DOI: 10.1002/cmdc.200600267

Dimerizable Redox-Sensitive Triazine-Based Cationic Lipids for in vitro Gene Delivery

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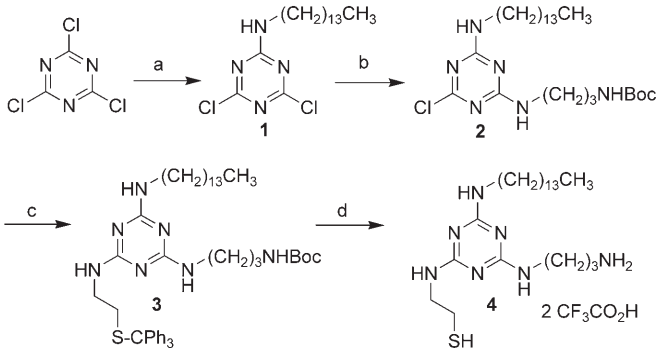
Dedicated to Prof. Pierfrancesco Bravo on the occasion of his retirement

Introduction of a missing gene into the cell nucleus (transfection), followed by its expression by the natural machinery of the cell, is a very well-established strategy to produce proteins. This process can also be used therapeutically to produce proteins needed to heal a certain pathological condition (gene therapy).[1] However, achievement of efficient transfection remains a challenging endeavor. A number of transfection methods have been developed, essentially based on the use of viruses,[2] chemical reagents (for example, cationic lipids[3] and polymers[4]), or physical methods (mechanical stimulation, electroporation, magnetic field induced, etc.).[5] All of these transfection strategies have advantages and drawbacks, but it is apparent that the use of chemical reagents is a very attractive option, because of their ready availability (many transfection reagents are commercial products), their versatility (they can be used to transfect different kinds of cells with genetic material having a wide range of dimensions), low toxicity for the operator (viruses can be pathogenic), and simple experimental use. The main drawback with chemicals as transfectants is connected with their generally low performance, both in terms of number of cells which are transfected and alive at the end of the process and in terms of duration of the transfection. For this reason, chemical transfection reagents must be effective and with limited cytotoxicity, but also cheap and readily prepared from inexpensive, nonexotic starting materials to be competitive and of potential practical use. With these premises, it is not surprising that calcium phosphate is still widely used to achieve transfection, particularly with "difficult" and resistant cells, in addition to popular "organic" reagents (which are generally cationic liposomes comprised of a cationic lipid and a neutral, helper lipid for example, cholesterol) such as Lipofectamine, DOTAP, and related compounds. Although a number of active ingredients for transfection have been recently described in the literature, few of them are truly innovative in terms of structure.

In this paper we present a conceptually new family of cationic lipids for transfection featuring a triazine scaffold, which allows for an easy derivatization, with three functionally different amino side chains: 1) a linear C12–C16 chain (the lipophilic tail), 2) a 3-propylammonium chain which acts as polar head, 3) a redox-sensitive dimerizable 2-thioethyl chain (according to the so-called disulfide-linker strategy).[6] The corresponding disulfide dimers have been synthesized and evaluated as well. The new triazine-based transfectants feature very simple preparation from inexpensive materials, and the triazine core allows for a smooth introduction of structural diversity by means of subsequent nucleophilic dechloroaminations of the starting trichlorotriazine by different amines. Most importantly, these triazine-based cationic lipids exhibit low cytotoxicity and high transfection efficiency on a variety of eukaryotic cells, even without any formulation with helper lipids. The lead thiol compound **4**, having a C14 chain, was synthesized in multigram amounts as portrayed in Scheme 1.



Scheme 1. a) $CH_3(CH_2)_{13}NH_2$, $NaHCO_3$, acetone/H_2O, RT (67%). b) H_2N-$(CH_2)_3NHBoc$, $NaHCO_3$, acetone/H_2O, 60 °C (97%). c) $Ph_3CS(CH_2)_2NH_2{\cdot}HCl$, DIPEA, benzene, 120 °C, sealed vial (65%). d) TFA/DCM (1:4), Et_3SiH, 5 °C (98%).

