Lipoic acid-derived amphiphiles for redox-controlled DNA delivery

Maxim Balakirev [1,3], Guy Schoehn [2] and Jadwiga Chroboczek [1]

Background: Intracellular release of free DNA from the vector complex is one of the critical steps limiting the efficiency of non-viral gene delivery. The complex should be stable enough to prevent DNA degradation but it should be destabilized inside the cell to allow DNA release and transcription. Destabilization and degradation of synthetic vectors is also required to reduce their cytotoxicity and augment the life-time of transfected cells.

Results: Here we describe new cationic amphiphiles made from the natural provitamin, lipoic acid, that reversibly binds and releases DNA, depending on the redox state of the lipoate moieties. In the oxidized state these amphiphiles condense DNA into homogeneous spherical particles, which, upon reduction, swell into DNA toroids with subsequent release of free DNA. Complex reduction and DNA release can be induced by various thiols as well as enzymatically, by thioredoxin reductase. Transfection with amphiphile–DNA complexes in vitro shows a several fold increase of transgene expression compared with DOTAP, and can be further augmented by attachment of the nucleus-targeting peptide to the amphiphile. The increase of transfection efficiency results from GSH- and NAD(P)H-dependent complex reduction and release of free DNA inside the cells.

Conclusions: The present work demonstrates the principle of a redox-controlled gene delivery system that uses the reversibility of thiol–disulfide exchange reaction. Our data suggest that the efficiency of synthetic vectors can be augmented by their controlled destabilization inside the cells. Being formed from the natural non-toxic compound lipoic acid, these cationic amphiphiles provide a new promising class of synthetic vectors for gene delivery.

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Introduction

Gene therapy and genetic engineering require reliable and efficient systems for delivery of exogenous genes to target cells. Among non-viral vectors, cationic polymers as well as cationic lipids are widely used as delivery agents for nucleic acids [1]. Though these synthetic vectors are versatile and safe, they are substantially less efficient than viruses. The efficiency of virus infection is based on the complex entry program involving receptor-mediated internalization, escape into the cytoplasm and trafficking of viral genome to the sites of expression. Viruses use cell functions such as acidification, proteolysis, phosphorylation, etc., to adopt their structure for the most efficient delivery of nucleic acid [2]. Similarly, the ideal synthetic vector should be able to use the intracellular environment and the cellular enzymatic and transport machinery to deliver and release DNA into the nucleus. In fact, some DNA delivery systems do work like this: branched cationic polymers [3], pH-sensitive cationic lipids [4], and vectors bearing amphipathic peptides [5] exploit endosomal acidification to disrupt endosomes and to escape into the cytoplasm. Furthermore, the attachment of a nuclear localization signal (NLS) to transfection complexes permits DNA to be transported to the nucleus by cellular importins [6]. DNA release from the vector complex is an important limiting step [7], that is often neglected. The complex should be stable enough to prevent DNA degradation and, at the same time, should be destabilized inside the cell to allow DNA release and transcription. Destabilization and subsequent degradation of synthetic vectors is also required to reduce their cytotoxicity and augment the life-time of transfected cells.

We describe here a new amphiphilic vector for gene delivery based on lipoic acid-derived polymer. This compound condenses DNA into stable virus-sized complexes, that enter efficiently cells and release DNA in a reducing intracellular environment. Being formed from the natural compound this vector might be biodegradable and non-toxic.

Results and discussion

Cationic amphiphiles from lipoic acid

The cationic amphiphiles capable of reversible DNA binding were synthesized from α-lipoic (6,8-thioctic) acid. This natural amphiphilic compound, a prosthetic group of the

