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# The Application of Nanoparticles in Gene Therapy and Magnetic Resonance Imaging

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## Abstract

The combination of nanoparticles, gene therapy, and medical imaging has given rise to a new field known as gene theranostics, in which a nanobioconjugate is used to diagnose and treat the disease. The process generally involves binding between a vector carrying the genetic information and a nanoparticle, which provides the signal for imaging. The synthesis of this probe generates a synergic effect, enhancing the efficiency of gene transduction and imaging contrast. We discuss the latest approaches in the synthesis of nanoparticles for magnetic resonance imaging, gene therapy strategies, and their conjugation and *in vivo* application.

### Keywords

nanoparticles; bioimaging; gene therapy; iron oxide; theranostic

## INTRODUCTION

The present review focuses on the use of nanoparticles for magnetic resonance imaging and gene therapy. The combination of nanotechnology, imaging techniques, and biomedicine has produced astonishing results in many fields, and now forms part of clinical practice in areas such as drug delivery, contrast agents, and hyperthermia cancer treatment (Cherukuri et al., 2010; McCarthy and Weissleder, 2008; Seale-Goldsmith and Leary, 2009; Veiseh et al., 2010; Wilson, 2008). Nanoparticles have also shown very promising results in other fields.

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Nanotechnology involves the synthesis and characterization of nanomaterials with new or improved features due to their size (Seale-Goldsmith and Leary, 2009). The use of nanoparticles is particularly important, since they can be functionalized with a variety of molecules that can provide a specific signal in different imaging techniques. The application of these materials to biomedicine leads to concepts such as nanomedicine and theranostics, that is, the combination of diagnosis and therapy in a single probe. Magnetic resonance imaging (MRI) is well suited to the diagnosis and monitoring of many diseases, thanks to its excellent anatomical resolution and the variety of physical methods available to obtain contrast. In addition, by using molecular probes and combination with other imaging techniques it is possible to overcome its relatively low sensitivity (Gould, 2004). Important advances have already been made in the use of nanoparticles in more traditional areas, such as drug delivery. We are interested on new approaches for the cure of diseases where conventional treatments have proven invalid: Gene and Cellular Therapy. The combination of this therapy with the concept of theranostics leads to the main point of this review, the use of nanomaterials for Gene Theranostics experiments. Gene therapy is used to treat a monogenic hereditary or acquired disease by the introduction of therapeutic genes through two types of vectors, integrative or non-integrative. Integrative vectors are safe modified viral vectors that enable a therapeutic transgene to be transferred to the genome of the target cell. With non-integrative vectors, the therapeutic transgene is not stably expressed in the target cell.

After a short introduction to magnetic resonance imaging, we summarize the main types of nanoparticles used as contrast agents or molecular imaging probes. We detail the synthetic approaches used to give the probes their physicochemical properties: functionalization of the surface to obtain physiologically stable nanoparticles and biofunctionalization of the probe so that it becomes active for molecular imaging. The application of gene therapy and alternative approaches is also addressed. We describe different techniques for cell labeling with probes and the methodology of magnetofection, which is closely related to some of the approaches followed in gene theranostics protocols (described in the last chapter).

## NANOPARTICLES AND MRI MRI

MRI makes it possible to visualize samples non-invasively. Its ability to image soft tissues and metabolic processes in humans and animals has made it a routine technique in medicine and animal research.

MRI uses a strong magnetic field to align the nuclear magnetization of (usually) hydrogen atoms in the sample. Radiofrequency (RF) radiation is used to rotate this magnetization to a plane perpendicular to the magnetic field, thus giving rise to a signal detectable by the scanner. In addition, gradients superimposed on the main magnetic field are used to locate the spatial position where the signal is coming from in such a way that the image corresponding to the object can be obtained as a Fourier-transform of the sampled data (Haacke, 1999).

MRI can yield images with several types of contrast. Ignoring contrast strategies that are beyond the scope of this review (e.g., diffusion or magnetization transfer), contrast depends mainly on spin density and relaxation times. Relaxation time is the time it takes nuclear magnetization to return to thermal equilibrium after perturbation by RF.

For example, in a spin echo acquisition protocol, signal is given by:

$$s \approx \rho\left(1 - e^{-T_R/T_1}\right) e^{-T_E/T_2}$$

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where ρ, $T_1$, and $T_2$ are spin density, longitudinal relaxation time, and transverse relaxation time, which depend on the sample, and $T_R$ and $T_E$ are repetition time and echo time, which are defined by the acquisition protocol. Useful images can thus be obtained in medical or research applications by measuring the different values of these parameters (spin density or relaxation times) in different tissues.

During imaging of water protons, spin density is proportional to water content. Relaxation times are tissue-dependent and can be modified using specific contrast agents. The most widely used contrast agents are based on gadolinium chelates and iron oxide nanoparticles. Although they lower both relaxation times, the former are used mainly as $T_1$-contrast agents and the latter as $T_2$-contrast agents, with the result that they increase or lower the MRI signal, respectively. Imaging techniques in which the iron oxide nanoparticles increase the MRI signal also exist, although they are beyond the scope of this review. Iron oxide nanoparticles can therefore be detected *in vivo* by MRI techniques. The biodistribution thus measured can be used to assess medical conditions or the response to treatment based on other phenomena, such as increase or decrease in blood flow or nanoparticle binding to a molecule of interest (Lowe, 2004; Strijkers et al., 2007).

