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Multifunctional Delivery Systems for Cancer Gene Therapy

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Abstract

This chapter examines key concepts with respect to cancer gene therapy and the current issues with respect to non-viral delivery. The biological and molecular barriers that need to be overcome before effective non-viral delivery systems can be appropriately designed for oncology applications are highlighted and ways to overcome these are discussed. Strategies developed to evade the immune response are also described and targeted gene delivery is examined with the most effective strategies highlighted. Finally, this chapter proposes a new way forward based on a growing body of evidence that supports a multifunctional delivery approach involving the creation of vectors, with a unique molecular architecture designed using a bottom-up approach.

Keywords: Cancer gene therapy, Multifunctional delivery systems, Non-viral gene delivery, Bio-inspired vectors, Tumour-targeted delivery

1. Introduction

Progress in the treatment of cancer in recent times has been unprecedented as cancer survival in the UK has doubled in the last 40 years, with 50% of adult cancer patients diagnosed in 2010–2011 in England and Wales predicted to survive 10 years or more [1]. Improvements in cancer screening techniques have led to improved prognosis through early detection, with breast cancer screening estimated to prevent up to 1,300 deaths per year; women who are diagnosed with the earliest stage of breast cancer have a 90% 5-year survival rate [2]. Likewise, if diagnosed early, prostate cancer responds well to treatments such as hormone therapy, with 65–90% of men diagnosed in stage 1 or 2 likely to live at least 10 years post diagnosis [3]. Despite improvements in screening and early detection methods, conventional treatment options are not always effective. Mainstay cancer treatment options including radiotherapy, chemother-

apy, and surgery are extremely arduous on patients, and often have only moderate success. Existing anticancer drugs are generally cytotoxic, but lack specificity for the target tumour, which results in severe side effects. Not only do these traditional therapies cause damage to healthy cells, they are rarely effective against all transformed cells, and sometimes lack potency entirely. Failure of treatment can result in disease recurrence, often in a more aggressive form, with chemo- and radio-resistant aggressive malignancies ensuing. Resistance to conventional therapy causes treatment failure in over 90% of patients with advanced metastatic cancer [4].

Gene therapy is an exciting research area that involves the delivery of genetic material into cells to alter their function. Diseases that arise consequential of anomalous DNA (e.g., cystic fibrosis [5, 6]) are appropriate for gene therapy intervention; as cancer's origins lie in DNA damage, this group of diseases is also particularly suitable for gene-based therapeutics [7]. The strategy of gene therapy is generally to replace or repair faulty genes by the transfer and insertion of corrective or therapeutic genes [8]. Alternatively, the strategy of gene therapy can be the supraphysiological expression of cytotoxic proteins, or the expression of proteins to affect metabolism of prodrugs, for direct or indirect cytotoxic effects, respectively [9]. Silencing of problematic genes is a nascent and very popular strategy that uses RNAi therapeutics to inhibit the expression of certain undesirable genes at the post-transcriptional level [10]. Some of the most common strategies of cancer gene therapy include suicide gene therapy, tumour suppressor gene therapy, antiangiogenic therapy, and cancer immunotherapy [11].

1.1. Tumour suppressor gene therapy

Mutation of tumour suppressor genes, such as p53, has been highlighted as a mechanism of proliferation and resistance in some cancers. Functional p53 interacts with other cellular pathways including the death-receptor pathways and caspases, as well as inhibiting anti-apoptotic mediators, such as the BCL family, leading to the suppression of tumour growth [12]. For this reason, the delivery of transgenes that encode tumour suppressor genes, including genes encoding p53, IL-2, EGFR, and E1A, has received considerable attention. Promising results have been obtained, with Senzer et al. reporting the systemic administration of targeted liposomal nanoparticles with an anti-transferrin receptor targeting moiety (TfR); they delivered the p53 tumour suppressor gene in complexes known as SGT-53 for advanced solid tumours in a phase I clinical trial [13]. SGT-53 was administered to patients with a range of advanced cancer types, including cervical cancer, thyroid cancer, and colorectal cancer. Median survival was 340 days and 7 of the 11 patients treated exhibited stable disease at 6-week assessment, with one patient reclassified from inoperable to operable due to significant tumour necrosis. The authors also demonstrated the tumour targeting ability of SGT-53 with biopsies of tumour tissue and normal tissue; normal tissue showing negligible exogenous p53 levels. However, the main aim of the study was to assess safety and further studies would be required to fully assess therapeutic effects.

1.2. Suicide gene therapy

Suicide gene therapy, also known as gene-directed enzyme-producing therapy (GDEPT), involves the administration of an enzyme-encoding transgene to the tissue, with a separate

administration of a relatively innocuous prodrug. Transgene expression produces the enzyme within cancerous cells and subsequently the prodrug is converted to its toxic form by this enzyme [14]. GDEPT strategies include cytosine deaminase/F-fluorocytosine (CD/5-FC), where the CD transgene metabolises 5-FC to 5-Fluorouracil (5-FU); herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) where HSVtk converts GCV to its cytotoxic triphosphate derivative; and *E. coli* nitroreductase/CB1954 (NTR/CB) where NTR activates the prodrug CB1954 resulting in toxicity to tumour cells [15]. Multiple GDEPT systems have made it to clinical trials and Sangro et al. reported on a recent phase 1 clinical trial of HSVtk/GCV in the treatment of advanced hepatocellular carcinoma. Intra-tumoural injection of the TK gene in a replication deficient adenovirus vector (Ad-TK) was followed with systemic administration of GCV in 10 patients [16]. Although the main aims of the trial were to assess feasibility and safety of treatment, anti-tumour effects were also assessed. Stabilisation of tumour was observed in 60% of patients, with two patients who received the high dose treatment showing tumour necrosis and one patient surviving for 26 months. Such therapies, which may be used in addition to radiotherapy, have the advantage of being activated only in the cancerous cells due to direct intra-tumoural delivery, reducing toxic side-effects to normal cells. A 'bystander effect' where neighbouring cells receive the toxic treatment through gap junctions has also been observed, which could be beneficial for therapy as reduced amounts of treatment are needed for the same therapeutic effect; conversely, this bystander effect may limit the potential of the therapy if neighbouring healthy cells receive the toxic treatment. However, problems in vector development still need to be overcome before these treatments are to be successful [17]. Despite progression to clinical trials, no GDEPT therapy has made it to the market and the use of such treatment strategies may be limited to locally available tumour sites due to the need for intra-tumoural injection.

1.3. Anti-angiogenic therapy

In contrast to suicide gene therapy and tumour suppressor therapy, which are quite specific in focus, targeting angiogenesis may attack the root of the greater tumour establishment. There are various 'classical' protein-based angiogenesis inhibitors, including receptor tyrosine kinase inhibitors, which block the activity of vascular endothelial growth factor (VEGF), and monoclonal antibodies against VEGF-A such as bevacizumab (Avastin®). However, as angiogenesis is required for normal function in the body, such as wound healing, complete blockade of angiogenesis is not desirable. In addition, it may seem that rather than kill tumours, inhibitor therapy may merely retard further tumour growth. Moreover, the existence of various resistance mechanisms to angiogenic inhibitors, including alternative signalling pathways poses major drawbacks to such therapy. Alternatively, a gene therapy approach targeting the genes behind the pro-angiogenic factors may be more suitable [18, 19]. Doan et al. described a gene silencing approach that halts the effects of the pro-angiogenic factors VEGF and kinesin spindle protein (KSP), which play a critical role in cellular proliferation [20]. Hep3B hepatocellular carcinoma cells were treated with a cocktail of anti-VEGF and anti-KSP siRNAs, and a significant reduction in both VEGF and KSP expression was observed; in vitro, this manifested reduced proliferation of the cells, assessed by WST-1 assay and clonogenic survival assay. The results demonstrate the potential for anti-angiogenic gene therapy, but translation to in vivo studies is required to establish this further.

1.4. Cancer immunotherapy

Cancer immunotherapy is the process of harnessing the immune system to attack cancer cells. Cancer cells present antigens, known as tumour-associated antigens (TAAs) or tumour-specific antigens (TSAs) on their surface; recognition of these antigens by immune cells has been exploited in the development of cancer DNA vaccines. Cancer vaccines may be prophylactic or therapeutic in their design, which would generate an active immune response specifically to tumours while also providing memory cells to control future recurrence [21]. DNA encoding the genes for TAAs is delivered to cancer cells that subsequently express the transgenic TAA, eliciting an immune response against the tumour cells and many cancer DNA vaccines are being assessed in clinical trials [22]. Chudley et al. reported on a phase I/II clinical trial of a DNA vaccine encoding a domain (DOM) from fragment C of a tetanus toxin linked to an HLA-A2-binding epitope from prostate-specific membrane antigen (PSMA) in patients with prostate cancer [23]. Following intramuscular administration of the DNA vaccine to 30 patients, 29 had a measurable CD4⁺ T-cell response and PSMA-specific CD8⁺ T cells were detected in 16/30 patients. As a result, PSA doubling time increased significantly from 11.97 months pre-treatment to 16.82 months denoting slower progression of the disease. Staff et al. reported on a phase I clinical trial in patients with colorectal cancer with a DNA vaccine [24]. The plasmid vaccine was administered by Biojector® and encoded human carcinoembryonic antigen (CEA), which is known to be over expressed by a large number of epithelial neoplasias, including colorectal cancer. No serious adverse effects were observed with the vaccine and of the 10 patients, 8 showed no evidence of disease at follow-up. However, despite the promising trial results and 4 DNA vaccines licensed for use in animals including Oncept® for Canine Melanoma [25], no product has made it to the market in humans. Many trials use direct injection of the vaccines to tumours and efficiency may be enhanced if delivery vectors were to be used, which could maximise transduction.

To date, cancer has been the most common disease focus for gene therapy, with 64% of all ongoing gene therapy clinical trials targeting a malignancy [26]. However, the progress of gene therapy beyond clinical trials has been disappointing, with only three products currently having made it to the market, namely, Gendicine®, Oncorine®, and Glybera®. Gendicine® and Oncorine®, which deliver p53 tumour suppressor genes for the treatment of head and neck cancer, are licenced in China; while Glybera®, used for the treatment of severe lipoprotein lipase deficiency, is the only gene therapy product licensed in Europe [27]. Despite the many promising therapeutic strategies for gene therapy, the common limiting factor has been the lack of a suitable delivery vehicle that has the ability to specifically target tumour cells, whilst being non-immunogenic and non-toxic. Consequently, a vast amount of research has therefore focused on delivery systems for gene therapy. In order for the potential of gene therapy to be realised, the focus needs to be on the design of an appropriate delivery vehicle that will meet all the demands in terms of functionality and satisfaction of regulatory bodies.

2. The biological barriers to gene delivery

The safest way to deliver gene therapy is by direct administration of the therapeutic to the target site. However, this is extremely inefficient, unreliable, and feasible only in tumours in

superficial sites. Generally, gene therapy approaches are delivered via the intravenous route; as nucleic acids are susceptible to degradation by nucleases and rapid clearance in systemic circulation [28], a vector is required to package, protect, and transport the genetic material to its site of action.

Viral vectors, derived from naturally evolved viruses capable of transferring their genetic material into host cells, remain the most efficient gene delivery agents [29]. However, difficulty in large scale production, limitation in size of DNA that can be carried, and concerns about mutagenesis, toxicity, and immunogenicity have hindered the progression of viral vectors [30, 31]. As a result, much research has focused on the design of non-viral vectors, which have the potential to circumvent the problems associated with viral vectors [32]. Non-viral gene delivery encompasses a wide variety of delivery systems including cationic polymers, liposomes, proteins, and peptides that have the ability to package nucleic acids and deliver them into cells [33]. However, transfection efficiency of non-viral vectors remains significantly lower than viral vectors [34], and many factors are to be considered and hurdles overcome when designing an efficient non-viral delivery system. Successful gene therapy relies largely on the development of an efficient vector that can overcome the various extracellular and intracellular barriers to deliver the genetic material to its target site [35].

