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Evaluation and optimization of DNA delivery into gliosarcoma 9L cells by a cholesterol-based cationic liposome

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Abstract

This paper reports results concerning the transfection of gliosarcoma cells 9L using an original cholesterol-based cationic liposome as carrier. This cationic liposome was prepared from triethyl aminopropane carbamoyl cholesterol (TEAPC-Chol) and a helper lipid, dioleoyl phosphatidyl ethanolamine (DOPE). The used concentration of liposome was not cytotoxic as revealed by the MTT test. TEAPC-Chol/DOPE liposomes allowed the plasmids encoding reporter genes to enter the nucleus as observed both by electron microscopy and functionality tests using fluorescence detection of green fluorescent protein (GFP) and luminometric measurements of luciferase activity. By changing the cationic lipid/DNA molar charge ratio, optimal conditions were determined. Further, improvement of the transfection level has been obtained by either precondensing plasmid DNA with poly-L-lysine or by adding polyethylene glycol (PEG) in the transfection medium. The optimal conditions determined are different depending on whether the transfection is made with cells in culture or with tumors induced by subcutaneous (s.c.) injection of cells in Nude mice. For in vivo assays, a simple method to overcome the interference of haemoglobin with the chemiluminescence intensity of luciferase has been used. These results would be useful for gaining knowledge about the potential for the cationic liposome TEAPC-Chol/DOPE to transfect brain tumors efficiently.

Keywords: Gliosarcoma cell; Cationic liposome; Gene therapy; Reporter gene

1. Introduction

In light of the low efficacy of available treatments for brain cancers, gene therapy appears to be a promising solution (for review see Ref. [1]). Among brain cancer models, one of the most studied is the gliosarcoma induced by 9L cells [2,3].This model has been used in several approaches of gene therapy, and attempts to treat gliosarcomas have been carried out, such as cytokine gene therapy [4,5] or the use of antisense oligonucleotides [6]. For such strategies, carriers are necessary for DNA transfer. Up to now, gene carriers are mostly of viral type. Despite their efficiency, viral vectors present serious drawbacks, because immunogenicity and insertional mutations cannot be excluded. As an alternative, nonviral gene carriers such as cationic liposomes have been developed [7,8] and seem attractive because they appear to be safe and are easy to handle. Since the first experiments by Felgner et al. [9], it has been shown that cationic lipids can efficiently transfect a variety of cell lines in culture. Transfections in vivo were successful and cationic lipid-mediated transfection has shown promise for brain cancer gene therapy [10,11]. Previous studies have demonstrated interest in cholesterol-based cationic liposomes for transfecting cancer cells [12,13]. Comparative experiments by Egilmez et al. [14] have shown the superiority of cationic liposomes derived from cholesterol over other cationic lipids in experiments in vivo.

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The original liposome developed for this work is based on 3-β[*N*-(*N*′,*N*′,*N*′-triethyl aminopropane) carbamoyl] cholesterol iodide (TEAPC-Chol). On one hand, cholesterol is known for its stabilizing effect on liposomal membranes; on the other hand, the ramified quaternary ammonium polar head of TEAPC-Chol has been chosen assuming that such a polar head will not be affected by the pH or charged components in transfection media. TEAPC-Chol forms stable liposomes when mixed with the helper lipid dioleoyl phosphatidyl ethanolamine (DOPE).

After analyzing the characteristics of the association between a DNA plasmid and TEAPC-Chol/DOPE liposome, cytotoxicity has been evaluated. Using a green fluorescent protein (GFP)-encoding plasmid, gene functionality was observed after liposome-mediated delivery of the DNA in 9L cells. The cytolocalization of the carried plasmid DNA was explored by electron microscopy to understand its behavior in the cellular environment. Using the luciferase reporter gene system, we investigated the ability of this cholesterol-based cationic liposome to transfect 9L cells in vitro and tumors induced in vivo. We focused our attention on studying conditions that improve the transfection efficiency, especially the cationic lipid/DNA molar charge ratio and the addition of polyethylene glycol (PEG) or poly-L-lysine.

2. Materials and methods

2.1. Reagents

Cationic lipid TEAPC-Chol was synthesized and characterized by Fourier Transform Infrared spectroscopy. DOPE was purchased from Avanti Polar lipids (Alabaster, AL). OPTIMEM, glutamax-containing DMEM medium, Trypsin–EDTA, and PBS were obtained from Life Technologies (Paisley, UK). Reagents were furnished by Sigma-Aldrich (Saint Louis, MI).

Plasmids encoding the β-galactosidase under the control of cytomegalovirus (CMV) promoter (pCMV-β) and the enhanced green fluorescent protein (pEGFP-N1) were purchased from Clontech (Palo Alto, CA); pUT650 containing luciferase reporter gene under the control of CMV promoter was purchased from Cayla (Toulouse, France). DNA oligonucleotide: 5′ FITC–GAAAGAAGAAGCCGAAGAAGAAGGAGAA was used for cytometry and purchased from Genosys (England).

2.2. Synthesis of TEAPC-Chol

3-β[*N*-(*N*′,*N*′,*N*′-triethyl aminopropane) carbamoyl] cholesterol iodide or Chol-O-CO-NH-$(CH_2)_3$-$N^+(C_2H_5)_3I^-$ was synthesized as follows: a solution of cholesteryl choloroformate (4.6 g; 10^{-2} mol) in 60 cm^3 of anhydrous ether was added dropwise into a solution of 3-diethylaminopropylamine (2.65 g; 2×10^{-2} mol) in 50 cm^3 of ether at 0 °C. After removal of hydrochloride by filtration, the solvent was evaporated by using a rotary evaporator. The synthesis of the quaternary ammonium iodide salt using the crude residual carbamate in 50 cm^3 of anhydrous tetrahydrofuran was carried out by refluxing 12 h with an excess amount of iodomethane (4×10^{-2} mol). After removal of the solvent and excess iodomethane, the recrystallization in absolute methanol yielded 3.4 g (49%) of a straw-colored powder of the pure cationic salt.

2.3. Liposome preparation

Cationic lipids TEAPC/Chol and amounts of DOPE at various w/w ratios were dissolved and mixed in chloroform and liposomes were prepared as described previously [13]. The preparations were controlled by negative staining electron microscopy indicating that liposomes are unilamellar. A mean size value of 108 nm±10 nm and a polydispersity factor of 0.2 were obtained by quasi-elastic light scattering (QLS) analysis.

