Journal of
Materials Chemistry B

REVIEW

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Cite this: J. Mater. Chem. B, 2017, 5, 7238

Biomaterials for polynucleotide delivery to anchorage-independent cells

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Anchorage-independent cells possess morphological features and cell membrane compositions that are distinct from adherent cells. They display minimal surface area, have a low rate of endocytosis and generally possess few proteoglycans which make it a challenge to deliver nucleic acids into them. Wide ranges of methods and materials have been developed to tackle the delivery obstacles for the polynucleotide-based therapeutics in modifying non-adherent cells. This article summarizes the techniques and biomaterials that have been utilized for transfection of anchorage-independent cells. First, physical techniques are briefly described along with particular applications for which they are well-suited. The structure–activity relationship of various biomaterial carriers of polynucleotides are then discussed with strategies employed to enhance their capability to transfect anchorage-independent cells. In conclusion, the authors' perspectives on different methods for polynucleotide delivery to primary human cells are compared, along with a discussion of their progression towards clinical trials.

Received 7th July 2017,
Accepted 9th August 2017

DOI: 10.1039/c7tb01833a

rsc.li/materials-b

I. Introduction to transfection technology

The majority of mammalian cells, with the notable exception of hematopoietic cells, typically grow and survive while anchored to the extracellular matrix (ECM) and neighbouring cells. Free-floating cells in the circulating blood, including immune cells, as well as metastatic cancer cells that migrate to secondary sites from the primary tumor site, do not require adhesion for survival,

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Professor J. Paul Santerre has published >170 peer reviewed publications and is a listed inventor on >60 patents in the area of medical polymers, biodegradation, protein and blood interactions with surfaces, surface modification, regenerative medicine, and drug delivery. He is a professor at the University of Toronto, and co-director of the Health Innovation Hub at the University of Toronto (a student focused entrepreneur training co-curricular program with >70 client health science and biomedical engineering start-up companies to date). He has received several awards for his innovation and industry related activity including the Governor General's award for Innovation in 2017.

though their life cycle is finite. Anchorage-dependent cells are routinely cultured *in vitro* as monolayers on artificial substrates. Upon contact with suitable surfaces, they flatten out and leave the suspension phase to initiate proliferation. Anchorage-independent cells, on the other hand, are maintained in cultures devoid of any substrate, with single cells or cell aggregates growing suspended in culture media. These fundamental differences in culture conditions lead to variations in cell morphology, surface area and membrane properties, which will affect penetration of macromolecules and nanoparticles. Coupled with differences in focal adhesion complexes (*e.g.*, integrins) and proteoglycan composition on plasma membranes, vastly different responses to transfection reagents are expected. In general, anchorage-independent cells are known to be more difficult to transfect than adherent cells.[1–3] One important reason is the flat, exposed surface of adherent cells that provides a larger surface area for transfection reagents to interact with. Furthermore, depending on the size of the transfection complex, gravitational settling of complexes on top of a cell monolayer increases the cellular contact, thereby enhancing the chances of cellular uptake and internalization.

