REVIEW

Department of Pharmaceutics[1], Faculty of Pharmacy, Alexandria University, Alexandria, Egypt; Department of Pharmaceutical Sciences[2], Appalachian College of Pharmacy (ACP), Oakwood, VA, USA; Department of Pharmaceutical Sciences[3], School of Pharmacy, University of Saint Joseph (USJ), Hartford, CT, USA

Non-viral gene delivery systems: hurdles for bench-to-bedside transformation

N. A. Helal[1], A. Osami[1], A. Helmy[1], T. McDonald[2], L. A. Shaaban[1], M. I. Nounou[3,*]

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**Corresponding author: Mohamed Ismail Nounou, MPSc., Ph.D., School of Pharmacy, University of St. Joseph, 229 Trumbull Street, Hartford, CT 06103, USA*
nounou@usj.edu

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Biologist and Nobel Prize winner James Watson's quote, "We used to think that our fate was in our stars, but now we know that, in large measure, our fate is in our genes", represents the initial food for thought that revolutionized the way medications and active pharmaceutical ingredients are defined (Rocholl 1996). This fate engraved in the genetic material, as mentioned in Watson's quote, fueled a tremendous revolution wave in gene therapy. Gene therapy is a promising technology for treating genetic and acquired diseases by modulating the expression of a specific gene in the pathological cells. This is achieved by introducing a DNA sequence or other nucleic acid material or oligonucleotides to the target cell (Kay, 2011). Moreover, gene therapy contributes to correction of genetic defects, expression of therapeutic proteins, and inhibition of the synthesis of malignant proteins. In this review article, different non-viral gene delivery systems and their applications are discussed in detail. We reviewed and tabulated over 90 papers and 50 patents from 2006 to date discussing non-viral gene delivery technologies, innovation, and bench-to-bedside transformation. Furthermore, we are going to shed light on the lack of standardization in the design and characterization of non-viral gene delivery systems worldwide, which is a major concern in this research's field. This review would aid in getting an eagle eye view through non-viral gene delivery technologies during the past 20 years. Such a view, capturing the advances, the hurdles, and experimental details, would aid expert researchers in tuning their experimentation strategies and help newcomers better initially design their studies to generate solid and comprehensive results that can be reliable and reproducible.

1. Introduction

The International Congress of Genetics introduced the term "gene therapy" in 1997. Additionally, Gregor Mendel used gene therapy techniques in the 1850s, and Ronald Fischer contributed heavily to the development of this field at the beginning of the 1990s (Cevher et al. 2012).

Theodore Friedman and Richard Roblin published a fundamental research paper that captured the readers' imagination and represented a major seed in gene therapy knowledge. They explained in their paper the concept of "good DNA" that could be used to replace defective DNA in people with genetic disorders (Friedmann and Roblin 1972). French Anderson (the father of gene therapy) conducted the first trial of gene therapy in 1990. The blood containing T-cells was taken from a 4-year-old girl and cultured with ADA (adenosine deaminase)-bearing retroviral vectors. Finally, the enhanced T-cells were injected into the girl. Another trial with the same procedure was performed on a 9-year-old girl in 1991 (Kohn 2000).

Gene therapy is useful in treating not only genetically inherited diseases, but also various diseases such as heart failure, stroke, and cancer (Pezzoli and Candiani 2013). The therapeutic plasmid is loaded *via* vector or carrier system. An optimal gene delivery system should meet three benchmarks: the carriers should protect the transgene from nuclease enzymes inside intracellular matrices, the carriers should transport the transgene from the plasma membrane to the target cell nucleus, and the carriers should not cause any toxic effects (Cevher et al. 2012). In summary, the carrier system needs to be safe and effective in delivering its genetic load to the target cell or organ.

Gene delivery methods can be physical (e.g. microinjection, electroporation and gene gun) (Meacham et al. 2014), chemical (e.g. lipid or nanoparticle carriers) (Midoux et al. 2009), or biological (e.g. ATP responsive carriers) (Mo et al. 2015). Furthermore, gene delivery systems can be classified as viral-based, non-viral-based, and combined hybrid systems. Viral gene delivery carriers utilize a modified non-virulent virus; and non-viral gene delivery carriers use cationic lipids, polymers, physical methods, and inorganic substances (Gardlík et al. 2005; Wivel and Wilson 1998). Both vectors may be used together and then called "hybrid vector" (Cevher et al. 2012; Gardlík et al. 2005).

1.1. Viral vs. non-viral gene delivery debate: History, progress, pros and cons

The reason of the high efficiency of viral gene delivery is their ability to escape from endosomes. Viral trafficking is a critical step to the viral escaping steps from endosomes (Daya and Berns 2008; Ramamoorth and Narvekar 2015). High immunogenicity and cytotoxicity are the main drawbacks of the immensely efficient transfecting system using viruses. The first related fatality of gene therapy clinical trial was related to the inflammatory reaction to the adenoviral vector injected to Jesse Gelsinger on Sept. 17, 1999. Gelsinger participated in a clinical trial conducted by the University of Pennsylvania (co-investigator, Dr. James M. Wilson) that aimed at developing a treatment for infants with severe genetic disorders. Gelsinger was subjected to an adenoviral vector containing a correction gene for ornithine transcarboxylase deficiency (Wilson 2009). He died at the age of 18. Gelsinger

suffered from a massive immune response triggered by the use of a high titer of the adenoviral vector used as the carrier system for gene transportation, leading to multiple organ failure. The severe immune reaction caused his death four days after the initiation of the study (Sibbald 2001). The tragic Gelsinger incident was a major setback to the gene therapy research field. The number of peer-reviewed original research articles and clinical trials drastically declined in the couple of years following this incidence (2000-2006). The field started to regain traction in 2006, after recovering from this incidence (Edelstein et al. 2004).

Another drawback for the viral vectors is the insertional mutagenesis, disrupting the expression of tumor suppression gene, or activating an oncogene leading to the malignant transformation of cells (Nault et al. 2015). However, three adenoviral-based drugs have reached the market: Gendicine®, Glybera® and Strimvelis®. Gendicine® is a recombinant Ad-p53 gene therapy for head and neck squamous cell carcinoma (HNSCC). Gendicine® was approved via the State Food and Drug Administration of China in October 2003 (Frew et al. 2008). The second gene therapy formulation to be marketed worldwide was Glybera® (alipogene tiparvovec). Glybera® was approved by the European Union (EU) as an engineered adeno-associated viral vector. Although Gendicine® was approved for sale in China in 2003 for cancer treatment, Glybera® (alipogene tiparvovec) was finally approved in Europe in 2012 for an ultra-rare genetic disease, familial lipoprotein lipase deficiency, nine years after Gendicine® (Ferreira et al. 2014). Glybera® was developed by UniQure Inc. UniQure went through major hurdles pushing alipogene tiparvovec through the clinical testing and approval process in Europe (Salmon et al. 2014; Scott 2015; Steinhagen-Thiessen et al. 2016). As mentioned earlier, in the early 2000s, the field of gene therapy was affected greatly by the multiple toxicity and death incidents during clinical trials in Europe and USA and went through its own roller coaster ride, trying to overcome past safety and other problems to prove its worth as a viable therapeutic option. In the time frame between 2003 and 2012, Glybera® was rejected by European regulators three times (Salmon et al. 2014). In spite of the hurdles, gene therapy was gaining momentum *via* technological advances reflected in improved engineered viruses, or "vectors" that help deliver the treatment along with the public need. Glybera® was finally approved in Europe in 2012. Glybera® was initially priced at around $1 million in Europe (Han and Ni 2015; Morrison 2015; Ronfard et al. 2017). Glybera® represented the initial spark in the field of gene therapy and has fueled the field since 2012. Another gene therapy, from GlaxoSmithKline™ (originally designed and developed by San Raffaele Telethon Institute for Gene Therapy and the biotech MolMed in Italy), is Strimvelis®, which was developed for a rare immune deficiency. It was approved in 2016 (European Commission) (Monaco and Faccio 2017; Ronfard et al. 2017; Schimmer and Breazzano 2016; Touchot and Flume 2017). Strimvelis® is an *ex-vivo* stem cell gene therapy designed to treat a rare inherited disorder (around 15 European children/year), severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) (Gardner 2016; Hoggatt 2016; Levenson 2016; Shorter et al. 2017). ADA-SCID is a homozygous disease resulting from a rare genetic disorder affecting children. It results in an ill-functioning immune system and inability to fight off common infections. Aside from big Pharma players like GlaxoSmithKline™, Glybera® inspired numerous gene therapy startups such as Spark Therapeutics, BioMarin Pharmaceutical, and Bluebird Bio (Carr and Bradshaw 2016; Cucchiarini 2016; Han and Ni 2015; Kotin and Snyder 2017; Morrison 2015; Ronfard et al. 2017; van der Loo and Wright 2016). Clinical data and initial controlled experimentation in humans from these startups suggest that gene therapy is potentially beneficial in multiple diseases, such as hemophilia, beta thalassemia, and sickle cell disease (Cucchiarini 2016; Ronfard et al. 2017). Furthermore, what could become the first gene therapy to win approval in the United States moved closer to market in 2015 with the first successful randomized, controlled trial for any gene therapy aimed at an inherited disease, when Spark Therapeutics announced that the medicine had succeeded in a late-stage clinical trial in treating an inherited eye disease (inherited retinal dystrophies) that can cause blindness (Bennett et al. 2016).

In spite of such continuing advancements and promising clinical data, it apparently took only five years for Glybera® to fizzle out after failing commercially. On April 2017, UniQure announced that it would no longer seek to renew marketing authorization of alipogene tiparvovec (Glybera®) after the Western world's first five-year conditional approval of the therapy is set to expire on October 2017 (European Biotechnology Life Science and Industry Magazine 2017). UniQure is set to pull the drug from the market and wind down all the related operations and infrastructure. The reason behind this decision is, unfortunately, not related to efficacy or safety, but due to weak sales and high price tag. Glybera® was a commercial failure. The decision brought a unique odyssey for UniQure to an end. Such a decision could be a set back to the field for some while and definitely would inspire the gene therapy researcher and scientists into exploring cheaper, more efficient, and safer options. Non-viral carriers could represent a cheaper and easier to manufacture option as compared to viral vectors with its unrealistic price tag (~ $ 1 million for Glybera®) (Han and Ni 2015; Morrison 2015; Ronfard et al. 2017).

On the other hand, non-viral gene delivery vectors are generally less toxic and immunogenic compared to viral vectors (Jones et al. 2013; Nayerossadat et al. 2012). They are safer and less expensive. However, non-viral gene delivery has low efficiency and less ability to target gene expression to the area of pathology impeding their use (Katz et al. 2013; Yang 2012). Non-viral gene delivery includes lipid-based vectors and cationic polymers. They have been used for the transfer of nucleic acids for many years. The cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-tri-methylammonium chloride) showed high efficiency in delivering RNA in various cell lines and, to a lesser extent, DNA transfection (Malone et al. 1989). Early examples of cationic polymers used for DNA transfer are PEI (polyethylenimine) and PLL (poly-l-lysine). Cationic polymers undergo diverse modifications that we will detail later in the review.

2. Requirements for a successful gene delivery

A successful delivery system depends on the vector or carrier used in the safe transport of the genetic material into its final cellular target destination. Vectors are vehicles that deliver the genetic material to target cells. Selectivity and efficiency in delivering a gene to the target cell along with minimal toxicity are the most crucial criteria. The lesson history taught, based on the failed and deadly clinical trials, is that safety perhaps has a leverage over efficacy (Smith and Byers 2002; Somia and Verma 2000; Stolberg 1999). Vectors should also be produced in large amounts with low cost. Carriers can largely vary in their transfection efficiencies and toxicity profiles. Furthermore, there is no one delivery vector that is ideal for the standards of a perfect gene delivery. The solution lies in the modifications that can be done to obtain a smart delivering vector, possessing considerable supply for the previously mentioned principles (Boeckle and Wagner 2006).

Among all vectors, viral vectors possess the highest transfection efficiency. However, it has detrimental side effects of cytotoxicity and a high ability to induce the body's immune response (Glover et al. 2005). Non-viral techniques for gene delivery are direct physical methods such as microinjection and particle bombardment, *i.e.* gene gun, electroporation, sonoporation, laser beam and magnetofection, and the chemical-based approaches such as non-viral carrier systems (Liposomes, lipoplexes, polymers, peptides, nanoparticles, etc). Physical methods are the easiest to implement and have acceptable transfection efficiency. Unfortunately, physical methods require numerous doses and cause damage to certain types of cells (Mali 2013).

Chemical methods are also generally easy to prepare, but their use is limited due to their insufficient transfection efficiency. As

a result, great attention should be directed to the use of hybrid systems. Using a viral vector along with one of the synthetic carriers can optimize the vector's efficiency with lower cytotoxicity. Polyethylene glycol was used to reduce the immune response and enhance the efficiency in viral-based systems using adenovirus (Wonganan and Croyle 2010). Along with hybrid systems, smart carriers have been introduced into the world of gene therapy. Smart polymers, which can display great changes in their properties or shape due to little changes or stimuli in the environment, can be the nucleus in the design of novel smart gene carrier systems. Smart carriers can modify their shape and characteristics to avoid many cell barriers easily (Dincer et al. 2005).

Non-viral gene delivery systems will be the focus of our review. An overview of the systems used, along with the recent novel advanced technologies involved in non-viral gene therapy, will be discussed. Research papers and registered patents since the comeback of gene therapy research from 2006 to date are surveyed, categorized, and tabulated in Appendix 1 (90 research papers) and Appendix 2 (54 registered patents; for appendices see online version). In these tables, research papers or patents are summarized based on the non-viral carrier system used, carrier system design, applications, efficacy testing, toxicity testing, expression readout techniques, reporter and therapeutic genes used, physicochemical characterization of the carrier system, study design, amount of genetic material used, and gold standards and controls used in each study. Such summary would easily aid researchers to effortlessly compare the various described carrier systems. These tables could be of value to experts and newcomers in the field of non-viral gene delivery research.

3. Non-viral gene delivery: Breaking down the barriers

The formulation of an effective gene delivery vector encounters many hurdles beginning from internalization and escaping degradation passing by safety perspectives and efficient transfection. In this part of the review, these problems, with suspected solutions, will be discussed. All different gene transfer methods should have the ability to conquer two major restraints: first, the need to carry the genetic material to its target with minimal risks (Jin et al. 2014; Nayerossadat et al. 2012), and second, the ability of the nucleic acid to penetrate cell membranes. For a high rate of cell transfection, these two conditions must be considered during formulation of the genetic vector besides overcoming extracellular barriers that include enzymatic degradation and rapid clearance after administration (Huang et al. 2015). *In-vivo* delivery remains one of the most complicated protests in gene therapy as the designed vector must escape the reticulo-endothelial system (RES) after systemic administration besides crossing many other barriers before reaching target cells (Jones et al. 2013a). For this reason, as shown in Appendices 1 and 2, many researchers bypass the use of serum in their *in-vitro* efficacy and safety testing and neglect any *in-vivo* experimentation of their novel systems.

Gene delivery barriers can be classified into four levels' barriers, as follows:

3.1. Blood level

The blood serum contains the enzyme nuclease. Nucleases degrade the extracellular nucleic acids if unprotected. Moreover, elements in the blood or serum proteins may complex with the polymer, forming an assembly that is phagocytosed (Raad et al. 2014).

3.2. Tissue level

Nonspecific interaction of the polymer with the extracellular matrix hinders the flow of the polymer carrying the genetic material (Al-Dosari and Gao 2009). Nonspecific interaction is an obstacle that can be affected differently according to the size of the vector. The large particle size hinders the movement of polyplex.

Blood and tissue barriers are named the extracellular barriers. Unwanted interactions at both levels can be avoided by some modifications on the vector's surface. Moieties such as polyethylene glycol (PEG), hydroxypropyl methacrylamide (HPMA), sugars, and proteins are used. For example, surface polyethylene glycol coating (PEGylation) effectively decreases the interactions of blood components and degradative enzymes with the vector surface, offering prolonged circulation in the blood. Furthermore, targeting ligands including proteins, antibodies, cell-penetrating peptides, sugars, and other small molecule ligands can be used to overcome these barriers (Jones et al. 2013; Tan et al. 2016).

3.3. Cellular level

Both polymer-based and lipid-based carriers carry positive charges (to enable polyplex or lipoplex formation with the negatively-charged DNA); therefore, adsorption can always take place between these cations and the negatively-charged cell membrane. Furthermore, there are two ways for a genetic material to enter any target cell, either by direct delivery "physical methods" or by endocytosis. Receptor-mediated endocytosis, which includes clathrin and caveolae mediated endocytosis, macro-pinocytosis, and others, is considered the most common uptake mechanism (Voigt et al. 2014). The problem regarding endocytosis lies in lysosomes attaching to the endosomes leading to degradation of the genetic material (Khalil et al. 2006). Also, phagocytosis is applicable for macrophages and dendritic cells. Therefore, endosomal escape of the carriers in non-viral systems is considered a big challenge for the intracellular delivery of nucleic acids. Endosomal escape of the carriers can be achieved via adding a cell-penetrating peptide, endosomal membrane rupture through pore formation, or proton sponge effect (Meng et al. 2017).

3.3.1. Endosomal escape strategies

3.3.1.1. The proton sponge effect

The proton sponge effect is the attraction of protons resulting in entrance of excessive chloride and water to the endosome along with the protons, hence increasing the endosome's size drastically and subsequently leading to their rupture (Behr 1997). The philosophy in the design of a successful non-viral gene carrier system is the inclusion of highly-protonated groups (such as primary, secondary, and tertiary amines) into the building blocks of the carrier system. Such protonated groups would aid the creation of proton sponge effect attracting water and chloride molecules along with the carrier system within the endosomes, leading to its rupture. Polyethyleneimine (PEI) has been designed by Behr et al. to generate the proton sponge effect *via* primary, secondary, and tertiary amines, and to subsequently generate enough buffering capacity to efficiently transfect genetic materials (Behr 1996; Boussif et al. 1995). The buffering capacity is essential in decreasing the acidic pH of the lysosomal environment. As a reflex mechanism, lysosomes tend to decrease the lysosome's pH to maintain its acidic environment in a continuous acidification process, leading to excessive increase of the lysosome's water content and subsequently size ending in its rupture ((Behr 1996; Boussif et al. 1995). PEI is considered a gold standard in non-viral gene delivery in terms of efficacy, mainly because of its buffering capacity and its proton sponge effect. For polymeric carriers without pH buffering properties, such as chitosan and poly-l-lysine (PLL), functional moieties are added to them to improve their buffering capacity and enhance their transfection efficiencies. Histidine is one of the frequently used moieties as a pH buffering enhancing functional group (Shi et al. 2013; Sun and Zhou 2016). Another agent which is widely used is chloroquine. Chloroquine is a lysosomotropic agent that prevents endosomal acidification and imparts buffering of the endosomal pH, competing with the continuous acidification process and subsequently leading to a peak rise in osmolarity and eventually osmotic lysis of the lysosomal vesicle. Chloroquine accumulates inside the acidic compartments of the cell, such as endosomes and lysosomes (Wolfert and Seymour 1998).

3.3.1.2. Cell penetration peptides (CPPs)

CPPs are short sequences of amino acids, mainly 10-30 residues (Madani et al. 2011). CPPs are able to cross the plasma membrane of living cells to facilitate the transportation of hydrophilic macromolecules such as proteins, peptides, and nucleic acids. Mostly, CPPs are classified into two main categories. The first is the cationic peptides that usually contain arginine and lysine residues, e.g., TAT peptide, penetratin and oligoarginines. The second is the amphipathic peptides that consist of both hydrophobic and hydrophilic segments (Madani et al. 2011).

3.3.1.3. Pore formation

Pore formation is another mechanism to facilitate the endosomal escape of non-viral gene delivery systems. This mechanism can be achieved via peptides such as GALA peptide (glutamic acid-alanine-leucine-alanine) (Nir and Nieva 2000). Pore-forming peptides become incorporated into the vesicle bilayer structure and accumulate to form a pore with a diameter ranging from 5 to 10 Å. GALA can only be added as an additional functional component to polyplexes or lipoplexes (Nir and Nieva 2000).

3.4. Nuclear level

Entering the nuclear envelope efficiently requires a molecule of a small mass and diameter. Molecules with a mass of less than 50 kDa and a diameter of few nanometers can cross the nuclear membrane by passive transport (Alberts et al. 2002). Comparing this to the size of the DNA, the particles that can pass the membrane passively are smaller than the transported DNA (Alberts et al. 2002; Khalil et al. 2006), a problem that can be overcome by an active transport system. A nuclear localization signal (NLS), mostly a peptide, is either coupled with the transported nucleic acid or attached on the surface of the carrier. NLS aids the entrance of the carrier by energy mediated nuclear translocation. It is also said that macromolecules may pass the nuclear membrane during the cell mitosis as the nuclear envelope cracks (Fortier et al. 2014). Sequence PKKKRKV in the SV40 (Simon Virus 40) Large T-antigen, Nucleoplasmin (AVKRPAATKKAGQAKKKKLD), EGL-13 (MSRRRKANPTKLSENAKKLAKEVEN), c-Myc (PAAKRVKLD) and TUS-protein (KLKIKRPVK) represent some examples of commonly used NLSs (Fortier et al. 2014).

4. Cytotoxicity face-off: Non-viral vs. viral systems

One of the main reasons for the existence of non-viral gene delivery carriers is their reputation as being safer than the ill-reputed viral vectors. In spite of reputation, non-viral carriers still have their share of toxicity. Aside from barriers and internalization issues, cytotoxicity of some used materials in non-viral gene delivery remains an obstacle that needs further investigation. Toxic effects of gene delivery reagents are mainly due to their cationic nature. Interaction between cationic polymers or lipids with the outer and inner cell membranes alter the integrity of these membranes (Juliano and Carver 2015). In some cases, simply adjusting the molecular weight may reduce polymer toxicity. In the case of PEI polymer where high MW PEI (greater than 25,000 Da) was found, it was seen to be toxic to cells while, polymers having medium to low MW (5,000–25,000 Da) were more efficient and less toxic. Moreover, nanoparticles have the ability to efficiently transfect post-mitotic cells *in vivo* and *in vitro* with no or low toxicity and are inert to immune responses. Finally, hybrid systems can always be the solution to many problems in terms of toxicity and efficacy. Combining different types of viral and non-viral carriers can lead to an advanced new system that may conquer many barriers (Fortier et al. 2014). Finally, non-viral carriers may have a better safety profile over viral vectors, but this does not mean that they do not show any toxicity. On the contrary, they may be as toxic and problematic as viral vectors. The key advantage of non-viral vectors is the versatility in its design and modulation, enabling researchers to easily tailor-design the carrier system to minimize and control the system toxicity profile.

5. Non-viral gene delivery carriers: Methods and techniques

The main core of gene delivery is to safely and effectively transfer the genetic material to host target cells. There are three main methods for delivering genetic material to the target cells: physical, chemical, and biologically-responsive methods (Nayerossadat et al. 2012).

5.1. Physical methods (direct delivery)

Physical methods are means by which nucleic acids are directly and rapidly delivered *via* different methods. Some of these methods are summarized as follows:

5.1.1. Microinjection

Microinjection is the injection of an intended gene, drug, or molecule into the skin using micro-needles. Purified DNA was successfully microinjected for the first time in 1980 (Kucherlapati et al. 1984). In addition, micro needles were used to deliver oligonucleotides, insulin, proteins, and RNA. A micro-needle is a mean that directly pervades the skin in the least possible intrusive way (Prausnitz and Langer 2008).

5.1.2. Gene gun

The target nucleic acid is precipitated onto gold or tungsten particles, then these particles are bombarded with a high velocity to the intended organ or cell using a biolistic device (gene gun) (Mellott et al. 2013). Particle bombardment was first known and applied in 1992 when DNA-coated tungsten molecules were delivered to plant cells (Finer et al. 1992). This method was proven to have a great role in gene immunization (Loehr et al. 2000). However, it must be understood that particle bombardment may cause tissue injuries (Mellott et al. 2013).

5.1.3. Electroporation

Electroporation is the application of an external electrical field that halts the cell membrane permeability temporarily, allowing the passage of any molecule (Young and Dean 2015). It is a simple, easily applied method that has undergone, in many studies, some modifications to provide considerable transfection efficiency. Moreover, electroporation can be applied to all types of cells and tissues. However, electroporation varies in its transfections for each type. As in the case of particle bombardment, electroporation may cause tissue damage in humans (Young and Dean 2015).

5.1.4. Sonoporation

Similar to electroporation, sonoporation is forming a pore in the cell membrane transiently *via* the application of ultrasound. This allows the target molecules to enter the cell (O'Neill and Li 2008). Many studies conveyed an enhanced gene transfer using ultrasound-assisted microbubbles (Rahim et al. 2006). However, many factors may impede the use of ultrasound-mediated gene delivery. These factors include tissue damages and that there is no known dose or limits to the areas affected by the ultrasound waves (Rahim et al. 2006).

5.1.5. Laser irradiation

Laser irradiation is applying pulsed laser waves using a laser source such as yttrium-aluminum garnet or argon ion or any other laser source. Therefore, a pore in the cell membrane is formed allowing plasmids to be transfected from the medium to the cytosol of the cell (Tao et al. 1987). It was first studied in 1986 using neodymium-doped yttrium aluminum garnet (Kurata et al. 1986). The laser irradiation method showed substantial transfection efficiency. The

use of laser irradiation may also be hazardous to the cells at certain concentrations (Kurata et al. 1986). Finally, laser irradiation is considered a viable mean for gene transfer to date.

5.2. Chemical carriers (inorganic particles, synthetic/ natural lipids, polymers, and peptides)

This group of carriers includes lipid and polymer-based vectors, in addition to nanoparticles and peptides. Chemical vectors have different rules including protection of genetic material from degradation, delivering it into target cells, enhancing the carrier safety profile, sustained release and/or enhancing transfection efficacy (Ramamoorth and Narvekar 2015). Nucleic acid/chemical ratio (N/P ratio in case of polymers, where N denotes Nitrogen on the polymer backbone and P denotes the Phosphors on the DNA backbone), solution pH, and cell membrane conditions are all factors that affect the transfection efficiency of a chemical-based delivery method. Chemical carriers rely on their cationic nature to efficiently complex the negatively charged nucleic acids (Guo and Huang 2012; Kim and Eberwine 2010).

5.2.1. Lipid and polymer

Lipid and polymer-based carriers can properly condense the negatively charged DN due to their high density of positive charges (Ibraheem et al. 2014). Preparation and toxicity of lipid-based carriers depend on their structure, with some toxic effects reported (Fischer et al. 2003). There are several types of lipid-based carriers, of which DOSPA (2,3-dioleyloxy-*N*-[2(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoro acetate), DOTMA (N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethyammonium chloride), and liposomes are the most important examples. Liposomes, in specific, are considered an important non-viral gene carrier system. In the case of liposomes, the genetic material can be conjugated to the external surface of the liposomes to form lipoplexes as in the case of the commercially available Lipofectamine™ (Felgner et al. 1995; Maruyama et al. 2007). In this case, liposomes have to be cationic in nature to conjugate the negatively charged genetic material. On the other hand, the genetic material can be encapsulated inside the liposomal vesicular structure. In this case, liposomes can be cationic, anionic, on nonionic in nature (Felgner et al. 1995). In addition, formulation and cytotoxicity of polymer-based carriers also depend on the compound structure, with generally low cytotoxicity (Fischer et al. 2003). Cationic polymers can be classified into natural-based polymers, such as chitosan, and synthetically manufactured, such as polyethylenimine (PEI) and poly-L-lysine (PLL) (Ibraheem et al. 2014).

5.2.2. Peptides

Peptides are short chains of amino acids that are considered as copolymers. Peptides have extensively vital roles in gene delivery and the role varies based on the type of peptide. Synthetic peptides, of lysine or arginine base, have a great role in condensing DNA (Boulikas 2016; Raad et al. 2014). Other peptides enhance the delivering of the genetic material to the cell or the nucleus (Raad et al. 2014; Zorko and Langel 2005). Qian et al. (2012) used TGN peptide in brain targeting. There are many other types of peptides with different properties that can overcome cell barriers (Raad et al. 2014).

5.2.3. Inorganic nanoparticles

Inorganic nanoparticles are another sub-category of chemical vectors for gene delivery. They are easily prepared and have moderate toxicity (Jin et al. 2014). Gold nanoparticles (AuNPs), carbon nanotubes (CNTs), silica nanoparticles, and quantum dots (QDs) are among the most important examples of this group.

5.3. Biologically-responsive methods

Designing a carrier that responds to the normal biological changes in the human body is the principal of the biologically-responsive (bioresponsive) methods. For example, the difference between ATP concentrations in the extracellular and intracellular matrices of a cell can be a target for modelling an ATP responsive polymer (Mo et al. 2014). A diverse biomolecule can act as a target for biologically-responsive methods, such as ATP and enzymes (Huang et al. 2013; Ramamoorth and Narvekar 2015).

6. Cargoes delivered by non-viral carriers

In non-viral gene delivery, genetic material transferred is mainly classified into the following types of nucleic acids (Midoux et al. 2009):

- Antisense oligonucleotides (AON) or related molecules synthesized chemically
- Large DNA molecules (Plasmid DNA; pDNA)
- RNA Interference (siRNA, miRNA, ShRNA)

6.1. Antisense oligonucleotides (AON)

First, an antisense oligonucleotide (AON) is a synthetic single-stranded deoxyribonucleotide, which is 15–30 nucleotides long (Chan et al. 2006). AON cause gene silencing *via* interfering with the production of the disease-causing protein. They fuse to their target messenger RNA, causing translational arrest *via* steric hindrance of ribosomal activity and induction of RNase H endonuclease activity (Chan et al. 2006). Selection of the AON sequence and incorporation of certain chemically-modified nucleotides in the AON largely affect its mechanism of action and the extent of modulating gene expression (Deleavey and Damha 2012). Furthermore, one AON has been approved for local administration to treat cytomegalovirus (Fomivirsen®, 1998; Grillone and Lanz 2001; Highleyman 1998a, b), while other AONs and small interfering RNA (siRNA) are in various stages of clinical development (Chan et al. 2006).

6.2. Plasmid DNA

A plasmid is a small, circular, extrachromosomal and double-stranded DNA molecule that differs from chromosomal DNA. pDNA is naturally found in bacterial cells and some eukaryotes. During bacterial division, all of the plasmids contained within the cell are copied to daughter cells. Bacteria can also deliver plasmids to one another *via* conjugation (Lodish and Zipursky 2001). The size of a commonly used pDNA ranges from a few hundred base-pairs (bp) to several thousand bps. In addition to the transgene, pDNA contains other regulatory signals such as the promoter, reporter, enhancer sequences, splicing, and polyadenylation sites, which have important roles in regulating gene expression (Williams et al. 2009). The selection of a suitable enhancer can improve transcription efficiency by several hundred folds (Noss et al. 2002). LifeTide® SW 5 (VGX Animal Health) is considered the only approved plasmid DNA for growth hormone releasing (Mignon et al. 2015). Many other human plasmid products are in different phases of clinical trials (Xu and Anchordoquy 2011).

6.3. RNA Interference (RNAi) using siRNA, miRNA, shRNA

RNA interference (RNAi) is a regulatory mechanism that occurs in the cytoplasm of most eukaryotic cells to control gene activity. Similar to AON design, the proper selection of siRNA sequence and incorporation of certain chemically-modified nucleotides can enhance its specificity and gene silencing activity (Dowler et al. 2006). RNA can also be transferred into cells by encapsulation in liposomes, or lipid-like nanoparticles (LLNs) (Li et al. 2016). Enhanced delivery of siRNA involves covalent conjugates to cell-penetrating peptides (CPPs) or protein transduction domains (Guan and Rosenecker 2017).

6.3.1. Mature micro RNA (miRNA)

miRNA is a short, 20-24 nucleotides, single stranded non-coding RNA that binds to partially complementary sites on their target

mRNA and inhibits mRNA after transcription. miRNA silences gene expression by affecting the stability of target mRNAs or via translational repression (Wilczynska and Bushell 2015).

6.3.2. Short hairpin RNA (shRNA)

shRNA is a synthetic RNA sequence that can induce gene silencing via RNAi. It consists of a stem that can vary from 25 to 29 bases and a loop of 4 to 23 nucleotides (Ge et al. 2010).

6.4. Toxicity and immune responses of cargoes delivered by non-viral carriers

Exogenous genetic materials, DNA or RNA, surely stimulate the immune system. This results in release of inflammatory cytokines which cause local and systemic inflammatory reactions. Conjugation with cationic lipids or polymers usually increases the immune response. Immune reaction and release of cytokines are mainly attributed to the endosomal toll-like receptors (TLR) transduction (Karikó et al. 2004). The intracellular receptors TLR3, TLR7, TLR8, and TLR9 are the main receptors involved in the identification of nucleic acid-like structures (Morozumi and Uenishi 2009). Receptors involved in immune response differ according to the genetic material. The recognition of the immune-stimulatory sequences in the plasmid structure (i.e. unmethylated CpG motifs) is usually mediated via TLR9, which can be reduced by decreasing the CpG content of the pDNA. RNAs are generally recognized by three main types of immune-receptors: TLR (3,7 and 8), protein kinase R, and helicases (Karikó et al. 2004). The incorporation of few 2- O-methyl modified bases into highly immune-stimulatory siRNA was found to evade the induction of the inflammatory cytokines *in vivo* (Judge et al. 2006). In case of AON, immune response occurs only if CpG sites were found on the AON sequence and is due to Toll-like receptor 9 (TLR9) mechanism.