As expected, because of the weak basicity of the melamine scaffold,[7] ^{19}F NMR titration experiments allowed us to establish that **4** incorporates two equivalents of CF_3CO_2H (TFA), therefore only the primary amine function and one melaminic nitrogen are actually protonated. Thus, the net cationic charge per molecule of **4** is two.

The disulfide dimer **8** was preferentially obtained not by oxidative dimerization of **4**, which was rather inefficient, but ex novo in four steps from cyanuric chloride (Scheme 2).

An analogous strategy was used to obtain the other derivatives **9**–**18**, each having a different lipophilic chain between C8 and C18 (Figure 1).

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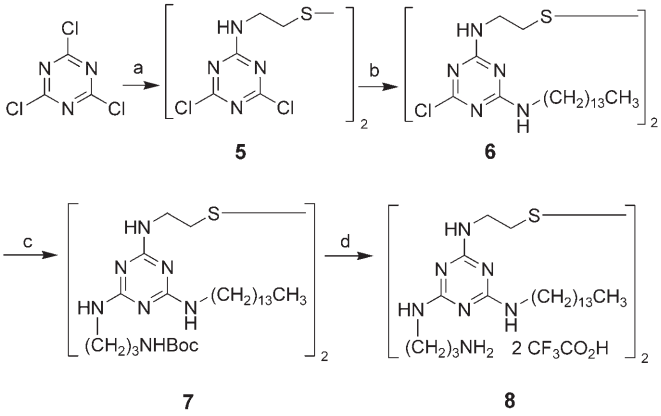

Scheme 2. a) ($HCl{\cdot}H_2NCH_2CH_2S)_2$, $NaHCO_3$, acetone/H_2O, RT (74%). b) CH_3-$(CH_2)_{13}NH_2$, $NaHCO_3$, acetone/H_2O, 60 °C (58%). c) $H_2N(CH_2)_3NHBoc$, DIPEA, benzene, 120 °C, sealed vial (75%). d) TFA/DCM (1:4), 5 °C (70%).

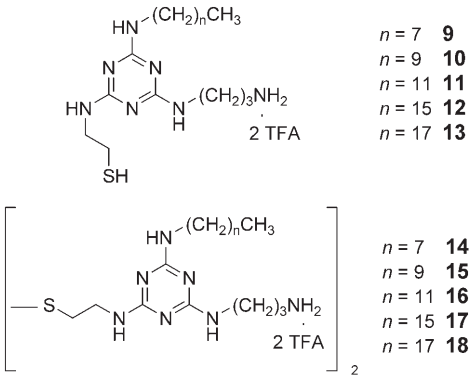

Figure 1. The complete array of triazine based cationic lipids.

The essential requirement for a transfection vector is a sufficiently strong and rapid DNA binding. As cationic lipid-based transfection efficiencies are known to be highly sensitive to the DNA:lipid ratio, in other words the charge ratio (CR) of the complex, we decided first to investigate by electrophoretic gel retardation assay, the complex formation between the DNA and molecule **4** (Figure 2). Thus, naked plasmid DNA (lane 2) was mixed with increasing amounts of compound **4** (lanes 3–9). As shown in Figure 2, plasmid DNA exists as a mixture of supercoiled and open, circular forms. It is apparent that the complex is effectively and completely formed at a CR $\geq$ 10 (lane 7) because free DNA, completely retained in the gel loading slots, disappeared. Therefore CR 10 was used as the upper limit for subsequent experiments evaluating the transfection efficiency of the new triazine-based cationic lipids.

The transfection efficiency and toxicity of the model compound **4** was tested on the NRK (Normal Rat Kidney Epithelial) cell line at different CRs (Figure 3). Toxicity was moderate for the whole range of CR tested. At the highest concentration of compound **4** (CR 10), corresponding to the most efficient compound/DNA complex formation, 70% of the cells survive (see above and Figure 3). Not surprisingly, the transfection efficiency was strongly dependent on the CR, and compound **4** proved to be a remarkably good transfecting agent at CR 10,