Transfection is the process of introducing exogenous polynucleotides into cells with the aid of a carrier. Polynucleotides, being macromolecular, highly hydrophilic/anionic in addition to displaying supramolecular assemblies, have little chance of entering cells on their own. All the genetic information of organisms is hard-wired in the form of a genetic code in DNA located in the nucleus of cells. Introducing functional genes composed of DNA molecules was the initial impetus behind developing effective transfection technologies. The central dogma of molecular biology states that protein codes in the form of genes contained in DNA are first transcribed into messenger RNA (mRNA), which is translocated to the cytoplasm for translation into proteins. In addition, a cellular process called RNA interference (RNAi) has evolved for regulating genes by silencing gene expression based on blockage and/or degradation of specific mRNAs.[4] This process, in addition to regulating aberrant gene expression, provides an innate defense mechanism against invading viruses. To express a protein, either plasmid DNA (pDNA) or mRNA can be introduced into cells. In order to silence unwanted or undesirable genes, antisense oligonucleotides (ASOs) or short interfering RNA (siRNA), both of which are chemically synthesized, can be delivered. ASOs are typically 16–20 nucleotides long single stranded DNA molecules, while siRNAs are typically 19–27 nucleotides long double stranded RNA molecules. ASOs (typically block translation) and siRNAs (typically degrade mRNA) work through different intracellular pathways and hence display different gene silencing potencies. MicroRNAs (miRNAs) are endogenous non-coding single stranded RNA molecules with 19–25 nucleotides and mis-matched base pairing that play a vital role in endogenous gene regulation and have been implicated in several diseases.[5] miRNAs, in addition to mediating gene silencing similar to the siRNA mechanism, are capable of regulating gene expression directly as well as regulating the expression of other mRNAs. In the cases where miRNAs themselves need to be downregulated, single stranded RNA molecules with sequences complementary to target miRNA, called anti-miRNA, could be employed. Alternatively, chemically synthesized miRNA mimics are introduced into cells for a direct effect. It is critical to understand the properties and functions of different nucleic acids to select the appropriate carrier for effective transfection (Fig. 1).

There is a pressing need to implement non-viral transfection of anchorage-independent cells due to substantial clinical ramifications contingent on their successful transfection. In the case of blood cancers, genetic modulation of malignant cells provides an alternative therapeutic modality that can overcome the limitations of conventional chemotherapy. Additionally, the genetic manipulation of normal immune cells, in a manner that enables them to eliminate diseased cells, opens up the possibility of immunotherapy of a broad range of diseases. Success with exploratory studies has paved the way for investigating non-viral transfection in clinical trials, and implementing the far-reaching clinical benefits of transfecting anchorage-independent cells. The current review comprehensively summarizes the techniques and materials that have been developed for the introduction of polynucleotides into anchorage-independent mammalian cells. The non-viral delivery approaches used for modifying blood and immune cells for biomedical applications have been described. The structure–activity relationship and chemical synthesis methods of relevant biomaterials, in the context of steps involved in polynucleotide delivery both *in vitro* and *in vivo*, have been discussed.

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Dr Hasan Uludağ has been with the University of Alberta since 1997, based at the Department of Chemical & Materials Engineering. Dr Uludağ is an elected Fellow of the IUS-BSE. Besides acting as the lead editor for Frontiers in Biomaterials, he is serving on the editorial board of 6 international journals. Dr Uludağ has published >170 peer-reviewed journal articles. Dr Uludağ obtained dual BSc degrees in Biomedical Engineering and Biology from Brown University in 1989. He then completed his PhD degree in 1993 at the Department of Chemical Engineering & Applied Chemistry at the University of Toronto.

II. Methods of transfection

To successfully deliver intact polynucleotides to cells, significant intracellular and extracellular barriers must be overcome. Initially, polynucleotides must be protected from biodegradation by extracellular (*e.g.*, serum and tissue-specific) nucleases. Once they reach the cell, polynucleotides should permeate through the anionic plasma membrane bilayer which is a dynamic structure composed of phospholipids, membrane proteins, and cholesterol held

