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Exosomes and Nanoengineering: A Match Made for Precision Therapeutics

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Targeted exosomal delivery systems for precision nanomedicine attract wide interest across areas of molecular cell biology, pharmaceutical sciences, and nanoengineering. Exosomes are naturally derived 50–150 nm nanovesicles that play important roles in cell-to-cell and/or cell-to-tissue communications and cross-species communication. Exosomes are also a promising class of novel drug delivery vehicles owing to their ability to shield their payload from chemical and enzymatic degradations as well as to evade recognition by and subsequent removal by the immune system. Combined with a new class of affinity ligands known as aptamers or chemical antibodies, molecularly targeted exosomes are poised to become the next generation of smartly engineered nanovesicles for precision medicine. Here, recent advances in targeted exosomal delivery systems engineered by aptamer for future strategies to promote human health using this class of human-derived nanovesicles are summarized.

1. Introduction

As the number of new cases of cancer diagnosed in Australia is estimated to be increased from 145 000 in 2019 to 150 000 by 2020, cancer has become a leading cause of death in Australia.[1] Intense efforts from both scientists and physicians across Australia have been devoted onward advancing studies to fill the gap in understanding the biology and pathogenesis underlying cancer development. Exosomes are membrane-bound bio-nanoparticles secreted or released by most cells into the extracellular space. There has been an increasing interest in such bio-nanoparticles over the past 10 years, evident from the publication number of only 324 papers in 2006 to a massive increase to 4603 publications in 2018 with the search term "exosome" based on title/abstract in PubMed. Among these studies, 151 articles published from 2007 to 2018 are from Australian institutions, with 33 papers published in 2018. Such a strong surge of interest is due to discoveries that exosomes play key roles in intercellular and intertissue communication mediated by virtue of cargos contained inside the membrane-confined space. Furthermore, exosome cargos, including proteins, mRNAs and miRNAs, are linked to the

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pathogenesis of many diseases and thus, they serve as attractive biomarkers for development of the next generation diagnostics for early detection of cancers, monitoring of disease progression and personalized guidance to therapy.[2] Since their original description over 30 years ago, exosomes are now known as a subtype of extracellular vesicles (EVs) with the size range 50–150 nm and can be harvested from cell culture or isolated from patient's body fluids which can be subsequently modified in vitro and transferred back to the same patient.[3] Exosomes are released from cells upon fusion of multivesicular body with the plasma membrane, different from microvesicles, which are formed and released by budding from the plasma membrane of cells.[4] Apart from exosomes and microvesicles, the nomenclature of EVs also includes microparticles, ectosomes, oncosomes, and apoptotic bodies.[5] Of note, the identification of EVs should meet the minimal requirements of guideline of Minimal Information for Studies of Extracellular Vesicles 2018, which was proposed by the International Society for Extracellular Vesicles.[5] Nevertheless, exosomes, the small EVs, are the subject covered in this Research News. Due to their prominent stability, long circulating half-life and favorable safety profile, exosomes emerge as promising drug delivery vehicles that are able to deliver cargoes into the cytoplasm with minimal toxicity.[6] Furthermore, exosomes possess a unique "homing" effect, i.e., the ability to target the cell type from which exosomes are produced.[7] Therefore, exosomes are poised to become a rising star as effective drug delivery vehicles.

The characteristic size (50–150 nm) of exosomes facilitates their accumulation in tumors via enhanced permeability and retention (EPR) effect.[8] However, the enhanced anticancer efficacy is best achieved when a surface targeting moiety is present to enable the active targeting of exosomal drugs. Indeed, exosomes have been shown to actively target specific cells via the receptor-mediated pathway.[9] In other words, the desired ligands engineered on the surface of drug-loaded exosomes can guide the exosomes to targeted sites where the corresponding cell surface biomarkers are overexpressed.[10] While antibodies and peptides are commonly used as targeting ligands, their clinical applications are hampered by several intrinsic disadvantages, including batch-to-batch variation, less specificity, immunogenicity, and instability.[11] One attractive alternative class of targeting ligands is aptamers, also known as chemical antibodies. Aptamers are single-stranded DNAs or RNAs that fold into specific 3D structures and thus bind to their targets with high affinity and specificity via the same mechanism as antibody–antigen binding.[12] Aptamers have several advantages over antibodies in precision medicine, including higher stability, superb tissue penetration, minimal toxicity, and devoid of immunogenicity.[13] From the translational research point of view, the key advantages of aptamers are their scalability and lack of batch-to-batch variations in pharmaceutical production, as they are chemically synthesized without the involvement of neither animals nor cultured cells.[14] The present Research News will highlight recent advances in targeted delivery of therapeutic agents to disease sites in vivo using aptamers and exosomes at the nanostructure level, with perspectives on key challenges and opportunities in the field.