2.1. Extracellular barriers

Although delivery vectors have the ability to protect the DNA from endonuclease attack, the vectors themselves may also be susceptible to recognition and clearance. In the systemic circulation, vectors may be rapidly cleared from circulation by the reticulo-endothelial system (RES), also known as the mononuclear phagocyte system (MPS) [36]. Many non-viral delivery vectors are cationic in nature, a desirable characteristic for condensing DNA and promoting cellular uptake. However, this cationic nature can be problematic for systemic administration due to interaction with blood components, such as serum proteins, which may result in opsonisation. Consequently, large aggregates are formed that cannot traverse cell membranes and may become lodged in microvascular networks or accumulate in MPS organs such as the liver or spleen [37]. Further to this, cationic systems may interact with cell membranes indiscriminately, affecting normal cells as well as cancerous cells, and strong cationic charges can induce damage of cellular membranes and apoptosis [38]. Thus, a balance must be reached in the design of a delivery vector such that the nucleic acid/vector complex has cationicity of appropriate magnitude, so as to permit proper association with target cells whilst preventing aggregation.

The circulation of gene therapy delivery systems is often cut short due to rapid hepatic metabolism and clearance, and often this clearance occurs before the particles can reach their target site to deliver the therapeutic. The use of 'stealth particles', such as those that contain polyethylene glycol (PEG), has been shown to increase the circulation time of various delivery systems by shielding the charge of the particles, reducing binding with serum proteins and aggregation, whilst evading the immune system [39]. Various strategies in evading this clearance have been employed, which will be discussed in a later section. However, if the vector can avoid clearance, extravasation from blood circulation needs to occur in order to reach the tumour cells, which can be hindered by the chaotic blood supply, poor permeability, and high interstitial pressure within the tumour [40].

2.2. Intracellular barriers

Surviving the systemic circulation and reaching the target cell is not the only hurdle faced by non-viral gene delivery systems. The nature of conventional gene therapy requires the genetic material to be transcribed by the cell, which requires delivery to the nucleus (or in the case of siRNA technology, delivery to the cytoplasm). A number of intracellular barriers exist that may impede this delivery include traversing the cell membrane, escape from the endosome, and release of the nucleic acid payload into the cytoplasm, followed by active transport to the nucleus with subsequent nuclear import.

2.2.1. Cell membrane/internalisation

Cell membranes are lipophilic anionic structures that are generally impermeable to large macromolecular anionic nucleic acids [32]. Non-viral gene delivery systems aim to complex nucleic acid cargo, thereby masking their native negative charge, to give an overall net cationic complex capable of interaction with cell membranes. Not only does this allow for electrostatic interactions between the vector and the membrane, it also condenses the DNA to a size suitable for cellular uptake (≤ 200 nm diameter). Various pathways of cellular uptake exist that are size dependent. For example, the cell penetrating peptide TAT, derived from the human immunodeficiency-1 virus (HIV-1) [41], enters cells via different routes depending on the size of the cargo. Larger cargoes of proteins or quantum dots that exceed the 500 Daltons restriction limit are internalised with TAT via the caveolae or macropinocytosis routes, and smaller cargoes such as peptides less than 30–40 amino acids via the clathrin route [42]. Further to this, the internalisation route may also depend on other factors such as cell type, receptors present on the cell, temperature, incubation time, concentration of the vector, and properties of the vector including cargo and linkage type [7, 43]. Different internalisation pathways also have an effect on the fate of the vector once inside the cell. As a result, much research has centred around elucidating the mechanisms involved in cellular uptake in order to improve the efficiency of gene therapy [44]. Endocytosis (clathrin-mediated, caveolae-mediated, or macropinocytosis) is thought to be the main uptake pathway for most gene delivery.

2.2.2. Clathrin-Mediated Endocytosis (CME)

CME is the most well-defined route of endocytosis and involves the internalisation of cargo via receptors on the cell membrane, such as proteoglycans, into vesicles known as clathrin coated pits, which are about 100–150 nm in diameter. These pits are transported via microtubules of the cell cytoskeleton deeper into the cell, where they form endosomes (acidic, degradative compartments that transport material back to the membrane for recycling, or to lysosomes for degradation). The term 'receptor-mediated endocytosis' is often used to describe CME, however, endocytosis via receptors does not exclusively occur via CME [45]. The addition of ligands, such as transferrin, to delivery systems has allowed for targeting to cancer cells overexpressing the transferrin receptor that binds to the ligand and facilitates internalisation via CME [46].

2.2.3. Caveolae-Mediated Endocytosis (CvME)

CvME is initiated by flask-shaped invaginations known as caveolae that have lipid-raft formations involving cholesterol and sphingolipids, which are around 50–200 nm in diameter. Internalisation occurs in an actin-dependent manner, forming a type of endosome known as a caveosome. Caveosomes are not as acidic or destructive as CME endosomes, but can still ultimately merge with the lysosomal machinery [47]. It has been observed that many commonly used cancer cells lines (e.g., PC-3 prostate cancer cells) lack the ability to form caveolae that may have significance for delivery systems relying on this route for internalisation [48]. Furthermore, it has been observed that caveolae may be upregulated in some cancer cells providing a possible target for delivery systems. Nguyen et al. reported that a polysorbitol-mediated transporter (PSMT) was used to deliver plasmid DNA encoding the p53 tumour suppressor gene into human cervical cancer (HeLa) cells and normal human diploid fibroblast (HDF) cells. PSMT entered cancer cells selectively via CvME with transgene expression resulting in cellular damage and apoptosis [49].

2.2.4. Macropinocytosis

Macropinocytosis involves the uptake of large amounts of fluid-phase materials. It occurs via an actin-driven mechanism that causes ruffling of the cell membrane to form protrusions that engulf the extracellular material into macropinosomes, which eventually merge with the endosomal pathway [50]. Anaka et al. reported that macropinocytosis was the main cellular uptake pathway of the peptide STR-CH2R4H2C when complexed with plasmid DNA and delivered to COS7 kidney fibroblast cells, attributing the position of arginine residues exposed on the surface of the complexes as the reason for this internalisation route [51].

2.2.5. Direct internalisation

Cationic vectors, especially those rich in arginine, have been observed to enter cells via non-endocytic routes through direct internalisation triggered by non-specific electrostatic interactions [52]. This form of internalisation is a more attractive route for non-viral gene delivery, as direct delivery into the cytoplasm avoids the endosome. In the case of arginine-rich cell penetrating peptides, an initial electrostatic interaction with the cell membrane is followed by formation of a peptide-cargo-phospholipid complex with the positively charged guanidinium group of arginine bound to the phosphate groups of the phosphatidylcholine (PC) and/or sphingomyelin (SM) of the outer leaflet of the cell membrane. A 'capacitor' is then formed between the cationic arginine residues and the anionic phosphatidylserine creating an electric field strong enough to form a reversible pore, which allows the CPP-cargo to pass through the membrane [53]. Arginine-rich peptides, such as octa-arginine (R8), have therefore been utilised for gene delivery due their strong cell penetrating ability. However, little is known about this entry route, and evidence suggests that vectors can enter cells via multiple mechanisms [54]. Understanding the various routes through which vectors can enter cells can aid the gene therapist in the design of vectors; ensuring appropriate size and charge, for example, can allow for targeted internalisation via a specific mechanism.

2.3. Endosomal entrapment

Following endocytosis, vectors may be trapped in the endosomal pathway. Endosomal entrapment poses a major limiting step to efficient gene therapy. The endosomal compartment provides cells with a way of regulating what enters and leaves the cell and material within the endosome can be either recycled to the cell membrane or progressed to lysosomes. It is essential that therapeutics escape the endosome in order to avoid degradation of the nucleic acid payload [55]. Endosomal escape can be achieved by different mechanisms and typically non-viral delivery systems are designed to facilitate this. The 'proton sponge' effect is exploited by polymers that contain amine groups such as polyethylenimine (PEI) or by histidine-rich peptides, which have been used in many delivery systems for gene therapy [56]. Fusogenic peptides evoke membrane destabilisation by interacting with anionic lipids in the endosomal membrane, thereby disrupting the membrane, allowing release of the endosomal contents [57]. INF-7 peptide is an example of a synthetic fusogenic peptide derived from influenza virus hemagglutinin protein, which enhances endosomal escape. Oliviera et al. report that the addition of INF-7 peptide to Lipofectamine for delivery of anti-kRas siRNA resulted in 3.5-fold improved gene silencing effect and subsequent reduction in kRas protein expression in C26 murine colon carcinoma cells in vitro when compared to Lipofectamine/siRNA complexes alone [58].

2.4. Intracellular trafficking

Following endosomal escape, the vector and its cargo must be delivered to the correct cellular compartment, i.e., DNA delivered to the nucleus, or siRNA assembly into RNA-induced silencing complexes (RISC) in the cytoplasm [59, 60]. However, this is not without its challenges due to the restricted movement of macromolecules in the cell, slowing the mobility of vectors towards the nucleus [61], while endonucleases may degrade any naked nucleic acid [62]. The vector is therefore required to protect and transport the nucleic acid through the cytoplasm in order to reach its target organelle. Movement in the cytoplasm is restricted due to overcrowding of organelles, the cell cytoskeleton and high protein concentrations, which collectively result in a major impediment to non-viral delivery of even relatively small cargoes [63]. A network of microtubules and associated motor proteins (dyneins and kinesins) are responsible for the maintenance of correct organelle location [64] and intracellular transport of vesicles, lysosomes, and endosomes [65]. If non-viral gene delivery vectors could utilise the microtubule network within the cell, it would serve as a direct route to the nucleus and transfection efficiencies may be greatly improved. Toledo et al. presented a recombinant fusion protein based on the dynein light chain LC8 that facilitated plasmid DNA uptake into HeLa cells and transported DNA via microtubules to the nucleus for GFP transgene expression [66].

2.5. Nuclear import

In the case of DNA gene therapy, once the vector reaches the nucleus, it must gain entry and deliver its genetic payload in order for the gene to be transcribed and elicit its effect. The nucleus is protected by a bilayer known as the nuclear envelope, and entry into the nucleus through the nuclear envelope is tightly controlled by the nuclear pore complex (NPC) [67].

The NPC only allows the passive entry of molecules that do not exceed 10 nm in diameter, which limits the entry of DNA; active traversing of the nuclear envelope is hence required. The NPC therefore poses the last major hurdle to gene therapy and is a huge rate-limiting step in transfection efficiency. The addition of short amino acid sequences known as nuclear localisation signals (NLS) to vectors has been useful in trafficking and facilitating nuclear entry [68]. The nuclear localisation signal from the simian virus 40 (SV40), large tumour antigen has been used to enhance transfection efficiency in many delivery systems. Wang et al. demonstrated that the addition of SV40 NLS to R8 resulted in a transfection efficiency of up to 80% as effective as jetPEI™ (transfection reagent) with no cytotoxic effects in HeLa cells [69].

Understanding the various barriers to gene delivery allows the rational design of delivery systems that can overcome these hurdles. The ideal non-viral gene delivery vector is a multi-functional system with the ability to condense DNA effectively, overcome the various intra- and extracellular barriers and must also be non-toxic and non-immunogenic. Furthermore, vectors can be designed specifically to exploit the characteristics of cancer cells and tumours, including the enhanced permeability and retention (EPR) effect associated with tumour vasculature; where gene therapy delivery systems exploit the permeability of the tumour vasculature to localise and accumulate in the tumour through passive diffusion [70]. Other factors, including tumour microenvironment, and the aberrant expression of certain enzymes and proteins commonly associated with cancer cells may also be targeted or exploited. Figure 1 represents how a multi-functional non-viral gene delivery system may be composed.

