Cellular delivery of CRISPR/Cas9 plasmid using multivalent cationic liposomes

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Background

Gene therapy techniques have been aimed at mitigating disease features of recessive and dominant disorders, as well as several cancers, cardiovascular, neuronal and immune diseases [1]. In recent years, the clustered regularly interspaced short palindromic repeats (CRISPR) and Cas9 protein system has been gaining recognition as a revolutionary tool for gene therapy. However, the implementation of CRISPR/Cas9 in the clinical setting remains difficult due to challenges in delivery high molecular weight DNA into cells. Various delivery strategies of nucleic acid-based therapeutics, including both viral and nonviral approaches have been tested for the delivery of CRISPR/Cas9 system. Although, the available delivery systems suffer from various limitations, such as immunogenicity and insertional mutagenesis for viral systems [2], and low transfection efficiency for nonviral systems [3]. Multivalent cationic lipids have been proposed as a promising nonviral vector strategy to effectively deliver nucleic acids into target cells. They are characterized by their higher membrane charge density, which has been suggested to facilitate endosomal escape, improving the transfection efficiency [4,5]. In this work, we explored the potential of multivalent cationic liposomes to deliver plasmid based CRISPR/Cas9 systems.

Methods

A nanocarrier was designed to deliver CRISPR/Cas9 plasmid into Human Embryonic Kidney (HEK) 293T cell line through the combination of a pentavalent cationic lipid (MVL5) with three different helper lipids, namely 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), monoleein (GMO) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). The multivalent cationic lipid-DNA complexes were prepared with cationic-to-anionic charge ratios (CR (+/-)) of 3 and 10 and characterized

regarding their size and charge by Dynamic Light Scattering (DLS). In order to study the *in vitro* suitability of these vectors to deliver CRISPR/Cas9 plasmid, a plasmid containing both Cas9/sgRNA and reporter green fluorescence protein (GFP) expression cassettes (PX458) was used to facilitate the detection of Cas9 expression by the GFP-expressing cells under flow cytometry and fluorescence microscopy. The cytotoxicity of MVL5-DNA complexes was also assessed using a standard colorimetric cell viability assay.

Results

The characterization of MVL5-based liposomes containing DNA plasmids of CRISPR/Cas9 system exhibited the formation of positively charged stable nanoparticles with sizes ranging from 85 to 120 nm (Figure 1a,b). Then, we proved that the plasmid PX458, which encodes Cas9 and sgRNA, can be successfully transfected into HEK 293T cells via MVL5-based lipoplexes in a concentration-dependent manner (Figure 1c). The transfection rates for the highest DNA concentration were superior to the most efficient commercial transfection reagent - Lipofectamine 3000. Regarding cell viability, multivalent CL-DNA complexes at CR(+/-) revealed to be safe enough for gene delivery, since no obvious cytotoxic impact was observed on HEK293T transfected cells, while at CR (+/-) 10 those formulations presented a moderate cytotoxicity (Figure 1d). Altogether, these results show that multivalent lipid-based lipoplexes are promising CRISPR/Cas9 plasmid delivery systems, and by further optimization and functionalization could achieve an effective and safe delivery system.