## Nanoparticles for MRI

A nanoparticle (NP) is a mesoscopic material with at least one dimension smaller than 100 nm. The most remarkable characteristic of this type of compounds is the new or enhanced physicochemical properties arising from its nanometric size, for example, the superparamagnetism in iron oxide nanocrystals or the surface plasmon resonance in gold nanoparticles. In addition, their high surface-to-volume ratio makes it possible to functionalize them with a variety of surfactants, ligands, and biomolecules. This functionalization also facilitates stabilization of the compound, interaction with specific biomolecular entities, and the introduction of a probe for a second imaging technique, for instance, a fluorophore for optical imaging or a paramagnetic complex for MRI (Doshi and Mitragotri, 2009; Gould, 2004; Portney et al., 2005).

The nanoparticles commonly used in MRI can be classified according to several parameters: their magnetic behavior means they can be superparamagnetic or paramagnetic; in terms of biodistribution, they can be classed as extracellular agents, blood pool agents, organ-specific agents, and cell-labeling agents (Rodriguez et al., 2008). Their core composition enables an enormous number of materials to be used, for instance iron oxides ($Fe_3O_4$, $Fe_2O_3$) and manganese oxides ($MnFe_2O_4$, $Mn_2O_3$), gadolinium oxide ($Gd_2O_3$), gold nanoparticles (Au-citrate, Au-$Fe_3O_4$, $Fe_3O_4$-Au, Au-Gd conjugate), quantum dots, and the up-converting nanophosphors ($NaXGdF_4$, X:Ho, Er, Y). For the sake of conciseness, we focus on the most commonly used materials for gene delivery, which are currently iron oxide nanoparticles and gold nanoparticles (Seale-Goldsmith and Leary, 2009).

## Iron Oxide Nanoparticles

Superparamagnetic iron oxide nanoparticles (SPIOs), mainly magnetite and maghemite, are the most important nanomaterials in MRI. These nanoparticles are used for a wide variety of applications. (1) Magnetic separation: The separation of biological entities from their environment is a key aspect of biochemical analysis and disease diagnosis (Gould, 2004). This well-developed technique can be used as an alternative to conventional centrifugal separation methods. (2) Medical imaging: Nanoparticles can be used as imaging probes for MRI and in many other imaging techniques after functionalization of the surface. In this way, dual probes have been synthesized for MRI, optical imaging, computed tomography, and positron emission tomography (Allen et al., 2005; Kim et al., 2006; Lee et al., 2007; Portney et al., 2005; Seale-Goldsmith and Leary, 2009; Sperling et al., 2008). (3) Targeted

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detection: Ideally, this technique can be used for detecting early signs of disease. One example is the detection of HIV viruses. The SPIOs are coated with gold and functionalized with an HIV antibody through an Au-S covalent bond. Despite the huge potential of this application, it requires further study before it can be applied in clinical practice (Gould, 2004). (4) Hyperthermia treatment: This technique is based on the fact that cancerous cells are more susceptible to temperature than normal cells. Increasing the temperature of the malignant cells to 42°C means that they can be selectively killed. To achieve this, iron oxide nanoparticles are injected into the tissue and an alternating magnetic field is applied. If the field is sufficiently strong and has the appropriate frequency, the NPs absorb the energy and heat the tissue, affecting mainly the cancer cells (Cherukuri et al., 2010; Everts et al., 2006). (5) Targeted drug delivery: SPIOs can be functionalized with one or several drugs that are released upon reaching the malignant tissue. Several approaches can be used, such as hydrolytic cleavage from the surface by pH variations or by temperature changes (Shubayev et al., 2009). One important feature is that, due to their magnetic behavior, these particles can be accumulated in the region of interest using an external magnet. A fundamental feature in the use of SPIOs for drug delivery is the size of the nanoparticle. If it is large, a strong magnetic force can be exerted against blood flow, with the result that more NPs reach the area of interest. However, if they are too large there is a high risk of clogging small capillaries, whose diameters are about several micrometers (Gould, 2004; Jiles, 1998; Lee et al., 2007). (6) Magnetofection: This technique makes it possible to increment the efficiency of gene therapy using magnetic nanoparticles and an external magnetic field over culture dishes. The most relevant characteristics and examples of this technique are reviewed below.

Superparamagnetic behavior is one of the most important features of nanoparticles. The magnetism in a solid is basically a function of the number of unpaired electrons on each atom and the orbital they occupy (Jiles, 1998). Bulk solids can be classified according to their response when an external magnetic field is applied. For example, if a magnetic field is applied to material with unpaired electrons, a small magnetization occurs and the solid is classified as paramagnetic. This is the case for $Gd_2O_3$ nanoparticles and supramolecular complexes containing a Gd nucleus. However, some nanoparticles are smaller than the magnetic domain. Thus, when an external magnet is applied, they have all their magnetic moments aligned, generating much greater magnetic susceptibility than paramagnetic materials (usually 100-fold higher). This effect implies a much stronger signal in magnetic resonance imaging than that produced by traditional paramagnetic contrast agents. This superparamagnetism has two experimental criteria which are no hysteresis for the magnetization curve and overlapping of the magnetization curves at different temperatures (Rodriguez et al., 2008; Shubayev et al., 2009). In Figure 1 we can see the magnetization curve shown by a superparamagnetic material in water with no hysteresis cycle.

