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Addition of ascorbic acid to the extracellular environment activates lipoplexes of a ferrocenyl lipid and promotes cell transfection

Burcu S. Aytar [a], John P.E. Muller [a], Sharon Golan [b], Shinichi Hata [c], Hiro Takahashi [c], Yukishige Kondo [c], Yeshayahu Talmon [b,*], Nicholas L. Abbott [a,**], David M. Lynn [a,***]

[a] Department of Chemical and Biological Engineering, University of Wisconsin - Madison, 1415 Engineering Drive, Madison, WI 53706, United States
[b] Department of Chemical Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel
[c] Dept. of Industrial Chemistry, Tokyo University of Science, Tokyo, Japan

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ABSTRACT

The level of cell transfection mediated by lipoplexes formed using the ferrocenyl lipid bis(11-ferrocenylundecyl) dimethylammonium bromide (BFDMA) depends strongly on the oxidation state of the two ferrocenyl groups of the lipid (reduced BFDMA generally mediates high levels of transfection, but oxidized BFDMA mediates very low levels of transfection). Here, we report that it is possible to chemically transform inactive lipoplexes (formed using oxidized BFMDA) to "active" lipoplexes that mediate high levels of transfection by treatment with the small-molecule reducing agent ascorbic acid (vitamin C). Our results demonstrate that this transformation can be conducted in cell culture media and in the presence of cells by addition of ascorbic acid to lipoplex-containing media in which cells are growing. Treatment of lipoplexes of oxidized BFDMA with ascorbic acid resulted in lipoplexes composed of reduced BFDMA, as characterized by UV/vis spectrophotometry, and lead to activated lipoplexes that mediated high levels of transgene expression in the COS-7, HEK 293T/17, HeLa, and NIH 3T3 cell lines. Characterization of internalization of DNA by confocal microscopy and measurements of the zeta potentials of lipoplexes suggested that these large differences in cell transfection result from (i) differences in the extents to which these lipoplexes are internalized by cells and (ii) changes in the oxidation state of BFDMA that occur in the extracellular environment (i.e., prior to internalization of lipoplexes by cells). Characterization of lipoplexes by small-angle neutron scattering (SANS) and by cryogenic transmission electron microscopy (cryo-TEM) revealed changes in the nanostructures of lipoplexes upon the addition of ascorbic acid, from aggregates that were generally amorphous, to aggregates with a more extensive multilamellar nanostructure. The results of this study provide guidance for the design of redox-active lipids that could lead to methods that enable spatial and/or temporal control of cell transfection.

1. Introduction

Cationic lipids have been investigated broadly as non-viral agents for the delivery of DNA to cells [1–6]. Early studies demonstrated that significant levels of cell transfection could be achieved by formulating cationic lipid/DNA complexes (lipoplexes) using lipids with relatively simple molecular structures (e.g., a simple cationic head group and several hydrophobic tails) [7, 8]. Over time, however, the structures and properties of lipids used for cell transfection have evolved to include chemical functionality designed to overcome or address a variety of intracellular barriers that limit cell transfection efficiency. For example, lipids have been designed to form lipoplexes that are stable in complex extracellular environments (including in the presence of serum proteins) [9–11] and/or release their cargo (DNA) in response to intracellular stimuli, including the presence of reducing agents [12–16] (e.g., glutathione), changes in pH [15, 17–19], or the presence of specific enzymes [20, 21]. The design of lipids that promote more efficient intracellular trafficking of internalized DNA has contributed significantly to the development of lipoplex-based approaches to DNA delivery.