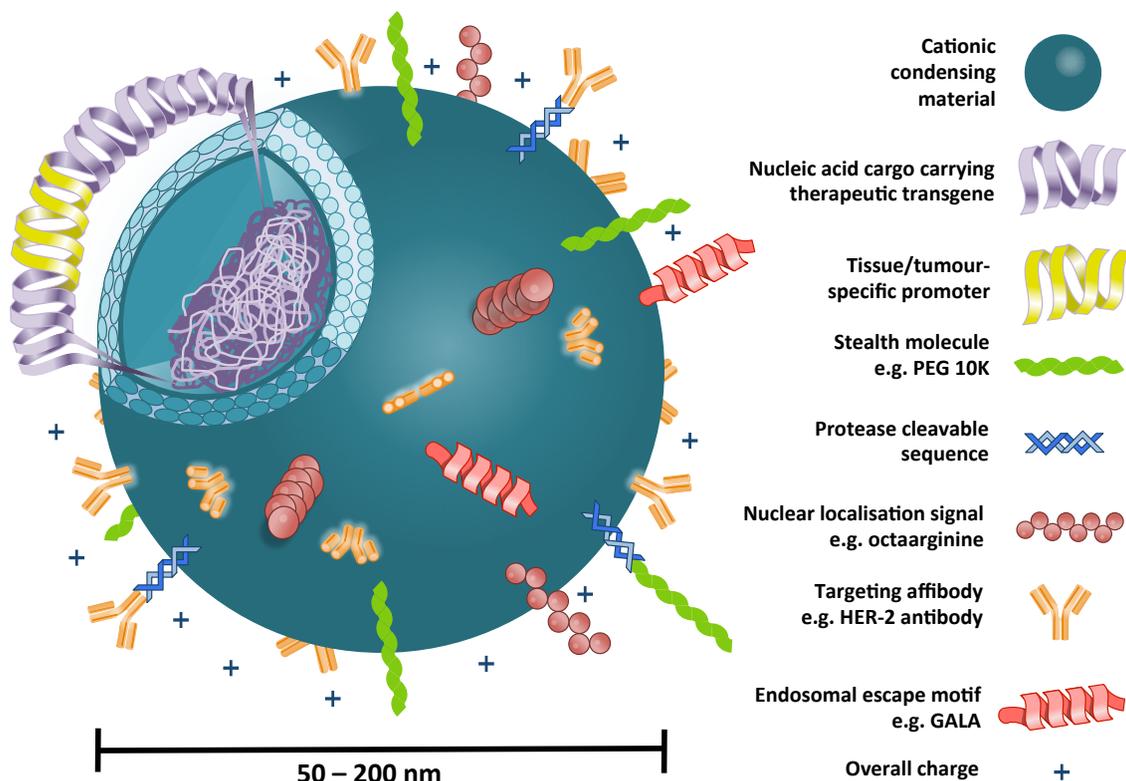


Figure 1. Schematic of a multi-functionalised vector for therapeutic transgene delivery.

Anionic plasmid DNA cargo is condensed using a cationic material such as poly-L-lysine or protamine. Vectors are functionalised with adjuncts to aid in evasion of the various barriers that are posed to gene therapy strategies, as highlighted above. The various functional groups will be discussed below.

3. Evading the immune response

When gene delivery systems are administered systemically they are usually cleared rapidly from circulation, mainly by Kupffer cells in the liver and macrophages in the spleen. This is a form of defence by the host designed to recognise and clear potentially harmful invaders from the system as quickly as possible, and involves a two-step process initiated by opsonisation with subsequent phagocytosis [71]. Opsonisation is the adsorption of foreign particles by opsonin proteins such as immunoglobulins, blood serum proteins, and complement proteins. Subsequently, macrophages may bind directly to the opsonised particle, engulf and remove it from circulation or the complement system may be activated, also leading to phagocytosis [72]. The characteristics of the particle in circulation play an important role in the recognition process and therefore are extremely important parameters to consider in the design of a delivery vector. Particles larger than the renal threshold of approximately 5,000 Daltons (usually greater than 200 nm hydrodynamic radii) are more likely to activate the complement system and are usually cleared more rapidly than their smaller counterparts. Surface charge, hydrophobicity, and the presence of certain functional groups are also important, with a more cationic nature favouring interaction with the anionic blood proteins and enhancing opsonisation [73].

Initial opsonisation of particles is critical to their subsequent removal, so if opsonisation can be reduced or avoided, then clearance may be circumvented. An extensively used method to overcome opsonisation is the utilisation of shielding groups or 'stealth' molecules that are generally long hydrophilic polymer chains. These are typically flexible and charge neutral, which can block the electrostatic and hydrophobic interactions between opsonins and the nucleic acid/vehicle complex, improving the stability of the particles in the systemic circulation. Various polymers have been used including polyacrylamide, poly(vinyl alcohol), poly(N-vinyl-2-pyrrolidone), and poly ethylene glycol (PEG) [72].

PEG is the most commonly used and effective polymer for stealth molecules; PEG is non-toxic, non-immunogenic, non-antigenic, highly water-soluble, and FDA approved. PEGylating a cationic complex shields the positive charge, thereby reducing interaction with blood components, and inhibiting clearance, allowing increased circulation time and opportunity for vectors to reach their target site. It also reduces non-specific binding to non-target cells and stabilises particles, reducing aggregation. The increased circulation time is highly desirable for passive tumour targeting, facilitated by the leaky tumour vasculature. Extravasation of vectors from the blood stream occurs with retention and accumulation in the tumour site by the EPR effect. It has been suggested, however, that repeat administrations may sensitise the immune system to PEG resulting in rapid clearance of PEGylated liposomes from circulation and

formation of anti-PEG antibodies [74, 75]. However, the validity of the assays used to test for anti-PEG antibodies have been questioned over flaws and lack of specificity [76].

In order for PEG to properly oppose the attractive forces between the opsonins and the cationic particle surface, it must have a sufficient surface coverage, which is usually correlated to the molecular weight, surface chain density, and conformation of PEG. It is generally held that sufficient stealth character is achieved with a molecular weight of 2,000 Daltons or more, with loss of flexibility in shorter chains being the probable cause for lack of stealth. As molecular weight increases, the blood circulation half-life also increases. Surface chain density and conformation are also important so that adequate surface coverage is achieved to avoid gaps where opsonins may bind, while also maintaining flexibility in the PEG layer responsible for the steric hindrance properties. By fine-tuning such properties of PEG, an improved biodistribution and the pharmacokinetic profile of the therapeutic may be achieved; such tunings have led to many different PEGylation strategies being developed [72].

3.1. The PEG dilemma

A major problem with the use of PEG for cancer gene therapy is that it may hinder gene expression by impeding the entry of the delivery system into tumour cells. The initial interaction of cationic delivery systems with cell membranes relies on electrostatic association, so masking by PEG may have an unfavourable effect. Further to this, the improved stability of PEGylated particles disrupts membrane fusion and may reduce the effects of fusogenic peptides either during cell internalisation or for endosomal disruption. The term 'PEG dilemma' was coined to describe the balance that must be struck between availing of the beneficial characteristics that PEG provides while not being limited by them. Appropriate vector design must ensure that an appropriate balance is struck between the facets that make PEG an attractive supplement to a vector and those that limit its effectiveness [77].

Various strategies have been employed in order to overcome the problems posed by the PEG dilemma. Once the PEGylated vector has survived in circulation and reaches its target cell, the PEG chain becomes redundant. By removal or detachment of PEG from the surface of the vector, interaction with the cell membrane can occur and initiate internalisation. One strategy that has gained much attention is the addition of targeting ligands to PEG that bind to cell surface receptors exclusive to the target cells, limiting endocytosis of the PEGylated delivery system to the target cells [78]. However, this may involve the introduction of a charged moiety onto the PEG, reducing the effectiveness of PEG in evading clearance. The bulky PEG chain may also still hinder the gene delivery system overcoming the various intracellular barriers discussed above. Therefore, the design of a detachable or reversible PEGylation has resulted in 'smart' delivery systems that can exploit different aspects of the intracellular or extracellular tumour environment, including pH, enzyme complement, or reduction, while also functioning as a targeting tool to direct vectors to tumours [79, 80].

3.2. pH-sensitive PEG linkers

Using linkages such as ester and hydrazine bonds, which are stable in circulation but hydrolysed in acidic conditions, is a promising way of creating a detachable PEG. The acidic tumour microenvironment may cleave off the PEG chain, thereby releasing the therapeutic at the target

site and allowing interaction of the cationic delivery system with cell membranes, initiating internalisation. Alternatively, the acidic pH within the endosome may also serve to cleave PEG from the delivery system after receptor-mediated endocytosis. This will unmask the vector allowing endosomal escape by, for example restoring fusogenic activity, facilitating cytosolic delivery and subsequent gene expression [79]. Fella et al. described a targeted polyplex system with PEG attached via an acid labile hydrazone linkage that afforded a 14-fold increase in transgene expression in HUH7 hepatocellular carcinoma tumours compared to the non-acid sensitive formulation. The system was able to protect the vector in the systemic circulation, facilitate entry to the cells via EGF-receptor mediated endocytosis, and exploit endosomal pH to execute the removal of PEG, which permitted release of the vector from the endosome [81].

3.3. Enzymatic cleavage of PEG

Various proteolytic enzymes are known to be secreted into the extracellular environment by cancer cells. The knowledge of specific enzymes and their substrates can then be exploited to tether PEG to a vector via an appropriate enzyme-cleavable linker. Matrix metalloproteinases (MMPs) are a family of proteases commonly secreted by tumours, degrading the extracellular matrix facilitating growth and progression of tumours [82]. Li et al. took advantage of the presence of MMP-7 proteases in the extracellular environment by functionalising polymeric nanoparticles with PEG via a MMP-7 cleavable linker for delivery of anti-luciferase siRNA. The authors reported a 2.5-fold increase in transfection efficiency in MDA-MB-231 breast cancer cells in the presence of MMP-7 *in vitro* compared to transfection efficiency in the absence of MMP-7 [83]. These results, however, would need to be further reinforced with *in vivo* studies in order to fully assess the pharmacokinetic profile of this system.

3.4. Reduction-sensitive PEG linkage

A reduction-sensitive linkage may be used to attach PEG to a vector using disulphide bonds. These bonds are susceptible to reduction by glutathione (GSH), a peptide with various functions within the cell such as antioxidant defence, metabolic processes, and regulation and maintenance of cellular redox status. The intracellular concentration of GSH is three orders of magnitude higher than in the extracellular compartment [84], which allows for reduction of the disulphide bonds and detachment of the bulky PEG chain once the functionalised vector is inside target cells. Alternatively, extracellular reduction may occur through the action of thiol-containing cell surface receptors [85]. Lei et al. described a targeted delivery system functionalised with PEG attached via a disulphide linkage. Polyethylenimine (PEI) nanoparticles that were functionalised with a reduction-sensitive linked PEG were twice as potent as their counterparts that lacked the reduction-sensitive linker in terms of GFP and RFP reporter gene delivery *in vitro* and *in vivo* in U87 glioblastoma tumours [86].

3.5. Copolymers

While the use of a cleavable linker for PEGylation has shown promise for gene delivery, issues may arise if the linker is not accessible for cleavage due to the shielding action of PEG. The nature of polymers renders them easily modifiable; changing the characteristics of the PEG

polymer itself by forming crosslinks may produce a degradable copolymer suitable for controlled release [79]. Fan et al. reported on a copolymer that comprised polyethylene glycol 5000 (PEG114), Vitamin E (VE), and thiocetic acid (TA), termed PEG114:VE:TA, which assembled into micelles with poly-disulfide crosslinks [87]. The copolymer resulted in improved thermodynamic and kinetic profile of the anticancer drug paclitaxel. Reduction of the disulfide crosslinks occurred in response to glutathione causing rapid disassembly of the micelles and accelerated drug release that resulted in approximately 3-fold higher plasma concentration than the non-crosslinked micelles leading to increased drug accumulation in the SKOV-3 human ovarian cancer xenograft mouse model. Although this study did not deliver gene therapy, it demonstrates the potential of modifying polymer crosslinks to achieve desirable characteristics for drug delivery.

