Review Article

A Review on the Application of Nanoparticles for Targeted Gene Delivery

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A B S T R A C T

Gene therapy is an attractive approach to treatment of diseases with genetic or non-genetic origins. This procedure is based on the delivery of genetic materials, mainly DNA or small interfering RNA (siRNA), to the target cells or tissues. Due to the presence of physical and chemical barriers in the internal environment and cells of the body such as degrading enzymes in the circulatory system or electrical charge of the cell membrane, transfection of the naked nucleic acids is inefficient. In order to overcome this problem, different types of gene transfer carriers were developed. Of note, nanoparticle-based carriers have attracted considerable attention owing to their particular properties. Nanoparticles (NPs) are available in different types, each with its own specific advantages and disadvantages. Some of their advantages such as their small size have made NPs a potential candidate for eliminating obstacles to the genetic material delivery. However, these NPs have several limitations. The current study aimed to introduce different types of NPs used in the delivery of genetic materials and examine the basic aspects of the fabrication, characterization, and functionalization of NPs. Further, it briefly summarized the advantages and disadvantages of each approach to gene delivery by means of NPs. Finally, it suggested some applications of the nanoparticle-based gene therapies in the clinical trials.

1. INTRODUCTION

Gene therapy is defined as the direct transfer of genetic materials to cells and tissues affected by either inherited or acquired diseases [1,2]. This procedure is considered to be a potential candidate for treatment or prevention of diseases resulting from defective gene expression [3]. This strategy involves introduction of genes (Figure 1) into the target tissues or cells to alter the expression of endogenous genes for therapeutic purposes or prevent further development of the associated disease [4]. Gene therapy is used not only for genetic disorders but also for other complex diseases such as viral infections (human immunodeficiency virus), autoimmune diseases (rheumatoid arthritis), coronary heart disease, cancer, diabetes, arterial disease, neurodegenerative disorders, hemophilia, AIDS, asthma, etc. [1,5].

The therapeutic molecules include nucleic acids,

antagonist oligonucleotides, and small interfering RNAs (siRNAs) facilitate the replacement of damaged gene or down regulation of undesirable gene expressions [3,6].

These molecules are large in size, easily degradable by enzymes, and characterized by anionic nature, characteristics that make their delivery quite difficult. In

this regard, carriers play a significant role in the gene delivery to the target cells or tissues [3]. Hence, the existence of nucleic acid-transferring vectors is necessary to facilitate the transport of nucleic acid molecules to the cells [1].
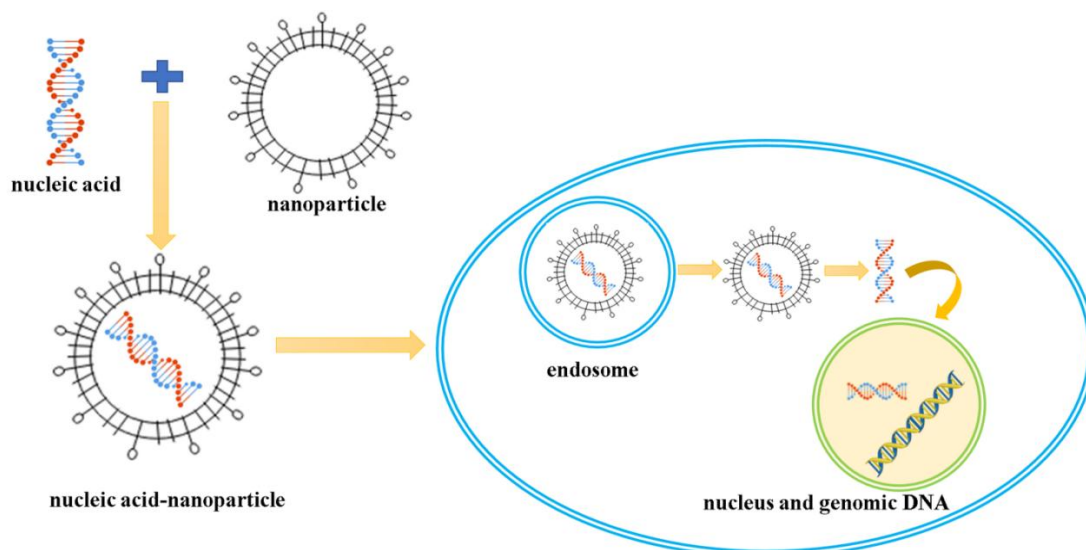
Figure 1. Nucleic acid delivery using nanoparticles

In general, there are two different methods of gene delivery depending on the carrier's characteristics namely the viral-mediated gene transfer and nonviral gene transfer using artificial carriers [7]. The effectiveness of the viral vectors in the delivery of nucleic acids is greater than that of non-viral ones. However, the carriers of viral vectors might considerably threaten the patients' health while the non-viral carriers of genes are inherently safer than the vectors of viruses [1,8].

In addition, these viral carriers have other limitations such as limited cell targeting and gene transport capacity as well as relatively high large-scale production costs. Non-viral carriers carry a wide range of nucleic acids, hence they are robust that can be used for large-scale production [3]. In ideal situations, gene transfer systems should be stable, biologically compatible, non-toxic, and highly efficient transfection systems. Nanoparticles (NPs) are ideal platforms that can be used among different nonviral nucleic acid carriers [7].

NPs, usually referred to as the dispersed or solid particles ranging from 1 to 100 nm in size, were found to be effective tools for gene delivery. NPs with quite small sizes can travel in the circulatory system and pass throgh many physiological barriers.