Extensive efforts have been made to synthesize different types of magnetic nanoparticles using colloidal chemical synthetic approaches. This is a key step in the development of superparamagnetic nanoparticles for biomedical applications, since features such as size and shape determine their behavior. Several methods are available for the synthesis of SPIOs and many interesting reviews on the topic can be found in the literature (Jin et al., 2009; Nune et al., 2009; Weinstein et al., 2009). We focus on the synthetic approach based on the decomposition of organic precursors, since this has proven to be one of the best ways to synthesize uniform and crystalline SPIOs. The approach involves the decomposition of an organic precursor, such as iron acetylacetonante or iron pentacarbonyl, in a solvent with a high boiling point to produce SPIOs with small size and narrow size distribution (Roca et al., 2006a,b). The reaction is carried out in the presence of a surfactant, usually oleic acid, which coats the surface of the NP. This molecule is bound to the surface of the nanoparticle through the carboxylic group whereas the hydrocarbon chain faces the solvent molecules in such a way that these NPs are only stable in organic solvents. A second modification is

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necessary to make them hydrophilic and useful for biomedical applications. Currently, this modification can be achieved using three alternatives: the micelle-like approach, the exchange-ligand approach, and chemical modification of the surfactant.

The micelle-like approach is based on the use of amphiphilic molecules, usually polymers, which camouflage the oleic acid surfactant. The new molecule forms a weak bond by van der Waals interactions between its hydrophobic side and the aliphatic chain of the surfactant whereas its hydrophilic head faces the solvent molecules. A wide variety of molecules have been used to this end, such as dextran, poly(ethylene glycol) (PEG), and different polymers (Rodriguez et al., 2008). The main advantage of this approach is the simplicity of the reaction. However, it presents two major drawbacks. One is the increase in nanoparticle size and size distribution depending on the polymer used. More importantly, the eventual desorption of the amphiphilic molecule from the surface may lead to destabilization of the SPIOs because of the weak interaction between the two molecules (Dong et al., 2010; Liu et al., 2009; Saha et al., 2010).

The exchange ligand approach is based on the use of hydrophilic ligands, usually small organic molecules with an affinity towards the surface of the SPIOs. Here, the SPIOs, which are coated with oleic acid, are mixed with a solution containing the ligand at a high concentration. The new ligand eventually substitutes the oleic acid, rendering water-stable NPs. The advantages of this approach are its simplicity and the versatility in the number and type of ligands that can be potentially used. In this regard, the best results have been obtained using dimercaptosuccinic acid as the exchange ligand (Dilnawaz et al., 2010; Valois et al., 2010). One of the problems with this method is the exchange, which, if not complete, leaves several molecules of the original surfactant on the surface, thus leading to stabilization problems. The second problem is the way the ligand is bound to the surface. Some of the new ligands would be coordinated through the appropriate functional groups whereas others would be adsorbed on the surface. This creates serious difficulties when a functionalization reaction must be carried out to bind a protein, for example.

The most recent approach involves chemical modification of the surfactant, in which a chemical reaction is carried out on the oleic acid structure by changing only that part of the molecule facing the solvent and not the bond between the surfactant and the molecule. Our group carried out such a modification in SPIOs using potassium permanganate and a transfer catalyst (Herranz et al., 2008a). With this two-phase approach, the surfactant double bond is cleavaged and renders a carboxylic acid, and oleic acid is transformed to azelaic acid (Fig. 2). In this way, the carboxylate group on the surface of the NP remains unchanged whereas a new carboxylic acid is created facing the solvent. The main advantage of this approach is the synthesis of SPIOs that are stable in physiological media without modifying the binding to the surface and ready for further functionalization through the newly generated functional group. Figure 3 shows transmission electron microscopy (TEM) images of the SPIOs as synthesized by decomposition of organic precursors (Fig. 3a) and after the chemical modification of the surfactant (Figs. 3b and 3c). It is possible to see the uniform spherical NPs obtained by decomposition of organic precursors and how the modification on the surfactant structure does not change either the shape or the lack of aggregation of the SPIOs.

The final step in the synthesis of nanoparticles that are active in biomedical imaging is functionalization with the biomolecule of interest, whether a protein, antibody, small molecule, or genetic material. The binding of the biomolecules to the surface can be obtained by covalent or ionic bonding, depending on the intended use of the molecule. If the purpose of the functionalization is to synthesize a bioconjugate in which the modification of the biomolecule must be kept to a minimum, thus avoiding any change in its functionality, an ionic binding approach should be followed. On the other hand, if a robust bond between

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the NP and the protein is required, then a covalent bond should be used. The approach will vary depending on the surfactant on the surface of the NP. Clearly, it is not the same to functionalize SPIOs coated with PEG with a small amine molecule or with a carboxylic acid. Consequently, synthesis of the final nanobioconjugate between the NP and the biomolecule is the subject of extensive research in many fields (Gao et al., 2009; Herranz et al., 2008b; Mulder et al., 2009).