6.5. Advantages and disadvantages of cargoes delivered by non-viral carriers

A main advantage for the use of non-viral gene delivery systems is their ability to carry large-sized DNA genetic material as compared to the limited packaging capacity of viral carrier systems (Thomas et al. 2003). When it comes to the type of genetic material, mRNA targeting *via* antisense therapy (AON, siRNA, shRNA and miRNA) was found to have many privileges over pDNA. Less amounts of the delivery vehicle are usually required. This leads to reduction in cost and cytotoxicity. In addition, the vector is easily designed, and the nuclear barrier and insertional mutagenesis can be escaped in contrast to plasmid DNA (Kutzler and Weiner 2008).

Nevertheless, the nuclear barrier can be a problem for some therapeutic applications of AON and the inability to express the gene of interest is a drawback of the antisense therapy (Elsabahy et al. 2011).

As for plasmid DNA, it can be used to express a missing gene and as an adjuvant therapy in vaccination. However, as previously mentioned, pDNA has the obstacle of the nuclear barrier, possibility of insertional mutagenesis, and difficulties in formulating it (Kutzler and Weiner 2008).

7. Carriers from the early synthetic polymers to the latest smart polymeric modifications

Over the past three decades, there is a wave of revolution of new strategies for non-viral gene delivery (Yin et al. 2014). More than 50 patents and 90 research papers describing novel strategies for delivering plasmid to target cells have been screened and tabulated in Appendices 1 and 2. This literature-extensive scan presents a time-span extending from 2006 into 2017. The bold and revolutionary introduction and marketing of Gendicine® in 2003 from China fueled the gene therapy field and fed confidence and encouragement to startups and researchers worldwide to follow-up and lead the field in the Western world. Screened patents and research papers illustrate different novel vectors such as polymers (linear and branched polymers, dendrimers and polysaccharides), thiol modified polymers, micelles, biodegradable nanoparticles, PEGylated polymers, cell-penetrating peptide grafted carriers, inorganic nanoparticles, and others. Furthermore, surveyed patents and papers also illustrate heterogeneous *in-vivo* and *in-vitro* evaluation methods, physicochemical characterization parameters, transfection tests, expression readout techniques, reporter and therapeutic genes, toxicity testing, genetic material doses, cell lines used in testing, animal's models, therapeutic purpose of non-viral delivery system, and even the controls or gold standards used in comparing their novel carriers with. Such heterogeneity and lack of standard or even consistent methodology protocols can be drawbacks in the progress of the field. The complete list of the surveyed patents and papers is categorized and tabulated in Appendices 1 and 2.

In this part of the review, the most common types of non-viral gene delivery carriers are discussed along with representative examples from the literature, either published as papers or registered patents over a time-span extending from 2006 into 2017.

7.1. Cationic polymers

7.1.1. Poly-l-lysine (PLL, the safety gold standard)

PLL is a synthetic linear polypeptide with repeated l-lysine residues (Fig. 1). It is one of the most studied cationic polymers in the field of gene delivery and is considered one of the commonly used gold standards with respect to safety (Lin and Lou 2012). PLL contains only primary amines, lacking any secondary or tertiary amines. This provides PLL with its high safety profile, but drastically affects its transfection efficacy (Lin and Lou 2012). PLL is responsible for the DNA condensation. The good binding of PLL to DNA is due to electrostatic attraction between its positive charges and the negatively-charged phosphate group (DNA) (Patil et al. 2011). Targeting of the malignant cells may be achieved through the electrostatic attraction between PLL's positive charge and negatively-charged malignant cell surfaces. On the negative side, complexes of PLL and DNA do not easily dissociate, making PLL of fair transfection efficiency compared to other polymeric carriers. Moreover, nonspecific interactions with cell membranes affect its cytotoxicity (Lin and Lou 2012). Therefore, to overcome PLL weak transfection efficiency, PLL is always found in a di-block or tri-block form with other polymers to balance the criteria of the delivering vector. Also, PLL is usually found PEGylated or modified with imidazole groups to enhance its transfection and toxicity profiles (Lin and Lou, 2012). Yu et al. (2007) designed PLL-grafted chitosan copolymer to enhance transfection and reduce cytoxicity *in vivo*. The good DNA binding of PLL and biodegradability and compatibility of chitosan cause PLL-grafted chitosan copolymer to yield better transfection efficacy compared to that of each polymer individually (Yu et al. 2011). Patil et al. (2011) developed a cationic PAMAM-PEG-PLL nanocarrier system for targeting cancer cells. PLL primary amines and PAMAM tertiary amines provide the advantage of a block copolymer with good DNA binding capabilities, transfection efficiency, endosomal escape, and cytoplasmic delivery of the genetic material (Patil et al. 2011).

7.1.2. Polyethyleneimine (PEI, the efficiency gold standard)

PEI is a three-member ring of ethyleneimine that, in the presence of a catalyst, polyethylenimine (PEI) is formed (Fig. 1). Linear or branched PEI is characterized by high-charge density and proton sponge effect (Liu et al. 2011b). Behr et al. were the first to detail the use of PEI in non-viral gene delivery in 1995 (Boussif et al. 1995). Their paper was a key milestone in the history of non-viral gene delivery technology. Prior to it, none of the available non-viral gene delivery carriers ever matched the efficacy of viral vectors transfection efficiency. Behr et al. demonstrated strong transfection efficacy with PEI empowered by its buffering capacity and its proton sponge effect coupled with high cationic charge imparted by its unique primary, secondary, and tertiary amine groups in

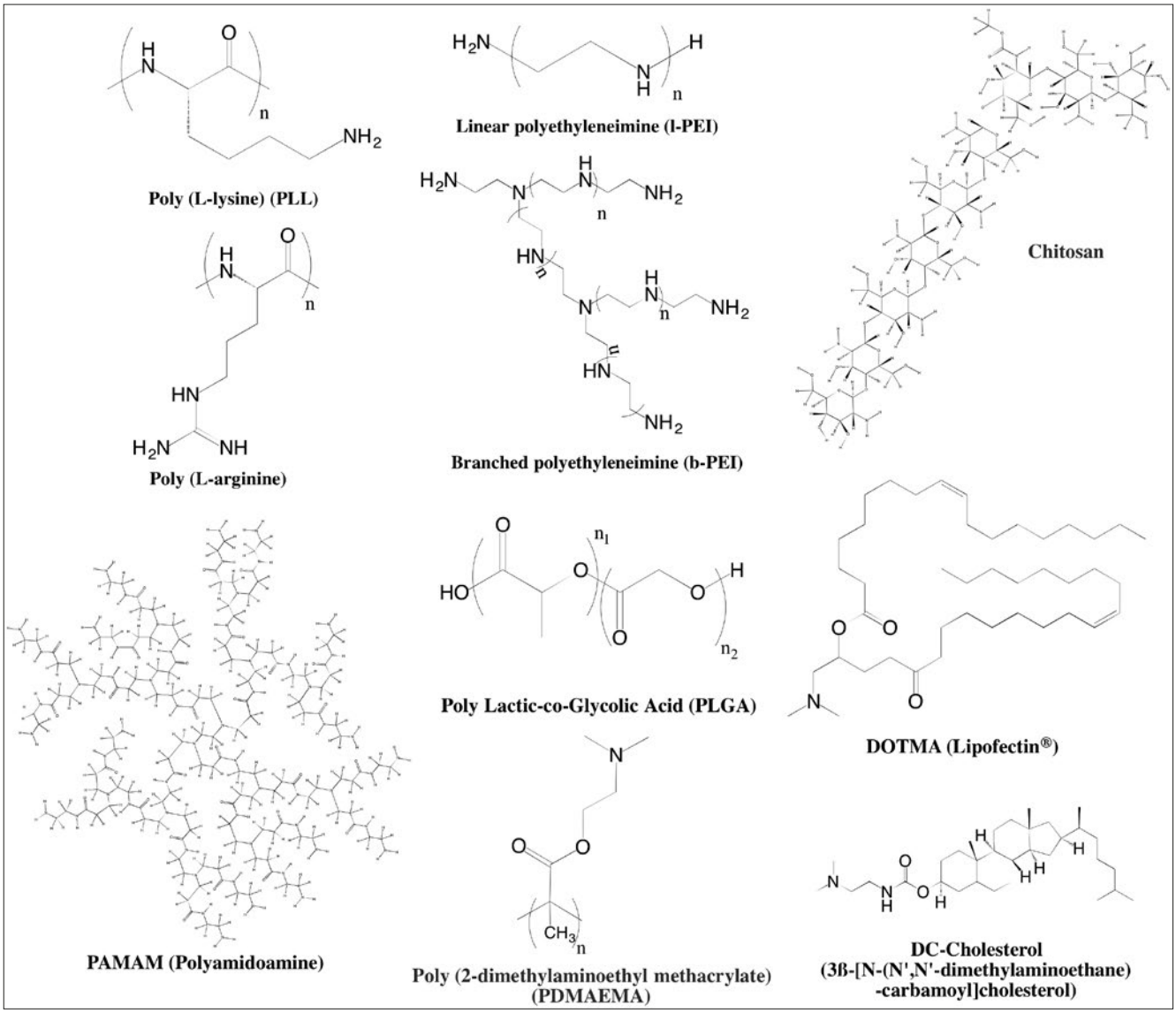

Fig. 1: Chemical structure of numerous polymers and lipids used in the formulation of non-viral gene carrier systems.

its structure backbone (Boussif et al. 1995). Since then, PEI is considered the gold standard in the non-viral gene delivery world with respect to efficacy. The proton sponge hypothesis shows that unprotonated amines inside the lysosomes attract protons; and, as a result, there is an increase in the influx of chloride ions and water. Finally, the repulsion between protons and the water influx leads to swelling and bursts of lysosomes (Benjaminsen et al. 2013). Although PEI is considered the gold standard in non-viral gene delivery when it comes to efficacy, it falls short when it comes to safety and toxicity pattern. Practically, the main reason that accounts for PEI efficacy (high positive change content) is also the source of its high toxicity profile. PEI can be classified according to its structure into linear and branched. Another classification is according to its molecular weight. According to PEI molecular weight, it can be classified into high and low molecular weight PEI. Low molecular weight (LMW) PEI has lower cytotoxicity than that of high molecular weight (HMW) PEI (Fang et al. 2014). However, due to its low gene transfection, LMW PEI is modified by a hydrophobic group to increase its gene transfection efficiency. PEI grafted copolymers using disulfide linkages are proven to enhance gene transfection and generate a smart bioresponsive and bioreducible non-viral gene carrier (Parhiz et al. 2013). On the other hand, HMW PEI exhibits a high DNA transfection efficiency. Unfortunately, HMW PEI has a high cytotoxicity due to its non-degradable linkers.

Li et al. (2014) designed a ternary complex mPEG-b-PLL-g-(ss-lPEI) in a human ovarian cancer therapy. LMW PEI was grafted to a PEG-PLL copolymer using the disulfide linkage to improve siRNA transfection and render the copolymer bioreducible and biodegradable. The bioreducible feature enables the carrier system to be solid and intact extracellularly and to disintegrate easily in the reducible environment intracellularly. Furthermore, they conjugated a monoclonal antibody (herceptin) that acts as a targeting ligand with PEG-PLL to enhance transfection efficacy in Skov-3 (cell line of ovarian cancer). mPEG-b-PLL-g-(ss-lPEI) reduced the cytotoxicity due to PEI proton buffering ability and PLL biodegradability (Li et al. 2014).

Branched PEI is considered the standard form of the polymer. Branched PEI consists of primary, secondary, and tertiary amines, all of which can be protonated. Therefore, it is of considerable buffering capacity and positive charge content and subsequently has a better condensation of DNA than that of linear PEI. Despite its high transfection efficiency, cytotoxicity of branched PEI is a major challenge as PEI toxicity increases with increasing the molecular weight or branching (Liu et al. 2011a).

Biodegradable disulfide linkages and ester groups are added to PEI to decrease its cytotoxicity. Like many other cationic polymers, PEI can be PEGylated to enhance its cytotoxicity profile due to polyethylene glycol shielding effect (Yang et al. 2015). Some patents, established for PEI, showed that PEI can be used

in chemotherapy and radiotherapy treatment. It showed promising potential as a polymeric carrier system to be used therapeutically and as a diagnosis tool (Chrony et al. 2010; Lynn and Miller 2012).

7.1.3. PAMAM (Poly(amidoamine) dendrimer)

Dendrimers have emerged in 1995 with a final 'dendrimer-like star-branched polymers' shape (Hirao and Yoo 2011). They have a unique three-dimension branched-architecture. Their nano-sized structure and cationic charge made them well suitable as promising candidates for non-viral gene delivery. Dendrimers are synthesized by capping the chains of a hydroxyl terminated six arm star polymer with dendrons (ring-opening). These produced "macro-initiators" are polymerized to produce the "DENDRIMER" polymer (Hirao and Yoo 2011). Poly(amidoamine) (PAMAM) dendrimers are hyper-branched polymers with molecular uniformity, narrow molecular weight distribution, defined size and shape characteristics, and a multifunctional terminal surface. The PAMAM dendrimer polymer was described by Tomalia et al. (1985) as "starburst'' dendritic macromolecule. PAMAM's design is based on spherical structure based on a sphere-like core and is characterized by an internal molecular design consisting of tree-like branching, with each external 'layer', or generation, containing exponentially more branching points. Based on this design language, PAMAM dendrimers may commercially exist in different forms of branching or, what is called, different generations (Fig. 1). PAMAM possess cationic primary amine groups at the surface. Such structure aids in efficient binding with DNA and siRNAs and increases their intra-cellular uptake by forming nano-sized complexes called "dendriplexes" (Kesharwani et al. 2012). PAMAM-dendrimer DNA complexes (dendriplexes) aid proficient cellular uptake by the endosomal escape mechanism as in the case of PEI (Majoros et al. 2008). Dendrimers are commercialized as SuperFect® and PolyFect®, which are known for high transfection DNA characteristics (Massadeh et al. 2015). As in the case of PEI, PAMAM dendrimers suffer from a high toxicity profile.

Kim et al. (2013) designed a bioconjugate system of arginine-grafted poly (cystamine bisacrylamide-diaminohexane) (ABP) and PAMAM (PAM-ABP/chimeric DNA polyplex) to treat type 2 diabetes via enhancing of exendin-4 expression. Furthermore, PAM-ABP enhances insulin secretion *via* activation of protein kinase K (PKA). In another study, PAMAM-Arg/DNA modification was clinically applied for ovarian cancer therapy (Jang et al. 2011). Furthermore, Kobyashi et al. (2016) used carbonyl reductase 1 (cbr1) DNA with a polyamidoamine dendrimer (PAMAM) to target ovarian cancer. In another example, Ayatollahi et al. (2015) designed PEGylated alkylcarboxylate-grafted PAMAM dendrimers (G4 and G5) which had a high transfection efficacy in mesenchymal stem cells (MSCs) and murine neuroblastoma (Neuro-2a) cell line (Ayatollahi et al. 2015).

7.1.4. Poly lactic-co-glycolic acid copolymer (PLGA)

PLGA is a biodegradable polymer that is used as a controlled drug delivery carrier (Fig. 1). PLGA has been approved by the US FDA for the use of drug delivery, diagnostics, and other applications (Lu et al. 2009). For this reason, PLGA is considered a gold standard with respect to safety. Furthermore, due to its copolymeric nature, varying the content of polylactic acid or polyglycolic acid in PLGA enables researchers to control the rate and extent of biodegradability of PLGA (Lu et al. 2009).

PLGA may be formulated as nanoparticles which can protect drugs from degradation and enhance their stability (Danhier et al. 2012). PLGA-based nanoparticles can improve pharmacokinetic and pharmacodynamics profiles (Danhier et al. 2012). Liu et al. (2012) designed mPEG-PLGA-b-PLL amphiphilic block copolymer for the delivery of adriamycin and siRNA for hepatic carcinoma treatment. Adriamycin-mPEG-PLGA-b-PLL or siRNA-mPEG-PLGA-bPLL nanoparticles showed negligible cytotoxicity and a high compatibility with live cells (Liu et al. 2012). PLGA-based nanoparticles were tested to target nucleic acids to hepato-cellular carcinoma with a good safety profile (Liang et al. 2011). Moreover, PLGA-based nanoparticles were tested in miRNA-related diseases such as diabetic, cardiovascular, and neurodegenerative diseases (Liang et al. 2011). Biodegradable PLGA developed higher gene inhibition efficiency than Lipofectamine in a study performed to treat lung cancer (Du et al. 2012).

7.1.5. Chitosan (the natural polymer)

Chitosan is a biodegradable cationic polymer that has free amino acids that can bind to negatively-charged moieties such as nucleic acids (Patel et al. 2010). It is composed of N-acetylglucosamine and glucosamine (Fig. 1) and can be obtained by alkaline deacetylation of chitin. Chitosan is found to be nontoxic, biocompatible, and biodegradable. Its degradation products by lysosomes are nontoxic, non-immunogenic, and non-carcinogenic. Due to its natural origin, chitosan and its derivatives present significant physicochemical and structural variability, leading in high variability in pharmaceutical formulations (Elsabee and Abdou 2013; Il'ina and Varlamov 2005; Patel et al. 2010). Chitosan has wide applications in the field of non-viral gene therapy due to its biocompatibility and low toxicity. However, it has low transfection efficiency (low endosomal escape) (Akbuga et al. 2016).

Mumper et al. introduced chitosan as a gene delivery carrier in 1995 (Centelles et al. 2008). Chitosan has reactive sites for ligand, conjugation, cross-linking, and modification by acetylation of the glucosamine monomers, affecting the charge and changing the weight of the carrier system. These modifications can enhance the efficiency of chitosan. Large numbers of clinical trials applied the bioconjugation technology to modify chitosan by adding a synthetic polymer such as PEI, forming Polyethylenimine grafted chitosan (PEI-g-chitosan) (Dang and Leong 2006). The main aim of such modification is the enhancement of the transfection efficacy of chitosan and the toxicity profile of PEI. PEI-g-chitosan-DNA complex was shown to be stable complex with high transfection efficiency and low cytotoxicity profile (Dang and Leong 2006).

Chitosan has been investigated intensively for non-viral gene delivery. A nanoparticle-chitosan complex was developed to overcome the instability barrier of RNA molecule in biological environment (Ragelle et al. 2016). Furthermore, chitosan's covalently-bonded hyaluronic acid nanoparticle was used for ocular diseases treatment (Zulliger et al. 2015).

7.1.6. PDMAEMA polymer (poly (2-(dimethylamino)-ethyl methacrylate)

PDMAEMA is a water soluble polycation (Fig. 1) (Synatschke et al. 2011). It can be easily synthesized by atom transfer radial polymerization technique (ATRP). It has a high transfection efficiency because it has the ability to escape from endosomes in the same fashion as PEI (Synatschke et al. 2011). Its cytotoxicity depends on the molar ratio of its co-polymeric subunits and the polymer concentration. As the N\P ratio increases, the cytotoxicity decreases (Qian et al. 2012). On the contrary, as the polymer concentration increases, the cytotoxicity increases (Rawlinson et al. 2010).

As for many polycations, having a net positive charge on their outer-structure, the positive charge enhances the DNA condensation and encapsulation into the polyplex. However, increasing the positive charge increases the cytotoxicity due to non-specific interaction with the negatively-charged cellular compartments as RBCs and proteins (Synatschke et al. 2011). Furthermore, the higher molecular weight of PDMAEMA leads to a higher transfection efficacy and cytotoxicity (Samsonova et al. 2011).

In studies with several polymers, polyethylene glycol has always been known for its shielding effect. PEG increases the relative colloidal and serum stability of PDMAEMA, prolongs the circulation time, and prevents protein adsorption into aggregates (Khalil et al. 2006). Furthermore, PEG-PDMAEMA complexes have reduced cytotoxicity compared to PEI due to lower surface activity of the complex (Qian et al. 2012).

Moreover, addition of surface ligands to enhance targeting of polymers to target tissue is a valid option to minimize the chance for non-specific interactions. For instance, brain targeting has been of

a major challenge due to restricted access to the blood brain barrier (BBB). TGN, a 12-aminoacid chain that revealed a significant brain transport, has been investigated to target brain cells (Qian et al. 2012). The tri-block polyplex of TGN-PEG-PDMAEMA is shown to have good condensation capacity, superior transfection efficiency, and markedly low cytotoxicity with efficient targeting to the brain (Qian et al. 2012). The transfection efficiency of TGN-modified polyplexes was higher than that of unmodified polyplexes both *in vitro* and *in vivo* (Qian et al. 2012).

7.2. Lipid-based carriers

Lipid-based molecules used in gene therapy delivery are classified into lipid nanoplexes (simple complexes of cationic lipids and negatively charged DNA), liposomes (either as complexes with negatively-charged DNA or DNA encapsulated within the liposomes), solid core lipid-based nanoparticles, and lipid-based nano-emulsions (Xue et al. 2015).

7.2.1. Lipid complexes (lipoplexes) and liposomes (vesicular structures) mediated gene transfer

The discovery of banghasomes (liposomes) by Bangham (named after him) on 1965 was a breakthrough in novel pharmaceutical nano-carriers (Bangham 1993; Bangham et al. 1965a, b).

Liposomes are self-assembling systems. The lipid molecules disperse spontaneously to conceal their hydrophobic tails interiorly and expose their hydrophilic heads in the aqueous compartment. Consequently, it inaugurates into a bilayer vesicular structure (liposome) with a particle size ranging from 20 nm to a few microns (Balazs and Godbey 2011). Liposomal DNA complexes are formed when a negatively-charged DNA molecule is complexed with a liposome structure. Liposomal DNA complexes are sometimes mistakenly called lipoplexes. Lipoplexes are complexes of cationic lipids (not in self-assembly liposomal vesicular form) and anionic DNA. In some cases, DNA is encapsulated inside the liposomal vesicular structure. In case of encapsulated DNA inside the liposomal vesicular structure, anionic, cationic, and non-ionic lipids can be used in the formulation of the liposomal structures. Generally, liposomes are formulated with charged lipids (cationic lipid) and a helper lipid (neutral lipid) such as cholesterol (Tai et al. 2017). Cationic liposomes establish sufficient Van der Waal forces and electrostatic binding with the poly anionic charged DNA to form the DNA/complex (Tai et al. 2017). Cationic liposomes are conjugated with neutral liposomes to enhance endolysosomal escape via membrane destabilizing effect of neutral liposomes (Balazs and Godbey 2011).

7.2.1.1. Mono-valent cationic lipids

DOTMA and DOTAP

N-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) is one of the first synthesized cationic lipids used in gene therapy (Fig. 1). DOTMA can form DNA complex or RNA complex (Felgner et al. 1987; Felgner and Ringold 1989). Moreover, the DOTMA/DNA complex is characterized by low toxicity and low immunogenicity (Zhdanov et al. 2002). DOTMA was mainly the spark for creation of the other remarkable cationic lipids, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) (Leventis and Silvius 1990) and 3β[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (Gao and Huang 1991) for enhanced efficiency. The only difference between DOTAP and DOTMA is that ether bonds linking the chains of to the DOTMA backbone are replaced by ester bonds in DOTAP. Ester bonds, which are hydrolysable, could render the lipid structure biodegradable and aid in reducing cytotoxicity.

DOTMA was conjugated with dioleoyl phophotidylethanolamine (DOPE) in a ratio 1/1 and commercialized it as Lipofectin®. DOPE enhanced the transfection efficacy of Lipofectin®. Many modifications have been imparted to DOTMA to reduce cytotoxicity and enhance transfection efficacy. DOTAP cannot be used in gene delivery alone due to its high positive surface charge density. Thus, combination of DOTAP with neutral helper lipids should be used to enhance transfection efficacy (Balazs and Godbey 2011).

Hatakeyama et al. (2009) designed an anticancer multifunctional envelope-type nano-device (MEND) conjugated with PEG-peptide-DOPE (PPD). The nano-device can be cleaved in a matrix metalloproteinase (MMP)-rich environment, with enhanced delivery and efficacy both *in vivo* and *in vitro* (Hatakeyama et al. 2009).

DC-Chol

3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol, or DC-Chol, was synthesized by Gao and Huang in 1991(Gao and Huang 1991). The cholesterol moiety is biocompatible and stable. Thus, DC-Chol has a higher transfection efficacy (2-4-fold) than Lipofectin® in chloramphenicol acetyltransferase expression (CAT assay) (Balazs and Godbey 2011). DC-Chol reduces the cytotoxicity up to four-fold compared to lipofectin®'s toxicity in some cell lines (Balazs and Godbey 2011). Combination of DC-Chol with DOPE enhances transfection efficiency and DNA-dissociation due to reduction in the lipoplex surface charge (Zuidam and Barenholz 1998) and aggregation (Ajmani and Hughes 1999).

7.2.1.2. Multi-valent cationic lipid (spermine group)

DOSPA (Lipofectamine®)

DOSPA, (2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N, N-dimethyl-l propanaminium trifluoroacetate), is another cationic lipid synthesized as a derivative of DOTMA. The structure is similar to DOTMA except for a spermine group which is bound *via* a peptide bond to the hydrophobic chains (Sitharaman 2011; Zhang et al. 2012). The addition of the spermine functional group leads to a tighter DNA compaction due to interaction between its hydrogen bonds and the DNA bases (Sitharaman 2011; Zhang et al. 2012).

The combination of DOSPA with DOPE at a 3:1 ratio is commercialized as a transfection reagent called Lipofectamine®. Since its launch in 1993, it became the most commonly used transfection reagent in gene therapy; cited in more than 50,000 scientific papers as the "Gold Standard" for non-viral gene delivery (Ondrej et al. 2007). The gold standard status was granted for the highlighted "high transfection" of DNA, siRNA, and miRNA. The transfection was into broad range of cells including difficult to transfect cells (Cardarelli et al. 2016), compared to alternative transfection reagents (DC-Chol/DOPE formulation) as demonstrated by Fumie et al. (2016).

Lipofectamine® reagents' types are divested to Lipofectamine® 2000, Lipofectamine® 3000, Lipofectamine Messenger MAX, Lipofectamine® LTX with Plus™ reagent, and Lipofectamine® RANIMAX reagent. Lipofectamine® 2000 reagent has delivered DNA or siRNA since 1999 with almost complete transfection performance for protein expression and gene silencing after 48-72 hours (Jensen et al. 2014). Lipofectamine® 3000 is advertised for its gentle low toxicity and "superior efficiency". It has an almost tenfold higher efficiency in the broad-spectrum reagents for the difficult- to transfect cells (Yu et al. 2016b). Lipofectamine® messenger MAX is highly efficient in delivering mRNA specifically to the neurons and the primary cell types (Goparaju et al. 2017). Furthermore, Lipofectamine® LTX with Plus™ reagent was used for transfection of highly sensitive cells (Sandbichler et al. 2013). Moreover, precisely transfected siRNA was conveyed by Lipofectamine® RNAiMAX Reagent to deliver siRNA into hES cells (Zhao et al. 2008). Most importantly and recently, Lipofectamine™ CRISPRMAX™ transfection reagent is providing a CRISPR-Cas9 protein delivery *in vitro* and *in vivo* (Zuris et al. 2015).

DOGS

Di-octadecyl-amido-glycyl-spermine (DOGS) has a structure similar to DOSPA. However, the chains in DOGS are saturated. The chains in DOGS are linked to the head group through a peptide bond and lack a quaternary amine as compared to DOSPA (Balazs and Godbey 2011). Behr et al. (1989) showed that DOGS

has double remarkable advancement by proving a highly efficient character in delivering catalase plasmid vector with no cytotoxicity. Eventually, DOGS was commercialized under the name Transfectam®. The head group of the DOGS facilitates DNA condensation and prevents pH-sensitive nucleases degradation *via* buffering the endosomal compartment (Ajmani and Hughes 1999). The conjugation of cationic liposomes (DODAC/DOPE) with PEG (polyethylene glycol) can enhance the transfection efficiency (Song et al. 2002), lower immunogenicity, and provide longer circulation time. Therefore, the inclusion of PEG in liposomes is known as stealth liposome. Nevertheless, Shi et al. (2002) remarked that PEGylation may limit the liposome advantage in cellular passive targeting.

7.2.2. Lipid-based solid-core nanoparticles

Solid-lipid core nanoparticles (SLN) were developed in the late 1990s in succession to liposomes and nano-emulsions discovery (Müller et al. 2002). SLN's average size is 40 to 1000 nm, and they have a spherical structure (Thatipamula et al. 2011). SLNs are containing 0.1 – 30 (% w/w) solid fat which is dispersed in an aqueous phase. Their lipid components remain stable, in nano range with respect to size, and solid at both body and ambient temperatures (Lima et al. 2013). The lipids which are used in formulation of SLNs may be triglycerides (compritol), partial glycerides, fatty acids (stearic acid, palmitic acid), steroids (cholesterol), and waxes (cetyl palmitate). Emulsifiers are used to disperse the lipid component (Das and Chaudhury 2011).

In comparison to liposomal structures, the solid-lipid nanoparticles have solid, lipophilic core regions. SLNs are used as a gene delivery system. The SLNs-DNA complexes are formed by the electrostatic interaction between the negative charges of the DNA and the positive charges of the lipid, resulting in a solid stable complex. Nevertheless, the solid core structure has difficulties in encapsulating DNA or RNA polyanionic structure. Correspondingly, few are used in clinical applications of gene delivery (Xue et al. 2015). However, Carrillo et al. (2013) showed that a cationic SLNs -DNA complex could be formed. In another example, Jin et al. (2011) modified the siRNA-PEG/SLN for brain tumor treatment, bypassing the blood brain barrier (BBB). siRNA-PEG/SLN showed no systemic toxicity (Jin et al. 2011). A novel study by Chen et al (2010) detailed multifunctional solid-lipid nanoparticles composed of anionic lipids dual-loaded with VEGF siRNA and doxorubicin as anticancer therapy. Also, Bae et al. (2013) and Su et al. (2012) reported novel solid-lipid nanoparticle formulations dual-loaded with siRNA and paclitaxel for cancer treatment.

7.2.3. Lipid-based nano-emulsions

Nano-emulsions are composed of two phases, oil and water phases. The size of oil-in-water droplets are typically in nano-range, ranging between 20 and 300 nm. The oil phase protects the RNA from degradation and is more efficient as compared to nanoparticles (Xue et al. 2015). Updated, few lipid nano-emulsions have been developed for RNA delivery. By convention, nano-emulsions are made of negatively-charged ingredients. Consequently, that makes it hard to stably encapsulate these RNA molecules. Another issue is that oil droplets cannot be small and uniform after inclusion of large molecules such as RNA. Thus, coalescence and Ostwald ripening in emulsions occur to the droplets after RNA inclusion (Souto et al. 2011). Nevertheless, clinical trials' efforts did not miss to tackle these limitations.

Kaneda et al. (2010) discovered a nano-emulsion that may consist of a perfluorocarbon core covered with positively-charged DOTAP for coupling with siRNA. Captivatingly, although the size of nanoparticles was 300 nm in diameter, it was able to deliver siRNA efficiently by the lipid raft mediated transport. Thought provokingly, this lipid nano-emulsion cellular uptake needs more investigation in more clinical trials. PEGylated poly(2-(dimethylamine) ethyl methacrylate)/DNA polyplex micelles showed higher transmission efficiency than unmodified lipid-based carriers (Shi et al. 2002).

7.3. Inorganic particles

Inorganic particles are nanoparticles which can easily cross the cell membrane to deliver their targets. They are readily prepared and their particle sizes are easily controlled. Inorganic particles include gold nanoparticles, silica, calcium phosphate, quantum dots, magnetic compounds, and others (Loh et al. 2016; Ramamoorth and Narvekar 2015; Tian et al. 2013). As discussed earlier, modifications and optimizations to carrier systems are critical for optimum efficiency. Modifications to inorganic carriers can be imparted through adding a positively-charged particle to complex with the genetic substance; through conjugation to a targeting ligand, a bioresponsive element that's responsive to a surrounding stimulus; or through the use of a cationic amphiphilic polymer between the inorganic particle and the genetic material (Loh et al. 2016). The biodegradability of calcium phosphate nanoparticles, along with the cationic polymer PLGA, showed high silencing efficiency in the treatment of intestinal inflammation (Frede et al. 2016a). Inorganic nanoparticles have been proven to overcome the deleterious consequences of systematic routes of delivery as they deliver their cargo to the target cells, making them a rising method for non-viral gene delivery (Frede et al. 2016b).

8. Smart carriers: A clever step forward towards biological intelligence

It wouldn't be possible to change the environmental factors that the polymer passes through, but it would be smart if we would take the advantage of knowing these factors to modify the polymer design to interact with such environmental factors. Modifying and adjusting the polymer is performed in a way that would help it crawl into the cells bypassing all the barriers. For example, temperature variabilities could be a triggering environmental factor. Responding to difference in temperature inside the cells is called thermo-responsiveness. Thermo-responsive polymers studies showed improved DNA condensation properties (Twaites et al. 2004). pH-responsive polymers are other examples of smart carriers. pH-responsive polymers are usually achieved by adding an acetal group. pH-sensitive sulfonamide/PEI system yielded impressive targeting to acidic extracellular compartments of tumors (Sethuraman et al. 2006). Dual responsiveness was also extensively studied. Adding a disulfide group along with an acetal group rendered polymers pH and reduction responsive (Zhang et al. 2014).