The high ratio of the surface area to the volume facilitates modification of the surface of particles with functional groups to control the pharmaco-kinetics and bio-distribution of the particles [9]. NPs are categorized into four main groups namely the polymer-based, lipid-

based, inorganic, and hybrid NPs (Figure 2) [1,4].

This study presents a review of the different types of NPs, ways of NP production, clinical development of gene-transferring NPs, and toxicity of NPs.

Figure 2. Gene delivery approaches
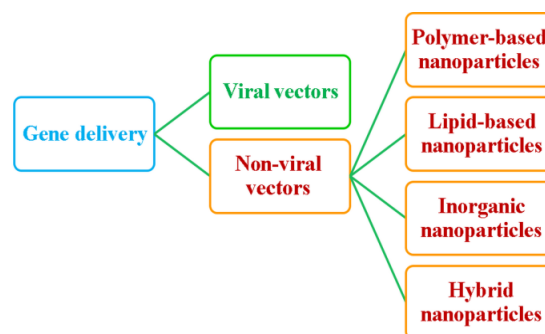
2. DIFFERENT TYPES OF NPS

2.1. Polymer-Based NPs

There is a positive charge in the spine in these polymers that supports their interactions with the negatively charged anionic nucleic acid materials. The binding of the cationic polymers to the DNA molecules can form nanometric complexes called the polyplexes [1]. Such NPs can be produced using biopolymers or synthetic

polymers [10]. Biopolymers are defined as the macromolecules produced from living organisms that are divided into three groups namely the proteins, polysaccharides, and nucleic acids [1].

Proteins with natural origin such as albumin, collagen, gelatin (a natural protein obtained from the degeneration of collagen with great biodegradability and biocompatibility in physiological conditions [11]), elastin, β-casein, fibronectin, zein, and silk protein are commonly used to produce biomaterial NPs [1,12]. A polypeptide polymer called the Poly-L-Lysine (PLL) is the earliest polymer as for non-viral gene delivery carrier [12]. Polysaccharides as another group of biopolymers are long molecules of carbohydrate that are made of repeating units of monosaccharide. These materials contain chitosan, pullulan, hyaluronic acid, alginate (a natural polysaccharide obtained from brown algae which is composed of alpha-l-guluronic acid and beta-d-mannuronic acid units [13]), dextran, cyclodextrin, heparin, and lignin [1,12]. Synthetic polymer-based NPs have also drawn considerable attention in recent years [14]. Polylactic Glycolic Acid (PLGA)-based NPs, polyethyleneimine (PEI), dendrimers, Polyethylene Glycol-Cationic Polylactide (PEG-CPLA) copolymers, and Polyion Complex Micelles (PIC) are some examples of synthetic polymers [1,12].

Polyethylenimine is able to condense the DNAs into a polyplex, hence widely used as a gene transferring vector [12]. There are two types of polyethylenimines available called the branched and linear with different molecular weights [3]. Dendrimer is a three-dimensional polymer characterized by a spherical structure with many branches. Polyamines, polyamides, or polyesters are the frequently used dendrimers. Polyamidoamines (PAMAMs) are also the most commonly used dendrimers [1,15].

2.2. Lipid-Based NPs

Cationic lipids, cationic solid lipids, cationic liposomes, cationic emulsions, lipidoids, and gemini surfactants are the lipid-based NPs generally used for gene delivery [1,12]. Cationic lipids are positively charged molecules of amphiphilic such as dioleoyl trimethylammonium propane (DOTAP) and Dioleylpropyl trimethylammonium chloride (DOTMA) [1]. Cationic liposomes are liposomes containing a positively charged lipid and a helper lipid that can keep nucleic acids from enzymatic deterioration in the bloodstream and interact with cell membranes with negative charge to intense internaling cell [1].

The main components used for fabrication of liposomes are 1,2-dioctadecanoyl-sn-glycero-3-phospho ethanolamine (DSPE) and dioleoylphosphatidylcholine (DOPC) or dioleoylphosphatidylethanolamine (DOPE) [7,9]. Solid Lipid NPs (SLNs) or cationic solid lipid core-shells are made from high-melting temperature lipid molecules as the core of the particle and surfactants as the shells around the particles [1]. Production of cationic emulsions involves a hydrophobic oil phase covered with cationic lipids [1]. Lipidoids or Lipidoid Nano-Particles (LNPs) are small molecules similar to the lipids that have recently been explored as the RNA interference gene transferring vehicles [12].

Gemini surfactants are basic structures composed of more than two polar head groups and two hydrophobic tails connected by a molecule as the space creator [12]. Gemini are found in four main types of surfactants namely the m-s-m (N, N-bis (dimethyl alkyl)-α, ω-alkanediammonium), peptide-stabilized, carbohydrate-based, and disulfide-bearing gemini surfactants [12].

2.3. Inorganic NPs

There are different types of inorganic NPs including Carbon Nano-Tubes (CNTs), graphene oxide, calcium phosphate NPs, Magnetic Nano-Particles (MNPs), silica NPs, Gold Nano-Particles (GNPs), silver NPs, and Quantum Dots (QDs) that are often used as the carriers for nucleic acid transportation [1,12]. In addition, zinc oxide NPs (ZnO NPs) are regularly utilized as different biomolecules delivery vehicles (gene, drug, etc.) [16]. CNTs are nanosized fibers with high specific surfaces [17]. Owing to their needle-like nanostructure, CNTs can traverse through the plasma membrane easily in an endocytosis way without causing considerable cell death [1]. They are usually insoluble materials that need surface functionalization for their stablization in solvents. Considerable attention has been paid to the CNTs (with either single- or multi-walls) owing to their wide applications in the field of gene therapy [9]. Magnetic NPs are another type of synthetic particles in submicron size that react with magnetic fields [1]. For example, super paramagnetic iron oxide NPs (Fe_3O_4) or SPIONs are used in these nucleic acid transferring systems [9].

