Chapter 1

General introduction and scope of thesis

Bhagyashree S. Joshi

University of Groningen, University Medical Center Groningen, Department of Biomedical Engineering, Antonius Deusinglaan 1, 9713AV, Groningen, The Netherlands
Intercellular communication and extracellular vesicles

For a long time, it was thought that there were two major pathways operating for intercellular communication, via direct cell-cell contact and via secretion factors. In the 1980s, small vesicles were shown to be released from cells, and thought to function as garbage bags shedding unwanted components out of the cells in bulk\(^1\). However, multiple studies in later years revealed that these vesicles contained various functional biomolecules and could interact with cells causing phenotypic changes\(^3\)–\(^10\). These vesicles now are collectively known as extracellular vesicles (EVs).

All prokaryotic and eukaryotic cells release EVs, i.e., lipid bilayer vesicles, into their extracellular environment\(^11\). Three main EV types exist which are categorized based on their biogenesis pathway\(^12\)–\(^14\) (Figure 1). (a) Exosomes are nanosized vesicles (~ 40 - 160 nm in diameter) of an endocytic origin. Formation of exosomes starts with inward budding of the plasma membrane followed by pinching off to form an endosome. The endosome matures in size and composition to form a late endosome. The limiting membrane of the late endosome forms inward invaginations that pinch off, resulting in the formation of intraluminal vesicles (ILVs; future exosomes). The vesicular compartment where ILVs are formed is called a multivesicular body (MVB). When an MVB fuses with the plasma membrane, the ILVs are released into the extracellular space and are then called exosomes. (b) Vesicles pinching off from the plasma membrane by outward budding form the second major type of EVs and are called microvesicles (MVs). MVs range in size from 50 nm to 1 µm in diameter, but are on average larger than exosomes\(^15\). (c) Apoptotic bodies are a third type of EVs, which are formed by outward budding of the plasma membrane from apoptotic cells.

Formation of exosomes, in particular, intersects with other intracellular vesicles and compartments, giving rise to an immense complexity in their composition both at the level of lipid bilayer (lipids, membrane proteins, glycosyl residues) and lumen (cytosolic proteins, DNA, small RNA, mRNA, metabolites) suggestive of a specificity of function\(^9\). EVs are suggested to play an important role in a myriad of physiological and pathological pathways, for example development, senescence, immune response, tumorigenesis, metastasis, neurodevelopment, and neurodegeneration\(^6\),\(^7\),\(^16\)–\(^22\). With the current isolation protocols, which produce heterogenous populations of EVs, it is difficult to classify the EVs. However, with the advance of isolation and detection technologies, more EV subtypes are being revealed\(^23\).
General introduction and scope of thesis

Figure 1 Biogenesis of different EV types. Extracellular vesicles are broadly classified in three categories – (a) exosomes are released from MVBs that fuse with the plasma membrane, (b) microvesicles are formed by outward budding of the plasma membrane, and (c) apoptotic bodies are large vesicles released from cells undergoing apoptosis. EE: early endosome, MVB: multivesicular body, ILV: intraluminal vesicle, N: nucleus.

The first step in the interaction of EVs with recipient cells is that of binding and uptake. Uptake can take place via fusion with the plasma membrane and/or endocytosis. In case of EV fusion with the plasma membrane, its cargo immediately reaches the cell cytosol. Upon endocytosis of EVs, the EV cargo needs to escape from endosomes in order to acquire access to the cytosol. The mechanism of cargo release from EVs is still unclear. In Chapter 2, we set out to understand the EV uptake and cargo release mechanisms. Using an analytical system involving molecular probes and correlated light electron microscopy (CLEM), we show that EVs are primarily taken up by cells via endocytosis. They do not escape endosomes by permeabilizing endosomes. Rather, the EV membrane fuses with the endosomal membrane for cargo exposure to the cytosol. This study along with others provides an important insight into the link between EV uptake and function.

