Chapter 14

Characterization and Investigation of Redox-Sensitive Liposomes for Gene Delivery

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Abstract

A number of smart nonviral gene delivery vectors relying on bioresponsiveness have been introduced in the past few years to overcome the limits of the first generation of gene carriers. Among them, redox-sensitive lipidic and polymeric vectors exploit the presence of disulfide bonds in their structure to take advantage of the highly reductive intracellular milieu and to promote complex unpacking and nucleic acids release after cellular uptake (disulfide linker strategy). Glutathione (GSH) has been often identified as the leading actor in the intracellular reduction of bioreducible vectors but their actual mechanisms of action have been rarely investigated in depth and doubts about the real effectiveness of the disulfide linker strategy have been raised. Herein, we outline a simple protocol for the preparation and investigation of nano-sized reducible cationic liposomes, focusing on their thorough characterization and optimization as gene delivery vectors. In addition, we carefully describe the techniques and procedures necessary for the assessment of the bioreducibility of the vectors and to demonstrate that the GSH-mediated intracellular cleavage of disulfide bonds is a pivotal step in their transfection process. Liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and of the reducible cationic lipid SS14 are reported as a practical example but the proposed protocol can be easily shifted to other formulations of reducible lipids/liposomes and to reducible polymers.

Key words Gene delivery, Nonviral vector, Liposome, Redox-sensitive vector, Glutathione, GSH depletion/repletion, Transfection efficiency, Cytotoxicity

1 Introduction

Gene delivery can be defined as the introduction of exogenous genetic material (i.e., DNA and RNA) into cells to control their protein expression [1]. The range of its therapeutic applications (gene therapy) has thoroughly expanded since the recent accomplishment of the Human Genome Project (HGP), leading to increased efforts in the investigation of gene delivery techniques. Aiming to obtain adequate delivery rates of nucleic acids to cells, two major classes of gene delivery agents have been developed so far, viral and nonviral vectors. Since their introduction in the late 1980s, nonviral synthetic gene vectors (transfectants) have been

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thoroughly developed and investigated as promising alternatives to viruses mainly because they are easy-to-use, cheap, and have a safer profile, thus often making them the vectors of choice for in vitro laboratory research [2]. Nonviral vectors for gene delivery can be divided into two major families, cationic lipids and cationic polymers. Both lipids and polymers can self-assemble with anionic nucleic acids forming nano-/micro-scaled particles respectively named lipoplexes and polyplexes, which can interact with the plasma membrane and mediate cellular uptake. However, to date, the lower efficiency in delivering the genetic material to the target cells as compared to viruses has limited the use of nonviral gene delivery vectors in clinics. In order to overcome the major bottlenecks hindering effective nonviral gene delivery, recently a new generation of polymeric and lipidic vectors relying on bioresponsiveness has been developed [2].

Among these new gene carriers, redox-sensitive vectors have received increasing attention, owing to their peculiar ability to exploit the reductive intracellular milieu to increase the release of nucleic acids from the complexes after cellular uptake, considered as one of the main open issues in nonviral gene delivery. In fact, by introducing disulfide bonds within the chemical structure of vectors, an approach known as the "disulfide linker strategy", their stability can be spatially controlled thanks to the gradient in redox potential existing between the extracellular and the intracellular environment. More precisely, disulfides are highly stable in the oxidizing extracellular environment but they are quickly reduced to sulfhydryls by the high levels of cytoplasmic glutathione (GSH, 1–11 mM), eventually causing the intracellular disassembly of reducible lipoplexes and polyplexes [3, 4]. A number of redox-sensitive lipidic and polymeric transfectants have been synthesized and studied so far but often the investigation of the actual mechanism of transfection has been overlooked and some authors raised doubts about the real effectiveness of the disulfide linker strategy in gene delivery, suggesting that other factors, such as changes in the ability to promote endosomal escape, may influence to a greater extent the overall efficiency of reducible vectors [5]. In this context, our group has recently developed several techniques and methodologies aimed at developing and optimizing redox-sensitive lipid-based systems for gene delivery and at adequately demonstrating the key role of bioreducibility in their transfection process [3, 4, 6, 7].

The overall goal of this book chapter is to outline a simple protocol for the characterization, optimization, and investigation of redox-sensitive liposomes as gene delivery vectors describing, as a practical example, the development of reducible liposomes composed by 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and SS14, a reducible *gemini* lipid previously synthesized and investigated by our group [3, 4, 6, 7]. After first outlining a quick method

to formulate and extrude nanometer-sized three-component unilamellar liposomes (16.7:33.3:50 molar ratio) DOPC/DOPE/SS14 in water), the first part of the protocol is focused on their overall physicochemical and biological characterization: (1) determination of the mean diameter and overall surface charge (ζ-potential) of liposomes and lipoplexes by Dynamic Light Scattering (DLS) and Laser Doppler Microelectrophoresis (LDM) techniques; (2) investigation of the ability to complex and condense DNA by fluorophore (SYBR® Green I)-exclusion assay; (3) evaluation of transfection efficiency and cytotoxicity using the Enhanced Green Fluorescent Protein (EGFP) as reporter gene (pEGFP-N1 plasmid) and AlamarBlue® as cell viability assay; and (4) identification of the best transfection conditions. The second part of the protocol highlights the key experiments necessary to assess if the presence of disulfides in the lipid structure really impart bioresponsiveness to the resulting lipoplexes: (1) evaluation of DNA release from lipoplexes in the presence of reducing agents; (2) transfection experiments in GSH-depleted cells.