2.4. Cell culture

Adherent tumoral cells 9L (rat gliosarcoma cells) were obtained from ATCC and grown on plastic ware at 37 °C in humid atmosphere containing 5% CO_2 in air. The culture medium DMEM containing glutamax was supplemented with 10% fetal calf serum (FCS) and 50 U/ml penicillin/streptomycin.

2.5. Formation of DNA/liposome complex and transfection protocol

For the formation of DNA/liposome complex, DNA (1μg/μl) and liposomes TEAPC-Chol (1.5 mM) were separately diluted in equal volumes of sterile water and then mixed to a final volume of 20 μl. The DNA/liposome complexes formed instantaneously.

9L cells were seeded at 70% confluence in six-well plates the day preceding the transfection. Best results are obtained with 8×10^5 9L cells per well. Just before transfection, the culture medium was replaced with 1 ml OPTIMEM without serum. Then DNA (3 nmol in nucleotide) and liposomes in the tested Lipid/DNA ratios were added. After 6 h, OPTIMEM was removed and replaced with the serum-containing culture medium, and the cells were incubated at 37 °C.

2.6. Gel retardation electrophoresis assay

DNA/TEAPC-Chol complexes were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 μg/ml) with a 0.04 M Tris–borate buffer, pH 7.4 at 60 V for 90 min. DNA was visualized by UV illumination. Five microliters of each complex was used. Wells with 12.5% serum were supplemented to a final volume of 8 μl with 1 μl of FCS.

2.7. Cytotoxicity assay

Cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent [MTT test]. Cells were seeded in 96-well plates at 5×10^3 cells per well. After 24 h of incubation, an increasing amount of TEAPC-Chol/DOPE was added to triplicate wells. After 6 h, the medium was removed and replaced with a serum-containing medium. After 48 h, MTT (1 mg/ml) was added (100 μl/well) to the cells and formazan crystals were allowed to form during 3 h and further dissolved in dimethyl sulfoxide (DMSO) (Sigma). Absorbance was measured at 570 nm on a microplate reader (Bio-Rad), and the percentage of viable cells was estimated by referring to the absorbance of untreated cells.

2.8. GFP observation

The day before transfection, 9L cells (5×10^4 per well) were seeded onto a four-well permanox slide Lab-Tek (Nunc, Napperville, IL). pEGFP-N1 (0.5 μg) complexed with TEAPC-Chol at optimal charge ratio was added to the cells following the transfection protocol described above. After 48 h of incubation, the media was removed and cells were rinsed twice with PBS followed by a fixation step with 4% paraformaldehyde (PFA) in PBS. Fluorescence expression was observed with a Zeiss Axiophot fluorescence microscope. Untransfected cells or cells with the plasmid alone were used as controls.

2.9. Observation of labeled plasmids by immuno-gold electron microscopy

For the preparation of digoxigenin-labeled plasmids, 0.4 μl of reactive digoxigenin (DIG-Chem-Link from Roche Diagnostics, Meylan, France) was added in a sterile vial to 2 μg of pCMV-β plasmid in a final volume of 20 μl. After incubation for 30 min at 50 °C in a waterbath, the reaction was stopped by adding 5 ml of the stop solution and stored at −20 °C before transfection.

Cells dissociated from the wells of plate were fixed at 4 °C for 20 min in 1% PFA and 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (PB). After washings, the cells were treated for 10 min with 0.05% $NaBH_4$ and 0.1% glycine in PB, both to block the free aldehyde groups and to permeabilize the cells. The cells were then rinsed in PB and pre-incubated in 0.2% acetylated BSA (BSA-C) in PB for 30 min. They were then incubated for 2 h at room temperature with diluted ultra-small gold-labeled anti-digoxigenin antibody Fab fragments (Aurion, Wageningen, The Netherlands) in 0.2% BSA-C in PB. The cells were rinsed in 0.2% BSA-C in PB, then in PB and refixed in 2.5% glutaraldehyde for 10 min. After washing in PB, the samples were post-fixed with 0.5% OsO_4 in PB for 20 min, washed in deionized water and treated with the silver enhancement reagent (Aurion). After washings in distilled water, samples were stained in 0.5% uranyl acetate in 50% ethanol, dehydrated in a graded series of alcohols, embedded in Epon resin, sectioned, and examined with a Philips EM300 transmission electron microscope operating at 60 kV.

2.10. Transfection level measurement

After the desired time of incubation, cells were twice washed with PBS and lysed with the lysis solution furnished in the chemiluminescence measurement Tropix kit (Applied Biosystems, Bedford, MA). The level of the transfection was estimated by measuring the quantity of expressed luciferase following the procedure of the supplier. Luminometric measurement was made using a BCL luminometer operating at integration mode for 10 s. The calibration was made by comparing with the luminescence of a luciferase solution (Sigma) of known concentration. Protein was titrated by using the Bio-Rad DC Protein assay kit (Hercules, CA) in order to normalize the results. The background level was measured and subtracted. Each transfection experiment was done in triplicate, and the mean is expressed in relative light units (R.L.U) per milligram of total protein±standard deviation or in relative transfection level, expressed in percentage terms.

2.11. Flow cytometry

9L cells were seeded onto 24-well plates (10^5 cells/well). The following day, 1 μg of pEGP-N1 or 28 mer oligonucleotide complexed to TEAPC-Chol/DOPE (1:1) at a charge ratio of 2 was incubated with cells following the transfection protocol. Commercial lipids associated with 1 μg of pEGP-N1 were used following the supplier protocol. After 24 h, adherent cells were trypsinized and washed twice with PBS. After resuspension in 1 ml of PBS, propidium iodide (PI) (Sigma) was added to evaluate the proportions of living cells and exclude dead ones. The cells were incubated with PI in the dark at room temperature for 15 min before flow cytometric analysis. For each sample 20,000 cells were analysed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL) equipped with argon ion laser tuned at 488-nm wavelength used for excitation. The emission filters used were 530±30 nm band pass for green fluorescence of EGFP, and 620 nm long pass for red fluorescence of PI. Data analysis was performed with System II software (Beckman Coulter). The software permitted to obtain the representation of the number of viable cells (selected by red fluorescence of PI) as a function of fluorescence intensity of EGFP or fluorescein (detected by green fluorescence).