## Gold Nanoparticles

Gold nanoparticles (AuNPs) have lately attracted a great deal of attention due to their use in biomedical imaging, although they have been used for many decades in other areas, such as glass pigments. One of the most important aspects of these compounds is the versatility of their synthesis. They can be prepared in a wide range of monodisperse sizes, from 2 nm to 250 nm and can be synthesized in a variety of shapes, which ultimately affect their properties. It is possible to obtain gold nanorods, nanoplates, branched nanostructures, and nanoparticles. Another important feature of AuNPs is their surface chemistry, in particular their ability to easily form covalent bonds with thiol groups, which enables their biofunctionalization with several molecules (Seale-Goldsmith and Leary, 2009; Sperling et al., 2008). The size and shape of AuNPs determine the basic characteristics of surface plasmon resonance, arguably the most important feature of this nanomaterial. This property is characterized by a strong broad absorption band in the UV-vis region arising from the coupling of incident electromagnetic radiation into a surface plasmon (described as a collective oscillation of the conduction electrons) at the interface between the particle and the medium surrounding the particle (Wilson, 2008). The resulting energy loss is manifested as an absorbance known as the surface plasmon band. This absorbance is responsible for the characteristic reddish color of colloidal gold. The reason why size and shape are so important is that the plasmon is confined to the surface and, as the diameter gets smaller, the energy required to collectively excite motion of the surface plasmon electrons increases (Jung et al., 1998). For example, AuNPs with diameters close to 5 nm strongly absorb at visible wavelengths with a maximum absorbance at 520 nm. In contrast, when the size is larger than 5 nm, the band appears at longer wavelengths, and by varying the particle size and shape, it is possible to tune the maximum absorbance from 520 nm to more than 1000 nm. In addition, this absorption is quite sensitive to the dielectric constant of the solvent. Media of high dielectric constants are effectively more polarizable (thus couple with the surface plasmon electrons more readily), and the energy required to collectively excite the electrons is decreased. In other words, the maximum in the spectrum shifts to lower energy. In this context, the AuNPs have an inherent sensing ability. Any compound adsorbed to the nanoparticle surface will manifest a color change proportional to the magnitude of the change in the refractive index near the nanoparticle surface (Kurihara and Suzuki, 2002).

Because of these characteristics, the range of applications of AuNPs in biomedical sciences is even larger than for SPIOs. (1) Immunostaining: Immunostaining is probably one of the oldest applications of AuNPs in biotechnology. It involves the labeling of specific molecules or compartments of cells with antibodies. Cells are typically fixed and permeabilized, and AuNPs conjugated with antibodies against the molecules of interest are added in such a way that the AuNP-antibody will bind to the antigen in the target regions. The AuNPs then provide excellent contrast for TEM imaging and can also be imaged with optical microscopy. This technique has several advantages over fluorescence labeling. AuNPs are more stable, as they do not suffer from photobleaching. In addition, immunostaining can be performed without permeabilization of the cells, in this case the AuNPs bind to the surface, leading to an aggregation of the nanoparticles that makes it possible to obtain a photoacoustic signal. The reason for this effect is that small aggregates of AuNPs can absorb light at wavelengths above the plasmon resonance, whereas monodisperse particles cannot.

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Consequently, there is a photoacoustic signal for aggregates of AuNPs, but not for single disperse AuNPs. In this case, the aggregates are formed because several of the functionalized particles bind to the regions where the right antigen is present, thus providing a photoacoustic signal (Mallidi et al., 2007). (2) Contrast Agents for MRI: Contrast agents represent one of the most recent applications of these nanoparticles, and it is still at an early stage. This is partly due to their chemical composition; AuNPs cannot be used directly in MRI without chemical modification using either Gd supramolecular complexes or iron oxide. In the Gd approach, the AuNPs are synthesized by one of the available methods. Their surface is then modified by binding a supramolecular ligand that ultimately complexes the gadolinium ion. This approach enables two goals: first, the particles become active for MRI, and second, the physical features of the gadolinium complex are improved by increasing the relaxivity value (Park et al., 2008, 2010; Warsi et al., 2010). Iron oxide is applied using an iron oxide core with individual gold nanoparticles on the surface or with a gold core and a layer of iron oxide surrounding the nanoparticle (Fig. 4) (Shevchenko et al., 2008). The figure shows TEM images of gold/iron oxide (core/hollow-shell) nanoparticles synthesized with different amounts of oleylamine and oleic acid, as indicated by the number in the upper right corner on each image. In panel A, when only oleylamine is used, the iron oxide shell is irregular and connected to the gold at a few points. The remaining panels show how the morphology of the iron oxide shell is improved by the addition of more oleic acid, with the best proportion at 1:1 (Shevchenko et al., 2008). This combination of materials is promising, because the final nanoparticle is a “triple probe,” that is, a contrast agent for imaging techniques such as MRI, optical imaging, and computed tomography (CT) (Cho et al., 2006; Cormode et al., 2010; Seino et al., 2006; Wu et al., 2010; Xianghong et al., 2010; Yuanpeng et al., 2010). (3) Contrast Agents for CT: As mentioned above, nanoparticles can also be used for X-ray CT, in which they provide a high signal-to-noise ratio, thus necessitating only short exposure times, which in turn help to reduce radiation damage to surrounding tissues (Boote et al., 2010; Cai et al., 2007; Kimet al., 2007; Popovtzer et al., 2008; Xiao et al., 2010). In this application, the AuNPs can be conjugated with antibodies or ligands that bind as specifically as possible to the target. When particles are injected into the bloodstream, some eventually bind via receptor–ligand interaction at the designated organ. These particles provide contrast for imaging and may resolve the structure of the organ. One drawback of this approach, common to all nanoparticulate agents, is that complicated chemical modifications must be carried out on the surface to ensure long circulation times. Otherwise, the nanoparticles will finish in the liver and spleen through the action of macrophages (Seale-Goldsmith and Leary, 2009; Wilson, 2008). This aspect is not so critical in gene therapy imaging protocols, as most of the approaches are *ex vivo*, as shown in following sections.