Calcium phosphate NPs are extensively used for gene transfection as the in vitro that have been thoroughly inspected as an advanced non-viral nucleic acid delivery. Application of silica NPs have been recently suggested as the non-viral vectors of in vivo gene delivery [1]. Metallic NPs, especailly gold NPs, are superior to their counterparts in terms of their simplicity of the synthesis method, high efficiency in gene transfection, and high capability of their surfaces in undergoing chemical modifications [1].

QDs are successfully used for in vivo and in vitro gene transfection. These vectors are approximately spherical semiconductor NPs characterized by core-shell structures [1]. QDs are also called nanocrystals mainly because they are nanometer-sized monodisperse crystals [12].

Graphene is an attractive nanomaterial. This allotrope of carbon enjoys favorable thermal, optical, and electrical advantageous properties. For instance, Graphene Oxide (GO) in protection of nucleotides from cleavage, makes it a proper gene delivery vector [1,18].

2.4. Hybrid NPs

Hybrid NPs are divided into two groups of multi-layer and Liposome-Polycation-DNA (LPD) NPs. LPD NPs are primarily manufactured through the spontaneous reorganization of the lipid layer around the polycationic DNA core, and arrangement of polycations and polyanions Layer-by-Layer (LbL) results in the fabrication of multi-layer NPs. Contrary to the cationic polypeptides like histone, poly-L-lysine, and protamine that are able to flexibly package the DNA molecule, polycations in multi-layer NPs are condensing polyanions (e.g., DNA) in highly compressed nanometric structures [1,19]. There are other types of hybrid vectors. For instance, theranostic nanomaterials comprises both organic and inorganic NPs and provides specific disease management nanosystems by combining different NP platforms (therapeutic and diagnostic capabilities) into one biocompatible and biodegradable NP [7]. In fact, theranostic nanosystems are multifunctional [20]. Of note, hybrids of polysaccharides and proteins are sometimes utilized to fabricate gene delivery carriers. NPs based on the core-shell structures of Albumin-chitosan-DNA are inquired for gene transportation purposes. Further, dendrimer-like hybrid silica NPs are functionalized nano-scale carriers that can be appropriate candidates for simultaneous and efficient delivery of different types of drugs or genes with different sizes [1]. A list of NPs that are developed for gene delivery puposes are summarized in Table 1.

3. FABRICATION OF NPS

Advancement of clinically felicitous NPs for gene therapy still faces many problems such as biocompatibility and biodegradation, aggregation of NPs in physiological fluids, non-specific adsorption by non-target tissues, inefficient extravasation to reach desired tissues, unwanted entrance to the target cells, and endosomal escape [4].

Fabrication of the commonly used NPs is elaborated in the following with the main emphasis on some approaches that help overcome the mentioned problems.

3.1. Polyethylenimine

PEI is a cationic polymer that is commonly used as an effective nucleic acid delivery vector. As observed in Figure 3, there are two branched or linear forms of PEI. Gene transferring efficacy and cellular toxicity of PEI originally depend on its size, molecular weight, and polymer:nucleic acid charge ratio. In this regard, several researches highlighted that PEI with higher molecular weight (> 25 kDa) was more toxic than small and medium-sized polymers (5-25 kDa), hence less effective at gene delivery [9].

Typically, aziridine monomers are polymerized in

TABLE 1. Different types of NPs developed for gene delivery

Type of NPs	Materials that have been used	Ref(s)
Polymer-based NPs	Collagen	[1]
	Elastin	[1],[12]
	Fibronectin	[1]
	Silk proteins	[1],[9],[12]
	Albumin	[1],[12]
	β-casein	[1]
	Zein	[1],[12]
	Gelatin	[12]
	Poly-L-lysine (PLL)	[12]
	Chitosan	[1],[7]
	Alginate	[1]
	Heparin	[1]
	Hyaluronic acid	[1]
	Pullulan	[1]
	Dextran	[1]
	Cyclodextrins	[12]
	Lignin	[12]
	Polyethylenimine	[1],[12]
	Dendrimers	[1],[15]
	Poly lactic-co-glycolic acid (PLGA)	[1]
	PEG-CPLA copolymers	[12]
	Polyion complex micelles (PICs)	[1]
Lipid-based NPs	Liposomes	[1]
	Cationic lipids	[1]
	Cationic solid lipids	[1]
	Cationic emulsions	[1]
	Lipidoids	[12]
	Gemini surfactants	[12]
Inorganic NPs	Carbon nanotubes (CNTs)	[1],[12]
	Magnetic NPs (MNPs)	[1],[12]
	Calcium phosphate	[1],[12]
	Silica	[1]
	Gold	[1],[12]
	Silver	[12]
	ZnO	[16]
	Quantum dots (QDs)	[1],[12]
	Graphene oxide	[12]

aqueous or alcoholic solutions in order to prepare branched polyethylenimine polymers. Initial concentration of the components and temperature of the reaction are two key regulators in the procedure of constructing PEI of different molecular sizes that finally lead to production of randomly branched polymers. Similarly, linear structures of polyethylenimine result from the polymerization of cationic ring and unrolled ethyl-2-oxazoline to poly (2-ethyl-2-oxazoline). In this procedure, partial hydrolysis with an acid or base catalyst lead to the generation of linear polyethylenimines. The molecular weight and degree of branching can be controlled by changing the conditions of each process. Essentially, such high-charge polymers are proved to be ideal vectors for condensation of nucleic acids and transfer of genes in vitro and in vivo. However, high charges can cause damages to the cells and tissues due to their toxic nature. In general, the effectiveness of the PEI-based vectors can be affected by some parameters namely

the positive charge density, degree of branching, molecular weight, cross-linking, buffering capacity, etc. These parameters directly affect the DNA binding properties of the PEI, surface charge magnitude, and size of the prepared compounds [3].

