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MINIREVIEW

Engineering protein toxins to modulate the intracellular trafficking of biologics into exosomes for third order drug targeting

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ABSTRACT

The successful use of mRNA as a vaccine to limit the effects of COVID-19 has highlighted the potential of RNA based drugs. However, the use of small interfering (si)RNA (or antisense oligonucleotides (ASOs)) as therapeutics remains limited by the availability of safe and robust drug delivery technology that can deliver intact RNA to the cytosol of tissues beyond the liver or muscle. New knowledge regarding the regulation of intracellular compartmentalisation associated with membrane trafficking has led to the identification of new problems and opportunities within the field of drug delivery. One of the technologies that has emerged is the use of attenuated toxins for the cytosolic delivery of macromolecules. Unlike charged lipids or synthetic polymers, these molecules do not display charge limited PKPD or toxicity profiles intimately linked to their ability to mediate transfection. Anthrax toxin uses intraluminal vesicles (ILVs) as an intermediary compartment *en route* to the cytosol of mammalian cells. These ILVs can be subsequently secreted as exosomes and herein is an opportunity to load selected nucleic acid or protein drugs into exosomes. Exosomes loaded in this manner have the potential to transport bioactive payloads across intercellular space to their target, whilst protecting their luminal cargo from hydrolytic enzymes or from the host's immune response before effecting cytosolic delivery. Following such a rationale, engineering biology may provide a valuable platform for third order drug targeting and facilitate the intracellular delivery of RNA drugs to cells and tissues beyond striated muscle and the liver.

Keywords: Antisense, siRNA, Toxin, Cytosol, Endocytosis, Exosomes, Biologics

INTRODUCTION AND RESEARCH GAP

The application of clinically safe and effective transfection systems has been highlighted using RNA vaccines to immunise against infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Forni and Mantovani, 2021). Current mRNA transfection technologies, whilst suited for transient expression within hepatocytes and striated muscle (Lu et al, 2003), have many limitations. The limitations of current intracellular delivery technology are often driven by vector pharmacokinetics and pharmacodynamics (PKPD), cargo nucleocytosolic delivery, stability, immunogenicity, and vector toxicity. Non-viral, nanoscale, advanced drug delivery systems have incorporated synthetic polymers, proteins, peptides, carbohydrates, lipids, and nucleic acids, forming either non-covalent complexes or covalent constructs. These systems have been extensively characterised over the last 30 years (Wu et al, 2020). Functional moieties that activate, release, protect or direct an active pharmaceutical such as a nucleic acid to an organ, tissue, cell, or subcellular compartment (organelle) have been proposed (Mousavizadeh et al, 2017). Notwithstanding, the intracellular fate of nanomaterial components must be considered to: 1) avoid the premature destruction of the drug; 2) facilitate the interaction of the drug with its target, and; 3) mediate the safe excretion or

degradation of system components from the body after homeostatic balance has been restored. Consequently, the prospect of using the body's own transport systems (*i.e.,* exosomes), to move material from the nucleocytosolic compartment of one cell into that of another, target cell, is appealing.

OPPORTUNITY: ENDOCYTOSIS AND ENDOLYSOSOMAL HYDROLASES

The endocytic system provides for many cellular needs. Endocytosis incorporates phagocytosis within specific populations of obligate or professional phagocytes, such as macrophages, and pinocytosis, within arguably every other cell type, with the possible exception of platelets. Endocytosis provides a mechanism for mitogenic signal attenuation, antigen cross-presentation, environmental remodelling, cell component recycling and the catabolism of extracellular material. As a result, it provides a defence against extracellular pathogens (Howel et al, 2006). Intracellular digestion occurs within the endolysosome, also referred to as the late endosome-lysosome hybrid organelle (Figure 1; Mullock et al, 2000), after the selective sorting and movement of cargo through early endocytic organelles or the secretory pathway. Trafficked cargo includes internalised solutes, trans-membrane proteins *i.e.,* receptors, which may originate from the plasma membrane or other organelles, or ligands (Mullock et al, 2000). Molecular sorting, membrane fission and fusion events regulate the temporal and spatial movement of cargo as well as membrane components, allowing the simultaneous execution of mutually exclusive anabolic and catabolic activities.

There exists within these membrane trafficking systems, phenomena differentiating the endocytic vesicular lumen from the interstitium, that is the fluid filled space existing between barriers such as cell membranes. An example of this might be the changes in pH used to "activate" a variety of advanced drug delivery systems *i.e.,* protonating amines within synthetic polymers or lipids (Hall et al, 2017), or triggering conformational changes within potentially endosomolytic proteins and peptides (Silva et al, 2019). Although well studied, the vesicular luminal pH (Murphy, 1988; Sipe et al, 1991; Sayers et al, 2019) is not the only phenomena associated with membrane trafficking that may be used for drug delivery. These phenomena are expanded upon in the subsequent section of this review.

