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Review Article

Development of Magnetic Nanoparticles for Cancer Gene Therapy: A Comprehensive Review

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Since they were first proposed as nonviral transfection agents for their gene-carrying capacity, magnetic nanoparticles have been studied thoroughly, both *in vitro* and *in vivo*. Great effort has been made to manufacture biocompatible magnetic nanoparticles for use in the theragnosis of cancer and other diseases. Here we survey recent advances in the study of magnetic nanoparticles, as well as the polymers and other coating layers currently available for gene therapy, their synthesis, and bioconjugation processes. In addition, we review several gene therapy models based on magnetic nanoparticles.

1. Introduction

1.1. Cancer & Current Therapy. Although huge efforts have led to advances in cancer treatment, this multifactorial and heterogeneous disease is still one of the major causes of death in the majority of countries [1–4]. Several factors influence the high death rate of cancer patients around the world, such as the genetic and phenotypic heterogeneity and the highly unstable genome of cancer cells, which ultimately leads to continuously emerging, nonheterogeneous cells from the tumor nest, and subsequent metastases [5].

Current cancer therapy encompasses a wide variety of treatments from systemic cytostatics to targeted therapy agents, most still in development phases, such as kinase inhibitors [6–9], antibodies [10, 11], small molecules, or cell- and antigen-based immunotherapies [12, 13]. Nevertheless, most approved therapeutics require systemic administration, which increases their toxicity and other clinical complications. One factor that eventually contributes to therapeutic toxicity is nonspecificity. Guidance of drugs to the desired tissue and specific target recognition are therefore major concerns faced by cancer researchers seeking less toxic side effects and improved efficiency of therapy.

Targeted strategies include ligand-receptor binding, antibody-antigen specificity, and some other forms of active targeting. For instance, several antibodies have been developed that are directed to specific tumor-associated antigens, either expressed uniquely by tumor cells or overexpressed compared with healthy tissue; this is the case of the HER2-specific antibody Herceptin [10], the VEGF-specific antibody Bevacizumab [11], and ganglioside-specific antibodies such as 14F7 [14].

Nanotechnology, and in particular a magnetic nanoparticle-based approach, is a promising tool for the guidance of therapeutic agents into tumor tissues.

1.2. Nanotechnology in Cancer Theragnosis. Use of nanotechnology is widespread in several fields of biomedicine due to its potential in controlled drug delivery systems [15, 16], for diagnostic agents [17] and biosensors [18], among other applications. The basis for their use in biomedicine centers on the combination of their distinctive physical properties, including their optical, electronic, and magnetic behaviors. These properties arise from the combination of two characteristics: a large surface area and the quantum confinement effect due to the nanoscale sizes. Nanoparticles

are usually coated with polymers or other coating layers that could endow additional functions.

One promising physical property of nanosystems in cancer biomedicine is their magnetism, especially superparamagnetism [19]. Two physical parameters theoretically define magnetic materials, magnetization or magnetic polarization, and the applied magnetic field (H). When materials show linear dependence between these two parameters as well as low maximum magnetization, they are classified as weak magnetic materials. In addition, these materials can be subdivided into paramagnetic and diamagnetic substances, depending on whether magnetization is positive or negative.

Strong magnetic materials differ from the above not only in their high magnetization but also in their complex behavior following exposure to an external magnetic field. This behavior is described by the hysteresis loop. Strong magnetic materials are classified as ferrimagnets, ferromagnets, and antiferromagnets, among others. There is a critical temperature value (Curie temperature and Neel temperature for ferrimagnets and antiferromagnets, resp.), below which magnetic polarization increases up to saturation. Saturation magnetization decreases as the temperature increases below these critical values. Ferromagnets show remanent magnetization when the external magnetic field is off. Above critical temperature, these materials become superparamagnetic, with a reversible magnetization curve (Figure 1) and zero magnetic moment when the magnetic field is removed; magnetic moments reach values more than 10 to 4 times larger than those for paramagnetics if an external magnetic field is applied.

Particles derived from the ferro/ferrimagnetic bulk state and nanosized to as small as 1 to 30 nm diameter, depending on the material, behave superparamagnetically [20]. Nanosystems in this state basically show linear dependence of magnetic susceptibility and applied magnetic field intensity, as paramagnets do, but almost constant magnetization once magnetic polarization is saturated. Superparamagnetic nanosystems show no hysteresis and negligible remanent magnetization.

Different areas or domains in which atoms are pinned in the same direction commonly form ferromagnetic materials. This phase separation is due to the gain of stability caused by a decrease in magnetostatic energy as new domains arise inside the bulk. The formation of new magnetic domains nonetheless requires a certain amount energy, which constrains the size resulting from the break-bulk. This is because the energy needed to form magnetic domains surpasses the magnetostatic energy, making the process infeasible. Below a certain size, a single-domain structure thus prevails. Therefore, magnetic nanoparticles due to their size show a single domain structure.

Magnetic nanoparticles are applied in cancer theragnosis as contrast agents [21–23], for example, for magnetic resonance images (MRIs). Superparamagnetic iron oxide nanoparticles (SPIONs) must nonetheless be able to evade the immune system to slow down their clearance from the bloodstream and other biological fluids. SPIONs are eliminated from the circulation mostly through the reticuloendothelial system (RES) and renal clearance mechanisms [24–29].

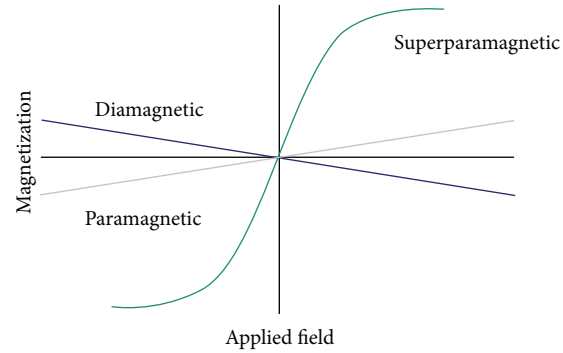

Figure 1: Magnetic behavior of materials as determined by relationship between magnetization and applied magnetic field.