PEIs with different molecular weights ranging from 430 to 800,000 Da were investigated in terms of their efficiency in gene transfer, and the transfection efficacy of 25 kDa PEI was found to be the best in vitro.

The cytotoxicity of the PEI results from the polymer aggregation on the surface of cells. On the contrary, less cytotoxicity of the low molecular weight PEI results from the diminished surface charge [21]. The stability of the PEI polyplex can be enhanced by modifying the periphery of the Polymer. The serum-tolerant capacity of the polyplex can be significantly improved by introducing hydroxyl groups. In addition, PEGylation as the most commonly used method can create a hydrophilic outer layer that lessens non-specific interactions with serum components and clearance by phagocytosis mechanism [7].
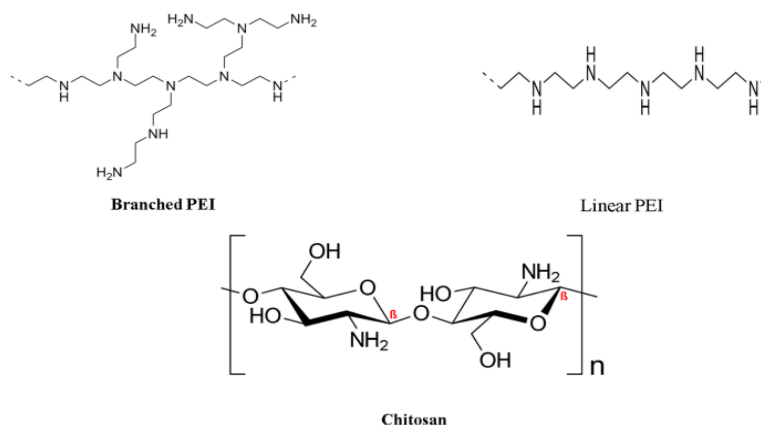

Figure 3. Structures of branched PEI, linear PEI [23], and chitosan [24]

3.2. Chitosan

Chitosan is derived from chitin which is the main component of cell walls of fungi, exoskeletons of crustacean and insect, and scales of fish. It is a positively charged polymer made of (1–4)-2-amino-2-deoxy-β-D-glucan (Figure 3) [22]. Owing to its cationic nature, the chitosan polyelectrolytes facilitate strong electrostatic interactions with mucus, mucosal surfaces with negative charges, and negatively charged macromolecules such as the DNA. In addition, presence of amine groups in the structure of chitosan made this biocompatible, biodegradable, and non-toxic polymer applicable as an attractive vector for non-viral gene transfer. Chitosan can form small stable particles (20 to 500 nm) with the plasmid DNA, and its binding efficacy depends on the molecular weight as well as the degree of deacetylation. Chitosan shows higher protective ability against DNase digestion and better biocompatibility than other polymers such as PEI. In the beginning, the efficacy of the DNA transfection by means of chitosan was slow; however, the transfection efficiency increased over time with lowering cytotoxic consequences in vivo [1].

Chitosan is a biologically degradable polymer, yet its limited transfection efficiency confines its application as a gene transferring vector [7]. Chitosan NPs can be chemically modified initiating from the base polymers or produced NPs. To this end, reactive hydroxyl and amino groups of chitosan are used at different temperatures in different alkaline conditions. Among the examples are alkylation, thiolation, carboxylation, quaternization, and PEGylation.

Chemical modifications is primarily performed as an additional process to improve the solubility, efficiency of encapsulation, and enzyme inhibition and adhesion properties [22].

3.3. Dendrimers

The role of dendrimers in gene, siRNAs, and antisense oligonucleotides transfer was also investigated in some studies. The positively charged surface groups of dendrimers can interact with negatively charged nucleic acids. Spherical nanoscopic polymers of PAMAM are a type of dendrimer generally used for nucleic acid transfection. Small dendrimers can provide better DNA binding efficiency than larger dendrimers.

In a study, a unique complex of PAMAM could cross the Blood-Brain Barrier (BBB). In fact, a peptide was derived from the glycoprotein of Rabies virus (named RVG29 peptide)[1] which is bound to PAMAM via bifunctional PEG as well as a system compounded with nucleic acids to generate PAMAMPEGRVG29/DNA NPs.

PAMAMPEGRVG29/DNA NPs confirmed more efficient crossing through the BBB than the

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[1] The sequence of RVG29 is YTIWMPENPRPGTPCDIFTNSRGKRASNGC

PAMAM/DNA in the in vitro BBB model57. It was taken up endocytotically by the endothelial cells of brain capillaries, a phenomenon that can be inhibited by free RVG29 [9].

3.4. Liposomes

Liposomal formulations optimized for gene transfer are usually composed of a complex of charged and neutral lipids (helper lipid), often DOPE or DOPC. These neutral lipids help form the lipid bilayer of liposome [9,12]. In DNA-binding studies mediated by N-(1-(2,3-dioleoyloxy) propyl)-N,N,N-trimethylammonium chloride (DOTAP), DNA was not efficiently combined with liposomes made without DOPE [9].