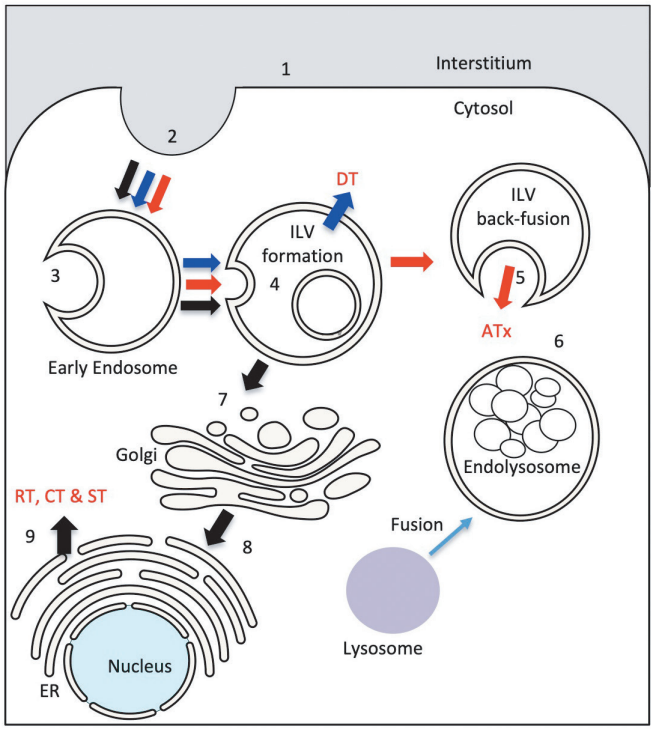
MEMBRANE DESTABILISING STRATEGIES FOR DRUG DELIVERY

Generally, exogenous macromolecules, such as proteins or nucleic acids cannot cross intracellular membranes, causing them to become enriched and compartmentalised within endocytic vesicles. The endocytic compartmentalisation of macromolecules is evident when albumin (covalently conjugated to a fluorophore) or a toxin a-chain, dislocated from its b-chain, is incubated with a population of cells. Here, the localisation, or entrapment and enrichment of the fluorescent albumin within endocytic puncta is profound. The effect of this enrichment of macromolecules within endocytic vesicles can be quantified if binary protein toxins are examined. If a toxin a-chain is fed to a cell with and without the associated b-chain, a dramatic reduction (up to 5 logs) of toxin a-chain toxicity relative to the holotoxin has been reported (Richardson et al, 2004; Dyer et al, 2016). These examples highlight the consequence and importance of cell compartmentalisation in relation to the development of drugs using gene editing technology, siRNA, ASOs, and other therapies that cannot readily cross biological membranes.