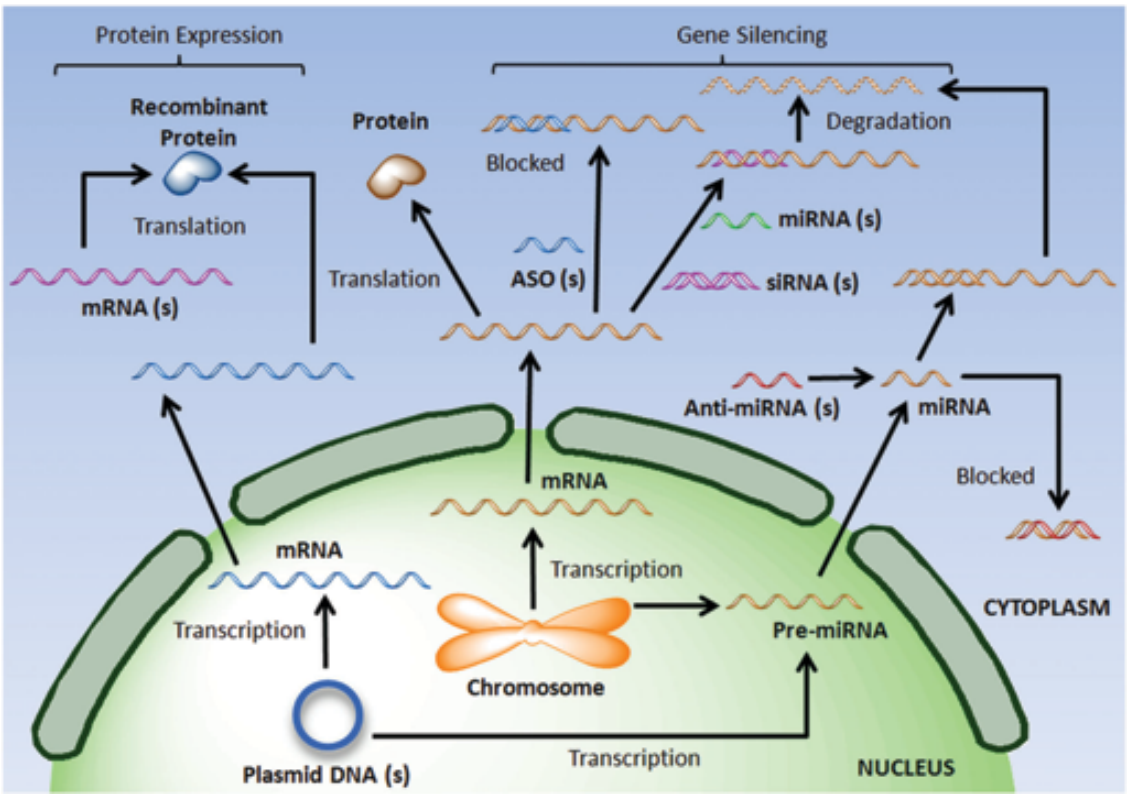

Fig. 1 An illustration of modes of action of both naturally occurring and chemically synthesized (exogenous) polynucleotides. Synthetic polynucleotides that can be delivered to cells are denoted with (s).

together primarily through their hydrophobic interactions. The desired result of transfection can then only be observed if the polynucleotide escapes intracellular degradation and carries out its function.[6] A method which delivers the polynucleotide to all target cells while causing minimal or negligible toxicity is desired. Even though numerous approaches have been developed for transfection, there is no universally effective material and/or method that functions optimally in all cells. Several different techniques still remain in use, not only because optimal procedures vary from one cell type to another, but also due to the need to deliver different types of polynucleotide to the cells. Categorically, transfection methods can be grouped into either physical or chemical (biomaterial-mediated) methods (Fig. 2).

II.A. Physical methods of transfection

The physical methods of transfection facilitate delivery of ''naked'' polynucleotides by creating pores in the cell membrane with physical forces such as electric or magnetic field, ultrasound waves and high pressure, among others.[7] The pores generated are expected to be transient in nature, thereby allowing polynucleotides to diffuse into the cell while restricting prolonged leakage of cellular content. The simplest way of polynucleotide delivery is direct injection into the cell cytoplasm or nucleus by means of specialized tools such as a glass microneedle, micropipette, and positioning manipulator. This technique was first demonstrated in 1980[8] and since then it has been refined with automated computer-guided microinjection systems that enable more control over precise positioning of cells and injection times.[9,10] However, only a single cell can be manipulated at a time, thereby limiting its use to single cell assays,[11,12] and production of recombinant cell lines.[13,14] This drawback has been overcome to a certain extent by fabrication of vertically aligned arrays of nanoneedles or nanofibres on a microchip.[15] This 'impalefection' technique may allow transfer of nucleic acid to several cells at once.[16] Biodegradable silicon nanoneedles have been employed to enhance impalefection efficiency.[17] Though this platform holds good potential for single cell manipulations,[18,19] it is only appropriate for small sample sizes and could be relatively labour-intensive. Furthermore, this technique appears to be more suitable for adherent cells rather than anchorage-independent cells *in vitro*. Impalefection is limited to superficial tissues for *in vivo* applications, and cannot be used systemically for transfection of circulating cells.