Cationic liposomes modified by grafting PEG or PEG-introduction methods demonstrated that in the presence of serum, the transfection efficiency of the conventional liposomal compounds was diminished while the related efficacy of the PEG-added compounds was retained. In addition, the transfection efficacy of the traditional gene delivery compounds considerably decreased during storage. However, the transfection efficiency remained stable for the PEG-containing liposomal gene transfer compounds even after storage for two weeks [25].

3.5. Gold NPs

Gold NPs (Au-NPs) possess flexible surfaces that support their functionalization. This allows nucleic acids to be directly combined with gold NPs. Coating of gold NPs with antibacterial Peptides (PEP) or Transactivators (Tat) of transcriptional peptides can be used for more efficient gene delivery to the stem cells [12].

For gold NPs, increasing the particle-to-DNA ratio (20:1) notably improved the transfection efficiency. Sandhu et al. studied gene transfer using gold NPs modified by N,N,Ntrimethyl(11-mercaptoundecyl) ammonium chloride and alkyl thiol in different chain sizes. Based on the best formulation for NP in their study, the transfection efficiency was about eight times more than that of the PEI [9].

3.6. Carbon Nanotubes

The small size of the CNTs and their chemical inactivity are attractive features for gene transfer but their hydrophobicity makes them less soluble in aqueous solution, thus limiting their applicability in biological systems. CNTs can be synthesized through covalent or non-covalent interactions to improve their dispersion and solubility. Oxidations and cyclo-additions are the two most ordinary covalent functionalization reactions. Another approach to non-covalent functionalization of CNTs is coating with amphipathic molecules such as Sodium Dodecyl Sulfate (SDS) or proteins [12]. Covalent modification of carbon nanotubes can be carried out using the 1,3-dipolar cycloaddition reaction of azomethine ylides. Both Single- and Multi-Walled Carbon Nanotubes (SWNT and MWNT) are functionalized with a pyrrolidine ring equipped with a free oligoethylene glycol moiety attached to the nitrogen at amino-terminal. Attachment of this functional group significantly increases the solubility of CNTs, especially in aqueous solutions [26].

4. MECHANISMS OF DNA-NP BINDING

One of the main applications of NP-based approaches is developing sensitive and specific medical diagnostics and delivery of nucleic acids to cells or tissues. Specific or non-specific molecular binding between nucleic acid molecules and NPs can provide high sensitivity. Covalent bindings between nucleic acids and NP surfaces are formed by anchor groups (–OH, –SH—COOH, or NH_2). In general, thiolated oligonucleotides can functionalize gold (Au) or silver (Ag) NPs to generate nucleic acid NP probes for specific recognition of complementary nucleic acid sequences in testing DNA mutations and polymorphism studies. In addition, non-covalent adsorption between nucleic acids and NPs result in non-specific interactions. Similar to the nucleic acid-protein interactions in vivo, this approach requires the affinity of non-covalent binding to control nucleic acid release in gene regulation or therapy. In this regard, a better understanding of the interactions between nucleic acids and NPs at the atomic level plays a crucial role in developing such approaches. Researchers declared that binding of short ss-DNA of 24-mer to 13 nm gold NPs could significantly prevent aggregation while complementary hybridized oligomers failed in stabilization of the gold NPs, thus resulting in aggregation of Au-NPs in saline mixtures [27].

Evaluation of the affinity with the deoxynucleosides-Au-NP binding confirmed the strong affinity between the four deoxynucleosides namely the Adenine, Thymine, Guanine, and Cytosine. Contrarily, the thymine showed the weakest affinity with the gold surface among the others. The negative charge distributed on the backbone of nucleic acid molecules mediate their adsorption on NP surfaces through the Fe-O-P interactions. Researches highlighted the crucial role of electrostatic interactions and hydrogen bonds formation in the adsorption of DNA/RNA to IONPs (iron oxide NPs) [28].

4.1. DNA−Gold NP Binding

Given that Au-NPs have negative charge on their surface, they are regarded as the selective vectors for delivery of ss-DNAs. The binding of the gold NPs to the ds-DNA is not favorable due to the higher repulsion between ds-DNA and negative charges on the surface of gold NPs. The longer ss-DNAs cause weaker interactions with Au-NPs, and binding of long ss-DNA molecules to gold NPs occurs just at high temperatures. In addition, the size of NPs is a significant part of interaction between

DNA and NPs [27,29].

4.2. DNA–Silver NP Binding

Silver NPs were functionalized to detect specific sequences of nucleic acids. Then, NPs of oligonucleotide-Ag provide ultrasensitive DNA detection systems. The results from several studies referred to the sequence-dependent interactions of DNA and Ag-NPs, thus suggesting the close affinity between nucleotides and silver NPs with variable attraction strength as the order C > G > A > T. It was also found that Ag NPs could efficiently bind to the ds-/ss-DNA molecules. As a result of the potential of negative zeta of the Ag-NPs, an electrostatic attraction between Ag-NPs and negatively charged DNA backbone would seem unlikely.

Accordingly, DNA-Ag complexes were formed through coordination coupling. Silver NPs could interact with N7 atoms of purines (A & G) and N3 atoms of pyrimidines (T & C) [30,31].