Smart carriers can be defined as those carriers that can exhibit significant changes in their physicochemical properties, size, integrity, or shape in response to different stimuli from the environment (Dincer et al. 2005).

Smart carriers can be classified according to the stimuli they respond to into:

- Carriers reacting to change in temperature – "thermo-responsive"
- Carriers reacting to change in pH – "pH responsive"
- Carriers reacting to change in reduction\oxidation states – "redox activity responsive"

Finally, bioresponsivness is not only limited to temperature, pH, or redox potential stimuli, but it can be designed to sense a limitless variety of stimuli such as glucose-sensing polymers for treatment of diabetes and interactive continuous measurement of blood sugar level (Yu et al. 2017).

8.1. Carriers reacting to change in temperature – "thermo-responsive"

The shape and solubility of the polymer can change due to temperature change. For any polymer delivering cargo, there is an upper critical solution temperature (UCST) or a lower critical solution temperature (LCST) (Fitzpatrick et al. 2012). Having a LCST, the polymer changes its state from the soluble liquid into a gel above this temperature. On the other hand, a polymer having an UCST is soluble when heated above such a temperature (Fitzpatrick et al. 2012). Studying these material properties helps in exploiting polymers for gene delivery optimization. Having a LCST, poly

(*N*-isopropylacrylamide) (PNIPAM) is the most commonly studied as the more responsive polymer. PNIPAM and phenyl boronic acid, along with the PAMAM dendrimer, were combined to make a smart polymer (Wang and Cheng 2016). Coupling the advantages of the proton sponge effect of PAMAM and the phase transition properties of pNIPAM developed an intelligent polymer that can release siRNA gradually in target cells only. The polymer showed high gene silencing at a low temperature (4 °C) that was not found at higher temperatures. The phenyl boronic acid has a great role in the polymer stability (Wang and Cheng 2016).

Furthermore, cooling of poly (*N*-isopropylacrylamide)/polyarginine complex below its LCST showed considerable gene expression. Targeting cancerous cells through thermo-responsive polymers has been widely studied. Tumor cells can normally have higher temperatures than normal cells or can be externally heated (Guo et al. 2014). Zintchenko et al. (2006) explained that heating a PNIPAM-based block copolymer caused polymer aggregation. Consequently, an increase in transfection efficiency was observed.

8.2. Carriers reacting to change in pH – "pH responsive"

Strong acids, having a low pKa, tend to lose their protons in a basic environment. On the other hand, strong bases accept protons in acidic environment. A pH responsive polymer contains a part that can accept or lose protons due to changes in pH environment. As a result, the polymer changes its shape either by getting larger or by shrinking and releases its contents at the intended place (Guo et al. 2014). It is known that the tumor intracellular compartments are more acidic than that of normal cells (Damaghi et al. 2015). Therefore, pH responsive polymers showed promising results in studies aiming to treat cancer.

In a study comparing the same polymer, one with an acid labile moiety and the other without, the results showed greater cellular internalization and endosomal escape after endocytosis by cancerous cells with the polymer comprising the carboxy dimethyl maleate (CDM, pH responsive acid hydrolysable moiety) (Tangsangasaksri et al. 2016). Not only polymers but also inorganic substances such as quantum dots (QD) can be used to target tumor cells via using pH responsive elements (Zhao et al. 2013). Zhu et al (2015) conjugated QDs with deoxy glucose, polyethylene glycol, and the amino acids, lysine and arginine. This pH responsive hybrid (with a hydrazone bond) quantum dots carrier demonstrated improved efficacy for delivering siRNA targeted to tumor cells.

8.3. Carriers reacting to change in reduction/oxidation states "redox activity responsive"

A redox responsive polymer is synthesized *via* incorporation of chemical moieties and/or bonds that are sensitive to a reducing or an oxidizing environment. Disulfide linkages are the most popular inserted moiety in many polymers to impart redox activity responsiveness. Their use was escalated as they were easily reduced in intracellular reducing environment compared to the oxidizing extracellular environment. Upon reduction of a disulfide-containing polymer, the polymer unpacks its cargo and releases its DNA genetic material load intracellularly (Cheng et al. 2011).

Yu et al (2016a) synthesized a glutathione responsive nano-complex that consists of gold-cysteamine (AuCM), plasmid DNA (pDNA), poly-TAT (pTAT), and hyaluronic acid (HA). The nano-complex showed that cells treated with glutathione displayed higher transfection efficiency than those without the reducing agent; even after coating the polymer with hyaluronic acid the transfection efficiency was not affected (Yu et al. 2016a). Tumor cells produce great amounts of oxidizing agents. Having this in mind, polymers sensitive to oxidizing environments were designed (Cheng et al. 2011). Ferrocenyl, selenium-based, poly (propylene sulfide), and thioester-responsive groups are all examples of oxidation responsive polymers (Ma et al. 2010).

Furthermore, Nounou et al (2010) have designed and synthesized novel reducible linear L-lysine modified copolymers (LLCs) as an alternative to high molecular weight poly (L-lysine) (PLL). LLC/pDNA polyplex showed exceptional stability during incubation with DNase I. Release of DNA from the polyplexes only occurred in the presence of the reducing agent dithiothreitol (DTT). The transfection efficiencies of the polyplexes showed that LLC polyplexes produced five times higher transfection efficiencies in HDF cells, three times higher transfection efficiencies in MCF-7 cells, and four times higher transfection efficiencies in MA cells as compared to the optimal PLL control. The LLC/pDNA polyplexes showed significantly lower cytotoxicities as compared to the control in HDF, MCF-7, and MA cells at certain N/P ratios. The results suggest that these novel LLCs are efficient, reducible, and biocompatible polymers for nonviral gene delivery (Nounou et al. 2010).

8.4. Smart hybrid systems

Formulating and designing a hybrid system can have many advantages and impart multifunctional properties to the carrier system. Not only can it help in passing many barriers, but also can assist in taking the benefits of each type of carrier and reducing its drawbacks. An et al. (2014) designed and formulated a four-arm three-dimensional PEG-b-poly disulfide histamine as a dual-bioresponsive polymer for non-viral gene therapy (An et al. 2014). The novel dual-bioresponsive carrier released its condensed DNA in the reducing environment of the cell (redox responsiveness) (An et al. 2014). This smart hybrid polymer also showed high transfection efficiency in MCF-7 and HepG2 cancer cells due to its acidic pH responsiveness. Similarly, PEG poly (2-(diisopropyl amino) ethyl methacrylate) poly (*N*-(2,2′-dithiobis (ethylamine)) aspartamide) "PEG-PAsp (AED)-PDPA" polymer has a pH sensitive part (PDPA) and a glutathione responsive part (PAsp (AED)) (Chen et al. 2014).

9. Non-viral gene therapy in cancer treatment: A glimpse into the future

Cancer is a major public burden all over the world. It is a chronic disease that largely affects one's quality of life. In 2015, the Centers for Disease Control and Prevention (CDC) reported that cancer was the second death-causing disease in the United States (National Center for Health Statistics (US). 2016). The American Cancer Society estimated that there will be 1,688,780 new cancer cases and 600,920 cancer deaths in 2017 (American Cancer Society, 2017). During the early stages of the disease, genetic changes such as activation of cellular proto-oncogenes or inactivation of tumor suppressor genes take place (Rakoff, 2006).

9.1. Current cancer therapies

There are a number of methods established to treat cancer. However, each one has drawbacks that impede its capabilities to cure the disease effectively and without side effects. The National Cancer Institute (NCI) of the National Institute of Health (NIH) in the US defines some cancer therapies as follows: Surgery might be the only treatment a patient with a solid tumor may need, it is not valid for cancers that spread or for blood cancers (U. S. National Cancer Institute, 2015). Radiotherapy works by disruption of the DNA of the cancer cell, but unfortunately radiation also affects healthy cells' genetic material. It causes different side effects according to the area exposed (American Cancer Society, 2017). Chemotherapy works on hindering the proliferation of cancerous dividing cells. Similar to radiotherapy, chemotherapy also affects normal cells that rapidly divide (Liu et al. 2015).

9.2. Gene therapy for cancer treatment

It is evident that there is a great need for a more specifically targeted method to treat cancer. Fixing, replacing, or deleting the mutated gene of a malignant cell is the application of gene therapy in cancer treatment. Gene therapy for treating cancer attempts either to modify tumor specific lymphocytes, deliver tumor suppressor genes, or to inhibit expression of oncogenes (Mulligan 1993). Another goal may be to introduce lethal genes to kill tumor cells (Mulligan 1993).

All methods of cancer gene therapy can be summed up into three main classes: immunomodulatory, corrective, and cyto-reductive therapies (Harrington et al. 2001; Spitzweg 2009; Spitzweg and Morris 2004). Immunomodulation is modifying the body's immune system to destroy cancer cells while corrective methods include the introduction of genes such as tumor suppressor genes. Finally, suicidal and anti-angiogenic strategies are examples for cytoreductive methods.

The first commercially available gene therapy worldwide approved by the State Food and Drug Administration of China in October 2003, Gendicine®, is a recombinant Ad-p53 cytoreductive gene therapy for head and neck squamous cell carcinoma (HNSCC) (Frew et al. 2008). This highlights the importance and potential of gene therapy in advancing novel therapeutics for cancer treatment. Furthermore, the cancer therapy field and the public need for innovative and successful treatment for malignant tumors can be a solid driving force for the gene therapy field.

9.3. Non-viral gene cancer therapy

Non-viral gene delivery systems were studied for the treatment of different types of cancer. Nanoparticles show a solid potential (Wang et al. 2015). Non-viral methods have advantages over the traditional methods for treating cancer; for instance, in treating cancer metastasis in terms of targetability and effectiveness. Wang et al. (2012) demonstrated that neural stem cell expressing suicide genes showed better effects in lung cancer brain metastasis. Research in this area is growing very quickly. However, all non-viral gene therapy methods are in experimental stages and the FDA has not approved any of them to the market yet (U.S. Food and Drug Admnistration, 2017). In this section, some examples of the current efforts cited in literature for different novel non-viral gene delivery systems, such as cationic polymers, inorganic particles, and smart responsive carriers used as anticancer, are discussed.

9.3.1. Cationic polymers

9.3.1.1. Poly-l-Lysine (PLL)

Liu et al. (2012) designed an amphiphilic block copolymer composed of conventional monomethoxy (polyethylene glycol)-poly (d,l-lactide-co-glycolide)-poly-(l-lysine) (mPEG PLGA-b-PLL) and used it in the formulation of nanoparticles with enhanced cellular uptake of encapsulated adriamycin or siRNA. These nanoparticles were tested on Huh-7 hepatic carcinoma. In another example, Mann and Kullberg (2016) synthesized a polyplex that consists of PEGylated poly-l-lysine core complexed with DNA. The polyplex is conjugated with Listeriolysin-O (LLO) and trastuzumab antibody *via* a disulfide bond. Listeriolysin-O (LLO) is a pore-forming protein that enables DNA to escape from endosomes into the cytoplasm while trastuzumab acts as specific targeting of over-expressing Her2 receptors. Finally, this novel polyplex showed promising efficacy against Her2-overexpressing breast cancer (Mann and Kullberg 2016).

9.3.1.2. Polyethyleneimine (PEI)

Ewe et al. (2016) synthesized a complex of tyrosine-modified low-molecular weight polyethylenimine (P10Y) and siRNA that had anticancer activity via surviving knockdown. In another example, Slobodkin et al. (2012) designed a linear biodegradable polyethyleneimine (LPEI) in a copolymer containing lipid oleoyl tetraethylene glycol carbonyl. This cross-linked multiblock showed reasonable anticancer activity.

Zamora et al. (2009) had revealed that PEI-RNAi- Wilms tumor gene 1 (WT1) antigen complexes inhibit B16-F10 lung metastases growth. Wilms tumor gene is a universal tumor antigen; therefore, it can be considered as a good therapeutic option. The polyplex was delivered to the lung of the mice in an aerosol system for *in-vivo* test.

9.3.1.3. Polyamidoamine (PAMAM, dense star)

Huang et al. (2011) have synthesized PAMAM-PEG-Angiopep/DNA nanoparticles to target glial cells and brain tumors. Angiopep-2 was responsible for specific low-density lipoprotein receptor-related protein-1 (LRP1) targeting that is expressed on BCECs and glial cells. Moreover, l-arginine-grafted-polyamidoamine dendrimer conjugated with epidermal growth factor receptor (EGFR) was synthesized to treat ovarian cancer with highly expressed EGFR (Jang et al. 2011).

9.3.1.4. Chitosan

Yao et al. (2014) formulated a nanocomplex consisting of Cis-aconitate-modified chitosan-g-stearic acid (CA-CSO-SA) micelles condensed with DNA. It was found that this nanocomplex has high transfection efficiency due to improved endolysosomal escape, making it an effective potential anticancer moiety for further research. In another example, Jin et al. (2008) developed a complex of imidazole ring-containing urocanic acid-modified chitosan (UAC). UAC was complexed with phosphatase and tensin homolog deleted on chromosome 10 (PTEN) genetic material. UAC was used to deliver PTEN to lung cancer using an aerosol formulation (Jin et al. 2008). The treatment of lung cancer was achieved *via* several mechanisms including formation of a nuclear complex between PTEN and p53, Akt-related signals knockdown, and cell cycle regulation suppression (Jin et al. 2008).

9.3.1.5. Poly lactic-co-glycolic acid (PLGA)

Du et al. (2012) designed and formulated biodegradable nanoparticles (NPs) consisting of triblock copolymers from monomethoxy-poly (ethylene glycol)-poly(lactic-co-glycolic acid)-poly-l-lysine (mPEG-PLGA-PLL) (Du et al. 2012). mPEG-PLGA-PLL/siRNA polyplex enhanced gene silencing efficiency and anticancer activity against lung cancer. In another interesting example, Shi et al. (2014) combined chemotherapy and gene therapy in lung-cancer treatment. They developed PLGA porous microparticles that are responsible for the delivery of doxorubicin and PEI25K/p5 that enhances apoptosis and tumor suppression.

9.3.1.6. Poly (2-(dimethylamino)-ethyl methacrylate) (PDMAEMA)

Zhang et al. (2014) used PDMAEMA in formulating a dual-responsive biodegradable galactosamine (Gal)-modified polymeric micelles that respond to change in pH (via acetyl group) and reduction (*via* disulfide linkage). Polymeric micelles were formulated as galactosamine poly(ethylethylene phosphate)-*a*-poly(ε-caprolactone)-*ss*-poly[2-(dimethylamino)ethyl methacrylate] (Gal-PEEP-*a*-PCL-*ss*-PDMAEMA) terpolymer that was loaded with doxorubicin and DNA.

9.3.2. Inorganic particles

In a US patent (US 20110229966 A1), Han *et al*. showed that a gold nanoparticles (AuNP) GDS system was more efficient in delivering antisense DNA than the liposome-based reagent providing better anticancer effect (Han et al. 2011). In another example, Kar et al. (2013) developed novel hybrid mesoporous silica nanoparticles (MSNs) with surface grafting of poly-l-arginine (MSN-PLArg). MSN-PLArg was used to deliver mCherry DNA plasmid and doxorubicin to HeLa and A549 cancerous cells (Kar et al. 2013).

9.3.3. Smart carriers in cancer gene therapy

9.3.3.1. Carriers reacting to changes in temperature "thermo-responsive"

Ma et al. (2014) designed and synthesized biodegradable and thermosensitive PLGA-PEG-PLGA hydrogels. PLGA-PEG-PLGA was used for sustained co-delivery of PLK1shRNA/polylysine-modified polyethylenimine (PEI-Lys) complexes and

doxorubicin (DOX) for osteosarcoma treatment *via* apoptosis induction (Ma et al. 2014).

9.3.3.2. Carriers reacting to change in pH "pH responsive"

Chen et al. (2013) designed and developed a pH-responsive cyclodextrin material conjugated with low molecular weight PEI to form a nano-vector that has higher efficacy and lower cytotoxicity when compared with PLGA-based counterpart, branched PEI (25,000 Da), and Lipofectamine 2000. The pH-responsive cyclodextrin material conjugated with low molecular weight PEI showed potential effect on lung adeno carcinoma.

9.3.3.3. Carriers reacting to change in reduction\oxidation states "redox-activity responsive"

An et al. (2014) designed a dual bioresponsive 4-arm three-dimensional poly (ethylene glycol)-b-poly (disulfide histamine). Their smart carrier is bioresponsive to changes in redox potential and pH. In this system, the dissociation of the plasmid from the carrier system occurred in the presence of glutathione and at pH 6.3 (cancerous cell intracellular acidic environment).

10. Cost effectiveness of gene therapy

Pharmacoeconomics is considered an invaluable decision-making process in non-viral gene therapy. An extreme example of the importance of Pharmacoeconomics is Glybera®. It took decades to get Glybera®, the first gene therapy in the Western world, to market; and apparently, with poor pharmacoeconomical planning, it took only five years to be out of the market after failing commercially.

Pharmacoeconomics acts as an alarm to double check the gene-therapy industrial cycle and a safety value in its success, especially in this critical initial priming phase of the gene therapy field. Currently, the capital investment from private and public markets on gene-therapy technology is around 4.3 billion dollars (Ledley et al. 2014). Economics of gene therapy and pharmacogenetics can be studied and evaluated via "two perspectives": the payer (patient) and the company perspective (Danzon and Towse 2002).

The cost-effectiveness evaluation of any gene therapy product is a critical step towards its success. It can be evaluated within two phases, the whole gene therapy industrial phase and the pharmacogenetic testing phase (Danzon and Towse 2002). Gene therapy approved drugs are the world's most expensive drugs in history (Regalado 2016b). Only three approved gene therapy drugs are now commercialized, including Glybera® (Su et al. 2012), Gencidine® (Westphal 2003) and Strimvelis® (Staton 2016).

Glybera®, the first gene therapy-drug in the Western World, is indicated for treatment of lipoprotein lipase deficiency (LPLD) and was launched in Germany and manufactured by UniQURE (Amsterdam). It costs around 1 million dollars (Han and Ni 2015; Morrison 2015; Ronfard et al. 2017). However, it was not commercially successful due to its extremely high price (Morrison 2015). Due to its commercial failure, on April 2017, UniQure announced that it will no longer seek to renew marketing authorization of alipogene tiparvovec (Glybera®) and is set to pull the drug from the market and wind down all the related operations and infrastructure (European Biotechnology Life Science and Industry Magazine 2017).

The first commercialized cancer gene therapy worldwide is the result of a Chinese entrepreneurship, "Gencidine®". Its commercially valued price in the whole course of treatment is $100,000 ($360 per injection). The development cost of Gencidine® was over $9.6 million used by the developing pharmaceutical firm (Shenzhen SiBiono GeneTech™) besides the numerous grants received from the Chinese government (Lenrosen 2012).

In 2016, the European Medicine Agency gave the veto to Strimvelis® (Scott and DeFrancesco 2016). Strimvelis® is a gene therapy which is indicated to treat patients with severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), (bubble boy syndrome), for whom no suitable human leukocyte antigen (HLA)-matched related stem cell donor is available (Hoggatt 2016). The product went through a long commercialization process. It was manufactured by Italy's San Raffaele Telethon Institute for Gene Therapy and the biotech MolMed (Milan, Italy), which was founded at the Telethon Institute with support from a charitable foundation (Regalado 2016c).GlaxoSmithKline (GSK) struck a deal with the Italian research partners to commercialize the product (Adams 2016b). The Strimvelis® price tag is 594,000 Euros ($665,000) (Adams 2016b).

The question of "Why gene therapy is so expensive?" is popping in everyone's mind in the scientific field. The idea revolves around the fact that gene therapy is tailored for each patient (not on-the-shelf used drug) (Adams 2016b; NL 2016). The precise stem cells are extracted from the bone marrow and mixed with the corrected gene, infused back to the patient (Fliesler and Farber 2016). Other than that, the therapy is given once, and a cure for life-time is granted (Regalado 2016a). Also, the company is responsible for following up the treated patients for years (NL 2016), and the patient is guaranteed their money back if the therapy failed (Regalado 2016c). Gene therapy (viral and non-viral) encompasses different architecture and scattered approaches in the industrial and the research cycle (Ledley et al. 2014). Subsequently, companies and investors conclude that further capital investment will also lead to non-reproducible clinical researches (no progressed and tangible products compared to investment). In spite of these hurdles, economical, logistical, and scientific, the field of gene therapy is still progressing in fast rhythm with a huge number of patents and clinical trials taking place (>350000 research papers, >160000 US patents, >1800 clinical trials and >50 biotechnology companies in the past 20 years) (Ledley et al. 2014). A massive capital investment went to non-viral gene delivery research and anticipated products *via* different companies as shown in Table 1 (Ledley et al. 2014).

Nevertheless, two ideas should be mind-seated for companies and researchers. First, the cost of these therapies will decline and will be a successful commercial business plan for companies in the long run with the fast evolving technologies, pharmaceutical research, and genetic engineering advances (Lenrosen 2012). Second, the ultimate key of reproducibility is standardizing the laboratory or manufacturing practices– precisely, protocols for cell culture, cryopreservation and storage (Thompson and Ehrhardt 2015).

Perhaps one of the main reasons for the disappointing failure of Glybera®, aside from the unreasonable price tag, is its limited target consumers. The drug is targeting a very rare genetic disorder for treatment of familial lipoprotein lipase deficiency (LPLD) in children (estimated to occur in approximately 1 in 250,000 people in the general population and has been described in all races) (Brunzell 1993; Ikeda and Takagi 2001; Tsukamoto 2007; Vidal Serra et al. 1990; Yoshida and Gotoda 1998). Even with the $1 million price tag, the product was set to fail due to the poor pharmacoeconomical planning. It is definitely of great importance to focus on rare diseases in the research and development of new therapies, but not in an emerging field as gene therapy. Perhaps Gencidine® pharmacoeconomical planning was more realistic and mature, as it targeted cancer. This ensured a large target consumer base and market, beside its acceptable price tag. Such factors justified the existence of Gencidine® in the market for over 14 years to date.

11. Lack of standardization in non-viral gene delivery

The non-viral gene delivery research field is a distinct special branch of novel drug delivery. Even in pharmaceutical technology journals, such as *Journal of Controlled Release* (JCR), a separate section is designated for non-viral gene delivery systems in each issue. Relativity in this field is a rate limiting step in the design and the formulation of the final carrier system. Initially, in the carrier system/DNA complex formation, standard traditional analytical units are not used. Mainly, the unit used is the N/P ratio, in which N refers to the nitrogen content on the positively charged carrier, and P refers to the phosphorus on the negatively-charged genetic material. The N/P ratio proved to be a flexible and logical analytical unit for the quantification of non-viral gene delivery complex

Table 1: Average capital investment of different non-viral gene products developed by different pharmaceutical companies for various applications

Company name	Capital investment	Product trade names/ gene	Indication	Approval
Adverum Biotechnology (Adverum Biotechnologies Company, 2016)	$ 55 million	ADVM-022 and ADVM-032	Age-related molecular degeneration	No /clinical trials
		ADVM-043 (rare disease)	AT1 deficiency	No /clinical trials
Calimmune (Carroll, 2015)	$ 15 million	Generex®/Ad5FGF-4	Cardiac microvascular insufficiency	No /clinical trials
Renova Therapeutics (Renova Therapeutics, 2017)	$ 7.52 million (Socaltec., 2014)	RT-100®/Ad5.Hac6	Heart failure	No /clinical trials
Audentes Pharmaceutical (Audentes Therapeutics Company, 2017)	$ 137.5 million	AT13	X-linked myotubular myopathy	No /clinical trials
		AT342	Crigler-Najjar syndrome	No /clinical trials
		AT307	CASQ2-related catecholaminergic polymorphic ventricular tachycardia	No /clinical trials
Gensigh Biologics (Crunchbase., 2017)	$ 36 million	Gn010	Ophthalmic diseases	No /clinical trials
		Gn030		No /clinical trials
		HORA-RPE65	Leber congenital amaurosis type 2	No /clinical trials
Horama Biologics (Horama Company., 2016)	€ 4 million	HORA-PDE6B and HORA-RLPB1	Retinitis pigmentosa	No /clinical trials
UniQure (UniQure., 2013)	$ 58 million	Glybera®/ engineered copy of the human LPL gene	Lipoprotein lipase deficiency	Yes (Withdrawal from market by 2018)
AveXis (Avexis gene therapy company, 2017)	$ 65 million (Regalado 2016a)	AVXS-101	Spinal muscular atrophy	No /clinical trials
Takaraa (Takara, 2010)	¥ 11 billion	TCR	Anticancer	No /clinical trials
		MAZ9	HIV	No /clinical trials
Orchard (Adams, 2016a)	$33.4 million	Strimvelis®/ human adenosine deaminase (ADA) cDNA sequence	Bubble boy Syndrome	Yes

as it provides an instant figure to illustrate the strength of the carrier system in complexing and shielding the genetic material. Furthermore, in traditional novel drug delivery, the drug itself can be easily analyzed in *in vitro*, *ex vivo*, *in vivo,* or by pharmacokinetic and pharmacodynamic evaluations. On the contrary, in non-viral gene delivery, the main aspect analyzed is the expression of the exogenously introduced gene, not the gene itself. For this main reason, a reporter gene is always used. Typically, a reporter gene is cloned with the genetic material sequence of interest into an expression vector that is then transferred into cells. Following transfer, the cells are assayed for the presence of the reporter gene by directly measuring the reporter protein itself or the enzymatic activity of the reporter protein. A good reporter gene can be identified easily and measured quantitatively when it is expressed. Bioluminescent reporters are ideal because they have a number of important features including instantaneous measurements, high selectivity and sensitivity, wide dynamic range, and, most importantly, no endogenous activity in host cells to interfere with quantitation. A widely-used reporter gene is the luciferase gene. The luciferase gene is not endogenous in human cells, providing a sensitive and distinctive quantification tool only specific to the exogenously delivered gene in question. These factors show the uniqueness of non-viral gene delivery field in the pharmaceutical technology and formulation research world.

In Appendix 1, 90 research papers have been summarized and tabulated. In this table, research papers are summarized based on their non-viral carrier system used, carrier system design, applications, efficacy testing, toxicity testing, expression readout techniques, reporter and therapeutic genes used, physicochemical characterization of the carrier system, study design, amount of genetic material used, and gold standards and controls used in each study. Such a summary would easily aid researchers to effortlessly compare the various carrier systems described and spot differences in key parameters used in the evaluation of non-viral gene delivery carriers. Figure 2 illustrates the heterogeneity in cell confluency, toxicity readout, physicochemical characterization techniques, reporter gene used, expression readout technique, and dose of DNA used in 90 papers surveyed from literature (2006-2017) discussing the design and characterization of different non-viral gene delivery formulations. This summarization shows the lack of standardization and the heterogeneity in the characterization of novel non-viral gene delivery carriers.

Heterogeneity in cell confluency during cell culture experimentation for *in-vitro* transfection efficacy and cell viability testing of non-viral gene delivery carrier is crucial and may lead to false-positive or false-negative results. Cell confluency below 70% may lead to high false-positive transfection efficiency results as the carrier system can easily enter the cell during mitosis (Honegger 2001; Johnson and Goldstein 1984; Phelan and May 2016; Phelan 2006, 2007; Wilson 1990). As shown in Fig 2, 57% of the papers did not report the level of confluency in their methodology, which is an important factor in the evaluation of non-viral gene delivery carriers, the reproducibility of the results and extrapolation of the results generated from one study to another. This would open the door for enormous variability in the generated results, even for the same carrier system, for different researchers. Furthermore, the effect of serum on the stability of the non-viral gene carrier system and the genetic material cargo is substantial. In spite of this critical important factor, many studies avoided the use of serum in their transfection and cell viability studies leading to positive false results (Appendix 1; see online version).

For reporter genes used (Fig. 2), 37% of the papers used green fluorescent protein (GFP) or enhanced green fluorescent protein (EGFP) as reporter genes. GFP and EGFP are distinct exogenous genes to the human cells and represent great qualitative visual tools to assess the transfection efficacy and the expression of the exogenous genetic material. On the contrary, quantitative evaluations could be a problem, as many cellular components fluoresce green in the background interfering with the fluorescence generated by the exogenous genetic material and its reporter gene (Doulatov 2003; Jia et al. 2004; Kavita and Burma 2008; Reddi et al. 1999).

For cell viability testing (Fig. 2), 56% of the papers rely mainly on MTT assay (Fig. 2). MTT assay is a colorimetric assay for assessing cell metabolic activity only, and uses it as a main parameter for assessing cell death. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of

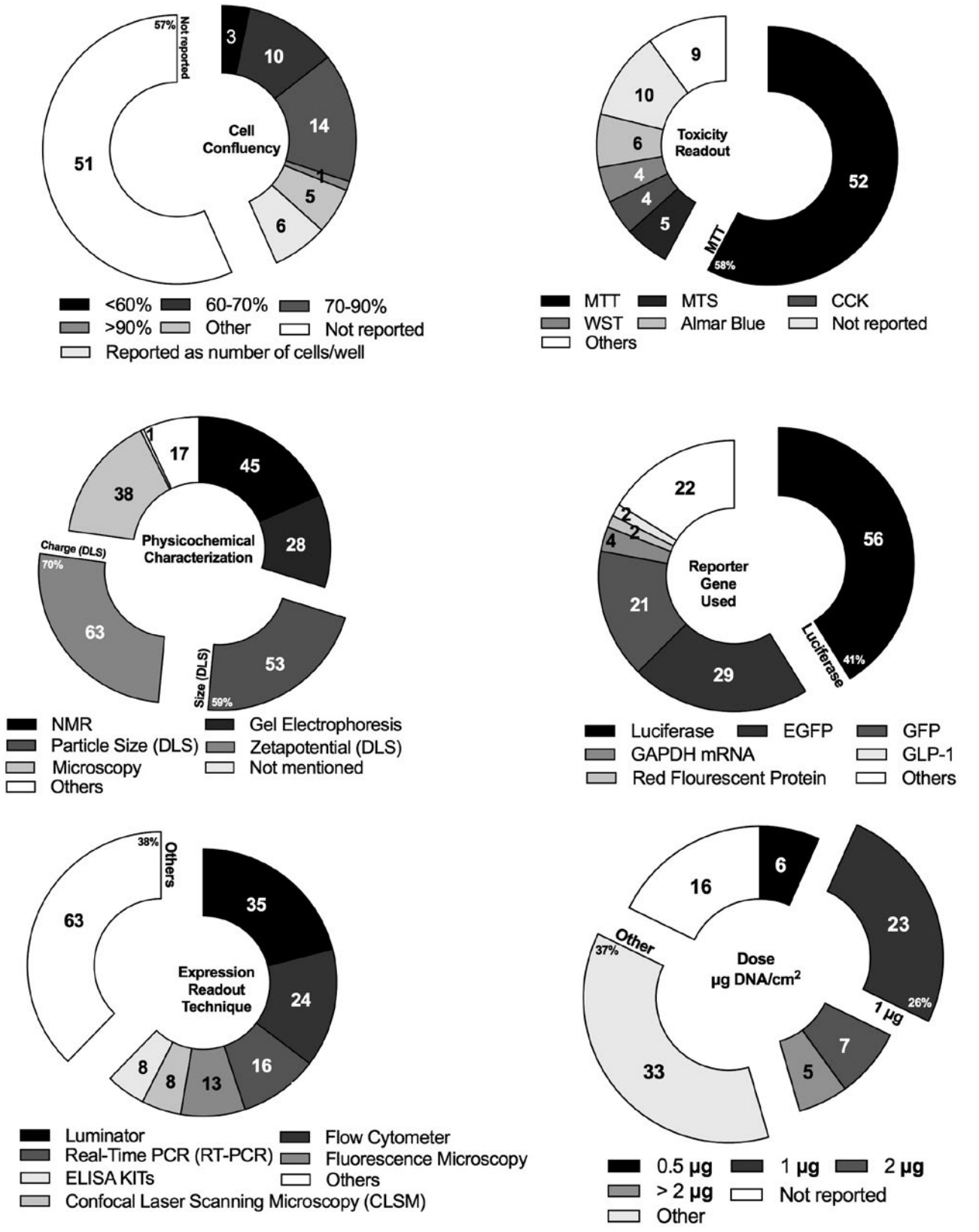

Fig. 2: Cell confluency, toxicity readout, physicochemical characterization techniques, reporter gene used, expression readout technique. and dose of DNA used in 90 papers surveyed from literature (2006-2017) discussing the design and characterization of different non-viral gene delivery formulations. This summarization shows the lack of standardization and the heterogeneity in the characterization of novel non-viral gene delivery carriers.

viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. In MTT assay, the collection and quantitation of the formed formazan crystals can be problematic and heterogeneous from one study to another due to the multiple washing steps (Nishida et al.

1992). Furthermore, relying only on MTT assay may not provide a realistic accurate measure of cell death.