The protocol described herein reports specific transfection conditions for the DOPC/DOPE/SS14 (16.7:33.3:50) liposomes and for MG63 cells as model cell line, anyway, it could be easily shifted to other formulations and types of reducible transfectants (e.g., polymers) and to other types of cells following the suggestions reported in the Subheading 4.

2 Materials

2.1 Cationic Liposome and Lipoplex Preparation and Characterization

1. DOPC (Avanti Polar Lipids, Alabaster, AL). Store at −20 °C.
2. DOPE (Avanti Polar Lipids). Store at −20 °C.
3. SS14 reducible cationic lipid [6].
4. Chloroform ($CHCl_3$).
5. Ultrapure water (dH_2O) with resistivity values greater than 5 MΩ-cm at 25 °C.
6. Plasmid DNA (pDNA) encoding for Enhanced Green Fluorescent Protein, pEGFP-N1 Control Vector (Clontech Laboratories, Mountain View, CA).
7. pDNA encoding for Gaussia Luciferase, pCMV-GLuc Control pDNA (New England Biolabs, Ipswich, MA).
8. SYBR® Green I (Sigma-Aldrich, St. Louis, MO).
9. LiposoFast™ apparatus equipped with two 1.0 mL gas-tight syringes, glass syringe, and polycarbonate membranes with pore size of 100 nm (Avestin, Ottawa, Canada; *see* **Note 1**).
10. DLS and LDM apparatus: Malvern Zetasizer Nano ZS apparatus (Malvern Instruments Ltd, Worcestershire, UK).

11. Disposable capillary cells for ζ-potential measurements (Malvern Instruments Ltd).
12. 1.5 mL and 15 mL polypropylene microcentrifuge tubes.
13. Polystyrene multiwell plates.
14. Absorbance and fluorescence microplate reader.
15. Rotary evaporator.
16. Vortex mixer.

2.2 Cell Culture

1. MG63, human osteosarcoma cell line (European Collection of Cell Cultures, ECACC, Salisbury, UK).
2. 4.5 g/L high-glucose Dulbecco's Modified Eagle Medium (DMEM), stored at 4 °C.
3. Fetal bovine serum (FBS), aliquoted under sterile conditions and stored at –20 °C.
4. 100× penicillin–streptomycin sterile solution: 10,000 U/mL penicillin and 10 mg/mL streptomycin, aliquoted under sterile conditions and stored at –20 °C.
5. 200 mM sterile L-glutamine, aliquoted under sterile conditions and stored at –20 °C.
6. 1 M sterile HEPES buffer, pH 7.0–7.6, stored at 4 °C.
7. 100 mM sterile sodium pyruvate in dH_2O, stored at 4 °C.
8. Complete cell-culture medium: high-glucose DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES buffer. Prepare complete cell-culture medium under sterile conditions and store at 4 °C. Prewarm it to 37 °C prior to use.
9. Dulbecco's Phosphate Buffered Saline (PBS), sterile solution. Store at 4 °C and prewarm it to 37 °C prior to use.
10. Trypsin-ethylenediamminetetracetic acid (EDTA) sterile solution: 0.5 mg/mL of porcine trypsin, 0.2 g/L of EDTA, aliquoted under sterile conditions and stored at –20 °C.
11. 1.5 mL and 15 mL polypropylene microcentrifuge tubes.
12. Polystyrene multiwell plates.
13. Benchtop centrifuge and microcentrifuge.
14. Cell-culture incubator.

2.3 Transfection Experiments and Post-Transfection Assays

1. Transfection medium: high-glucose DMEM supplemented with 10% FBS, 2 mM of L-glutamine, 10 mM HEPES, and 1 mM of sodium pyruvate (*see* **Note 2**). Prepare transfection medium under sterile conditions and store at 4 °C. Prewarm it to 37 °C prior to use.

2. 10× AlamarBlue® solution (Thermo Fisher Scientific, Waltham, MA).
3. Fixation buffer: 4% (w/v) paraformaldehyde (PFA) in PBS. Store at −20 °C.
4. 1.5 mL polypropylene microcentrifuge tubes.
5. Polystyrene multiwell plates.
6. Flow cytometer (FCM).

2.4 Glutathione Depletion/Repletion Studies

1. 2′,7′-dichloro-dihydrofluorescein-diacetate (DCFH-DA, Thermo Fisher Scientific).
2. Glutathione Assay Kit (Sigma-Aldrich).

2.4.1 GSH Quantification

3. 5% (w/v) 5-sulfosalicylic acid in dH_2O (SSA, Sigma-Aldrich).
4. Working mixture (without GSH Reductase): 40 μg/mL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 1× Assay Buffer (Glutathione Assay Kit, 100 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA).
5. GSH Reductase-NADPH solution: 0.5 U/mL of GSH Reductase, 0.16 mg/mL of β-nicotinamide adenine dinucleotide 2′-phosphate (NADPH) in 1× Assay Buffer.
6. BCA Protein Assay Kit (Thermo Fisher Scientific).
7. 1.5 mL and 15 mL polypropylene microcentrifuge tubes.
8. Polystyrene multiwell plates.
9. Absorbance and fluorescence microplate reader.