Approaches that utilize high pressure for polynucleotide transfer into cells are gene gun (biolistic gene transfer), jet injection and hydrodynamic injection (hydroporation). In biolistic gene transfer, target cells are bombarded with DNA-coated heavy metal particles (gold or tungsten), driven by water vaporized under high-voltage electric spark[20] or helium discharge[21] for cell penetration. Factors that influence transfection efficiency, such as size and density of particles, force of bombardment, gene gun instrumentation, and particle to DNA ratio, need to be optimized for different cell types.[22] It is highly efficient and leads to long-term expression of the introduced gene,[23] but is limited in

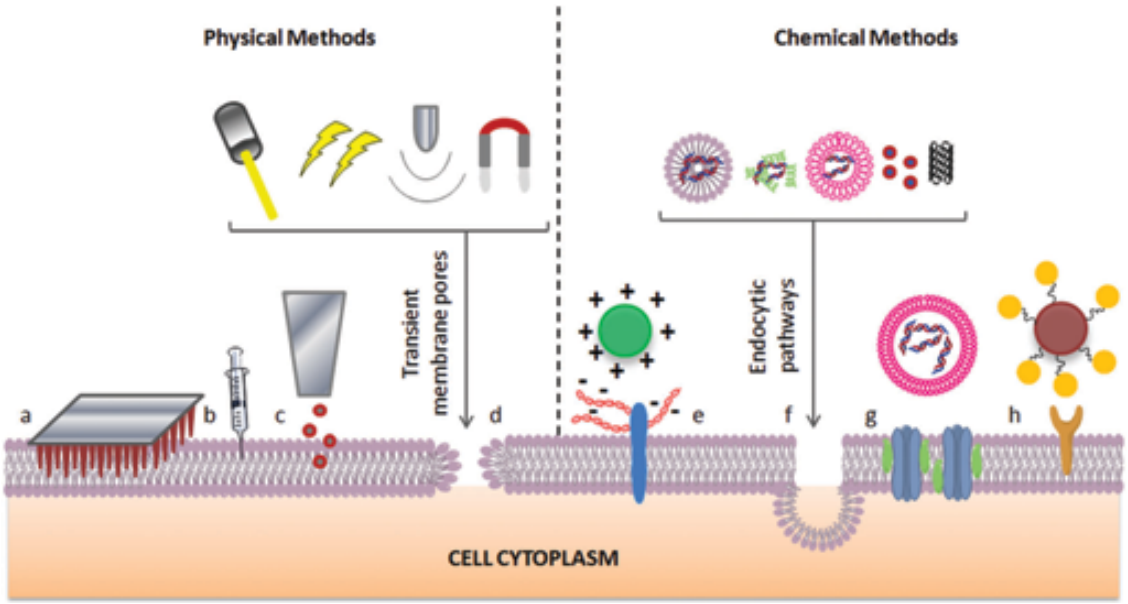

Fig. 2 Routes of polynucleotide delivery in physical and chemical methods of transfection. Physical methods involve direct delivery by (a) impalefection, (b) microinjection, (c) gene gun, or through (d) transient membrane pores generated by applying electric or magnetic fields, sound waves or laser. In the case of chemical methods, (e) cationic carriers interact with negatively charged glycosaminoglycans (GAGs) on the cell surface for cell entry, (f) cell-penetrating peptides (CPPs), carbon nanotubes (CNTs), quantum dots (QDs), superparamagnetic iron oxide nanoparticles (SPIONs), polymer and lipid nanoparticles are taken up *via* endocytic pathways, (g) large (>500 nm) lipid-based particles are internalized through lipid rafts, and (h) ligand-associated carriers undergo receptor-mediated endocytosis.