Synthesis of AuNPs has developed in such a way that it allows for a wide variety of sizes and shapes, which all involve the reduction of a gold compound, typically $HAuCl_4$ or $AuCl_3$, and the coating of the surface with an appropriate surfactant, which can be an organic molecule, a polymer, or a peptide. Gold can be reduced by the same molecule acting as surfactant, for example, citric acid, or by a different molecule, in which case the reducing agent is usually sodium borohydride. As with SPIOs, it is possible to synthesize AuNPs in an organic phase and then modify them for transference to water. However, better results are obtained using citric acid as both the reducing agent and the surfactant; the negative charge from the citric acid makes it possible to obtain stabilized nanoparticles in a single step. Once synthesized, they are ready for biofunctionalization with no further modification of the surfactant. This final functionalization can be performed in two ways: by ionic or covalent binding to the surfactant, for example, through an amide bond to the citric acid, or by covalent binding through thiol groups. As thiol groups show a high affinity towards gold surfaces, the most used functionalizations employ ligands bearing a thiol group at one end and a hydrophilic group at the other. Thus, besides remaining stable in water, they are ready

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for further functionalization, as occurs with SPIOs. PEG is often used as a ligand, as it reduces nonspecific adsorption of molecules to the particle surface by providing colloidal stability for steric reasons. The approach is similar when binding proteins, antibodies, and other biomolecules. These can be attached by covalent amide bonds to the carboxylate on the surface or through any of the thiol groups from cysteine amino acids. Although these synthesis techniques are relatively well established, bioconjugation of AuNPs is still not easy, mainly to avoid aggregation effects or unspecific binding during the conjugation reaction. In many conjugation protocols, the number of attached molecules per gold nanoparticle is only a rough estimate, as no standard method for determining the surface coverage of particles modified with molecules has yet been established (Wilson, 2008).

## GENE THERAPY

### Introduction to Gene Therapy

Gene therapy emerged in 1980s as a new approach for the treatment of monogenic diseases. It involves modification of the genetic content of the cell through the introduction of genes using delivering integrative or non-integrative vectors. This approach makes it possible to permanently correct the defective gene and thus provide a definitive cure.

Gene therapy can be used to treat different hereditary or acquired diseases. In the case of monogenic recessive diseases caused by the absence of a gene function, the use of gene therapy for the restoration of the defective gene will expectedly lead to stable expression of the therapeutic transgene in the target cells. If the expression of the therapeutic transgene confers a selective or proliferative advantage to the target cells, prognosis will be much better. When gene therapy is used to treat cancer, the objective is the selective elimination of the cancerous cell. In this case, the success of treatment will depend on the efficiency of transgene delivery. The success of gene therapy depends on the transgenes to be expressed and the delivery vectors used. Therapeutic transgenes can substitute their endogenous counterparts that are not able to express certain proteins or that express a non-functional form of the protein. Transgenes can also be used to inhibit expression of a pathologically active endogenous gene or to express a toxic protein able to kill the cell in which it is expressed. Such would be the case of tumor cells that could be modified to express suicide transgenes able to eliminate the tumor under the stimulation of a prodrug.

We can classify gene therapy depending on the delivery vector used. As explained above, vectors can be integrative or nonintegrative. Integrative vectors are usually viral vectors (gamma retroviruses, lentiviruses, foamy viruses, adeno-associated viruses). For non-integrative vectors, either viral (adenoviruses, herpesviruses, poxviruses, vaccinia viruses) or nonviral vectors can be used (Fig. 5). Nonviral vectors, mainly based on lipids or cationic polymers, can be useful when there is no requirement for high or long-term expression of the transgene, or when the aim is to trigger an immune response to the tumor. When high and long-term expression is required, viral vectors will be more useful, as they provide higher and more stable levels of transgene expression, even if they are non-integrative. The vector of choice will always depend on the disease. Retroviral vectors based on the Moloney murine leukemia virus (MoMLV) were the first vectors used in clinical trials assessing gene therapy of monogenic diseases. Today, they remain the vector of choice, together with adenoviruses. If we review the gene therapy clinical trials over the past 20 years, 24% were carried out using adenoviruses, 21% using retroviral vectors, and 18% using naked plasmid DNA (data obtained from http://www.wiley.co.uk/genmed/clinical/). Nevertheless, despite the large number of clinical trials of gene therapy carried out to date, no protocol or product has been approved by the FDA for general use.

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These protocols can be carried out following *in vivo* and *ex vivo* strategies (Fig. 6). *In vivo* gene therapy consists of the direct infusion of genes into the patient. In this case, the vectors of choice are replication-incompetent viruses that are unable to induce pathogenesis. *Ex vivo* gene therapy consists of the in vitro modification of previously obtained patient cells. After the introduction of the desired therapeutic transgene, the modified cells are infused back into the patient. The second strategy is much more effective than that of *in vivo* protocols, thus becoming the protocol of choice in most of the gene therapy clinical trials.

## Gene Therapy Strategies

During the seventies, an important advance was made in the field of molecular biology with the discovery of restriction enzymes. These proteins were able to cut fragments of DNA and enabled researchers to cut and paste the fragments as required. These new findings established the basis for gene transference and genetic modification of the cell genome. The first human trial of gene transfer was carried out in the late 1980s, although its objective was merely to gene-mark the target cells (Culver et al., 1991; Rosenberg et al., 1990). The same group later carried out the first gene therapy study in a patient with adenosine deaminase deficiency, which rendered the patient susceptible to continuous infections (Blaese et al., 1995).