One factor that influences nanoparticle clearance is their hydrodynamic size. In general, particles between 10 and 100 nm have the longest circulation time [30]; by contrast, 200 nm diameter particles tend to be eliminated by the RES, and those with diameters <10 nm are removed mainly by renal filtration.

Nanoparticle size also affects their ability to extravasate from the bloodstream into tumor tissues, through the socalled transcytosis process [31, 32]. This process depends on particle size, with an upper limit of ~150 nm. Another important factor is the chemical nature of the nanoparticle surface [33]. For instance, positive surface charge allows nanoparticles to adhere nonspecifically to cell membranes whilst negative surface charge leads to adsorption of plasma proteins, thus increasing uptake and clearance via the RES [34, 35]. Furthermore, plasma protein interaction with negatively and positively charged nanoparticles induces different biological responses [36]. Hydrophilic and neutral surfaces endow nanoparticles with enhanced stealth, as they interact less with blood proteins. The rationale and evidence for the distinct factors that affect nanoparticle-biological interfaces have been reviewed elsewhere [37].

Passive SPION-based cancer theragnosis relies on specific features of the tumor microenvironment. These characteristics allow nanoparticles to permeate tumor tissue without extra active forces, though nanoparticle concentration can be increased if such forces are used. The disorganized, leaky, damaged vasculature of tumor tissue due to accelerated angiogenesis facilitates SPION permeation of the tumor microenvironment [38]. Once inside tumor tissue, nanoparticles are retained due to the poorly developed lymphatic vessels; this phenomenon is known as the enhanced permeability and retention effect (EPR) (Figure 2). This high grade of retention in tumor tissues allows most nanoparticles to be taken up by tumor and stromal cells, including immune cells. According to some reports, upper limits for macromolecule size to show the EPR effect could be up to 1-2 μm, as for *Lactobacillus* sp. [39], which accumulate preferentially in tumor tissues.

Modification of the tumor microenvironment is indeed another way to achieve better nanoparticle uptake by malignant cells. These approaches intend to increase the EPR effect

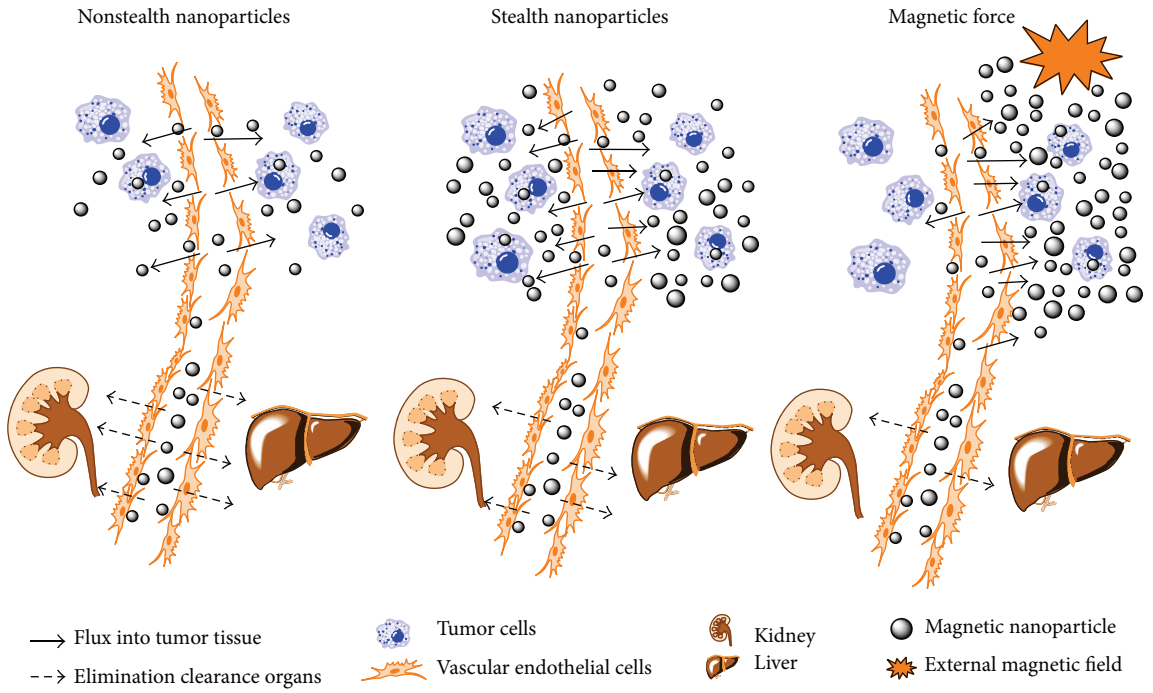

Figure 2: The enhanced permeability retention effect (EPR) in a scenario of magnetic nanoparticle-based cancer therapy. Due to the weak tumor vasculature, nanoparticles are able to permeate the tumor microenvironment, although clearance organs and the reticuloendothelial system (RES) eliminate most. Polymers/moieties endow nanoparticles with stealth and specificity and thus with RES-evading capacity; nanoparticle concentration in tumor tissue consequently increases. This nanoparticle accumulation in targeted tissue further increases as an external magnetic field is applied.

inherent to solid tumors; for instance, application of a single 15 Gy-radiation dose in a syngeneic mouse breast tumor model doubled the accumulation of iron oxide nanoparticles in tumor tissue [40]. This effect is associated with decreased interstitial pressure and the subsequent increase in vascular permeability and thus provides a new tool to improve drug delivery into the tumor.