2.12. Transfection level enhancement by poly-L-lysine and PEG

Poly-L-lysine hydrobromide (MW: 19500) was obtained from Sigma and diluted in water before use. Starting from a

stock solution of 50 μl containing 0.975 mg of poly-L-lysine, four aliquots of 5 μl containing, respectively, 19.5, 3.9, 0.78, and 0.15 μg were prepared. Before complexation with TEAPC-Chol, 1 μl of each aliquot was added to 1 μg of plasmid DNA. Concentrations of polylysine were chosen assuming that 1 μg of polylysine corresponds to 4.7 nmol of positive charge.

PEG, MW: 8000, was obtained from Sigma and diluted at 50% wt/v in sterile water. Just before transfection, various amounts of PEG 50% were added to the transfection medium to give desired percentages. Transfection was done as described above.

2.13. Tumor transfection

Five-week-old nude (NMRI) mice were purchased from Harlan (Belgium) and housed in microisolator cages. 9L cells were trypsinized and collected during exponential growth, washed twice in serum-free DMEM, and resuspended in PBS. Three subcutaneous (s.c.) tumors were established by injecting 2×10^6 cells in 100-μl PBS into the flanks and the abdomen of nude mice and grown to 0.5–1 cm in size. For intra-tumoral injections, animals were anesthetized using Avertine, 500 μl/mice. In a final volume of 100 μl, 20 μg of pUT650 plasmid (1 μg/μl) was complexed with TEAPC-Chol/DOPE (3 mM) for 15 min at the indicated charge ratio and injected. After 48 h, tumors were removed and frozen for analysis. Each tumor was measured, weighed, crushed, and homogenized in 200 μl of lysis solution supplemented with 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/ml leupeptin. Centrifugation at 18,000×*g* was used to eliminate aggregates. Supernatants were collected, and the luciferase activity was measured using the chemiluminescence assay Tropix kit.

For these assays, because of the presence of blood in the tumor lysates, the intensity of emitted light at 562 nm by luciferase was underestimated due to the absorption by haemoglobin presenting two peaks at 540 and 570 nm as reported by Colin et al. [15]. To overcome this problem, we used a simple two-step method to correct the measurements of the luciferase intensity [16]. This protocol allowed to avoid perfusion of tumors which was not easy to accomplish.

3. Results

3.1. Efficiency of complex formation

Seven different cationic lipid/DNA charge ratios were analyzed by gel retardation in the absence of serum. A typical result obtained by electrophoresis is shown in Fig. 1A. In this example, movement of an increasing fraction of the plasmid in the gel is retarded as the amount of TEAPC-Chol/DOPE is increased, demonstrating that the association between negatively charged plasmid DNA and the cationic lipid TEAPC-Chol/DOPE is based on electrostatic interactions of charges of the two components. As no running band of DNA is observed for ratios from 1.5, complete complexation is achieved at a charge ratio of between 1.3 and 1.5.

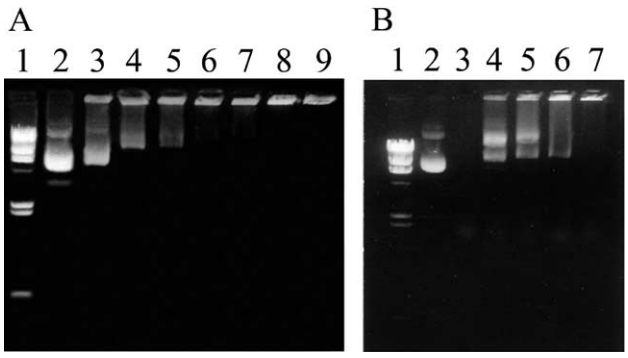

Fig. 1. Gel retardation assay of DNA/liposome complexation. (A) In medium without serum. Lane 1: molecular weight marker (lambda *Hin*dIII); lane 2: plasmid DNA alone; lanes 3–9: plasmid DNA complexed with TEAPC-Chol/DOPE at molar charge ratio X=0.5 (lane 3), X=1 (lane 4), 1.3 (lane 5), X=1.5 (lane 6), X=1.7 (lane 7), X=2 (lane 8), and X=4 (lane 9). (B) In medium containing serum. Lane 1: molecular weight marker (lambda *Hin*dIII); lane 2: plasmid alone; lane 3: FCS (13%); lanes 4–7: plasmid complexed with TEAPC-Chol/DOPE in the presence of FCS (13%) at charge ratio X=0.5 (lane 4), X=1 (lane 5), X=2 (lane 6), and X=4 (lane7).

One important parameter for any synthetic vector is the ability to carry nucleic acids efficiently in the presence of serum, especially for in vivo transfection. To obtain information about the effect of serum on the stability and the association of lipid with DNA in the complex, gel retardation assay has been repeated in the presence of serum (Fig. 1B). With 12.5% serum, the complete complexation requires a greater amount of TEAPC-Chol/DOPE, as the complexation is not achieved at the charge ratio of 2 but rather between 2 and 4. This behavior suggested a competition between charged serum components and the DNA in the binding to TEAPC-Chol/DOPE liposomes. Thus, an excess of lipid may entrap serum proteins that interfere with cationic lipid/DNA association.

In any case, the yield of the formation of DNA/TEAPC-Chol/DOPE complex can reach 100% when the molar charge ratio is appropriately chosen, even in the presence of serum.