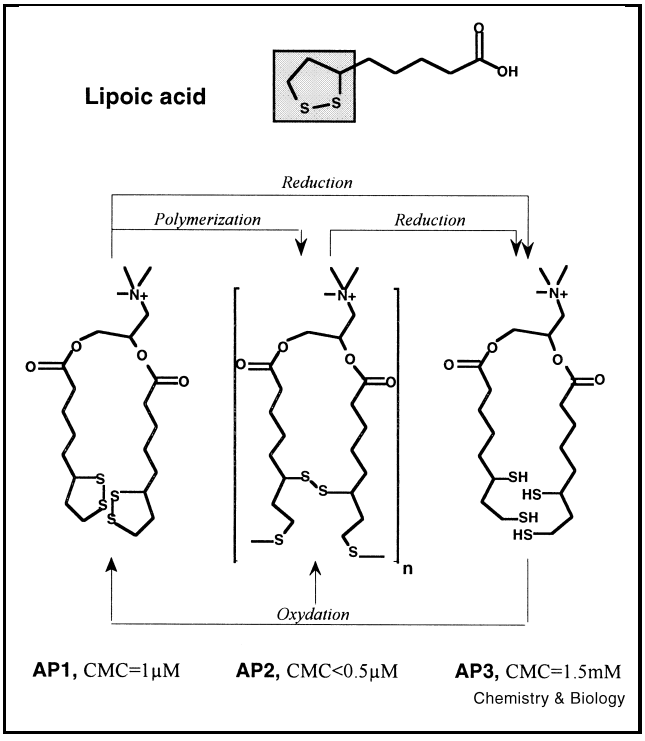

Figure 1. Chemical structures of α-lipoic acid and amphiphiles **AP1–AP3**. 1,2-Dithiolane ring is shown inside the box. CMCs were determined from titration of 1 μM ANS with amphiphiles. The onset of increase of ANS fluorescence (370/470 nm) corresponded to CMC.

α-keto acid dehydrogenases, is widely used in anti-oxidant therapy of liver diseases and diabetes-associated dysfunctions [8]. The characteristic structural feature of lipoic acid (Figure 1) is the presence of a constrained 1,2-dithiolane ring that is thermodynamically unstable and possesses a high tendency to thiol–disulfide exchange and self-polymerization [9–12]. We synthesized the dilipoyloxy-substituted analogue of cationic lipid DOTAP, amphiphile **AP1** (Figure 1). Thiolate-catalyzed polymerization of **AP1** at alkaline pH resulted in the cationic polymer **AP2**, while complete reduction of **AP1** and **AP2** resulted in compound **AP3**. Polymerization as well as reduction of **AP1** was accompanied by the loss of the UV absorption at 333 nm, which is characteristic of the five-membered ring cyclic disulfides [10,11]. Comparison of the critical micelle concentrations (CMCs) of the synthesized amphiphiles revealed significant differences in their aggregation properties. Thus, the presence of the micelles was observed at all concentrations of **AP2** studied, whereas **AP1** showed a well-defined CMC at 1 μM, and **AP3** had a millimolar CMC resulting from the increased polarity of reduced lipoate moiety [13]. Consistent with the difference in CMC, the reduction of **AP1** and **AP2** with DTT resulted in micelle solubilization, whereas aerobic oxidation of **AP3** solution induced micelle formation (not shown). The observed micelle dynamics provided an interesting amphiphilic system where the aggregation state of the amphiphile is controlled by redox inter-conversion of the dithiolane ring.

Redox-controlled DNA binding

The binding of cationic amphiphiles to DNA shows significant similarity with the micelle formation process. In both cases the cooperative interaction is driven by hydrophobic association of the nonpolar tails of the amphiphile [14]. To study the interaction of plasmid DNA (pDNA) with compounds **AP1–AP3**, pDNA was titrated with amphiphiles in the presence of propidium iodide (PI). Comparison of the apparent association constants revealed that polymer **AP2** ($K_A^* > 10^7$ M^{-1}) binds DNA 10 and 2000 times more strongly than amphiphile **AP1** ($K_A^* = 9 \times 10^5$ M^{-1}) and **AP3** ($K_A^* = 5 \times 10^3$ M^{-1}), respectively (Figure 2A). The difference in K_A^* was consistent with that in CMCs, showing a dramatic decrease in association constant for the most polar compound **AP3**. The pDNA binding was also more efficient for the polycation **AP2** than for the mono-cation **AP1**. The observed difference in the DNA binding affinity of compounds **AP1–AP3** provided a redox-controlled mechanism of DNA release (Figure 2A,