Various strategies have been studied to endow nanosystems with stealth (Figure 2). For instance, when the proportion of hydrophilic and hydrophobic polymers is changed with negligible variation in particle size, the charges and core composition of spontaneously self-assembled micelles hindered their elimination by RES [41]. When tracked *in vivo*, some of these micelles accumulated less in liver, while they were more concentrated in blood at 1 h after intravenous injection, as compared with PEGylated micelles.

2. Synthesis of Superparamagnetic Iron Oxide Nanoparticles

According to the LaMer theory [42], nucleation and growth processes for the formation of uniform nanoparticulates can be divided into three phases. As the monomer concentration increases, the system accumulates energy during phase I. Once a supersaturated concentration is reached, the system has sufficient energy for a nucleation burst that will be homogeneous; hence, a narrow-size dispersed colloid is obtained. The presence of seeds such as crystallites in the medium

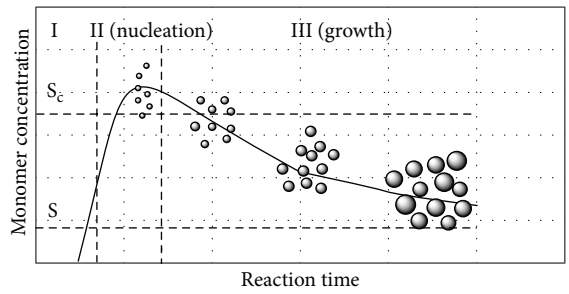

Figure 3: The LaMer theory describes the synthesis of monodisperse nanoparticles. S and S_c are supersaturation and critical supersaturation, respectively.

would induce heterogeneous nucleation, increasing the range of nanoparticle diameters (Figure 3).

One of the most popular chemical methods for SPION synthesis is based on coprecipitation of iron compounds [43–47]. Two approaches can be used; the first takes advantage of oxidizing agents such as nitrates [43] to induce partial oxidation of ferrous hydroxide whereas the second takes place in an oxygen-free environment in which an alkaline suspension is added to an aqueous solution of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions [44]. Using the second approach, Massart obtained magnetic nanoparticles of ~10 nm [44]. Using the first approach, however, Sugimoto et al. obtained larger magnetic nanoparticles with diameters from 30 to 200 nm [43].

Among factors affecting these methods, pH and ionic strength contribute the most to the efficiency and quality of the chemical reactions [43–47]. These parameters affect nanoparticle size, in turn an indicator of their dispersion stability.

Due to the properties of aqueous microdroplets that allow the confinement of growth and agglomeration processes, the microemulsion approach renders more uniform and smaller nanoparticles [48, 49]. Assembly of surfactant-stabilized aqueous microdroplets into an oil suspension that contain iron precursors leads to controlled size SPION synthesis as the precipitation of iron oxides occurs inside the confined space [50]. Another suitable method for synthesis of uniform nanoparticles is based on polyol. When reduced in a polyol solution, metallic ions precipitate as metallic nanoparticles with size ~100 nm [51, 52]. Sun et al. reported similar results when they synthesized 3 to 20 nm-diameter monodisperse magnetic nanoparticles by thermal degradation of iron (III) acetylacetonate in phenyl ether, in the presence of alcohol, oleic acid, and oleylamine at 265°C [53]. Other methods are based on dendrimer platforms onto which SPION can be precipitated uniformly [54] and on the use of high-energy ultrasound waves [55, 56]; the application of these waves induces hot empty spots that in turn randomly emit energetic waves that eventually lead to a reduction in nanoparticle size. Magnetic nanoparticles have also been synthesized by electrochemical deposition of metal on a cathode, produced by reduction of metal ions dissolved from the anode [57, 58], similar to polyol-based synthesis.

Emerging tools for magnetic nanoparticle synthesis use pyrolysis either by spraying a solution of Fe^{3+} salt and reducing agents and subsequent vaporization of the droplets obtained [59, 60], or by CO_2 laser heating of flowing gases containing iron precursors [61, 62]. When a critical concentration of nuclei is reached due to pressure and laser intensity, the process of homogeneous nucleation of nanoparticles begins. Iron pentacarbonyl is usually used as a precursor for synthesis of γ-Fe_2O_3 nanoparticles by the laser-induced pyrolysis method. Table 1 summarizes current methods for magnetic core synthesis.

2.1. SPION Functionalization. SPIONs are coated with polymers to confer colloidal suspendability. Polymers, such as hydrophilic polymers, act as stabilizers or endow nanoparticles with biocompatibility [63]. There can also be coupling of physical properties of superparamagnetic core and polymeric matrix. This coupling would depend on an external magnetic field whose power is transferred to the surrounding matrix, inducing conformational changes. The combination of magnetic nanoparticles with polymers sensitive to temperature changes induced by an external magnetic field is thus of interest [64–67].

Drug delivery systems combined with magnetic nanosystems have received constantly increasing attention (Figure 4). The possibility of summing properties increases the efficiency of drug delivery as well as the evasion of the immune system and RES. One approach that has received particular attention and effort is the magnetoliposome [65, 68–73]. Magnetic