application to small areas and superficial tissue. Accordingly, it has been employed extensively for delivering DNA vaccines in preclinical models and human clinical trials[24–26] as well as antibody production.[27–29] The primary drawback of this technique is the toxicity associated with accumulation of tungsten particles or high cost in the case of gold particles. Polymeric nanoparticles have been explored as an alternative,[30–32] but more comprehensive studies need to be conducted to understand their efficacy. The underlying principle of the jet injection approach is the same as the gene gun, except that it uses a stream of liquid containing polynucleotides instead of particles. Jet injectors have been used widely for vaccination[33–35] and therapeutic protein delivery.[36,37] It has primarily been investigated for DNA vaccination[38] and gene therapy for certain cancers,[39,40] excluding hematological malignancies, and skin diseases,[41] due to its limited applicability to a small area. Hydrodynamic injection is a method for gene transfer in animals, involving rapid intravenous injection of a large volume of nucleic acid solution which leads to reversible cardiac congestion and an increased hydrodynamic pressure in the inferior vena cava. Organs linked to the inferior vena cava, mainly the liver, are able to take in the nucleic acid solution due to perforations created in the cell membrane by the elevated pressure.[42–44] While this technique has been established to target different solid tissues,[45–47] only one example of such a study reported observation of gene expression in anchorage-independent antigen-presenting cells in spleen along with the liver.[48]

Electroporation or electro-permeabilization involves the application of short and intense electric pulses to living cells that result in reversible pores in the cell membrane permitting entry of large and/or anionic macromolecules.[49] This approach was first demonstrated in mouse L cells cultured in suspension in 1982.[50] Small changes in experimental conditions and target cell characteristics lead to significant variations in gene transfer efficiency, and hence optimization of these parameters is required for different cell types.[51] Electroporation-mediated gene transfer has been used in several tissues[52] and solid tumors for therapeutic purposes.[53,54] In the case of anchorage-independent cells, it has been utilized extensively to modify dendritic cells and T cells *ex vivo*, as described later, for immunotherapy. In an extensive survey of studies on siRNA transfection in different types of leukemic cells *in vitro*, 42% of the studies reported the use of electroporation, which was the most common approach to modify cells for experimental manipulations.[55]

Magnetofection involves guiding polynucleotide-coated superparamagnetic iron oxide particles to desired sites by applying a magnetic field.[56,57] This enriches the nucleic acids in the vicinity of cells, thereby enhancing cellular uptake of these particles through non-specific endocytosis.[58] Magnetic field mediated gene transfer has been explored for the delivery of nucleic acids into "hard-to-transfect" adherent cells[59,60] and solid tumor models,[61] but still needs to be evaluated more comprehensively for anchorage-independent cells.

Ultrasound mediated gene transfer or sonoporation, as the name suggests, utilizes ultrasound waves that reversibly permeabilize the cell membrane through the formation of cavitation bubbles,[62,63] thereby allowing entry of the macromolecules. Although the transfection efficiency is lower than electroporation, the use of ultrasound for gene delivery offers the advantages of being safe, relatively non-invasive (*i.e.*, clinically acceptable), and not causing any tissue damage at reasonable

