Customisation strategies and loading of EVs to treat neurological disease

Ella L. Johnston[1] & Johannes F. Weber[1], Smriti M. Krishna[1], Craig Newton[1], Ian Nisbet[1], Xenia Sango[1], Mitch Shambrook[1], Louise White[1,2], David N. Haylock[1]

[1]VivaZome Therapeutics Pty Ltd, Victoria, Australia [2]SeerPharma Pty Ltd, Victoria, Australia

Introduction

Extracellular vesicles (EVs) have enormous therapeutic potential. EVs naturally contain various cargo including nucleic acids, proteins, and other biological material, and can have intrinsic anti-inflammatory and pro-angiogenic properties.

Furthermore, EVs can be customised in order to enhance their natural therapeutic abilities. Several strategies for customising EVs include surface and luminal EV modifications. Surface modifications may enable the targeting of EVs to particular cell types or tissues to enhance their ability to directly transfer cargo and to modify pathological states with reduced off-target effects and increased efficiency.

In addition to surface modifications, there are currently several strategies that enable the loading of specific cargo into EVs, including:

- Small molecule drugs
- Nucleic acids
- Proteins

Loading techniques include:

- Incubation/passive diffusion
- Transfection
- Electroporation
- Sonication
- Extrusion
- Freeze-thawing
- Endogenous loading

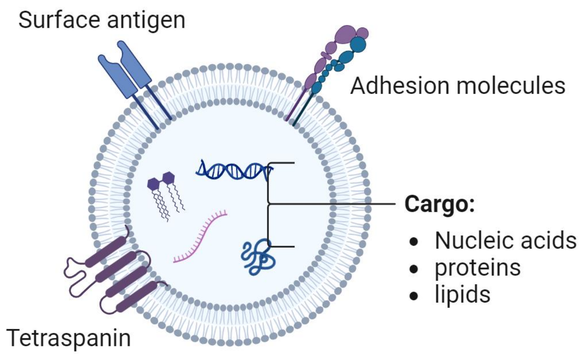

Figure 1. Schematic of a modified EV containing cargo such as surface antigens, adhesion molecules, tetraspanins and luminal cargo including nucleic acids, proteins, and lipids.

Luminal cargo loading efficiencies

Cargo loading techniques can be used to load nucleic acids, proteins, and small molecules into EVs. However, depending on the type of molecule being loaded, some techniques may be more efficient than others. Some factors affecting loading of cargo into EVs include:

- Technical specifications for each method (i.e. buffer composition, temperature)
- Cargo type
- EV source
- EV purity and isolation method
- Charge (EV surface charge/cargo charge)
- Cargo concentration

As a small-scale case study, we compared the efficiency of loading doxorubicin, a small molecule drug, into EVs from different publications that used various methods (Table 1). This comparison exemplified the differences in loading efficiencies of different cargo loading techniques, and that an orthogonal and holistic approach is required for EV customisation.

Table 1. Small molecule loading: comparison of reported loading efficiencies.

Method for small-molecule loading (doxorubicin, 543.52 g/mol)	Reported loading efficiency (μg/10^{10}EV)
Incubation	**9.06** (1)
Electroporation	**0.055** (2)
Extrusion	**3.22** (3)
Endogenous	**13.6** (4)