The first success in gene therapy was obtained in Italy (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002) in a clinical trial that was carried out on children suffering from X chromosome-linked severe combined immunodeficiency (SCID-X1). These patients have a deficiency of the gene coding for the gamma chain, an indispensable protein in the development of T and B lymphocytes, leading them to suffer from lifethreatening infections. The disease was known as the “bubble boy disease” after a boy with X-linked SCID who lived for 12 years in a plastic, germ-free bubble during the 1980s. The therapeutic transgene was introduced into bone marrow stem cells *ex vivo* using a modified gamma retroviral vector. These modified cells were then infused into the patients. After a few months, the patients became immunocompetent, demonstrating that the therapy can constitute a definitive cure. This success was also achieved by an English group following a similar protocol (Gaspar et al., 2004). Since then, there has been a continuous increment in the number of gene therapy clinical trials (Fig. 7) (Aiuti et al., 2002a,b; Ott et al., 2006), with more than 1500 carried out worldwide. USA has the highest number of protocols, followed by the United Kingdom, Germany, and France. Most protocols were indicated for cancer (64.5%), but a high percentage have also addressed the treatment of monogenic diseases (7.9%) or gene marking (3%) (data obtained from http://www.wiley.co.uk/genmed/clinical/).

Despite these successes, negative effects of gene therapy have been observed in the last few years. The use of integrative modified viral vectors has not proven sufficiently safe in terms of insertional oncogenesis. The integration pattern of the retroviral vectors used in these trials is not random but targets the promoter or enhancer region of the transcribed genes (De Palma et al., 2005). This potential toxicity is reminiscent of the disease associated with the parental virus on which the vector-system is based. The therapeutic retroviral vectors can become deleterious if they integrate close to an oncogene. This effect was observed for the first time in 2003 in the originally successful SCID-X1 gene therapy protocol (Hacein-Bey-Abina et al., 2003). Other groups have shown similar adverse effects (Baum et al., 2006; Howe et al., 2008; Stein et al., 2010). These results highlight the need to improve gene therapy protocols and, more importantly, delivery vectors.

## Gene Therapy and Nanoparticles

Successful gene therapy depends on two important aspects. (1) Efficient and safe delivery of genes to the target cell in vitro and *in vivo*. To achieve this goal, it is necessary to improve

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transduction efficiency, viral titer when using viral gene therapy, or transfection efficiency when using nucleic acids. (2) Effective monitoring of modified cells or modifying agents by noninvasive imaging techniques. This will allow tracking of gene delivery and gene expression.

These aspects and others are being addressed in new approaches, one of which involves magnetic nanoparticles. In gene delivery, the nanoparticles used in MRI present important advantages over other imaging techniques, such as fluorescence, luminescence, or PET, which have been also used in gene therapy.

In the last few years, many groups have reported the use of nanoparticles to complex and deliver viral vectors (e.g., adenoviruses, retroviruses) and nucleic acids, leading to the emergence of new approaches known as magnetofection and theranostics. Magnetofection is a viral and non-viral approach that uses superparamagnetic nanoparticles to improve gene delivery under a magnetic field. Theranostics combines therapeutics with diagnostics and covers several fields, including personalized medicine, pharmacogenomics, and molecular imaging to develop efficient new targeted therapies with an adequate risk/benefit ratio. Furthermore, theranostics aims to monitor the response to treatment and to increase efficacy and safety.

## Magnetofection

Magnetic nanoparticle transfection methods are based on principles developed in the late 1970s for magnetically targeted drug delivery. Mixing genetic material with nanoparticles and applying a magnetic field increases sedimentation of the complex and the kinetics of transfection, as well as the concentration of vectors at the cell surface.

Nanoparticles can be used to complex both nucleic acids (non-viral magnetofection) and viral vectors (viral magnetofection). Magnetic vector complexing involves the standard preparation of the gene vector prior to the mix between these vectors and the magnetic particles. Most published results reveal the need for additional agents to form the complex between the nanoparticles and the gene delivery vector. In the case of DNA, polymers can act as a link between the particles and the nucleic acid (Bryson et al., 2009; Kamau et al., 2006). Iron oxide nanoparticles (SPIOs) have been coated with transfection agents such as polyethyleneimine (PEI) and coupled to DNA. When the mix is performed under a magnetic field, either permanent or pulsating, transfection efficiency increases 40-fold over standard conditions (Kamau et al., 2006). With this approach, a minimal DNA dose is sufficient to achieve high transfection levels, thus offering new highly efficient alternatives to integrative vectors and avoiding adverse effects related to their integration (see above).

In the case of viruses, some authors have taken advantage of the strong biotin–streptavidin interaction. Retroviral and lentiviral vectors can be produced using new modified envelope proteins to which a biotin motif has been added (Hughes et al., 2001; Kaikkonen et al., 2009; Lesch et al., 2009, 2010; Weber et al., 2009). This new protein can be joined to the streptavidin nanoparticles, leading to a stable complex that can be isolated.

Several studies have shown that magnetofection can enhance the transduction efficiency of adenoviruses (Bhattarai et al., 2008; Kamei et al., 2009) and retroviruses (Chan et al., 2005; Haim et al., 2005; Hofmann et al., 2008; Shin and Shea, 2010; Weber et al., 2009). In particular, transduction of lentiviral vectors improves markedly in the presence of magnetic fields when combined with magnetite nanoparticles (Haim et al., 2005). Magnetofection can also enhance the transduction efficiency of measles virus, a paramyxovirus, by 30–70 fold (Morishita et al., 2005) in both receptor-positive and receptor-negative cells.