3.2. Evaluation of cytotoxicity

To know the consequences of adding lipidic molecules on 9L cells, cytotoxicity of TEAPC-Chol/DOPE liposomes was evaluated by MTT assay. Three formulations of DOPE/TEAPC-Chol/liposome with ratios (wt/wt) of 0.5:1, 1:1, and 2:1 have been tested (Fig. 2). For this assay, liposomes at ratios of 2:1 and 1:1 present the same IC_{50} of 52 μM; however, the best cell viability at low concentrations of liposomes are obtained with formulation of DOPE/TEAPC-Chol at the ratio of 2:1. Before 20 μM any formulation gives

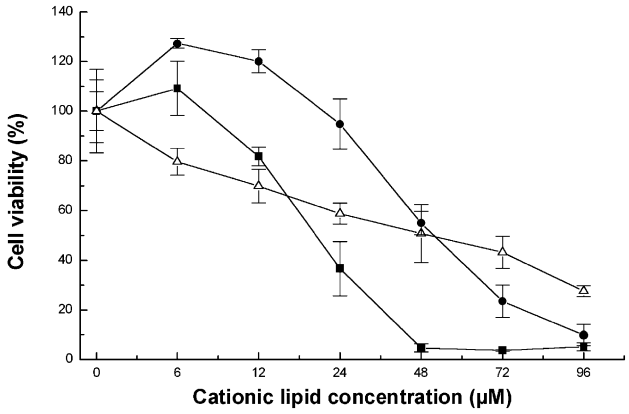

Fig. 2. Viability of 9L cells in the presence of cationic liposomes with three different formulations of TEAPC-Chol and DOPE, evaluated by MTT assay and expressed by the percentage referred to untransfected cells. Close circles: TEAPC-Chol/DOPE at ratio (wt/wt) 2:1. Close squares: TEAPC-Chol/DOPE at ratio 1:1. Open triangle: TEAPC-Chol/DOPE at ratio 1:2. Each point is an average of three experiments±standard error.

a significant cytotoxicity (less than 50% viable cells). This critical concentration is above threefold the concentration used for in vitro experiments (6 μM). This demonstrates that TEAPC-Chol/DOPE liposomes have a low cellular toxicity and are suitable for transfection experiments on 9L cells. Moreover, a small positive effect on cell growth has been observed for TEAPC-Chol/DOPE (2:1) and (1:1) at the lowest values corresponding to the range of concentrations used for further in vitro experiments.

3.3. Expression of GFP

Using a plasmid encoding the GFP, we checked the functionality of the GFP plasmid delivered by TEAPC-Chol/DOPE liposome inside the 9L cells. Observations of the cells 48 h after transfection clearly showed that vectorization with TEAPC-Chol/liposome preserved the functionality of the plasmid, whose expression is shown in typical cell fields (Fig. 3B). Controls with untransfected cells or cells with the plasmid alone did not present any fluorescence (Fig. 3A).

3.4. Localization of the plasmid DNA after transfection

The cellular pathway of plasmid DNA carried by TEAPC-Chol/DOPE liposomes was investigated by transmission electron microscopy (TEM) using immuno-gold labeling to distinguish the exogenous plasmid DNA from the cellular DNA. The plasmid labeled with digoxigenin is revealed by a gold-labeled antibody against digoxigenin. An enhancement of the gold particle sizes was performed with silver reagent. Finally, DNA can be observed among the cellular DNA as dense black spots. Results of TEM observations of 9L cells after transfection are shown in Fig. 4.

Many gold particles are present inside the cell, and clusters of plasmids can be found in the nucleus. All of the controls with untransfected cells, treated with the silver reagent alone or treated with antibody alone plus silver reagent, did not show any black spots. The same was observed when cells incubated with plasmids alone were treated following the protocol described. These results indicate that plasmids carried by TEAPC-Chol/DOPE liposomes penetrated inside the nucleus.

3.5. In vitro transfection level

The ability of TEAPC-Chol/DOPE complexed with DNA to transfect 9L cells in vitro was examined using pUT650, a plasmid containing the luciferase reporter gene under the control of the cytomegalovirus promoter. To

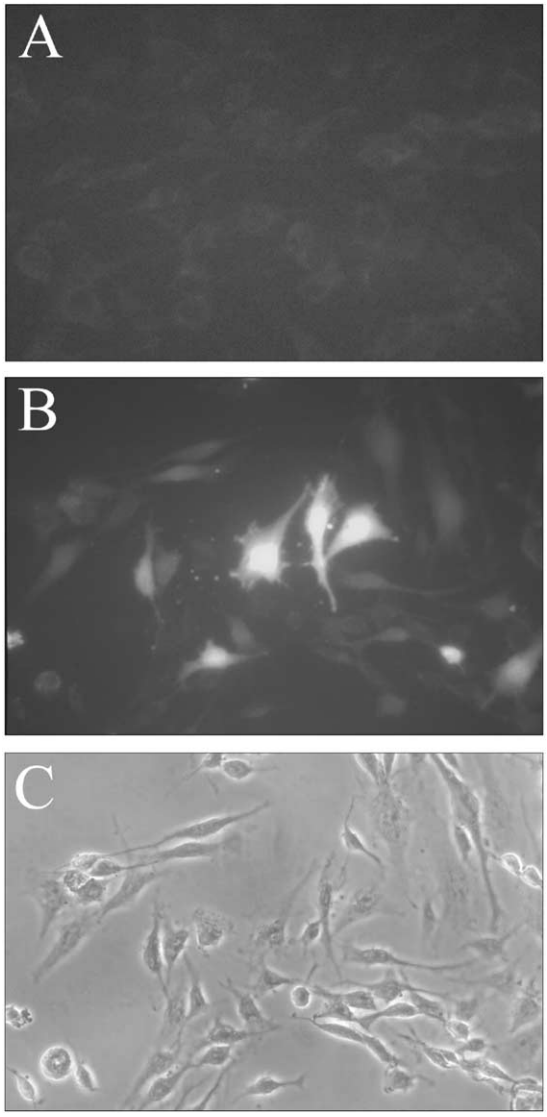

Fig. 3. Expression of gene encoding for GFP transferred into 9L cells by cationic liposomes. The pictures were obtained 48 h after transfection. (A) Cells with plasmid pEGFP-N1 alone observed in epifluorescence. (B) Cells transfected with plasmid pEGFP-N1 carried by TEAPC-Chol/DOPE (1:1) observed in epifluorescence. (C) Same field observed by phase contrast.