3 Methods

3.1 Preparation of Tri-Component Cationic Liposomes

1. Prepare stock solutions of cationic lipids by dissolving in $CHCl_3$ DOPC, DOPE, and SS14 in three separate glass vials to a final concentration of 20 mM (*see* **Note 3**).
2. Prepare the lipid ternary mixture at a DOPC/DOPE/SS14 molar ratio of 16.7:33.3:50 by adding the correct amounts of lipid solutions in a round-bottomed flask using a glass syringe (*see* **Note 4**) and mix well. For example, for 1 mL, add 167 μL of 20 mM DOPC, 333 μL of DOPE and 500 μL of SS14.
3. Use a rotary evaporator at 40 °C to remove $CHCl_3$ up to the formation of a dry lipid film on inner surface of the flask.
4. Dry under vacuum overnight to completely remove the organic solvent.
5. Add dH_2O to a final total lipid concentration of 20 mM (*see* **Note 5**).
6. Hydrate the lipid film by vortexing thoroughly until a clear solution, containing large multilamellar vesicles, is obtained (*see* **Note 6**).

7. Freeze/thaw eight times (or at least five times) and bring to room temperature (RT).
8. Transfer the lipid dispersion (maximum 1 mL) in one of the gas-tight syringes (loading syringe).
9. Mount two 100 nm-pore polycarbonate membranes (*see* **Note 7**) and both the loading syringe and the empty receiving syringe onto the LiposoFast™ apparatus.
10. Extrude 27 times (*see* **Note 8**) the lipid dispersion.
11. Harvest the obtained liposome dispersion in a sterile plastic vial and store at 4 °C (s*ee* **Note 9**).

3.2 Characterization of Liposomes

3.2.1 Dynamic Light Scattering (DLS)

1. Dilute 50 μL of liposome suspension 1:20 in dH_2O (*see* **Note 10**) inside a disposable 12 mm square polystyrene cuvette.
2. Measure particle size using a DLS apparatus checking that the polydispersity index (PDI) of the Cumulant analysis is below 0.2 (*see* **Note 11**).

3.2.2 Laser Doppler Microelectrophoresis (LDM)

1. Transfer 750 μL of diluted liposome suspension, the same used for DLS measurement, into a disposable capillary cell for ζ-potential measurements.
2. Measure ζ-potential using a LDM apparatus and verify that the obtained value is positive (*see* **Note 12**).

3.3 Preparation and Characterization of Lipoplexes

3.3.1 Lipoplex Preparation

1. Dilute pDNA (pEGFP-N1) in dH_2O (*see* **Note 10**) to a final concentration of 0.04 μg/μL, corresponding to a phosphate (PO_4) concentration of 121.2 μM (DNA solution) (*see* **Note 13**).
2. Dilute liposome suspension in dH_2O so that the final concentration of net positive charges is X times higher than PO_4 concentration in the DNA solution (*see* **Note 14**), where X is the desired charge ratio (CR, +/−).
3. Mix equal volumes of DNA solution with liposome suspension and incubate at RT for 30 min (lipoplex suspension, *see* **Note 15**).

3.3.2 Evaluation of DNA Condensation

1. Prepare 20 μL of lipoplex suspension at different CRs (1–10) as described in Subheading 3.3.1 and 20 μL of pDNA at 0.02 μg/μL in dH_2O (CR 0).
2. Add 100 μL of dH_2O (*see* **Note 10**), containing 2× SYBR® Green I and incubate for 10 min at RT (*see* **Note 16**).
3. Add 100 μL of dH_2O containing 2× SYBR® Green I to 20 μL of dH_2O to prepare a blank sample.
4. Place 35 μL aliquots of the resulting solutions in triplicate in the wells of a black polystyrene 384 well plate (*see* **Note 17**).
5. Read fluorescence by using a fluorescence microplate reader with an excitation wavelength $\lambda_{ex} = 497$ nm and an emission wavelength $\lambda_{em} = 520$ nm.

