HIJACKING NATURE'S OWN COMMUNICATION SYSTEM: EVALUATION OF EXTRACELLULAR VESICLES AS A SIRNA DELIVERY VEHICLE.

Stephan Stremersch[1], Kevin Braeckmans[1,2], Roosmarijn E. Vandenbroucke[3], Stefaan De Smedt[1], Koen Raemdonck[1]

[1]Ghent Research Group on Nanomedicines, Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Ottergemsesteenweg 460, Ghent, Belgium; [2]Center for Nano-and Biophotonics, Ghent University, Ghent, Belgium; [3]Inflammation Research Center, VIB – Ghent University, Ghent, Belgium

E-mail: Stephan.Stremersch@UGent.be

Abstract

In order to exploit the therapeutic potential of small interfering RNA (siRNA), it is key to overcome the various intra- and extracellular barriers imposed by the human body. To this end, siRNA therapeutics are commonly packaged in an appropriate nanosized drug carrier.[1] Recently it was discovered that extracellular vesicles (EVs), i.e. lipid membrane-sealed nanosized particles, act as nature's own nucleic acid delivery system.[2] EVs are secreted by every cell type and have been shown to contain a variety of biological molecules, including miRNA, which can be transferred to other cells leading to phenotypic changes. For this reason, interest has surged towards evaluating these vesicles as a new personalized drug delivery platform for therapeutic nucleic acids, such as siRNA. Yet, to date a major impediment in using EVs as a carrier for siRNA in the clinic is the lack of a suitable procedure for efficient and reproducible siRNA loading.[3]

In this work we aimed to develop and thoroughly characterize methods for loading isolated EVs with siRNA. EVs were purified from conditioned cell culture medium derived from a B16F10 melanoma cell line by (density gradient) ultracentrifugation. The presence of EVs was confirmed by means of cryo-TEM imaging, immunoblot detection of EV-specific markers and via their typical size and buoyant density.

In a first effort towards intravesicular loading of exogenous siRNA, we critically evaluated a previously reported method based on electroporation of an EV/siRNA mixture with the aim to induce transient pores in the EV membrane, hence allowing the siRNA to migrate through the lipid bilayer. Using this approach, siRNA encapsulation efficiencies up to 25% were reported.[4] Importantly, duplication of these experiments in our hands under identical experimental conditions revealed that the afore-mentioned siRNA encapsulation was largely due to unspecific aggregate formation, independent of the presence of extracellular vesicles.[5] The latter aggregates resulted from the interaction of multivalent cations, released from the metal electrodes in the electroporation cuvettes, with hydroxyl anions present in the electroporation buffer and were shown to co-precipitate siRNA. After blocking aggregate formation no significant encapsulation of siRNA could be measured. Taken together, these results demonstrate the necessity for alternative methods to load EVs with siRNA and the importance of including the correct controls to properly assess loading efficiencies in biological vesicles.

Next, we developed a new loading approach in which siRNA modified with a cholesterol moiety, was used to ally siRNA to the EV lipid membrane. The association of siRNA on the surface of EVs was shown using different methods based on gel electrophoresis, an antigen-specific bead based assay and iodixanol density gradient ultracentrifugation. Moreover, we clearly demonstrated that this approach required the use of chemically stabilized siRNA, due to the presence of significant nuclease activity in the isolated EV sample. Furthermore, we could confirm that EV cell uptake was not affected by the siRNA incorporation and compared the functional siRNA delivery capacity with an anionic, fusogenic liposome in a monocyte/DC cell line (JAWSII). Interestingly, we observed that the fusogenic liposomes clearly outperformed the EVs in terms of siRNA delivery. Finally, we also compared the gene silencing capacity of the cholesterol-siRNA inserted in the EV membrane with that of the endogenously present,

intravesiclular miRNA's. Therefore, a total miRNA profiling of the purified EVs was done by a nCounter® miRNA expression assay. Target mRNA's in JAWSII cells of the most abundant miRNA's identified in the melanoma EVs were evaluated for specific post-transcripitional gene suppression and compared to specific gene silencing of the pan-leucocytic marker CD45 via the loaded cholesterol siRNA.

To conclude we can state that electroporation, in contrast to previous reports, is not a feasible technique for loading siRNA in isolated EVs. Instead we developed a new approach based on a cholesterol modified siRNA to efficiently and reproducibly load EVs with exogenous small nucleic acids (graphical abstract). Finally, we compared the functional siRNA delivery potential between EVs and a classic, fusogenic liposome and between exogenous siRNA and endogenous miRNA.

References

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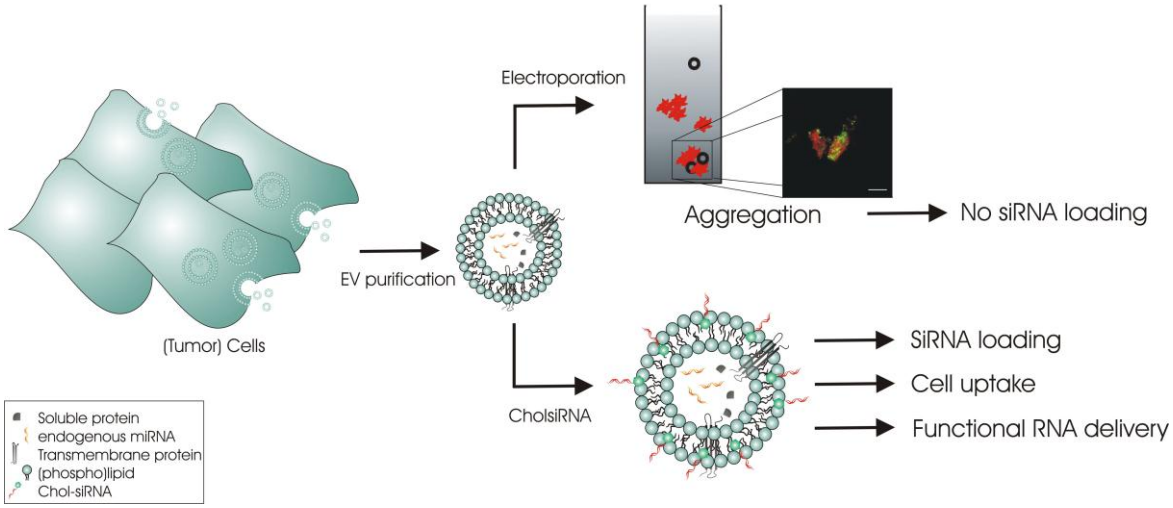
Figures



<u>Graphical abstract</u>: EVs released by B16F10 melanoma cells were purified via density gradient ultracentrifugation. Next, two methods for exogenous siRNA loading were evaluated. Electroporation appeared not a feasible loading technique, cholesterol mediated siRNA loading on the other hand, provided efficient and reproducible loading. Finally, these cholesterol-siRNA loaded EVs were evaluated for cell uptake and functional siRNA delivery.