In contrast to efforts to design functional lipids that promote the intracellular trafficking of DNA, relatively few studies have reported on the design of lipids that can be transformed (or "activated" toward transfection) in response to externally-controlled stimuli applied in *extracellular* environments (e.g., so as to influence extents to which complexes are either internalized or not internalized by cells) [22–24]. This latter approach could provide lipoplexes that enable control over the timing of the delivery of DNA to cells (i.e., "temporal control" of transfection) or, alternatively, allow delivery of DNA to sub-populations of cells within a larger population (i.e., "spatial control" of transfection

* Corresponding author.
** Corresponding author. Tel.: +1 608 265 5278.
*** Corresponding author. Tel.: +1 608 262 1086.
E-mail addresses: ishi@tx.technion.ac.il (Y. Talmon), abbott@engr.wisc.edu (N.L. Abbott), dlynn@engr.wisc.edu (D.M. Lynn).

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by spatially-controlled delivery of an activating agent). The design of lipids that offer the ability to achieve external control over the timing and the locations at which DNA is available to cells could potentially find use in a broad range of applications, ranging from basic biomedical research (e.g., the design of transfected cell arrays) [25–29], engineering of tissues with complex tissue architectures [30–33], and, potentially, for the development of new gene-based therapies. In this current study, we report a step toward the realization of general and facile principles for spatial and temporal control over the lipoplex-mediated delivery of DNA to cells. Our approach is based on the use of a redox-active ferrocene-containing cationic lipid.

The study reported in this paper exploits the physicochemical properties of the redox-active ferrocene-containing lipid bis(11-ferrocenylundecyl)dimethylammonium bromide (BFDMA, Fig. 1) [34–36]. This lipid can be reversibly cycled between its reduced state (net charge of +1) and its oxidized state (net charge of +3) by either oxidation or reduction of the ferrocene groups present at the end of each hydrophobic chain [35–41]. Our past studies have demonstrated that the oxidation state of BFDMA significantly affects the interaction of this lipid with DNA [39, 40] and the efficiency with which lipoplexes of BFDMA and DNA transfect cells [37, 38, 41]. In particular, our past studies have identified a range of lipid concentrations over which lipoplexes formed from reduced BFDMA mediate high levels of transgene expression *in vitro*, whereas lipoplexes formed from oxidized BFDMA (over the same range of concentrations) yield negligible levels of transgene expression [37, 38, 41]. Our previous physical characterization experiments also reveal that the oxidation state of BFDMA influences the zeta potentials and nanostructures of lipoplexes formed from BFDMA [39, 40].

The oxidation state of ferrocene-containing surfactants and lipids can be transformed readily and reversibly using electrochemical methods or by using chemical oxidizing and reducing agents [34–46]. In a recent study, we reported the use of glutathione (GSH) to chemically reduce the ferrocenium groups within lipoplexes formed using DNA and oxidized BFDMA (in 1 mM aqueous Li_2SO_4 solution at pH 5.1) [41]. We demonstrated that when these transformed lipoplexes were subsequently introduced to COS-7 cells *in vitro*, they mediated high levels of transgene expression (i.e., levels that were comparable to the levels of transgene expression mediated by lipoplexes formed from reduced BFDMA and DNA). This past study demonstrated that "inactive" lipoplexes of oxidized BFDMA and DNA can be activated by treatment with a chemical reducing agent. However, while these past studies represent a significant step toward the realization of principles that could be used to exert spatial and temporal control over transfection using ferrocene-containing lipids, a number of important issues remain to be addressed. First, as mentioned above, in our previous studies chemical reduction was performed (i) in a simple electrolyte solution (1 mM aqueous Li_2SO_4, as opposed to cell culture media) and (ii) prior to the introduction of the lipoplexes to cells [41]. Second, we also observed that a large molar excess of GSH was required to achieve rapid transformation of oxidized BFDMA within lipoplexes (transformation occurred over ~90 min in the presence of a 10-fold molar excess of GSH, but within seconds to minutes in the presence of a 50-fold molar excess of GSH).