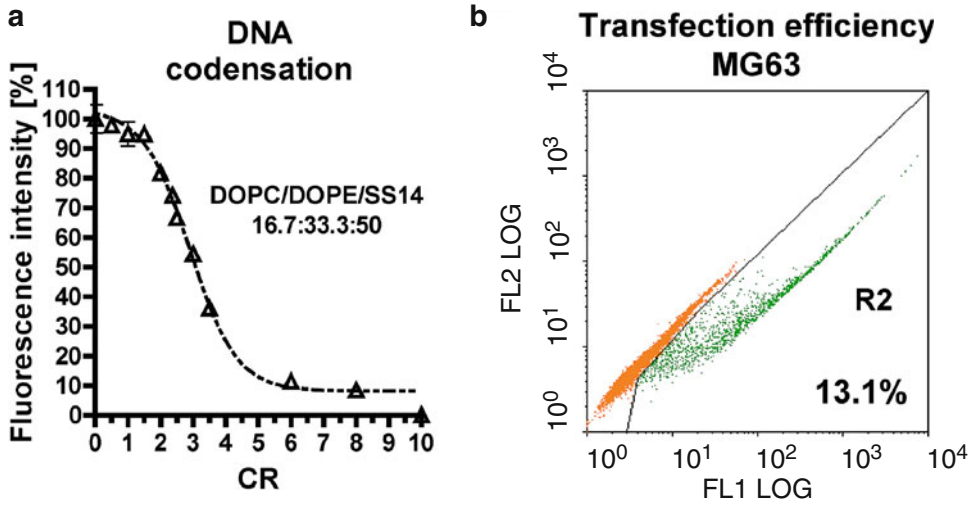

Fig. 1 (**a**) Evaluation of DNA condensation by 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 liposomes as a function of CR by fluorophore (SYBR® Green I)-exclusion assay. Results are presented as fluorescence % with respect to uncomplexed DNA; adapted from [3]. (**b**) Example of cytofluorimetric analysis; a FL1 (*green fluorescence*) vs. FL2 (*orange fluorescence*) dot plot of MG63 transfected cells is reported. EGFP-expressing cells appear as a population delineated by region 2 (R2) (where FL1 > FL2) identified by the analysis of mock-transfected (pCMV-GLuc) cells

6. Identify the minimal fluorescence plateau, corresponding to maximum pDNA condensation, by plotting samples average fluorescence signal (subtracted of blank average signal) as a function of CR (*see* **Note 18**). An example of a typical condensation curve is reported in Fig. 1a for DOPC/DOPE/SS14 (16.7:33.3:50) liposomes.

3.3.3 Lipoplex Characterization

1. Prepare 100 μL of lipoplex suspension at the desired CR as described in Subheading 3.3.1 and dilute them 1:10 in dH_2O.
2. Measure particle size and ζ-potential of the obtained diluted lipoplex suspensions as described in Subheadings 3.2.1 and 3.2.2 (*see* **Note 19**).

3.4 Transfection Experiments

3.4.1 Cell Transfection

1. Seed MG63 cells at a density of 1.0×10^4 cells/cm^2 (*see* **Note 20**) in a 12-well cell-culture plate containing 800 μL/well of complete cell-culture medium (*see* **Note 21**).
2. 24 h after seeding, remove old medium, rinse cells with 800 μL of PBS (*see* **Note 22**) and add 800 μL/well of transfection medium.
3. Prepare 72 μL of lipoplex suspension at different CRs as described above, using both pEGFP-N1 and pCMV-GLuc.
4. Add 16 μL/well of lipoplex suspension at different CRs (pDNA dose: 0.32 μg/well). It is recommended to prepare at least 4 replicates per CR.

5. Place cells in a cell-culture incubator (37 °C, 5 % CO_2, humidified atmosphere).
6. Facultative: after 4 h of incubation, remove lipoplex-containing medium and add complete cell-culture medium (*see* **Note 23**).

3.4.2 Cytotoxicity Assay

1. 48 h after transfection (*see* **Note 24**), remove medium and add 800 µL/well of 1× AlamarBlue® (diluted in complete cell-culture medium, *see* **Note 25**).
2. After 2 h (*see* **Note 26**), for each sample, transfer 100 µL of AlamarBlue®-containing medium into a black polystyrene 96 well plate (*see* **Note 17**) and read fluorescence with $\lambda_{ex} = 540$ nm and $\lambda_{em} = 585$ nm using a fluorescence microplate reader.
3. Subtract the average fluorescence of blank samples (100 µL of 1× AlamarBlue® incubated in empty wells) from the fluorescence signal of the samples.

Average the net fluorescence signal of each quadruplicate and calculate cell viability as:

$$\text{Viability}\,[\%] = \frac{\text{Sample fluorescence}}{\text{CTRL fluorescence}} \times 100$$

and cytotoxicity as:

$$\text{Cytotoxicity}\,[\%] = 100\% - \text{Viability}\,[\%]$$

where CTRL fluorescence is the net fluorescence signal of positive controls (non-transfected cells).

3.4.3 Preparation of Samples for Cytofluorimetric Analysis

1. After AlamarBlue® assay (*see* Subheading 3.4.2), rinse cells with 500 µL of PBS.
2. Add 100 µL/well of trypsin-EDTA solution.
3. Incubate at 37 °C for 3 min (*see* **Note 27**).
4. Once cells are detached, add 250 µL of complete cell-culture medium to block trypsin activity.
5. Mix by pipetting and then transfer 350 µL of cell suspension into a clean 1.5 mL polypropylene microcentrifuge tube.
6. Rinse the well with 350 µL of complete cell-culture medium and transfer them in the same tube of **step 5** to collect the remaining cells.
7. Centrifuge the tube containing cell suspension at 1000 × *g* at 4 °C for 5 min.
8. Remove 650 µL of supernatant and add 450 µL of PBS.
9. Centrifuge the tube at 1000 × *g* at 4 °C for 5 min.
10. Discard the supernatant.
11. Resuspend cell pellet in 300 µL of fixation buffer.