4.3. DNA-CNT Binding

Different nanomaterials of carbon were studied with the main focus on the DNA-NP binding, and the findings revealed that single-walled CNTs were bound to ss-DNAs. Molecular simulation studies also suggested that torsional and electrostatic interactions of DNA backbone may drive wrapping of DNA molecule around SWNTs in order to form compact helices that can be used in gene therapy approaches [32,33].

5. THE APPLICATION OF NPS IN GENOME EDITING

In recent years, a versatile genome editing system known as CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (Cas9)) has emerged as an efficient tool to carry out precise mutations and gene targeting. It can perform gene replacements, gene deletions/ insertions, and single base pair conversions [34].

Since the nuclear genome is the target of CRISPR/Cas9 complex, the components of this system are required to be transported to the nucleus. In this regard, CRISPR/Cas9 is needed to pass through the barriers of cellular and nuclear membrane and nanocarriers can fulfill this purpose. For example, nanocarriers based on polymers such as block polymer PEG-b-PLGA, lipid-based NPs, chitosan, and PEI or Au-NPs can transfer the CRISPR/Cas9 complex into nucleus [35].

6. DEVELOPMENT OF NPS FOR GENE DELIVERY AND CLINICAL APPLICATIONS

Gene therapy is the methodology of correcting genetic errors in living organisms either by delivery of exogenous integrating/non-integrating nucleic acids or modification of the gene expression to prevent or treat the disease. Recently, a wide variety of NPs have been functionalized for delivery of DNA and RNA to the cells or tissues of interest. These novel Nano-systems can be utilized as alternatives for viral vectors. Gene delivery through nanomaterials enjoys several advantages such as lower immune response than that of viral vectors, highly flexible design, low cytotoxicity, and feasibility of targeted gene delivery to the cells and tissues in vivo and in vitro [36].

Two basic approaches to gene therapy are (i) introduction of a functional gene in order to recover the function of related defective gene and (ii) antisense technology that is the delivery of interfering RNAs such as siRNA, micro RNA (miRNA) or short hairpin RNA (shRNA) to modulate post transcriptional gene expression by degrading the mRNA of interest or repressing its translation, or through Antisense Oligo-Nucleotides (AONS) that are single stranded nucleic acids directly finding their complementary sequences of mRNAs without the aid of auxiliary cellular mechanisms. The second approach is usually employed to treat tumors or other genetic disorders caused by upregulation of specific genes [36,37].

Nanomaterials are the delivery vectors for small RNA molecules [37]. For novel nanomaterials, the approaches to gene delivery are still developing. In this respect, the key challenges are how to make a balance between transfection efficiency, targeting specificity, particle size, biodegradability, and cytotoxicity as well as their short- and long-term fates in the environment [38].

Application of nucleic acid delivery in clinics is still in its infancy, and the FDA has not yet approved the NP-based gene therapy [1,4]. In the following, several cases of clinical applications of NPs in gene transfer are introduced (Figure 4).

A study took into account the HER2 expression based on some cancerous cells as a principal for developing the targeted NPs. HER2 is a tumor marker gene that is commonly upregulated in certain tumors such as ovarian and breast cancer. A monoclonal antibody called the Herceptin (HER) can selectively recognize HER2 and target the HER2+ cancerous cells [7]. Magnetic antifouling iron oxide NPs (IONPs) coated with block copolymer poly(ethylene oxide)-block-poly (γ-methacryloxypropyltrimethoxysilane) (PEObPγMPS) was also utilized to improve cell targeting by reducing non-specific uptake. Attachment of Herceptin, the antibody of HER2 or a single-chain fragment (ScFv) of anti-epidermal growth factor receptor (ScFvEGFR) antibody to the IONPs coated with PEO-b-PγMPS resulted in HER2- or EGFR-targeting IONPs (anti-HER2-IONP or ScFvEGFR-IONP). In vitro studies demonstrated that anti-HER2-IONPs could specifically bind to SK-BR-3, a HER2 overexpressing breast cancer
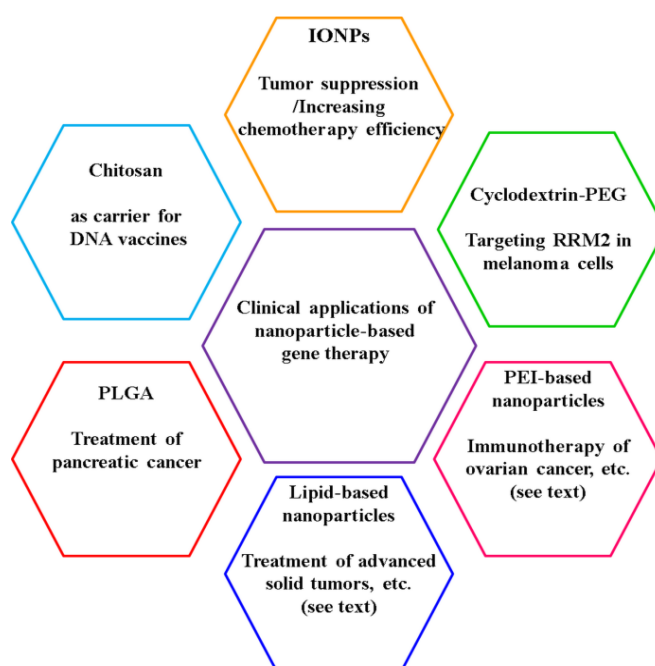
Figure 4. Clinical applications of nanoparticle-based gene therapy