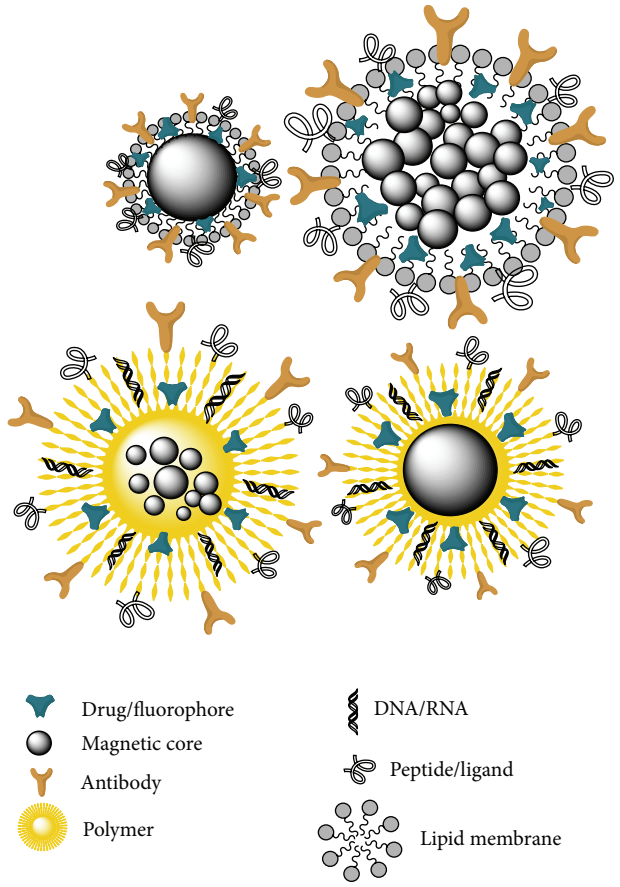

Figure 4: Scheme of magnetic nanoparticles based on current reports. Two main types of magnetic nanocarriers are depicted: magnetoliposomes with a lipid membrane structure surrounding either a single magnetic core or a group of them, and a biodegradable polymer encapsulating a single magnetic core or group. Hydrophilic/hydrophobic balance defines the type of drug that can be adsorbed, including genetic cargo. Molecular moieties can be attached electrostatically or covalently.

nanoparticles can be embedded in the lipid bilayer or inside the liposome, depending on the coating hydrophobicity [68–73]. The former strategy might destabilize the bilayer by physical movement rather than by heat dissipation; a lower magnetic nanoparticle concentration is therefore needed to induce drug release. Nanoparticle size must be controlled to avoid further destabilization of the magnetoliposome, and an appropriate coating is mandatory. The second approach is more feasible due to the availability of hydrophilic layers, but a larger number of magnetic nanoparticles are needed to achieve the temperature increase and thus the lipid layer destabilization that leads to drug release.

Using an external magnetic field, Mikhaylov et al. showed that ferri-liposomes with SPION clusters encapsulated inside liposomes were able to reach tumor tissue in an immunocompetent tumor-bearing FVB/N mouse model [68]. Administration of the cysteine cathepsin inhibitor JPM-565 embedded in ferri-liposomes showed tumoricidal activity, as they targeted not only tumor but also stromal cells. Moreover,

Table 1: Summary of synthesis methods for magnetic nanoparticles and their features.

Method	Temperature (°C)	Solvent	Size range (nm)	Morphology
Coprecipitation	20–90	Water	15–200	Spherical or rhombic
Microemulsions	20–50	Organic	4–12	Spherical or cubic
Hydrothermal synthesis	220	Water-ethanol	520	Spherical
Sol-gel synthesis	200–400	Organic	20–200	Spherical
Electrochemical deposition	70–100	Organic	3–8	Spherical
Sonochemical method	25	Water	10–30	Spherical or rod shaped
Polyol method	120–280	Organic	5–40	Spherical
Thermal decomposition	100–320	Organic	3–20	Spherical
Spray pyrolysis	400–700	Organic	5–60	Spherical but aggregated
Laser-induced pyrolysis	1100	Organic	5–30	Spherical less large
Biomimetic synthesis		—	50–100	Spherical cluster, cubo-octahedral

better MRI sensitivity was observed, indicating another function of these ferri-liposomes.

In another approach, the ability of iron oxide to dissipate heat locally after exposure to an alternating magnetic field was combined with the cargo-carrying property of liposomes [74]. A distinct concept was tested in which magnetic nanoparticles were not inside the liposome, but embedded side by side in a calcium alginate hydrogel microparticle for temperature-controlled drug release.

To design an early diagnostic method and targeted therapy for pancreatic cancer, Deng et al. obtained ultrasmall superparamagnetic iron oxide nanoparticles onto which doxorubicin and antimesothelin antibody were inserted [75]. By targeting mesothelin-overexpressing pancreatic tumors, these nanoparticles showed not only correct resonance contrast but also specific targeting in two models. Dimercaptosuccinic acid (DMSA)-coated magnetic nanoparticles have likewise been tested for their ability to absorb IFN-γ [76] and their therapeutic efficiency in tumor immunotherapy. These nanoparticles functioned correctly as an MRI agent [77] and also markedly reduced tumor growth in a mouse pancreatic tumor model (Pan02) [78]. IFN-γ attachment to the nanocarriers did not affect its function, as different immune cell subsets such as T cells and macrophages were detected in tumor niches.

Although a variety of functionalization methods have been studied thoroughly, a general methodology can be followed and materials used in the processes will depend on the aim of the nanosystem. Most magnetic nanosystems are coated with a layer that supplies the magnetic core with a biocompatible, modifiable platform. If the goal is to use the nanosystem as a diagnostic agent, for instance in MRI, no other chemical processes will be needed, although further modifications can be used to increase SPION ability to avoid the RES.

Current and future perspectives address not only specific targeting of nanosystems to tumor tissue but also transport and controlled release of cargo; additionally, more complex chemical modifications have been exploited to achieve these goals. Some of these alterations include electrostatic or covalent insertion of molecules. The chemical process basically involves three types: direct conjugation, often used to conjugate drugs or fluorescent molecules (Figure 5) [79–81]; linker-mediated conjugation, which is suitable for ligand attachment (Figure 6) [82–86]; and physical interaction-mediated adsorption, best for assembling therapeutics such as siRNA onto nanoparticle surfaces (Figure 7) [87–89]. Other methods can also be used, such as click chemistry [90, 91] and hybridization [92, 93].

2.1.1. Making SPIONs Feasible for Gene Therapy. In addition to the superparamagnetic features of SPION, other key properties must be present in the nanocarrier for correct cargo delivery. For further conjugation, polymers must endow SPION with reactivity to nucleic acids. This reactivity might be based on physical interaction, that is, electrostatic forces, or on reactive residues through which polymer and nucleic acid react. Polymers onto which DNA or RNA will be encapsulated must have a positive surface charge for the electrostatic conjugation of nucleic acids; alternatively, both polymer and nucleic acid must be modified with reactive chemical residues for covalent conjugation. Although both strategies have been used, the former is preferred, as it does not modify nucleic acid integrity.