ultrasound intensities.[64] Over the years, ultrasound contrast agents such as compressible gas-filled microbubbles with a stabilizing lipid or polymer coating have been employed to enhance gene transfer efficiency.[65,66] Application of ultrasound induces oscillation of the gas core, which ultimately implodes at sufficiently high ultrasound intensities. The impact of implosion can temporarily disrupt the membranes of cells in the vicinity, thereby enabling nucleic acid entry into cells. For example, the treatment of anchorage-independent murine bone marrow-derived dendritic cells (BMDCs) with perfluorobutane microbubbles loaded with antigen mRNA lipoplexes resulted in modest yet significant levels of transfection *in vitro*. These results were also validated *in vivo* when mRNA sonoporated BMDCs were administered as a vaccine in a melanoma mouse model, in which induction of T cell proliferation and reduction in tumor load was observed.[67] However, the efficacy of the mRNA lipoplexes alone was not assessed, which is required to determine if the positive results observed are a consequence of using sonoporation or of the lipoplexes, which also possess the capability of transfecting anchorage-independent cells, as discussed in a later section.

Another noninvasive method, known as optoporation or laserfection, makes use of laser beams with sufficient intensity to generate transient holes in cell membranes.[68] The shock waves resulting from laser irradiation can permeabilize several cells in its vicinity.[69] However, this also leads to varying levels of gene transfer to cells, contingent on their distance from the shock wave.[70]

Microfluidic devices have been recently investigated for shear-induced cellular uptake of polynucleotides. Cells suspended in a solution along with polynucleotides are passed through microchannels with diameters 30–80% smaller than the cell diameter. Compression and shear forces reversibly deform the cell membrane allowing diffusion of polynucleotides into the cell.[71,72] Additionally, microfluidic chips have been explored for polynucleotide delivery in conjunction with electroporation, optoporation, and hydrodynamic gene transfer.[73] However, in these cases, the diameter of microchannels is designed to be slightly bigger than the cell diameter, so that the polynucleotide uptake is not solely due to membrane deformation and shear forces. The use of a microfluidic electroporation system yielded 60% transfection efficiency and >75% viability in anchorage-independent chronic myeloid leukemia (CML) K562 cells.[74,75] While this method is suitable for genetic manipulation of cells in suspension *in vitro*, it cannot be used for direct *in vivo* applications.

A clear advantage of physical techniques is that they can bypass some of the barriers to polynucleotide delivery without introducing additional reagents (possibly inducing cytotoxicity) into the system. Polynucleotides are not usually required to attach to the cell membrane or interact with it in any way for successful transfer. The cytoplasmic delivery of naked polynucleotides with physical methods may circumvent the endosomal processing, and thereby possibly avoiding lysosomal degradation. Since these methods use mechanical forces to transiently permeabilize cells, they are not impacted by cell type specific behaviour and can be applied to all cells alike. The pitfall, though, is the limited capacity for scale-up and any cytotoxicity caused by excessive loss of membrane integrity. While microinjection, gene gun, and jet injection manipulate single cells, the other methods can cater to a larger, yet restricted, number of cells. Practical considerations such as the need for specialized equipment, skilled operators, and costs associated with them should be considered. It is difficult to rank the physical methods based on reported transfection efficiencies,[76] and preferential use of one over another depends on the specific application. Currently, they are more suitable for the administration of polynucleotide-based vaccines to superficial tissues and generating cell lines for commercial production of recombinant proteins. Clinical application of physical methods faces several hurdles, such as toxicity and invasive procedures required to access non-superficial tissues. Clinical immunotherapy of hematological malignancies, as well as a few other diseases, has relied on *ex vivo* manipulation of patient cells by physical methods, followed by re-introduction of the modified cells into patients. In particular, electroporation has been used widely for *ex vivo* mRNA or siRNA transfection of anchorage-independent natural killer (NK)[77,78] and T-cells[79–81] in animal studies, some of which have proceeded to clinical trials (Table 1). In an ongoing clinical trial (ClinicalTrials.gov Identifier: NCT01974479), *ex vivo* expanded allogeneic NK cells in suspension are electroporated to introduce mRNA coding for the anti-CD19 chimeric antigen receptor (CAR) to treat B-lineage acute lymphoblastic leukemia (B-ALL).[77] Similarly, plasmid DNA electroporation of anchorage-independent autologous T cells to generate CAR-T cells *ex vivo* has been examined in clinical trials for cancer immunotherapy.[82,83] In an effort to facilitate "off-the-shelf" therapy with mismatched donor T cells in suspension, the first clinical application of universal anti-CD19 CAR-T cells, generated by electroporation of mRNA coding for a site-specific gene-editing enzyme, was recently reported.[84]