2. Aptamer Emerges as a Prominent Targeting Ligand for Nanodelivery

The aptamer-guided drug delivery heralds a new era of targeted delivery of therapeutics to cancer in nanomedicine. The in vitro screening process for aptamer selection, systematic evolution of ligands by exponential enrichment (SELEX), provides a powerful tool for not only selection of aptamers against a target as small as an element, but also for the isolation of cell- or organ/tissue-specific aptamers.[15] Indeed, the terms "aptamer" and "SELEX" were originated in 1990, when RNA molecules were found to form stable surfaces by folding into "pockets" for specific binding to small molecules (e.g., organic dyes).[16] The aptamer research has grown steadily since then. The first aptamer taken into the clinic as therapeutic agent is an RNA aptamer against vascular endothelial growth factor (VEGF).[17] As a milestone in aptamer research, this VEGF antagonist, Pegaptanib, with the commercial name as Macugen, gained FDA approval for the treatment of neovascular (wet) age-related macular degeneration in 2004.[18] Over the past three decades, the SELEX has evolved into highly sophisticated, efficient, and versatile aptamer selection methods that are capable of not only selecting cell-specific aptamers using live cells in vitro,[19] but also generating organ-specific aptamers in live animals.[20] A group at Deakin University in Melbourne, has led the development of nucleic acid aptamers for cancer therapeutics and diagnostics in Australia. This group was the first in the world to have developed RNA aptamers against cancer stem cell (CSC) markers and demonstrated the feasibility of using aptamers to effectively deliver nanoencapsulated chemotherapeutic agent to CSCs in vivo.[21–23] Their 19 nucleotide RNA aptamer against epithelial cell adhesion molecule cancer (EpCAM) interacts specifically with breast, colorectal, and gastric cancer cells that express EpCAM with binding affinity at $\approx 55 \times 10^{-9}$ M. Upon binding to cell surface EpCAM, a known marker for CSCs, the aptamer is efficiently internalized.[21] The swift internationalization (endocytosis) of aptamers establishes a robust route to deliver cargos inside cells across the natural barriers, e.g., the plasma membrane. Such an aptamer-based molecular shuttle effectively circumvents the plasma membrane to deliver several classes of payloads that are not able to pass the membrane barrier, such as medium and large sized nanoparticles (over 100 nm), molecules that are hydrophilic and/or carry net negative charges. The EpCAM aptamer was not only specific but also more sensitive than conventional EpCAM antibody in detection of EpCAM in formalin-fixed paraffin-embedded primary breast cancer tissues.[22] In addition, this aptamer is able to detect EpCAM in clinical samples with chromogenic staining, indicating the potential for future aptamers-based histopathological diagnosis.[22] It has been well established that at the site of tumor, molecules including therapeutic agents, travel within the tumor tissue mainly via random diffusion.[24] Therefore, a targeting molecule with a smaller size has distinct advantages over a larger counterpart as it can effectively diffuse and penetrate into the entire tumor mass. To this end, Duan and coworkers have successfully developed two novel RNA aptamers against the AC133 epitope and the CD133 protein, including the smallest 15 nucleotide RNA aptamer.[25] These CD133 aptamers exhibited high sensitivity, remarkable tumor