EV cargo loading strategies

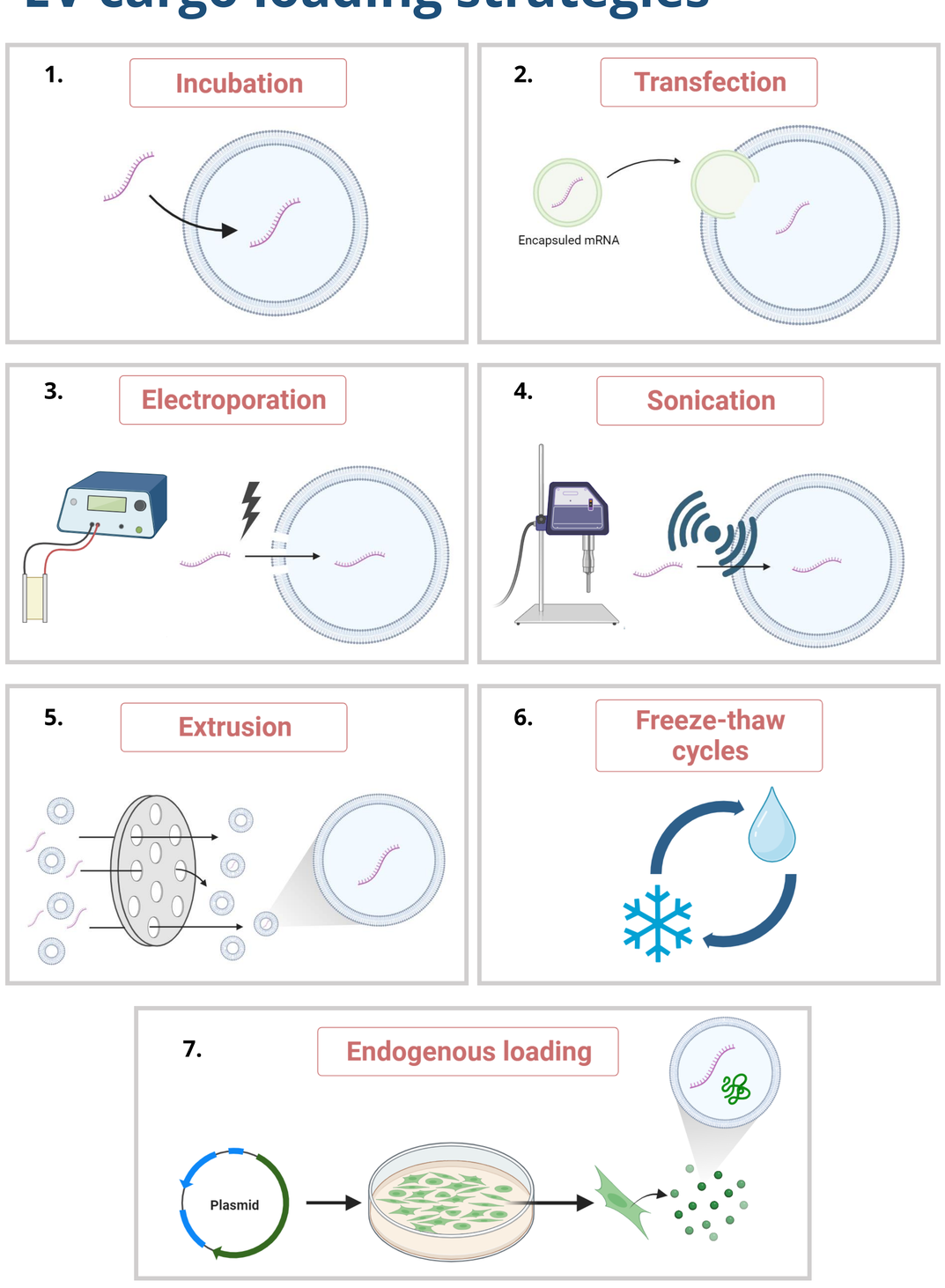

Figure 2. Schematic diagrams of EV loading strategies. 1. Incubation of EVs with the cargo of choice results in binding and passive uptake of cargo. 2. Transfection involves the use of a transfection reagent, such as Exo-Fect to deliver the cargo of choice into the EV lumen. 3. Electroporation of EVs uses electricity to generate pores in the EV membrane, allowing for cargo to enter the lumen. 4. Sonication works using a similar principal to electroporation, but uses sound waves to perturb the EV membrane and load cargo. 5. Extrusion is performed by pushing cells or EVs through small pores in the presence of cargo, resulting in membrane fragments reforming to generate EVs containing packaged cargo. 6. Freeze thaw cycles can also be used to disrupt EV membranes, and when performed in the presence of cargo, can result in packaging. 7. Endogenous loading involves modification of cell lines from which EVs are produced, with the modified cellular cargo being loaded into EVs.

Surface modification and targeting

In addition to loading cargo into the lumen of EVs, surface modifications can also contribute to the therapeutic potential of EVs, in addition to enhancing their ability to target to particular tissues or cell types. Surface modification of EVs may include tethering of peptides or ligands to the membrane using EV-bound receptors, or click chemistry techniques (Figure 3). Additionally, charge modification can enhance electrostatic interactions with molecules being modified on the surface of EVs, and synergistically enhance targeting of EVs.

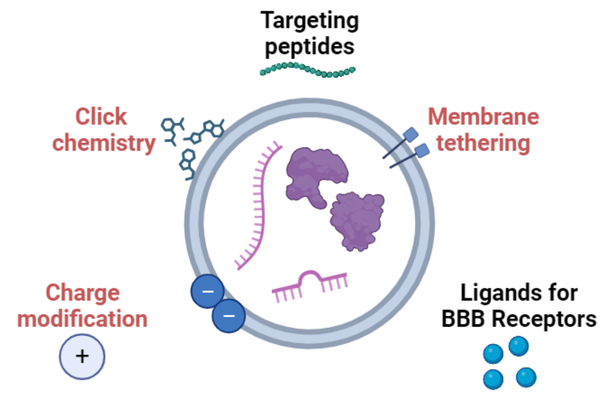

Figure 3. Schematic of EV surface modifications and targeting strategies. Targeting of EVs to particular cell types or tissues (black text), such as the CNS, can be achieved by the addition of targeting peptides or ligands for the BBB. Surface modifications of EVs (red text) can be used to achieve targeting, and can include membrane tethering and click chemistry, as well as charge modifications.

Summary

EVs have enormous therapeutic potential due to their customisable nature. However, in addition to challenges with EV customisation, we must also confirm effective EV modification and functionality. Customisation of EVs has many considerations and allows us to generate targeted and specified EV therapeutics (Table 2).

Table 2. Advantages and considerations for customised EVs.

Advantages	Considerations
Targeted delivery of luminal cargo.	Cargo loading efficiency can vary depending on methods used.
	Effective cargo release into recipient cells.
Cargo protection from degradation.	Effects of loading on native EV cargo and intrinsic properties.
Reduced immune response compared to other nanoparticle therapeutics.	Technical challenges with optimising the manufacture, purification, and characterisation of EVs.
Minimised off-target effects with EV targeting.	Limited cargo capacity.

References

(1) Chen C, Li Y, Wang Q, Cai N, Wu L, Yan X. Single-particle assessment of six different drug-loading strategies for incorporating doxorubicin into small extracellular vesicles. Anal Bioanal Chem. 2023;415(7):1287-98. **(2)** Schindler C, Collinson A, Matthews C, Pointon A, Jenkinson L, Minter RR, et al. Exosomal delivery of doxorubicin enables rapid cell entry and enhanced in vitro potency. PLoS One. 2019;14(3):e0214545. **(3)** Jang SC, Kim OY, Yoon CM, Choi DS, Roh TY, Park J, et al. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. ACS Nano. 2013;7(9):7698-710. **(4)** Farhat W, Yeung V, Kahale F, Parekh M, Cortinas J, Chen L, et al. Doxorubicin-Loaded Extracellular Vesicles Enhance Tumor Cell Death in Retinoblastoma. Bioengineering (Basel). 2022;9(11).

Images generated using BioRender.