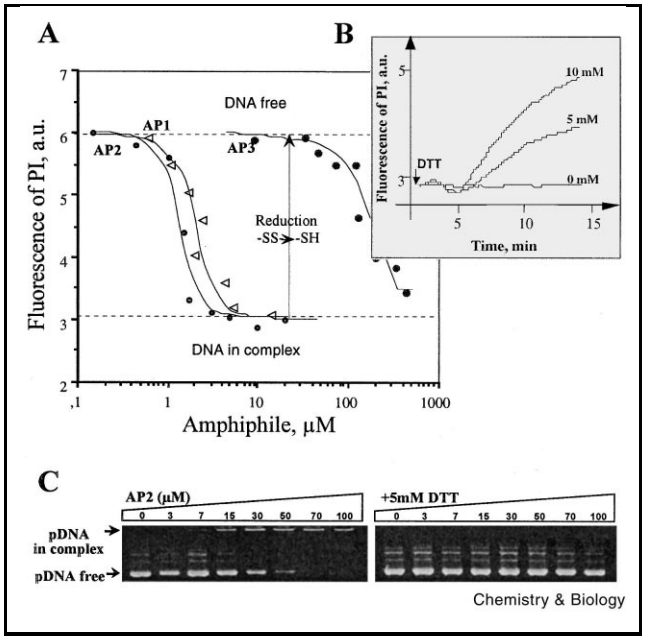

Figure 2. Reversible binding of amphiphiles to pDNA.
(A) Amphiphile binding prevents intercalation of PI into DNA and results in decrease of PI fluorescence (535/617 nm). Apparent association constants (K_A^*) for **AP1–3** were estimated from DNA titration curves (2.5 μM of pCMV-luc phosphate) as described in Section 4. **(B)** Reduction of **AP2**–pDNA complex by DTT induces the release of free DNA and increase in PI fluorescence.
(C) Prevention of **AP2**–pDNA complex formation by DTT, shown by gel electrophoresis.

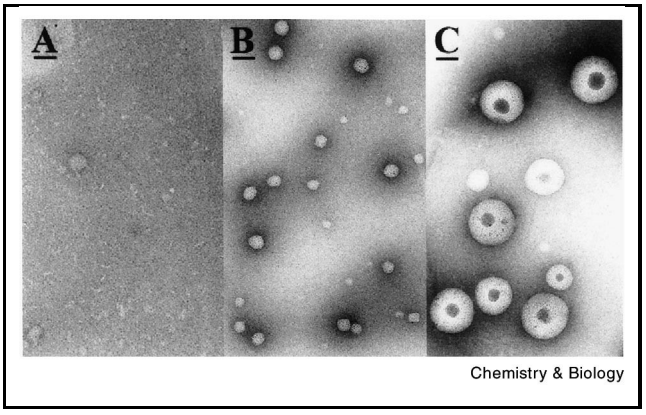

Figure 3. Electron micrographs of amphiphile condensates formed in 50 mM HEPES pH 7.4. **(A)** Micelles observed with 0.5 mM **AP2**. **(B) AP2**–pDNA complexes formed after addition of 0.2 mM pCMV-luc. **(C) AP2**–pDNA complexes 10 min after addition of 10 mM DTT. Similar structures were observed with **AP1**. Bars are 50 nm.

arrow). Indeed, the DTT reduction of **AP1** and **AP2** destabilized their complexes with pDNA resulting in the release of free pDNA, that was shown for **AP2** by PI fluorescence (Figure 2B) and agarose gel electrophoresis (Figure 2C).

The amphiphile–pDNA complexes were further investigated by negative stain electron microscopy. Amphiphile **AP3** either alone or with pDNA did not show any characteristic structures. In contrast, the solutions of **AP1** and **AP2** revealed the presence of non-symmetrical micelles with a prevalence of wormlike structures of about 4×20 nm (Figure 3A). Addition of pDNA resulted in the formation of a homogeneous population of spherical complexes having a mean diameter of 32 ± 7 nm ($n = 180$) (Figure 3B). This size was consistent with that of 40 nm obtained by quasi-elastic light scattering (not shown) and suggested a monomolecular condensation of 6 kb pDNA within the complex. The most striking effect was observed upon reduction of these pDNA complexes with DTT: the spheres swelled and transformed into a population of toroids with a mean diameter of 100 ± 20 nm ($n = 86$) (Figure 3C). Thereafter, these assemblies disappeared with characteristic time dependent on DTT concentration. Although never seen with cationic amphiphiles, quite similar toroidal morphology has been shown for polycations-condensed DNA [15], sperm cell DNA [16], and virus DNA [17]. Recently, based on the kinetic mechanism of toroid formation and on the observation of toroidal structures in phage lysates, a toroid model for DNA organization in bacteriophage head has been proposed, which is an alternative of the existing 'spool' model [18]. Interestingly, assuming that the observed spherical complexes (Figure 3B) are, in fact, the 'spools' of pDNA, our EM data suggest that toroids can appear as an intermediate structure of 'spools' unfolding.