cell line, but it failed to bind to the HER2 underexpressing MDA-MB231 cell line. Furthermore, ScFvEGFR-IONPs exhibited strong reactivity with EGFR-positive MDA-MB231 human breast cancer cell line but not with an EGFR-negative human breast cancer cell line, i.e., MDA-MB453. Transmission electron microscopy was employed to demonstrate the internalization of NPs targeting the receptors of cancerous cells. Of note, non-specific uptake of IONPs by macrophages of RAW264.7 mouse was reduced for both antibody-conjugated and non-antibody-conjugated NPs in vitro. The produced IONPs exhibited long persistence in blood circulation with the half-life of 11.6 hours in serum and lowered accumulation in spleen and liver of mice. Administration of ScFvEGFR-IONPs into the circulatory system of mice bearing EGFR-Positive breast cancer 4T1 mammary carcinoma showed a reduction in the magnetic resonance imaging signals in the tumors at 24 hours after administration due to the accumulation of the targeted IONPs [39]. These targeted IONPs can be complexed with therapeutic nucleic acids and applied as site specific gene delivery vectors to carry the nucleic acids for disease treatment purposes.

Davis et al. reported he first gene delivery system based on the NPs, called CALAA-01 in a Phase I clinical trial for cancer. CALAA01 is made of a polymer containing cyclodextrin, siRNA that target M2 subunit of ribonucleotide reductase (RRM2), PEG stereostabilizer agent, and ligand that target the transferrin for attachment of NPs to the transferrin receptors that are upregulated on the cancer cells 71. According to the results, the systemic administration of this "drug" carried the siRNA

component into the melanoma cells and potentially showed the antiproliferative effect on the multiple types of cancerous cells [4].

IONPs are a group of NPs that can carry the targeted nucleic acids, basically in the form of plasmid DNA or siRNA, in order to regulate the altered expression of genes resulting from the carcinogenesis process. It should be noted that the IONPs have the potential to improve the efficiency of gene therapy. The complexes of IONP-gene make feasible the delivery of nucleic acids to the organ of interest such as a tumor and function against the tumor either directly or indirecly.

In the direct delivery, IONPs that are bound to siRNA (siPLK1) act on a cell cycle-specific serine/threonine kinas (pololike kinase-1) and two peptides (MUC1 and) are injected into tumor-bearing mice. Then, the IONPs accumulate in the tumor, efficiently silence PLK1, and suppress the tumor by increasing apoptosis.

In the indirect delivery, IONPs that carry the phosphatase and tensin homolog (PTEN) gene increase the sensitivity of A549 / CDDP lung cancer cells to cisplatin treatment, indicating that PTEN can be effectively utilized against cisplatin-resistant lung cancer cells [40].

Inorganic MNPs are commonly used for gene delivery. Typically, MNPs in combination with a delivery platform encapsulate nucleic acids and facilitate their uptake by cells. However, the efficacy of the MNPs as nucleic acid carriers or drug delivery vectors depends on the modification of the outer surface of the NPs to permit binding of target molecules. The desired therapeutic molecules are attached to the NPs by cleavable linkers or

electrostatic attraction between the magnetic NP and target molecule. Novel reseraches have been conducted on how to find ultra-small and biologically compatible magnetic NPs that assist genetically modified cells such as macrophages and monocytes with efficient uptake by tumors after systemic administration [41].

In gene therapy, silica-based carriers are preferred to other non-viral/viral vectors due to their high safety level, flexibly modifiable surface and structure, great stability, and affordable costs. Silane is a versatile material with high combinatory features with lipids, polymers, and inorganic NPs. Silica NPs provide high loading capacities, efficient nucleic acid interaction and protection, specific tissue targeting, and cargo releasing. In this regards, several gene therapy approaches have been recently developed based on the silica NPs, e.g., forward and reverse transfection as well as sedimentation agents (non-porous NPs). Silica-based gene therapy yielded promising results both in vitro and in vivo for therapy or imaging purposes [42].

PEG – PEI - Cholesterol (PEGPEIcholesterol) was successfully developed as a carrier for gene transfer in the immunotherapy of epithelial ovarian with upregulated cytokine interleukin 12. Moreover, some gene therapy approaches with the main focus on the NPs are commonly employed in clinical trials such as PEI-based NPs which is used for treatment of ovarian, bladder, and pancreatic cancers, lipid-based NPs for treatment of advanced solid tumors, transthyretin amyloidosis, hepatocellular carcinoma, liver metastases, and lung cancer [4].

Poly (lactic-co-glycolic acid) (PLGA) is a synthetic polyester with favorable properties including thermoplasticity, biocompatibility, and aliphatic nature. Specific formulations were proposed based on this polymer and its relevant homopolymers, poly (lactic acid) (PLA), and poly (glycolic acid) (PGA). The potential of the PLGA NPs as nanocysytems for drug delivery was proved for many therapeutic agents including proteins, antioxidant drugs, anti-inflammatory, antibiotics, antiseptics, and chemotherapeutic agents. Then, it can be suitable for targeting tumors and/or DNA [43]. For example, clinical evaluation of the PLGA-based NPs are under consideration for the treatment of pancreatic cancer [4].

Chitosan-based NPs can used for delivery of both DNA and siRNA. Studies highlighted the potential of chitosan NPs as the DNA vaccine carrier and adjuvant for effective immunization through a non-invasive nasal route [44].