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Figure 1. Aptamer-guided nanodelivery of siRNA to CSCs in vivo. A) Secondary structure for the longer strand of chimera, an 18 nt RNA EpCAM aptamer covalently linked with the guide strand of a 27 mer survivin siRNA. B) The passenger strand of the 27 mer survivin siRNA with 2 nt DNA at the 3′-end and a fluorophore at the 5′-end. C) A stable aptamer-siRNA chimera with Gibbs' free energy of –42.6 kcal mol^{-1}. D) The predicted 21 mer survivin siRNA after being processed by Dicer. E) Confocal micrographs show effective targeting of CSC by aptamer-guided in vivo RNAi in NOD/SCID mice bearing orthotopic MCF-7/Adr tumor model. F) Representative enlarged microscopy images from (E) depicting internalization of chimera into $EpCAM^+/CD44^+/CD24^-$cells. A–F) Reproduced with permission.[36] Copyright 2015, Ivyspring International Publisher.

penetration, and prolonged retention compared to the antibody counterparts.[25]

In agreement with other studies that have demonstrated superior performance of aptamers compared with that of antibodies,[26] an EpCAM aptamer developed by Duan and coworkers was also shown to perform better than an EpCAM antibody in tumor penetration, homogeneous distribution and retention in vivo using a mouse xenograft tumor model.[27] In this study, pharmacokinetic performance of the EpCAM aptamer in vivo for a targeted drug delivery has been significantly optimized via chemical modification (base modification and PEGylation) of the EpCAM aptamer. The improved half-life of aptamers in vivo is suggestive of future clinical application as molecular imaging agents.[27] Of note, aptamers themselves can be used as therapeutics in blood clotting,[28] or local treatment,[29] or inducing antitumor effect.[30] Furthermore, aptamer-mediated delivery systems have been broadly applied in cancer therapies, including aptamer-drug conjugates,[31] aptamer-nanoparticle/polymer-drug delivery system,[32] and aptamer-mediated cancer nucleic acid therapy.[33] Doxorubicin, one of the most effective chemotherapeutic agents for the treatment of a variety of solid cancers, has been extensively employed in aptamer-drug conjugates for optimized therapeutic outcomes.[34] These conjugates have been produced by either covalent or noncovalent conjugation through intercalation.[34] Through self-assembly by noncovalent integration of doxorubicin into engineered DNA–RNA hybrid EpCAM aptamer, this drug-aptamer conjugate was able to effectively target colorectal CSCs, internalize and deliver the drug to the nuclei of CSCs in vivo.[35] In addition, Duan lab pioneered aptamer-guided in vivo delivery of siRNA into CSCs by engineering a chimera of EpCAM aptamer and a 27 mer Dicer substrate survivin siRNA (**Figure 1**A–F). Thus, effective targeted delivery of a high dose of siRNA to CSCs of drug-resistant xenograft breast cancer in vivo was accomplished (Figure 1E,F).[36] In this combinatorial therapy using aptamer-siRNA chimera and free doxorubicin, the breast CSCs were eliminated in vivo, resulting in the suppression of tumor growth and prolonged survival of chemoresistant tumor-bearing mice.[36]

To apply aptamers in brain diseases, a bifunctional aptamer was generated by fusing an anti-EpCAM aptamer with an aptamer against transferrin receptor.[37] Since transferrin receptor is highly expressed on the surface of the cells lining on the blood–brain barrier, the EpCAM aptamer can piggyback on transferrin aptamer to cross the blood–brain barrier, and therefore, delivering payload into the brain.[38]

Aptamers are now able to be transformed into a multifunctional molecular probe for early detection, in vivo imaging, and targeted delivery.[39] The great supremacy in development of technology for new aptamers and second generation analogs, such as X-Aptamers (XAs), SOMAmers, locked nucleic acids (LNAs),[40] suggests aptamers are promising as a theranostic tool in clinical setting in near future. Recently, DNA aptamers were selected against EpCAM expressed on primary lung cancer cells, and then successfully applied to isolation and detection of circulating tumor cells in blood samples of lung

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cancer patients.[41] Aptamers have also been used to design electrochemical aptamer-based biosensors for the detection of specific cancer cell markers in blood samples.[42]