12. Store at 4 °C until cytofluorimetric analyses are performed.

3.4.4 Evaluation of Transfection Efficiency by Cytofluorimetry

1. Transfer cells from Subheading 3.4.3, **step 12** in a round bottom 12 × 75 mm tube (compatible with the available FCM instrument).
2. Load the sample on the FCM.
3. Analyze at least 1.0×10^4 events exciting cells at $\lambda_{ex} = 488$ nm and measure fluorescence at $\lambda_{em} = 520$ nm (green fluorescence) and $\lambda_{em} = 575$ nm (orange fluorescence) to enable correction for autofluorescence by diagonal gating as described below and as shown in Fig. 1b.
4. Using a cytometry software, create a dot plot and graph green fluorescence (FL1) on the X axis and orange fluorescence (FL2) on the Y axis. Using mock-transfected cells (cells transfected with an empty plasmid or a plasmid encoding a nonfluorescent protein such as Luciferase, herein pCMV-GLuc, *see* **Note 28**), identify and define the region of positive (green fluorescent) cells, on the right of the population of mock-treated cells which should lie nearby the diagonal of the quadrant (the percentage of cells present in the defined positive region should be lower than 1 % in all mock-treated samples).
5. Use the identified region to calculate the percentage of positive (green fluorescent) cells of all the samples.

3.4.5 Choice of the Best Transfection Conditions

1. Identify the best CR for transfection experiments taking into account both cytotoxicity and transfection efficiency results.
2. Among low cytotoxic conditions, a good compromise between high transfection efficiency and low cytotoxicity should be chosen. For 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 liposomes, CR 5 was chosen.

3.5 Lipoplex Disassembly in a Reducing Environment

1. Prepare 16 μL of lipoplexes at the identified working CR as described in Subheading 3.3.1 and incubate at RT for 30 min.
2. Add 304 μL (1:19 dilution) of aqueous solution of 10 mM GSH or 10 mM GSSG containing 2× SYBR® Green I.
3. Place 3 × 100 μL aliquots (*see* **Note 17**) of the resulting solutions in a black polystyrene 384 well plate and monitor the fluorescence ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 520$ nm) using a fluorescence microplate reader every 30 s for at least 2 h.
4. Plot the samples average fluorescence signal (subtracted of blank average signal) as a function of time. An increase in fluorescence signal of samples containing GSH, compared to GSSG, indicates that the reduction of lipoplexes components (i.e., SS14) by the reducing agent led to the release of nucleic acids (Fig. 2a, *see* **Note 29**).

3.6 Glutathione Depletion/Repletion Studies

GSH depletion/repletion studies are aimed at proving that the transfection process of redox-sensitive liposomes is strictly dependent on the intracellular reduction by the reducing environment, specifically by the high levels of reduced GSH. Results of GSH depletion/repletion studies for DOPC/DOPE/SS14 (16.7:33.3:50 molar ratio) lipoplexes prepared at CR 5 are reported in Fig. 2.

3.6.1 Transfection Experiments After Glutathione Depletion/Repletion

The complete experimental procedure for GSH depletion/repletion experiments is outlined in Fig. 2b.

1. Seed MG63 cells in T25 flasks (*see* **Note 30**) at a density of 1.0×10^4 cells/cm^2 in 5 mL of complete cell-culture medium (*see* **Note 31**).
2. Seed MG63 cells in 24-well plates at a density of 1.0×10^4 cells/cm^2 in 400 μL of complete cell-culture medium (*see* **Notes 30** and **31**).
3. After 8 h, supplement medium with BSO to a final concentration of 0.05 mM (*see* **Note 32**).
4. After 20 h, wash cells with PBS and add fresh complete cell-culture medium supplemented with either 0.05 mM BSO, 1 mM NAC, or 0.2 mM Vit-C (*see* **Note 32**) and incubate in a cell-culture incubator.
5. After further 20 h (this time point is defined as t_0 in Fig. 2), wash cells with PBS, add fresh transfection medium and transfect cells by adding 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 reducible lipoplexes prepared at CR 5 (100 μL/flask for T25 flasks and 8 μL/well for 24-well plates, *see* Subheading 3.4.1) and incubate in a cell-culture incubator for 48 h (t_{final}).

3.6.2 Cell Processing for Subsequent Assays

1. At t_0 and t_{final}, trypsinize cells in T25 flasks (*see* Subheading 3.4.3) using 300 μL of trypsin-EDTA and add 1 mL of complete cell-culture medium to block trypsin activity.
2. Collect cells in 1.5 mL polypropylene microcentrifuge tubes (*see* **Note 33**).
3. Divide t_{final} samples (transfected cells) into two aliquots.
4. Centrifuge cells for 5 min at 4 °C at $1000 \times g$ and wash the obtained pellets with 500 μL of PBS.
5. Fix cells in half the aliquots of t_{final} samples in fixation buffer as described in Subheading 3.4.3 and store samples at 4 °C for the following cytofluorimetric analysis.
6. For t_0 samples and the remaining aliquots of t_{final}, resuspend the obtained cell pellets in 150 μL 5% SSA.
7. Freeze-thaw twice using liquid nitrogen and incubate for 5 min at 4 °C (*see* **Note 34**).