The production of copolymers, which combine the characteristics of more than one polymer, has shown promise where a balance is struck between PEGylation and copolymer reducible characteristics. Recently, Lai et al. presented a reducible copolymer comprising poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide)-SS-P[Asp(DET)] (P(EPE)-SS-P[Asp(DET)]), which possesses a redox potential-sensitive disulfide linkage between the P(EPE) polymer and the cationic block P[Asp(DET)]. The copolymer was used to deliver the pGL4 DNA vector for luciferase expression, and a 2-fold increase in transfection efficiency was observed compared to delivery with non-reducible copolymer counterparts in MDA-MB-231 breast cancer cells *in vitro* [88]. However, much work is yet to be done to elucidate the exact characteristics and polymer design needed to produce optimal transfection efficiencies.

Systems that combine two or more mechanisms for masking delivery vectors while improving uptake have been investigated, with PEG being combined with other polymers or peptides. Huang et al. designed a multifunctional delivery system that uses a combination of an MMP-sensitive linkage, a pH-sensitive mask to quench the cationic charge of nona-arginine (R9), and PEG to improve steric stabilisation in circulation [89]. The masking peptide was pH-sensitive with an isoelectric point (pI) of 6.4, affording the masking peptide a negative charge at physiological pH, which interacts with the cationic R9 cell-penetrating peptide (CPP). However, a tumour's acidic environment neutralises the masking peptide, allowing the cationic nature of R9 to come to the fore. Cleavage of the PEG by MMP-2 allowed the CPP-cargo complex to enter cells. The authors used *in vivo* imaging to demonstrate the specificity of these nanoparticles to target human hepatocellular carcinoma cell (BEL-7402) xenografts.

Although PEGylation provides a means of enhancing circulation times, allowing vectors to reach their target site, reliance on the EPR effect as a means of passive targeting may not be as reliable as initially thought. The variability displayed in tumour biology as well as the disordered and discontinuous tumour vascular structure means that the accumulation of delivery vectors by the EPR effect may not give a tumour-wide distribution. Although it may give an added advantage, total reliance on the EPR effect cannot give reliable results and so there is a need for an active targeting strategy [90].

4. Targeting in non-viral systems

Early approaches to gene therapy involved direct introduction of genetic material into locally accessible tumours. While this proved useful in some cases, the invasive nature of these methods renders them impractical for internal and disseminated tumours. Therefore, a systemically administered cancer gene therapy vector that can target tumours is ideal. Traditional chemotherapeutic cytotoxic drugs cause such harsh and debilitating side effects because they affect rapidly dividing cells and do not differentiate between normal or cancerous cells. In order to avoid these off-target effects, it is necessary to target the therapeutic directly to the cancer cells without affecting normal healthy cells. Improved knowledge of cellular, metabolic, and signalling pathways essential for tumour growth has led to the identification of targets on cancer cells [91]. Different types of cancerous cells tend to have distinct characteristics, which set them apart from normal cells, meaning that a range of potential molecular targets exists that can be targeted to direct gene therapy towards tumours. Cancer cells typically overexpress certain proteins on their surface, which may be exploited through targeting strategies; commonly overexpressed proteins include integrins [92], transferrin receptors [46], epidermal growth factor receptors (EGFR) [93], folate receptors [94], and proteoglycans [95], and targeting such receptors has been found to increase the specificity and efficacy of drug delivery, while reducing side effects [96]. Active targeting using ligands that target overexpressed receptors specific to cancer cells is therefore an attractive targeting strategy for systemically administered non-viral gene delivery and much research has focused around this.

4.1. Targeting ligands

Through phage display techniques, ligands for specific receptors commonly overexpressed on cancer cells have been successfully identified and incorporated into vectors [97]. When the delivery vector reaches the tumour environment, the overexpressed receptors bind the ligand on the surface of the vector and it is subsequently internalised via receptor-mediated endocytosis. This strategy serves to both target the cells and facilitate uptake by cells, but the endocytic pathway used can depend on the targeting ligand and cell type [98]. RGD peptide is a commonly used peptide targeting ligand. It is a tripeptide of Arg-Gly-Asp that was derived from fibronectin, which mediates cell attachment. RGD peptide is involved in cell adhesion to cell surface integrins [99]. Integrin receptors, such as $\alpha\beta3$ integrin and related αv -integrins, are commonly upregulated on the surface of angiogenic endothelial cells and can have a profound effect on the ability of tumours to survive and progress through regulation of stemness, metastasis, and resistance [100]. This activity makes integrins valuable for targeting aggressive cancers and various strategies have been used to incorporate RGD into non-viral delivery systems for targeting angiogenic tumours [101]. Kim et al. presented a tumour-targeting, RGD-conjugated, bio-reducible polymer for the delivery of vascular endothelial growth factor (VEGF) siRNA. The RGD-functionalised vectors showed 20–59% higher cellular uptake in MCF-7 breast cancer cells and PANC-1 pancreatic cancer cells compared to non-targeted vectors. In addition, MCF-7 and PANC-1 cancer cells had significantly reduced VEGF gene expression (51–71%) and cancer cell viability (35–43%) compared with control [102]. Nie

et al. described a dual functionalised system that boasts two targeting ligands, namely RGD and B6 peptide, which target transferrin receptors. Transfection efficiency of the dual targeting system resulted in 8- and 4-fold higher luciferase reporter gene expression compared to single targeted control formulations with either B6 or RGD in DU145 and PC3 prostate cancer cells, respectively [103].

When formulating a targeted non-viral gene delivery system, there are a number of factors to be considered that may have an effect on the overall efficiency of the delivery system, such as ligand density and positioning on the surface of the vector, vector size, and choice of targeting ligand [90]. Vector ligand density should be optimised to ensure efficient binding to receptors. Furthermore, the binding of a ligand to its substrate may facilitate binding of neighbouring receptors in a thermodynamically favourable way [104]. In addition, the strategies used to link the targeting moieties to vectors, as well as many other factors including relative specificity, expression of target receptors, and physiological factors need to be considered in order to improve effectiveness and avoid interference or loss of biological activity [105].

One such problem with active targeting is that it is dependent on the expression of certain receptors by tumour cells. In breast cancer, oestrogen, progesterone, and human epidermal growth factor (HER) receptors have been identified and targeted. However, 15% of breast cancers, termed triple negative, are defined by a lack of these receptors. Absence of these receptors makes such cancers difficult to target and treat, hence patients with triple-negative disease have poorer prognoses [106]. Therefore, there is a need for a more general targeting strategy that targets the common characteristics of cancer cells and is not reliant on the expression of any one receptor. This would also broaden the scope of disease states that may be treated using any individual gene therapy strategy, making them more marketable for the pharmaceutical industry. Additionally, drug resistance can develop if mutation of cancer cells affects the expression of the target receptors. The receptors may be down-regulated resulting in reduced targetability and subsequent reduced cellular uptake of the vectors, or conversely up-regulation of receptors could render the vector inefficient. Receptors may also be expressed in different isoforms, altering their recognition of the targeting moiety [107]. Moreover, heterogeneity of tumours may result in different levels of receptor expression within a single tumour [108].

Although ligand-targeted vectors have proven to be safe and efficacious in preclinical models, it has not yet been unambiguously proven that targeting ligands contribute to the efficacy of vectors, and it seems that targeting ligands do not cause localisation within the target tissue, but rather provide benefits in terms of internalisation to target cells and retention at the target site once the delivery system has arrived [109]. While this method of targeting may enhance non-viral gene delivery systems, it has not completely met expectations and other targeting strategies have been explored.

4.2. Targeting at the transcriptional level

In an attempt to overcome these problems with the targeting of proteins expressed by cancer cells, it has been suggested that targeting the upstream genetic causes of dysregulated genes may be more successful [110]. Regulation of gene expression at the transcriptional level for

cancer gene therapy can occur in a cell-specific manner with a focus on tissue-specific and tumour-specific promoters, or alternatively the use of inducible promoters, which allow gene expression to be controlled exogenously by factors such as heat or radiation. The major drawback with tissue-specific promoters, however, is that toxic gene expression occurs in all cells in the tissue, both healthy and cancerous, which limits use of this method to tissues that are not critical to the survival of the patient such as thyroid or prostate [111]. Use of inducible promoters to drive transgene expression requires the activation by exogenous factors, but the tumour specificity that this strategy affords may be useful in supplementing the potency of other therapies, such as the use of a radiation-inducible promoter for enhancement of iNOS transgene expression [112]; this dual approach to therapy can limit toxic effects in normal cells. However, for simplicity, we will focus solely on tumour-specific promoters.

The complex interplay of various factors involved in gene expression is often altered in cancer cells, and through exploiting this genetic signature of cancer, reduced off target effects and toxicity should result. Certain genes are upregulated in cancer through the over activation of transcription factors, which activate the upstream promoter of these genes. This can then be exploited to give tumour-specific targeting by using promoters that are activated by transcription factors known to be overexpressed in cancer cells to drive expression of the transgene in tumour cells only. Tumour-specific promoters are sub-categorized as follows: cancer specific promoters, tumour-type specific promoters, tumour microenvironment-related promoters, and tumour vasculature-related promoters, and are extensively reviewed by [113] and [114].

4.2.1. Cancer-specific promoters

The identification of genes that are expressed in cancer cells only may lead to targeting of cancer gene therapy in a broad sense regardless of cancer type. One such example is telomerase, involved in telomere maintenance, which is considered crucial in the progression and immortalisation of cancer cells and is expressed in the vast majority of cancers [115]. Telomerase expression is regulated by the human telomerase reverse transcriptase subunit promoter (hTERTp), which was recently used by Xie et al. in a non-viral delivery system. The hTERTp promoter was used to drive expression of a transgene amplification vector VISA (VP16-GAL4-WPRE integrated systemic amplifier) to target a phosphoprotein that is enriched in astrocytes (PEA-15) in advanced breast tumours. PEA-15 is known to affect signal-regulated kinase (ERK) in the cytoplasm, thereby inhibiting cell proliferation and inducing apoptosis [116]. Transgene expression was found to be highly specific, inducing cancer-cell killing in breast cancer cell lines (T47D, MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-361, MDA-MB-453, BT474, 4T1, SKBR-3) in vitro without affecting normal mammary epithelial cells (184A1 and MCF-10A). Furthermore, an in vivo study in a MDA-MB-231 xenograft mouse model demonstrated that the expression of PEA-15 driven by the hTERTp driven VISA vector prolonged mouse survival more effectively than PEA-15 driven by cytomegalovirus (CMV) promoter whilst showing no acute toxicities. The authors demonstrated that the use of the hTERT promoter achieved targeting and selective cell kill in triple negative MDA-MB-231 breast cancer cells, a selectivity that was lacking when transgene expression was promoted by the CMV promoter [116].

Survivin is a protein that functions in the inhibition of apoptosis, therefore, its overexpression in cancer cells can facilitate uninhibited growth. It is known to be upregulated in cancer cells and expression is controlled by various transcription factors including nuclear factor kappa B (NF- κ B), Runx2 and the Ras family that bind to the survivin promoter, triggering expression [117, 118]. The survivin promoter (pSURV) has therefore been incorporated into gene delivery systems to drive transgene expression preferentially in cancer cells. Qu et al. used pSURV to drive the expression of the herpes simplex virus thymidine kinase (HSVtk) gene for suicide gene therapy. The authors used pSURV/GFP to demonstrate that gene expression occurred in HepG2 hepatocellular carcinoma cells, while no gene expression was observed in LO2 normal human liver cells. Apoptotic rates of up to 55% were achieved in HepG2 cells with pSURV/HSVtk demonstrating the possibility of this system for suicide gene therapy. However, further in vivo studies need to be carried out to properly assess the targeting ability of this system [119].