Once DNA/RNA is encapsulated onto SPION, the complex must assure cargo stability and integrity. Nucleic acids are very labile in the bloodstream and tissue microenvironment, due to nucleases that recognize the 2-hydroxyl residue of the sugar ring and catalyze alkaline hydrolysis [94]. Cell uptake also plays a pivotal role in DNA elimination, for example, in liver endothelial cells [95]. In addition, the encapsulation must hamper TLR7/8 recognition of DNA/RNA [96, 97] to avoid an immune response to nanocarriers, leading to early elimination from circulation.

Once at the tumor site, nanoparticles must be able to permeate the tumor matrix. Polymers used for gene cargo should protect nucleic acid from the acid microenvironment that frequently surrounds tumor tissues. Tumor cells must subsequently take up most of the nanocarriers that, once internalized, must evade lysosomal degradation to deliver the cargo into the cytosol.

Current problems with gene therapy in cancer are associated with poor expression of the therapeutic agent, due to

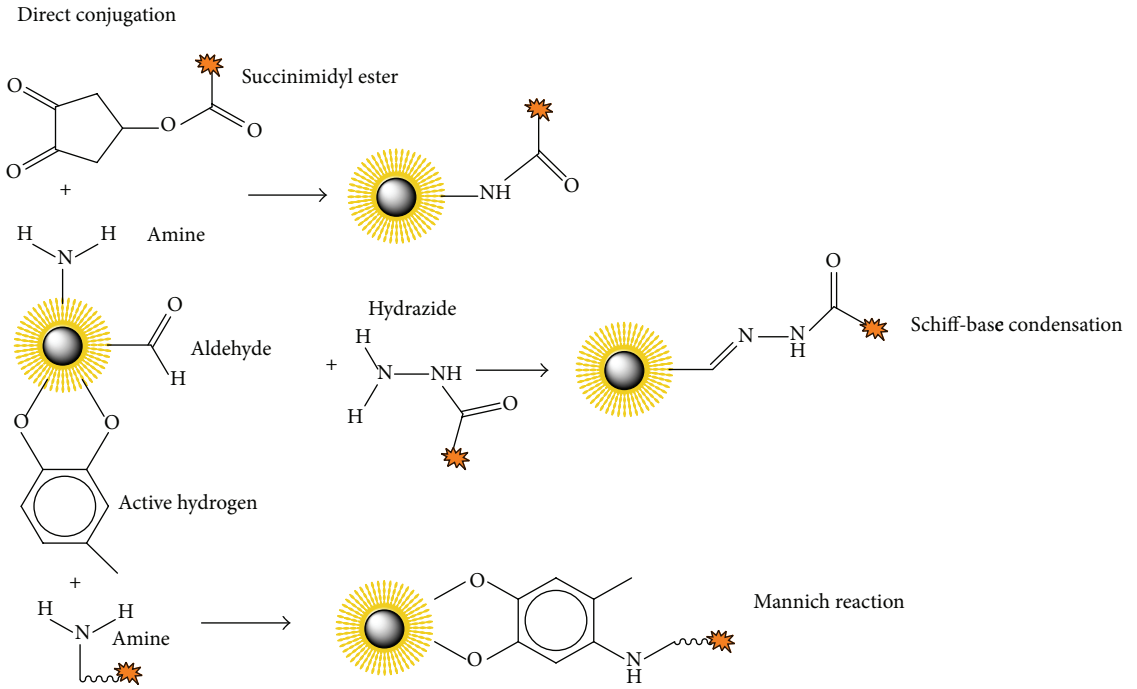

Figure 5: Representation of direct conjugation synthesis.

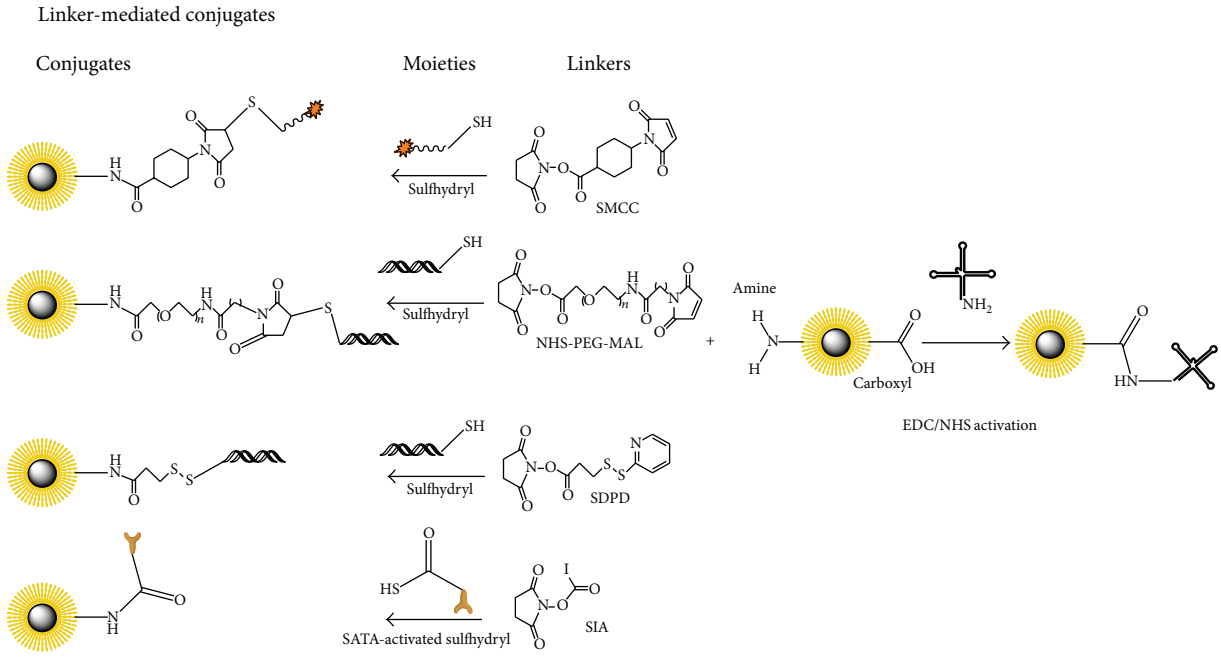

Figure 6: Representation of indirect conjugation through linkers.