The amount of therapeutic DNA used is also a major point of discrepancy. 26% of the papers used 1 microgram of genetic material along with 37% not reporting the amount of genetic material used (Fig. 2). All of these discrepancies in the evaluation and characterization of the non-viral gene carrier systems and their *in vitro*, *ex vivo*, *in vivo,* or pharmacokinetic and pharmacodynamic evaluations lead to significant lack of standardization. Such lack of standardization is considered a major setback in the field of non-viral gene therapy and imposes significant challenges in result reproducibility across researchers.

Moreover, such lack of standardization generates hurdles in extrapolating data from one study to another, even for the same carrier system as indicated in Table 2. For Table 2, six different polymeric carriers used for non-viral gene delivery had been chosen to be screened. The six polymers have been evaluated by different researchers in literature. The six polymers evaluated and tested by different researchers in different laboratories are summarized with respect to localization, transfection efficacy, cytotoxicity, and physicochemical characterization. Table 2 significantly highlights the discrepancies across researchers with respect to generated results and conclusions even for the same tested non-viral carrier system. Kim et al. (2009) showed that relative transfection efficiency of ABP polymeric carrier was the same as PEI without serum, 4 to 5 times as PEI with serum, and the same as PEI with and without cloroquine. On the contrary, Lee et al. (2012) showed that 200 µg of ABP polyplex at a N/P ratio of 1/10 was higher than 200 µg PEI polyplex at N/P ratio of 1/1 at 3 to 7 days of injection. In another example, describing chitosan-PEI carrier system, Taranejoo et al. (2016) showed that chitosan-PEI polyplex had a higher transfection efficiency (around 28-fold) than PEI at N/P ratio 10. On the contrary, Mohapatra ans Wang (2015) showed that the transfection efficiency of polyplex was higher than lipofectamine, but significantly lower than PEI: DNA (10:1) at 1 µg DNA/ml. Such discrepancies and lack of standardization could lead to contradictory and conflicting results which, subsequently, can be considered as a setback to the whole non-viral gene therapy field.

Table 2: Polymers designed and tested for non-viral gene delivery by different investigators. Each polymer shows different efficacy and safety results with each investigator

Peptide	Ref.	Uptake	Localization	Transfection test	Transfection efficiency	Cytotoxicity test	Cytotoxicity results	Physicochemical characterization
Arginine-grafted bioreducible poly(disulfide amine) (ABP) polymer	Kim et al. (2009)	~ 1.4 times of pDNA.	Nucleus	Luciferase assay via a luminometer. Cellular uptake via BD FACScan analyzer.	Relative transfection efficiency was the same as PEI without serum. Relative transfection efficiency was 4 to 5 times as PEI with serum. Relative transfection efficiency was the same as PEI with and without cloroquine.	MTT assay	Polyplexes at N/P ratio of 40 or more had relative cell viability similar to (CBA-DAH) (around 100%, compared to PEI of ~0%).	SEC Particle size via DLS Zeta-potential via DLS Gel electrophoresis
	Lee et al. (2012)	~7.2 times of pDNA		IL-6 and hEPO protein were measured with Quantikine rat IL-6 ELISA kit and Quantikine-hEPO ELISA kit, respectively. Cellular uptake of YOYO-1 iodide-labelled phEPO was measured via flow cytometry.	Plasma hEPO protein of 200µg polyplex at N/P ratio of 1/10 was higher than 200µg PEI polyplex at N/P ratio of 1/1 at 3 to 7 days of injection.	Not tested		Gel electrophoresis Particle size via DLS Zeta potential via DLS
c(RGDyK)-poly(ethylene glycol)-polyethyleneimine (RGD-PEG-PEI)	Zhan et al. (2012)	Not tested		EGFP expression via fluorescent microscope The volume and area of the tumour was determined via MR imaging. Mitotic delay through cell cycle distribution was evaluated via FACS analysis.	The transfection efficiency of RGD–PEG–PEI nanoparticle was lower (2 fold) than RGD–PEG–PEI + 10nM CDX–PEG–PLA micelle (36.04%).	MTT assay	The U87 cell viability of RGD–PEG–PEI nanoparticle (~100%) was higher than RGD–PEG–PEI+ 10nM CDX–PEG–PLA micelle (~57.5%).	DLS
	Wamg et al. (2014)	~ 5 times of pDNA	Endo/lysosomal compartments.	GFP via flow cytometry YOYO-1-labeled DNA via fluorescence spectrophotometer.	The transfection efficacy of GNR-DSPEI-PEG RGD/DNA complex (97.1 ± 3.4%) with NIR laser irradiation was higher than the GNR-DSPEI/DNA complex (66.1 ± 3.6%) without NIR laser irradiation and the GNR-DSPEI/DNA complex (~85%) with NIR laser irradiation.		The cytotoxicity of GNR-DSPEI/DNA complex (cell survival: 90%) was lower than GNR-DSPEI-PEG RGD/DNA complex (cell survival: 80%) (Compared to PEI-25 kDa of ~60% cell survival).	^{1}H NMR Zeta potential via DLS TEM UV–Vis–NIR spectrophotometer Thermogravimetric analysis (TGA).
Arg-rich cell penetrating HIVTAT-(47-57) peptide 47YGRKKRRQRRR57	Ho et al. (2001)	~ 100% of pDNA	Not tested	Kinetic (real time) flow cytometry analysis. Flow cytometry (FACS) analysis *in-vivo* and *in-vitro* Fluorescent confocal microscopy analysis.	PTD-4 peptide had a higher intracellular concentration (5 fold) than TAT peptide *in-vitro*. PTD-4-peptide- and TAT-peptide had the same percentage of transduction (100% of cells present in whole blood).	Not tested		Fluorometric analysis Modelling molecular surface and peptidyl bond via the LINUS and GRASP programs.
	Takeshima et al. (2003)	~ 80% or of pDNA	Cytoplasm & Nucleus	GFP analysis & flow cytometry. Intracellular distribution of fluorescent-labelled cationic peptides was examined via confocal microscopy.	Tat-(47–57) had peptide cellular uptake (~17.5nmol/mg) higher than BF2d (~7.2 nmol/mg) and MG2d (~2 nmol/mg) in Hela cells at 15 µM.	MTT assay	BF2d peptide had a lower cytotoxicity (cell survival: ~90%) than Tat-(47–57) (~60%) and MG2d (~20%) in Hela cells at 100 µM.	RP-HPLC Ion-spray mass spectroscopy.

Peptide	Ref.	Uptake	Localization	Transfection test	Transfection efficiency	Cytotoxicity test	Cytotoxicity results	Physicochemical characterization
	Ignatovich et al. (2003)	~ 250% of pDNA	Cytoplasm	B-Galactosidase assays Luciferase assay Bradford assay Fluorescein-labeled peptides was detected via confocal microscopy Human apoA-I gene expression was determined via RT-PCR and the sandwich enzyme-linked immunosorbent assay *in-vivo*, respectively.	pCMVL-K_8 at N/P ratio of (1:5) had higher β-galactosidase expression (1.2 fold) than pCMVL-Tat at N/P ratio of (1:5) for HepG2 cells.		Not tested	HPLC. Electrospray ionization-mass spectrometry Electrophoretic mobility shift assay (EMSA).
Bioreducible DMAEMA (rPDMAEMA)	You et al. (2007)	Not tested		Luciferase assay	rPDMAEMA at N:P ratios ranging from 15 to 25 had higher luciferase transfection efficacy (3 to 4 fold) than PDMAEMA at N/P ratios ranging from 15 to 25 polyplexes. PEI polyplexes had a higher luciferase transfection efficacy (10 to 20-fold) than rPDMAEMA polyplexes. The luciferase expression of PDMAEMA, rPDMAEMA and PEI was elevated by (460–580 fold), (200 fold) and (45-80 fold), respectively in the presence of chloroquine enhancement.	MTS assay in B16F10 cells.	rPDMAEMA was less toxic than PDMAEMA by 2 fold at 20 µg/mL and 10 folds at 50 µg/mL.	Size exclusion chromatography (SEC). ^{1}H NMR Hydrodynamic diameters via DLS Zeta potential via DLS
	Zhu et al. (2012)	Not tested	Endosomes.	Luciferase assay (luminometer). Cellular uptake of Cy5-labeled DNA via confocal laser scanning microscopy (CLSM). GFP assay.	rPDMAEMA polyplexes had higher luciferase transfection efficacy (28 fold) than PDMAEMA-PEG-PDMAEMA polyplexes at a N/P ratio of 6/1. PEI 25k Da polyplexes had higher luciferase transfection efficacy than rPDMAEMA polyplexes at N/P ratio of 12/1.	CCK (cell counting kit) assay in COS-7 cells.	Reducible and nonreducible PDMAEMA had similar cell viability. Cell viability of rPDMAEMA at N/P 12/1 and 25 kDa PEI at N/P 10/1 was 80% and 70%, respectively.	Zeta potential via DLS Particle size via DLS Agarose gel electrophoresis Gel permeation chromatography (GPC) Colloidal stability was assessed via DLS and agarose gel retardation assays in the presence DTT over the time.
Chitosan- polyethyleneimine	Taranejoo et al. (2016)	Not tested	Late endosomes/ lysosomes	GFP expression was evaluated via fluorescence microscope and quantified by flow cytometer. Intracellular localization of FITC-GCS-ss-PEI was evaluated via CLSM.	Polyplex had higher transfection efficiency (around 28 fold) than PEI at N/P ratio 10.	MTT assay in HEK 293T cells.	Polyplex had cytotoxicity lower than PEI. IC50 values of polyplex and PEI were 434.4 ± 45.7 µg/m and 262.4 ± 30.2 µg/ml respectively. There was no significant difference between the cell viability of the polyplex and Lipofectamine™ 2000.	FT-IR Buffering capacity measurement Particle size via DLS Zeta potential via DLS Morphological examination of particles via SEM Redox responsive properties were evaluated by measuring the amount of released DNA in the presence of GSH and heparin via gel electrophoresis.
	Mohapatra et al. (2015)		Endosomes in the cytoplasm.	Luciferase expression *In-vivo* and *in-vitro* tomato protein expression Xenogen imaging *In-vivo* magnetic resonance imaging (MRI)	The transfection efficiency of polyplex and PEI in 3T3 cells was lower than Lipofectamine at (0.5 µg DNA/ml). The transfection efficiency of polyplex was higher than lipofectamine, but significantly lower than PEI: DNA (10:1) at 1 µg DNA/ml. The transfection efficiency of polyplex (5:5:1 and 7:3:1) was 10-fold more than Lipofectamine and PEL: DNA (10:1) at 2 µg DNA/ml.	WST assay *In-vivo* toxicity test was assessed via determination of nanoparticles accumulation in liver, spleen, lung and prostate	Cell viability of CS-Mag micelles (7:3, 5:5 & 3:7) ranged from 75-100% and 80-100% compared to 80-100% and 65-85% for PEI for concentrations ranging from 40-80 ug/ml in prostate cancer (PC3) and HEK-293 cell lines respectively.	TEM DLS NMR FTIR Gel electrophoresis Laser confocal microscopy

Peptide	Ref.	Uptake	Localization	Transfection test	Transfection efficiency	Cytotoxicity test	Cytotoxicity results	Physicochemical characterization
	Zhu Manman et al. (2014)	Not tested		Luciferase expression	The transfection efficiency of polyplex was 23 fold more than PEI (2KDa) and PEI (25KDa) for Hela cells.	MTT assay	Polyplex had lower cytotoxicity than PEI (25KDa)	^{1}H NMR
Poly β-amino ester and polyethylene glycol (PBAE-PEG)	Zhiqing et al. (2012)	Not tested		GFP assay. *In-vivo* pharmacokinetics and tissue distribution of the peptide ELISA (for hBDNF protein)	The polyplex (NP-TB2) had transfection efficacy (86%) comparable to the commercial kit Lipo-Plus (80%).	MTT assay	Cell viability was higher than 80%.	Zeta potential Particle size analysis TEM
	Zugates et al. (2011)			Luciferase expression β-galactosidase expression.	Poly (β-amino ester)s polymer had a higher efficacy 34 folds than PEI. DNA uptake of poly(β-amino ester)s was 5 folds higher than free plasmid. DNA internalization of poly(β-amino ester)s was 30 folds higher than free plasmid.		Polyplex had lower cytotoxicity than PEI. The cell availability of PEI was only 3%.	Zeta potential. DLS in the presence of serum. PicoGreen assay. Gel electrophoresis. ^{1}H NMR.

12. Trends and a view toward the future: Lessons from past failures

The gene therapy initiative represents a dream for not only researchers and pharmaceutical companies, but also the public. It provides the hope for a complete cure of any disease rather than the traditional symptomatic treatment. In the past 15 years, gene therapy has been the cover story of *Time Magazine*, which is not a scientific journal targeting the public audience, over 12 times (Time Magazine 2017). Due to the major problems and drastic incidences of viral vectors in gene therapy, like the death of Jesse Gelsinger on Sept. 17, 1999 (Wilson 2009), gene therapy research has been slightly shifted towards the non-viral carriers. In spite of the viral gene therapy problems, the first three commercialized gene therapy products, Gendicine®, Glybera® and Strimvelis®, were based on viral carriers. Due to the commercial failure of Glybera® in April 2017, UniQure announced that it will no longer seek to renew marketing authorization of alipogene tiparvovec (Glybera®) and is set to pull the drug from the market and wind down all the related operations and infrastructure (European Biotechnology Life Science and Industry Magazine 2017). The failure of Glybera® was not related to a lack of efficacy or safety, but its commercial irrelevance, limited target customers, and unrealistic price tag. Glybera® may have a negative influence on the advancement of the field of gene therapy on the short term, but perhaps would aid in advancing non-viral gene carriers over their viral counterpart in the long run. The non-viral gene delivery system has several advantages over the viral gene delivery system including easier manufacturing, better safety profile, and smoother transition from bench-to-bedside.

A major setback in the field of non-viral gene therapy is the lack of standardization in the methodology and experimentation involved in system characterization and safety and efficacy testing. Out of 90 surveyed research papers from the literature over the past 15 years, the efficacy and safety evaluation of non-viral gene carriers was evaluated *in vitro*, in the presence and absence of serum, in only 8 research papers. The surveyed research papers and patents in the literature over a time-span extending from 2006 into 2017 revealed that there is a lack of standardization in numerous parameters in non-viral gene delivery systems evaluation and validation. Evaluating the transfection efficiency and cytotoxicity profile of each polymer has a decisive role in the choice of the non-viral carrier in any study. A cornerstone to truly knowing the advantage of any polymer over the other is having a standard technique for measuring each carrier's efficacy and toxicity. Thus, it is not rational to compare two polymers when they had not undergone the same tests and conditions. Figure 2 proves that there is no standard protocol for the design and evaluation of non-viral gene delivery systems. Finally, we need standardized guidelines to be followed by all formulators and investigators worldwide for the design and evaluation of non-viral gene carrier systems (Figure 3). Figure 3 represents a seed for the logical steps in the design and evaluation of non-viral gene delivery systems. The uniform evaluation parameters for non-viral gene delivery systems would aid ease of comparison of different systems and, subsequently, the advancement of the whole field of non-viral gene therapy. Such standardization would facilitate the non-viral gene therapy field transformation from bench-to-bedside and, subsequently, the generation of marketed products based on non-viral carriers.

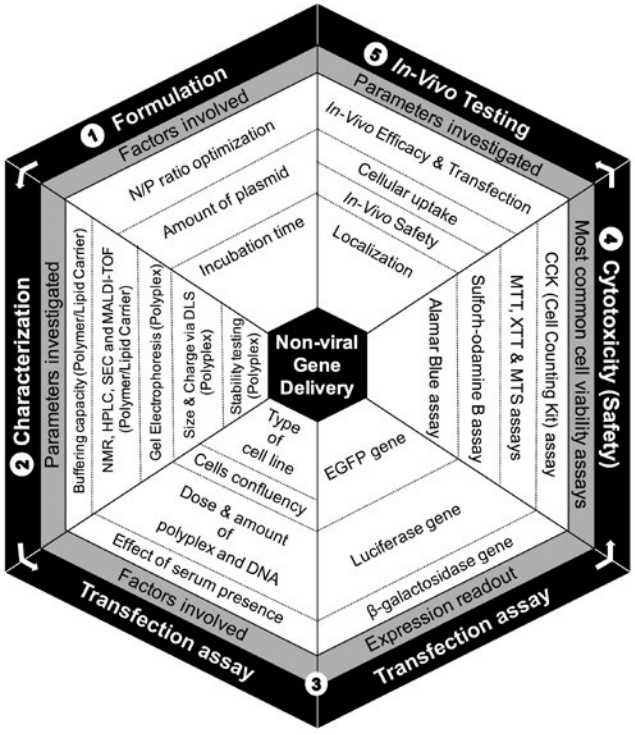

Fig. 3: Different phases of the characterization and *in-vitro* and *in-vivo* testing of non-viral gene delivery formulations

List of abbreviations

A2780	Human ovarian carcinoma cell line
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
A549	Human lung carcinoma cell line
AA	α-Amylase
ABP	Androgen-binding protein
ADA	Adenosine deaminase deficiency
AF4	Asymmetric flow field-flow fractionation

AFM	Atomic force microscopy
ALT	Alanine aminotransferase
AON	Antisense oligonucleotides
ARPE-19	Human retinal pigment epithelial cell line
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATRP	Atom transfer radial polymerization technique
AUC	Analytical ultracentrifugation
AuCM	Gold cysteamine
AuNP	Gold nanoparticles
B16F10	Mouse melanoma cell line
BBB	Blood brain barrier
BCA	Bicinchoninic acid assay
BCEC-1	Bovine caruncular epithelial cell line
BLI	Bioluminescence imaging
BMAAD	Polybutadiene-block-poly(methacrylic acid)-block-poly(2-(dimethylamino)ethyl methacrylate)
bp	Basepairs
BxPC3	Human primary pancreatic adenocarcinoma
C2C12	Mouse myoblast cell line
CA-CSO-SA	Cis-aconitate-modified chitosan-g-stearic acid
CAC	Critical aggregation concentration
CaP	calcium phosphate
CAT	Chloramphenicol acetyltransferase
CBA	Cystamine bisacrylamide
cbr1	Carbonyl reductase 1
CDC	Centers for disease control and prevention
CDM	Carboxy dimethyl maleate
CeO_2	Cerium dioxide
CFP	Cyan fluorescent protein
CGL	Cystathionine-gamma-lyase
CHO-K1	Chinese hamster ovary
CLSM	Confocal laser scanning microscopy
CM-PLH	Carboxymethyl poly (L-histidine)
CMV	Cytomegalovirus
CNTs	Carbon nanotubes
COS-7	African green monkey SV40-transformed kidney fibroblast
CPPs	Cell penetrating peptides
CPT	Camptothecin
Cryo-TEM	Cryogenic transmission electron microscopy
CT-26	Colon adenocarcinoma Cell Line
DAH	Diaminohexane
DC-Chol	3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DLS	Dynamic light scattering
DMAEMA	2-(dimethylamino) ethyl methacrylate
DMD	Duchenne muscular dystrophy
DMPE	Dimyristoyl-snglycero-3-phosphatidylethanolamine
DNA	Deoxyribonucleic acid
DODAB	Dimethyldioctadecyl ammonium bromide
DOGS	Di-octadecyl-amido-glycyl-spermine
DOHH-2	Haematopoietic and lymphoid tissue
DOPE	Dioleoyl phosphatidyl ethanolamine
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate
DOTAP	1,2-bis(oleoyloxy)-3-(trimethylammonio) propane
DOTMA	N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride
DOX	Doxorubicin
DPH	1,6-diphenyl-1,3,5-hexatriene
DSC	Differential scanning calorimetry
DSDA	Disulfide diacrylate
DTT	Dithiothreitol
EDA	Ethylenediamine
EFTEM	Energy filtering transmission electron microscopy
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ELP	Elastin-like polypeptide
EMSA	Electrophoretic mobility shift assay
ESI-MS	Electrospray ionization mass spectrometry
F98 cells	Rattus norvegicus, brain glioblastoma
FA	Folic acid
FACSC	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
FR	Folate receptor
FRET	Förster (Fluorescence) resonance energy transfer
FT-IR	Fourier transform infrared
Gal	Galactosamine
GALA	Glutamic acid-alanine-leucine-alanine
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCS	Grafting glycol chitosan
GFP	Green fluorescent protein
GPC	Gel permeation chromatography
GSK	GlaxoSmithKline
H1299	Human non-small cell lung carcinoma
H9C2	Rat cardiac myoblast
HA	Hyaluronic acid
HDF	Human dermal fibroblasts
HEK	Human embryonic kidney (modified)
HeLa	Human cervical carcinoma
HepG2	Hepatocellular carcinoma
HES	Hydroxyethyl starch
HLA	Human leukocyte antigen
HMW	High molecular weight
HNSCC	Head and neck squamous cell carcinoma
HPLC	High performance liquid chromatography
HPMA	Hydroxy propyl methacrylamide
HT1080	Human fibrosarcoma
HuH7-Luc	Hepatocellular carcinoma with luciferase reporter gene
HUVEC	Human umbilical vein endothelial cells
Jurkat	Immortalized line of human T lymphocyte
kDa	KiloDalton
L929	Mouse C3H/An connective tissue
LCST	Lower critical solution temperature
LDH	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
LLC	Linear Lysine Copolymer
LLNs	lipid-like nanoparticles
LLO	Listeriolysin O
LMW	Low molecular weight
LPEI	Linear poly ethyleneimine
LPLD	Lipoprotein lipase deficiency
LRP1	Lipoprotein receptor-related protein-1
Luc	luciferase
m109	Murine lung carcinoma cells
MA	Mouse adipose stromal cells

MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MALS	Multi-angle light scattering
ManNPs	Mannose receptor-targeted nanoparticles
MBs	Microbubbles
MC3T3- El	Clonal murine calvarial preosteoblast
MCF-7s	Human breast adenocarcinoma cells
MDCK	Canine kidney cells
MDTD	3-methylidene1,9-dioxa-5,12,13-trithiacyclo-pentadecane-2,8-dione
MEND	Multifunctional envelope-type nano device
MG-63	Human osteosarcoma cell line
MG2d	Magainin 2 derivative
miRNA	Mature micro RNA
MLNP	Multi-layered polymer nanoparticle
MMP	Metalloproteinase
MRC5	Human lung fibroblast cell line
MS1s	Mile seven pancreatic cell line
MSCs	Mesenchymal stem cells
MSNs	Mesoporous silica nanoparticles
MTT	Microculture tetrazolium
N/P ratio	Nitrogen/Phosphorus ratio
NCI	National cancer institute
ND	Not detected
Neuro 2A	Mouse neuroblastoma cell line
NIBS	Non-invasive back scatter technology
NIH	National institute of health
NIH3T3	Mouse embryonic fibroblast cell line (3-day transfer, inoculums 3 x 105 cells)
NIR	Near Infra-red Spectrophotometry
NIT-1	Insulinoma pancreatic β-cell line
NLS	Nuclear localization signal
OEI	oligoethylenimine
OTMCS	N-octyl-N-quaternary chitosan
OVCA	ovarian cancer cell line
P10Y	low-molecular weight polyethylenimine
PAA	Propyl acrylic acid
PALS	phase analysis light scattering
PAMAM	Polyamidoamine
PANC-1	Pancreatic cancer cells
Panc-28	Pancreatic Cell lines
PAsp(DET)	Polyaspartamide derivative that have repeated aminoethylene's units
PAT	Proximity activated targeting
PBAE/ PbAE	Poly(β-amino ester)
PBPC	Poly(allylamine)-benzophenone-pyridyldithio-carboxylate
PC-12	Pheochromocytoma of the rat adrenal medulla
PCL	Poly(ε-caprolactone)
PCR	Polymerase chain reaction
PDMAEMA	Poly (2-(dimethylamino)-ethyl methacrylate)
pDNA	Plasmid DNA
PEEDEPE	2-[2-(prop-2-enoyloxy)ethyl]disulfanyl-4-ethyl prop-2-enoate
PEEP	Poly(ethylethylene phosphate)
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PET	Positron emission tomography imaging
PGMA	Poly(glycidyl methacrylate)
phEPO	Human erythropoietin
PKA	Protein kinase
PLGA	Poly(lactic-co-glycolic acid)
PLL	Poly-l-lysine
PLN	Polycation lipid nanocarrier
PNIPAM	Poly (N-isopropylacrylamide)
PPC	PEG-PEI-Cholesterol
PPD	PEG-peptide-DOPE
PPGMA	Poly(propylene glycol methacrylate)
PTX	Paclitaxel
PUR	Polyurethane
QCM-D	Quartz crystal microbalance with dissipation
QDs	Quantm dots
QELS	Quasi-elastic light scattering
R9	Arginine 9
RBCs	Red blood cells
RES	Reticulo-endothelial system
RFPs	Red fluorescent proteins
RGD	Arginyl glycyl aspartic acid
rHSA	Recombinant human serum albumin
RNA	Ribonucleic acid
RNAi	RNA interference
RP-HPLC	Reversed phase high performance liquid chromatography
RPE	Retinal pigment epithelium
RPLL	Reducible poly(L-lysine)
RT-PCR	Real-Time PCR
S-180	Sarcoma cell line
SAM	siRNA–ABP–MB
Saos-2	Sarcoma osteogenic cell line
SCID	Severe combined immunodeficiency
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
ShRNA	Short hairpin RNA
siRNA	Small (or short) interfering RNA
SKOV-3	Ovarian carcinoma cells
SLNs	Solid lipid nanoparticles
SLS	Static laser light scattering
SMA	Smart multilayered assembly
SPC-A1	Human lung adenocarcinoma
SPION	Super paramagnetic iron oxide nanoparticle
SPN	Smart polymer nanoparticle
SVR	Mouse pancreatic islet endothelial cells
TA	Triamcinolone acetonide
TAMEL	Targeted and modular exosome loading
TAT peptide	Transactivator of transcription peptide
TEGDA	Tri(ethyleneglycol) diacrylate
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
TGC	Thiolated glycol chitosan
TGN	12-aminoacid peptide chain with significant brain transport ability
TLR	Toll-like receptor
TNBS	Trinitrobenzenesulphonic acid
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TSTA	Two-step transcription amplification
U-2OS	Human osteosarcoma cell line
U87	Glioblastoma cell line
U87-MG	Human glioblastoma-astrocytoma
UAC	Urocanic acid-modified chitosan
UCST	Upper critical solution temperature
US	Ultrasound
UTMD	Ultrasound-targeted micro-bubble destruction
UV–Vis–NIR	Ultraviolet-visible-near infrared
VEGF	Vascular endothelilal growth factor

VIPER	Vector iopamidol, protamine and ethiodized oil reagent
VSMC	Aortic vascular smooth muscle cells
WST	Water soluble tetrazolium salts
WT1	Wilms' tumor gene 1
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
γ-CD	γ-cyclodextrin

Conflicts of interest: None declared.

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Appendix 1: Papers on non-viral gene delivery between 2006 and 2017