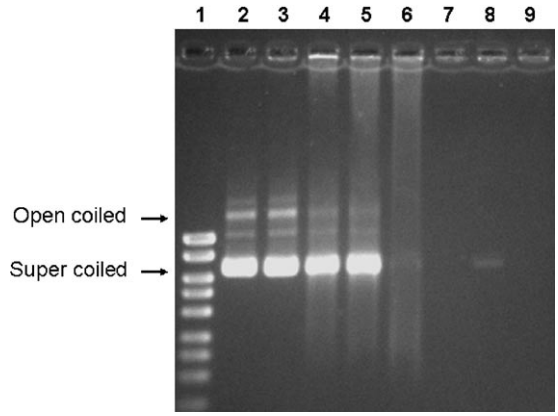

Figure 2. Electrophoretic gel retardation assay showing DNA binding gel patterns for lipoplexes prepared using compound **4**/DNA at different charge ratios (CRs). From the left to the right: lane 1, molecular weight marker; lane 2, naked plasmid; lane 3, CR 0.2; lane 4, CR 1; lane 5, CR 2; lane 6, CR 5; lane 7, CR 10; lane 8, CR 15; lane 9, CR 20. The details of treatments are described in the text.

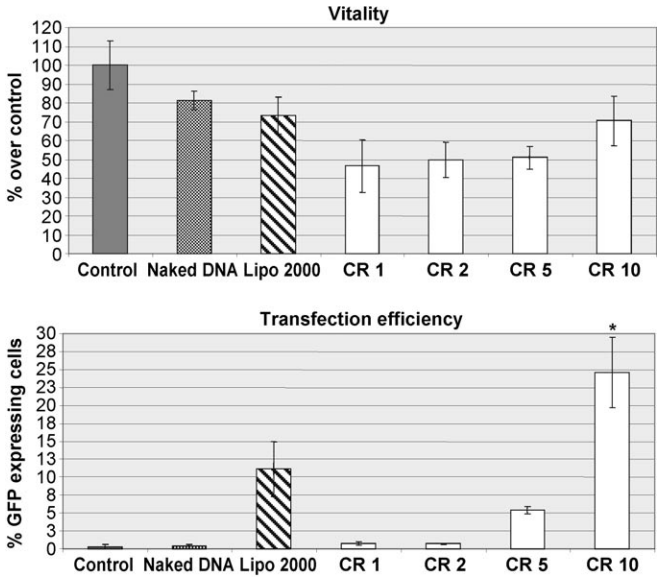

Figure 3. In vitro cytoxicity and transfection efficiency of compound **4** at different charge ratios (CRs) in NRK cell line. The vitality and the transfection efficiency of the same cationic lipid were compared to that of Lipofectamine 2000 (Lipo2000), (* $P < 0.05$ versus Lipo2000). Increasing levels of GFP expression with increasing amounts of compound **4** in NRK cells are associated to constantly low cytotoxicity.

considerably superior to Lipofectamine 2000 (Lipo2000). Lower CRs were inefficient, probably as a consequence of ineffective lipoplex formation.

The transfection efficiency and toxicity for the thiols **4** and **9**–**13**, and for the disulfides **8** and **14**–**18** were tested on the PC3 (Human Prostatic Cancer) cell line at CR 10 (Figure 4). The transfection with naked DNA alone produced almost no toxicity nor measurable fluorescence. The array of triazines showed a modest toxicity, intermediate between DOTAP and Lipo2000. The transfection profile showed a Gaussian bell-type trend, with the top performance corresponding to the C14 com-