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A direct link may exist between both compounds (the nanoparticle and the biological compound). The mix of an appropriate proportion of polyelectrolyte-coated magnetic nanoparticles and gene vectors at an appropriate ratio in salt-containing medium could be sufficient to achieve the association by means of saltinduced colloid aggregation (Plank et al., 2003a,b). After a short incubation period, the newly generated “magnetic vectors” are added to the cells on magnetic culture plates. These plates are commercially available (Chemicell, Berlin, Germany) or can be home-made using standard culture plates and strong Nd-Fe-B magnets.

Nowadays, there are commercially available magnetic compounds that make it possible to enhance direct DNA transfection, siRNA delivery, and viral uptake of cells in the presence of a magnetic field (OZbiosciences http://www.ozbiosciences.com/magneto-fection-4.html; Chemicell http://www.chemicell.com/home/index.html). In some cases, the absorption of the virus into the cell takes place by a different mechanism in the presence of the magnetic field, thus allowing the transduction of non-permissive cells.

## GENE THERAPY IMAGING WITH NANOBIOCONJUGATES

Synthesis and imaging are the two main differences between magnetofection and the methods we will review in this section. Synthesis requires the design and purification of a stable bioconjugate, and not only a simple mixture of the different components in the culture dish. Imaging is an integrated part of the results, as is the efficacy of gene therapy, which is not usually taken into account in magnetofection experiments (McCarthy and Weissleder, 2008). It is clear, therefore, that a multidisciplinary approach is required, involving professionals from fields as diverse as chemistry, molecular biology, physics, and engineering.

Binding between the biomolecule and the nanoparticle to obtain a stable bioconjugate, which is central to these projects, can be performed in two ways namely covalent and ionic binding, each of which has advantages and drawbacks. A covalent bond is a specific and strong attachment that prevents possible separation of the bioconjugate components. Several reports examine this approach (Cheon and Lee, 2008; Everts et al., 2006; Huh et al., 2007; Lewin et al., 2000; Räty et al., 2006). However, covalent bonding is limited in that modification of biomolecular structure to make a specific covalent bond and strong attachment to the surface of the nanoparticle could alter functionality. In addition, to make such an attachment, several time consuming steps are required for modification of the nanoparticle and biomolecule (Cheon and Lee, 2008). Ionic bonding requires a strong charge on the surface of the nanoparticles. This is achieved using a technique for functionalization of the nanoparticles (see above). In this case, the advantages and drawbacks differ from those of the covalent approach. A robust, yet less strong nonspecific attachment is achieved, although with no modification of the structure of the biomolecule to enable separation if needed. Thus, the functionality of the biomolecule is unlikely to change during the process; however, depending on the specific characteristics of the system the components could separate before the biomolecule reaches the cell. The ideal situation would be a combination of both methods, and this can be achieved to some extent under conditions of careful chemical synthesis.

Once synthesized, the nanobioconjugate must fulfill certain requirements. To improve the efficiency of genetic transfer, the nanoparticle must improve the contact between the cells and the genetic material—viral or nonviral—on the surface. Therefore, the bioconjugate cannot be too stable in the culture media, as this would prevent deposition of the nanoparticles on the cells. Furthermore, the relaxivity and magnetic susceptibility values for

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the nanobioconjugate should be determined under appropriate physiological conditions to determine their applicability in MRI.

## Iron Oxide Nanobioconjugates

An interesting example of the use of magnetic nanoparticles for the synthesis of a stable nanobioconjugate in gene therapy was developed by Räty et al. in the field of non-invasive imaging of baculovirus biodistribution *in vivo* by MRI (Räty et al., 2006). The nanoparticles used were commercial ultrasmall superparamagnetic iron oxide nanoparticles (USPIO). As the authors used a covalent approach, both the nanoparticle and the virus capsid had to be functionalized. The virus was conjugated to avidin proteins, thus displaying a high affinity towards biotinylated compounds (Räty et al., 2004). The NPs were functionalized with biotin through amide formation with the surfactant using O-benzotriazole-*N,N,N',N'*-tetramethyl-uroniumhexafluorophosphate (HBTU) as a coupling reagent. These authors followed a viral approach for the transduction of HepG2 cell lines using baculoviruses, which are effective genetransfer vectors in vertebrate cells (Airenne et al., 2000). Atomic force microscopy was used to characterize binding between the virus and the nanoparticles (Fig. 8). The results confirmed that nanoparticles of the estimated size were bound to the virus surface with an average of 1-2 virions per particle. The USPIO nanoparticles were detected in the size range of 42 6 7 nm in conjunction with viral particles (ranging from 200 to 300 nm in length and 24 to 27 nm in width). Unfortunately, no data were reported for the relaxivity and magnetization values, so there is no way of knowing how the binding of the virus and the agglomeration of the NPs affected these parameters. Transduction assays in HepG2 cell lines showed that USPIO coating of the avidin-displaying baculovirus increased both transduction efficiency and transgene expression level. When avidin-displaying viruses were conjugated with the nanoparticles before injection, viruses were detected in the ipsilateral ventricle of rat brain. After intraventricular injection into the rat brain, the animals underwent MRI scans at 4.7 T and time points of 2 h and 1, 3, 6, and 14 days after the injection. Using gradient echo sequences, which render tissue more susceptible to the field-dephasing effects of iron oxide, the signal was further enhanced, although at the cost of anatomic reference points (Fig. 9a). Using spin-echo sequences, however, sufficient contrast effects were detected with less blooming effect and better anatomical acuity (Fig. 9b). In these studies, the signal measured in MRI corresponds to the injected nanobioconjugate. The most typical method involves Prussian blue staining to detect iron in the tissue and to compare the results with suitable controls, including tissue without nanoparticles and tissue where non-functionalized nanoparticles were injected. The authors found no detectable deposition of iron in choroid plexus cells in negative controls. Consistent staining of cuboid epithelial cells of the choroid plexus was observed in tissue sections from the ipsilateral side, although not from the rest of the brain. Besides, functionalization of the viruses did not change their capacity to transduce choroid plexus cells. This was achieved by assessing the expression of the nuclear-targeted LacZ transgene using b-galactosidase staining of cryosectioned rat brains. This indirect approach to detecting transduction is somewhat less useful than viruses encoding a fluorescent protein (usually GFP), which serves as a probe for cell transduction and as a second imaging signal, in such a way that the nanobiconjugate would be a dual probe for MRI and optical imaging.