II.B. Chemical methods of transfection

Chemical carriers are rationally designed to prevail over extracellular and intracellular obstacles that prevent polynucleotide entry into cells. The carriers essentially leverage the anionic charge of polynucleotides to either electrostatically condense them to a relatively smaller size suitable for internalization, or encapsulate them behind a physical barrier, or in some cases adsorb them onto surfaces. This masks the negative charge on polynucleotides as well as forming a physical barrier in some cases to protect them from endonucleases. Internalization is undertaken through various endocytosis mechanisms or interaction with anionic glycosaminoglycans (GAGs) on the cell surface or lipid rafts in the case of larger lipophilic particles (Fig. 2). The delivery vehicles and their payload subsequently experience a rapid pH drop (typically 7.2–7.4 on the cell surface) down to ~6 in early endosomes, and a further reduction to 4–5 as the endosomes mature into late endosomes and lysosomes, where degradation occurs. The biomaterials need to possess attributes that enable endosomal escape to release their payload intracellularly. In the case of *in vivo* application, the route of administration is important as it identifies additional hurdles

Table 1 Summary of clinical trials employing electroporation for *ex vivo* genetic manipulation of anchorage-independent cells

Clinicaltrials.gov/ clinical trial no.	Cells modified	Nucleic acid	Gene	Rationale
NCT00012207	Autologous T cells	pDNA	Anti-CD20/CD3ζ chimeric receptor	Modified T cells specifically target CD20+ cells in patients with relapsed or refractory NHL
NCT00621452	Autologous T cells	pDNA	Anti-CD20/CD28/4-1BB/CD3ζ chimeric receptor	Combining two costimulatory molecules (CD28 and 4-1BB) augments T cell activity in patients with relapsed or refractory MCL or indolent B-cell NHL
BB-IND#11411	Autologous T cells	pDNA	Anti-CD19/CD3ζ chimeric receptor	Genetically re-directed T cells can specifically recognize and kill CD19+ B-lineage lymphoma cells
BB-IND#8513	Autologous T cells	pDNA	Anti-CD20/CD3ζ chimeric receptor	Genetically re-directed T cells can specifically recognize and kill CD20+ B-lineage lymphoma cells
NCT01974479	Allogeneic NK cells	mRNA	Anti-CD19/4-1BB/CD3ζ chimeric receptor	Chimeric receptor on NK cells enhances their cytotoxicity against B-lineage ALL cells, as they highly express CD19 on their surface
NCT02117518	Autologous T cells	mRNA	Peptide/MHC-1/CD3ζ chimeric construct (peptide: insulin β chain 10–18 or IGRP 265–273 or IGRP 222–230)	Modified T cells target autoreactive CD8+ T cells, showing specificity to the particular peptide/MHC-1 complex expressed on modified T cells, to eliminate the autoreactive CD8+ T cells in T1D patients
NCT02315118	Autologous T cells	mRNA	CD16/4-1BB/CD3ζ chimeric receptor	Modified T cells boost antibody-dependent cell cytotoxicity of an anti-CD20 antibody (Rituximab) in CD20+ B-cell CLL and B-cell NHL
NCT03166878 NCT02799550	Allogeneic CD19-specific T cells	mRNA	Specific TALEN to disrupt TCR and MHC-1 expression	Disruption of TCR and MHC-1 molecules on allogeneic CD19-specific T cells prevents host-mediated immunity and GVHD upon infusion in patients with CD19+ relapsed or refractory lymphoma or leukemia
NCT01355965 NCT01897415	Autologous T cells	mRNA	Anti-mesothelin/4-1BB/CD3ζ chimeric receptor	Chimeric receptor on T cells induces anti-tumor activity in solid tumors overexpressing mesothelin
NCT00730613	Autologous T cells	pDNA	Anti-IL-13/CD3ζ chimeric receptor	Specific recognition and killing of glioblastoma multiforme cells by IL-13 specific T cells
NCT02624258 NCT02277522	Autologous T cells	mRNA	Anti-CD19/4-1BB/CD3ζ chimeric receptor	Recognition and killing of CD19+ lymphoma cells in HL patients