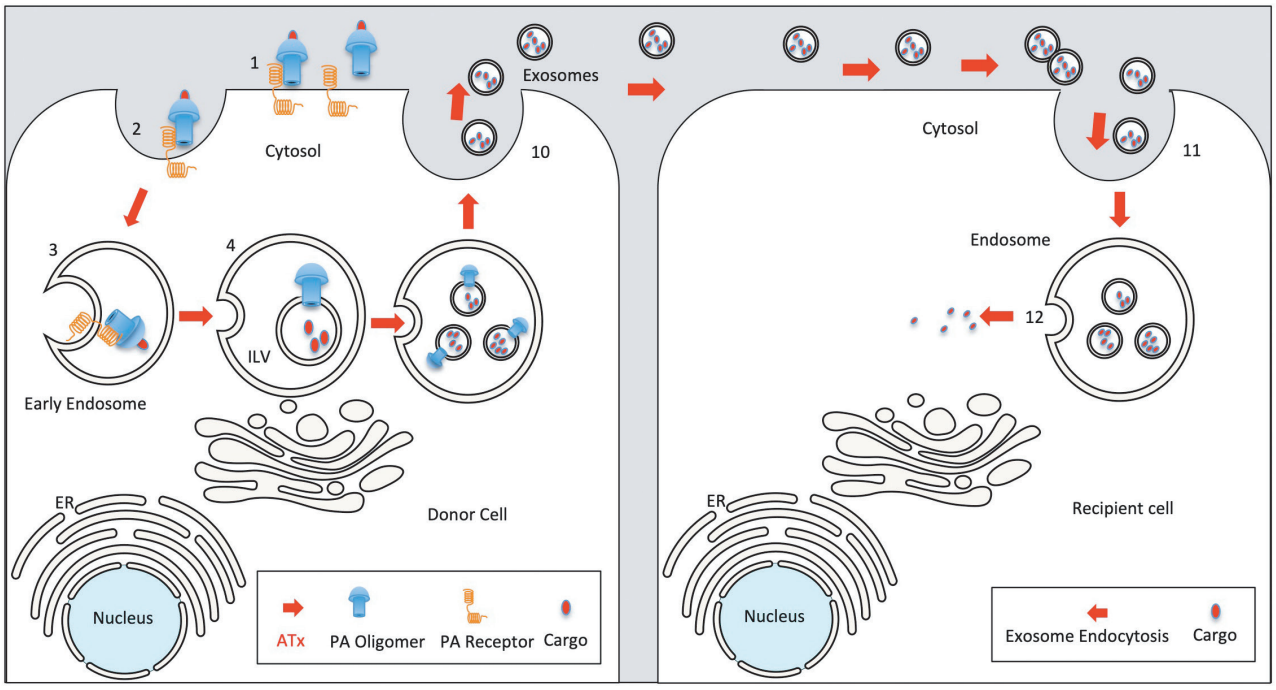
Technologies that destabilise cell membranes to achieve the nucleocytosolic delivery of exogenous large molecules have the potential to be minimally invasive. However, membrane selectivity poses a challenge, as an early endosome has been documented to either mature into a late endosome and eventually an endolysosome (Rink et al, 2005), or use carrier vesicles to move cargo between these two organelles (Gruenberg, 2001). Rupturing the endolysosome results in the release of lysosomal enzymes into the cytosol, many of which retain activity at cytosolic pH. This phenomenon has been reported to induce apoptosis *in vitro* (Kagedal et al, 2001) and is further complicated by the variation in physiology used as "triggers" (such as variations in luminal pH) (Murphy, 1988) across the endomembrane system, and by vector PK-PD (Nishikawa et al*,* 1996). Vector intracellular compartment targeting, and membrane specificity are issues highlighted by the extensive work evaluating polycations as components of non-viral DNA delivery systems. Polycations such as poly(ethyleneimine) (PEI) (Florea et al, 2002), poly(L-lysine) (PLL) (Richardson et al*,* 1999) or chitosan (Carreño-Gómez and Duncan, 1997), amongst others, have the capacity to kill cells. In the instance of PLL, toxicity has been reported in the scientific literature since the 1950s (Nevo et al, 1955) and at higher PLL concentrations, non-endosomal membrane damage was evident (Richardson et al, 1999). Consequently, to optimize bioavailability whilst minimizing toxicity, strategies limiting membrane destabilisation to specific compartments must be implemented once the limitations of PKPD have been alleviated. Notwithstanding, variations in the pH of early endosomes and late endosomes between different populations of cells further complicate this strategy (Murphy, 1988; Sipe et al, 1991; Sayers et al, 2019).

THE PEG DILEMMA AND OTHER HURDLES

The effect of reducing the density of positive charges within a nucleic acid containing particulate to increase its biocompatibility and bioavailability has been subject to extensive study and experimentation. One of the tools used to achieve this end has been poly(ethylene glycol) (PEG). PEG has been used extensively within the pharmaceutical industry and is a substance generally regarded as safe. In addition, it is very hydrophilic, imparting "stealth" properties hiding and stabilising molecules and materials it is conjugated to. An example of this might be the PEGylated liposomes (such as DOXIL® (Caelyx®)) made by Tibotec Therapeutics (Green and Rose, 2006). PEG also has utility during RNA delivery via solid lipid nanoparticles and this has been recently reviewed (Schoemaker et al, 2021). In summary, reducing the density of positive charges reduced toxicity, as well as transfection efficiency. This