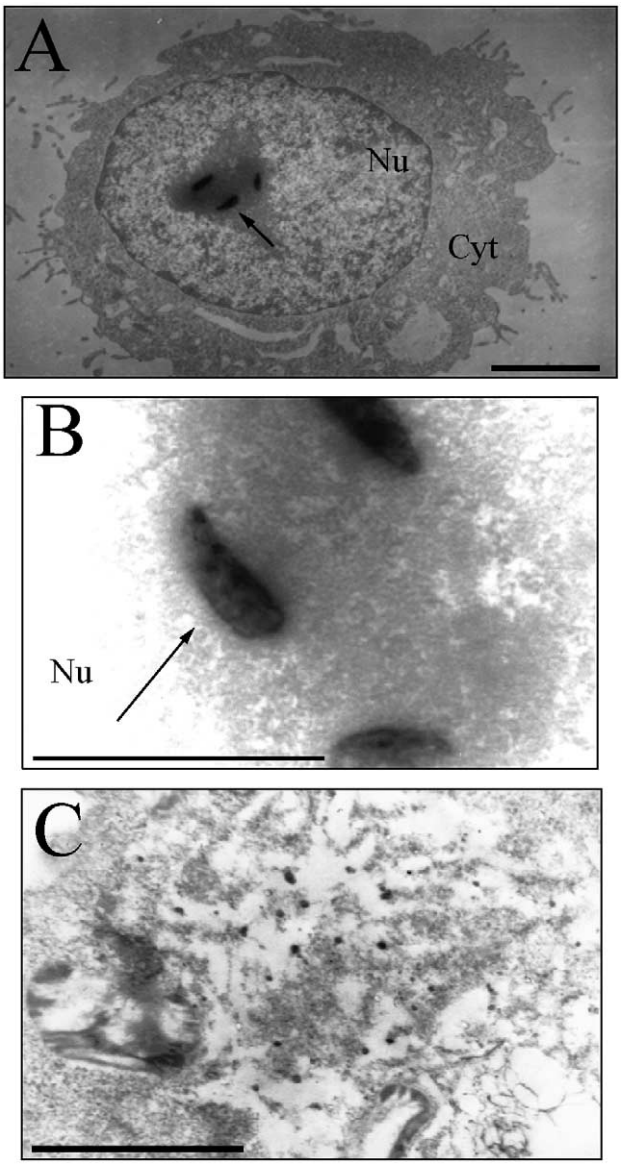

Fig. 4. Pathway in 9L cells at 24 h after transfection of plasmids pCMV-β vectorized by cationic liposomes TEAPC-Chol/DOPE (1:1) observed by TEM. Plasmids, indicated by arrows, were labeled with digoxigenin, detected by antibodies conjugated with gold particles enhanced by silver reagent. (A) Whole cell observed at magnification ×6800. (B) The same field, focused on the nucleus at magnification ×42000. (C) Focused view of another nucleus at magnification ×27,000. Bars represent 2 μm for (1A) and 1 μm for (B) and (C).

determine the optimal charge ratio of the complex for an efficient transfection, seven cationic lipid-to-DNA molar charge ratios have been tested. Results for TEAPC-Chol/DOPE liposome (1:1) (w/w) are shown in Fig. 5. The control presented a very low luciferase activity of the cells transfected with the DNA alone, while the transfection level varied with the charge ratio of the lipid/DNA complexes added to the cells. Increasing the ratio from 0.5 to 2 resulted in more than 18-fold increase in luciferase activity. The three higher ratios show a significant decrease in efficiency as a function of the molar charge ratio, indicating that the best ratio is 2 for maximum transfection efficiency. These results are consistent with the previous observation in gel retardation assay regarding the fact that the charge ratio should be higher than 1.3 for optimum complex formation.

The same experiments have been repeated with two other formulations of liposome TEAPC-Chol/DOPE (1:0.5 and 1:2) (data not shown). The better transfection level has been obtained for a molar charge ratio lip+/DNA equal to 2 for TEAPC-Chol/DOPE (1:0.5) or 4 for TEAPC-Chol/DOPE (1:2). However, the maximum transfection efficiency has been obtained with TEAPC-Chol/DOPE (1:1) used at the lipid/DNA charge ratio of 2; thus, this ratio and formulation of the liposome have been used for further experiments connected with this work.

To have more information about the efficiency of TEAPC-Chol to transfect 9L cells, we used flow cytometry to determine precisely the percentage of cells positive for fluorescence of expressed GFP or for an FITC-labeled oligonucleotide. In complement to luminometry measurements made with a pool of cells, flow cytometry provided useful individual data for each cell. Transfections of 9L cells with three currently used monocationic lipids have been performed to compare the efficiency between TEAPC-Chol and these reagents (Table 1). The data illustrated the advantage to use TEAPC-Chol/DOPE liposomes to carry the plasmid DNA inside 9L cells in comparison to three currently used monocationic lipids.

The behavior of TEAPC-Chol/DOPE in the presence of serum has been investigated in transfecting 9L cells in media containing increasing amounts of serum. As for previous experiments, five different lipid to DNA charge ratios have been used (Fig. 6). A maximum value of transfection level was observed for each concentration of serum

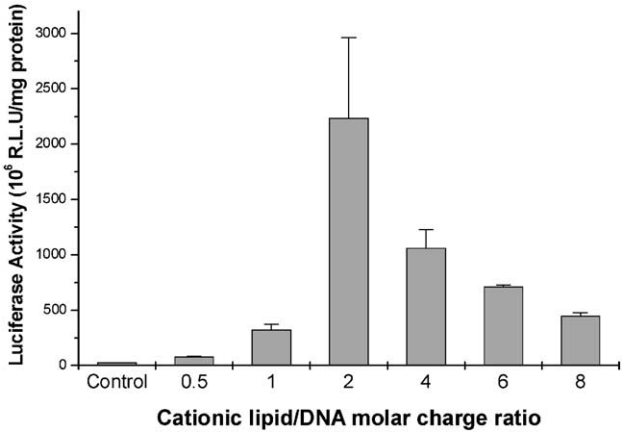

Fig. 5. Transfection level of 9L cells by plasmid pUT650 delivered by cationic liposomes TEAPC-Chol/DOPE (1:1), evaluated by luciferase activity as a function of the cationic lipid/DNA molar charge ratio. Cells: 10^8/well; plasmid: 1 μg; cationic lipid in the range 1.5–12 nmol; transfection medium for the first 6 h: OPTIMEM without serum. After 6 h OPTIMEM was replaced with the serum-containing culture medium DMEM. The activity was expressed in RLU/mg protein per 10 s. Nonvectorized DNA is used as control.