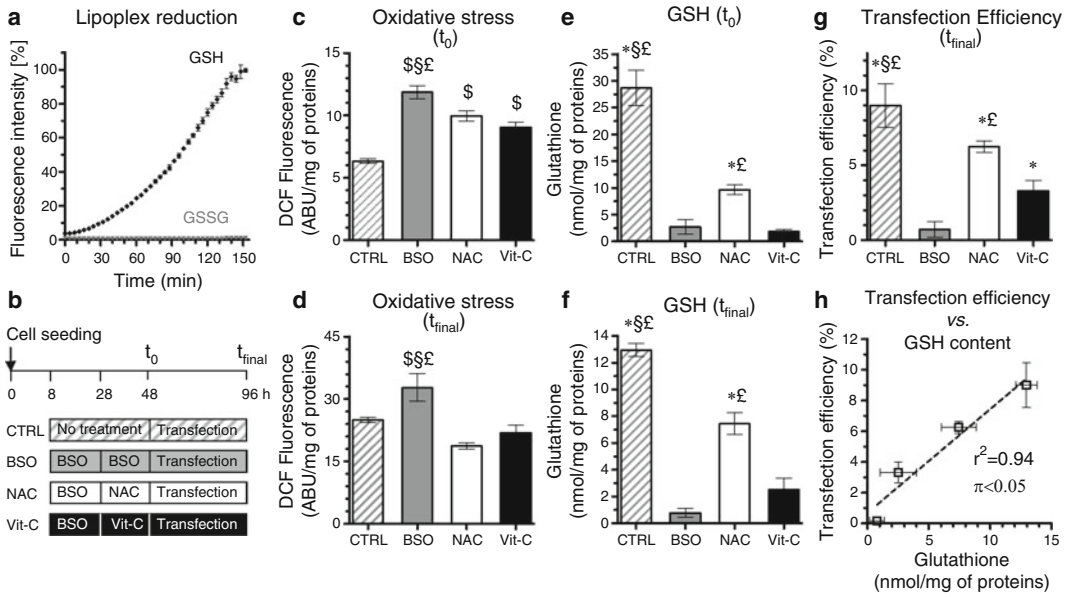

Fig. 2 (**a**) Disassembly of 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 lipoplexes at CR 5 in the presence of GSH or GSSG. Results are presented as fluorescence % with respect to uncomplexed DNA. (**b**) Outline of the experimental procedure of glutathione depletion/repletion studies. Four groups were analyzed: untreated CTRL, BSO-, NAC-, and Vit-C-treated cells. Following pharmacological treatment (t_0), cells were transfected for 48 h (t_{final}) with 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 lipoplexes at CR 5. Oxidative stress and GSH content were measured at t_0 ((**c**) and (**d**), respectively) and after transfection ((**e**) and (**f**), respectively). (**g**) Transfection efficiency, expressed as % of positive (*green fluorescent*) cells. (**h**) A linear correlation between GSH content and transfection efficiency was observed. Results are expressed as mean ± SEM ($n=3$). \$ $p<0.05$ vs. CTRL; *$p<0.05$ vs. BSO; § $p<0.05$ vs. NAC; £ $p<0.05$ vs. Vit-C. From [3]

8. Centrifuge the extracts for 10 min at 4 °C at 10,000 × *g* to pellet precipitated proteins.
9. Keeping samples on ice, collect the supernatants in new polypropylene microcentrifuge tubes and measure their volume (*see* **Note 35**). Supernatants can now be stored at −80 °C.
10. Add to the protein pellets a volume of 25 mM NaOH equal to the corresponding measured volume of supernatant and resuspend them (*see* **Note 36**).
11. Evaluate transfection efficiency of the PFA-fixed samples by cytofluorimetry as described in Subheading 3.4.4.

3.6.3 Evaluation of Oxidative Stress

1. At t_0 and t_{final} wash cells cultured in 24-well plates with 500 µL of PBS and incubate with 10 µM DCFH-DA in PBS (500 µL/well) for 15 min at 37 °C (*see* **Note 37**).
2. Wash cells twice with 500 µL of PBS.
3. Lyse cells by adding 300 µL of 0.5 % (v/v) Tween 20 in 50 mM Tris–HCl, pH 7.5, and incubate for 15 min on ice.

4. Keeping the samples on ice, detach cells from the well surface with the help of a cell scraper and collect lysate in 1.5 mL polypropylene microcentrifuge tubes.
5. Centrifuge samples for 5 min at 4 °C at 1000 × *g* to pellet cell debris.
6. Place 200 μL of the resulting supernatants in a black 96-well plate and measure the fluorescence at $\lambda_{em} = 530$ nm and exciting at $\lambda_{ex} = 485$ nm using a fluorescence microplate reader (*see* **Note 38**).
7. Measure protein content of the supernatants by BCA assay, according to manufacturer's instructions.
8. Normalize fluorescence results over the total protein content of each cell lysate sample.

The obtained normalized sample fluorescence is an index of cellular oxidative stress (*see* **Note 37**) [8]. Oxidative stress levels at t_0 and t_{final} for MG63 cells treated with BSO, NAC, and Vit-C and transfected with DOPC/DOPE/SS14 (16.7:33.3:50) reducible lipoplexes (*see* Subheading 3.6.1) are shown in Fig. 2c, d**,** respectively. Results show that, at t_0, oxidative stress levels of BSO-treated cells increased by almost two-fold with respect to untreated cells (CTRL) while the antioxidant treatment with NAC and Vit-C equally alleviated BSO effects. At t_{final}, 48 h post-transfection, oxidative stress levels in NAC- and Vit-C-treated groups were equal to CTRL while those of BSO-treated cells were still higher ($p < 0.05$).