3. Nanoengineering of Aptamer-Targeted Exosomal Delivery

Australian scientists have made significant contributions to exosomes research through identification of novel roles of exosomes in the pathogenesis of neurodegenerative diseases,[43] tumorigenesis and metastasis,[44] development of methodologies for isolation, characterization, and loading process of exosomes,[45] exosomal proteomic profiling,[46] as well as identification of exosomal biomarkers for disease diagnosis.[47]

Recent advances in aptamer research have contributed to the growing exosomes research in development of a new generation of targeted delivery systems. As a class of targeting ligands, the small size of aptamers favors the travel of aptamer-guided nanosystems through microvasculature and interstitium, facilitating their efficient tissue penetration.[48] For aptamer-nanoparticle drug delivery, amphiphilic polymers are good candidates for stabilization of the surface chemistry and drug encapsulation, leading to the generation of a stable and protective surface-engineered nanoplatforms in the circulation. These aptamer-guided nanoparticles however still face the challenge of nonspecific uptake by phagocytic cells of the reticuloendothelial system after systemic administration. Several approaches have been attempted to circumvent this limitation, including smart engineering of aptamers and nanoparticles with optimal particle size and surface characteristics.[49] To this end, exosomes guided by engineered-aptamers seem able to afford enhanced specificity and efficacy in targeted therapies.[50] Moreover, exosomes have been shown to be capable of escaping phagocytosis,[51] having prolonged circulation half-life,[52] and reduced immunogenicity.[53] The combination of superior performance of aptamers and the aforementioned advantages of exosomes leads to a targeted drug delivery system with much improved therapeutic outcomes than synthetic nanoparticle-based systems with or without aptamers. Current generations

Figure 2. A) Transfection as a typical method for the manufacture of targeted exosomes. B) Modification of exosomal amine groups with a terminal alkyne followed by conjugation of azide-fluor 545 to exosomes using click chemistry. B) Reproduced with permission.[59] Copyright 2014, American Chemical Society.

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of targeted exosomes delivery systems have been produced by transfecting donor cells with corresponding recombinant virus or plasmid,[54] by fusion with targeting liposomes,[55] or by chemical conjugation.[56] The typical approach to harvest targeted exosomes relies on the target proteins or peptides specifically expressed on the cell membrane through gene transfection (**Figure 2**A). However, this method faces several challenges including existence of potential safety hazard, variable transfection efficacy, mutagenesis occurrence, and insufficient targeting capabilities.[57] Moreover, this bioengineering strategy is time consuming, and the whole process needs to be repeated every time a new molecular identity to be targeted.

There is an urgent need for developing a simpler, faster, more effective and importantly, scalable method to translate these nanovesicles from laboratory bench to clinic. One commonly adopted approach is to produce nanoplatforms with predesigned functional groups for subsequent modification of aptamers on their surface. Chemical conjugation often entails between carboxylic groups on the nanoplatforms and amino groups at the 3′ end of aptamers.[58] In addition, "click chemistry" was applied to functionalize targeting ligands on the surface of exosomes where alkyne groups can be introduced followed by a conjugation with azide-fluor 545 (Figure 2B).[59] This approach is indeed beneficial for bioconjugating small- and macro-molecules to exosomes with the benefits of short reaction time, minimal variation of size and properties of exosomes, compatibility in aqueous buffers and high specificity.[60] Most recently, Tian et al. engineered exosomal surface with targeting ligands using bio-orthogonal copper-free azide-alkyne cycloaddition, providing a targeting delivery-based exosomes for cerebral ischemia therapy.[61] To increase circulation time and achieve targeted delivery of exosomes to a desired site of action, a CSC marker, e.g., CD44, which is highly enriched in a variety of tumors,[62] may be a target of interest to synthesize an anti-CD44 aptamer for CD44 targeted exosomes, as observed with other types of nanoparticles.[63] Recently, a method for production of aptamer-mediated exosomal delivery of paclitaxel has been reported as a rapid, economic, and scalable method that could meet the clinical requirements in the future.[64] In this study, the cancer cell-targeting extracellular nanovesicles were produced in just ≈1 h by mechanical extrusion of ≈10^7 dendritic cells grafted with a lipidated ligand which is a conjugate of nucleolin-targeting aptamer AS1411 and cholesterol–poly(ethylene glycol) (PEG). Subsequently, the nanovesicles were loaded with paclitaxel, demonstrating effective delivery and enhanced treatment efficacy compared with free paclitaxel in vitro and in vivo.[64] Indeed, to exploit the specific biochemical feature of exosomal lipid bilayer for surface nanoengineering, a lipidated ligand has been commonly utilized.[65,66] Pi et al. amended the orientation of arrow-shaped RNA for advanced controlling RNA loading and aptamer surface engineering on membranes of exosomes for specific cell targeting in vivo. This strategy was able to deliver therapeutic siRNA and miRNA to target cancer cells, and hence, inhibiting tumor growth in prostate and breast cancer models.[65] Locating membrane-attaching cholesterol to the tail of RNA arrows led to external presentation of RNA aptamers on the exosomes; whereas, adhering the cholesterol at the arrowhead resulted in efficient internal loading of RNA nanoparticles in the exosomes (**Figure 3**). As a result, these RNA-engineered vesicles were able to knock down the expression of survivin, and suppress tumor growth without any significant toxicity.[65] Most recently,