Redox-controlled DNA delivery

The transfection efficiency of synthesized amphiphiles **AP1–AP3** was assessed on cultured cells, in comparison with their structural relative, DOTAP. Since reporter gene expression depends on promoter and cell type, different plasmids (pSV-lacZ, pGL3-luc, and pCMV-luc) as well as different cell types (HeLa, A549, and BHK) were used. Irrespective of the nature of either reporter gene or cell type, polymer **AP2** resulted in a 2–3-fold enhancement in expression over transfection with DOTAP, whereas amphiphile **AP1** was less efficient and no expression was observed with **AP3** (Figure 4A). Recently, we have shown that the peptide **pI** bearing NLS of adenovirus fiber protein can mediate the translocation of pDNA complex into the nucleus [19]. In order to improve intracellular targeting of the **AP2**–pDNA complex, **pI** was attached to **AP2** in situ by using of the **pI**-thiolate ion as an inducer of **AP1** → **AP2** polymerization. The resulting polymer **AP2(pI)** provided a 6–10-fold increase in transgene expression compared with DOTAP.

To determine whether this increase in transfection efficiency was a result of increased pDNA uptake into the

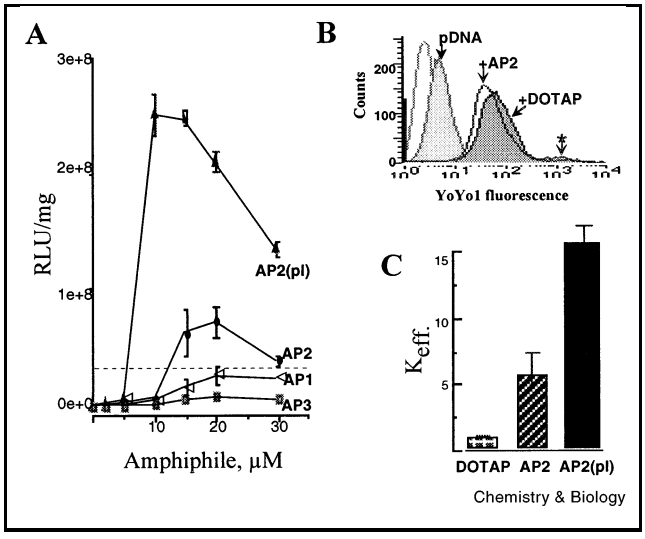

Figure 4. Transfection of HeLa cells with amphiphile–pDNA complexes. Complexes were formed with 1.5 μg of pCMV-luc and indicated concentration of amphiphiles. **(A)** The luciferase expression was measured in RLU/mg of cell protein 24 h after transfection ($n = 4$, for all amphiphiles), the maximum level of DOTAP transfection is shown by the dotted line. **(B)** Cellular uptake of YoYo-1-labeled pCMV-luc measured by flow cytometry. Cells were incubated 1 h with plasmid alone or with the complexes **AP2**–pDNA or DOTAP–pDNA (arrows). The asterisk indicates the population of dead cells that appeared after transfection with DOTAP. **AP2(pI)** provided pDNA uptake similar to **AP2**. **(C)** Relative efficiency of amphiphile transfection $K_{eff.}$ is calculated as described in Section 4, $K_{eff.}(\text{DOTAP}) = 1$.