3.6.4 GSH Quantification

1. Add in duplicate 10 μL of known concentrations of GSH in 5% SSA (GSH standards, 3.125–100 μM) and of unknown samples (supernatants collected in Subheading 3.6.2**, step 9**) in a transparent 96-well plate (*see* **Note 39**).
2. Add in duplicate 10 μL of 5% SSA as reagent blanks.
3. Add 150 μL of working solution (without GSH Reductase) to each well and incubate for 15 min at RT. Measure absorbance at $\lambda = 412$ nm (OD_{412}), using an absorbance microplate reader (*see* **Note 40**).
4. Add 50 μL of GSH Reductase-NADPH solution to each well with a multichannel pipette and mix by pipetting.
5. Measure OD_{412} for 10 min at 1 min intervals.

Calculation of Reduced GSH Content

1. Subtract the average OD_{412} of the reagent blank replicates from the OD_{412} of all standards and unknown samples recorded at Subheading 3.6.4, **step 3**.
2. Plot the average blank-corrected OD_{412} for each GSH standard against its concentration and fit a linear standard curve.
3. Calculate the reduced GSH concentration of each unknown sample using the standard curve.

4. Measure protein content of the samples obtained at Subheading 3.6.2, **step 10** by BCA assay, according to manufacturer's instructions (*see* **Note 41**).
5. Normalize the measured reduced GSH concentrations of each sample with the corresponding protein concentrations; reduced GSH content will be expressed as mmol of GSH/mg of proteins.

Calculation of Total Glutathione (GSH and GSSG)

1. Subtract the average OD_{412} of the reagent blank replicates from the OD_{412} of all the measurements recorded at Subheading 3.6.4, **step 5**.
2. For each standard and sample, calculate the ΔOD_{412}/min (*see* **Note 42**) by fitting a linear trend line (OD_{412} vs. time). ΔOD_{412}/min is represented by the slope (angular coefficient) of the fitted linear trendline.
3. Plot the average ΔOD_{412}/min for each GSH standard against its concentration and fit a standard curve.
4. Calculate the total glutathione concentration of each unknown sample using the standard curve.
5. Normalize the measured total glutathione concentrations of each sample with the corresponding protein concentrations (*see* Subheading 3.6.4.1, **step 4**); total glutathione content will be expressed as mmol of (GSH+GSSG)/mg of proteins (*see* **Note 43**).

Reduced GSH levels at t_0 and t_{final} for MG63 cells treated with BSO, NAC, and Vit-C and transfected with DOPC/DOPE/SS14 (16.7:33.3:50) reducible lipoplexes (*see* Subheading 3.6.1) are shown in Fig. 2e, f, respectively. As expected, results show that only incubation of GSH-depleted cells with NAC significantly restored GSH levels at t_{final} (57% repletion in GSH content compared to BSO-untreated CTRL). Taking into account the transfection results in the four groups (Fig. 2g), a linear correlation between GSH content and transfection efficiency ($r^2 = 0.94$) could be observed (Fig. 2h). Inversely, oxidative stress levels and transfection efficiency did not correlate at all ($r^2 = 0.35$), demonstrating the pivotal role of intracellular GSH levels in the transfection process of 16.7:33.3:50 (molar ratio) DOPC/DOPE/SS14 reducible lipoplexes.

4 Notes

1. LiposoFast™ is a manually powered extruder designed for researchers who use only small amounts of liposomes. Vesicles (lipid emulsions) prepared with LiposoFast™ are repeatedly extruded through a porous polycarbonate membrane forced back and forth by specially modified gas-tight syringes [9]. The apparatus can be autoclaved in order to produce sterile liposomes.

2. Transfection experiments in antibiotic-free medium are recommended since the increase in cell membrane permeability by cationic liposomes during transfection could lead to higher antibiotic uptake and consequently increase the cytotoxicity.
3. The use of glass or stainless steel in the presence of organic solutions is recommended; the use of vials made of polymeric materials should be avoided as impurities could leach out of the container.
4. The preparation of 16.7:33.3:50 (molar ratio) formulation is here reported as an example, but desired molar ratios can be easily obtained by changing the volume ratio of the starting lipid solutions.
5. The final physicochemical and transfection properties of the obtained liposomes can be strongly influenced by the aqueous solution where they are prepared; other buffers such as PBS and 10 mM Hepes buffer, pH 7, can be used. The use of buffers containing EDTA is not recommended as it could cause liposome aggregation.
6. If vortexing is ineffective, bath sonicate the lipid dispersion for 2–5 min, until clarity is obtained.
7. The use of two stacked polycarbonate membranes helps yielding monodisperse, nanometric-sized liposomes. Liposomes of different dimensions can be obtained by simply using polycarbonate membranes with different pore sizes (e.g., 50, 100, 200, 400 nm; available from Avestin).
8. An odd number of passages is recommended (at least 21) to finally have extruded liposomes in the, initially empty, receiving syringe, thus avoiding contamination with unextruded vesicles which might remain inside the loading syringe.
9. Usually liposomes are stable up to 1 year at 4 °C. However it is recommended to periodically verify liposome stability by measuring their mean size (hydrodynamic diameter) and ζ-potential (*see* Subheading 3.2).
10. For both characterization experiments and lipoplex formation, the dilution of liposome formulations in the same aqueous solution where they were prepared, is recommended.
11. PDI is a dimensionless parameter that evaluates the width of the particle size distribution. A high PDI indicates a large variability in the particle size. If PDI is higher than 0.2 or mean size is much higher than membrane pore size, it may be necessary to extrude liposomes again. In these cases, lipid concentration during extrusion should be reduced.
12. A positive ζ-potential is expected for cationic liposomes in dH_2O.