Extracellular vesicles as biological delivery vehicles

Due to their functional cargoes, including miRNAs, proteins, mRNAs and bioactive lipids, EVs are being exploited as therapeutic delivery vehicles. In addition to their innate therapeutic potential, EVs can be used to deliver exogenous drugs of interest, thus acting as drug delivery vehicles. Using nanoparticles for drug delivery is not a new paradigm. Many nano-formulations such as liposomes, polymersomes, nanorods, nanocrystals, and micelles have been developed as drug delivery
vehicles\textsuperscript{34-38}. However, most of these formulations contain synthetic ingredients, which can evoke a strong immune response in the body\textsuperscript{39}. EVs, being endogenous in origin and exposing CD47 (a ‘do not eat me’ signal\textsuperscript{40}) at their surface, are non-immunogenic\textsuperscript{41}. Furthermore, their target cell specificity, stability in bodily fluids, and efficient therapeutic delivery make EVs attractive drug delivery candidates\textsuperscript{42}. Multiple studies show favorable functional outcomes with EV-drug formulations in applications such as cancer regression, neurodegenerative disease abatement, and immune response activation\textsuperscript{42}. Several methods exist to load cargo into/onto EVs, while the method to be employed mainly depends upon the inherent characteristics of the molecule of interest. In Chapter 3\textsuperscript{43}, we give a comprehensive overview of strategies used so far to achieve efficient loading of different cargo species e.g. RNA, DNA, protein, lipids etc. However, high cargo loading in EVs does not always translate into a high functional response in the recipient cells. Therefore, we further recapitulate hitherto reported methods to stimulate release or ‘unloading’ of cargoes in target cells, to advance EV drug delivery potential. Finally, we suggest an experimental approach to facilitate EV characterization, including its correlation with function.

Chapter 4 and 5\textsuperscript{44} together provide an example of EVs as a therapeutic protein delivery system as a proof-of-concept therapeutic intervention for Huntington’s disease (HD), a debilitating neurodegenerative disease. Many neurodegenerative diseases, including HD, are caused by aggregation of proteins\textsuperscript{45, 46}. HD is a monogenic disorder in which the gene encoding huntingtin (Htt) harbors mutations leading to an unusual expansion of CAG (glutamine) repeats resulting in the presence of a large polyglutamine stretch in Htt. The aggregation-prone mutant Htt gradually forms protein aggregates which eventually disturb neuronal functioning and inevitably lead to cell death\textsuperscript{47}. Molecular chaperones, in particular DNAJB6, have shown promise as therapeutic candidates as they can efficiently suppress protein aggregation in vitro as well as in vivo\textsuperscript{48, 49}. However, naked proteins as therapeutics face the challenge of rapid degradation and clearance from circulation when administered systemically\textsuperscript{46}. As EVs offer excellent ‘Trojan horse’ cloaking and protective properties in addition to specificity in targeting\textsuperscript{9}, we hypothesized that EVs can act as excellent vehicles for DNAJB6 as a proof-of-principle treatment of HD.

Cytosolic overexpression of the cargo of interest is a popular method of choice for protein loading into EVs\textsuperscript{50-55}. A major concern with this approach, however, is the
possible cell malfunction and induction of unwanted metabolites and biomolecules while increasing the expression of a certain protein in the cell. Therefore, when using EVs as protein delivery vehicles, a comprehensive assessment of the overall proteomic fingerprint of the EVs is of utmost importance. Hence, in Chapter 4 we present a proteomic analysis of EVs derived from cells overexpressing DNAJB6 (wild type and mutant). With this study, we aim to assess the fitness of EVs loaded with DNAJB6 through cytosolic overexpression by assessing the presence of other chaperones, deleterious proteins, and differential protein expression specifically induced upon the overexpression of DNAJB6. We find that these EVs do not contain proteins that could be assigned to any particular disease, whilst containing additional beneficial chaperones. Therefore, we conclude that they appear fit as DNAJB6 delivery vehicles. After this preliminary validation, Chapter 5 evaluates the potential therapeutic effect of DNAJB6 loaded EVs. Here, we show that EVs derived from DNAJB6 overexpressing neural stem cells are taken up by recipient cells. In cells expressing expanded polyQ tracts the DNAJB6-loaded EVs can significantly suppress polyQ aggregation \textit{in vitro} and \textit{in vivo}, specifically in the R6/2 HD mouse model. Our results are in line with various reports that have shown the potential of EVs for the treatment of neurodegenerative diseases, for example Alzheimer’s disease\cite{56, 57}, and demyelinating diseases\cite{58}.

