**Cationic lipids and polymers for gene delivery**

Gabriele Candiani¹, Daniele Pezzoli¹, Alessandro Volonterio¹, Roberto Chiesa¹, Monica Sani²

1) Dipartimento di Chimica, Materiali e Ingegneria Chimica “Giulio Natta”, Politecnico di Milano
2) Istituto di Chimica del Riconoscimento Molecolare (ICRM) – CNR, Milano

**Abstract** — Gene therapy, aiming to eradicate causes rather than symptoms of diseases, is believed to be the therapy of the future. A new promising area for the application of gene therapy is the emerging field of regenerative medicine, where gene delivery can be used to enhance or modify cell functions in an organ/tissue or in engineered substitutes. The aim of gene therapy is to achieve the expression of transgenes in target cells, for as long as required, in an appropriately regulated form, without side effects. The main strategies for the delivery of nucleic acids in gene therapy involve viral-mediated and non-viral methods. Non-viral synthetic vectors feature a high safety profile but they are still less efficient than their viral counterparts in mediating gene expression. To overcome this limitation, the incorporation of disulfide bonds has been proposed as an efficient stimuli mechanism in gene delivery to exploit the high difference in redox potential existing between the reducing intracellular space and the oxidizing extracellular milieu. Inspired by this disulfide-linker strategy, we describe herein the development of different reductible cationic and polymeric non-viral gene delivery systems: a reducible cationic lipid, SS14, a series of reducible SS14-based liposomes and a family of reducible dithio-oligopeptides.

I. INTRODUCTION

Introduction of an exogenous gene into eukaryotic cells, followed by its expression by the cellular machinery, is a well-established strategy to produce proteins. When exploited therapeutically to heal certain pathological conditions, this process is named gene therapy. Gene therapy has become one of the most intensively developing fields for current clinical research because it offers new treatment possibilities for many common inherited and acquired human diseases. The generic aim of gene therapy is to achieve the expression of transgenes in the target cells for as long as required, in an appropriately regulated form, without side effects.

Given that both the nucleic acids and biological cell surfaces are negatively charged, spontaneous entry of naked DNA inside cells is unlikely to be an efficient process (Giordano et al., 2006; Pezzoli et al., 2007). Therefore, a major hurdle to the continued development and therapeutic application of nucleic acids is the lack of suitable vectors for their delivery. The principal strategies for gene delivery involve viral-mediated and non-viral methods. Non-viral gene therapy is based on the use of plasmid expression vectors and chemical or physical DNA delivery systems. The benefits of using synthetic vectors are their ability to complex and deliver nucleic acids of virtually any size, their high safety profile and ease of preparation. However, the levels of gene expression mediated by non-viral vectors, commonly named as transfectants, are typically lower compared to their viral counterparts. Non-viral vectors are mainly of two kind: cationic lipids and cationic polymers. Felgner et al. (1987) and Wu et al. (1988) were the first to introduce cationic lipids and cationic polymers respectively as novel gene delivery systems. Since then, a number of polymer- and lipid-based non-viral vectors have been developed and several of them are currently under investigation as potential tools for gene therapy.

In order to overcome the limitations of currently available non-viral vectors, the use of stimuli-responsive carriers can offer novel alternatives for the optimization of gene delivery. Redox potential has been proposed as an efficient stimuli mechanism in gene delivery because of the high difference ($10^2-10^3$ fold) existing between the reducing intracellular space and the oxidizing extracellular milieu. The rapid disulfide linkages reduction by the high intracellular levels of glutathione (GSH), the main intracellular reducing agent, induces fast reducible lipo/polyplex dissociation and efficient DNA release, yielding increased gene expression, as described by the disulfide linker strategy represented in Fig. 1 (Tang and Huges, 1998; Candiani et al., 2010).