Panel A: The Intracellular trafficking of select protein toxins



Panel B: Cytosolic delivery via an exosome intermediate

Figure 1 Panel A: Upon receptor binding (1), cargo is enriched within coated pits (2) and after transport to the early endosome (3), cargo encounters an acidic environment. Here integral membrane proteins destined for destruction are enriched upon an internal membrane invagination, which eventually dislocates from the limiting membrane to form an intraluminal vesicle (ILV) (3). Low pH triggers conformational changes in Atx and DT toxin b-chains, leading to b-chain membrane insertion and toxin a-chain translocation (4). Cargo such as Atx a-chains that are within the ILV lumen maybe released into the cytosol after an ILV back fusion event (5). Material following the default endocytic pathway is subject to enzymatic destruction (6) within the endolysosome after the fusion of lysosomes to the late endosome. Equivalently ricin toxin, shiga or cholera toxin derived material may be trafficked to the Golgi (7) and eventually the ER (8) prior to a-chain transit over structures such as the Sec61p translocon, into the cytosol (9). **Figure 1 Panel B** continues this journey where cargo loaded into ILVs may be secreted by the exosome donor cell, as exosomes (10). Spanning intercellular space, exosome maybe subject to endocytic capture by recipient cells (11) prior to membrane fusion and cargo release (12). Adapted from: Gruenberg 2001; Abrami et al, 2004; Richardson et al, 2004; Abrami et al, 2013; Bissig and Gruenberg 2014; Dyer et al, 2015; Zhan et al, 2015; Rabideau and Pentelut 2016; Feron and Richardson 2018.

phenomenon was termed the "PEG dilemma" on account of examining the effects of introducing PEG blocks within a PEI-PEG di-block co-polymer (Bernkop-Schnürch et al, 2018). It has since been hypothesized that eliminating the use of strongly positive or negative charges could circumvent the PEG dilemma. This has been attempted through various means including the development of multivalent *N*-acetylgalactosamine (GalNAc) ligands (Huang et al, 2017). In addition to this, GalNAc interacts with the asialoglycoprotein receptor, highly expressed by hepatocytes, which contributes to its enrichment within endocytic structures (Weigel, 1980). Vesicle mis-fusion or leakage during fusion may account for the cytosolic release reported clinically, as no other mechanism of vesicular escape is obvious (Ladokhin 1997; Engel and Walter, 2008).

SOLUTIONS FROM EVOLUTION?

The licencing of a variety of nucleic acid drugs that rely on viral delivery technology has been noted. These range from Gendicine (2004) (Peng, 2005), to Glybera (2012) (Keeler and Flotte, 2019), and Strimvalis (2016) (South et al, 2019). Other clinically relevant medicines also include the AZ COVID-19 vaccine (formerly AZD1222), which utilises a modified chimpanzee adenovirus to deliver mRNA encoding a fragment of the SARS-CoV-2 spike protein to muscle cells via intramuscular injection (Knolla and Wonodia, 2021). In addition to the well-studied viruses used as transfection vectors, the biosphere contains an abundance of structures that have evolved to overcome compartmentalisation and endolysosomal destruction, such as proteinaceous toxins (Zhan et al, 2015). Protein toxins often contain architecture that subverts membrane trafficking to enable the dissemination of the toxins a-chain(s) across intercellular space, from one cell to another (Abrami et al, 2013).

Cells deploy various strategies to utilise compartmentalisation and the associated topological protection of luminal material, for the intercellular transit of labile, antigenic, and biologically active material. Exosomes shed by a donor cell and fusing to a recipient cell exemplify one such system that facilitates intercellular nucleocytosolic communication (Doyle et al, 2019). This further highlights the evolutionary importance of overcoming topological barriers to transit biological material across membranes. Cell-to-cell communication has also been repurposed by Anthrax toxin, shown to hide lethal factor (LF) inside exosomes to temporally extend its activity and to deliver it to the cytosol of distant populations of cells (Abrami et al, 2013).

Exosome membrane composition is thought to be related to cell origin and target specificity. Exosome biogenesis may be partially regulated by proteins such as apoptosis-linked gene 2- interacting protein X (ALIX), responsible for back-fusion between exosome precursor *i.e.,* intraluminal vesicle (ILV), and the limiting membrane of the multivesicular body which, from a biochemical perspective, is similar to that of the late endosome (Bissig and Gruenberg, 2014). However, the development of exosome-based delivery systems for clinical application remains hindered by cell selectivity, exosome stability and aggregation, and the efficient and non-destructive loading of the exosome lumen with biologic drugs (Doyle et al, 2019).

LOADING EXOSOMES WITH THERAPEUTIC CARGO

Much attention has been focused upon the potential of enriching drugs such as siRNA or mRNA in exosomes. Examples include iExosomes loaded using electroporation (Kamerkar et al, 2017), extrusion (Luan et al, 2017) or the overexpression of material fused to endogenous exosome membrane proteins (Gee et al, 2020). The application of energy during extrusion or electroporation can disorganise exosome membrane proteins, which may be critical to the fusion of the exosome with the recipient cell.