all of the biological barriers mentioned. A variety of polymers has been developed for use in gene therapy, in which chemical modification often leads to increased gene transfection both *in vitro* and in *in vivo* models.

Polyethylenimine (PEI), one of the most popular polycations for gene transfection, has been modified with the amino acids arginine (Arg), lysine (Lys), and leucine (Leu) [98]. Intravenous administration of these modified PEI complexed with a β-galactosidase expression vector improved gene expression efficiency by threefold compared with the unmodified PEI polyplex. In another report, although the oligo (benzylethylenimine)-b-polyethylenimine (OBzEI-PEI) DNA complex profile was similar to its linear PEI counterpart, OBzEI-PEI showed higher gene transfection

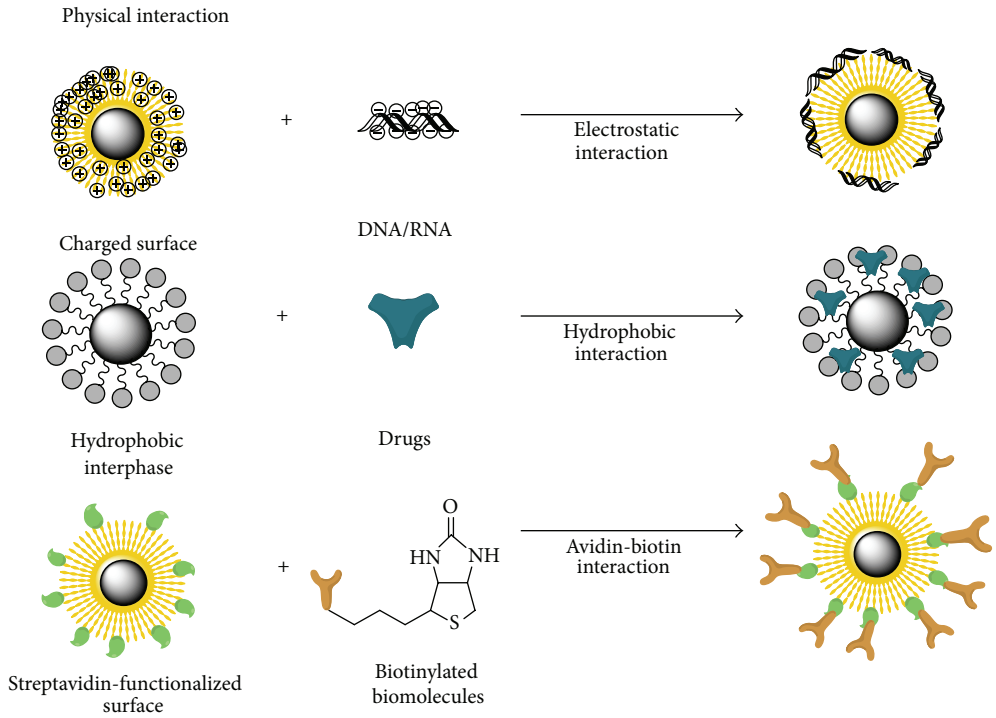

Figure 7: Representation of physical interactions.

efficiency in several cell lines despite decreased cell uptake of plasmid [99]; the OBz-EI moiety also contributes to the sponge effect of PEI, mediated by the pH-sensitive membrane-disrupting activity.

In an attempt to elucidate the effect of the hydrophilic/hydrophobic balance in PEI on function, Fan et al. reported that transfection of HeLa cells with copolymers of different degrees of hydrophobicity showed that Pluronic addition enhances gene expression [100]. This effect is apparently associated with homogeneous plasmid distribution in the cytoplasm due to the greater hydrophilic/hydrophobic ratio of Pluronics; in contrast, a lower ratio promoted nuclear localization.

Linear PEI appears able to transfect cells efficiently *in vitro* and *in vivo*. Goyal et al. synthesized a set of linear PEI nanoparticles (lPEI, 25 kDa) by varying the percentage of the crosslinker 1,4-butanediol diglycidyl ether (BDE) [101]. Compared with linear PEI and other available transfection agents, all BDE-crosslinked linear PEI showed higher transfection efficiency in terms of specific siRNA silencing of GFP (Green Flourescent Protein). Although both polymers, 25 kDa branched PEI (b-PEI) and 22 kDa linear PEI (l-PEI), are able to package DNA and siRNA, there are important differences between them. First, the b-PEI/DNA complex is less efficient than its l-PEI/DNA counterpart in the transfection of the luciferase gene. Whereas the bPEI/siRNA complex induces gene knockdown, the l-PEI/siRNA silencing effect is negligible [102], presumably due to poor stability of the complexes.

In addition to the sponge effect of PEI, its combination with other moieties that add new or enhanced properties to complex has been exploited exhaustively. One goal of such a strategy is to integrate gene therapy with other current therapeutics. When ataxin-1 siRNA, in complex with PEGylated chitosan modified with the cell membrane-penetrating peptide TAT, was delivered *in vitro* to neurodegerative spinocerebellar ataxia (SCA1) cell cultures, ataxin-1 was downmodulated at 48 h after transfection [103]. Similarly, PEI-PEG (polyethylene glycol) copolymers modified with the TAT peptide and loaded with doxorubicin not only packaged DNA but also delivered doxorubicin after acid cleavage and penetrated the cell membrane, mediated by TAT [104]. Adsorption of sulfamerazine (SA)-PEG-NGR on the surface of the nanopolyplexes provided double targeting to tumor vascular endothelial cells and tumor cells.