4.2.2. Tumour-type specific promoters

Many different types of cancer overexpress various genes, which are characteristic of that tumour type, and the promoters responsible for this expression can then be exploited for tumour-type specific targeting. Osteocalcin is a protein normally found in the bone matrix but has been found to be elevated in cancers such as ovarian and prostate cancer and is associated with the progression and formation of bone metastases; McCarthy's group used the human osteocalcin promoter (hOC) for tumour-limited gene expression. It has been shown that hOC has strong promoter activity in cancer cells, with transcription factors such as Runx2 involved in gene upregulation [120, 121]. In this case, hOC was used to drive the expression of inducible nitric oxide synthase expression in PC-3 and DU145 prostate cancer cells. The authors demonstrated significant delay in tumour growth with no toxic side effects in vivo, highlighting the potential for hOC to target prostate cancer tumours [122]. The advantage of using this tumour-type specific promoter is that it may facilitate the targeting of the primary tumour, as well as disseminated metastatic lesions that are often the most aggressive and hardest to treat forms of cancer. Figure 2 represents the targeting strategy of a tumour-type specific promoter that is activated in cancerous cells but not in normal cells.

Figure 2 summarises active internalisation of gene delivery vector and initiation of transgene expression in a non-transformed and a transformed cell. Gene delivery vectors are commonly functionalised using an antibody that targets HER-2 [123], while the human osteocalcin promoter has been employed to drive inducible nitric oxide synthase gene expression in prostate and breast cancer cells [122].

4.2.3. Tumour microenvironment-related promoters

The tumour microenvironment provides a unique environment that provides ideal conditions for growth and progression of tumours. Hypoxic conditions are often associated with chemo- and radio-resistance in tumours, and hypoxia is thought to be a key element for the cancer stem cell niche [124]. Various genes have been identified to be upregulated in the hypoxic environment with hypoxia response elements (HREs) working in concert with transcription factors, such as HIF-1, to activate transcription in response to hypoxia. Fujioka et al. reported

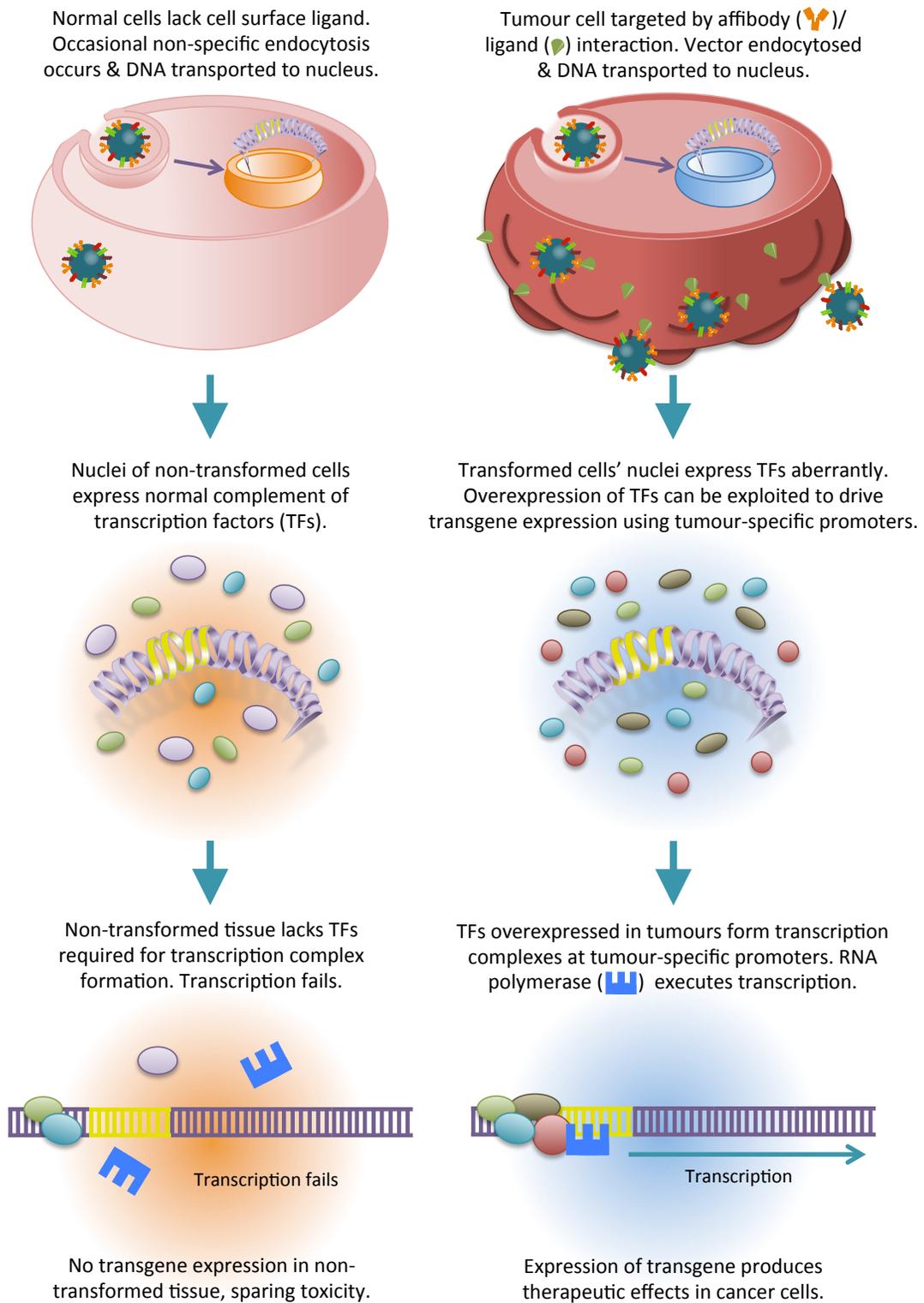


Figure 2. Targeted therapeutic transgene expression using affibodies and a tumour-specific promoter.

the construction of a vector combining a hypoxia response promoter with the CMV promoter (HRE-CMV) that resulted in a 2-fold increase in apoptotic gene expression compared to

expression driven by CMV alone. In vivo, BCL-2 shRNA activity driven by the HRE-CMV promoter in hypoxic colon 26 tumours resulted in tumour volume reduction that was significantly greater than when bcl-2 shRNA was driven by CMV alone [125]. Although this study demonstrates the action of the HRE promoter for treatment in hypoxic tumours, the authors used intra-tumoural injections to deliver the vector, which does not give an indication of the tumour targeting specificity of this strategy.

4.2.4. Tumour vasculature-related promoters

The ability of tumours to trigger angiogenesis for increased tumour blood supply has been associated with more aggressive tumours, metastases, and poor prognosis. Identification of the genes involved in this process has led to the use of promoters that can be exploited for targeting. One such example is VEGF, which has been shown to have a major role in tumour angiogenesis by activating tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (kinase insert receptor (KDR) in humans/Flk-1 in mice). KDR was found to be overexpressed in activated endothelial cells of newly formed vessels and strongly associated with invasion and metastasis in human malignant diseases [126]. Wang et al used the KDR promoter to drive thymidine kinase (TK) gene expression, which activated the prodrug ganciclovir (GCV) for suicide gene therapy. The authors demonstrated that the KDR promoter and TK/GCV showed a targeted killing effect on transfected human umbilical vein endothelial cells (HUVEC). Cells transfected with KDR-TK were 2- to 5-fold more sensitive to GCV compared to non-transfected HUVEC and HepG2 cells [127]. Again, however, confirmation of these impressive in vitro results in an in vivo setting using systemic delivery is required to validate tumour targetability and efficacy of the suicide gene/prodrug system.

5. RNA interference

Most of the strategies of cancer gene therapy discussed so far have involved introduction of therapeutic transgenes. An alternative strategy that is gaining considerable attention in the cancer gene therapy field involves inhibiting expression of problematic genes. Inhibition of gene expression can be facilitated by RNA interference (RNAi) that binds to mRNA. RNAi, discovered by Fire and Mello in 1998 [128], can be defined as a mechanism of gene-silencing produced by small RNAs. These RNAs include endogenous miRNA and exogenous siRNA or shRNA and their gene silencing activity is highly dependent on gene sequence [129]. These small RNAs then recruit cellular proteins, such as the RNA-induced silencing complex (RISC), to elicit their effect either through degradation of the mRNA or blocking the translation of mRNA [130, 131]. RNAi interference is therefore a highly attractive approach to cancer gene therapy and is currently a major research focus.

5.1. siRNA and shRNA

siRNA is a short (usually 21-bp) double-stranded RNA with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides. siRNA exerts its effect by directly

incorporating into RISC, where its guide-strand binds to and cleaves the complementary mRNA with a perfect match. The cleaved mRNA is subsequently released and the siRNA guide-strand-bound RISC is free to bind to another mRNA and start a new round of cleavage [132]. However, the short half-life of siRNA has resulted in production of shRNA, which has been developed as an alternative RNA molecule. Transcription of shRNA occurs in the nucleus from an expression vector that bears a short double-stranded DNA sequence with a hairpin loop. This shRNA transcript is then processed by RNase enzymes and incorporated into RISC in the cytoplasm [133].

The use of siRNA and shRNA to silence unfavourable genes that are overexpressed in cancer has gained much attention. Multidrug resistance (MDR) genes, responsible for resistance to chemotherapeutics have been problematic in the treatment of cancer and associated with poor prognosis. By silencing these genes using siRNA, it has been possible to improve response to conventional treatments. For example, Chen et al. used siRNA to silence the MDR1 gene in doxorubicin resistant MCF-7 breast cancer cells, which resulted in 85–90% reduction in MDR1 gene expression and subsequently sensitisation of 70% of cells to doxorubicin [134]. Another approach is to target and silence pro-angiogenic genes such as the Notch pathway. Yang et al. used a non-viral delivery system to deliver siRNA for silencing the Notch-1 gene in breast cancer and found that transfected MDA-MB-231 cells exhibited significantly decreased expression of Notch-1, inhibited cell proliferation, and increased cell apoptosis [135]. One advantage of using siRNA to down-regulate overexpressed proteins is that non-specific delivery is often less toxic than the delivery of plasmid DNA that encodes genes such as IL-2 and TNF-alpha. However, to limit any toxicity that does exist, many groups have added targeting ligands to the delivery systems to increase tumour specificity [136].

5.2. MicroRNA

MicroRNAs (miRNA) are highly conserved short non-coding RNAs that negatively regulate a wide range of physiological processes at the post-transcriptional level including apoptosis, proliferation, and migration [137]. Initially, miRNA is transcribed in the nucleus as a primary transcript (pri-miRNA), which is processed to give a two-nucleotide overhang at its 3' and is termed a pre-miRNA. Pre-miRNA is subsequently exported to the cytoplasm where it is further cleaved and mature miRNA is loaded into RISC to elicit its effect [138]. miRNAs can be either oncogenic or tumour suppressive in nature and as a result, may be overexpressed (e.g., miR-132, miR-20, and miR-17-92 family) or underexpressed (e.g., miR-34a and miR-126) in cancer cells making them targets for cancer gene therapy. A vast amount of information has been obtained in recent years on many different miRNAs and their role in cancer and with cancer stem cells, and by characterising their function, it may be possible to exploit them in cancer gene therapy [139, 140].