NK: natural killer, MHC-1: major histocompatibility complex class 1 molecules, NHL: non-Hodgkin's lymphoma, MCL: mantle cell lymphoma, IGRP: islet-specific glucose-6-phosphatase catalytic subunit-related protein, T1D: type I diabetes, CLL: chronic lymphocytic leukemia, TALEN: transcription activator-like effector nucleases, TCR: T-cell receptor, GVHD: graft-*versus*-host-disease, HL: Hodgkin's lymphoma.

that particles may encounter. For example, in the case of systemic administration, particles that are less than 10 nm in size are rapidly filtered by the glomerular basement membrane of kidneys for excretion,[85,86] thereby drastically reducing their bioavailability. Additionally, it is advantageous to have carriers equipped with the ability to identify cells of interest.

Some anchorage-independent cells are easily accessible due to their presence in the circulatory system, but targeting specific cells may be necessary to prevent undesirable side-effects that arise from the genetic manipulation of non-targeted cells. Other anchorage-independent cells might be buried in deep tissue compartments, as suggested for some of the 'stem' cells that are located in the bone marrow, spleen or adipose tissue. In this case, being able to seek the appropriate cell will be paramount to improve the delivery efficiency. Furthermore, dilution of the nucleic acid vehicle in circulation presents another challenge in transfecting anchorage-independent cells *in vivo*. Extensive efforts have been invested into designing and developing nucleic acid carriers that are stable in the bloodstream, specifically recognize target cells, facilitate endosomal release, and convey cargo to its site of action intracellularly, as elaborated in the following section.

II.B.i Modified polynucleotides for delivery without biomaterials. The modification of nucleic acids has been primarily explored in the context of therapeutic utility, due to the requirement of large doses and limited clinical efficacy of unmodified polynucleotides.[87] The distinct molecular features of nucleic acids that define target specificity (nucleotide sequence) and pharmacokinetic behaviour (chemical and structural architecture) serve as an advantage for the rational design of such drugs. Chemical modifications of all three nucleic acid components, sugar-phosphate backbone, sugar moiety and heterocyclic base moiety (Fig. 3), and their combinations have been investigated.

Phosphorothioate (PS) backbone linkages created by replacing one non-bridging phosphate oxygen with a sulphur atom[88] in ASOs drastically increase their resistance to serum nucleases as well as their affinity to serum albumin, thereby prolonging the circulation half-life,[89] leading to more favorable biodistribution and subsequently increased cellular uptake.[90,91] Incorporation of PS linkages in siRNA does not alter its potency[92–94] but can lead to cytotoxicity when the number of PS linkages is high.[95] Instead of S, replacement with boron or acetic acid to create boranophosphate (BP) and phosphonoacetate (PACE) linkages, respectively, also imparts nuclease stability[96,97] while retaining siRNA or ASO activity.[98,99] Uncharged nucleic acids have been synthesized by replacing the sugar-phosphate backbone with *N*-(2-aminoethyl)glycine in peptide nucleic acids (PNA) and non-ionic phosphoramidate bonds to link furanose sugars in phosphoramidate morpholino oligomers (PMO). Since the nitrogenous bases are the same as naturally occurring nucleic acids, they follow base pairing rules. Their neutral backbones confer enhanced nuclease resistance[100,101] and target binding affinity.[102,103]