To increase PEI/DNA complex stability, He et al. established PEI/DNA complexes with hyaluronic acid (HA), previously disulfide bond-modified or unmodified [105]. The resulting complexes not only showed less cytotoxicity but also increased transfection efficiency in HA receptor-positive HepG2 and B16F10 cell lines.

An appropriate balance of modified primary PEI amines is a key factor that affects DNA complexation, transfection, and PEI cytotoxicity. When carboxylated with bromoacetic, 6-bromohexanoic, 10-bromodecanoic, and 16-bromohexadecanoic acid, the higher degree of PEI substitution or a longer alkyl chain was associated with decreased DNA binding capacity [106]; cytotoxicity decreased concomitantly and transfection efficiency increased. In another attempt to hinder the intrinsic cytotoxicity of PEI, Patnaik et al. modified b-PEI/DNA by adsorption of hexametaphosphate [107]. The resulting polyplex protected DNA from

DNases *in vitro* and efficiently transfected cells, either with a GFP gene expression vector or with siRNA.

Despite its limited solubility in physiological media, the linear polysaccharide chitosan has been thoroughly studied as a gene delivery agent [108]. Toh et al. obtained succinylated chitosans that were more efficient for gene transfection compared with unmodified chitosan, with lower cytotoxicity [109]. Chitosan has also been used as a polymeric material for developing theranostic nanocarriers. Na et al. designed a chitosan-based nanocomposite carrying Cy5.5 and paclitaxel for live imaging and cancer treatment, respectively [110]. Theranostic nanoparticles require long-term stability in the bloodstream and rapid uptake by tumor cells [111]. Other cationic polymers used for gene transfection include HBP-DEAPA 60, a biodegradable amine modified hyperbranched polyester whose transfection efficiency was studied in A549 cells [112].

EPR underlies the passive targeting of most nanocarrier-based cancer therapy although active targeting is needed to increase the efficiency of these approaches. In the development of a gene-carrier platform, some strategies for polymers feasible for gene therapy require modification with targeting moieties. Moieties such as antibody [113–115], peptides [116–118], aptamers, and ligands [119] endow nanosystems with tumor specificity. Apart from the EPR phenomenon and magnetic field application in the case of magnetic nanocarriers, modification with targeting moieties consequently increases nanocarrier concentration in tumor tissue and thus the *in vivo* efficiency of gene therapy.

2.1.2. SPIONs as Gene Nanocarriers. The magnetic field-dependent concentration of magnetic nanocarriers in a desired site has drawn attention to their potential as gene carriers. Magnetic nanocarriers not only protect genetic cargo from degradation, but also deliver this cargo correctly into targeted cells, thus resolving some problems of gene therapy. Since magnetic nanoparticles were first proposed as a feasible nonviral transfection agent [120, 121], magnetofection (magnetic field-guided transfection) has been studied thoroughly in *in vitro* and *in vivo* models.

Coating magnetic nanoparticles with PEG-g-PEI copolymer rendered suitable nanocarriers for MRI function *in vivo* in lung and liver [122]. These nanoparticles delivered a siRNA against the CD44 variant 6, a putative marker for metastatic behavior of gastric tumors, intracellularly into the SCG-7901 cell line. Nevertheless, when tested in nude mice bearing both SCG-7901 and A375-derived tumors, PEG-g-PEI-SPION/siRNA complexes were unable to infiltrate tumor tissue and thus did not contrast tumors adequately. PEG-g-PEI-SPION delivered the IL-10 gene efficiently [123]; IL-10 expression triggered by magnetofection in primary vascular cells (HUVEC) conditioned the medium to induce downmodulation of TNF-α-triggered PAI-1 expression in these cells. PAI-1 is responsible for some vascular pathologies such as inflammation and atherosclerosis.

Further modification of PEG-g-PEI-SPIONs by attachment of a neuroblastoma-specific ligand (GD2 single chain antibody) and BcL-2 siRNA was delivered *in vitro* and *in vivo* to the SK.N-SH human neuroblastoma cell line [124]. The downodulation of BcL-2 induced an increase in cell apoptosis rates and reduced tumor growth when the nanoparticule complex was injected subcutaneously into SK-N-SH tumor-bearing female mice.

Lee et al. developed thermally crosslinked SPION that were subsequently modified with branched PEI of 1800 Da [125]. The p53 plasmid/PEI-coated SPION nanoplexes had a hydrodynamic diameter ranging from 100 to 130 nm; *in vitro* transfection of wild-type tumor suppressor p53 suppressed tumor cell proliferation. Another report demonstrated the suitability of low molecular weight PEI for gene therapy by alkylating PEI 2 kDa and absorbing it onto magnetic nanoparticles [126]. These nanoparticles were taken up *in vitro* by fluc-4T1 murine mammary cancer cells (which express luciferase), as determined by confocal laser imaging. Once injected into the tumor, alkyl-PEI 2 kDa-magnetic nanoparticles conjugated with siRNA specific for the eluciferase gene, partially silenced luciferase activity, even after a single shot. Second and third intratumor injections further decreased the luciferase activity.

An interesting approach took advantage of the unique properties of the socalled yolk-shell nanocapsules. These nanosystems are composed of a magnetic core, interstitial hollow spaces, and a shell. Zhang et al. proposed a Fe_3O_4 magnetic core and a PEI-coated fluorescent mesoporous SiO_2 shell [127]. Nanoplexes obtained by attachment of β-actin siRNA to the yolk-shell nanosystems efficiently silenced β-actin in HeLa cells. In addition, the fluorescent shell allowed nanoparticle tracking simultaneously with the magnetic field-dependent guidance.