A single miRNA may have several varied targets to which it could bind and bring about gene silencing. miRNA-34a, known to be down-regulated in various cancers, has been shown to be a potent tumour suppressor that has various targets including the Notch pathway, BCL-2, survivin, c-Myc, and c-Met transcription factors [141]. Hu et al. demonstrated the value of miR-34a-mediated tumour suppression with the *in vivo* systemic administration of a non-viral

miR-34a delivery system. Nanoparticles were used to deliver the miR-34a using a tumour-targeting and penetrating bifunctional CC9 peptide (CRGDKGPDC) conjugated to β -cyclodextrin-polyethylenimine in a PANC-1 pancreatic cancer xenograft model; the miR-34a-loaded particles significantly inhibited tumour growth and induced cancer cell apoptosis [142]. Conversely, the inhibition of some miRNAs using complementary miRNA antagonist oligonucleotides (anti-miRNAs) can be an attractive gene therapy strategy to neutralise miRNA function. miR-132 acts as an angiogenic switch at the endothelium, inducing tumour neovascularization. Anand et al. reported the systemic administration of anti-miR-132 containing liposomes incorporating an integrin $\alpha v\beta 3$ -targeting cyclic RGD peptide to inhibit angiogenesis [143]. The authors demonstrated that anti-miR-132 blocked the action of mi-132 on angiogenesis induced by a VEGF-secreting ID-8 ovarian carcinoma in mice, and significantly reduced tumour burden and angiogenesis in an MDA-MB 231 xenograft model of human breast carcinoma when compared to treatment with scrambled miR-132. There is a huge potential of miRNA therapeutics for cancer. However, miRNA gene therapy is still in its infancy and more research is required to elucidate the exact pathways and possible targets available.

The active targeting of cancer gene therapy is hugely important for efficiency and safety. Yet despite the plethora of characteristics that can be targeted, active targeting remains elusive in many non-viral gene delivery systems. A move towards a combination of targeting strategies in one delivery system may hold promise for improved specificity using non-viral vectors.

6. The future: Molecular engineering

Strategies involving PEGylation and the use of targeting ligands have shown great promise for cancer gene therapy in overcoming certain hurdles, but in order to maximise the efficiency of non-viral delivery, vectors must have the ability to overcome all the barriers to gene delivery. Recent research in the field has focused on the development of vectors for nucleic acid delivery that efficiently evade the barriers to gene delivery highlighted above, and provoke adequate transgene expression in vivo following systemic delivery.

6.1. Multifunctional Envelope-type Nano Devices (MENDs)

Harashima et al. presented a multifunctional envelope-type nano device (MEND) that was produced on the concept of 'programmed packaging' with a rational design to overcome barriers to delivery and assembly into nano-sized vectors. Generally, a MEND comprises a DNA core condensed using a cationic polymer such as poly-L-lysine (PLL), which is wrapped in a separate lipid envelope fortified with various functional attachments including targeting ligands, PEG, and groups facilitating cellular uptake and endosomal escape [144]. One of the first MEND systems described consisted of a PLL DNA condensing core, surrounded by a lipid envelope, and functionalised with stearylated octaarginine (R8) to promote cellular uptake to deliver anti-luciferase siRNA [145]. The gene silencing effect of the MEND was found to be comparable to that of the transfection reagent Lipofectamine 2000, without any detectable

cytotoxicity and further optimisation of this system to include protamine as the DNA condensing agent resulted in a 70% silencing effect in transfected COS7 fibroblast cells.

The nature of the MEND system renders it relatively easy to modify in order to optimise transfection efficiency. When the lipid component of egg phosphatidylcholine (EPC) and cholesterol were replaced with the fusogenic lipids DOPE and cholesteryl hemisuccinate (CHEMS), respectively, an overall 2-fold improvement was observed due to the optimisation of the lipid component [146]. Furthermore, functionalization of MEND with octa-arginine R8 (R8-MEND) for enhanced cellular uptake resulted in a transfection efficiency of more than 80% in HeLa cells [147]. The addition of pH-sensitive endosomal escape motifs to MEND, such as INF7 derived from the HA2 protein of the influenza virus envelope, has also proven beneficial, and the combination of INF7 with R8 resulted in the production of R8/INF7/MEND. In vivo administration of R8/INF7/MEND to ICF mice produced luciferase expression 240-fold higher in liver and 115-fold higher in spleen than that of R8-MEND alone, demonstrating the importance of optimising functionality of the MEND system [148].

In a similar approach, the pH-sensitive fusogenic peptide GALA was incorporated into a MEND system as an endosomal escape enhancer in a system that comprised of R8 and an MMP-cleavable PEG functionality. Increased gene silencing effect was observed for delivery of anti-luciferase siRNA in HeLa-luc cells in vitro when compared to an unmodified MEND [149]. In addition to this, an in vivo study using a HT1080-luc xenografted model demonstrated that the cleavable PEGylated GALA/R8/MEND exhibited efficient luciferase gene knockdown in comparison to PEG-MEND, which was unable to cause any gene knockdown.

Incorporation of targeting motifs has also proved useful in MEND systems. The addition of RGD peptide to MEND, which is a targeting ligand for integrins, resulted in significant tumour growth delay in OS-RC-2 human renal carcinoma bearing mice when RGD-MEND was used to deliver anti-VEGF siRNA in vivo [150]. GALA peptide was also used as a targeting ligand for sialic acid-terminated sugar chains on pulmonary endothelium as reported by Kusumoto et al. [151]. Following intravenous administration of GALA-MEND delivering antiCD31 siRNA in vivo, approximately 50% inhibition of lung metastasis in a Murine melanoma B16-F10 mouse model was observed when compared with control groups. Examples of MENDs and the various functionalities that have been employed are detailed in Table 1.

| Name | Condensing material and nucleic acid cargo | Lipid Envelop | Endosomolytic component | Nuclear localisation component | Other functional groups | Activity |
|--------------|---|---|---|--------------------------------|-------------------------|---|
| R8/INF7-MEND | Protamine (plasmid DNA for luciferase transgene expression) | Egg phosphatidylcholine (EPC), cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphocholine | INF7 peptide derived from N-terminal domain of the HA2 protein influenza virus envelope | Protamine | - | Luciferase transgene expression levels 240-fold higher in liver and 115-fold higher in spleen |

| Name | Condensing material and nucleic acid cargo | Lipid Envelop | Endosomolytic component | Nuclear localisation component | Other functional groups | Activity |
|---------------|---|---|--|--------------------------------|--|---|
| | | (DOPC), and dioleoylphosphatidyl ethanolamine (DOPE) | | | | than that of the R8-MEND <i>in vivo</i> [148]. |
| GALA/PPD-MEND | Stearylated octahistidine (STR-H8) (anti-luciferase siRNA condensation) | DOPE, 1,2-dioleoyl-3-trimethylammonium-propane(DOTAP), Chol | pH-responsive fusogenic GALA peptide derived from HA2 | N/A | (MMP-cleavable PEG) | Intratatumoral injection of PPD/ GALA- MEND HT1080-luc into human fibrosarcoma mouse xenografts resulted in more efficient luciferase gene silencing compared with unmodified MENDs <i>in vivo</i> [149]. |
| RGD-MEND | Protamine (anti VEGF siRNA) | YSK05 (pH dependent cationic lipid), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), Chol | YSK05 (YSK05 consists of two linoleyl fatty acid chains and a tertiary amino group, which are responsible for pH-responsive fusigenicity in endosomes) | N/A | Cyclo (RGADPK) (cRGD) peptide ligand for $\alpha V\beta 3$ integrin PEG | Significant tumour growth delay was observed in OS-RC-2 human renal carcinoma bearing mice when RGD-MEND was used to deliver anti-VEGF siRNA <i>in vivo</i> [150]. |
| GALA-MEND | Polyethylenimine (PEI) (anti-CD31 siRNA) | EPC, Chol | GALA peptide | N/A | GALA peptide (ligand for sialic acid-terminated sugar chains on pulmonary endothelium) PEG | Murine melanoma B16-F10 lung metastasis significantly inhibited by approx. 50% compared with control groups |

| Name | Condensing material and nucleic acid cargo | Lipid Envelop | Endosomolytic component | Nuclear localisation component | Other functional groups | Activity |
|------|--|---------------|-------------------------|--------------------------------|-------------------------|--|
| | | | | | | following intravenous administration of GALA-MEND delivering anti-CD31 siRNA <i>in vivo</i> [151]. |

Table 1. MEND non-viral delivery systems, their components and applications to gene therapy

The potential of controlled intracellular delivery using the MEND system was also highlighted by Toriyabe et al. who used stearylated-octahistidine (STR-H8) as a pH-responsive component to facilitate the efficient release of siRNA in the cytoplasm [152]. STR-H8 was used to complex anti-luciferase siRNA and delivered using a conventional R8/GALA functionalised MEND. The authors demonstrated that luciferase gene knockdown was significantly higher in HeLa-GL3 cells treated with the STR-H8 MEND than with a MEND containing stearylated octarginine (STR-R8) to condense the siRNA. This may be explained by more efficient decondensation and release of siRNA from STR-H8 in the cytoplasm, which was confirmed by a RiboGreen assay showing siRNA release efficiency from STR-H8 was much higher than siRNA release from STR-R8 at pH7.4 (intracellular pH). It is clear, therefore, that PEGylation and unpacking of DNA are important considerations in the development of MEND systems, and with further optimisation and characterisation, MENDs have great promise as effective non-viral gene delivery agents.

6.2. Bio-inspired systems

Viral vectors still remain the most efficient gene therapy delivery vehicles with no non-viral delivery system producing comparable gene delivery potencies. Viruses have evolved naturally to infect and transfer their genetic material into host cells [153]. Understanding the various mechanisms by which viruses elicit delivery of genetic material has led to exploitation of viral peptide motifs by gene therapists and molecular engineers [154]. Functional peptide motifs derived from viruses have been engineered and incorporated into a wide range of bio-inspired non-viral delivery systems with great success, thereby benefiting from the viruses' expertise, while circumventing immunogenicity and safety concerns associated with viruses [35, 154]. Peptides are an attractive alternative to polymer and lipid-based non-viral vectors as they are less toxic, easily synthesised, and only weakly activate the complement system therefore enhancing safety [155]. The main peptides of interest are generally classified according to their function, i.e., DNA condensing peptides, cell penetrating peptides, endosomolytic peptides, and nuclear location sequences [156].

6.3. DNA-condensing peptides

Cationic peptides containing lysine or arginine residues interact electrostatically with the negatively charged phosphate backbone of DNA, condensing and packaging DNA into complexes with a net positive charge, which protects DNA from degradation and allows interaction with cell membranes [157]. Examples include histones, including H2A, which are natural basic proteins [158], μ (mu) peptide derived from adenovirus [159], and TAT peptide from HIV-1 [160]. Condensing peptides alone have a limited role because they cannot overcome many of the barriers to gene delivery; although some peptides, such as TAT, have cell penetrating properties, which makes them more attractive options in vector development [161]. However, the unpredictable nature of interactions between peptides and nucleic acids remains an issue and further research is needed for optimisation of vectors [157].

6.4. Cell Penetrating Peptides (CPPs)

Cell penetrating peptides (CPPs) are generally short peptides that have the ability to cross the cell membrane via various mechanisms including endocytic pathways or through direct translocation, without the need for receptors or other carriers [162]. Such peptides have been shown to deliver various cargoes to a range of cell types; peptide sequences are easily modifiable to optimise properties such as cargo transport or subcellular targeting [163]. Natural peptides exhibiting this penetrating activity include Penetratin (RQI-KIYFQNRRMKWKK), derived from the third helix of the homeodomain of Antennapedia [164], and TAT (GRKKRRQRRR) derived from HIV-1 [41]. Both have regions of basic amino acids and an alpha-helical conformation with the ability to translocate a cargo across cell membranes, which highlights the potential application of CPPs in gene therapy. Novel CPPs have since been derived and include peptides with a wide range of structures and characteristics; however, generally CPPs are cationic/basic, amphipathic, or hydrophobic in nature [156].