13. DNA phosphate density (and then negative charge density) is 3.03 nmol of PO_4/μg of DNA.
14. SS14 is assumed to carry 4 positive charges per molecule while DOPC and DOPE are neutral in dH_2O at pH 7.
15. In these works, lipoplexes were always prepared at RT.
16. SYBR® Green I is the DNA stain of choice for these experiments because it has been shown to be much less mutagenic and much more sensitive than ethidium bromide [10].
17. It is recommended to prepare a triplicate of each sample to take into account experimental and instrumental variability. The use of black polystyrene microplates is also suggested for fluorescence analysis as they minimize light scattering and well-to-well crosstalk and have low background fluorescence.
18. Maximum DNA condensation is often necessary to obtain efficient lipoplexes; if a plateau is not observed, test higher CRs.
19. Size (hydrodynamic diameter) and ζ-potential measurements of polyplexes are recommended: to assure DNA complexation and lipoplex interaction with negatively charged cell surfaces, positively charged lipoplexes are necessary (when using cationic liposomes as transfection reagents).
20. Transfection efficiency and cytotoxicity of lipoplexes are strongly cell-dependent. If high cytotoxicity or poor transfection results are observed, optimal cell seeding density and/or lipoplex dose should be identified [11].
21. If using other cell lines or primary cells, the appropriate culture medium should be chosen, according to existing literature. Presence of serum in the culture medium may affect the transfection efficiency of nonviral gene delivery vectors, therefore sometimes it could be preferable to carry out transfection experiments in serum-free transfection medium.
22. Washing step can be avoided to reduce cell detachment if culture medium is the same before and during transfection.
23. Medium change after 4 h can be carried out to reduce cytotoxicity and is often necessary in case of transfection in serum-free medium.
24. GFP expression usually peaks at 24–48 h post-transfection, even though longer incubation times could be necessary for some liposomal formulations.
25. AlamarBlue® is a nontoxic, nondestructive cell growth indicator. The use of AlamarBlue® as cell viability assay allows to test the same samples in the following cytofluorimetric analysis.
26. Increase/decrease incubation time if too low/too high (saturated) signal is observed.

27. To facilitate cell detachment gently tap the plate, then check by optical microscope. Put the plate again at 37 °C for 1–2 more min, if necessary.
28. Mock transfected cells are a negative control used to determine any nonspecific effects that may be caused by the transfection reagent or processes such as background fluorescence and autofluorescence of transfected cells.
29. In some cases the simple presence of a reducing agent such as GSH is not sufficient to lead to lipoplex disassembly and nucleic acid release. In these cases, in order to demonstrate the reducibility of the complexes, it is necessary to add a counter ion such as heparin in the reducing solution. If DNA is released in the presence of heparin and GSH but not of heparin and GSSG, nucleic acid release from lipoplexes in reducing environment can be considered effective. A DNA/heparin w/w ratio of 1 is often appropriate for this purpose, but it should be optimized specifically for each transfectant.
30. 6-well culture plates can also be used; smaller size wells are not recommended since a high number of cells is necessary for the following assays. Cells are also seeded in 24-well plates to allow the evaluation of the oxidative stress levels by DCFH-DA assay.
31. Prepare enough samples considering the number of treatments investigated and the fact that cells will be analyzed at two different time points, t_0 (immediately before transfection) and t_{final} (48 h after transfection).
32. BSO is a glutathione depletor, NAC is a glutathione repletor and Vit-C is an antioxidant. The concentrations of BSO, NAC, and Vit-C to be used for these experiments are cell-dependent. It is recommended to optimize concentrations for each cell type used in order to obtain adequate levels of GSH depletion/repletion together with low cytotoxicity.
33. Approximately $0.5–1.0 \times 10^6$ and $2.0–3.0 \times 10^6$ cells should be obtained from a T25 flask at t_0 and t_{final}, respectively.
34. A sonication step could be added to facilitate cell rupture but usually the freeze-thaw step in 5 % SSA is enough to efficiently lyse cells and release GSH.
35. The volume should be around 140–145 μL.
36. The addition of 25 mM NaOH is necessary to guarantee complete protein resuspension. Samples can be now stored at –80 °C.
37. The nonfluorescent fluorescein derivative DCFH-DA is relatively resistant to oxidation, but upon cellular uptake, it is deacetylated to form DCFH whose oxidization by intracellular oxidants lead to the formation of highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) [8].

38. Supernatants can be now stored at −80 °C directly in the 96-well plate.
39. Do not exceed 10 μL volume.
40. Measuring the absorbance at 412 nm before the addition of GSH Reductase and NADPH allows to quantify the intracellular levels of reduced GSH and not of the redox couple GSH-GSSG.
41. 25 mM NaOH is compatible with BCA assay. In case of using different assays for protein quantification, check the compatibility.
42. Since the signal of samples with high concentrations of glutathione could saturate within the 10 min of reading, it is important to exclude saturated values when fitting the linear curve.
43. Both reduced and total glutathione should be measured. If it is not possible to adequately quantify reduced GSH owing to low signal, total glutathione can be taken into account. The technique for the quantification of total glutathione is in fact more sensitive since it exploits a kinetic assay in which GSH causes a continuous reduction of DTNB and the GSSG formed in the process is recycled by glutathione reductase, thus strongly increasing the absorbance signal.

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