By combining minicircle β-galactosidase DNA with magnetic nanoparticles coated with a stearic acid-modified low molecular weight PEI, Gao et al. were able to target the liver [128]. As early as 3 h after intrabiliary infusion, accumulation of magnetic nanoparticles was evident according to T_2-weighted images. Immunohistochemical analysis of β-galactosidase expression and Prussian blue staining clearly demonstrated that magnetic nanoparticles accumulated in liver tissue and efficiently induced β-galactosidase expression.

In addition to PEI, other polymers have been used extensively to coat magnetic nanoparticles. Poly(maleic anhydride-alt-1-decene) modified with dimethylamino propylamine was used to coat hydrophobic magnetic nanoparticles [129]. The presence of alternating hydrophilic and hydrophobic chains provides an interface for interaction with the hydrophobic surface of magnetic nanoparticles and carboxylic residues for further conjugation of protamine, a cell-penetrating peptide. The resulting magnetic nanosystem not only was less cytotoxic compared with popular gene transfection agents such as PEI and lipofectamine but also more efficiently silenced the GFP gene in U251 cells in a serum-containing medium.

To treat adenoid cystic carcinoma, Miao et al. developed PEI-coated Fe_3O_4 nanoparticles onto which the pACTERT-TRAIL plasmid, a human telomerase reverse transcription promoter-driven TRIAL expression plasmid, was adsorbed electrostatically [130]. The nanocomplex triggered TRIAL expression in SACC-83 cells *in vitro* and *in vivo* and ultimately increased the apoptosis rate.

The commercially available magnetic nanocarrier CombiMAG was also tested for its gene delivery capacity. Kong et al. demonstrated that the magnetoplex composed of CombiMAG and IGF-1R-specific shRNA was delivered efficiently into A549 cells *in vitro*, with inhibition of proliferation, adhesion, and chemoresistance [131–134]. A magnetoplex CombiMAG bearing the plasmid pGFPshIGRR1 that drives the concomitant expression of green fluorescent protein and the IGF-R1-specific shRNA was injected into mice via the tail vein; as early as 48 h post-injection, GFP expression peaked in several organs including heart, kidney, and lung [135]. IGFR1 expression decreased as early as 48 h after injection, as determined by western blot of explanted tumor tissue and immunohistochemistry analysis.

An interesting approach paired magnetic nanocarriers and the ultrasound microbubble technology [136]. Lipid microbubbles were coated with PEI-attached magnetic nanoparticles and a dsRed DNA plasmid was subsequently adsorbed. In a dorsal skin-fold chamber model to visualize vessel network by intravital microscopy in BL6 mice, injection of the pdsRed/PEI-coated NPs/microbubble complex delivered the genetic cargo only when both magnetic and ultrasound forces were applied in the dorsal skin. No other vascular walls were affected, indicating precise, site-specific, and ultrasound-controlled delivery of genetic cargo through the vasculature system.

Others nanocarriers suitable for DNA/siRNA delivery were obtained by coating a magnetic core with lipoids, a cationic lipid [137]. By monitoring GFP expression or luciferase activity in luciferase siRNA-treated cells, lipoid-coated magnetic nanoparticles were found to deliver both nucleic acids. The efficiency of gene expression and siRNA-mediated luciferase silencing increased greatly when an external magnetic filed was applied. Mok et al. developed a magnetic nanosystem composed of pH-sensitive branched PEI to exploit the low pH normally found in the tumor microenvironment [138]. Once conjugated with the tumor-specific peptide chlorotoxin and a siRNA against GFP, the nanosystem silenced GFP; in addition, its efficiency was highly dependent on the acidic conditions surrounding tumor cells.

To silence the epidermal growth factor receptor (EGFR) in tumor-infiltrating vessels, the nanocompound LipoMag was used as carrier for EGFR-specific siRNA [139]. When tested *in vivo*, LipoMag complexed with EGFR siRNA reduced the tumor burden in nude mice inoculated with MKN-74 or NUGC-4 as compared with commercially available PolyMag.

An important hurdle for gene therapy is gene transfection efficiency in physiological conditions, where soluble factors can hinder appropriate delivery of the nanovector. Magnetic nanoparticles developed by physical interaction between previously polyacrylic acid-coated magnetic nanoparticles and PEI demonstrated increased gene expression in the presence of 10% fetal bovine serum when an external magnetic field was applied [140].

One emerging gene nanocarrier is derived from the combination of magnetic nanoparticles and viral agents. Tested on HEK293T cells, both heparin- and streptavidin-coated magnetic nanoparticles onto which adenovirus vectors (AVVs) were adsorbed directly or through a biotin-Ni-His_6 link, respectively, enhanced AVV-driven GFP expression when a magnetic field was applied [141, 142]. Strikingly, a heparin-coated Fe_3O_4/AVV-driven neuron growth factor (NGF) expression nanocomplex was more efficient than NGF itself in neurite elongation, as seen in the pheochromocytoma of PC12 rat adrenal medulla cells [141]. For the *in vitro* transfection of poorly transfectable human neuron stem cells, the AVV-His_6-Ni-biotin/streptavidin magnetic nanoparticles showed enhanced internalization and gene expression compared with AVV alone [142]. Sapet et al. enhanced *in vivo* gene expression in rat hippocampal neuronsusing a type 5 AVV carrying a CMV-driven GFP expression cassette associated with magnetic nanoparticles [143].

3. Conclusions and Future Challenges

Magnetic nanoparticle technology offers an enormous field for gene therapy, especially in cancer treatment. Finding new polymers or improving those currently available as gene carriers is the goal of scientists; the development of new synthesis methods has accelerated this process. Important questions remain to be addressed, such as the escalation of magnetic nanocarrier manufacture and cytotoxicity to attain regulatory approval. Once these two elements are resolved, clinical trials can be undertaken.

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