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Targeted polymeric micelles for siRNA treatment of experimental cancer by intravenous injection	• The gene delivery system is a nanosized block copolymeric nano-micelles. • The block copolymer consists of a siRNA binding segment comprising thiol groups, a hydrophilic nonbinding segment, and a cell-surface binding peptide.	Poly (ethylene glycol)-block-poly (L-lysine) (PEG-b-PLL) comprising lysine amines modified with 2-iminothiolane (2IT) and the cyclo-Arg-Gly-Asp (cRGD) peptide on the PEG terminus	Anticancer therapy	200nM siRNA	siRNA
PLGA-based gene delivering nanoparticle enhance suppression effect of miRNA in HePG2 cells	Biodegradable poly(d, l-lactide-co-glycolide) PLGA forming a complex with polyethylenimine (PEI) in the form of nanoparticles.	PLGA/PEI nanoparticles	Treatment of diabetic, cardiovascular and neuro-degenerative diseases.	miRNA expression: 1µg miRNA GFP: 2µg miRNA	miRNA
A family of bioreducible poly(disulfide amine)s for gene delivery	• Five novel poly (disulfide amine)s which have different lengths of polymethylene spacer [–$(CH2)_n$–] in the main chain and the side chain. • Poly(cystaminebisacrylamide-spermine) (poly(CBA-SP)), poly (cystaminebisacryl-amide-triethylenetetramine) (poly(CBA-TETA)), and others.		Not reported	1.0µg/mL pCMV-Luc	pDNA
A mPEG-PLGA-b-PLL copolymer carrier for adriamycin and siRNA delivery	Amphiphilic block copolymer composed of conventional monomethoxy (polyethylene glycol)-poly (D, l-lactide- co-glycolide)-poly (L-lysine) (mPEG PLGA-b-PLL) NPs with enhanced cellular uptake of contained adriamycin or siRNA drug.	mPEG-PLGA-b-PLL	Anticancer therapy	Not reported	siRNA Adriamycin
A new pathway for developing *in-vitro* nanostructured non-viral gene carriers	• Triblock nanostructure consists of polylactide-poly(ethylene glycol)-polylactide(PLA–PEG–PLA) with the encapsulated DNA. • The system formed a non-woven nano-fibrous DNA containing scaffold	Tri-block copolymer (polylactidepoly(ethylene glycol)-polylactide, L8E78L8)	Repairing fractured bones		pDNA
A paradigm change: Efficient transfection of human leukemia cells by stimuli-responsive multi-compartment micelles	• Multi-compartment pH-responsive micelles which consists of triblock terpolymers, including polybutadiene-block-poly (methacrylic acid) blockpoly(2-(dimethylamino) ethyl methacrylate) (BMAAD), are capable of undergoing pH-dependent changes in charge stoichiometry. • The structure has a patchy shell including amphiphilic (interpolyelectrolyte complexes, MAA and D) and cationic patches cationic patches (excess D).		Not reported	1µg pDNA	
A pH and redox dual responsive 4-Arm poly(ethylene glycol)-block-poly(disulfide histamine) copolymer for non-viral gene transfection *in-vitro* and *in-vivo*	4-arm poly (ethylene glycol)-b-poly (disulfide histamine) pH and redox dualresponsive copolymer that have a neutral surface under physiological conditions, with enhanced cellular uptake of the polyplexes in tumor cells.	4-arm poly(ethylene glycol)-b-poly(disulfide histamine)	Anticancer therapy	• 2mg DNA (*In-vitro* GFP (flow cytometry) • 30µg of pCMV-Luc (*In-vivo* luciferase expression assay)	
A pH-responsive cyclodextrin-based hybrid nanosystem as a non-viral vector for gene delivery	A pH-responsive cyclodextrin material and low molecular weight polyethylenimine (PEI) integrate to form a nanovector that has higher efficacy and lower cytotoxicity when compared with PLGA-based counterpart, branched PEI (25,000 Da) and Lipofectamine 2000.	Cyclodextrin based –low molecular weight PEI nanosystem.	Lung Adeno carcinoma	1.0 nmol ASON	Antisense oligonucleotide (ASON)
A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles *in-vitro* and *in-vivo*	Multifunctional envelope-type nano-device (MEND) conjugated with PEG-peptide-DOPE (PPD) can be cleaved in a matrix metalloproteinase (MMP)-rich environment, with enhanced delivery both *in-vivo* and *in-vitro.*		Anticancer therapy	2.4µg of siRNA	siRNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
Luciferase assay	HeLa-luc	Yes	Not reported	MTT assay	Female BALB/c nude mice	• ^{1}H NMR • Particle size by DLS • Zeta potentials of micelles by laser doppler electrophoresis • Imaging of the siRNA complexes via Atomic force microscopy • Fluorescence measurement • Effect of the reducing agent DTT on polyplex integrity. • Ellman's assay • Blood stability and micro distribution of micelles by intravital real-time confocal laser scanning microscopy (IVRTCLSM) imaging of micelles	2012	[1]
• GFP expression assay • miRNA expression by gene-specific primers and reverse transcriptase	HepG2 (human hepatocellular carcinoma cell)	No	50-70%		Hepatic carcinoma bearing mice	• Gel retardation assay • Hydrodynamic diameters via DLS • Transmission electron microscopy • Energy dispersive spectroscopy • Zeta potential via DLS • Agarose gel electrophoresis • Ultraviolet-visible spectrophotometer	2011	[2]
• Luciferase assay visualized via luminometer and quantified via BCA protein assay kit.	• Hela • C2C12 (Mouse myoblast cell line)		Not reported		Not tested	• Size Exclusion Chromatography • Gel electrophoresis assay • Zeta potential & size via DLS • ^{1}H-NMR • Acid-base titration	2009	[3]
• Cellular uptake of Cy5-siRNA mPEG-PLGA-b-PLL NPs was evaluated via confocal laser scanning microscopy (CLSM) • Evaluation of release of nanoparticles by dialysis method. • Cellular binding of Cy5-siRNA mPEG-PLGA-b-PLL NPs was measured via flow cytometer analysis • A fluorescence imaging system for evaluation of *in-vivo* delivery of adriamycin or siRNA	Huh-7 hepatic carcinoma cells	Not tested	~ 95%		Female Balb/c mice	• ^{1}HNMR • Gel Permeation Chromatography (GPC) • FTIR • Zeta potential via DLS • Particle size via DLS	2012	[4]
GFP expression	Pre-osteoblastic MC3T3 cells		Not reported	Not tested		• DNA size and conformation via DLS and static laser light scattering (SLS) • Transmission electron microscopy (TEM)	2006	[5]
• EGFP expression was quantified via flow cytometry • Confocal laser scanning microscopy • Cellular uptake of YOYO-1 NPs was measured via flow cytometry	• HEK-293 • Jurkat (Immortalized line of human T lymphocyte) • L929 cells	Yes	10^5 cells per well in 12-well plates	Alamar-Blue assay	Not tested	• Asymmetric flow field-flow fractionation (AF4) • Cryogenic electron microscopy (cryo-TEM) • Analytical ultracentrifugation (AUC) • Confocal laser scanning • microscopy (CLSM) • Size and zeta potential (DLS)	2013	[6]
• *In-vitro* GFP (flow cytometry) • *In-vivo* luciferase expression assay	• COS-7 • NIH3T3 • MCF-7 • HepG2		60%–70%		4 to 6-week-old male Balb/c nude mice with HepG2 tumor	• Agarose gel retardation • TEM • Particle size via DLS • Zeta potential via DLS • ^{1}H NMR	2014	[7]
• RT-PCR • Western blot analysis of Bcl-xl protein • Cell apoptosis analysis by flow cytometry • Cellular uptake of Ac-aCD NPs-containing ASONCy3 was visualized via CLSM and quantified on the fluorescence intensity of CLSM images. • Fluorescence-activated cell sorting (FACS) analysis of transfection efficiency of ASONCy3-containing NPs.	A549 cells	Not tested	80%	MTT assay	Not tested	• TEM • Scanning electron microscopy (SEM) • Fluorescence spectroscopy • *In-vitro* release and degradation study via fluorescence spectroscopy • FT-IR • ^{1}H NMR • Zeta potential via DLS • Particle size distribution via DLS	2013	[8]
Luciferase gene silencing via luminometer	• HeLa cells • HT1080 cells	Yes	Not reported	Not tested	male BALB/c nude mice with HT1080-luc cells	• Particle size analysis (DLS) • Zeta potential (DLS)	2009	[9]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Acetylation of PAMAM dendrimers for cellular delivery of siRNA	Bioreducible cationic acetylated Polyamidoamine (PAMAM) dendrimer to reduce cytotoxicity		Not reported	100nM	
AmphiphilicMethoxy Poly(ethylene glycol)-b-poly(ε-caprolactone)-b-poly(2 dimethylaminoethyl methacrylate) cationic copolymer nanoparticles as a vector for gene and drug delivery	Methoxypoly (ethylene glycol)-b-poly (epsilon-caprolactone)-b-poly (2-dimethyl-aminoethyl methacrylate) (mPEG-b-PCL-b-PDMAEMA) nanoparticles as the co-delivery vector of hydrophobic drugs.		Anticancer therapy	1 µg plasmid pDNA	pDNA
Anti-CD22 antibody targeting of pH-responsive micelles enhances small interfering RNA delivery and gene silencing in lymphoma cells	• Novel delivery system in a micellar form comprised of i) an internalizing streptavidin-conjugated monoclonal antibody (mAb-SA) directed against CD22 and ii) a biotinylated diblock copolymer containing both a positively charged siRNA condensing block and a pH-responsive block to facilitate endosome release. The modular design of the carrier facilitates the exchange of different targeting moieties and siRNAs to permit its usage in a variety of tumor types. • pH sensitive polymers used: poly(dimethylaminoethyl methacrylate) Polymers used are[poly(DMAEMA)] macro chain transfer agent and corresponding poly[(DMAE-MA)-b-(BMA)-co-(DMAEMA)-co-(PAA)]			GAPD: 10 nmol/l siRNA Interferon genes *OAS* and *STAT1* expression: 15nmol/l siRNA	siRNA
Arginine-grafted bioreducible poly(disulfide amine) for gene delivery systems	Arginine-grafted bioreducible -redox sensitive- hyperbranched poly (disulfide amine), having the unique properties of bioreducible polymers and the advantages of arginine residues	Arginine-grafted bioreducible poly(disulfide amine) (ABP)	Not reported	0.5µg pDNA	pCMV-Luc pDNA
Avidin–biotin interaction mediated peptide assemblies as efficient gene delivery vectors for cancer therapy	Peptide based TAC bioconjugates formed by the connection of biotin-functionalized peptides bio-R8, which condenses DNA and enhances internalization, and bio-CREKA, target receptors on MCF-7 cells *in-vitro*.	Avidin /biotin peptides	Anticancer therapy	Luciferase activity: 1µg pDNA RT-PCR: 2µg RNA	pDNA
Biodegradable hybrid recombinant block copolymers for non-viral gene transfection	• Thermo-responsive polyplexes elastin-like polypeptide (ELP) segment and a diethylenetriamine (DET) modified poly-L-aspartic acid segment • The biodegradable hybrid recombinant block copolymers showed appreciable transfection efficiency with low cytotoxicity		Not reported	2.5 µg/mL pGL4	
Biodegradable branched polycationic polymers with varying hydrophilic spacers for non-viral gene delivery	• Biodegradable branched polycationic polymers with varying hydrophilic spacer lengths. • Hydrophilic spacers can be incorporated into polycationic polymers to reduce their cytotoxicity and enhance their degradability for non-viral gene delivery	Polycationic polymers such as polyethylenimine (PEI) and poly (dimethylaminoethylmethacrylate) (PDMAEM) with varying hydrophilic spacer lengths		5µg of pDNA	
Biodegradable nanoparticles of mPEG-PLGA-PLL triblock copolymers as novel non-viral vectors for improving siRNA delivery and gene silencing	Efficient delivery of siRNA via the use of biodegradable nanoparticles (NPs) made from mono methoxypoly(ethylene glycol)-poly(lactic-co-glycolic acid)-poly-l-lysine (mPEG-PLGA-PLL) triblock copolymers		Anticancer therapy	0.8µg siRNA in each well	pCMV-eGFP pDNA
Biodegradable polymeric vectors for gene delivery to human endothelial cells	Biodegradable poly (beta-amino esters) for gene delivery to human endothelial cells.	Poly beta amino esters	Cardiovascular diseases	Luciferase assay: 0.3 or 0.6 DNA µg per well GFP: 3ug DNA per well	pDNA
Biodegradable tri-block copolymer poly(lactic acid)-poly(ethylene glycol)-poly(L-lysine) (PLA-PEG-PLL) as a non-viral vector to enhance gene transfection	The tri-block copolymer PLA-PEG-PLL combined the characters of cationic polymer PLL, PLA and PEG: the self-assembled nanoparticles (NPs) possessed a PEG loop structure to increase the stability, hydrophobic PLA segments as the core, and the primary ε-amine groups of lysine in PLL to electrostatically interact with negatively charged phosphate groups of DNA to deposit with the PLA core		Not reported	1µg DNA	
Bioreducible cross-linked nanoshell enhances gene transfection of polycation/DNA polyplex in-vivo	• Nanoshell of polycation and DNA. • Polycation consisting of bioreducible polymer. • Glutathione (GSH) can act as a stimulus to open the nanoshell after it has entered the cell.	PEG-based hyperbranched bioreducible polymer		*In-vivo* luciferase transfection 20µg DNA	
Bioreducible cross-linked polymers based on G1 peptide dendrimer as potential gene delivery vectors	Cationic polymers that consists of low generation (G1) peptide dendrimer and disulfide-containing bond that cleaved in cellular reductive environmentto release the genetic material	Bioreducible disulfide linked lysine based dendrimers (P1, P2, P3)		1µg pDNA	

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• d1EGFP transgene expression via flow cytometry • Confocal imaging	U87 (Malignant Glioma Cells) U87-d1-EGFP		1.5×10^5 cells/well in 12 well plates	MTS assay		• Complex size analysis (DLS) • Zeta potential • Confocal microscopy • ^{1}H NMR • Measuring complex formation via picogreen dye exclusion. • Measuring buffer capacity via titration of acetylated dendrimers	2009	[10]
• EGFP expression via flow cytometry • Confocal laser scanning microscope	HEK-293T		Not reported	MTT assay		• Particle size via DLS • Gel retardation assay • TEM • Zeta Potential via DLS • HPLC	2010	[11]
• Expression quantitation via Flow cytometry • GAPD mRNA(glyceraldehyde-3-dehydrogenase) levels via quantitative reverse transcription (qRT)-PCRGAPD • Interferon genes OAS and STAT1 expression by qRT-PCR assay.	• DoHH2 cells • HeLa-R CD22+ cells			Not tested	Not tested	• Zeta Potential via DLS • Particle size via DLS • Microscopy • Evaluation of pH-dependent endosomolytic activity by red blood cell hemolysis assay.	2011	[12]
• Luciferase assay via a luminometer and a BCA™ protein assay reagent kit • Cellular uptake via BD FACScan analyzer	• NIH3T3 • C2C12		70-80%	MTT assay		• Size exclusion chromatography • Particle size via DLS • Zeta-potential via DLS • Agarose gel electrophoresis	2009	[13]
• Luciferase activity • pDsRed2-N1-p53 plasmid expression via laser scanning microscopy • Western blotting analysis. • Inhibition of tumor growth • Reverse Transcription Polymerase Chain Reaction (RT-PCR).	• MCF-7 • HEK-293T • HeLa		6×10^4 cells/well in 24-well plates		4-6 weeks old normal male Chinese Kun Ming (KM) mice	• Particle Size • Zeta potential • Agarose gel retardation assay	2012	[14]
Luciferase assay measured by a luminometer	COS-7 cells		50-60%	Cell Titer-Blue® assay	Not tested	• Particle size via DLS • Zeta Potential via DLS • ^{1}H NMR • Gel retardation assay	2012	[15]
EGFP expression via flow cytometry	CRL 1764 rat fibroblasts		80-90%	MTT assay		• Zeta potential • Band Retardation with Gel Electrophoresis • Hydrodynamic polyplex sizes by DLS • ^{1}H NMR • Size exclusion chromatography (SEC) • Hydrogen ion titration	2010	[16]
• EGFP expression via FACS Calibur flow cytometer. • RT-PCR analysis • Cellular uptake via confocal laser scanning microscopy and fluorescence microscopy	SPC-A1 and SPC-A1-GFP lung cancer cells		Not reported			• SEM photograph • Zeta potential • HPLC • Gel retardation assay	2013	[17]
• Flow cytometry • Luciferase assay • GFP expression via FACSC	Human umbilical vein endothelial cells (HUVEC)			Cellular metabolic activity measurement		• Gel-permeation chromatography (GPC) • Particle size analysis (DLS) • Zeta potential (DLS) • Permeation chromatography	2006	[18]
EGFP expression via FACS Calibur flow	• HepG2 • HeLa cell lines		70–80%	MTT assay		• Zeta potential • Gel retardation assay • ^{1}H NMR • Heparin replacement for evaluation of DNA-NPs complex • Stability of PLA-PEG-PLL in human plasma via agarose gel electrophoresis	2011	[19]
• Luciferase activity • Hemolysis test to ensure biocompatibility in blood • Protein BSA adsorption assay	• HEK-293T • HeLa (Human Cervical Carcinoma cell line)		30 000 cells per well on 48-well	MTT assay	Male ICR mice	• Particle size analysis (DLS) • Zeta potential measurement • Agarose gel electrophoresis	2014	[20]
• Luciferase assay • Fluorescent microscopy assays (FMA)	• HEK-293 • U-2OS(Human Osteosarcoma cells)		70-80%	Cell counting kit (CCK)-8 assay	Not tested	• DLS • Transmission electron microscopy (TEM) • Zeta potential • Agarose gel electrophoresis • ^{1}H NMR • HPLC • Ethidium bromide displacement assay to evaluate DNA condensation ability	2014	[21]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Bioreducible liposomes for gene delivery: From the formulation to the mechanism of action	• Bioreducible liposomes containing a redox-sensitive triazine-based geminisurfactant, SS14 and helper lipids for effective transfection • Bioreducible lipoplex example: DOPC/DOPE/SS14 (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dimyristoyl-snglycero-3-phosphatidylethanolamine (DMPE), triazine-based gemini surfactant, SS14)			80ng/cm^2 of pDNA	
Bioreducible polymers as a determining factor for polyplexdecomplexation rate and transfection	Polyplexes composed of a mixture of PLL and Reducible PLL (RPLL) to understand the relationship between decomplexation rate, intracellular localization, and transfection efficiency	Poly(L-lysine) (PLL: MW ~7.4 kDa) and reducible PLL (MW ~6.7 kDa)		0.5µg DNA/well	
Bioreducible polypeptide containing cell-penetrating sequence for efficient gene delivery	• Chemical linked linear bioreducible polypeptides comprised of CHK6HC and CR8C (xPolyK6, xPolyK6-R81 and xPolyK6-R82) for efficient gene delivery via disulfide linkage. • CHK6HC and R8 have the ability of DNA condensation • DNA is released from the system by glutathione stimulation			1 µg of pGL-3	pGL-3
Bioreducible POSS cored star-shaped polycation for efficient gene delivery	• Star-shaped bioreducible gene carriers that has a relatively narrow molecular weight distribution • Polymer: polyhedral oligomericsilsesquioxane - poly (2-dimethylamino)ethyl methacrylate consists of POSS core and eight disulfide-linked PDMAEMA arms **POSS-(SS-PDMAEMA)$_8$**		Treatment of cardiovascular diseases	0.5 mg/mL pDNA	pRL-CMV pDNA
Co-delivery of drug and DNA from cationic dual-responsive micelles derived from poly(DMAEMA-co-PPGMA)	• A series of cationic **poly(DMAEMA-co-PPGMA)** copolymers was used to form micelles at physiological temperature. • The hydrophobic PPGMA interior allows a cell-sensitizing drug such as paclitaxel to be incorporated while the cationic and hydrophilic PDMAEMA corona allows the complexation of anionic DNA to form a nano-sized polyplex.		Not reported	0.5 µg/µL of pRL-CMV	pRL-CMV & Paclitaxel
Co-delivery of TRAIL gene enhances the anti-glioblastoma effect of paclitaxel in vitro and *in-vivo*	• A novel anticancer strategy which is a combination of low concentration of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene to paclitaxel • Tumor-targeted gene carrier RGD-PEG-PEI nanoparticles and brain-targeted micelle CDX-PEG-PLA • CDX, a peptide that binds to nicotine acetylcholine receptors (nAChRs) to inhibit the growth of a tumor • c(RGDyK) is a cyclic arginine–glycine–aspartic acid– D-tyrosine–lysine which binds to integrin (such as αvβ3) to inhibit the growth of a tumor	CDX–PEG–PLA micelle & RGD–PEG–PEI nanoparticles	Intracranial glioblastoma	EGFP expression: 2µg of pEGFP-N2 *In-vivo* transfection: 0.8 mg/kg pDsRed-N1	pEGFP-N2 pDNA
Cytoplasm-responsive nanocarriers conjugated with a functional cell-penetrating peptide for systemic siRNA delivery	Diblock biodegradable polymeric nanocarriers (methoxy polyethylene glycol-polycaprolactone (MPEG-PCL) conjugated with a cytoplasm-responsive cell-penetrating peptide (CPP) CH2R4H2C). **(MPEG–PCL–CH2R4H2C)**		Anti-tumor effect	1µg siVEGF	siRNA
Degradable gene delivery systems based on pluronics-modified low-molecular-weight polyethylenimine: preparation, characterization, intracellular trafficking, and cellular distribution	Polyethylenimine grafted with nonionic amphiphilic surfactant polyether-pluronic that facilitates and controls intracellular trafficking **Pluronic-PEI conjugate**		Not reported	3µg of pGL3	pDNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• EGFP expression via flow cytometer • BCA protein assay to measure protein content • Glutathione assay kit to measure GSH levels	• U87-MG (Human glioblastoma-astrocytoma) • MG63 (Humanosteosarcoma cells) • COS-7		Not reported	Alamar Blue assay		• DLS • Zeta potential	2010	[22]
• Luciferase assay • BCA protein assay • Cellular uptake via flow cytometry • Intracellular trafficking of polyplexes via laser scanning confocal microscope • Evaluation of intracellular glutathione concentration	• MCF7 • HEK-293		1 - 5× 10^5 cells/well of a 6-well plate	MTT assay		• Agarose gel electrophoresis assay • Laser scanning • Confocal microscopy • ^{1}H NMR • Particle size via Zetasizer • Surface charges	2013	[23]
• Luciferase assay • BCA protein assay • Cellular uptake by flow cytometry and confocal microscopy	• HELA • COS-7		~ 80%			• Particle size analysis • Zeta potential • Acid base titration assay • Agarose cell retardation assay • TEM • Confocal microscopy • HPLC • Size-exclusion chromatography and multiangle laser light scattering (SEC-MALLS) • ^{1}H NMR	2013	[24]
• EGFP expression • Luciferase assay via luminometer • Determination the concentration of protein in cells via bicinchoninic acid assay	• HepG2 • COS7		Not reported			• Agarose gel electrophoresis • Gel permeation chromatography • Zeta potential • Particle size • ^{1}H NMR • ^{13}C NMR • ^{29}Si NMR • UV transilluminator • BioDco-It imaging system (UVP Inc.) • Examination of morphology of nanocomplexes via atomic force microscopy (AFM) system and a Nanoscope Multimode III AFM instrument	2014	[25]
Luciferase assay via luminometer and a commercial kit	Cos7 cells		5 × 10^4 cells/well	Not tested		• ^{1}H NMR • DLS • Gel retardation assay • Zeta potential	2013	[26]
• EGFP expression via fluorescent microscope • Determination of the tumor volume and area via MR imaging. • Evaluation of mitotic delay through cell cycle distribution via FACS analysis • Confocal images of brain tumor sections	U87 (Glioblastoma cells)	No	2 × 10^4 cells/well	MTT assay	4-6 weeks of age Male Balb/c nude mice	• DLS • Atomic forcemicroscopic technique	2012	[27]
• VEGF silencing assay via a mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit • RNase resistance of siRNA complexes • Cellular uptake via flow cytometry • Tumor distribution of Alexa-dextran/CPP carrier in tumor-bearing mice • Examination tumor volume in mice after MPEG–PCL–CH2R4H2C/siVEGF complex injection	S-180 sarcoma cells	Yes	70-80%	WST-8 assay	IV injection of MPEG–PCL–CH2R4H2C/siVEGF complex via the tail vein of S-180 tumor-bearing mice	• Zeta potential • Agarose gel electrophoresis • HPLC • Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry • Fourier transform infrared spectroscopy (FT-IR) • Gel permeation chromatography (GPC) • ^{1}H NMR • Determination of complex formation with plasmid via SYBR Green exclusion assay • The complexation of MPEG–PCL with CH2R4H2C (CPP) was detected via the ninhydrin test	2013	[28]
• Luciferase assay via Micro-BCA protein assay kit • Cellular uptake via Green QDs and confocal microscope	HeLa cells		60–70%	Cell proliferation assay via enzyme-linked immunosorbent assay plate reader	Not tested	• ^{1}H NMR • Degradation of PP (polymer Particles) assay • The size and zeta potential via electrophoretic light-scattering • Spectrophotometer • Agarose gel retardation assay • DNA protection and release assay • Confocal microscope • Gel permeation chromatography	2012	[29]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Delivery of two-step transcription amplification exendin-4 plasmid system with arginine-grafted bioreducible polymer in type 2 diabetes animal mode	• Arginine-grafted bioreducible **poly (disulfide amine) polymer (ABP)** as a carrier contains Exendin-4 and conjugated with TSTA system • TSTA system consists of dual plasmids (pβ-Gal4-p65 and pUAS-SP) that also have advantages of signal peptide (SP) to facilitate its secretion in ectopic cells or tissue. **ABP/TSTA-SP-exendin-4**		Type 2 diabetes	1.5 µg	Dual DNA including: • pβ-Gal4/p65 plasmid • pUAS-SP-exendin-4 plasmid
Design, synthesis and evaluation of spermine-based pH-sensitive amphiphilic gene delivery systems: Multifunctional non-viral gene carriers	• Spermine-based pH-sensitive amphiphilic carriers which are able to form stable complexes with plasmid DNA. • pH-sensitive polymers: [N,N′-Bis(oleoyl-cysteinyl)b-analinyl-a-lysinyl]histinyl-histidine spermine monoamide (SHHKACO), N,N′-Bis(oleoyl-cysteinyl-histidinyl)lysine spermine monoamide (SKHCO), [N,N′-Bis(oleoyl-cysteinyl)lysinyl]histinyl-histidine spermine monoamide (SHHKCO), N,N′-Bis(oleoyl-cysteinyl)lysine spermine monoamide (SKCO), [N,N′-Bis(oleoyl-cysteinyl-histidinyl)b-analinyl-a-lysinyl] histidine spermine monoamide (SKAHCO), [N,N′-Bis(oleoyl-cysteinyl)lysinyl]-histidine spermine monoamide (SHKCO), [N,N′-Bis(oleoyl-cysteinyl)b-alaninyl-a-lysinyl] histidine spermine monoamide (SHKACO) and [N,N′-Bis(oleoylcysteinyl)(-alanyl-a-lysyl)]-sperminemonoamide (SKACO).	Not reported	1 mg pCMV-GFP	plasmid pCMV-GFP pDNA
Development of anionic bubble lipopolyplexes for efficient and safe gene transfection with ultrasound exposure in mice	• Ternary complex that consists of pDNA, cationic polymer, and an anionic bubble liposome • The anionic bubble lipopolyplexes helped in efficient and safe gene transfection with ultra sound exposure	Anionic bubble lipopolyplex		*In-vitro* Luciferase assay: 10µg pDNA Erythrocyte aggregation experiment: 5µg pDNA *In-vivo* luciferase assay: 50µg of pDNA	pDNA
Dextranation of bioreducible cationic polyamide for systemic gene delivery	Polycondensation reaction of bis-(p-nitrophenyl)-3, 3'- dithiodipropanoate and 1, 4-bis(3-aminopropyl) piperazine was performed and followed by dextranation to form bioreducible polycation triblock copolymer with multiple disulfide bonds **Dex-SSBAP-Dex**	Dextran-SSBPA-dextran triblock copolymer	Not reported	1µg DNA	
Dual responsive, stabilized nanoparticles for efficient *in-vivo* plasmid delivery	• Copolymer combines reversible hydrophobization for enhanced polyplex stability in extracellular environments and statistical hydrophilization for efficient plasmid and siRNA release inside cells. • Copolymer: Ternary, amphiphilic block copolymers containing a sheddable hydrophobic PCL block, pH-sensitive oligoamine tetraethylenepentamine (TEPA)-decorated poly(glycidyl methacrylate) (PGMA) block, and hydrophilic oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA) segment.		Brain targeting	Not reported	
Dual targeting effect of Angiopep-2-modified, DNA-loaded nanoparticles for glioma	• Polyamidoamine (PAMAM) bound to Angiopep-2 via PEG. • PAMAM-PEG-Angiopep/DNA nanoparticles target glial cells and brain tumors	**PAMAM-PEG-Angiopep2**	Brain glioma treatment	Intracellular trafficking: 0.8 ml LysoTracker Green DND-26 (1 mM) *In-vitro* transfection 5µgpORF-TRAIL Biodistribution in brain tumor-bearing mice 50 mg DNA	pORF-TRAIL pDNA
Efficient gene delivery system mediated by cis-aconitate-modified chitosan-g-stearic acid micelles	• Cis-aconitate-modified chitosan-g-stearic acid (CA-CSO-SA) micelles condense DNA to form nanocopmlex • The nanocomplex has a high transfection efficiency due to improvedendolysosomal escape	Cis-aconitate-modified chitosan-g-stearic acid (CA-CSO-SA)	Anticancer therapy	1µg DNA	pDNA
Efficient gene transfection by histidine-modified chitosan through enhancement of endosomal escape	Histidine Modified chitosan was synthesized by introducing histidine via disulfide bond to improve the transfection efficiency by improving endosomal escape	Histidine-modified chitosan	Not reported	Cellular uptake: 1µg/mlpDNA Luciferase assay: 6µg/ml pDNA	

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• GLP-1 and exendin-4 expression via ELISA • Exendin-4 expression according to the ratios between pβ-Gal4/p65 and pUAS-SP-exendin-4 plasmids • Rat/mouse insulin ELISA (Millipore) • *In-vivo* anti-diabetic study via measuring blood glucose, exendin-4, GLP-1, and insulin concentrations • Biodistribution assessment in mice	• A549 (Non-small cell lung carcinoma cells) • NIT-1 (Insulinoma cells)			• MTT assay • Assessment of liver toxicity *in-vivo* via serum levels of aspartate amino-transferase (AST) and alanine transaminase (ALT)	6 weeks male C57BL/6J mice	• DLS • Zeta potential. • Particle size • DNA quantification assay via Quant-iTPico Green assay kit	2012	[30]
• Luciferase assay via BCA kit • GFP expression via FACSCalibur flow cytometer • Cellular uptake via the green fluorescence of YOYO-1	U87 MG cell line		5×10^4 cells/well	MTT assay	Not tested	• Agarose gel electrophoreses • ^{1}H NMR • Particle size analysis	2011	[31]
• *In-vitro* and *in-vivo* luciferase assay was examined via a luminometer and was quantified via protein quantification kit • Cellular uptake of using fluorescein-labeled pDNA and LysoTracker Red DND-99 was measured via confocal microscope • Erythrocyte aggregation experiment via phase microscopy • Luciferase and glyceraldehyde 262 3-phosphate dehydrogenase (GAPDH) mRNA expression was measured via Lightcycler 2.0 real-time PCR system in both separated parenchymal and non-parenchymal liver cells	The human vascular endothelial cell line (EAhy926)		Not reported	• WST-1 assay • Assessment of liver toxicity *in-vivo* via determination of serum levels of aspartate amino-transferase (AST) and alanine transaminase (ALT)	Female ICR mice	• Particle size via DLS • Zeta potential via DLS • TEM • GelRed nucleic acid Gel stain were used for pDNA visualization via ImageQuant LAS4000 162 system	2014	[32]
• GFP fluorescence intensity via BAC protein assay • *In-vitro* and *in-vivo* luciferase assay via a luminometer and luciferase assay kit	• MCF-7 • SKOV-3 (Ovarian carcinoma cells)		60–70%	Alamar Blue assay	4-6 week male Balb/c nude mice	• Agarose gel electrophoresis • Zeta potential • ^{1}H NMR • Particle size	2014	[33]
• Luciferase assay • The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene activity	HeLa cells		> 80%	Not tested	Right lateral ventricle of mice	• TEM • Particle size distribution via DLS • Evaluation of stability of polyplexes via fluorescence quenching of YOYO-labeled pDNA	2013	[34]
• *In-vitro* transfection was visualized via fluorescence microscopy analysis by using propidium iodide • Intracellular trafficking was evaluated via CLSM by using LysoTracker Green DND-26 and EMA fluorescence. • Bio-distribution of EMA-labeled DNA in brain tumor-bearing mice • In situ tumor apoptosis was detected via TUNEL assay kit • Evaluation of the pharmacodynamics of brain tumor-bearing mice	C6 glioma cells	No	70-80%	MTT assay	ICR mice and nude mice	• TEM • Particle size via DLS • Zeta potential via DLS • NMR	2011	[35]
• EGFP expression was visualized via fluorescence microscope and quantified via flow cytometry with and without inhibitors • Cellular uptake of RITC-labeled CA-CSO-SA was visualized via CLSM and quantified via flow cytometry • Intracellular distribution of RITC-labeled CA-CSO-SA was visualized via CLSM	HEK-293	Yes	Not reported		Not tested	• ^{1}H NMR • Particle Size via DLS • Zeta potential Via DLS • TEM • Agarose gel electrophoresis	2014	[36]
• Cellular uptake of fluorescein-labeled pDNA was evaluated by quenching the extracellular fluorescein of trypan blue via flow cytometry analysis. • Intracellular distribution was evaluated via Nikon Eclipse Ti inverted confocal microscope • Luciferase assay was visualized via luminometer and quantified via Bradford protein assay.				PicaGene Luciferase assay		• Particle size via DLS • Zeta potential via DLS • Agarose gel electrophoresis • Electrospray ionization mass spectrometry (ESI-MS) • Histidine quantification by ninhydrin test • Determination of buffer capacity • UV-vis spectrometer	2010	[37]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Efficient GLP-1 gene delivery using two-step transcription amplification plasmid system with a secretion signal peptide and arginine-grafted bioreducible polymer	• Glucagon-like peptide (GLP-1) encoding dual plasmid system conjugated with arginine- grafted bioreducible polymer (ABP) as a gene carrier • It is an ABP/TSTA (SP-GLP-1) system for novel GLP-1 gene delivery system • TSTA (SP-GLP-1) consists of pβ-Gal4-p65 and pUAS-SP-GLP-1	ABP (Arginine- grafted Bioreducible Polymer)	Type 2 diabetes therapy	4 µg pDNA	pDNA (TSTA (SP-GLP-1))
Efficient, dual-stimuli responsive cytosolic gene delivery using a RGD modified disulfide-linked polyethylenimine functionalized gold nanorod	• The system is composed of gold nanorod (GNR) based gene delivery system • Efficient NIR (near infra-red) laser-triggered controlled release system based on intracellular reductive environment (high GSH content) and NIR irradiation	Gold nanorod- disulfide cross-linked with short polyethyleneimine-polyethylene glycol-RGD peptide.	Not reported	3µg/well DNA	pDNA
Enhanced incretin effects of exendin-4 expressing chimeric plasmid based on two-step transcription amplification system with dendritic bioreducible polymer for the treatment of type 2 diabetes	• Dendrimer consisting of two-step transcription amplification (TSTA) system • PAM-ABP/chimeric DNA complex elevated insulin level via high expression of exendin-4	Arginine-grafted poly (cystaminebisacrylamide-diaminohexane) (ABP) bound to poly (amido amine) (PAMAM) dendrimer to form (PAM-ABP) complex	Type 2 diabetes	1.5µg exendin-4 DNA	
Enhanced siRNA delivery using a combination of an arginine-grafted bioreducible polymer, ultrasound, and micro bubbles in cancer cell	An ultra sound sensitive micro-bubble consists of an arginine-grafted bioreducible polymer (ABP), micro bubbles (MB) with human serum albumin (HAS) shell and perfluorocrownether gas core, and siRNA (SAM) for targeting vascular endothelial growth factor (VEGF) in a human ovarian cancer cell line	SAM complex (siRNA-ABP-MB)	Ovarian cancer treatment	<u>FACS analysis:</u> 10nM siRNA/well <u>VEGF ELISA assay:</u> 50nM siRNA/well	siRNA
Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg	Polyamidoamine (PAMAM) dendrimer grafted with basic L-arginine residues (PAMAM-Arg) for enhancing gene delivery to primary neuronal cells	Arginine-grafted PAMAM dendrimer	Neuronal disorders	1µg/well DNA	HMGB1 shRNA-expressing plasmid.
Enhancement of gene delivery using novel homodimeric Tat peptide formed by disulfide bond	Cationic liposome conjugated with cysteine moiety to form homodimeric and cyclic TAT peptide by introduction disulfide bond between cysteine molecules	Cationic lipids conjugated with modified TAT	Not reported	<u>Luciferase assay:</u> 0.3 µg of DNA <u>EGFP expression assay:</u> 0.3 µg per well	pcDNA-Luc
EphA2 targeting peptide tethered bioreducible poly (cystaminebisacrylamide–diamino hexane) for the delivery of therapeutic pCMV-RAE-1γ to pancreatic islets	PEGylated poly (cystaminebisacrylamide - diamino hexane) in conjugation with targeting peptide GGGCHVLWSTRC (Eph) for EPhA2 and EPhA4 receptor targeting		Treatment of type 1 diabetes	<u>Cellular uptake assay:</u> 2µg/well of YOYO-1 labelled pDNA <u>Luciferase assay:</u> 0.5µg DNA/well	pDNA
Folic acid modified cationic γ-Cyclodextrin-oligoethylenimine star polymer with bioreducible disulfide linker for efficient targeted gene delivery	γ-cyclodextrin (γ-CD) core and multipleoligoethylenimine (OEI) arms with folic acid (FA) linked by a bioreducible disulfide bond for efficient targeted gene delivery		Anticancer therapy	0.1 mg/mL with an equal volume of polymer solution	