We also note, in this context, that while GSH served as a useful model reducing agent in our initial studies, this molecule is produced in significant concentrations inside cells and is also present at lower concentrations in extracellular environments [47, 48]. In the study reported in this paper, we demonstrate that the rapid and efficient chemical reduction of oxidized BFDMA within lipoplexes can be achieved at significantly lower concentrations using ascorbic acid (vitamin C) as a chemical reducing agent. Ascorbic acid (AA) is a well-known and biologically important chemical reducing agent, but in contrast to GSH it is not synthesized naturally by humans or primates [49]. Below, we demonstrate using cell-based experiments and physicochemical methods of characterization that AA can be used to chemically transform lipoplexes of oxidized BFDMA (via reduction of ferrocenium groups) to "activate" these lipoplexes both in cell culture media and *in the presence of cells* (i.e., by adding small amounts of AA to lipoplex-containing media in which cells are already growing).

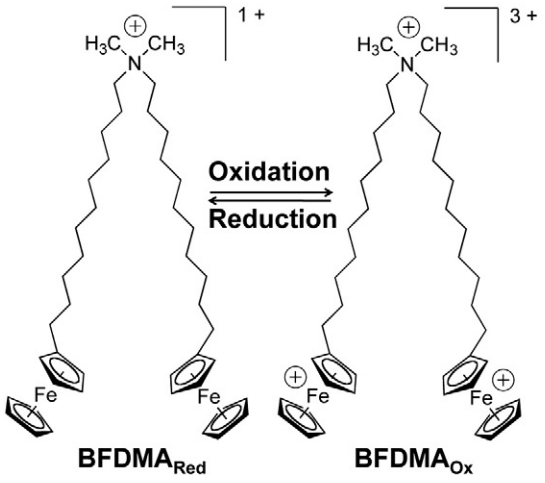

Fig. 1. Molecular structure of bis(11-ferrocenylundecyl)dimethylammonium bromide (BFDMA).

2. Materials and methods

2.1. Materials

BFDMA was synthesized using methods described previously [35]. Ascorbic acid, heparin, and lithium sulfate monohydrate were purchased from Sigma Aldrich (St. Louis, MO). Dodecyltrimethylammonium bromide (DTAB) was purchased from Acros Organics (Morris Plains, NJ). Plasmid DNA constructs encoding enhanced green fluorescent protein [pEGFP-N1 (4.7 kb), >95% supercoiled] and firefly luciferase [pCMV-Luc, >95% supercoiled] were purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM cell culture medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), Lipofectamine 2000, Lysotracker Red, Wheat germ agglutinin (WGA)-Alexa Fluor 488, and Hoechst 34580 were purchased from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kits were purchased from Pierce (Rockford, IL). Glo Lysis Buffer and Steady-Glo Luciferase Assay kits were purchased from Promega Corporation (Madison, WI). Cy5 Label-IT nucleic acid labeling kits were purchased from Mirus Bio (Madison, WI). Glass inset dishes used for laser scanning confocal microscopy (LSCM) were purchased from MatTek (Ashland, MA). Deionized water (18 MΩ) was used to prepare all buffers and salt solutions. All commercial materials were used as received without further purification, unless otherwise noted.

2.2. General considerations

Electrochemical oxidation of BFDMA was performed as described previously [37–39, 41]. UV/vis absorbance values of lipoplex solutions were monitored using a Beckman Coulter DU520 UV/vis Spectrophotometer (Fullerton, CA). Zeta potential measurements were performed using a Zetasizer 3000HS instrument (Malvern Instruments, Worcestershire, UK). Fluorescence microscopy images used to evaluate the expression of enhanced green fluorescent protein (EGFP) in cell transfection experiments were recorded using an Olympus IX70 microscope and were analyzed using the MetaVue version 7.1.2.0 software package (Molecular Devices; Toronto, Canada). Luminescence and absorbance measurements used to characterize luciferase expression and total cell protein were performed using a PerkinElmer EnVision multilabel plate reader (Luciferase: Em, 700 nm cutoff. BCA: Abs 560 nm). For LSCM experiments, DNA was