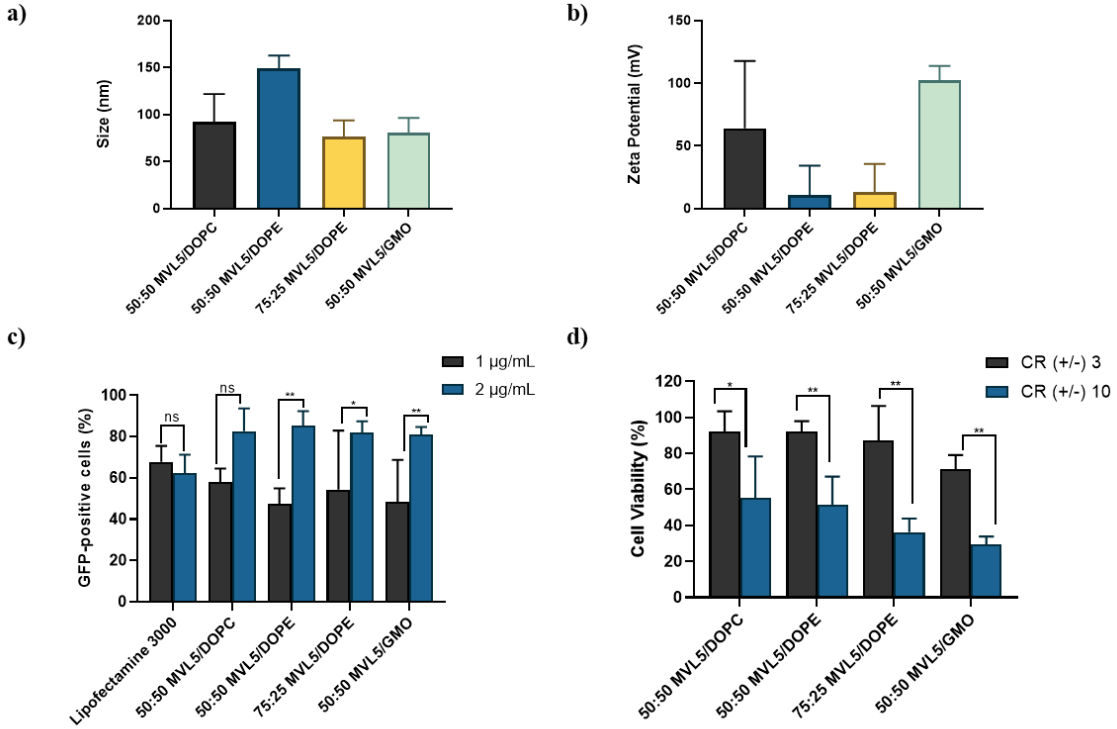

Figure 1. Characterization and *in vitro* studies of MVL5-based liposomes containing a CRISPR/Cas9 plasmid system a, b) DLS measurements of multivalent cationic liposomes incorporating PX458 plasmid at CR (+/-) 3, regarding to size and zeta potential, respectively c) *In vitro* transfection efficiency of MVL5-based lipoplexes at a CR (+/-) of 10 d) Cytotoxicity profile of MVL5 lipoplexes evaluated by the MTT assay.

Conclusion

Plasmids for the CRISPR/Cas9 system were encapsulated in liposomes comprised of MVL5 combined with a helper lipid (DOPC, DOPE or GMO) and were shown to induce expression of Cas9 in human cells *in vitro.* Although MVL5-based formulations also showed considerable cytotoxicity at the best performance formulations, their known composition opens significant opportunities for further optimization, either by tuning the cationic-to-anionic CR (+/-) to lower cytotoxicity, or by surface modifications to improve transfection efficiency *in vitro* and *in vivo* (e.g PEGylation, biomarkers ligands) even further, which is difficult to achieve for the available commercial transfection reagents.

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References

[1] Naldini, L. Gene Therapy Returns to Centre Stage. *Nature* **2015**, *526* (7573), 351–360. https://doi.org/10.1038/nature15818.

[2] Glass, Z.; Lee, M.; Li, Y.; Xu, Q. Engineering the Delivery System for CRISPR-Based Genome Editing. *Trends Biotechnol.* **2018**, *36* (2), 173–185. https://doi.org/10.1016/j.tibtech.2017.11.006.

[3] Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: Classification, Preparation, and Applications. *Nanoscale Res. Lett.* **2013**, *8* (1), 102. https://doi.org/10.1186/1556-276X-8-102.

[4] Bouxsein, N. F.; McAllister, C. S.; Ewert, K. K.; Samuel, C. E.; Safinya, C. R.

Structure and Gene Silencing Activities of Monovalent and Pentavalent Cationic Lipid Vectors Complexed with SiRNA. *Biochemistry* **2007**, *46* (16), 4785–4792. https://doi.org/10.1021/bi062138l.

[5] Lin, A. J.; Slack, N. L.; Ahmad, A.; George, C. X.; Samuel, C. E.; Safinya, C. R. Three-Dimensional Imaging of Lipid Gene-Carriers: Membrane Charge Density Controls Universal Transfection Behavior in Lamellar Cationic Liposome-DNA Complexes. *Biophys. J.* **2003**, *84* (5), 3307. https://doi.org/10.1016/S0006-3495(03)70055-1.