**Blood-brain barrier and EVs**

Although there is considerable development of drug candidates for brain disorders, their delivery to the brain forms a major bottleneck due to the presence of the blood-brain barrier (BBB)\cite{59-63}. This vascular filter system controlling the exchange of substances between the blood and brain, primarily consists of endothelial cells, selectively keeping toxins away from the brain\cite{64, 65}. However, during this process most of the drugs are also denied access, resulting in less than 1% of traditional drugs reaching the brain\cite{66}. Five major pathways operate for molecular transport across the BBB depending upon the nature of biomolecules (Figure 2)\cite{67}. These include\cite{67} – (i) Transcellular lipophilic transport: passive transport of small lipophilic compounds. (ii) Paracellular aqueous transport: passive transport of small hydrophilic substances through the tight junctions between two adjacent cells. (iii) Carrier mediated transport (CMT): Protein-mediated transport exploiting members of the solute carrier transporter family, facilitating amino acid, glucose and nucleoside transport. (iv) Receptor mediated transcytosis (RMT): transcellular receptor-mediated transport.
(e.g., via transferrin receptor, insulin receptor). (v) Adsorptive transcytosis: transcellular charge-mediated transport (e.g. cationic nanoparticles, cationic proteins).

Figure 2 Mechanisms of transport across the blood-brain barrier. Small lipophilic molecules can traverse via passive diffusion through the cells, i.e., transcellular route (i). Small hydrophilic molecules can cross the endothelial cell layer by passive diffusion in between cells i.e. paracellular route (ii). Solute transporters can facilitate transport of selected solutes across the endothelium (iii). When biomolecules selectively interact with the receptors present at the apical side of the brain endothelium, receptor mediated transcytosis can take place (iv). Charge interactions promote adsorption of charged molecules/entities which can then be transported via transcytosis (v).

CMT, RMT and adsorptive pathways have been majorly exploited for drug transport to the brain. For example, conjugation of a drug with a targeting peptide can target RMT for their transport across the BBB. Likewise, polymeric and lipidic nanoparticles decorated with homing motifs provide tools for RMT based transendothelial transport. In recent years, EVs have proven to be efficient carriers...
of therapeutics for the treatment of neurodegenerative disorders\textsuperscript{56, 74-76}. However, in most studies EVs had to be either modified with a targeting peptide or injected directly into the brain\textsuperscript{56, 77}. A more straightforward approach would be to intravenously administer EVs with a capacity to cross the BBB. As discussed earlier, EVs possess inherent homing capacity which depends upon the source cells that EVs are derived from\textsuperscript{78}. For example, lung and brain tumor cell derived EVs are preferentially transported to lungs and brain respectively\textsuperscript{7}. Corroborating these results, EVs derived from macrophages and endothelial cells have recently been observed to show BBB crossing capacity to reach the brain\textsuperscript{74, 76}. However, their transport mechanisms across the BBB remain elusive. Neural stem cells have also been shown the propensity to traverse across the BBB \textsuperscript{79-82}. Thus, in \textbf{Chapter 6\textsuperscript{83}}, we use EVs derived from neural stem cells to carry a protein cargo across an in vitro BBB. We show that EVs interact with heparan sulfate proteoglycans and are internalized by dynamin-dependent endocytosis and eventually end up at the basolateral compartment (brain side). This way, protein cargo loaded into the EV lumen can be transported across the endothelial monolayer. These data suggest that EVs derived from NSCs show promise for crossing the BBB, which may find use in therapeutic interventions for brain disorders.

\textbf{Aim of this thesis}

Extracellular vesicles are players in cell-cell communication, influencing vital functions in physiology as well as pathology. Importantly, due to their amenability to modifications, including loading with a cargo of interest, they also show promise as drug delivery vehicles. In this thesis, emphasis is placed on using EVs as vehicles of biomolecular therapeutics for neurodegenerative diseases, in particular Huntington’s disease, to decrease protein aggregation in cellular and mouse HD models. The main goal is to establish a comprehensive picture of EV contents, EV content release in target cells, EV transport across the BBB, and finally functional effects of EVs loaded with therapeutic protein cargo in \textit{in vitro} and \textit{in vivo} HD models. This knowledge will be important for converting EVs into nanomedicine for neurodegenerative disorders.
References