Table 1
Comparison of transfection levels in 9L cells by TEAPC-Chol/DOPE and three currently used monocationic liposomes

DNA	Liposome	Transfected cells (%)
pEGFP		0.30±0.08
+	Lipofectin	8.06±0.40
+	DC-Chol/DOPE	4.90±0.74
+	DMRIE-C	3.28±0.18
+	TEAPC-Chol/DOPE	12.53±0.60
Oligonucleotide		0.54±0.10
+	TEAPC-Chol/DOPE	57.3±4.7

Percentage of transfected 9L cells positive for the fluorescence of GFP or FITC-labeled oligonucleotide vectorized by cationic liposomes TEAPC-Chol/DOPE and lipofectin, DMRIE-C (Life Technologies). DC-Chol was synthetized as indicated by Gao and Huang [18]. Cells: 10^5/well; plasmid pEGFP-N1 and oligonucleotide: 1 μg; cationic lipid: 6 nmol for TEAPC-Chol/DOPE and DC-Chol/DOPE; otherwise as indicated by the suppliers; incubation time, 24 h; other conditions, as described in text. Each point is an average of three experiments±standard error.

used. This peak was shifted toward higher charge ratios when transfection was carried out in media containing increasing amounts of serum. Despite a decrease of the transfection efficiency as a function of the serum concentration, the transfection efficiency falls more than twofold only for media containing 50% of serum. Consequently, increasing the lipid/DNA charge ratio can improve the transfection efficiency in the presence of increasing concentrations of serum.

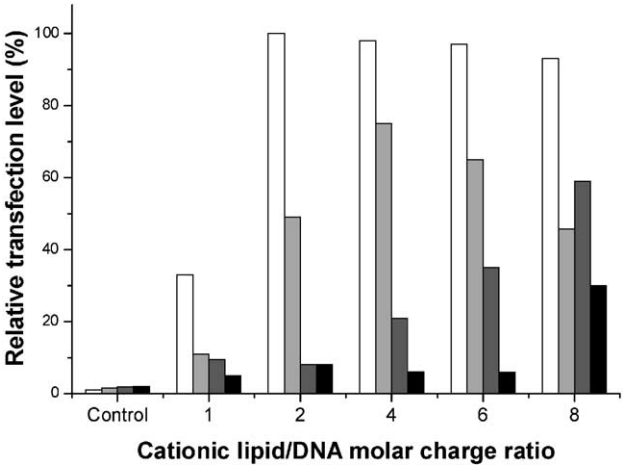

Fig. 6. Transfection level of 9L cells by plasmid pUT650 delivered by cationic liposomes TEAPC-Chol/DOPE (1:1), evaluated by luciferase activity as a function of the molar charge ratio in presence of increasing concentrations of FCS. Cells: 10^8/well; plasmid: 3 nmol. Cationic lipid in the range 1.5–12 nmol. Transfection medium for the first 6 h: OPTIMEM without serum, white bars; OPTIMEM with 10% FCS, light grey bars; OPTIMEM with 25% FCS, dark grey bars; OPTIMEM with 50% FCS, black bars. After 6-h transfection, media was replaced with the serum-containing culture medium DMEM. The activity was expressed by the percentage of relative transfection level referred to the maximum transfection level obtained without serum. Nonvectorized DNA is used as control.

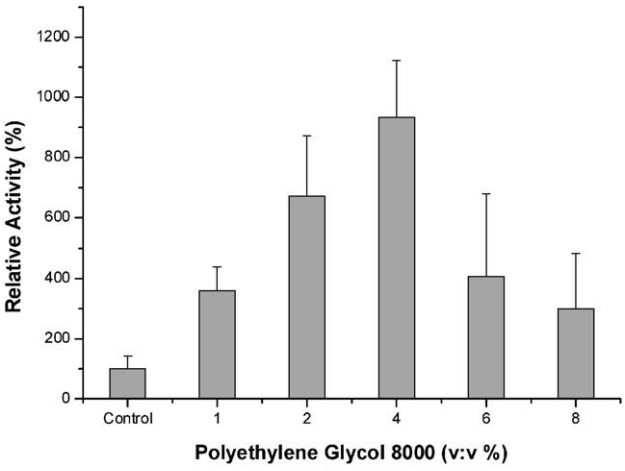

Fig. 7. Transfection level of 9L cells by plasmid pUT650 delivered by cationic liposomes TEAPC-Chol/DOPE (1:1), evaluated by luciferase activity as a function of increasing concentrations of PEG 8000 expressed as percentage (wt/v) added to the media containing 10% FCS before transfection. The plasmid DNA vectorized without PEG in the media is used for control (100% relative activity).

3.6. Enhancement of transfection efficiency by addition of PEG and poly-L-lysine

To increase the entry of TEAPC-Chol/DOPE/DNA complex inside 9L cells in the presence of serum, we investigated the influence of PEG on the transfection level (Fig. 7). In the transfection media supplemented with 10% serum, five different concentrations of PEG have been added. In comparison with the control, results show a clear improvement of the transfection level with all tested PEG concentrations in a concentration-dependent manner. A peak was

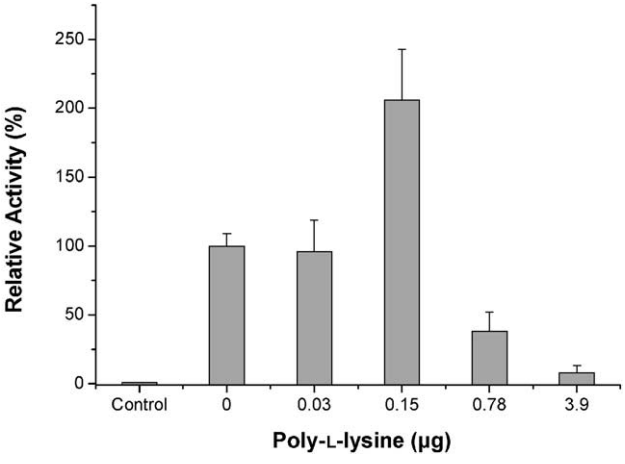

Fig. 8. Transfection level of 9L cells by plasmid pUT650 delivered by cationic liposomes TEAPC-Chol/DOPE (1:1), evaluated by relative luciferase activity as a function of the poly-L-lysine amount added to 1-μg plasmid DNA assuming that 0.65 μg of polylysine corresponds to 3.13 nmol of positive charge. Nonvectorized DNA is used for control.

obtained with 4% PEG where an increase of about 10-fold of the transfection level was observed.