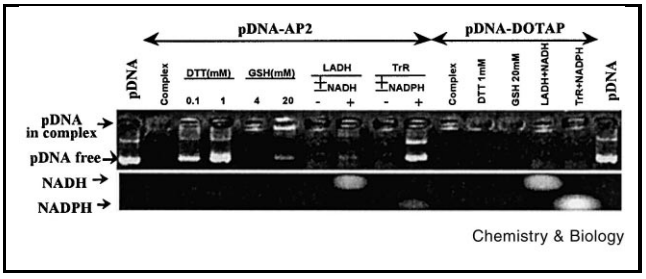

Figure 5. Release of free DNA from the complex with amphiphile. Complexes were formed with 0.125 μg of YoYo-1-labeled pCMV-luc and 0.8 μg of AP2 or 0.5 μl of DOTAP, which corresponds to conditions of complete pDNA condensation. The complexes were reduced for 30 min under indicated conditions, and analyzed by gel electrophoresis. The final concentrations of enzymes were 10 U/ml of LADH ± 0.5 mM NADH or 1 U/ml of TrR ± 0.5 mM NADPH. Note that the DNA in complex shows little fluorescence due to YoYo-1 self-quenching. The TrR reduction of the **AP2**–pDNA complex occurs only in the presence of NADPH and is accompanied by NADPH consumption shown by decrease of NADPH fluorescence.

cells, pDNA was labeled with YoYo-1 dye, and transfected cells were analyzed by flow cytometry. Unexpectedly, polymers **AP2** and **AP2(pI)** were found to bring 2–3 times less pDNA than DOTAP (Figure 4B). The lower uptake of **AP2**–pDNA is likely due to the absence of an excess of cationic charge in the complexes, resulting in an inefficient interaction with the cell membrane. These data together suggest that pDNA, once translocated into a cell within the complexes with **AP2** and **AP2(pI)**, allowed 6 and 16 times more efficient gene expression, respectively, than pDNA delivered by DOTAP (Figure 4C).

Subsequent experiments attempted to elucidate the mechanism of the observed enhancement of gene expression with **AP2**. Cellular factors, known to participate in lipoate redox-turnover [8,20,21], were assessed for their ability to reduce and to destabilize the **AP2**–pDNA complex in comparison with DTT (Figure 5). At cytoplasmic concentrations (1–4 mM), glutathione (GSH) did not destabilize the complex, whereas some complex reduction and release of pDNA was observed at 20 mM, a concentration of GSH observed within the cell nucleus. NADH-dependent reduction of the complex by lipoamide dehydrogenase was inefficient, which is consistent with a high avidity of this enzyme for lipoamide [20,21]. In contrast, mammalian thioredoxin reductase (TrR) [21] rapidly reduced the **AP2** complex that was accompanied by pDNA release and NADPH consumption (Figure 5). To our knowledge this is the first demonstration of enzymatically catalyzed release of DNA from a synthetic vector complex. It should be noted that none of the reducing conditions destabilized the DOTAP–pDNA complex.

Next, we determined whether the changes in intracellular GSH and NAD(P)H concentrations would affect **AP2**-mediated transfection. The depletion of intracellular GSH by inhibiting its de novo synthesis with BSO decreased the efficiency of early gene expression, whereas augmentation of intracellular GSH by means of membrane-permeable GSH mono-ethyl ester (GSH-OEt) had the opposite effect (Figure 6A,C). Diamide (DA), that depletes both GSH and NADPH pools via a GSH-reductase catalyzed reaction [22], dramatically decreased the **AP2** transfection efficiency (Figure 6B,C). The inhibition with BSO and DA was not due to a general toxic effect since none of these compounds inhibited DOTAP transfection (not shown). Duroquinone (DQ), that oxidizes NADH via a diaphorase reaction [22], fully abolished **AP2**-mediated transfection (Figure 6B,C), though in this case DOTAP transfection was also significantly inhibited.