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• Level of GLP-1 mRNA transcription was evaluated via RT- PCR • The level of GLP-1 was measured via ELISA • Insulin level was measured via insulin ELISA	• NIH3T3 • NIT-1 insulinoma cell		70 - 80 %			• Agarose gel electrophoresis • Particle size • Zeta-potential	2012	[38]
• GFP quantification via flow cytometry • YOYO-1-labeled DNA released from GNRs-based complexes was quantified via fluorescence spectrophotometer upon exposure to either NIR irradiation or GSH or both combined • Cellular uptake of GNRs was determined via a dark-field microscope and ICP–MS, respectively. • Intracellular distribution of the FITC labeled GNRs was determined via CLSM	The U-87MG (human glioblastoma cell) cell line		Not reported	MTT assay		• ^{1}H NMR • Zeta potential via DLS • TEM • UV–Vis–NIR spectrophotometer • Thermogravimetric analysis (TGA)	2014	[39]
• The level of exendin-4 was measured via exendin-4 enzyme immunoassay kit • Insulin level was measured by rat/mouse insulin ELISA. • Intracellular signaling pathway in exendin-4-stimulated pancreatic β-cells was measured via cAMP Parameter assay kit and PKA kinase activity kit • *In-vivo* exendin-4 expression assay • Blood glucose levels was measured via a one-touch blood glucose meter • Free fatty acid level was measured via serum/plasma fatty acid and glycerol detection kits	• NIT-1 (Insulinoma pancreatic β-cell line) • HeLa		60-70%	• MTT assay • Liver toxicity was assessed via ALT or AST enzyme assay kit	Diet-induced obese (DIO) male C57BL/6J mice	• Particle size via DLS • Zeta potential via DLS	2013	[40]
• Cellular uptake was measured via fluorescence-activated cell sorting (FACS) analysis • VEGF protein was measured by using a human VEGF ELISA kit	A2780 cells		30-40%			• Zeta Potential (DLS) • Particle size analysis (DLS) • The concentration (number of MB/mL) of MB was measured via light microscope using a hemocytometer • Stability of MB was assessed via determination of particle size and concentration of MB at storage temperature (4° C) and body temperature (37°C). • The stability and degradation of the polyplex was determined via gel retardation assay in the presence of US • SAM complex formation was assessed via CLSM • siRNA loading capacity of MBs was assessed by measuring the fluorescence of the Cy3-labeled siRNA using a TECAN infinite® 200 Pro series fluorometer	2013	[41]
• Luciferase activity via luminometer • GFP expression • Immunocytochemistry assay	Neuro 2A cells (Mouse neuroblastoma cell line)		Not reported	MTT assay	Not tested	• Agarose gel electrophoresis	2006	[42]
• Luciferase assay via luminometer • The protein content was measured via a bicinchoninic acid (BCA) assay • EGFP expression assay via fluorescence microscope	• MCF-7 • VSMC (Aortic vascular smooth muscle cells)					• Gel Retardation Assay • Reverse-phase HPLC	2011	[43]
• Luciferase assay • DNase Protection Assay • Cellular uptake assay via BD FAC scan analyzer • Expression of pCMV-sRAE-1γ was detected via anti-V5-AP antibody and colorimetric detection	• MS1s (Mile Seven pancreatic cells) • NIH3T3 • ᶠmouse embryonic fibroblast cells) • HEK-293T		70~80%			• ^{1}H-NMR • MALDI-ToF • Size-exclusion chromatography • Hydrodynamic radius of polyplexes via DLS • Polyplex size • Agarose get electrophoresis	2012	[44]
• Luciferase activity was visualized byluminometer and quantified via bicinchoninic acid assay • pEGFPN1expression via CLSM	A549 (Human KB carcinoma cell line)	No	Not reported			• ^{1}H NMR • UV−Vis Spectroscopy • The concentration of released folic acid via UV−vis spectroscopy • Gel retardation assay. • Particle size via DLS • Zeta potential via DLS	2013	[45]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Galactosylated reduction and pH dual-responsive triblock terpolymer Gal-PEEP-a-PCL-ss-PDMAEMA: a multifunctional carrier for the targeted and simultaneous delivery of doxorubicin and DNA	• Dual-responsive biodegradable galactosamine (Gal)-modified polymeric micelles consists of galactosamine (Gal), poly(ethylethylene phosphate) (PEEP), poly(3-caprolactone) (PCL), and poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) blocks (**Gal-PEEP-a-PCL-ss-PDMAEMA terpolymer**) The components were linked by both reduction and pH dual-responsive bonds (Click reaction) and bound to DNA and doxorubicin to form Gal-PEEP-a-PCL-ss-PDMAEMA/DNA/DOX micelles.		Treatment of malignant solid tumor	Not reported	doxorubicin and pDNA
Human Erythropoietin gene delivery using an arginine-grafted bioreducible polymer system	Arginine-grafted bioreducible poly(disulfide amine) (ABP) polymer gene vector for *in-vivo* transfer of human erythropoietin plasmid DNA (phEPO) to produce long-term, therapeutic erythropoiesis	Arginine-grafted bioreducible poly(disulfide amine) (ABP)	Anemia treatment in case of chronic kidney disease (CKD) and cancer	Reverse transcriptase-PCR: 200 µg phEPO Cellular uptake: 2 µgYOYO-1 iodide-labeled *phEPO*	human erythropoietin plasmid DNA (phEPO)
Hydroxyl PAMAM dendrimer-based gene vectors for transgene delivery to human retinal pigment epithelial cells	• PEGylated (PAMAM) dendrimers conjugated with triamcinolone acetonide (TA) that acts as a nuclear localization enhancer **(PAMAM-TA)** • Dendrimer-gene complex enhances cellular uptake and transfection efficacy of retinal pigment epithelium (RPE)		Blinding disorders treatment via ocular gene therapy	2µg/well pDNA	pDNA
In-Vitro and *In-Vivo* gene transfection using biodegradable and low cytotoxic nanomicelles based on dendritic block copolymers	• A redox-cleavable star cationic dendritic copolymers PCL-b-PDMAEMA consists of poly(ε-caprolactone) (PCL) core and poly(2 (Dimethylamino) ethylmethacrylat) (PDMAEMA) arms (**PCL-b-PDMAEMA**) • PDMAEMA is able to self-assembly to form nanoscale micelles		Anticancer therapy	GFP expression: 1 µg of pDNA *In-vivo* Luciferase: assay 50 µg of pDNA for each mouse	Plasmid pC-MV-GFP pDNA
Macrophage-specific RNAi targeting via 'Click', mannosylated polymeric micelles	• Endosomolytic, pH-responsive and mannose receptor-targeted nanoparticles (ManNPs) micelle for siRNA delivery • ManNPs consist of a pH-responsive block which is able to disrupt endosomes at low pH, a cationic block for condensation of nucleic acids, and an azide-displaying block for conjugation of targeting motifs by click chemistry **(Triblock copolymers)**		Anticancer Atherosclerosis treatment	50nM siRNA per well	siRNA
Manipulating the pH response of 2,3-diaminopropionic acid rich peptides to mediate highly effective gene silencing with low-toxicity	• The pH responsive carrier system is composed of 2, 3-diaminopropionic acid rich peptides • Peptide was modified by replacing histidine with Dap and its methylated derivatives to assess their effect on the pH response of the peptide		Treatment of atherosclerosis, coronary artery occlusion, diabetes and tumor growth	Not reported	pDNA siRNA
Matrix metalloproteinase responsive, proximity-activated polymeric nanoparticles for siRNA delivery	• Gene delivery system is a pH-responsive, smart polymeric nanoparticles (SPN) with matrix metalloproteinase (MMP)-7-dependent proximity-activated targeting (PAT). • The polymer forming MMP-7-responsive PAT-SP is PEG-peptide-pDMAEMA **(PEG-pep-pD)**		Treatment of tumors and metastases	50nM siRNA	siRNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• Competitive binding assays • Cellular uptake was evaluated via live cell imaging system • GFP quantitation via flow cytometry	HeLa cells HepG2 (Hepato-cellular Carcinoma cell line)					• ^{1}H NMR • FT-IR spectroscopy • Agarose gel electrophoresis • GPC • Zeta potential • Particle size distribution via DLS under different pHs and reductive condition. • TEM • The critical aggregation concentration (CAC) was measured via spectrofluorometer	2014	[46]
• Hematocrit level were determined via the microhematocrit method • Whole blood reticulocyte counts were measured via BD FACS Calibur. • The plasma concentrations of IL-6 and hEPO protein were measured with Quantikine rat IL-6 ELISA kit and Quantikine hEPO ELISA kit, respectively. • Quantification of real time reverse transcriptase-PCR • Cellular uptake of YOYO-1 iodide-labeled phEPO was measured via flow cytometry.	Bone marrow (BM) cells cultures			Not tested	male SD rats	• Gel electrophoresis assays • Particle size via DLS • Zeta potential via DLS	2012	[47]
• EGFP expression via CLSM • Luciferase assay visualized by luminometer and quantified via BCA protein assay kit • Cellular uptake of Cy5-labeled plasmid was assessed via flow cytometry • Cellular uptakes of BiD-TA-Cy5 and Cy3-labeled plasmids were imaged via CLSM	ARPE-19 (Human retinal pigment epithelial cell line)	Yes		Dojindo CCK8 assay	Not tested	• Fluorescence spectroscopy. • ^{1}H NMR • HPLC • Particle size via laser Doppler anemometry • Zeta potential via laser Doppler anemometry • TEM • Gel retardation assay • Buffering capacity was measured via microprocessor-based pH meter	2015	[48]
• EGFP expression was imaged via spectro fluorophotometer quantified via BCA protein assay kit • *In-vivo* Luciferase assay via BCA protein assay kit	• MCF-7 • SKOV-3			MTT assay	Nude mice	• ^{1}H NMR • TEM • Agarose gel assay analysis • Zeta potential • Particle size • Critical micelle concentration (CMC) was measured via FLS-920 fluorescence spectrometer	2014	[49]
• Quantification of real time reverse transcriptase PCR • Transfection efficacy of FAM-siRNA was measured via flow cytometry and CLSM • Kinetics of ManNP-mediated FAM-siRNA delivery into primary macrophages was assessed via CLSM • The ability of pH-dependent disruption of endosomal membranes and cytosolic delivery of the cargo was assessed via erythrocyte hemolysis assays	• Immortalized human macrophages (THP 1) • Human breast cancer cell lines (MDA-MB-231 and MDAMB- 468)			Flow cytometry detection of green fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity		• ^{1}H NMR • HPLC • ^{13}C NMR • Agarose gel assay analysis • TEM • Hydrodynamic diameters via DLS • Zeta potential via DLS	2013	[50]
• Luciferase activity • Conformational transition of modified peptide was detected via far-UV circular dichroism • GAPDH silencing expression was assessed via densitometry of Western blots • Cellular uptake of Cy3 labelled peptide/siRNA complexes was assessed via live cell confocal microscopy	• MCF-7 cell line • A549 cells • Monocyte THP-1 cells	No		MTT assay	Not tested	• RP-HPLC • MALDI-TOF mass spectrometry • Acylations and deprotections were confirmed by trinitrobenzenesulphonic acid (TNBS) test	2013	[51]
• Stability of PAT-SPN/DNAFAM/DNACy5 was determined via Förster (Fluorescence) resonance energy transfer (FRET)-based assay and agarose gel electrophoresis • Luciferase assay • Cellular uptake of PAT-SPN/DNA^{FAM} via flow cytometry • Cellular uptake of PAT-SPN/DNA^{Cy5} via CLSM • PH-dependent hemolysis of erythrocytes for PAT-SPN/DNA was conformed via hemolysis assay • Endo-lysosomal escape of PAT-SPN/DNACy5 via confocal imaging	• MDA-MB-231 cell line • R221A-Luc mammary tumor cells	Yes		Cell viability of R221A-Luc mammary tumor cells was assessed via luciferase assay		• HPLC • Liquid chromatography-mass spectrometry (LC-MS) • Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) • Gel permeation chromatography (GPC) • Particle size via DLS • TEM • ^{1}H NMR • UV-vis Spectroscopy • Zeta-potential via DLS	2013	[52]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Melittin-grafted HPMA-oligolysine based copolymers for improved gene delivery	• The gene delivery system is a peptide-based block copolymer • The first block copolymer conjugated with melittin peptide was N-(2 hydroxypropyl) methacrylamide (HPMA). • The second block copolymer that bound to DNA was methacrylamido-functionalized oligolysine monomer (MaAhxK10) and HPMA The resultant vector was melittin-grafted diblock copolymers **poly[(HPMA-g-Melittin)-b-(HPMA-MaAhxK10)]**		Gene delivery to the brain	Not reported	pCMV-Luc pDNA
Mixtures of poly(triethylenetetramine/cystaminebisacrylamide) and poly(triethylenetetramine/cystaminebisacrylamide) polyethylene glycol for improved gene delivery	Nontoxic bioreducible polycation consists of branched disulfide-containing poly(amidoethyleneimines) (SS-PAEIs) and cystaminebisacrylamide (CBA) in conjugation with PEG to improve carrier performance in the presence of serum	p(TETA/CBA)5k-g-PEG2k	Not reported	0.5µg DNA	
Multifunctional triblock nanocarrier (PAMAM-PEG-PLL) for the efficient intracellular siRNA delivery and gene silencing	• A triblock poly(amido amine)-poly (ethylene glycol)-poly-l-lysine **(PAMAM-PEG-PLL)** nanocarrier for the delivery of siRNA • **PLL** has cationic primary amine groups for electrostatic interaction with negatively charged siRNA • **PAMAM** dendrimer has tertiary amine groups to give the proton sponge effect • **PEG** enhances nuclease stability in blood serum		Anticancer therapy	RT-PCR assay: 1µM siRNA Other experiments: 2µg of total RNA	siRNA
Multi-layered nanoparticles for combination gene and drug delivery to tumors	• Multi-layered polymer nanoparticle (MLNP) consists of poly (lactic-co-glycolic acid) PLGA with surface polyethyleneimine (PEI) and functional peptides modified antennapedia (mAP and iRGD) • The system was loaded with plasmid or camptothecin (CPT) for synergistic effect			11.6ng/uL pDNA	pDNA
Nonviral gene delivery to human ovarian cancer cells using arginine-grafted PAMAM dendrimer	• L-Arginine-grafted-polyamidoamine dendrimer bond to epidermal growth factor receptor (EGFR) **(PAMAM-Arg)** • PAMAM-Arg/EGFR antisense gene complex treats ovarian cancer with highly expressed EGFR			1µg DNA	pDNA
Novel reducible linear L-lysine-modified copolymers as efficient non-viral vectors	• Reducible linear L-lysine modified copolymers (LLCs) as an alternative to high molecular weight poly (L-lysine) (PLL) • DNA is released from LLC/pDNA only in the presence of reducing agent dithiothreitol (DTT)		Not reported	2µgDNA	pCMV-Luc pDNA
Paclitaxel-conjugated PEG and arginine-grafted bioreducible poly (disulfide amine) micelles for co-delivery of drug and gene	The cationic arginine-grafted bioreducible poly (disulfide amine) (ABP) conjugated with paclitaxel or gene via PEG to form micelles	Arginine-grafted bioreducible poly (disulfide amine)	Anticancer therapy for Kaposisarcoma	Luciferase assay: 1µg DNA Cellular uptake of YOYO-1 labeled plasmid DNA: 0.5µg of YOYO-1 labeled plasmid DNA	pDNA or paclitaxel
PDMAEMA-grafted core–shell–corona particles for nonviral gene delivery and magnetic cell separation	Star shaped hybrid polymer that consists of: • γ-Fe2O3 that coated with silica layer bearing an atom transfer radical polymerization (ATRP) initiator • 2-(dimethylamino) ethyl methacrylate (DMAEMA) that polymerized with core-shell nanoparticles leads to formation of **core-shell-corona hybrid nanoparticles** **(γ-Fe2O3-silica-PDMAEMA)**		Not reported	1µg DNA	pDNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• The membrane-lytic activity of carrier was assessed via hemolysis assay • *In-vivo* luciferase assay was visualized by luminometer and quantified via BCA protein assay kit • GFP expression via flow cytometry	• HeLa cells • PC-12 (Pheochromocytoma of the rat adrenal medulla)			Propidium iodide (PI) as a cell viability marker	7-9-week-old female C57/Bl6 mice	• Size exclusion chromatography (SEC) • Modified amino acid analysis. • Hemolysis assay • Particle size via DLS • Zeta potential via DLS • Transmission electron microscopy (TEM) • Gel retardation assay	2013	[53]
• Luciferase assay was visualized by luminometer and quantified via BCA protein assay kit • Polyplex stability was evaluated via agarose gel electrophoresis via rabbit serum	• CT-2 (Colon Adenocarcinoma cells) • SVR (Mouse pancreatic islet endothelial cells)		70%	MTT assay	Not tested	• ^{1}H NMR • Molecular weight analysis via fast protein liquid chromatography (FPLC) • MALDI-TOF analysis • Buffering capacity was determined via acid base titration • Zeta potential via DLS • Particle size via DLS • TEM	2010	[54]
• Cellular uptake of fluorophore-labeled siRNA was assessed via confocal microscopy • BCL2 gene expression • RT-PCR assay	A2780 (Human Ovarian Carcinoma cell line)		50-70%			• ^{1}H NMR • MALDI-TOF • Gel permeation chromatography (GPC) • Particle size via DLS • ZetaPotential via DLS. • Confocal microscopy measurement • Agarose gel electrophoresis	2011	[55]
• Luciferase assay was visualized by luminometer • *In-vitro* release of plasmid and CPT was measured via dialysis technique • Percent cellular transfection was measured via GFP expression. • Synergistic effect of CPT and pTRAIL that were delivered via CT2 MLNPs by Chou-Talalay analysis • Antitumor activity was evaluated by measuring the tumor size via traceable digital vernier calipers (Fisher) *in-vivo* • Therapeutic effect was evaluated via terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL)	• Human embryonic kidney 293 (293T) • Human glioblastoma (U87) • Human colorectal adenocarcinoma (HCT116) • Human breast adenocarcinoma (MDA-MB-231)	No		Cell titer blue cell viability assay	4 weeks male athymic nude mice	• Particle size and morphology via scanning electron microscopy (SEM) • Particle hydrodynamic diameter via DLS • Zeta potential via DLS	2014	[56]
• Luciferase assay via luminometer • Thymidine incorporation in SK-OV3 cells was evaluated by analyzing 3H-radioactivity via a liquid scintillation counter	SK-OV3 (Ovarian carcinoma cells)	Yes		Sulforhodamine B assay	Not tested		2011	[57]
• Luciferase assay was visualized via chemiluminometer and quantified a BCA protein assay kit • EGFP via fluorescence microscopy • The release of The EMA-labeled pDNA from LLC/pDNA was confirmed via CLSM • The release of The EMA-labeled pDNA from LLC/pDNA was confirmed in the presence of DTT via fluorescence spectroscopy • DNase I protection assay via agarose gel electrophoresis	• HDF (Human dermal fibroblasts) • MCF-7s (Human Breast Adenocarcinoma Cells) • MA (Mouse adipose stromal cells)		Not reported	• MTT assay • LDH Assay	Not tested	• ^{1}HNMR • Size exclusion chromatography (SEC) • MALDI-TOF • Particle size via DLS • Zeta potential via DLS • Gel retardation assay • Stability testing in serum	2010	[58]
• Luciferase assay • ELISA assay for IL-12 • Cellular uptake of YOYO-1 labeled plasmid DNA was measured via BD FACScan analyzer • Antitumor activity was evaluated in the presence of glutathione inhibitor via the colorimetric MTT assay	• MCF-7 • A549 cells			MTT assay		• Size exclusion chromatography (SEC) • The critical micelle concentration (CMC) was measured via UV spectroscopy • Gel electrophoresis • Zeta potential via DLS • Particle size via DLS	2012	[59]
• EGFP expression via flow cytometry • EGFP expression was measured after using the magnetic activated cell sorting system (MACS™)	• CHO-K1 • L929					• Particle size via non-invasive back scatter technology (NIBS) • Zeta potential via laser doppler micro-electrophoresis • Hydrodynamic radius measurement via DLS • The effect of temperature and pH on the system was evaluated via turbidity measurement • Asymmetric Flow Field-Flow Fractionation (AF4) • Size Exclusion Chromatography • FT-IR spectroscopy • Vibrating sample magnetometry • TEM • Thermogravimetric Analysis • Turbidity measurements via titrator	2013	[60]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
PEG-detachable cationic polyaspartamide derivatives bearing stearoyl moieties for systemic siRNA delivery toward subcutaneous BxPC3 pancreatic tumor	• PEG bound to a cationic polyaspartamide derivative, poly(N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide) (Pasp(DET)) by disulfide linkage with a flanking **stearoyl moiety to form stearoyl PEG-SS-Pasp (DET) copolymer** • The plasmid is released in reductive condition (i.e. GSH presence) via disulfide cleavage		Pancreatic cancer treatment	Quantification of siRNA in blood circulation: 50µg siRNA/mouse Antitumor activity measurement: 100µg siRNA/mouse	siRNA
PEGylated poly (2-(dimethylamino) ethyl methacrylate)/DNA polyplex micelles decorated with phage-displayed TGN peptide for brain-targeted gene delivery	• TGN bound to the surface of PEGylated poly(2- (dimethylamino) ethyl methacrylate) PEG-PDMAEMA through maleimide by covalent bond to form TGN-PEGPDMAEMA. • TGN ligand consist of 12 amino acid • TGN enhance the blood brain barrier (BBB) targeting • PEG-PDMAEMA bound to DND to form polyplexes	TGN-PEG-PDMAEMA PEGylated poly(2-(dimethylamino) ethyl methacrylate	Brain targeting	<u>EGFP expression:</u> 3µg pDNA per well <u>*In-vivo* luciferase assay:</u> 50 mg DNA/ mouse	pDNA
PEGylated poly(amine-co-ester) micelles as biodegradable non-viral gene vectors with enhanced stability, reduced toxicity and higher *in-vivo* transfection efficacy	Nanosized micelles based on cationic, amphiphilic PEG-PPMS (poly(ω-pentadecalactone-co-N-methyldiethyleneamine-co-sebacate) block copolymers with higher transfection efficiency		Anticancer therapy	Not reported	
Photodegradable neutral–cationic brush block copolymers for non-viral gene delivery	Brush block copolymer possesses cationic poly (N,N-dimethylaminoethyl methacrylate) PDMAEMA for pDNA compaction, poly(glycidyl methacrylate) (PGMA) as the backbone, and hydrophilic poly (ethylene oxide) (PEO) for improved polyplex-forming, stability, and effectively complexing pDNA into nanoparticles and releasing pDNA under UV irradiation	Poly(ethylene oxide)-b- poly(glycidyl methacrylate)-g- poly(N,N-dimethylamino-ethyl methacrylate) **PEO-b-P(GMA-g-PDMAEMA**	Not reported	200ng DNA/well	
PLK1shRNA and doxorubicin co-loaded thermosensitive PLGA-PEG-PLGA hydrogels for osteosarcoma treatment	• Biodegradable and thermosensitive PLGA-PEG-PLGA hydrogels is used for sustained co-delivery of PLK1shRNA/polylysine-modified polyethylenimine (PEI-Lys) complexes and doxorubicin (DOX) • PLK1shRNA/PEI-Lys and DOX induced the synergistic apoptosis of osteosarcoma tumors	PLGA-PEG-PLGA hydrogels	Osteosarcoma treatment	Not reported	PLK1shRNA and doxorubicin