![Fig. 1. Schematic representation of the disulfide linker strategy in gene delivery experiments.](image-url)

In the light of these findings, we will herein describe the development of a new reducible cationic gemini-like surfactant, SS14, its formulation with helper lipids to obtain reducible liposomes and their application to gene delivery. Moreover we will discuss the synthesis and the characterization of a family of reducible dithio-oligopeptides, based on a central lysine (K, Lys) chain and two cysteine (C, Cys) residues (CK₄C), as novel gene delivery system.
II. MATERIALS AND METHODS

A reducible cationic, triazine-based, *gemini*-like surfactant, SS14, was previously synthesized by our group (Candiani et al., 2007; Candiani et al., 2008) and its structure is reported in Fig. 2.

Fig. 2. Chemical structure of the reducible cationic, triazine-based, *gemini*-like surfactant, SS14.

SS14-containing liposomes were prepared by repeated extrusions through polycarbonate filters of 100 nm pore diameter, obtaining monolamellar aggregates of low polydispersity in size. Three formulations with different combinations of DOPC, DOPE and SS14 were chosen: DOPC/DOPE/SS14 29.2:58.3:12.5, 50:25:25 and 16.7:33.3:50 molar ratios.

Dithio-oligopeptides featuring a central Lys chain of 6, 10 and 14 residues and two side Cys residues (CK<sub>6</sub>C, CK<sub>10</sub>C and CK<sub>14</sub>C; Fig. 3) were assembled on a 433A Applied Biosystem peptide synthesizer, using the stepwise solid phase Fmoc method on 2-Chlorotrityl chloride resin on a 0.1 mmol scale.

Fig. 3. Structure of the dithio-oligopeptide family CK<sub>n</sub>C.

Size and surface charge of liposomes, lipoplexes and polyplexes were determined by Photon Correlation Spectroscopy (PCS) and Laser Doppler Velocimetry (LDV). Lipoplexes and polyplexes were prepared by adding an aqueous stock solution of plasmid DNA (pEGFP-N1 or pGL3) to a lipid, liposome or oligopeptide suspension at the desired concentration, yielding different Charge Ratios (CR, +/-). The DNA binding ability of the different vectors was assessed fluorimetrically by monitoring the displacement of SYBR-Green I from DNA. In parallel, lipoplex and polyplex disassembly in presence of a reducing agent (10 mM GSH vs GSSG) was evaluated by fluorimetry as well.

For transfections, U87-MG, Cos-7, MG63 and HeLa cells were seeded, 24 hours later medium was replaced by complete medium (DMEM additioned with 10% Fetal Bovin Serum, FBS) containing lipo/polyplexes. 48 h post-transfection cytotoxicity was evaluated by AlamarBlue® assay. EGFP expression was estimated by fluorescence-activated cell sorter (FACS, Becton Dickinson) and analyzed using WinMDI 2.9 software. Luciferase activity was measured using a luminometer and normalized to the total cell protein content. Statistical analysis was performed by ANOVA test. Significance was retained when p<0.05.

III. RESULTS AND DISCUSSION

A. SS14-based gene delivery systems.

SS14, in working conditions, condenses pDNA above its critical micelle concentration (CMC ~ 35 μM). We decided to prepare SS14-based liposomes to produce reducible liposomes exploiting the helper lipids ability to increase transfection efficiency. By monitoring the displacement of SYBR-Green I from DNA we noticed a negative trend of fluorescence in function of CR that plateaued at its lower limit beyond CR6 for SS14 alone and beyond CR5 for DOPC/DOPE/SS14 liposomes. Because of the high redox potential difference existing between the reducing intracellular space and oxidizing extracellular milieu (10<sup>2</sup>–10<sup>3</sup> fold), we demonstrated the ability of glutathione (GSH), mimicking intracellular reducing milieu, to reduce SS14-based and liposome-based lipoplexes and release DNA (Fig. 4).

![Lipoplex reduction](image)

Fig. 4. DNA release after lipoplex reduction by GSH in DOPC/DOPE/SS14 50:25:25 liposomes.