Amphipathic peptides are composed of both hydrophobic and hydrophilic domains in primary or secondary conformation. The secondary structure produces an alpha-helical structure with the hydrophobic residues such as leucine, glycine, or tryptophan localised on one face of the helix and the hydrophilic residues such as lysine, arginine, or histidine localised on the other. This amphipathic structure has been shown to be essential for passage across the cell membranes [165]. Structural changes of amphipathic peptides contribute to their binding affinity for cell membranes, and insertion of hydrophobic portions of the peptide into the membrane are important for interaction with the lipid membrane and subsequent uptake [54]. However, although amphipathic peptides have shown much promise, not all CPPs rely on this amphipathic nature for internalisation. For example, in the case of Penetratin, it is the positive charges rather than the helical structure that is responsible for cell penetration [166]. Therefore, increasingly, attention has been paid to developing simple linear peptides rich in cationic amino acids such as arginine. Cationic CPPs are composed mainly of basic amino acid residues including arginine, lysine, and histidine [167] and electrostatically bind to various anionic species present on the extracellular surface of the cell membranes, e.g., lipid head groups or proteoglycans such as heparin sulphate [168].

It has been reported that peptides containing arginine residues have stronger cell penetrating ability than peptides comprising lysine and histidine, with the guanidine moiety possessed by arginine being held as crucial for cell entry [169]. As a result, arginine-rich peptides have been extensively researched in order to characterise their activity [170–172]. The discovery that the basic portion of TAT responsible for the cell penetrating activity is rich in arginine residues prompted much research into the characterisation of mechanisms involved in the cellular entry of arginine-rich peptides [41]. It has been elucidated that the exact peptide sequence involved is not as crucial as the length of sequence and number of arginine residues incorporated, with between 6 and 15 arginine residues required for optimum activity [54]. In a study carried out by Wender et al., it was observed that truncated versions of TAT with arginine residues replaced with alanine exhibited reduced cellular uptake, but a 9-mer oligoarginine peptide (R9) was 20-fold more efficient than TAT [173]. Further to this, Mitchell et al. used peptides composed of multiple arginine residues termed oligoarginines, labelled with fluorescein to demonstrate that negligible cell uptake was exhibited with fewer than 6 arginine residues, but that when peptides of 7 arginines or more were tested, fluorescence increased as a function of peptide length up to 15 arginine residues, beyond which no increase in fluorescence was observed. Peptides containing more than 15 arginine residues can still penetrate cells, although this happens at a reduced efficiency and with toxicity to cells [169].

CPPs have the ability to enter any cell they come in contact with and this lack of specificity is problematic for gene therapy [174]. The use of 'smart' delivery vectors with 'activatable' CPPs (ACPs) has been explored, where a CPP is connected to a neutralising polyanion via a cleavable linker, reducing the overall charge and non-specific electrostatic uptake by cells. Enzymes produced in cancerous cells, such as MMPs, can then cleave the linker and allow the CPP to enter cancer cells [175]. For example, Mei et al. reported an ACP that includes a masking sequence of anionic E8 (sequence: EEEEEEEE) to shield the cationic nature of R8 [176]. The mask was linked to R8 by a MMP-2 sensitive linker; when the ACP nanoparticles were in the tumour environment, which overexpresses MMP-2, the mask was cleaved exposing R8 to tumour cells allowing tumour specific uptake. The authors used *in vivo* imaging to demonstrate this, while also showing lower ACP nanoparticle distribution in other tissues.

Another strategy for targeting and cell specificity has focused on the use of cell-penetrating-homing peptides (CPHPs) [97, 177] that combine targeting and cell penetration abilities. Kondo et al. described a CPHP known as RLW (peptide sequence: RLWMRWYSPRTRAYG) found through systematic selection from a random peptide library that had the ability to selectively target and penetrate A549 non-small cell lung cancer cells via an unknown mechanism thought to involve specific RLW ligand receptors on A549 cells [178]. Gao et al. demonstrated that when RLW was anchored onto poly(ethyleneglycol)-poly(ϵ -caprolactone) (PEG-PCL) nanoparticles loaded with infrared dye (DiR) cellular uptake was 2-fold higher in A549 cells than in umbilical vein endothelial cells *in vitro* [179]. Further to this, *in vivo* imaging showed the RLW nanoparticles targeted A549 xenografts specifically over U87 xenografts, with only low levels seen in normal organs in comparison to PEG-PCL nanoparticles functionalised with R8, which evoked DiR accumulation in all tissues. The specificity of CHCPs is a great asset; however, elucidation of the exact mechanism of how CHCPs work and a broader spectrum of activity may be more attractive so that a peptide may be used to treat more than one cancer type.

The cargo being carried by the vector must also be considered when designing a vector, as CPPs interact with various cargoes in different ways. For example, TAT mediates internalisation by at least two distinct pathways. Large cargoes, e.g., proteins, enter via caveolae endocytosis and macropinocytosis leading to endosomal entrapment, whereas small cargoes, e.g., peptides, enter slowly by endocytosis and rapidly by transduction by an unknown mechanism that gives direct access to the cytosol [42]. As endosomal entrapment is a major barrier to transfection, CPPs have been functionalised with endosomolytic peptides. Liou et al. described a fusion peptide that combines R9 for cell penetration and hemagglutinin-2 (HA2) for endosomal escape; the resulting vector was tagged with red fluorescent protein (RFP) for imaging purposes [180]. Significantly more RFP was detected *in vitro* when A549 human lung carcinoma cells were treated with the R9-HA2 peptide in comparison to R9 alone.

Problems with CPPs, such as humoral immune response induction, as seen in studies with Penetratin [181], and stability need to be addressed. Amino acids exist in different isoforms with variable susceptibility to degradation by proteases in serum. The L-isoform found in abundance in nature is sensitive to degradation, but the D-isoform is more resistant due to the altered stereochemistry that affects protease recognition. The use of the D-isoform of amino acids has therefore been suggested as a modification to render CPPs protease-resistant, enhancing stability [182]. However, further characterisation of the structure-activity relationship of individual CPPs is needed to allow the tailoring of specific CPPs to particular intracellular targets, optimising efficiency and reducing side effects [183].

6.5. Endosomolytic peptides

The harsh endosomal environment can lead to degradation of peptides and their cargo, as CPPs, such as TAT and oligoarginines, lack the ability to escape the endosome unaided, resulting in poor transfection efficiencies [184]. Histidine-rich peptides are usually endosomolytic in nature and can facilitate endosomal escape through the proton sponge where the protonation of imidazole groups in histidine-rich peptides facilitates buffering of the endosome causing endosomes to swell and burst, releasing their contents [185, 186]. Another mechanism employed by histidine-rich peptides is the 'flip-flop' effect, which may operate depending on the number of histidine residues or their arrangement in a peptide [187]. In a study conducted by Lo et al., the addition of 10 histidine residues to TAT increased luciferase transgene expression up to 7,000-fold in the human glioma cell line U251 *in vitro* [188]. Bafilomycin A1, a known inhibitor of the proton sponge effect for endosomal escape, in turn inhibited transfection significantly, supporting the idea that the activity of histidine as an endosomal escape motif could improve the transfection efficiency of TAT. However, *in vivo* administration of the TAT-histidine peptide/DNA complexes to deliver the luciferase reporter gene into the brain of rats showed 5-fold lower expression than was achieved using PEI 25 kDa/DNA complexes, suggesting more work needs to be done to ensure *in vitro* results translate to the *in vivo* setting. One example of a histidine-rich peptide that has shown great promise is H5WYG, derived from the HA2 subunit of haemagglutinin (HA) protein of the influenza virus. H5WYG causes endosomal escape through the proton sponge effect, when the histidine residues become protonated at around pH 6. H5WYG is unaffected by the

presence of serum that gives it an added advantage of being suitable for *in vivo* gene delivery [189]. Asseline et al. reported a 2-fold increase in luciferase mRNA levels when H5WYG was added to an antisense oligonucleotide (2'-Ome RNA705) targeting aberrant splicing of luciferase pre-mRNA in HeLa pLuc705 cells [190].

Fusogenic peptides have also been of great importance in facilitating endosomal escape [191]. Pore formation may be mediated by cationic amphiphilic peptides that bind to the lipid bilayer of the endosomal membrane, causing internal stress or tension leading to pore formation. Fusogenic peptides are known to adopt an amphipathic α -helical structure when pH drops to around 5 within the endosome, causing interaction with the phospholipid membrane and endosomal disruption [156, 192]. This fusogenic activity also allows these peptides to interact with cell membranes and facilitate internalisation, giving some fusogenic peptides a dual function with ability to package nucleic acid to avoid degradation and be delivered into the cytoplasm of the cell. One such example is RALA (WEARLARALARALARHLARALARAL-RACEA), a 30 amino acid fusogenic peptide with a cationic nature [193]. It is composed of a hydrophilic arginine (R) region that facilitates condensation of anionic complexes, e.g., DNA; a hydrophobic leucine (L) region that interacts with lipid membranes; and an alanine (A)-rich region that gives the peptide amphipathicity. This structure allows RALA to maintain α -helical conformation at low pH, enabling endosomal escape. The design of RALA was informed by the understanding of two similar peptides, namely GALA (WEAALAEALAEALAEHLAEALAEALAEALAA) and KALA (WEAKLAKALAKALAKHLAKALAKALKACEA), peptides that were in turn derived from the HA2 subunit of the influenza virus, with GALA being the first cell penetrating amphipathic peptide demonstrated to possess fusogenic activity [192]. However, GALA carries an overall negative charge and therefore cannot be used for delivery of DNA alone. KALA was derived by substituting the glutamic acid (E) in GALA with lysine (K); the resulting derivative was positively charged, and thereby more suitable for delivery of DNA. This E to K substitution resulted in improved interaction with negatively charged cell membranes and allowed condensation of negatively charged DNA cargoes [194]. RALA was derived by substituting lysine residues with arginine (R), which conferred a lower toxicity [172, 193].

6.6. Nuclear Localisation Sequences (NLSs)

Intracellular trafficking of nucleic acid cargo and entry into the nucleus is crucial for transgene expression. The use of nuclear localisation sequences (NLS) has proved beneficial in improving the efficiency of vectors. NLSs help traffic vectors towards the nucleus and facilitate entry through the nuclear envelope in association with the importin pathway [62]. Classical nuclear localisation signals, such as the NLSs from simian virus 40 (SV40), large tumour antigen (PKKKRKV), and Rev peptide (RRNRRRRWRERQRQ), consist of short stretches of basic amino acids [195]. Such NLSs have the ability to bind DNA in order to facilitate nuclear entry. Elder et al. used atomistic molecular dynamics to investigate the effect of peptide chemistry and sequence on DNA binding behaviour, focusing on the NLS from SV40 [196]. By analysing the conformational entropy and free energy of binding, the authors found that replacing arginine with lysine reduced binding strength by eliminating arginine–DNA interactions, but

placing arginine in a less sterically hindered location has little effect on polycation–DNA binding strength. This strong binding ability of arginine is important for an NLS because nucleic acids need to be bound and protected from degradation by nucleases in the cytosol before reaching the nucleus.