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• The intracellular uptake of Cy5-labeled siRNA complexes was measured via flow cytometer • Vascular endothelial growth factor (VEGF) endogenous gene silencing was quantified by real-time PCR assay (gene silencing) • Cy5-labeled siRNA complexes were quantified in blood circulation via intra-vital real-time confocal microscope • Antitumor activity was measured by determine the volumes of xenograft tumors	BxPC3 Human primary pancreatic adenocarcinoma				7 week female BALB/c nude mice	• Zeta potential via DLS • Particle size via DLS • Gel electrophoresis • Disulfide bond cleavage determination was evaluated via zeta potential measurement in reducing condition	2012	[61]
• Intracellular distribution of YOYO-1-labeled TGN-PEG-PDMAEMA/DNA polyplexes via confocal microscopy • In-vitro EGFP expression via fluorescent microscope • In-vitro EGFP expression was confirmed with luciferase assay • Brain targeting of TGN-PEG-PDMAEMA/DNA labeled by EMA was evaluated via Maestro in vivo imaging system (CRI, MA) in injected mice • Distribution of TGN-PEG-PDMAEMA/pEGF polyplexes in the brain was assessed via fluorescence microscope in injected mice • *In-vivo* luciferase assay was quantified via a BCA protein assay	BCEC		80-90%		Male BALB/c mice Nude mice	• Gel permeation chromatography (GPC) • ^{1}H NMR • Particle size via DLS • Zeta potential via DLS • Agarose gel electrophoresis	2013	[62]
Luciferase activity was visualized by luminometer and quantified via BCA protein assay In-vivo luciferase assay. The stability of micelle in physiological condition was evaluated by in-vitro release study of DNA from the PEGPPMS micelles	239T cells hela cells		60–70%		Nude mice	• Gel red exclusion assay • Particle size via DLS • Zeta potential via DLS • TEM • The stability of DNA/PEG-PPMS micelles was evaluated by measurement of particle size in serum	2014	[63]
• Luciferase transfection via BCA protein assay kit and microplate reader with and without UV radiation • GFP assay with and without UV radiation	HepG2 cells		Not reported		Not tested	• ^{1}H NMR • Gel retardation assay was measured before and after UV radiation. • Particle size • TEM • Zeta potential • Gel electrophoresis analysis was assessed before and after UV radiation	2014	[64]
• Luciferase assay • Cell cycle analysis via FACS • Antitumor activity was measured by determine the volumes of tumors • The apoptosis of osteosarcoma cells in vitro was determined via flow cytometry • Apoptosis genes including; Bcl-2, BAX, caspase-3, caspase-9 and the housekeeping gene b-actin expressions were evaluated via quantitative real-time PCR tests • The mechanism of PLK1 on cell cycle regulation was assessed by quantitative real-time PCR of cyclin dependent proteins *in-vitro* or *ex-vivo* tumor masses • The organ damage and the tumor pathology analysis were assessed via histological analysis of hematoxylin/eosin stained tissues. • TUNEL assay	• Saos-2 • (Sarcoma osteogenic cell line) • MG-63 (Human osteosarcoma cell line)				5-week-old male BALB/C nu/nu nude mice	• ^{1}H NMR • Gel permeation chromatography • The sol-gel phase transition behaviors investigation • *In-vivo* and *in-vitro* gel degradation • Biocompatibility of hydrogel was evaluated via examination of inflammatory response in rats. • Histology and Tunel assay	2014	[65]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Poly(b-amino ester) as a carrier for si/shRNA delivery in lung cancer cells	Biodegradable poly (beta amino ester) **(PAE)** carrier consists of low molecular weight PEI and PEG.		Lung cancer treatment		siRNA Akt1 shRNA
Poly(galactaramidoamine) is an efficient cationic polymeric non-viral vector with low cytotoxicity for transfecting human embryonic kidney (HEK293) and murine macrophage (RAW264.7) cells	• The carrier system is composed of Poly(galactaramidoamine) (PGAA) cationic co-polymer (dimethyl-meso-galactarate and penta ethylenehexamine) • PGAA electrostatically bound to plasmid DNA to form nano sized particles	Poly(galactaramidoamine) (PGAA) copolymer	Not reported	1 µg of pDNA/well	pDNA
Poly-L-Arginine grafted silica mesoporous nanoparticles for enhanced cellular uptake and their application in DNA delivery and controlled drug release	Well defined poly-L-arginine grafted silica mesoporous nanoparticles (MSN-PLArg) with high grafting density (MSNs) is a pH sensitive vehicle for delivering doxorubicin to cancerous cells	poly-L-arginine grafted mesoporous nanoparticles (MSN)	Anticancer therapy	0.5 µg of pDNA	
Polymeric delivery of therapeutic RAE-1 plasmid to the pancreatic islets for the prevention of type 1 diabetes	• Bioreducible polymeric carrier consists of cationic polymer poly (cystaminebisacrylamide–diamino hexane) (p(CBA-DAH)) and PEG. • P(CBA-DAH) targets the over expressed EphA2 and EphA4 in the pancreatic islets, while PEG improves the stability of the system in the serum. • Eph-PEG-p(CBA-DAH) complex contains plasmid coding for soluble RAE-1γ.	p(CBA-DAH)	Treatment of type 1 diabetes	40µg of DNA per mouse	
Primary cardiomyocyte targeted bioreducible polymer for efficient gene delivery to the myocardium	• Disulfide-linked bioreducible polymer consists of primary cardio-myocyte (PCM) specific peptide and **poly(CBA-DAH)** polymer ((*N, N′*-Cystaminebisacrylamide (CBA), (1, 6-diaminohexane (DAH)). • PCM-modified bioreducible poly(CBA-DAH) inhibits Fas siRNA gene expression which leads to inhibit cardio-myocyte apoptosis	PCM-poly (CBA-DAH) (PCM-PCD)	Treatment of heart diseases	Luciferase assay: 0.5µg/well pDNA Cellular uptake: 1.0µg pDNA	• pDNA • siRNA
Reducible poly (2-dimethylaminoethyl methacrylate): Synthesis, cytotoxicity, and gene delivery activity	• The carrier system is composed of oligomer, which consists of reducible poly (2-dimethylaminoethyl methacrylate) (DMAEMA) for efficient gene delivery • DMAEMA was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization	rPDMAEMA	Not reported		pDNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• EGFP silencing expression via FACS and confocal microscopy • Intracellular tracking of rhodamine-labeled Akt1 shRNA via confocal microscopy • Semi-quantitative RT-PCR • Apoptosis and necrosis of Annexin V-FITC and PI stained A549 cells was measured via FACS • Cell proliferation assay at different time intervals of PEI25KeshAkt treatment • Tumor growth reduction due to Akt1 protein knock-down was assessed via soft agar assay • Cell migration and invasion assay • *In-vivo* aerosol delivery of PAEe-Akt1 shRNA complexes was determined via western blot analysis • Invasion and migration of Akt knocked-down A549 cells was assessed via transwell cell culture inserts assay and confirmed via western blot assay	Human lung adenocarcinoma cell line (A549)			• MTS assay • Trypan blue exclusion assays	BALB/c mice	• ^{1}H NMR • Gel permeation chromatography with multi-angle laser scattering (GPC-MALS) • Agarose gel electrophoresis • Energy-filtering transmission electron microscopy (EFTEM)	2008	[66]
Luciferase assay was visualized by luminometer and quantified via BCA protein assay	• Human embryonic kidney cells • (HEK293) • Murine macrophage (RAW264.7) cell			MTS assay	Not tested	Size measurements via DLS	2012	[67]
• The cellular uptake of MSN-PLArg was assessed via • epifluorescence microscopy • Transfection efficiency of mCherry-Plasmid using MSN-p (LArg) was determined and quantified via counting transfected cells versus total number of cells per field via microscopic images and FACS	• HeLa cells • A549 cells			MTT assay		• FT-IR • ^{13}C CP-MAS NMR • Agarose gel electrophoresis • Zeta potential via DLS • Particle size via DLS • Transmission Electrum Microscopy (TEM) • Scanning Electrum Microscopy (SEM) • Size-exclusion chromatography (SEC) • Thermogravimetric Analysis (TGA) • Nitrogen adsorption and desorption studies	2013	[68]
• Luciferase assay was visualized by luminometer and quantified via luciferase assay kit • The efficacy of system to reduce diabetes incidence was evaluated by measuring different parameters in mice such as body weight, survival rate and blood glucose levels • The insulitis level was measured by computer morphometry of hematoxylin-eosin stained pancreatic cell and insulitis grading system • Immunohistochemistry was performed to examine the rate of infiltration	Not tested			Not tested	Female 6-week-old non-obese diabetic mouse modle (NOD)	• Gel retardation assay	2012	[69]
• Luciferase assay was visualized by luminometer and quantified via luciferase assay kit • Fas gene silencing expression via flow cytometry • Quantitative real-time PCR • The apoptotic cells were measured via flow cytometry using PI staining • The cellular uptake of YOYO-1 labeled plasmid DNA was examined via a BD FACScan analyzer	• NIH 3T3 • H9C2 cells (Rat cardiac myoblast)	Yes		MTT assay	Not tested	• Size exclusion chromatography (SEC) • ^{1}H NMR • UV/Vis spectrophotometry • Particle size via DLS • Zeta-potential via DLS	2010	[70]
Luciferase assay	• Mouse melanoma B16F10 cell line • a panel of human pancreatic cancer cell lines (Panc-1, AsPC-1, COLO-357, BxPC-3, MiaPaCa, and Panc-28)			MTS assay		• Size exclusion chromatography (SEC) • ^{1}H NMR • Refractive index increments of the polymers via Optilab differential refractometer and used in SEC analysis. • The static light scattering (SLS) measurements of polyplexes • Hydrodynamic diameters via DLS • Zeta potential via DLS	2007	[71]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Reversibly shielded DNA polyplexes based on bioreducible PDMAE-MA-SS-PEG-SS-PDMAE-MA triblock copolymers mediate markedly enhanced nonviral gene transfection	• The carrier system is composed of triblock copolymer that can reach peri-nuclear region • The triblock copolymer consists of two PDMAEMA and PEG linked by disulfide bond reversibly shielded with DNA in polyplex	PDMAEMA-SS-PEG-SS-PDMAEMA	Not reported	Luciferase assay: 1µg of plasmid DNA per well Cellular uptake: 1µg of Cy-5 labeled plasmid DNA per well	pCMV-Luc pDNA
Self-assembled carboxymethyl poly (L-histidine) coated poly (b-amino ester)/DNA complexes for gene transfection	• The carrier system is composed of CM-PLH/PbAE/DNA complex. • The CM-PLH/PbAE/DNA complex consists of pH-sensitive carboxymethyl poly (L-histidine) (CM-PLH) and poly (b-amino ester) (PbAE) • Plasmid is electrostatically adsorbed to coatings of CM-PLH/PbAE/DNA complex	Poly beta amino esters (CM-PLH)		EGFP expression: 0.8 mg DNA Luciferase assay: 50 mg DNA per mouse	pDNA
siRNA delivery from an injectable scaffold for wound therapy	• Smart polymer nanoparticle (SPN) consists of pH-responsive 2-dimethylaminoethyl methacrylate (DMAEMA) and 2-propyl acrylic acid (PAA) diblock copolymer • PH-responsive, siRNA-loaded nanoparticles into a biodegradable polyurethane (PUR) scaffold		Wound therapy	Not reported	siRNA
Smart multilayered assembly for biocompatible siRNA delivery featuring dissolvable silica, endosome-disrupting polycation, and detachable PEG	• A **smart multilayered assembly SMA** was designed to feature a siRNA-loaded core, a transiently core-stabilizing silica interlayer, an endosome-disrupting polycation interlayer PAsp-(DET), and a biocompatible poly reduction responsive detachable PEG shell • SMA was synthesized via a layer-by-layer assembly technology		Not reported	1.25 mg siRNA/kg mouse	
Systemic siRNA delivery to a spontaneous pancreatic tumor model in transgenic mice by PEGylated calcium phosphate hybrid micelles	Nano carrier consists of PEG-CCP (poly (ethylene glycol)-block-charge-conversional polymer) with calcium phosphate (CaP) hybrid micelles		Pancreatic tumors	20 µg siRNA/ injection	
Triolein-based polycation lipid nanocarrier for efficient gene delivery: characteristics and mechanism	• Polycation lipid nanocarrier (PLN) consists of dioleoyl phosphatidylethanolamine (DOPE), cetylated PEI and triolein • Triolein enhanced formation of the H_{II} phase that leads to enhance the gene transfection efficiency		Not reported	1µg/well pDNA	pDNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• Luciferase assay was visualized by luminometer and quantified via luciferase assay kit • Cellular uptake of Cy5-labeled DNA via CLSM. • GFP expression assay	COS-7		70%	CCK assay		• Zeta potential via DLS • Particle size via DLS • Agarose gel electrophoresis • Gel permeation chromatography (GPC) • Colloidal stability and reduction-triggered deshielding and unpacking of DNA polyplexes were assessed via DLS and agarose gel retardation assays in the presence of 150mMNaCl and/or 10 mM DTT over the time	2012	[72]
• EGFP expression fluorescent microscope was quantified via flow cytometry • The percentage of cellular uptake of YOYO-1 positive cells and fluorescence intensity was quantified via flow cytometry • Intracellular distribution of YOYO-1-DNA in B16-F10 cells was assessed via confocal microscopy • Luciferase expression level via BCA Protein Assay Kit.	• HEK293 cells • B16-F10 cells (Musmusculus skin melanoma)		70-80%	MTT assay	5-week old Balb/c mice	• ^{1}H NMR • TEM • Agarose gel retardation electrophoresis assay was performed with and without the presence of serum or heparin • Particle Size via DLS • Zeta potential via DLS • Erythrocyte agglutination was observed by microscopy	2011	[73]
• Qualitative analysis of FAM-labeled siRNA via flow cytometry • Gene silencing was measured via real-time reverse transcriptase–polymerase assay • Wound healing was evaluated via scanning electron microscope imaging	human cervical carcinoma cells	No	Not reported	Not tested	mice	TEM pH-dependent activity Red blood cell hemolysis assay	2013	[74]
• Luciferase assay via a luminescence microplate reader • Cellular uptake of Cy3-siRNA PICs was measured via flow cytometric analysis • Intracellular distribution of Cy3-siRNA was assessed via CLSM • The intracellular distribution of Cy3-siRNA was quantified by calculating the co-localization ratio of Cy3- siRNA pixels with LysoTracker green pixels. • *In-vitro* and *In-vivo* VEGF gene silencing activity was measured via RT-PCR	HuH7-Luc			• Cell counting Kit-8 assay • Hematological toxicity assay was assessed by measuring different parameters such as the level of ALT, AST, RBC, WBC and hemoglobin	Balb/c nude mice (male, 6-weeks-old	• Transmission Electron Microscopy (TEM) • Confocal microscopy (CLSM) • Zeta potential via DLS • Particle size via DLS • Size exclusion chromatography (SEC) • ^{1}H NMR • Agarose gel electrophoresis	2012	[75]
• *In-vitro* and *in-vivo* luciferase gene silencing assay • Bio-distribution and tumor accumulation of Alexa647-siRNA was evaluated by fluorescent imaging using *in-vivo* imaging system (IVIS) • Histological examination of tumor was performed via staining with Hoechst and observation of cellular nuclei by a confocal laser scanning microscope (CLSM) • *In-vivo* bioluminescence reduction of siScr-loaded hybrid micelles in transgenic mice was quantified after luciferin injection	HeLa-Luc (Human cervical cancer cell line)			• Water soluble tetrazolium salt (WST-8) • Inflammatory cytokine levels and hematological parameters, the levels of AST, ALT, and ALP	Transgenic mice	• Transmission Electron Microscopy (TEM) • Size particles via DLS • Zeta potential via DLS	2014	[76]
• Qualitative and quantitative EGFP expression via flow cytometry • Luciferase assay • The localization of the fluorescently labeled PDC was evaluated via CLSM • Endocytosis of PDC via the clathrin-mediated pathway was confirmed by transfection efficiency in the presence of specific inhibitors such as chlorpromazine (Chlor) and filipin III	SPC-A1 (Human Lung adenocarcinoma)	Yes		MTT assay	Not tested	• Particle size distribution and Zeta potential by DLS • Morphology of particles via atomic force microscopy (AFM) • Agarose gel electrophoresis	2011	[77]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Tumor targeting RGD conjugated bio-reducible polymer for VEGF siRNA expressing plasmid delivery	• The gene carrier is tumor targeting bio-reducible polymer, **PA-PEG1k-RGD** • PA-PEG1k-RGD consists of cyclic RGDfC peptides which are conjugated with PAM-ABP via PEG • Arginine-grafted bio-reducible poly (cystaminebisacrylamide-diaminohexane, CBA-DAH) "ABP" was conjugated with dendrimer type poly (amido amine) (PAMAM) to form PAM-ABP (PA)		Anticancer therapy	Luciferase assay: 1µg/well pDNA GFP assay: 2 mg/well pDNA	
Tumor-targeted redox-responsive nonviral gene delivery nanocarriers based on neutral-cationic brush block copolymers	• The carrier system is a neutral-cationic brush block copolymer, which condenses pDNA into nanoparticles via disulfide linkage to form polyplex micelles • The novel neutral-cationic brush block copolymer is poly[oligo(ethylene glycol) monomethyl ether methacrylate-co-folic acid methacrylate]-b-poly[2-(2-(2-bromo-2-methylpropanoyloxy)-ethyl) disulfanyl) ethyl methacrylate-g-2-dimethylamino-ethyl methacrylate], P(OEGMA-co-FAMA)-b-P(BSSMA-g-PDMAEMA) • The gene delivery system targets folic acid and release plasmid by the degradation of disulfide bond (bioreducible) under reductive conditions			200 ng/well pDNA	
Ultrasound-assisted siRNA delivery via arginine-grafted bioreducible polymer and microbubbles targeting VEGF for ovarian cancer treatment	• Polymeric siRNA carrier system consists of arginine-grafted bioreducible polymer (ABP), microbubbles (MBs), and ultrasound technology (US) to form (SAM) complexes • SAM has high siRNA uptake and VEGF protein knockdown in vitro and *in-vivo* by ultrasound activation		Ovarian cancer therapy	3µg VEGF or luciferase siRNA in 50µl injection volume	siRNA
Tumoral gene silencing by receptor-targeted combinatorial siRNA polyplexes	• Polymeric siRNA carrier system consists of PEGylated folate-conjugated oligomers (Polycationic oligomers) that target folate receptor (FR)-overexpressing tumors • Oligomer is an oligoaminoamide-based sequence defined polycationic oligomer that was synthesized via solid-phase assisted synthesis • Oligomer can form polyplexes with anionic siRNA by disulfide linkage		Anticancer therapy	Cellular uptake: 2.5µg of siRNA GFP assay: 500ng of siRNA Biodistribu-tion study: 50µg of Cy7-labeled siRNA	
Main-chain degradable single-chain cyclized polymers as gene delivery vectors	• Single-chain cyclized polymeric nanoparticles with gene transfection capabilities • Three different single-chain cyclized polymers were synthesized via reversible addition-fragmentation chain transfer polymerization (RAFT) including: non-degradable poly(DMAEMA-co-TEGDA) (P1), crosslinker-degradable poly(DMAEMA-co-DSDA) (P2) and backbone-degradable poly (DMAEMA-co-MDTD-co-TEGDA) (P3)		Not reported	0.25µg/ well pDNA	plasmid DNA
An albumin-mediated cholesterol design-based strategy for tuning siRNA pharmacokinetics and gene silencing	• The gene delivery system consists of recombinant human serum albumin (rHSA) and cholesteryl-modified siRNA to form **rHSA/siRNA complex**. • rHSA/siRNA exhibited reduced nuclease degradation and enhanced hepatic accumulation and gene silencing.			EGFP: 2.5 µL siRNA Bio-distribution of Cy7-labeled siRNA 1.5 mg/kg siRNA	siRNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• Luciferase assay was visualized by luminometer and quantified via BCA protein assay • GFP via fluorescence microscopy and a multimode microplate reader • Competition studies were done with free RGD peptides • Cellular uptake of PA-PEG$_{1k}$-RGD was evaluated by GFP expression and BCA protein assay in the presence of a 10-fold excess of free RGDfC peptides • Cellular uptake of YOYO-1labeled pshVEGF was measured via BD FACScan II • Gene silencing efficiency was evaluated via determination of VEGF level via human VEGF ELISA kit	• PANC-1 (Pancreatic Cancer Cells) • HUVEC (Human Umbilical Vein Endothelial Cells) • HEK-293					• Gel retardation assay in the presence of reductive condition (DTT) • Particle size by DLS • Zeta-potential by DLS • ^{1}H NMR	2014	[78]
• Luciferase assay was visualized by luminometer and quantified via BCA protein assay kit • GFP expression via flow cytometry	• HeLa cells • HepG2 cells					• ^{1}H NMR • Gas permeation chromatography (GPC) • UV-vis spectroscopy • Gel retardation assay • Particle size distribution via DLS • Zeta potential via DLS	2014	[79]
• Cellular uptake of siRNA was measured either in serum free and serum containing media or different MB: cell ratios via FACS analysis • Gene silencing efficiency was evaluated via determination of VEGF level via human VEGF ELISA kit • The efficacy was evaluated *in-vivo* by measuring different parameters such relative Body weight and tumor size of mice	A2780 cells		40%		Female tumor bearing nude mice (nu/nu)	• Stability of SAM in serum was evaluated by confocal microscopy	2014	[80]
• Cellular uptake of Cy5-labeled siRNA was measured via flow cytometry • Immuno-TEM of FR-mediated endocytic pathway of TCP1 in FR-overexpressing KB cells • Luciferase assay • Mitotic aster formation was evaluated via a axiovert 200 fluorescence microscope • The level of mRNA of EG5 gene was measured via quantitative real-time polymerase chain reaction (qRT-PCR) • Bio-distribution of Cy7-labeled siRNA was evaluated via *in-vivo* imaging system (IVIS) • Gene silencing mediated by EG5-siRNA was measured via qRT-PCR *in-vivo* • Blood biochemistry was examined by measuring different parameters such as; alanine aminotransferase (ALT), asparate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine	• M109 cells • MCF-7 cells	No	80–90%		L1210 tumor-bearing mice	• Zeta potential via DLS • Particle sizes via DLS • Transmission Electron Microscopy (TEM) • Gel retardation assay	2016	[81]
• luciferase assay • GFP expression	HeLa cells	Yes	10,000 cells per well seeded in 96 well plates	Alamar Blue® reduction assay	Not tested	• Gel permeation chromatography (GPC) • ^{1}H-NMR spectroscopy • Degradation experiment was performed by GPC and ^{1}H NMR in the presence of glutathione and hydrazine • UV spectroscopy	2016	[82]
• EGFP-silencing via flow cytometry • Tumor necrosis factor alpha response of rHSA/siRNA and siRNA was determined via ELISA • Bio-distribution of Cy7-labeled siRNA was evaluated via in-vivo imaging system (IVIS) and data reported as mean fluorescence intensity (MFI) in mice • The serum level of Factor VII was measured via colorimetric Biophen VII assay kit	H1299 cells	Yes (2%FBS)	Not reported	Not tested	NMRI mice	• Gel retardation assay • Serum stability assay was detected via 8% polyacrylamide gel electrophoresis	2016	[83]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Gene delivery and immunomodulatory effects of plasmid DNA associated with branched amphiphilic peptide capsules	• The gene delivery system consists of **Branched Amphiphilic Peptide Capsules (BAPCs)** • Pre-formed water filled "conformationally constrained" BAPCs were nucleation cores for the DNA that coat the surface of the peptide capsules to form BAPCs-DNA nanoparticles		BAPCs-DNA has immunomodulatory properties for DNA vaccines	*GFP:* 2.5µg of pDNA *In-vivo:* 40µg pDNA per mice	pDNA
Polymeric nanoparticles for non-viral gene therapy extend brain tumor survival in-vivo	• Gene delivery system consists of a poly(β-amino ester) **(poly(1,4-butanediol diacrylate-co-4-amino-1-butanol) end-modified with 1-(3-aminopropyl)-4-methylpiperazine, PBAE)** that formed nanoparticles with herpes simplex virus type I thymidine kinase (HSVtk) DNA • Combining the administration of ganciclovir and PBAE/HSV-tk nanoparticles leads to significant potential to treat malignant gliomas		Gene therapy for extended brain tumor	*In-vitro:* 600ng/well pDNA *In-vivo:* 26 µg DNA with 780 µg PBAE	
Novel PEI/poly-γ-glutamic acid nanoparticles for high efficient siRNA and plasmid DNA co-delivery	Dual nucleic acid co-delivery system (DDNPs or γ-DDNPs) consists of PEI and polyanionic γ-PGA (polyglycolicacid) that complex with DNA/siRNA to form spherical nanoparticles with about 200 nm diameter	PEI/ γ-PGA	Not reported	2 µg DNA and 0.5 µg siRNA/well	siRNA and pDNA
A biodegradable polyethylenimine-based vector modified by trifunctional peptide R18 for enhancing gene transfection efficiency in vivo	Gene delivery system was synthesized by crosslinking low-molecular-weight (LMW) PEI with N-octyl-N-quaternary chitosan (OTMCS) and trifunctional peptide (RGDC-TAT-NLS) R18 for specific tumor targeting to form OTMCS-PEI-R18 system	**(LMW) PEI with OTMCS**		2.5µg/ well pDNA	pDNA
Bioreducible PEI-functionalized glycol chitosan: a novel gene vector with reduced cytotoxicity and improved transfection efficiency	A new gene vector is synthesized by grafting glycol chitosan (GCS) into branched low molecular weight polyethyleneimine (LMW PEI) via disulfide linkage to form (GCS-ss-PEI) small nanoparticles	**GCS-ss-PEI**		1µg of GFP DNA per well	
A novel tyrosine-modified low molecular weight polyethylenimine (P10Y) for efficient siRNA delivery in vitro and in vivo	Gene delivery system is a novel tyrosine-modified low-molecular weight polyethylenimine (P10Y)		Anticancer therapy	0.4µg siRNA per well	siRNA

Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• GFP assay was monitored by confocal microscopy and quantified via fluorescence activated cell sorting (FACS) • *In-vivo* evaluation was performed by measuring tumor size progression and survival rate • The level of intracellular IFN-γ was measured by flow cytometry and expressed as percentage of CD8+IFN-γ+ cells of total CD8+ T cell	HeLa cells	Yes	60%	• Cell death exclusion propidium iodide (PI) • *In-vivo* toxicity was evaluated by measuring: aspartate (AST) and alanine (ALT) transaminases, and lactate dehydrogenase (LDH)	C57BL/6 mice	• Reversed phase HPLC • Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) • Atomic force microscopy (AFM) • Zeta potential via DLS • Transmission Electron Microscopy (TEM)	2016	[84]
• GFP expression before and after lyophilization • The rats were examined daily for neurological impairment followed by histological analysis of their brains • The percentage of survival of 9l gliosarcoma-bearing rats • The intra-tumoral distribution and transfection ofCy5-labeled GFP DNA in brain was evaluated via fluorescence microscopy • Immunohistochemistry of the brain was examined via confocal microscopy (CLSM) and captured with Axiovision software	F98 cells		Not reported	MTS assay Rats were examined for signs of pain and distress, including ruffled fur, dehydration, hunched position, weakness, lethargy, immobility, lack of coordination, labored respiration, or cyanosis daily	Not tested	• Gel permeation chromatography (GPC) • ^{1}H NMR • Transmission Electron Microscopy (TEM) • Hydrodynamic diameter before and after lyophilization via DLS • Zeta potential before and after lyophilization via DLS	2015	[85]
• The cellular uptake of FITC-labeled (PEI) and Alexa Fluor-647 labeled (siRNA) nanoparticles was visualized via CLSM and quantified via flow cytometry • GFP silencing was quantified via flow cytometry	HeLa cells		60%–80%	MTT assay		• Agarose gel electrophoresis • UV-spectroscopy • Particle size distribution via DLS • Zeta potential via DLS • The morphology and the size were examined via Transmission Electron Microscopy (TEM)	2016	[86]
• pEGFP-N2 expression • *In-vitro* and *in-vivo* luciferase assay was evaluated via a luminometer and quantified via a BCA protein assay kit • Cellular uptake of FITC-labeled OTMCS-PEI-R18 /DNA complexes was determined via confocal microscopy (CLSM)			*In-vitro* transfection 80% Cellular uptake: 60%		Athymic nude mice (males, 4-6 weeks old, 16–18 g)	• Particle size and zeta potential via DLS • Agarose gel retardation assay • MALDI-TOF mass spectroscopy • ^{1}H NMR • Degradation of OTMCS-PEI-R18 was evaluated by measurement of molecular weight via GPC • Buffering capacity measurement • Resistance of OTMCS-PEI-R18 to DNase I digestion and sodium heparin was evaluated via electrophoresis	2016	[87]
• GFP expression was evaluated via fluorescence microscope and quantified by flow cytometer • Intracellular localization of FITC-GCS-ss-PEI was evaluated via confocal microscopy (CLSM)	HEK 293T	No	70%		Not tested	• FT-IR • Buffering capacity measurement • Particle size via DLS • Zeta potential via DLS • Morphological examination of particles via Scanning Electron Microscopy (SEM) • Redox responsive properties were evaluated by measuring the amount of released DNA in the presence of GSH and heparin via gel electrophoresis	2016	[88]
• Luciferase assay via luminometer • EGFP gene expression was quantified via FACS analysis. • Cellular uptake of fluorescently labeled siRNA was visualized via flow cytometry • The level of mRNA after knockdown was measured via RT-qPCR • Western blotting was performed to determine the level of protein of pooled lysates • *In-vivo* efficacy was evaluated via measuring all three dimensions of tumor size, the level of siRNA and target gene knockdown • TNF-α and INF-γ serum levels were determined via ELISA kits • Blood serum markers were measured for biocompatibility studies • *Ex-vivo* erythrocyte aggregation assay • The biodistribution of in-vivo P10Y/siRNA complex was evaluated via autoradiography and quantified by Image J software (intraperitoneal and intravenous injection)	• MV3 cells • PC-3 cells	Yes	Not reported	• Lactate de-hydrogenase (LDH) assay • Colorimetric WST-1 assay	Athymic nude mice	• ^{1}H NMR • Agarose gel electrophoresis • Zeta potential via phase analysis light scattering (PALS) • Particle size via PALS • *In-situ* atomic force microscopy (AFM)	2016	[89]

Paper title	System description	Carrier/Polymer	Therapeutic purpose	Amount of genetic material	Plasmid/Drug
Cationic lipid-nanoceria hybrids, a novel nonviral vector-mediated gene delivery into mammalian cells: investigation of the cellular uptake mechanism	Non-viral gene delivery vector consisting of dimethyldioctadecyl ammonium bromide (DODAB) and nanoceria (CeO_2) hybrids. DODAB-modified CeO_2 Nanoparticles (CeO2/DODAB) compact the pDNA for efficient gene transfection into the selected cell lines	**CeO2/DODAB nanovectors**	Not reported	EGFP: 1 µg pEGFP-N1 for 6 hrs Cellular uptake: 1 µg/well Cy3-labeled DNA	pDNA

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Expression readout	Cell line(s)	Serum during transfection	Cell confluency	Cytotoxicity readout	*In-vivo* testing	Physicochemical characterization	Year	Ref
• Luciferase assay • *In-vitro* and *in-vivo* EGFP expression was visualized via inverted fluorescence microscope and quantified via flow cytometry • Red fluorescent protein (RFP) gene expression was visualized via inverted fluorescence microscope and quantified via flow cytometry • Cellular distribution of Cy3-labeled DNA was evaluated via confocal laser scanning microscopy • The Luciferase assay was performed in the presence of endocytosis inhibitors to determine cellular uptake pathways of the vector • Transfection Index (TI) of the individual transfection reagents was measured by calculating the product of transfection efficiency and cell viability	*HEK293 cells* *MCF-7 cells* *HepG2 cells*		50–60%	• CCK-8 assay • *In-vivo* biochemical analysis	Posterior tibialis muscles of six-week-old male ICR mice	• Energy dispersive X-ray spectroscopy • FT-IR spectroscopy • The amount of DODAB bound to the nanoceria surface was measured via elemental analysis • Transmission Electron Microscopy (TEM) • UV-visible spectroscopy • Hydrodynamic size via DLS • Zeta potential via DLS • Gel retardation assay • DNase I protection assay	2016	[90]

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Appendix 2: Patents for non-viral gene delivery 2009-2017

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
CN 103087314 A	Preparation method of biodegradable polyurethane gene transfection reagent	Tongji University	DNA	Not tested	• 293T cells • H Indian G2 cells • MC3T3 cells • SK0V3 cells • MCF-7 cells	Biocompatible and degradable polyurethane gene carrier reagent for mammalian cell lines transfection which contains reducible disulfide linkages.	Polyurethane biodegradable polymer containing disulfide linkage.
US 20110076767 A1	Magnetically-driven biodegradable gene delivery nanoparticles formulated with surface-attached polycationic complexes	The Children's Hospital of Philadelphia			• Cultured rat aortic smooth muscle (A10) • Bovine aortic endothelial cells (BAEC)	Gene delivery system consists of a matrix-forming agent which made a complex with a magnetic field-responsive polyelectrolyte-amphiphilic agent (poly (ethyleneimine) carboxylate) and other biomaterials.	Polyethylenimine (PEI) modified magnetic nanoparticles which respond to magnetic field.
US 7914714 B2	Methods and apparatus using electrostatic atomization to form liquid vesicles	The Regents of the University of Colorado			Not mentioned	• The genetic delivery system consists of liquid vesicles. • The liquid vesicles consist of three layers including; first layer the inner aqueous layer that has the encapsulated active material, second layer that is miscible with the first layer and has a lipid encapsulating agent or an organic solvent, and the third layer has a targeting or steric stabilizing agent.	
US 20090324726 A1	Non-viral gene therapy using chitosan-containing nanoparticles	Fernandes Julio C,Winnik Francoise		Adjuvant-induced Arthritis (AIA) rat model	Not mentioned	Nanoparticle system consists of chitosan attached with biodegradable PEG for autoimmune disease treatment.	Folate-PEG-chitosan
US 20070036867 A1	Controlled and sustained gene transfer mediated by thiol-modified polymers	University of South Florida		Mice	• HEK 293 cells • MDCK cells	Sustained released cross-linked thiolated chitosan with plasmid DNA.	Thiolated chitosan
EP 2396365 A2	Reducible polymers for non-viral gene delivery	University of Houston		Not mentioned	• Human dermal fibroblasts (HDFs), • human breast adenocarcinoma cells • (MCF-7s) metastatic mouse breast cancer cells (4TIs)	• Biodegradable linear copolymers contain reducible linkage along its backbone bound to biomolecule to form nanoplex. • Biomolecules release from nanoplex by reduction of polymer.	Synthesized linear lysine-modified copolymer
US 7635734B2	Photochemical activation of surfaces for attaching biomaterial	The Children's Hospital of Philadelphia	• DNA • RNA	Male Sprague-Dawley rats (450-500 g)	Rat aortic smooth muscle cells (A10 cells)	Magnetically-responsive polymer consists of ultra-small iron oxide nanocrystals which were dispersed in chloroform.	poly(allylamine)-benzophenone-pyridyldithio-carboxylate (PBPC)
US 20130065942 A1	Nucleic acid-lipopolymer compositions	Egen, Inc.	DNA	• Mouse glioma model • Women with recurrent ovarian cancer	Brain parenchyma of mice	Lipopolymer includes a cationic polymer backbone (polyethyleneimine) covalently bound to cholesterol (cholesteryl chloroformate) and polyethylene glycol (methoxypolyethyleneglycol-propionic acid N-hydroxysuccinimidyl ester) in the form of nanoparticle. **(PEG-PEI-Cholesterol (PPC)**	
EP 2496268 A1	Nanoparticle-based gene delivery systems	Chung-Ang University & Industry Academy Cooperation	• single-stranded DNA • Antisense DNA • ShRNA	Mouse model	• HEK 293T, human embryonic kidney cells • Hela cells	The gene delivery system consists of a nanomaterial, an oligonucleotide covalently linked to the surface of the nanomaterial as a universal binding partner.	Gold nanoparticles (AuNP)
US 8097277 B2	Charge-dynamic polymers and delivery of anionic compounds	Wisconsin Alumni Research Foundation	DNA	Not tested		• Dynamic charge state cationic polymers bound to removable functional group. • Cationic polymer bound to anionic molecule (DNA) in the form of micro- or nanoparticles. • Positive charge of cationic polymers decreased by removing one or more removable functional group(s) in the target cell.	PEI

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Gene therapy of genetic diseases	• Green Fluorescent Protein (GFP) • Luciferase assay	Alamar Blue assay	Yes		• ^{1}H NMR • Gel permeation chromatography (GPC) • FT-IR	2013	[1]
• Anticancer • Diagnosis	GFP				• Transmission electron micrograph • Particle size • Gel electrophoresis	2011	[2]
Not mentioned	Not tested				• Conductivity • Droplet diameter measurement	2011	[3]
Autoimmune diseases, characterized by over-expression of folic acid receptors on a cell surface.	• In-vivo β-galactosidase expression (by X-gal) • Measurement of rat's ankle size change • EGFP • Human IL-1Ra ELISA detection • Measurement of PGE_2 level in the sera of rats.	Not tested		Yes	• ^{1}H NMR • UV spectroscopy • Gel permeation chromatography (GPC)	2009	[4]
• Gene therapy • Tissue engineering	• *In-vivo* an *In-vitro* EGFP expression by flow cytometry • Immunoblotting assay	MTT assay	No		• Particles size via DLS • Zeta potential via DLS • Agarose gel electrophoresis • Transmission electron microscopy (TEM) • Electrophoretic light scattering	2007	[5]
Not mentioned	• Luciferase assay • EGF fluorescence detection • Cellular uptake of EMA-labeled confocal microscopy • DNase protection assay		Yes		• Particle size via DLS • Zeta potential via DLS • Gel retardation assay • ^{1}H NMR • Size exclusion chromatography (SEC) • Gel retardation assay • MALDI-TOF	2011	[6]
Cardiovascular disease treatment	• GFP transgene expression • Luciferase gene expression • Cellular uptake of labeled PAA-BzPh was evaluated via fluorescence microscopy	Alamar Blue assay			• Particle size via photon correlation spectroscopy • HPLC • Size exclusion chromatography • Gel electrophoresis	2009	[7]
Anticancer therapy	• Luciferase expression • In-vivo and in-vitro IL-12 ELISA assay	Not tested		Yes	• ^{1}H NMR • MALDI-TOF mass spectrometric analysis • DLS • Stability tests such as DNA concentration, particle size, osmolality and gene expression • Electrophoretic experiment	2013	[8]
• Anticancer therapy • Anti-apoptosis	• Luciferase Assay • Knockdown of Mcl-IL expression was assessed via EGFP • B-catenin protein expression analysis with Western blot assay. • Western blot assay was done for Anti-MCL-IL and anti-p53 monoclonal antibodies	Trypan blue assay	No		• Agarose gel electrophoresis • The extent of DNAzyme annealing to the gold nanoparticles was assessed via UVtransilluminator	2012	[9]
Therapeutic, diagnostic, and prophylactic agent(s)	Luciferase expression	MTT assay	Yes		• Hydrodynamic diameter via DLS • Zeta potential via DLS • Gel retardation assay • NMR • Gel Permeation Chromatography (GPC). • Elemental analysis • Agarose gel electrophoresis	2009	[10]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
US8829109 B2	Cleavable modifications to reducible poly(amido ethylenimine)s to enhance nucleotide delivery	University of Utah Research Foundation	• DNA • siRNA	Not tested	H9C2 cells	• The delivery system consists of modified biodegradable poly(amido ethylineimine) copolymers • Polyethylene glycol (PEG) covalently bonded to a branched poly (triethyenetetramine/ cystamine bisacrylamide) copolymer (poly (TETA/ CBA)) • The nucleic acid is released due to changing in redox potential between the extracellular and the intracellular environment	Poly (TETA/CBA) -PEG_{3400}-RGD RGD (hydrophilic PEG spacer)
US20120035320 A1	Polyacridine nucleic acid delivery peptide complexes	University of Iowa Research Foundation	DNA	ICR male mice	• 3T3 cells • HepG2 cells • DC-SIGN Cells • CHO cells	• Potent *in-vitro* gene delivery was achieved through the linkage of melittin to polyacridine DNA binding anchor using biodegradable disulfide bond. The released melittin improved gene transfer by lysis of endosomal membranes.	
EP 2298829 A1	Triblock copolymers for cytoplasmic delivery of gene-based drugs	École Polytechnique Fédérale De Lausanne	siRNA	mice	HeLa cell	• The delivery system is biodegradable triblock copolymer comprising: 1) A hydrophilic block 2) A hydrophobic block 3) A positively charged block that bound to a negatively charged molecule. • The system has the ability to self-assemble into a supramolecular structure such as a micelle or a vesicle.	Triblock copolymer poly(ethylene glycol)-poly(propylene sulfide)-peptide (PEG-PPS-peptide)
US 20110306657 A1	Polyalkyleneimine-graft-biodegradable polymers for delivery of bioactive agents	Agency for Science, Technology and Research	DNA	Male Wistar rats		• A graft and biodegradable poly (alkyleneimine) polymer that consists of at least two poly (C2-C6 alkyleneimine) side chains and a biodegradable polymer main chain. • The side and main chain attached to each other by a covalent bond.	The poly(alkylene-imine)-graft-chitosan polymers
US 8846850 B2	Amphiphilic macromolecules for nucleic acid delivery	Rutgers, The State University of New Jersey	siRNA	Not mentioned	• U87 glioma cells • U87-Luc cells	• Amphiphilic macromolecules that formed a complex with lipids to form liposomes. • The delivery system is used for delivering nucleic acids to cells.	• Cationic amphilphilic macromolecules CAM-lipid complexes • CAMs are hydrophobic acyl chains and hydrophilic poly(ethylene glycol) (PEG) • Lipid system consists of 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) (ratio 1:1).
US 8592385 B2	Polymer micelle complex including nucleic acid	The University of Tokyo	pDNA	Not mentioned	HeLa cells	• Polyion complex consists of a block copolymer. • The block copolymer is polyethylene glycol that binds to polycation via a disulfide linkage.	PEG-SS-P (Asp(DET))
US 20120065242 A1	Polymeric nanoparticles for siRNA delivery using charge interaction and covalent bonding	• Kim Meyung • Ick Chan Kwon • Kuiwon Choi • Myung S. Huh • Seung Y. Lee • So Jin Lee	siRNA	mice	• Melanoma cell line • SCC7 cancer cells	Stable polymer attached to siRNA via charge interaction and biodegradable covalent bond to form a stable polymer-siRNA nanoparticles.	Thiolated glycol chitosan (TGC)

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Not mentioned	Luciferase activity				• Particle size via DLS • Zeta potential via DLS • Gel permeation chromatography (GPC) • Gel electrophoresis	2009	[11]
Treatment of hereditary disorders, cancers	• Luciferase expression was quantified by bioluminescence imaging (BLI) • (*In-vivo* and *in-vitro*) • RBC hemolysis assay • Polyacridine-Melittin Hemolysis Assay		Yes		• Particle size (quasi-elastic light scattering (QELS)) • The size and shape of polyplex via Atomic Force Microscopy • Zeta potential • ^{1}HNMR • LC-MS • RP-HPLC Analysis • Asylum atomic force microscope (AFM) • MALDI-TOF MS analysis • Atomic Force Microscopy Analysis • Agarose gel electrophoresis • Autoradiography	2012	[12]
Treatment of cancer, restenosis, scar formation and postsurgical adhesions	• GAPDH enzyme activity assay (Ambion) • Cellular uptake of plypolex via fluorescence-activated cell sorting (FACS) • siRNA GAPDH downregulation assayin the presence and absence of chloroquine				• Particle size via DLS • Zeta potential via DLS • ^{1}H-NMR • Gas permeation chromatography • Gel electrophoresis • Fluorescence reduction assay • Fluorescence microscopy (Cell internalization studies in the presence and absence of serum)	2011	[13]
Not mentioned	*In-vivo* and *in-vitro* luciferase activity				• Gel retardation assay • Zeta potential • ^{1}H-NMR • ^{13}C-NMR • Gel permeation chromatographs • Intracellular trafficking studies • Agarose gel electrophoresis	2011	[14]
	• EGFP • Luciferase silencing assay • Cellular uptake of Cy5-labeled plasmid via confocal microscope images.	MTS assay			• Gel Electrophoresis • Zeta potential via DLS • Hydrodynamic radius via DLS • Differential scanning calorimetry (DSC) • Transmission Electron Microscopy (TEM).	2014	[15]
	Luciferase assay	Not tested			• ^{1}H NMR • Gel Permeation Chromatography (GPC) • Agarose gel electrophoresis • Ethidium bromide assay • Zeta potential via DLS • Particle size via DLS	2013	[16]
Anticancer therapy	Red fluorescent proteins (RFPs) expression suppression effect test (*in-vivo*)				• Agarose gel electrophoresis	2012	[17]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
WO 2013021056 A1	Method for the controlled intracellular delivery of nucleic acids	Ludwig-Maximilians-Universität München	pDNA	mice	• Murine N2A neuroblastoma cell lines • Human HUH7 hepatoma cell lines	• A cationic polymer attached to hydroxyethyl starch (HES) by charge interaction and covalent bond. • The polymeric complex is shielded by HES. • The polymeric complex is deshielded via removing HES by amylase enzymatic cleavage.	Polyethylenimine (PEI) attached to hydroxyethyl starch (HES)
WO 2011112902 A2	Nonviral gene delivery vector iopamidol, protamine, ethiodized oil reagent (viper)	The Board of Trustees of the Leland Stanford Junior University	DNA	• Rat • Rabbits	• Hepatoma cells • Hepatocytes • 293T cells	• The system is composed of protamine, plasmid DNA, ethiodized oil and iopamidol. • Protamine facilitates cell entry and acts as a DNA-condensing agent for protection. • Iopamidol acts as a visualizing agent for gene delivery. • Ethiodized oil provides selectivity for tumor uptake.	Vector iopamidol, protamine and ethiodized oil reagent (VIPER)
WO2011154331 A1	Polymers for delivery of nucleic acids	• F. Hoffmann-La Roche Ag • Ludwig-Maximilians-Universitaet Muenchen	• siRNA • DNA	mice	• Neuro2A cells • HUH7 cells • IGROV cells	• Polyamide polymers consist of defined oligo (alkylene amino) acids that are attached to each other by covalent bonds. • Polyamide polymers terminated by a free carboxyl group in one end and a free amine group on the other end. Polyamide polymers used as a delivery reagent for nucleic acids by proton sponge effect.	
US 7939505 B2	Amino acid lipids and uses thereof	Marina Biotech, Inc.	siRNA	mice	• A549 cells • 9L/LacZ cells • HepG2 cells	• A group of amino acid lipids contain an amino acid residue and one or more lipophilic tails. • A liposomal system consists of amino acid lipids form a bilayer vesicle. • The outer layer of the liposome is protected by PEGylation. • The outer layer also attached with a ligand for specific targeting. • The liposomal system has a condensed RNA nanoparticle. • The active agent is released by an acid-labile linker.	
WO 2011026641 A1	Disulfide-linked polyethyleneglycol/peptide conjugates for the transfection of nucleic acids	Curevac Gmbh	mRNA	mice	• L929 cells • HeLa cells • CHO cells	• Disulfide-linked polyethyleneglycol/peptide conjugates for the transfection of nucleic acids. • The reducing conditions in the cell lead to the release of the RNA from polymeric carrier complex.	Peptide conjugated with PEG via disulfide linkage.
CN103626996 A	Guanidinylation SS-PAAs polymer as well as preparation and application thereof	Shenyang Pharmaceutical University	• RNA • DNA	Not mentioned	MCFlOA cells	• The guanidinylation SS-PAAs gene vector polymer consists of cross-linking agent with a dual-acroloyl structure and a guanidinylation reagent. • It enhances transfection efficacy and membrane permeability.	A disulfide-containing amino guanidine amine polymer poly (SS-PAAs)
CN103665169 A	Three-function peptide-modified gene carrier as well as preparation method and application thereof	Shanghai Ocean University	DNA	Mouse B16 melanoma xenograft model	Hela cells	Gene carrier system consisting of biodegradable chitosan is conjugated with modified polyethylenimine	Amphiphilic chitosan-modified polyethyleneimine was conjugated with a three-functional polypeptide
WO 2014059446 A1	Biodegradable hydrogel for polynucleotide delivery	Case Western Reserve University	siRNA	Non-human animals (e.g., rodents, arthropods, insects, fish)	• HEK 293 cells • DeGFP HEK293 cells • Human MSCs (hMSCs)	• Biodegradable photocrosslinked hydrogel that includes neutral DEX- HEMA which binds with cationic LPEI-GMA macromers by ester linkages. • It's capable of forming electrostatic interactions with siRNA. • Hydrogel polymers swell and release bioactive molecules gradually. In situ forming hydrogels were controlled via the density of hydrolysable ester groups in the materials.	