Collectively, our data suggest that GSH-and NADPH-dependent reduction of **AP2**–pDNA complexes inside the

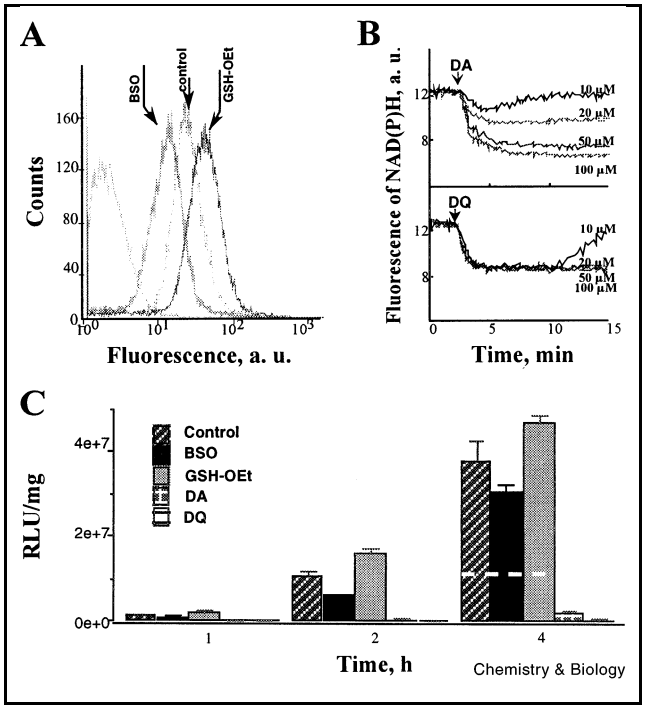

Figure 6. Effect of intracellular GSH and NADPH on **AP2**–pDNA transfection. **(A)** HeLa cells were incubated with either 2 mM BSO for 18 h or with 10 mM GSH-OEt for 1 h, stained with 1 μM of CMFDA, and intracellular GSH was analyzed by flow cytometry. **(B)** Changes of intracellular NAD(P)H after addition of the indicated concentration of DA or DQ were determined fluorometrically (340/460 nm) using HeLa cells suspended in PBS (2×10^5 cells/ml). **(C)** HeLa cells were treated with 2 mM BSO, or 10 mM GSH-OEt, or 100 μM DA, or 100 μM DQ and transfected with **AP2**–pCMV-luc for 1 h. Luciferase activity was measured at the indicated times ($n=3$), the maximum level of DOTAP transfection is shown by the white dotted line.

cells results in release of free pDNA and increases the efficiency of transgene expression. This effect depends on the concentration and redox potential of the reducing agents (Figure 5). The lowest redox potential in the cell seems to be within the nucleus, where it is required for DNA synthesis and DNA repair as well as to maintain a number of transcription factors in a reduced state [23]. The concentration of GSH has been reported to be ~4 mM in the cytoplasm and ~20 mM in the nucleus [24], corresponding to redox potentials of approximately −228 and −249 mV (calculated by the Nernst equation, assuming a GSH/GSSG ratio of 100:1 [25]). This suggests that, even in the absence of specific nuclear targeting, the reduction of **AP2**–pDNA complex and release of pDNA would occur 4–5 times more efficiently in the nucleus than in the cytoplasm.

Several disulfide and polydisulfide-containing vectors have been proposed for gene delivery [26,27]. The use of lipoic acid for the construction of a cationic vector shows important advantages compared to other approaches: (1) a constrained 1,2-dithiolane ring of lipoate undergoes self-polymerization under very mild conditions; (2) the amphiphilic nature of lipoate allows the construction of an amphiphilic vector capable of transporting DNA across the cell membrane; (3) release of DNA from the complex with lipoic acid-derived polymer proceeds at a thiol concentration comparable with that within the nucleus; (4) thiolate-induced polymerization of the 1,2-dithiolane ring can be used to attach specific ligands to the delivery vector; (5) lipoic acid is a naturally occurring pro-vitamin [8], and it demonstrates no cytotoxicity even at the highest concentration applied; (6) therapeutic application of lipoic acid is approved by FDA. The cationic amphiphiles of lipoic acid provide a several fold increase of transgene expression in vitro, and may prove particularly valuable as transfection vectors for in vivo gene delivery.