The ability of TEAPC-Chol/DOPE to carry DNA into 9L cells was enhanced by mixing plasmid DNA with poly-L-lysine before complexation with TEAPC-Chol/DOPE and cell transfection. Four different quantities of poly-L-lysine have been used, each one furnishing various amounts of positive charges interacting with the negative charge of the plasmid DNA. In comparison with the control, results as indicated by Fig. 8 showed an enhancement of the transfection efficiency when 0.15 μg of poly-L-lysine was added to 1 μg of DNA. Distribution of the results indicated a concentration dependence of the enhancement, suggesting a process implying accurate electric charge interactions.

3.7. In vivo gene transfer

To determine transfection efficiency in vivo, we induced gliosarcoma tumors in nude mice by s.c. injection of 9L cells. For those experiments, the plasmid DNA complexed with TEAPC-Chol/DOPE (1:1) was directly injected in the tumors. With indications from in vitro transfections, the influence of the lipid/DNA charge ratio was tested for in vivo experiments. With the total complex volume limited to 100 μl for the chosen DNA dose (20 μg), precipitation occurred when plasmid was associated with cationic liposomes at high lipid/DNA charge ratios (X=4, 6, or 8).

Previous experiments with various batches of plasmid and cationic liposomes at different concentrations demonstrated that complex precipitation is a recurrent effect for critical concentrations of the components (data not shown). Despite those restrictions, we investigated the transfection efficiency in vivo with three different lipid/DNA molar charge ratios: 0.5, 1, and 2 (Fig. 9). Controls with PBS solution indicated undetectable luciferase activity while the unvectorized plasmid gave a nonnegligible luciferase activity. Nevertheless, the plasmid complexed with TEAPC-Chol/DOPE at a charge ratio of 1 showed a significant improvement of more than sixfold in comparison to the naked plasmid. Ratios of 0.5 and 2 gave an identical or inferior result, demonstrating that the charge ratio is a critical parameter for efficient in vivo transfections. With a less important dose of DNA (15 μg) and a final complexation volume of 150 μl, the same results have been obtained (data not shown).

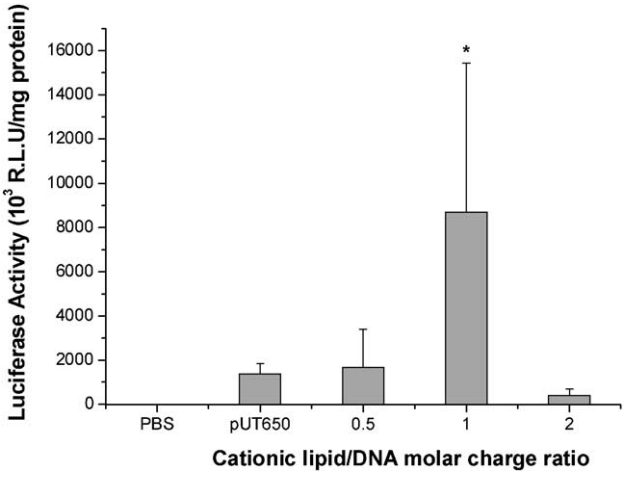

Fig. 9. Transfection level of in vivo 9L tumors by injection of plasmid pUT650 vectorized by cationic liposomes TEAPC-Chol/DOPE (1:1) and evaluated by luciferase activity as a function of cationic lipid /DNA molar charge ratios. Phosphate buffer saline and non-vectorized pUT650 DNA (20 μg) are used as controls. Error bars correspond to confidence interval at 5% of four to six tumors. *: Statistical significance by Mann–Whitney U-test with P=0.05.

4. Discussion

The research of carriers is still a challenge for an efficient transfer of foreign genetic materials into cancer cells. While viral vectors present safety problems, nonviral vectors are a promising alternative. However, many features of cationic lipidic vectors have to be improved.

For DNA transfer into cells, several important conditions must be fulfilled. The efficiency of the carrier to transport DNA must be high. The DNA has to be protected against enzymes in the extracellular medium. The complex DNA/carrier must be internalized into the cell and the functionality of DNA has to be preserved for the expression of the gene in the cell. Finally, the carrier must not be cytotoxic. In the present work, liposomes formulated from an original cationic lipid, TEAPC-Chol, in association with DOPE, were used as the DNA carrier into a brain cancer model.

The results have shown that TEAPC-Chol/DOPE yields a 100% association with a plasmid DNA at a molar charge higher than 1.3. TEAPC-Chol/DOPE also exhibits a very low cytotoxicity as revealed by the MTT assays. Localization using TEM shows that plasmids can efficiently penetrate into the nucleus when they are delivered by TEAPC-Chol/DOPE. This process is a key step necessary for gene expression and successful transfection. Moreover, results using GFP or luciferase show that the vectorized plasmids are fully functional.

An important result to emphasize is that the cationic lipid/DNA molar charge ratio is a key point for transfection. If the liposome bilayer structure is conserved, the neutral charge ratio lip+/PO_2^- should be 2 because only half the number of lipid molecules are in the outer side of liposomes interacting with DNA. On the contrary, if the liposome structure is destroyed, all the cationic lipid molecules would be associated with DNA and the neutral charge ratio would be 1. In the light of this, a molar charge ratio in the range of 1 to 2 probably leads to the formation of a heterogeneous distribution of complexes. This heterogeneity was remarked in the studies of Cao et al. [13] and Zabner et al. [17]. The various molar charge ratios used in this work showed variable transfection levels in cells, indicating a correlation between the structure of the complexes and the transfection

level. A similar behavior was observed with liposomes prepared with DC-Chol, a cationic lipid close to TEAPC-Chol [18]. A small excess of positive charges is probably necessary for liposomes/DNA complexes to bind to the cellular membranes, but this is not the only factor involved in the cellular transfection. For transfection of CaSki cells with oligonucleotides, Lappalanaien et al. [19] found that oligonucleotide/liposome complexes were rapidly and effectively taken up during endocytosis and transported into the perinuclear area; however, the charge of the complexes affects only the intracellular distribution of oligonucleotides. These results indicate that the value of the optimum charge ratio for an efficient transfection depends on the nature of the lipidic vector, the carried DNA, and the cell line. Thus, optimization is a necessary step for an efficient delivery. It is worth noting that at the optimal charge ratio, comparison experiment showed the advantage of using TEAPC-Chol/DOPE over other formulations. Otherwise, the uptake of the DNA does not correlate with the gene expression as demonstrated by Ruponen et al. [20], and the values observed for GFP expression could reflect this situation. The use of a FITC-labeled oligonucleotide indicated the ability of TEAPC-Chol to deliver DNA inside 9L cells with 57% of positive cells.