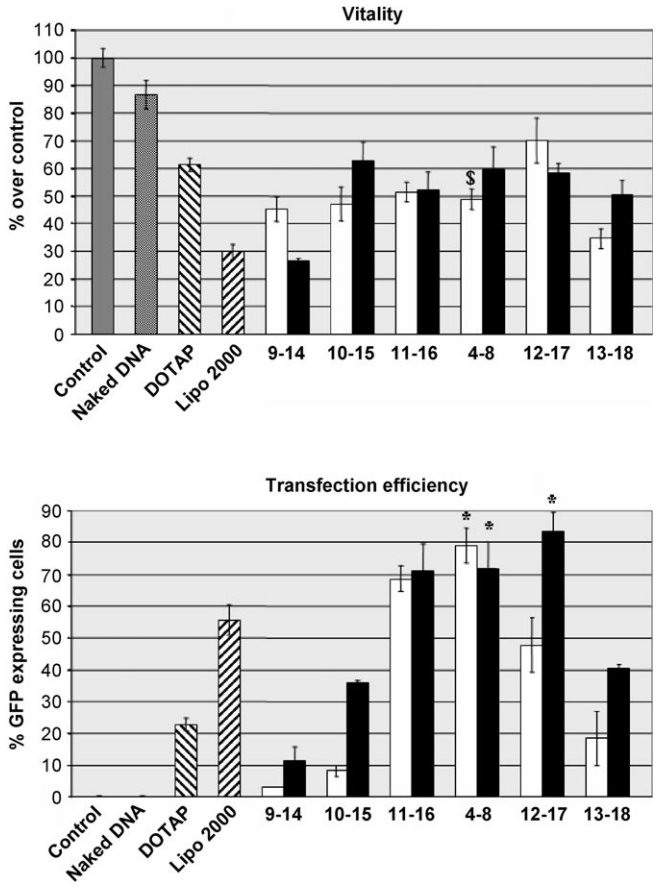

Figure 4. In vitro cytoxicity and transfection efficiency of compounds **4**–**18** at charge ratio (CR) 10 in PC3 cell line. The vitality after transfection was compared to that of the commercially available DOTAP, one of the less cytotoxic transfectants ($ $P < 0.05$ versus DOTAP). The efficiency of the cationic lipids was then evaluated and compared to that of Lipofectamine 2000 (Lipo 2000) (* $P < 0.05$ versus Lipo2000).

Table 1.
Cellular toxicity and transfection efficiency of compound **4** at CR 10 on various cell lines.

Cell line	Transfectant	Vitality (% of control)[a]	Transfection efficiency (% of total)[a]
HeLa	Lipo2000	70 ± 13	4 ± 1
	Compound **4**	76 ± 13	11 ± 2*
MG63	Lipo2000	34 ± 7	22 ± 2
	Compound **4**	31 ± 5	71 ± 2*
COS-7	Lipo2000	58 ± 3	27 ± 2
	Compound **4**	77 ± 5	44 ± 8*
P19	Lipo2000	94 ± 3	2 ± 1
	Compound **4**	104 ± 12	6 ± 1*

[a] Each value represents the mean ± S.E.M. * $P < 0.05$ versus Lipo2000.

pound **4** in the thiol series, and a delayed curve with the top for the C16 compound **17** for the disulfide series. Particularly remarkable are the results obtained with the thiols **4** and **11**, and the disulfides **8**, **16**, and **17**, which showed the best compromise between toxicity and transfection efficiency, at a level which is superior to that of Lipo2000 and DOTAP. Dimeric disulfides with a lipophilic side chain shorter than C10 are not very effective, whereas for the monomeric thiol structures the lipophilic side chain must be at least C12.

Fully consistent with the findings in the reporter gene expression profile observed using epifluorescence microscopy (Figure 5, A1–C1), the percentage of GFP reporter gene-expressing COS-7 cells transfected with compound **4** was higher than for the Lipo2000-transfected cells (Figure 5, A3–C4; Table 1). Moreover, transfection reagents can affect cell physiology. Light microscopy analysis of cells transfected with compound **4** (Figure 5, C2) showed that the morphology of the non transfected cells (Figure 5, A2) was preserved.

To confirm the ability of compound **4** to enable high transgene expression and display low cellular toxicity in different cell lines, we tested it at CR 10 in comparison to Lipo2000 (Table 1). In vitro transfection assays were also carried out using HeLa, MG63, COS-7, and P19 cell lines. Consistent with previous results on the PC3 cell line, compound **4** showed higher transfection efficiency than Lipo2000 preserving equally low toxicity.[8]

The morphology of the nanoparticles formed by the lipoplexes DNA/**4** at CR 10 was investigated by SEM analysis. As shown in Figure 6, the resulting structures are rather heterogeneous both in shape and size, suggesting that the nanoparticles are formed by a variable amount of DNA plasmid.