Significance

The success of gene therapy depends significantly on reliable and efficient systems for delivery of nucleic acids to target cells. We describe here a new principle for gene delivery that improves the efficiency of synthetic vectors. The delivery system mimics the cell invading strategy of some viruses, which, although stable in the extracellular medium, undergo stepwise disassembly inside the cells. We have synthesized an SS-crosslinked amphiphilic polymer that forms homogeneous virus-sized complexes with pDNA, stable under extracellular redox conditions. This polymer, which is formed from the natural coenzyme lipoic acid, undergoes GSH- and NAD(P)H-dependent reduction inside the cell, releasing DNA from the complex. The reduction proceeds most efficiently under redox conditions found within the cell nucleus, thus providing the mechanism for preferential DNA liberation in this organelle. The release of free DNA augments its availability for cellular transcription enzymes and, as a result, increases the efficiency of transgene expression. The use of lipoic acid for the construction of delivery vectors shows several important advantages, one of which is the possibility of attaching different ligands during amphiphile polymerization. We have used the thiolate-catalyzed polymerization of lipoate moiety to attach the NLS of adenovirus fiber to the polymer, thus increasing the vector efficiency. The same approach can be used to introduce other specific ligands for tissue or organ targeting of transgene DNA. Since the amphiphiles of lipoic acid are nontoxic to cells, they may prove particularly valuable as delivery vectors for in vivo gene therapy.

Materials and methods

Materials

Chemicals were purchased from Sigma and Aldrich, DOTAP from Boehringer Mannheim, and YoYo-1 from Molecular Probes. The plasmids pSV-lacZ, pGL3-luc, and pCMV-luc were prepared as described [19]. Adenovirus fiber peptide (**pI**, AKRARLSTSNPVYPYEDESC) was synthesized using Fmoc chemistry. Recombinant rat TrR [21] was kindly provided by Dr. E. Arnér. GSH-OEt was synthesized as described [28].

Synthesis of AP1 (N-[1-(2,3-dilipoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate)

Lipoic acid anhydride was synthesized as described [11,12]. Dimethylamino-1,2-propanediol (40 mg, 0.3 mmol) was added to 4 ml of a 0.25 M solution of lipoic acid anhydride in chloroform containing 50 mg (0.4 mmol) of 4-(dimethylamino)pyridine. The mixture was stirred overnight under nitrogen at 20°C, quenched with methanol, concentrated, and chromatographed on HPLC (silica gel, 3% methanol in chloroform), affording 105 mg of *N*-[1-(2,3-dilipoyloxy)propyl]-*N,N,*-dimethylamine (70% yield). Then, *N*-[1-(2,3-dilipoyloxy)propyl]-*N,N,*-dimethylamine (75 mg, 0.15 mmol) was added to dimethyl sulfate (20 mg, 0.16 mmol) in 3 ml of dry chloroform and stirred at 20°C under nitrogen. After 20 h the reaction mixture was concentrated and chromatographed on HPLC (chloroform/methanol/acetic acid, 95/5/1) yielding 67 mg of final product (87%). The structure was confirmed by UV and IR spectra, MS *m/z*: 511 (MH^+), and element analysis (C,H,N,S≤±0.4%). Since **AP1** was unstable and underwent partial polymerization upon drying, it was stored in chloroform at 4°C in the dark.

Polymerization reaction (AP2, AP2(pI))

The polymerization conditions were similar to those described earlier [11,12]. The solution of **AP1** (1 mg, 0.002 mmol) in chloroform was evaporated and the residue was dried in vacuum (20 h, 20°C). The film was dissolved in 50 μl of DMSO, and the resulting solution was injected into 500 μl of TNE buffer (25 mM Tris, 140 mM NaCl, 0.5 mM EDTA, pH 8.5). After bubbling nitrogen, polymerization was induced by addition of DTT (30 μl of 2 mM solution) or fiber peptide **pI** (1 mg, 0.0004 mmol) and the reaction was allowed to proceed at 37°C for 48 h under nitrogen atmosphere.

Synthesis of AP3

Amphiphile **AP3** was prepared by reduction of either **AP1** or **AP2** with TCEP immobilized on agarose (Bond-Breaker™, Pierce), or in situ with 100 mM DTT in TNE solution under nitrogen.

CMC determination

The CMC of the amphiphiles was determined in TNE buffer using a protocol modified from [29]. The increase in steady-state fluorescence (AMINCO-2 fluorimeter, excit./emmiss.: 370/470 nm) was measured for 1 μM ANS with varying concentrations of amphiphiles. For **AP3** 50 mM

DTT was present throughout the measurements to prevent aerobic oxidation.