Transfection experiments carried out in the presence of increasing amounts of serum showed a shift toward higher values of the molar charge ratio giving the best transfection level. The lipid bilayer of the liposome is a preferential target of serum proteins as shown by Li et al. [21]. Highly negatively charged molecules in the serum interacting with cationic lipidic vectors lead to DNA release and degradation [22]. The excess of charged liposomes in transfection experiments in the presence of high serum concentration could be a key point for explaining the better observed results. Thus, a great amount of positively charged lipidic molecules may protect DNA from release and degradation due to competition by negative serum components. The gel retardation assay results are relevant with this hypothesis. McLean et al. [23] have demonstrated in an in vivo study a serum-induced aggregation of DOTIM-cholesterol liposomes vectors. As these aggregates exhibit a larger size than free complexes, the penetration into tissues by the endocytosis way, in particular into endothelial cells, would be easier, resulting in a better transfection levels with high serum concentration. On this assumption, in the presence of serum, an excess of liposome in the complex may enhance the aggregation of the vectors and lead to improve transfection. Nevertheless, the constraints that the lipidic vector undergoes in the conditions of an intravenous injection or in a simple cell transfection in vitro are quite different and remain to be explored.

Another point to be discussed is the role of the liposome formulation of cationic lipid TEAPC-Chol and the helper lipid DOPE. On one hand, DOPE is necessary for the formation of the bilayer structure, but is also endosomolytic [24]. On the other hand, cholesterol brings rigidity to the liposome structure, but is also known to modulate the hydrocarbon chain fluidity of the lipidic molecules of the cell membrane [25]. Thus, TEAPC-Chol associated with DOPE at ratio 1:1 may depict a good balance between rapid self-aggregation and a persistent resistance to serum. Because rare informations have been published about interactions between cationic lipids and serum proteins, further investigations are necessary to identify which proteins are involved and which structures and parts of the cationic lipid molecule are important. Despite the hypothesis explaining the better transfection levels with a higher molar charge ratio, serum interactions still induce a global decrease of transfection efficiency. However, increasing the molar charge ratio is a way to minimize the influence of serum over TEAPC-Chol/DOPE/DNA complexes.

Regarding in vitro results, transfection levels of 9L cells can be enhanced by adding PEG 8000 in the transfection media or using poly-L-lysine associated with DNA. In both kinds of experiments, transfection levels have been improved in a dose-dependent manner.

The entry in the cytoplasm is the first important step for liposome-mediated transfection. Yang et al. [26] showed that a low concentration of PEG could induce liposome aggregation, increasing the association between cell membranes and lipid/DNA complexes. This better association results in better penetration of the complexes, as demonstrated by Ross and Hui [27]. Our observations on 9L cells agreed to the conclusions of Yang et al. [26]. Finally, PEG seems to be an important factor to enhance the cytoplasmic penetration.

The nuclear transport of the DNA following the cytoplasmic penetration is also another limiting step. Recently, the role of poly-L-lysine in the nuclear transport process has been investigated [28]. Assuming that poly-L-lysine can mimic a nuclear localization signal (NLS), we attempted to increase the ability of DNA to enter the nucleus by adding poly-L-lysine to the plasmid. Thus, it may be that the observed improvement of the transfection is due to a better nuclear importation of the DNA contributed by polylysine. Zauner et al. [28] proposed such a role for polylysine using primary human fibroblasts. Apart from polylysine-mediated nuclear transport, alternative explanations may also be suggested. Protection of the DNA from a cytoplasmic degradation could be a possible explanation for improvement by poly-L-lysine. The ability of the poly-L-lysine to condense DNA may also enhance the transfection level by facilitating the DNA penetration in cells.

Another important result concerns in vivo experiments. Regarding transfection of the tumors induced by 9L cells, results are not relevant to in vitro assays done with high serum concentration. Surprisingly, the best result was obtained with the molar charge ratio of TEAPC-Chol/DOPE to DNA complex of 1 and not with the ratio of 2 or higher as for the in vitro transfection in the presence of serum. However, these observations must take into account the precipitation of the complex occurring at high molar charge

ratios. Clark et al. [29] have reported the same behavior common to cationic lipids. Felgner [30] mentioned that trying to mimic in vivo conditions by adjusting in vitro assays leads to very unexpected results. The behavior of the lipid/DNA complex in the intratumoral environment is probably the key point in relation to a change of structure and properties, which may affect the permeation of DNA. As proposed by Clark et al. [29], complexation of plasmid DNA with cationic lipid may improve DNA retention in the tumor and thus its penetration in cells. However, an excess of cationic lipid in the tumoral tissue could lead to a stronger retention but at the same time appears unfavorable to DNA expression as observed. One would think that the conditions of an efficient in vivo transfection should be obtained by an accurate balance between the antagonist effects due to Lip+/DNA ratios. On the other hand, as in the case of muscle where Schwartz et al. [31] have demonstrated that naked DNA alone could penetrate efficiently into brain cells, a nonnegligible luciferase activity was observed in 9L cells transfected with the plasmid alone. However, free DNA is not protected from degradation so that the level was not as high as in the case of DNA associated with liposome.

For those experiments in vivo, it is also worth noting that the interference of haemoglobin with the luciferase measurement of tumor samples has been overcome by an original approach [16]. The values of the luciferase activity are reproducible and do not fluctuate with the uncontrolled amounts of haemoglobin present in the analyzed samples, regardless of the haemoglobin quantity.

In conclusion, results of this work demonstrated that cationic liposomes prepared from TEAPC-Chol are promising vectors to carry plasmid DNA efficiently into gliosarcoma 9L both in vitro and in vivo. Optimization of the transfection levels can be achieved with an appropriate choice of the lipid/DNA molar charge ratio in combination with the use of poly-L-lysine or PEG.

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