Exosome fusion to the limiting membrane of the recipient cell has been reported to occur as an explosive fusion event between the exosome membrane and the limiting membrane of a late endocytic structure, and to require the lipid lysobisphosphatidic acid (LBPA). Yet the exact mechanism by which exosomes fuse to the membrane of the recipient cell has yet to be fully characterised (Joshi et al, 2020). It is of note that the family of proteins responsible for mediating vesicle fusion during endocytosis and exocytosis (soluble *N*-ethylmaleimide-sensitive factor attachment proteins receptors (SNARE) proteins) can participate in both "kiss and run" and "explosive" vesicle fusion (Urbina and Gupton, 2020).

ANTHRAX TOXIN AND EXOSOMES

It has been reported that attenuated anthrax toxin utilises exosome precursor vesicles (ILVs) to deliver a membrane impervious a-chain (LF and oedema factor (EF)) to the cytosol (Abrami et al, 2004). This system has been adapted and can deliver a variety of cargo *e.g.,* the removal of EF and LF amino acids beyond residue 225 to generate LFn, with high efficiency and low toxicity (Dyer et al, 2015). The in-frame fusion of other functional domains has resulted in the cytosolic delivery of several types of cargo, from diphtheria toxin a-chain to single chain antibody fragments and nucleic acid binding domains shown to deliver antisense oligonucleotides and siRNA (Rabideau and Pentelut, 2016). As the a-chains of anthrax toxin have been previously reported to be found in exosomes, it was reasoned that it may be possible to "trap" cargo in ILVs prior to inducing their secretion as exosomes (Abrami et al, 2013; Feron and Richardson, 2018). This is something that has been explored and a selection of cargo has been documented inside mammalian cell-derived vesicles of the predicted morphology and size (between 60-200nm). The latter (exosomal) system has the advantage of being able to hide luminal cargo from enzymatic destruction and has the potential to hide immunogenic material whilst in transit to the target cell though may operate with a reduced efficiency relative to the former. Balancing cargo stability against transfection efficiency may also help determine which of these technologies is best suited for a given application *i.e.,* as part of a medicine, to treat a particular disease.

Engineering protein toxins to subvert endomembrane trafficking for drug delivery is described in Figure 1. Cargos

delivered by recombinant attenuated anthrax toxin may include LFn-DTA, siRNA, ASOs and the gene editing protein Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (Cas9) (Feron and Richardson, 2018). Further work is required to: ascertain payload specific bioactivity, quantitate and optimise drug loading efficiency, and determine treatment dose response, all resulting in the *in vivo* exemplification of the concept. This strategy may be limited by the need to match the pathology targeted to the accessible cell population (Sancho-Albero et al, 2019). Further steps remain to be taken to unlock the therapeutic potential of exosomes as a drug delivery vehicle, including controlling exosome toxicity (Zhu et al, 2017), increasing exosome stability (Jeyaram and Jay, 2019) and reducing exosome aggregation (Bosch et al, 2016).

CONCLUSIONS

Insight into the regulation of membrane trafficking provides new opportunities for drug delivery and specific rate limits have been identified. Understanding how membranous barriers are overcome in nature provides additional opportunities for both direct cytosolic delivery and exosome-mediated delivery of biologics to the cytosol of mammalian cells. Using tools that have evolved, such as modified protein toxins to load large therapeutic molecules into exosomes, may further augment and enhance the potential of biomimetic engineering to develop new medicines.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

ALiX: Apoptosis-linked gene 2- interacting protein X
ASO: Antisense oligonucleotide
ATx: Anthrax toxin
CT: Cholera Toxin
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
COVID: Coronavirus disease
Cas9: CRISPR-associated protein 9
DT: Diphtheria Toxin
EE: Early Endosomes
GalNAc: N-Acetylgalactosamine
ILV: Intraluminal vesicle
LE: Late Endosomes
LF: Lethal Factor
LFn: Amino acids 1-255 of LF
LFn-DTA: LFn-diphtheria toxin a-chain
LBPA: Lysobisphosphatidic acids
LAMP1: Lysosome Associated Membrane Protein 1
MVB: Multivesicular Body
EF: Oedema Factor
PK-PD: Pharmacokinetics and Pharmacodynamics
PEG: Polyethylene glycol
PLL: Poly-L-Lysine
PEI: Polyethylimine
PA: Protective Antigen
RT: Ricin Toxin
RNAi: RNA interference
SARS CoV-2: Severe acute respiratory syndrome coronavirus 2
ST: Shiga Toxin
siRNA: Small interfering RNA
SNARE: Soluble N-ethylmaleimide-sensitive factor attachment proteins receptors

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