Several other proteins derived from viruses are excellent at traversing the intracellular network and facilitating nuclear import [197]. The TAT, Rev, and Rex proteins of the retroviruses contain arginine-rich NLSs, which have the ability to shuttle to and from the nucleus. Herpes simplex virus (HSV) type 1 tegument proteins, known as VP13/14, are also arginine-rich and act in a similar way [198, 199]. The arginine-rich portion of these proteins is responsible for the nuclear import, with leucine-rich portions, known as nuclear export signals (NES), being responsible for the shuttling between the nucleus and cytoplasm. Arginine-rich NLSs have been shown to use importin β pathway with no involvement of importin α pathway [200]. Importin β is not only involved in nuclear import but is also a potential adaptor for movement along microtubules, which may enhance trafficking of arginine-rich peptides to the nucleus, as well as entry to the nucleus [201]. Identification of exact binding sites and utilisation of such mechanisms may be the key to improving transfection efficiencies for peptide delivery vectors.

Incorporation of such sequences has proven to be useful in vector design. Hatefi et al. demonstrated that the addition of Rev (RRNRRRRWRERQRQ) to their fusion peptide KALA-2H1-NLS-TP facilitated cargo delivery to the nucleus by utilising microtubules for nuclear localisation [186]. Non-classical NLSs, such as M9 from human mRNA binding protein hnRNP A1, have also shown promise for non-viral vector functionalisation [202]. These NLSs lack stretches of basic amino acids and do not enter the nucleus via the importin pathway. M9 binds to the transportin receptor that results in nuclear localisation and has shown the ability to transport the vector towards the nucleus by shuttling between the nucleus and cytoplasm [203–205]. These properties make M9 an attractive NLS for gene delivery [65]. A number of viruses are known to exploit host microtubule machinery to facilitate access to the nucleus [206], but little is known about the exact mechanisms and binding domains used by viruses, and further study is required to elucidate exact peptide sequences involved that may be incorporated into non-viral vectors for rational design to achieve enhanced transfection efficiencies [207]. For example, the motif sequence contained in the adenoviral capsid hexon (E3-14.7K peptide: VVMVGEKPITITQHSVETEG) was conjugated to plasmid DNA and promoted microtubule-mediated transport of the DNA, resulting in 2.5-fold increase in transfection efficiency in HeLa cells compared to plasmid DNA only [208]. Incorporation of this sequence into a non-viral vector may therefore improve transfection efficiency.

Problems have been encountered where binding of an NLS with DNA renders the NLS unable to bind to the importins that allow passage through the NPC. Using a basic NLS to condense and deliver DNA alone has not been successful because they do not bind DNA strongly enough and the complexes are generally broken down in the cytoplasm [209]. Covalent conjugation of an NLS to DNA has been problematic as this may render the NLS or the DNA non-functional, as demonstrated when covalent bonding of SV40 did not increase nuclear localisation of pDNA [68]. Various binding strategies have been used to improve this, as well as using condensing agents such as histones that also possess nuclear localisation properties [210], but generally, NLSs are used to supplement other delivery systems rather than as stand-alone vectors.

6.7. Designer Biomimetic Vectors (DBVs)

An exciting approach to the multifunctional vector has been the introduction of recombinant production of bio-inspired fused protein sequences, each coding for a discrete motif with an explicit barrier evasion function [211]. Termed designer biomimetic vectors, these vectors are rationally designed to incorporate several motifs with distinct functions, and could be a step towards the production of 'artificial viruses'. The previous strategies discussed involving different components of a multi-functional system being conjugated together by various attachments may not be ideal for production of gene delivery systems. Simple conjugation of certain peptides has also led to alteration in the function of the peptides [212], therefore conjugating all the desired components together may be problematic. Production of DBVs using recombinant DNA technology allows the fusion of discrete motifs in a relatively simple process that should not affect the functional operation of the motifs. This would circumvent any problems involved with complex conjugation reactions to attach different components and ultimately could be more cost effective and reproducible in a large scale industrial setting. The production process involves introduction of plasmids, which have been engineered to contain the desired motifs for the protein, into competent bacterial cells. The bacteria then utilise the plasmid to produce the fusion proteins, which are subsequently extracted and purified. The use of this recombinant DNA technology allows the specific design of the vector at the molecular level, which can be tailored to enhance and optimise gene delivery [213]. Examples of multi-functional recombinant vectors are detailed in Table 2.

| Name | Nucleic acid condensation | Endosomolytic component | Nuclear localisation | Targeting motif | Other | Activity |
|-----------------|---|-------------------------|--------------------------------------|---|---|--|
| Tetra-H2A(TH) | Four tandem repeats of human histone H2A peptide (TH) | GALA peptide | N/A (anti-luciferase siRNA delivery) | Anisamide (AA) to target cancer cells that overexpress sigma receptor | PEGylated, cathepsin D cleavage sites in the TH for digestion in endosome compartment, DOTAP and Chol Lipid envelope surrounding TH | TH produced a higher silencing efficiency in HT60-luc cells <i>in vitro</i> and <i>in vivo</i> than the NPs assembled with protamine as the nucleic acid condensing agent [218]. |
| KALA-2H1-NLS-TP | Two repeating units of histone H1 (2H1) | KALA peptide | NLS from Rev protein of HIV virus | ZR-75-1 peptide (RVCFLWQ DGRCVF) | - | Transfection efficiency of luciferase comparable to PEI 25 kDa with preferential targeting to ZR-75-1 |

| Name | Nucleic acid condensation | Endosomolytic component | Nuclear localisation | Targeting motif | Other | Activity |
|----------------------------------|---|-------------------------|---|--|---|---|
| | | | | | | breast cancer cells [186]. |
| FP-(DCE) _n -NLS-CS-TM | Arginine-histidine (RH) with general structure (RRXRRXHHXH HX) _n | Arginine-histidine (RH) | M9 (included in FP-(DCE) _n -NLS-CS-TM for DNA delivery; absent in FP-(DCE) _n -CS-TM for siRNA delivery) | HER2 targeting affibody to target SKOV-3 | - | Significant cell death observed in SKOV-3 cells treated in vitro with FDT/BCL2-siRNA in combination with FDNT/pSR39 plus GCV [216,217]. |
| DBV | μ peptide derived from adenovirus | H5WYG | Rev | HER2 affibody | Targeting moiety attached via cathepsin substrate (CS) for cleavage in the endosome | DBV-mediated iNOS gene delivery resulted in a maximum of 62% cell killing and less than 20% clonogenicity in ZR-75-1 breast cancer cells <i>in vitro</i> [215]. |

Table 2. Recombinant multifunctional non-viral delivery systems, their components, and application in gene therapy

Recently the Gandehari and Hatefi groups have reported the design and development of recombinant fusion proteins for targeted gene delivery [34, 211]. The DBVs are produced by fusing the desired motif sequences, usually composed of a DNA condensing motif (DCM), endosomal disruption motif (EDM) and nuclear localisation motif (NLS) [34, 214]. Sadeghian et al. described a fusion protein comprised of two repeats of histone H1 for DNA condensation, H5WYG pH responsive fusogenic peptide for endosomal escape and the simian virus 40 (SV40) large T-antigen NLS for a nuclear localization [211]. The fusion peptide was complexed with the pGL3 plasmid for luciferase expression to form nanoparticles; the nanoparticles transfected Chinese hamster ovary (CHO) cells efficiently in vitro. However, this system lacked a targeting motif that is highly desirable in the design of gene delivery system.

Soltani et al. recently described a delivery system known as KALA-2H1-NLS-TP, which is composed of two repeating units of histone H1 (2H1) to efficiently condense DNA into nano-sized particles, a synthetic pH-dependent endosome disrupting motif (KALA) to promote escape from endosomes, a cyclic targeting peptide (TP) selected from a phage display library to target antigens on the surface of ZR-75-1 breast cancer cells, and an NLS from the Rev protein of HIV to facilitate translocation of DNA towards the cell nucleus [186]. The authors demonstrated that the recombinant vector had a high rate of gene transfection efficiency compared to vectors that lacked one or more functional motifs, and targeted the ZR-75-1 cells. Besides

the ability to target, the developed multifunctional vector was able to disrupt endosomal membranes, reach the nucleus by utilizing microtubules, and transfect efficiently while showing no detectable toxicity. McCarthy et al. presented similar results using a DBV for the delivery of iNOS gene therapy targeted to breast cancer [215].

Canine et al. described a biopolymer termed FP-(DCE)_n-NLS-CS-TM that contains repeating units of arginine and histidine to condense pDNA and lyse endosome membranes (DCE), a HER-2 targeting affibody to target cancer cells (TM), a pH responsive fusogenic peptide (FP) H5WYG to destabilize endosome membranes and enhance endosomolytic activity of histidine residues, and a nuclear localization signal (NLS) M9 to enhance translocation of pDNA towards the cell nucleus. A cathepsin D enzyme substrate (CS) was also engineered in between targeting motif and NLS to facilitate dissociation of the targeting motif from the biopolymer inside late endosomes where cathepsin D is abundant [216]. The authors demonstrated the functioning of each motif in the polymer resulting in successful transfection of SKOV-3 and GFP transgene expression.

The production of these recombinant vectors renders it relatively easy to change their characteristics by sequence modification. Canine et al. further demonstrated that by modifying the sequence of the biopolymer FP-(DCE)_n-NLS-CS-TM, it was possible to fine tune the vector for either delivery of plasmid DNA to the nucleus or delivery of siRNA to the cytoplasm [217]. It was reported that inclusion of the M9 NLS rendered the biopolymer (FP-(DCE)_n-NLS-CS-TM) suitable for delivery of plasmid DNA to the nucleus but not for delivery of siRNA. However, exclusion of the NLS from the biopolymer (FP-(DCE)_n-CS-TM) rendered it more suitable for delivery of siRNA to the cytoplasm but not for nuclear delivery of plasmid DNA in SKOV-3 human ovarian cancer cells. This study demonstrates the possibility of not only targeting specific cells for gene delivery, but also the ability to target intracellular compartments depending on the nature of the therapeutic to be delivered.

Wang et al. presented a novel recombinant protein tetra-H2A (TH) derived from histone H2A that was developed to replace protamine as a conditionally reversible, nucleic acid condensing agent. The recombinant protein comprised of four tandem repeats of human histone H2A peptide, interspersed with cathepsin D cleavage sites and a pH-responsive fusogenic peptide GALA to facilitate the endosome escape of the cargo. The recombinant protein, tetra-H2A (TH), was able to condense siRNA into a stable complex that was in turn coated in a cationic lipid with a high degree of PEGylation, forming Lipid-tetra-H2A-Hyaluronic acid (LHH) nanoparticles [218]. This design was developed in order to mimic lipid-enveloped viruses to replicate the transfection abilities of viruses in vivo. The histone-containing polymer demonstrated an enhanced intracellular release of the cargo and an increased anti-luciferase siRNA silencing efficiency in vitro compared with the protamine-containing polymer in H460-luc human lung carcinoma cells. Furthermore, in vivo gene silencing by tumour-targeted anti-luciferase siRNA was evaluated in H460-luc xenograft-bearing mice with the histone-containing nanoparticles loaded with anti-luciferase siRNA resulting in ~66% silencing of luciferase expression, significantly higher than that of the protamine-mediated knockdown (34%). This study demonstrates the importance of efficient release of the genetic payload for efficient gene therapy; through optimisation of each component of the multifunctional vector, it may be

possible to maximise transfection efficiency. However, these vectors are still in the early stage of development and much research is needed. Many of these findings serve only to confirm the theory behind the design of the vector, and further in vivo work with therapeutic transgenes is ultimately required.

7. Conclusion

There is a huge potential for cancer gene therapy, which, in some respect, has yet to be realised. However, the lack of gene therapy products making it onto the market is disappointing when one considers the number of research groups involved and clinical trials underway. It seems that until a suitable delivery system for cancer gene therapy becomes available, the potential of this field will never be reached. The difficulty in this is the number of hurdles to overcome and the extremely high standards of safety and efficiency expected by regulatory authorities. Nevertheless, the development of multifunctional systems and a move towards the creation of artificial viruses may finally help cancer gene therapy to progress to a viable and successful cancer treatment.

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