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
• Anticancer therapy • Treatment immune diseases, neuronal diseases, infections, and inflammatory diseases.	• *In-vitro* luciferase assay • *In-vivo* luciferase expression in lung and tumor tissues of N2A tumor mice • Cellular uptake of Cy5-labeled plasmid via flow cytometry.	MTT assay	Not tested		• Zeta potential • Particle size • ^{1}H-NMR • Size exclusion chromatography (SEC) • Multi-angle light scattering (MALS) • HPLC • Enzymatic degradation of HES was evaluated via quartz crystal microbalance with dissipation (QCM-D) • Erythrocyte aggregation assay. • Copper Assay (Photometric analysis) via UV-vis Spectroscopy System • The enzymatic activity of pancreatic α-amylase (AA) was assessed via the Phadebas® Amylase test.	2013	[18]
Hepatocellular carcinoma treatment	• Luciferase activity (in rats and rabbits) • Bioluminescence imaging (BLI) of injected HCC rat model • Positron emission tomography imaging (PET)	Not tested		Yes	Not mentioned	2011	[19]
Anticancer treatment	• Erythrocyte leakage assay • *In-vivo* and *in-vitro* luciferase reporter gene silencing assay • Luciferase reporter gene expression • EGFP	MTT assay	No	Yes	• DNA gel-shift assay • RNA gel-shift assay • Particle size via DLS • Gel electrophoresis • ^{1}H NMR • ^{13}C NMR • Ion exchange chromatography	2011	[20]
Treatment of disorders related to modulation of gene expression or activity in a subject	• β-galactosidase expression • LacZ gene knockdown activity *in-vitro* assay • *In-vivo* RT-PCR Assay • ApoB gene knockdown activity *in-vitro* assay • SYBR™ Gold assay • Serum Cholesterol Assay (blood and serum) • *In-vivo* assay for influenza viral titer knockdown in mouse	Not tested			• Transmission electron microscopy • Particle size analysis	2011	[21]
Treatment of Hashimoto's thyroiditis and Addison's disease	• Luciferase expression *in-vitro* and *in-vivo* • Cytokine stimulation assay • Cellular uptake of Alexa Fluor 647 labelled mRNA was evaluated via confocal microscopy				• Zeta potential via DLS • Hydrodynamic diameters via DLS • SDS PAGE gel electrophoresis • Gel shift assay	2011	[22]
Gene therapy	• FANCF silencing efficiency • EGFP via flow cytometry	MTT assay	Yes		• Particle size via DLS • Zeta potential via DLS • GPC	2014	[23]
Anticancer therapy	Luciferase expression		No		• ^{1}H NMR	2014	[24]
• Anticancer therapy • Tissue engineering	• Luciferase expression • Noggin gene expression • Alkaline phosphatase (ALP) activity • BSP and PPAR-γ expression were examined by qRT-PCR • Confocal fluorescent photomicrographs • siRNA release kinetics (fluorescein isothiocyanate (FITC)) • Bioactivity of released siRNA	MTS assay			• ^{1}H NMR • Rheological properties • Determination of swelling • *In-vitro* degradation of the hydrogels	2014	[25]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
WO2014053654 A1	Polymer synthesis	National University of Ireland, Galway	DNA	mice	• Mouse 3T3 fibroblasts • Rat adipose derived stem cells • PC12 pheochromocytoma • Human RDEB keratinocytes • Type VII collagen-null keratinocytes (RDEBK) • Rabbit-adipose derived stem cells (ADSCs) • Neu7 astrocytes	• A biodegradable cationic and disulfide hyperbranched polymer. • The system had a better transfection and cell metabolic activity when compared to conventional dendrimer and liposome based transfection agents.	2-(dimethylamino) ethyl methacrylate (DMAEMA), 2-{[2-(prop-2-enoyloxy) ethyl]disulfanyl4ethyl prop-2-enoate (PEEDEPE) Polyethylene glycol (PEG) (meth) acrylate.
CN103588749 A	Novel methacrylamide monomer, preparation method of pH sensitive polycation genetic vector of methacrylamide monomer and application thereof	Anhui University		Not tested	• human kidney epithelial cells (293 cells) • African green monkey kidney fibroblasts (C0S-7)	• The carrier system consists of methyl acrylamide monomer and pH sensitive polycationic gene vector. • The synthetic polymer has acid-sensitive group and ortho ester linkage. • It enhanced transfection efficacy via enhancing the endosomal escape.	A novel N- ((2- (2- dimethylamino) ethoxy) - [1, 3] dioxane -4-methyl) methacrylamide is banded with polycationic polymer
CN103554492 A	Thiourea modified polyethyleneimine copolymer as well as method thereof	Anhui University Changchun Institute of Applied Chemistry & Chinese Academy of Sciences	RNA		• HeLa cells • IX IO4 cells	• The thiourea modified polyethyleneimine copolymer reduced the high electric density of PEI which leads to cytotoxicity reduction. • The modified PEI attached with genetic material by hydrogen linkage in a nano system.	Thiourea modified polyethyleneimine copolymer.
WO 2014121050 A1	Functionalized DNA dendrimers for gene delivery to cells	Genisphere, Llc, Lankenau Institute of Medical Research	DNA	NOG mice	• LAPC4-AS cells (androgen sensitive human prostate cancer cells) • LAPC-4AS cells • LAPC-4AI cells	• Dendrimers attached to DNA. • Targeting molecules such as antibody or a peptide were covalently bound to DNA- dendrimers.	DNA dendrimer including a targeting moiety
US8968746 B2	Complexation of nucleic acids with disulfide-cross-linked cationic components for transfection and immunostimulation	The Regents of the University of California	RNA	mice	• HepG2 cells • B16F10 cells	Polymeric carrier consists of cationic components (disulfide-cross linked cationic peptide) and binds with nucleic acid (RNA).	Disulfide-cross linked cationic peptide$CR_{12}C$ (Cys-Arg_{12}-Cys)
WO 2010093420 A2	Ultra-small super paramagnetic iron oxide nanoparticles and uses thereof	University of Houston	DNA		• OVCA cells • HUVECs cells	The delivery system is gold coated iron oxide nanoparticle comprising: 1) Functionalized iron oxide nanoparticle cores 2) A layer of silica around the core 3) An outer gold-silver alloy nanoshel 4) A targeting ligand bound to the nanoshell by linkers such astriethylene glycol polymer or a polyethylene glycol polymer.	Gold coated iron oxide nanoparticle
US20110182979 A1	*In-vivo* non-viral gene delivery of human vascular endothelial growth factor following islet transplantation	Baylor Research Institute	pDNA		Not mentioned	• Ultrasound-targeted micro-bubble destruction (UTMD) enhances revascularization, and increases the efficacy of the grafted cells in liver transplantation. • Human insulin and C-peptide levels were measured. • The microbubble complex consists of lipid shell containing gas, pDNA and promoter sequence cytomegalovirus (CMV). • Ultrasound destroys the micro-bubble (liposome) releasing the DNA into the liver cells directly.	Liposome (l, 2-dipalmitoyl-sn-glycero-3 -phosphatidylcholine and l, 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine glycerol)

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Not Mentioned	• Luciferase expression activity analysis • GFP • VII collagen (Green) transfection activity followed by western blot. • Immunoblot of RDEB keratinocytes	AlamarBlue® assay			• Zeta potential via DLS • Particle size via DLS • Agarose gel electrophoresis • Transmission electron microscopy (TEM) • ^{1}H NMR • Gel permeation chromatography (GPC)	2014	[26]
	• Luciferase expression • EGFP expression • EB exclusion experiment • Heparin replacement experiment	MTT assay			• Particle size via DLS • Agarose gel electrophoresis • ^{1}H-NMR • ^{13}C-NMR • GPC • Stability test was done by ^{1}H-NMR analysis at different times.	2014	(27)
Treatment of cancer, tissue engineering and other gene-related diseases	• Luciferase expression • GFP expression			No	• ^{1}H-NMR • Infrared spectroscopy	2014	[28]
• Treatment of human papillomavirus (hpv)-infected cells • Anticancer therapy (prostate cancer) • Cervical precancerous lesions treatment.	• *In-vitro* and *in-vivo* GFP expression • Cyan Fluorescent Protein (CFP) expression • Luciferase expression	TUNEL assay	No	Yes	• HPLC • Gel electrophoresis	2014	[29]
Immunostimulating agent	• *In-vitro* and *in-vivo* luciferase expression. • B-cell immune response detection in vaccinated mice. • Cytokine production of the hPBMCs was measured. • Detection of an antigen-Specific Immune Response (B-Cell Immune Response) • Detection of an antigen specific cellular immune response by ELISPOT.	Not mentioned			• Zeta potential via DLS • The hydrodynamic diameters via DLS	2015	[30]
• *In-vivo* magnetic resonance imaging • Treating primary or metastatic cancers • Ablating atherosclerotic plaque	• luciferase expression • *In-vivo* IL-12 expression with an enzyme-linked immunosorbent assay (ELISA)	MTT assay		No	• Transmission electron microscopy (TEM) • Particle size via DLS • Zeta potential via DLS • Fourier Transform-Infrared (FT-IR) • X-Ray Diffraction (XRD) • ^{1}H NMR • HPLC • MALDI-TOF • Gel retardation assay • Scanning electron microscope with an energy-dispersive spectrometer (SEM/EDS) • Inductive Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) • Ninhydrin assay	2010	[31]
Diabetes treatment	• GFP and hVEGF expression • Immunohistochemical analysis • Measurement of serous human VEGF levels by ELISA kit (*in-vivo*) • Reverse transcription polymerase chain reaction (RT-PCR)	Serum of alanine amino transferase (ALT) and aspartate amino transferase (AST) levels were measured after polyplex administration			The mean diameter and concentration of the microbubbles were measured with a particle counter	2011	[32]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
WO 2014051318 A2	Short interference RNA gene delivery system for systemic circulation	Industry-University Cooperation Foundation Hanyang University	RNAi		Squamous Cell Carcinoma (SCC7)	The system consists of polyethylene glycol (PEG) and arginine 9 (R9) peptide which bound to nucleic molecule in nano-system.	Polyethylene glycol (PEG) and (arginine) peptide R9 (PEG-R9)
CN103505744 A	Gene delivery system of targeting brain capillary endothelial cell	Fudan University	DNA	• Rat • AD model mice	BCEC cells	• It is a targeted brain capillary endothelial cells release gene delivery system. • The system consists of PEG covalently bound to poly-β- amino ester which is in nano system. • Plasmid DNA has therapeutic gene which is a neurotrophic factor gene. • The gene is entrapped or adsorbed loading the gene delivery system.	Poly β-amino ester and polyethylene glycol
US20060093674A1	Biodegradable cross-linked cationic multi-block copolymers for gene delivery and methods of making thereof	• Gregory Slobodkin • Majed Matar • Jason Fewell • Khursheed Anwer		mice	African green monkey kidney cells (Cos-1 cells)	• It is a cationic multi-block lipopolymer. • A linear polyethyleneimine (LPEI) which is biodegradable cross-linked cationic multi-block copolymer with lipid moiety. • L-PEI units covalently bound to each other by the hydrophilic biodegradable linkers. • The cationic multi-block copolymers improve gene delivery via enhancing the adhesion to biological surfaces and cellular localization.	A linear polyethyleneimine (LPEI) which is biodegradable cross-linked cationic multi-block copolymer conjugated with the lipid
US RE43612 E1	Biodegradable poly(β-amino esters) and uses thereof	Massachusetts Institute of Technology		Non-human animal (e.g., a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig).	• NIH 3T3 cells • COS-7 (green monkey kidney) • HepG2 (human Hepatocarcinoma • CHO (Chinese Hamster Ovary) cell lines	• Biodegradable, pH-responsive and biocompatible poly (beta-amino esters), salts and derivatives polymercomplexed with polynucleotide in nano system. • The system consists of microparticles for delivery of encapsulated agents by endocytosis. • The system provides sustained release.	Poly β-amino ester polymers.
US 8071082 B2	End-modified poly (beta-amino esters) and uses thereof		siRNA	Not tested	• COS-7 cells • HeLa cells	An amine terminated poly (beta-amino ester) was reacted with an acrylate electrophilic reagent such as acrylamide to generate an end-modified Poly (beta-amino ester)	End-modified Poly (beta-amino ester)
WO 2014133351 A1	Composition for gene delivery comprising chitosan and liquid crystal formation material	Chong Kun Dang Pharmaceutical Corp.		mice	Human prostate cancer cell line (PC-3, ATCC)	• Gene delivery system is a liquid crystal that consists of gene, sodium lauryl sulfate, chitosan, a liquid crystal hardener and a phospholipid. • The particle size is in the range of 200-300 nm.	Chitosan
WO 2014134627 A1	Methods for modulating development and function of photoreceptors cells	The Schepens Eye Research Institute, Inc.	cDNA	Rd7 mice	Not mentioned	• The gene delivery system consists of nile red poly(lactide-co-glycolide) (PLGA) in the form of nanoparticle. • This invention is based on using a modifier gene for ocular disease treatment.	PLGA
US 8153155 B2	Arginine-conjugated bioreduciblepoly(disulfide amine) polymers for gene delivery system	University of Utah	• pDNA • siRNA	Not tested	• C2C12 cells • NIH3T3 cells • PC-3 cells	• Cationic arginine-grafted bioreducible poly(disulfide amine) (ABP) bound to plasmid in a nano system. • ABP releases the plasmid in the cytoplasm due to biodegradation in reducing condition.	Arginine grafted bioreducible poly(disulfide amine) (ABP)

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Anticancer treatment	• Luciferase expression • Cellular permeability of PEG-R9 / FITC-siVEGF was evaluated via flow cytometry • Fluorescence Activated Cell Sorter (FACS) • *In-vivo* antitumor efficacy	MTT assay	Yes		• Zeta potential via DLS • Particle size via DLS • analyzer • Agarose gel electrophoresis • Flow cytometry • TEM	2013	[33]
Brain nerve treatment of degenerative diseases	• EGFP expression. • *In-vivo* pharmacokinetics and tissue distribution of the peptide. • Cellular uptake of 5-carboxy-fluorescein (5-FAM) labeled peptide via flow cytometry. • ELISA (for hBDNF protein). • *In-vivo* imaging for EMA-labeled plasmid DNA. • *In-vivo* Morris water maze test was performed for evaluation ofTB-NP-hBDNF treatment on rats with spatial learning and memory impairment improvement.		No	No ELISA (Yes)	• Zeta potential • Particle size analyzer • TEM	2012	[34]
Anticancer treatment	• In vitro luciferase • β-galactosidase expression.	• Total protein assay • Cell proliferation assay • CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)	Yes		• Particle size • Zeta potential • ^{1}H NMR • Agarose gel electrophoresis • UV-spectrophotometer.	2012	[35]
Therapeutic, diagnostic, or prophylactic agents	• Luciferase expression • EGFP • Relative transfection efficiencies were measured via cell protein assay (Pierce) kits. • Cellular uptake of Cy5-labeled plasmid was assessed via flow cytometry.	MTT assay	No		• Particle size analysis • Zeta potential • Gel permeation chromatography (GPC) • Scanning electron microscope (SEM) • Fluorescence microscopy • Agarose gel electrophoresis • Scanning electron microscope (SEM)	2012	[36]
• Inherited and acquired genetic diseases treatment • Tissue engineering	• Luciferase expression • Cellular Uptake Assay via β-galactosidase expression.		Yes		• Zeta potential via DLS • Particle size was measured via DLS in the presence of serum • Binding of Polymer-DNA was measured via picoGreen assay • Agarose gel electrophoresis • ^{1}H NMR • Gel permeation chromatography (GPC)	2011	[37]
Anticancer therapy	The inhibitory effect against target protein expression was evaluated via ELISA kit *in-vitro* and *in-vivo*.	Not mentioned	No	Yes	• Particle size of liquid crystals via Electrophoretic Light Scattering Spectrophotometer (ELS) • Electric charge analyzer • Structures of liquid crystals via cryo transmission electron microscopy (Cryo-TEM)	2014	[38]
Ocular disease treatment	• *In-vivo* GFP expression • Electro-retinograms of scotopic analysis • RT-PCR	Not mentioned	No		• Particle size analysis • Size distribution • Morphology • Zeta potential • Photomicrographs	2014	[39]
Anticancer therapy	• Luciferase activity • Cellular uptake of YOYO-1-labeled plasmid was assessed via flow cytometry. • Subcellular confocal images of cytoplasmic siRNA release from the delivery system. • VEGF expression using ELISA • BCA™ Protein Assay • Electrophoretic Mobility Shift Assay	MTT assay	Yes		• Particle size via DLS • Zeta potential via DLS • Agarose gel electrophoresis • Gel retardation assays	2012	[40]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
US 20110263025 A1	Biodegradable poly disulfide amines for gene delivery	University of Utah Research Foundation	pDNA		• HeLa cells • C2C12 cells	• Different Poly (disulfide amine)s including: poly(disulfide amine)s include poly(N,N′-cystaminebisacrylamide-spermine), poly(N,N′-cystaminebisaciylamide-N,N′-bis(3-aminopropyl)1,3-propanediamine), poly(N,N′-cystaminebisacrylamide-N,N′-bis(3-amino-propyl)ethylenediamine), poly(N,N′-cystaminebisacrylamide-N,N′-bis(2-aminoethyl)-1,3-propanediamine), and poly(N,N′-cystaminebisacrylamide-triethylenetetramine) that bound to plasmid by electrostatic attraction. • Poly (disulfide amine)s are attached to each other via Michael addition.	
US8952133 B2	Non-viral vectors for delivering polynucleotide to target tissue	Gencia Corporation	Not mentioned		Transgenic non-human animals	Modified polynucleotide that was coated by fusion proteins (A mitochondrial transcription factor (TFAM)) comprising a targeting signal to the brain.	Modified polynucleotide-binding proteins
US 20150064116 A1	Multilayered magnetic micelle compositions and methods for their use	University of South Florida (A Florida non-profit corporation)	DNA	mice	• HEK 293 cells • 3T3 cells • PC3 cells	• The targeting system is a micelle that consists of three layers. • The first inner layer is a hydrophobic super paramagnetic iron oxide nanoparticle (SPION) core while the first coating is a cationic polymer and the second coating is a polynucleotide. • The gene delivery system enhances the endosomal escape of plasmid by proton sponge effect.	Chitosan and PEI based polymers
US 20110305685 A1	A supramolecular approach for preparation of size controllable nanoparticles	University of California Board of Directors			• NIH 3T3 mouse fibroblast cell line • U87 brain cancer cell lines • MCF7 breast cancer cell lines	• Au-SNP with enhanced photothermal effectthat bound to a target-specific ligand (i.e. RGD). • Convenient, flexible and size-controllable supramolecular nanoparticles (SNPs). A molecular recognition system based on adamantane (Ad) and β-cyclodextrin (CD) was employed to assemble three molecular building blocks (i) n-Ad-PAMAM (ii) CD-PEI, and (iii) Ad-PEG.	
US20150093433 A1	Targeted and modular exosome loading system	Northwestern University	RNA	Not mentioned		• Targeted and Modular Exosome Loading (TAMEL) system consists of RNA binding domain and an exosome targeting domain. • The disclosed exosomes may comprise novel proteins, polypeptides, or peptides.	
WO2016027699 A1	Cationic lipid for nucleic acid delivery	• Nof Corporation • Hokkaido University • National University Hokkaido University		4-week-old male mice	• Prokaryotic and eukaryotic cells • Animal and plant cells	• A cationic lipid that has higher intracellular delivery efficiency than that of a conventional cationic lipid. • The cationic lipids used in the lipid membrane structure includes1,2-Dioleoyl-3-dimethylaminopropane (hereinafter, referred to as "DODAP") and, 1,2-Dilinoleoyl-3-dimethylaminopropane (hereinafter, " referred to as the DLinDAP "), and the like. • The amino group contained in the cationic lipid is changed to protonated cationic group with decreased pH. Subsequently, pH responsive properties are imparted to the lipid membrane structure.	
US20150064160 A1	Engineered primate cystine/cysteine degrading enzymes as antineogenic agents	Board of Regents, The University of Texas System	DNA	Male nude mice	• Chinese hamster ovary cells (CHO-K1) • Rat pituitary cells (GH1) • HeLa S3 cells • Rat hepatoma cells (H-4-II-E)	• A protein with L-cyst(e)ine degrading enzyme activity such as modified cystathionine-γ-lyase comprising one or more amino acid substitutions and capable of degrading L-cyst(e)ine (modified primate Cystathionine-gamma-lyase (CGL) enzyme(s)). • The protein with L-cyst(e)ine degrading enzyme is coupled with PEG polymer. • The enzyme is coupled to PEG via one or more amino acid residues such as lysine or cysteine.	
US 20150086612 A1	RNA formulation for immunotherapy	Biontech Ag	• DNA • RNA	mice	• COS cells • K562 cells • HEK293 cells • HELA cells • Yeast cells • Insect cells	• The delivery system is nanoparticles that are lipoplexes comprising 1, 2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and 1, 2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE) • Nanoparticles may comprise one or more of the following: pH dependent compounds, cationic polymers such as polymers containing histidine and/or polylysine, wherein the polymers may optionally be PEGylated and/or histidylated, or divalent ions such as Ca^{2+}.	
CN103025356 B	Optimized *in-vivo* delivery system with endosomolytic agents for nucleic acid conjugates	• DNA Medical Company • the French Curie Institute • The French National Science Research Center	• DNA • RNA		MRC5 cell lines	• A nucleic acid molecule bound to the inner promoting endocytic molecules such as cholesterol or tocopherol (coDbait). • PEI covalently bound to nucleic acid - cholesterol. • Radiation or administered DNA damaging antineoplastic agents were also used.	

Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Not Mentioned	• Luciferase expression • Cellular uptake of YOYO-1-labeled plasmid DNA was assessed via flow cytometry.				• ^{1}H NMR • Particle size via DLS • Agarose gel electrophoresis • Fast protein liquid chromatography • Determination of buffering capacity by acid-base titration. • Size exclusion chromatography (SEC)	2011	[41]
Anticancer therapy	Not mentioned		No		Not mentioned	2015	[42]
Anticancer therapy (prostate cancer)	• EGFP expression • Luciferase expression • In-vivo and in-vitro tomato protein expression • Xenogen imaging • *In-vivo* magnetic resonance imaging (MRI)	• WST cytotoxicity assay • *In-vivo* toxicity test by determination of nanoparticles accumulation in liver, spleen, lung and prostate • Rate of degradation of SPION by Prussian blue for iron detection.	Yes	No	• TEM • DLS • NMR • UV-Vis spectroscopy • FTIR • Gel electrophoresis • laser confocal microscopy	2015	[43]
Anticancer therapy	• EGFP • Luciferase assay • Pore formation of the damaged cell was assessed via inverted fluorescence microscope	Not mentioned	Yes		• ^{1}HNMR • Zeta potential via DLS • Particle size via DLS • Hydrogel gel electrophoresis • Transmission electron microscopy (TEM)	2016	[44]
Not mentioned	EGFP	Not mentioned				2015	[45]
	• Luciferase assay • Biophen FVII chromogenic assay	Not mentioned			• Zeta potential via DLS. • Particle size via DLS • ^{1}HNMR • Gel exclusion chromatography	2016	[46]
Treatment of cancer	• Human Cysthionine-γ-Lyase and Human Cyst(e)inease expression • Anti-tumor activity was examined in xenograft tumor	MTS assay	Yes		• Ion-exchange chromatography • Gel exclusion chromatography polyacrylamide gel electrophoresis • Affinity chromatography • Immunoaffinity chromatography • Isoelectric focusing • SDS-PAGE • HPLC • Mass spectrometer coupled with electrospray ionization	2015	[47]
Immunotherapy	• Luciferase assay *in-vivo* and *ex-vivo* • Cellular uptake of the uptake of Rhodamine labeled Cy5-RNA or F4-rho was assessed • Serum concentrations of IFNa and TNFa were measured via ELISA. • Cellular uptake of Luc-RNA via *in-vivo* Bioluminescence Imaging (BLI).	Survival rates via Kaplan-Meier survival curves	Not mentioned	Yes	• Zeta potential via DLS • Particle size via DLS • HPLC • Asymmetrical flow field-flow fractionation	2015	[48]
Anticancer therapy	• Cellular uptake of coDbaitcy3 was measured via confocal microscopy. • Anti-tumor activity was examined in xenograft tumor	Local and systemic toxicity in mice was determined such as focal necrosis, ischemic local inflammation and survival rates examination.	Not mentioned		• Agarose gel electrophoresis • Zeta potential via DLS • Particle size via DLS	2015	[49]

Patent Number	Title	Owner	Genetic material	*In-vivo* testing	Cell line	System description	Carrier\Polymer
WO2016025469 A1	Prevention of muscular dystrophy by crispr/cas9-mediated gene editing	The Board of Regents of The University of Texas System	DNA		• HeLa cells • Hepatoma cells	The disclosure reports means of myo-editing-mediated exon skipping from somatic tissues in mice to human DMD patients-derived iPSCs (induced pluripotent stem cells.	Liposomes and/or nanoparticles
WO2015042585 A1	Nanoparticle-mediated gene delivery, genomic editing and ligand-targeted modification in various cell populations	Rensselaer Polytechnic Institute		Not mentioned	MC3T3- El cells	• An improved nanoparticle for transfecting cells consists of polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide. • The polyplex was surrounded with silica coating. • Cationic polymer could be poly(ethylenimine) and/or poly(L-arginine). • Anionic polymer could bepoly(D-glutamic acid).	
US9345775 B2	Compositions for targeted delivery of siRNA	Arrowhead Madison Inc	siRNA	mice	Not mentioned	• RNAi polynucleotides electrostatically bound to liposomes and stable nucleic acid-lipid particles (SNALPs). • Delivery polymers provide membrane penetration function for movement of the RNAi polynucleotide from outside the cell to inside the cell.	siRNA-galactose cluster or cholesterol or PEG conjugate and masked Poly (Vinyl Ether)
CN105745221 A	Delivery system for functional nucleases	President and members of Harvard University	• RNA • DNA		HeLa reporter cell line	• Functional effector proteins include TALE effector proteins e.g., TALE transcriptional activators and repressors, as well as TALE nucleases. • The delivery system is cationic lipid-mediated with extra negatively charged Cre recombinase protein or other proteins. • Liposomes are also mentioned as possible carrier system.	
CN 201180006572	Sustained-release nucleic acid matrix compositions	Boli Pi too Ltd.	ssDNA	Not mentioned		• The extended release delivery system consists of biodegradable polyester (PLGA for example), a sterol, phosphatidylethanolamine fatty acid moiety of at least 14 carbon atoms, 14 carbon fatty acid portion of the phosphatidylcholine, a nucleic acid agent and PEG. **PLGA-PEG-PLGA**	

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Therapeutic purpose	Expression readout	Cytotoxicity assay	Presence of serum in cytotoxicity assay	Presence of serum in efficacy assay	Physicochemical characterization	Year	Ref.
Duchenne muscular dystrophy (DMD)	• GFP expression in-vivo via the T7E1 assay • Histological examination of muscles • Immunofluorescence detection of dystrophin & dystrophin expression *in-vivo* • Histological and Western blot analysis of muscle • Serum creatine kinase (CK) measurement which is a marker for muscular dystrophy	Not mentioned	No		• Agarose gel electrophoresis	2016	[50]
Not mentioned	• Luciferase assay • Sclerostin expression • β-catenin expression • mCherry gene expression • Cellular uptake of Fluorescein isothiocyanate (FITC) labeled polyplex was determined • SOST expression via ELISA and quantitative real-time PCR (qPCR) assays		Yes		• Particle size via DLS • Complex formation was confirmed via fluorescence reduction of EtBr-tagged DNA	2015	[51]
	• Serum ApoB levels was determined via ELISA. • Plasma Factor VII (F7) activity determination • Silencing of apoB gene expression in-vivo.	• Serum levels of liver enzymes and cytokines • Slight elevations of ALT and AST levels were measured in mice. • The levels of TNF-α and IL-6 were measured in serum via ELISA.			• Gel electrophoresis • Zeta potential via DLS • Particle size via DLS • Anion exchange HPLC • RP-HPLC • DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence was measured for confirmation of polymer synthesis	2016	[52]
Treatment of genetic diseases	• Gene silencing • EGFP expression was quantified via qRT-PCR *in-vivo* and *in-vitro*. • Immunohistochemistry analysis.	Not mentioned			• Gel electrophoresis • Fluorescence microscopy	2016	[53]
Not mentioned	GFP expression	Not mentioned			• Optical microscope examination. • Gel electrophoresis	2015	[54]

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