Amphiphile–DNA complex formation

PI incorporation into DNA was used to measure amphiphile–pDNA complex formation. An aliquot (<3 μl) of amphiphile was added to the solution of pDNA (pCMV-luc, 0.7 μg) in 100 μl of TNE, pH 7.4. The complex was formed for 30 min and then diluted with 900 μl of 0.2 μM PI in TNE. The final concentration was 2.5 μM of pDNA phosphate and 0–100 μM of amphiphile (for **AP2**, concentration of monomers). The fluorescence (excit./emmiss.: 535/617 nm) was measured, and apparent association constants (K_A^*) for **AP1–3** were estimated from titration curves (Figure 2) assuming 1:1 amphiphile:phosphate stoichiometry and using the simplified equation: $K_A^* = 1/(C^* - 0.5 \times C_{DNA})$, where C^* is the concentration of amphiphile at the transition point, and C_{DNA} is the total DNA concentration. For gel electrophoresis, the complex was prepared with 21 μM pCMV-luc and different concentrations of amphiphiles in 100 μl of TNE and analyzed on 1% agarose gel.

Transmission electron microscopy

The samples were prepared with 5 μg of amphiphile with and without 1 μg of pCMV-luc in the absence and in the presence of DTT (from 0.1 to 50 mM) in 20 μl of 50 mM HEPES, pH 7.4. Negative staining with 1% sodium silicotungstate and low-dose electron microscopy were performed as described [30].

Cell transfection, pDNA uptake measurements, and cytotoxicity assay

HeLa, A549, and BHK cells were cultured and transfected in 24-well plates (1.5 μg DNA/well, 4 h) as described [19]. β-Galactosidase gene expression (pSV-lacZ) was detected histochemically using X-Gal. Luciferase activity (pGL3-luc, and pCMV-luc) was measured by a luminescence assay [19]. Each transfection experiment was done several times in duplicate and results were expressed as light units per mg of cell protein (RLU/mg). Protein content was measured with the Bio-Rad Protein Assay reagent. To measure pDNA uptake, transfection was performed with YoYo-1-labeled pDNA (30 ng of YoYo-1 per μg of pCMV-luc, ~1 molecule per 100 bp). After 1 h of incubation with pDNA complex, cells were washed three times with PBS, detached with Versen solution and analyzed on a FACScan using Cell Quest software (Becton Dickinson). Under these conditions all membrane-bound pDNA was removed since additional treatment of the cells with lipase:TCEP:DNase (2 U/ml:50 μM:50 U/ml in PBS) did not change the intensity of cell-associated pDNA fluorescence. The relative efficiency of transfection was expressed as $K_{eff.}(\text{vector}) = k^* \times \text{RLU(vector)}/F_{YoYo-1}(\text{vector})$, where RLU(vector) is a luciferase activity, $F_{YoYo\text{-}1}$(vector) is the fluorescence of YoYo-1-labeled pDNA in the cells, and $k^* = F_{YoYo\text{-}1}(\text{DOTAP})/\text{RLU(DOTAP)}$ is a normalizing coefficient. Cell transfections under different redox conditions were carried out as described above except that the time of transfection was 1 h. Cytotoxicity of the amphiphiles was determined by a colorimetric assay of cell proliferation (WST-1, Boehringer Mannheim), and flow cytometry analysis (PI staining).

pDNA complex reduction

The reduction of pDNA complexes was performed in 10 μl of TNE buffer for 30 min at 37°C. The reaction contained 0.125 μg of YoYo-1-labeled pCMV-luc with 0.8 μg of **AP2** or 0.5 μl of DOTAP. The final concentrations of enzymes were 10 U/ml of LADH ± 0.5 mM NADH or 1 U/ml of TrR ± 0.5 mM NADPH. The reaction was analyzed on 1% agarose gel.

Measurements of cellular redox state

Intracellular GSH concentration was measured by flow cytometry. After incubation with BSO or GSH-OEt, HeLa cells were labeled with 1 μM CMFDA (Molecular Probes) and analyzed on a FACScan. The concentration of intracellular NAD(P)H after addition of DA or DQ was determined fluorometrically (340/460 nm) [22] using HeLa cells in PBS (2×10^5 cells/ml).

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