We tested cytotoxicity and transfection efficiency of SS14-based gene delivery systems on U87-MG, Cos-7, MG63 and HeLa cell lines in presence of serum. Lipoplexes formed at CR12 with SS14 alone showed the best compromise between high transfection efficiency and low cytotoxicity while CR5 was the optimum for liposome formulations in all cell lines tested. Chemico-physical characterization of lipoplexes in these conditions of complexation highlighted the formation of polydisperse particles for SS14 and DOPC/DOPE/SS14 29.2:58.3:12.5 (P.I.=0.6 and 0.32 respectively) and monodisperse particles for DOPC/DOPE/SS14 50:25:25 and 16.7:33.3:50 (P.I.<0.1) with the mean diameter always of ~300 nm.

The introduction of DOPC and DOPE in liposome formulations increased transfection efficiency up to 7-fold (p<0.05) reaching high levels of transfection efficiency, in
particular 33.4 ± 0.9% at CR5 in U87-MG cells (Fig. 5) for DOPC/DOPE/SS14 16.7:33.3:50 (Candiani et al., 2010). On the other hand, liposome formulations were more cytotoxic.

**Fig. 5.** FACS graph of transfection efficiency of DOPC-DOPE-SS14 16.7:33.3:50 liposomes at CR5 on U87-MG cells.

**B. CK\textsubscript{n}C-based gene delivery systems.**
Given the redox sensitive nature of the CK\textsubscript{n}C family of polymerizable dithio-oligopeptides, we demonstrated that oxidative polymerization was effective only in presence of DNA, as assessed by MALDI TOF Mass Spectroscopy. DNA complexation and oxidative polymerization resulted in a nanoscaled monodisperse polyplex population (PI<0.3) with a diameter that increased with CR and decreased with Lys chain length, while Z potential increased with both CR and Lys chain length (Fig. 6).

**Fig. 6.** Mean diameter and Z potential of CK\textsubscript{n}C polyplexes in function of CR (A) and of CK\textsubscript{n}C polyplexes in function of lysine chain length (B).

By monitoring the displacement of SYBR-Green I from DNA, a negative trend of fluorescence in function of CR was noticed, with a plateau reached beyond CR3 for each peptide. Since between the reducing intracellular space and the oxidizing extracellular environment a high redox potential difference exists, we demonstrated, as already described for SS14-based gene delivery systems, the ability of GSH to enable DNA release.

Transfection activity, tested in Cos-7 cell line in presence of 10% FBS, increased with CR reaching a plateau for CR\textless{}50. Finally, Lys chain length influenced polyplex effectiveness: CK\textsubscript{6}C was inefficient in gene transfection compared to CK\textsubscript{10}C and CK\textsubscript{14}C (p>0.05; Fig. 6).

**Fig. 7.** Transfection efficiency of CK\textsubscript{n}C oligo-peptides in function of CR in Cos-7 cells.

It is worth noting that transfection efficiency was not affected by the presence of serum and that cytotoxicity was very low in all conditions.

**CONCLUSIONS**
Concluding, we demonstrated that the developed reducible cationic surfactants, cationic liposomes and oligopeptides can reversibly condense DNA forming highly stable serum-resistant nanoscaled lipo/polyplexes able to enter inside the cells and efficiently release DNA with a reduction-driven lipoplex disassembly mechanism. These features render the developed reducible gene delivery systems very attractive reagents for future in vivo gene delivery applications. Moreover, the disulfide linker strategy adopted for the synthesis of these vectors could be a key approach in the application of gene delivery to regenerative medicine of liver and kidney, in which the intracellular levels of GSH are even higher than in other tissues.

**ACKNOWLEDGMENT**
We wish to thank Politecnico di Milano - Grant: 5 per Mille Junior (Development of smart surfaces for gene-eluting stents - SURGES) and MIUR - FIRB Futuro in ricerca (Surface-associated selective transfection - SAST, RBFR08XH0H) for economic support.
REFERENCES