The critical micelle-forming concentration (cmc) is a fundamental parameter in the evaluation of the biological activity of cationic lipids as no micelles should be present during DNA condensation. The cmc of **4** (Figure 7) was found to be approximately 25 mM, well above the concentration used in all the assays (in our conditions CR 10 corresponds to 303 μM). This means that mixing **4** at 303 μM with DNA at 60.6 μM, should result in the encounter of individual molecules, leading to clean monomolecular collapse of DNA.

In summary, we have described a conceptually new class of reagents for gene delivery, consisting of cationic lipids having a *N*-substituted melamine scaffold supporting three side chains bearing 1) a lipophilic C10–C16 chain, 2) a protonated primary amine polar head, and 3) a dimerizable redox-sensitive thiol function. The dimeric disulfides have been also synthesized. Thiol **4** is extremely effective in transfecting different cell lines in vitro, with a combined toxicity/efficiency profile which is nearly always superior to commercially available and popular transfection reagents, such as DOTAP and Lipofectamine 2000. The easy synthesis in multigram amounts from inexpensive and commercially available starting materials, combined with a user-friendly (no formulation or liposome preparation is required) and effective protocol for cell transfection render these triazine-based compounds very attractive reagents for gene delivery applications.

Many issues are currently under investigation, such as the identification of other triazine-based lead transfectants, the mechanism of action of the new reagents, the role of the triazine scaffold, the possibility to transfect primary cells, the influence of serum, the effect of helper lipids, and the use of these cationic lipids for in vivo gene delivery.

