is effective in other monolayer cells and suspension cells. Table III shows that two other monolayer cells, Vero (monkey cells) and HeLa (human cells), could be effectively transfected by LPLL using a protocol which was optimized for the mouse L929 cells. However, two suspension cell lines, mouse RDM4 and human CEM, showed little transfection activity. Both RDM4 and CEM cells are lymphoid cells which are known to be resistant to transfection [16,17].

TABLE II

The effect of post-treatment on transfection efficiency

Mouse L929 cells (approximately 40% confluent) were incubated with complexes of LPLL-DNA (66 μg and 10 μg, respectively) or lipofectin/DNA (40 μg and 10 μg, respectively) for 3 h. Cells were washed and either scraped or detached by trypsin or EDTA treatment and recultured in a new plate for 2 days. CAT activity of the cell extract was determined as described in Materials and Methods.

Transfection reagent	Post-treatment	CAT activity (% of max)
Lipofectin	none	10.4
	scraping	31.4
LPLL	none	0.8
	scraping	100
	trypsin	2.7
	EDTA	2.0

TABLE III

Comparison of transfection efficiency for different cell lines

L929, Vero, HeLa, RDM4 and CEM cells were transfected with LPLL (66 μg)-pSV2CAT (10 μg) complex and assayed for CAT activities following the standard procedures described in Materials and Methods.

Cell line	CAT activity (% of max)
L929	100
Vero	71.3
HeLa	43.2
RDM4	0.7
CEM	0.5

Discussion

The long-term goal of the project is to develop a target-specific and efficient vehicle for DNA transfection. Such a vehicle would be very useful for genetic therapy treatment currently under active investigation. Polylysines are suitable for this purpose for the following reasons. First, the polymer readily complexes with DNA of different sizes, both linear, circular and supercoiled [18,19]. A vehicle composed of polylysine would be a general carrier for DNA. Secondly, polylysine contains many primary amino groups which can be conveniently used for conjugation with lipids or ligands. The cationic oligomer or polymer itself is not a good carrier for DNA transfection; a lipophilic derivative significantly enhances the transfection activity (Table I and Ref. 8). Polylysine-conjugated drugs, although potent in drug action, are usually non-specific. Additional conjugation with ligands, such as antibody or hormones, is needed for the target specificity of the vehicle [9,10]. The third advantage of using polylysine as a DNA carrier is its relatively low toxicity to cells. This is particularly the case for poly(L-lysine) which is degradable by the target cells. Lastly, the polymer is commercially available in different polymeric sizes and is relatively inexpensive. We have thus chosen polylysine as the starting point of the project.

As shown in Table I, the native polylysine, no matter L- or D-isomer, showed very little activity for DNA transfection; only the phospholipid conjugates exhibited a high level of activity. This result clearly demonstrates the importance of lipophilicity of the transfection reagent. While the mechanism by which the lipophilic polylysine mediates the DNA transfection is not known, it is unlikely that the large macromolecular complex can actually enter the cytoplasm by directly penetrating the plasma membrane. A more plausible mechanism is that the DNA-lipopolylysine complexes bind to the cell

surface and are internalized to the cellular endosomes or lysosomes. The phospholipid moiety of the complex could conceivably cause a perturbation in the membranes of the endosomes or lysosomes, leading to a release of the complex from these organelles into the cytoplasm. Liposomes containing cationic lipids mediate the delivery of DNA into cells probably with a similar mechanism [4]. Although there is not enough experimental data at present to support the hypothesis, the mechanism is consistent with the observation that both poly(L-lysine) and poly(D-lysine) are similarly effective in transfection (Table I). Since poly(D-lysine) is not biodegradable, it implies that the release of the DNA-polymer complex does not require the degradation of the polymer, which takes place in the lysosome compartment. Since poly(L-lysine) is less toxic than the poly(D-lysines) [20], we have chosen to perform the detailed investigations with the poly(L-lysine) conjugates.

The dose-response curves for LPLL (Fig. 1) and for DNA (Fig. 2) are both bell-shaped. This is not a surprise in view of the polyvalent nature of DNA and polylysine. Optimal level of complex formation between the polyvalent binders requires a balance in the concentrations of both binders. The presence of excess of either binder would result in fewer and/or smaller complexes. The optimal amounts of LPLL and DNA are such that the positive-to-negative charge ratio of the complex is approximately 6. This means that the complex contains a net positive charge (Fig. 3) which would allow an efficient binding of the complex to the negatively charged cell surface. Furthermore, the complex can probably tolerate the presence of other negatively charged macromolecules such as serum proteins. This is probably the reason why the action of lipopolylysine transfection reagent was only slightly inhibited in the presence of 10% calf serum (Fig. 5). The lipofectin reagent, on the other hand, was very sensitive to the presence of serum; little activity could be detected when 10% calf serum was added (Fig. 5).

The data in Table II clearly demonstrate the importance of mechanical scraping for the maximal transfection activity of lipopolylysine. It has been reported that scraping alone can help load the cells with macromolecules [21]. Mechanical shearing during the scraping procedure could produce transient membrane damage to allow a more efficient entry of the complex into the cytoplasm. Alternatively, the cells could be stimulated to undergo more active endocytosis as a part of the membrane repair mechanism. Interestingly, the transfection activity of lipofectin was also enhanced by scraping the cells, suggesting that the two reagents share a common mechanism of action. The enhancement effect of cell scraping does not imply that an efficient transfection requires an intermittent period of time during which the cells must be in suspension. This is because the cells which were detached from the plate by a treatment of trypsin or EDTA did not show a high level of transfection (Table II). In fact, suspension cells such as mouse RDM4 and human CEM cells were not transfected well with the lipopolylysine reagent (Fig. 5). These lymphoid cells are known to be relatively resistant to transfection; they are usually transfected with the electroporation method [22–24].

The present LPLL reagent contains an average of 16 lysine residues and 2 phospholipid groups. While this reagent works well for DNA transfection, it is possible that polylysines of greater or shorter length may also work. Conjugates with more or fewer phospholipid groups could also be active in transfection. Furthermore, the location of the phospholipid groups on the polylysine chain is probably random for the present LPLL reagent. Specific location such as at either or both ends of the polylysine molecule could possibly give a different transfection activity. These possibilities are currently under investigation.

In summary, we have described a new transfection reagent which is composed of a simple conjugate of a cationic polymer and phospholipids. The reagent works well for the DNA transfection of a number of monolayer cells, even in the presence of 10% serum. The transfection protocol is relatively simple and non-toxic to cells. The transfection activity of the new reagent is about 3-fold higher than that of the lipofectin, a commonly used reagent for DNA transfection. This new reagent should also be useful in the construction of a target-specific delivery vehicle for DNA.

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