Figure 3. A novel strategy for functionalization of exosomes with targeting ligand. A) Cholesterol labeling of the arrowhead or arrowtail of the three-way junction (3WJ) RNA. B) 2D structure (left) and native PAGE for testing 3WJ assembly. C) Electron microscopy images of extracellular vesicles. D) The siRNA loading and RNA aptamer decoration for the vesicles. A–D) Reproduced with permission.[65] Copyright 2018, Springer Nature.

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the diacyllipid–aptamer conjugate composed of a diacyllipid tail and aptamer sgc8 via a PEG linker was developed to generate a promising exosomal platform for cancer targeting.[66] Specifically, functionalization of exosomes was achieved by simply mixing the 5-carboxyfluorescein (FAM)-labeled diacyllipid-sgc8 ligand with exosomes at 37 °C for 30 min followed by washing using ultracentrifugation. The sgc8-targeted exosomes displayed a selective and dose-dependent toxicity with target cells.

4. Summary and Outlook

Exosomes produced naturally by human cells are emerging as a new generation of exceptionally protective nanoplatform for efficient drug delivery. They can carry not only therapeutics but also molecular imaging agents to function in the precision therapeutics and/or diagnostics. Due to a unique structure encompassing an aqueous core and a membrane highly enriched with lipid rafts, exosomes are an ideal nanoplatform for loading both hydrophilic and lipophilic agents. However, despite currently promising studies about exosomes, several major challenges remain, including lack of characterization of exosomes derived from different sources, low exosomal yield and encapsulation efficiency and lack of advanced purification techniques with high efficiency. Further molecular- and nano-engineering of exosomes will provide insights for future effective and precision medicine of devastating diseases including cancer and neurodegenerative disorders. As targeted exosomal delivery is a fast developing field, aptamer-mediated exosomal delivery is becoming attractive for preparation of smart nanodelivery systems in terms of easy operation, high performance at the nanoscale, enhanced efficacy as well as safety and low cost. In fabricating targeted exosomal delivery systems, ligands are engineered for their attachment to the membrane of exosomes to target specific cells or tissues. Nearly three decades after the initial development of SELEX technology, aptamer has emerged as a powerful tool in theranostic applications. The unique pharmacokinetic properties of aptamers confer distinct advantages for future clinical development, as evident from the increasing number of aptamers that have entered clinical trials. Most aptamers have been utilized to guide nanoparticles, therapeutic and imaging agents to target locations in several promising anticancer preclinical studies, whereby they are able to modulate tumor retention and biodistribution, as well as to minimize nonspecific uptake into other organs once particle size and surface characteristics are optimized. In this context, exosomes appear to be outstanding biological platforms that would match with aptamers very well to form the next generation of targeted delivery systems. With concerted efforts from researchers in diverse disciplines such as material scientists, molecular and cell biologists, pharmaceutical scientists and clinicians, the aptamer-targeted exosomal drug delivery system will become a new theranostic frontier for future nanomedicine.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

aptamers, exosomes, nanoengineering, precision therapeutics, targeting ligands

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