6.1. The Effect of the Size of NPs on Gene Therapy

The size of NPs is considered as a significant factor in their in vivo/vitro applications that also affects their cellular delivery, efficacy of transfection, bio-distribution, and cytotoxicity. The NPs of 200 nm or less in size typically benefits endocytosis mediated by

clathrin, and those of more than 200 nm in size are usually transferred through caveolar endocytosis. As shown, the NPs with the size of 100 nm and less efficiently enter a wide variety of cell lines while the NPs of 50 nm in size are the optimum size for uptake by cells. However, the impact of NP size on the transfection efficiency is, to some extent, conflicting. There are reports on the higher efficiency of transfection with smaller NPs (< 200 nm) while some others show the better transfection efficacy of larger NPs (> 200 nm). In addition, smaller NPs have larger surface areas capable of exposing higher percentage of molecules on their surface that led to high cytotoxicity of these NPs. Furthermore, small NPs, particularly NPs coated by biocompatible polymers (such as PEG), were found to be persistant in the blood circulatory system for a long time, and NPs conjugated to the targeting ligands showed better cellular penetrance. Overall, it can be concluded that the NPs with the size of 100 nm and smaller coated by biologically compatible polymers and attached to the targeting ligands can play a critical role in ensuring the success of nucleic acid transfer in clinical concerns [45].

7. TOXICITY OF NPS

NPs have great potential for improving gene therapy; however, their toxicity-related risks are inevitable. Determination of the toxicity and safety profile of a NP system for clinical utilization can be significantly challenging given the variety of factors involved including the size, shape, composition, stability, surface chemistry, electromagnetic properties of the nanopartciles as well as the genetic and existing conditions of the intended individual. Depending on the composition and size of the NPs, they can induce irreversible cell damages through oxidative stress and/or organelle damage [9]. Moreover, NPs can induce intracellular calcium (Ca^{2+}) perturbation in homeostasis, thus resulting in molecular actions attributed to energy and metabolic imbalances as well as cellular dysfunction [46].

The physicochemical properties of the developed NPs notably affect how they interact with the target cells and determine their potential overall toxicity. A better understanding of these properties can facilitate the fabrication of safer less toxic NPs [46].

Indeed, most inorganic NPs are biologically toxic. To be specific, while lipid-based and hybrid NPs are toxic in high doses, NPs based on polymers are less toxic to cells [1]. For example, the toxicity of the CNTs depends on their size. In animals, while single-walled CNTs are taken up easily by macrophages, the multi-walled CNTs have a carcinogenic effect similar to that of the asbestos after injection into a peritoneal cavity. However, when accumulated in the liver in the long run, single-walled CNTs will cause disturbance in some biochemical

parameters in the form of alanine transaminase, aspartate transaminase, LDH, malondialdehyde, and glutathione and alter the organ indices among the laboratory animals [47].

There are evidence of the adverse health effects of long exposure to NPs on the brain (neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease), cardiovascular diseases (Hypertension, atherosclerosis, thrombosis, arrhythmia, vasoconstriction, heart disease), lungs (emphysema, asthma, cancer), gastrointestinal system (colon cancer, Crohn's disease), and skin (dermatitis, autoimmune disease) [9].

8. CONCLUSION

Gene delivery to cells or tissues is a critical step in gene therapy of diseases with genetic or non-genetic origin in order to treat or alter the molecular mechanisms that cause different diseases. Transferring the genetic materials to the cells is bound to a number of biological and other limitations. To overcome these limitations and facilitate gene delivery process, noanoparticle-based carriers have been recently developed. These types of carriers are found in a wide variety including polymer-based, lipid-based, inorganic, and hybrid NPs that can be fabricated through different approaches and functionalized for different purposes. Despite the positive characteristics of each NP, they have some advantages and disadvantages that should be simultaneously taken into consideration in special gene therapy approaches. One of the main problems in the application of NPs as the gene delivery carriers is their toxicity. In this respect, a balance should be made between the advantages and disadvantages of each NP which is a critical step in transferring genetic materials by NPs that may result in the development of specific and safer clinically applicable gene therapy approches that are promising in treating many diseases.

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NOMENCLATURE

Au-NPs	Gold-Nanoparticles
CNTs	Carbon nanotubes
CPLA	Cationic polylactide
CRISPR	Clustered regularly interspaced short palindromic repeats
DOPC	Dioleoyl phosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DOTAP	Dioleoyl trimethylammonium propane
DOTMA	Dioleoyl propyl trimethylammonium chloride
ds-DNA	Double-stranded DNA
DSPE	1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine

EGFR	Epidermal growth factor receptor
FDA	Food and drug administration
GNPs	Gold Nanoparticles
GO	Graphene oxide
HER	Herceptin
IONPs	Iron oxide Nanoparticles
LbL	Layer-by-layer arrangement
LNP	Lipidoid Nanoparticles
LPD	Liposome-polycation-DNA Nanoparticles
miRNA	Micro RNA
MNPs	Magnetic Nanoparticles
MWNTs	Multi-walled carbon nanotubes
NPs	Nanoparticles
PAMAM	Polyamidoamine
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEG-b-PLGA	Polyethylene glycol-b-poly (lactic-glycolic acid)
PEI	Polyethyleneimine
PGA	Poly glycolic acid
PIC	Polyion complex micelles
PLA	Poly lactic acid
PLGA	Polylactic glycolic acid
PLL	Poly-L-lysine
PTEN	Phosphatase and tensin homolog
QDs	Quantum dots
RRM2	M2 subunit of ribonucleotide reductase
RVG29	Peptide derived from the glycoprotein of rabies virus
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLNs	Solid lipid Nanoparticles
SPIONs	Super paramagnetic iron oxide Nanoparticles
ss-DNA	Single-stranded DNA
SWNTs	Single-walled carbon nanotubes

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