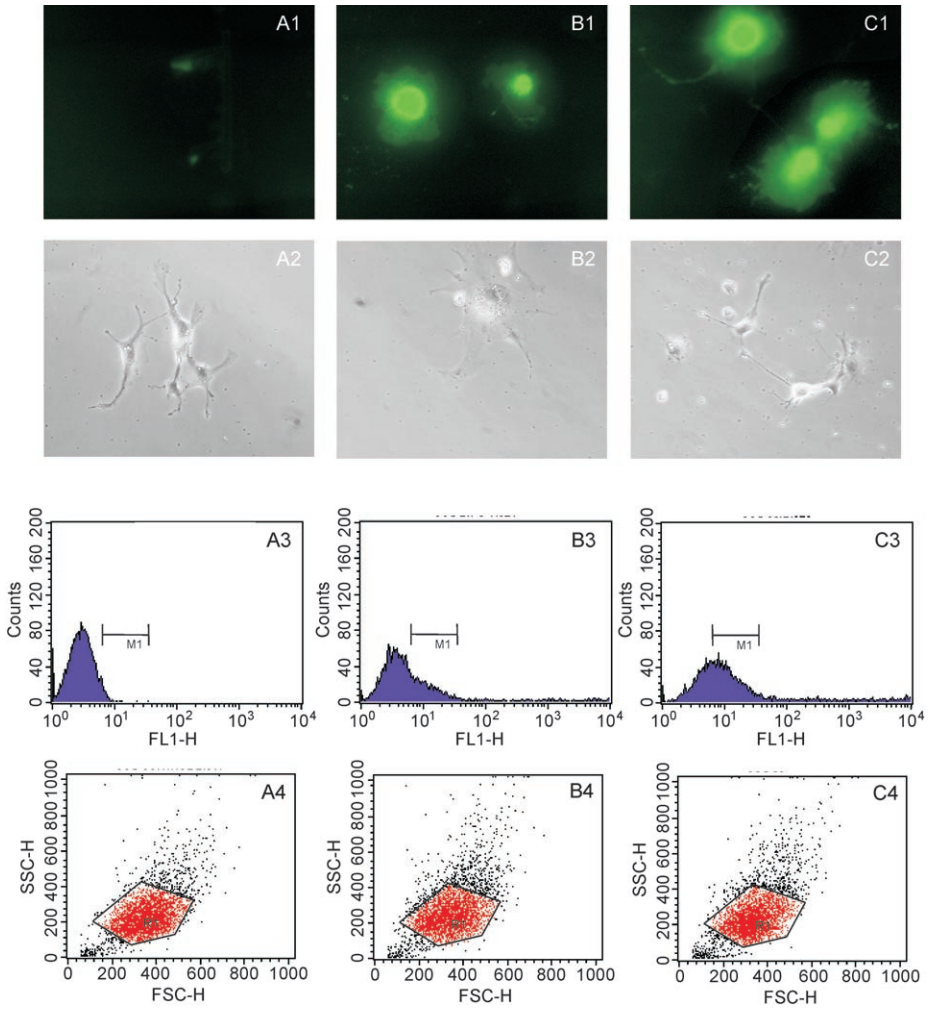

Figure 5. A) Control, B) Lipo2000, C) Compound **4** at CR 10; Row 1: fluorescence microscopy, Row 2: inverted microscope; Rows 3 and 4: FACS analysis. Uptake and expression of GFP-containing plasmid DNA by COS-7 cells transfected either with Lipo2000 (B) or compound **4** (C) evaluated by fluorescence microscopy (B1 and C1, respectively) and by FACS analysis (B3–B4 and C3–C4, respectively). Picture A1 shows a pale cellular autofluorescence, A2 cell morphology, and A3–A4 FACS analysis of COS-7 cell line in the absence of transfectants (control). In A4–C4 FACS data were plotted as cell size (FSC) as a function of granulosity (SSC).

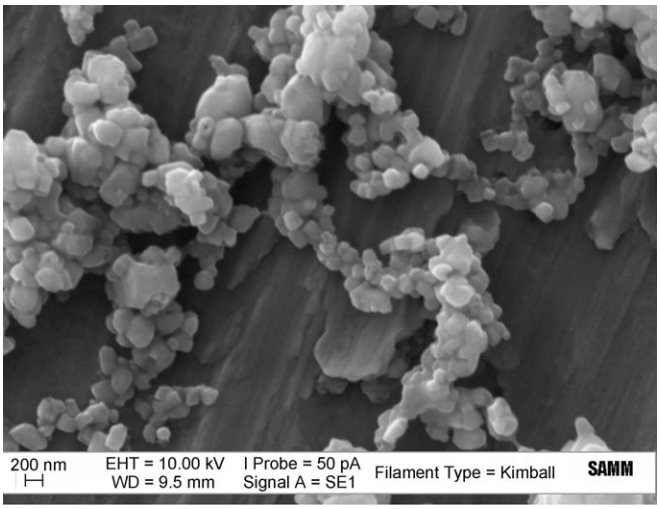

Figure 6. Scanning electron microscopy (SEM) of lipoplexes generated mixing compound **4** and DNA at CR 10 illustrating the heterogeneity of the discrete nanoparticles.

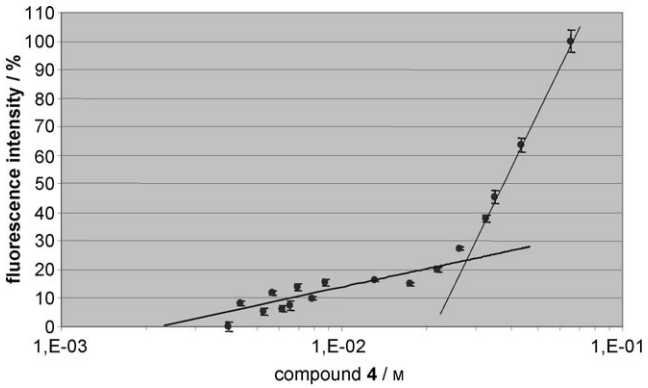

Figure 7. Determination of the critical micellar concentration (cmc) of compound **4**. The fluorescence of *N*-phenyl-1-naphthylamine (NPN) was plotted against detergent concentration.

Experimental Section

Full experimental details for the preparation and characterization of the triazine-based thiols and disulfides **4**, **8**, **9**–**18**, as well as for the transfection experiments is provided in the Supporting information.

Acknowledgements

We thank the European Commission (Marie Curie European Reintegration Grant "TRANSFECTAZINE" MERG-CT-2005-029132 and Integrated Project "STROMA" LSHC-CT-2003-503233), Politecnico di Milano, and C.N.R. for economic support. The Authors wish to thank the staff of Laboratory of Biocompatibility and cell culture—BioCell, Politecnico di Milano (Italy) for their technical support.

Keywords: amphiphiles · DNA · thiols · transfection · triazines

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Received: November 16, 2006
Published online on December 27, 2006