A similar approach was recently published (Huh et al., 2007), where the strategy adopted was the hybridization of an adenovirus with magnetic nanoparticles into a single bioconjugate with dual functional capabilities of target-specific MRI and gene delivery. The authors used a mix of manganese and magnetism-engineered iron oxide (MnMEIO) as magnetic nanoparticles. MnMEIO is particularly attractive, as it has a very high mass magnetization value of 110 (emu/g of magnetic atoms) and exceptional MR contrast effects ($R_2$ value of 358 $s^{-1}mM^{-1}$), although the introduction of manganese can increase toxicity.

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Synthesis consisted of the thermal reaction of manganese chloride and iron tris(2,4-pentadionate) in hot organic solvents containing oleic acid and oleylamine as surfactants. The hydrophobic nanoparticles obtained were transferred to water by ligand exchange using 2,3-dimercaptosuccinic acid as a hydrophilic ligand. With this approach, 12 nm monodisperse NPs were obtained with a saturation magnetization value of 110 emu $g^{-1}$ and a negative zeta potential (Sun et al., 2004).

The biological component of the nanobioconjugate was an adenovirus with target-specificity to cells with overexpressed Coxsackievirus B adenovirus receptor (CAR), which is known to facilitate binding and entry of adenoviruses to the host cells. The two components were bound using covalent coupling based on sulfo-succinimidyl(4-N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) cross-linker. First, the capsid lysine residues of the adenoviruses were converted into maleimide groups by reacting them with the cross-linker. These groups were allowed to react with a large stoichiometric amount of MnMEIO nanoparticles, which resulted in the formation of adenovirus-MnMEIO hybrid nanoparticles by way of the nucleophilic addition of a surface thiol group from the dimercaptosucccininc surfactant on the NPs. This example demonstrates the need for several functionalization steps, both with the nanoparticles and the virus, when a covalent bond is desired. In this work, much effort was devoted to covalent bonding between the surfactant and the viral capsid. However, surprisingly, due to the ligand exchange approach followed for binding of dimercaptosuccinic acid to the NPs, the virus is likely to be attached to a molecule of surfactant that is adsorbed on the surface and not bound to the particle through the carboxylic acid. Some of the most remarkable results in this work can be seen in Figure 10. TEM pictures of the nanobioconjugate consisting of several MnMEIOs on the viral capsid. Besides, in vitro MRI of the nanobioconjugate-treated cells revealed dark MR signals specifically from the CAR-positive U251N cells, whereas no MR contrast was observed from the control cells. These results indicate successful eGFP gene delivery in which intense green fluorescence is only observed from the CAR-positive cells (Fig. 10c). Finally, TEM analysis of the labeled cells (Fig. 10d) shows particulate accumulations, which are internalized through CAR-mediated endocytosis processes forming multiple endosomes (solid circles) or in the proximity of the nucleus (dashed circles). Unfortunately, the authors did not provide data for the transduction efficiency of the cell lines with the use of the nanobioconjugates.

Several authors report the use of superparamagnetic nanoparticles in gene theranostics experiments (Bhattarai et al., 2008; Jin and Ye, 2007; Morishita et al., 2005; Rharny M et al., 2009). The second approach in the synthesis and purification of nanobioconjugates for gene theranostics should be ionic binding between the nanoparticle and the virus. This newer and more flexible approach has many advantages, although it is still under development.

## Gold Nanobioconjugates

Although gold nanoparticles have been used for gene delivery and imaging, this approach is much less developed in MRI, mainly due to the need for a second modification of the NPs to be active. In drug and gene delivery, AuNPs are a well-established methodology that has been examined by several authors in recent years (Cho et al., 2009; Conde et al., 2010; Ghosh et al., 2008; Jin and Ye, 2007; Sperling et al., 2008). Recently, Kamei et al. (Kamei et al., 2009) published an interesting paper, halfway between magnetofection and nanobioconjugate synthesis, which showed a good example of how gene therapy can be improved using nanoparticles and how gold nanoparticles can improve the potential of several imaging techniques. The approach involves amagnetic iron oxide (g-$Fe_2O_3$ or $Fe_3O_4$) core with smaller gold nanoparticles (hydrodynamic diameter of 240 nm) immobilized on its surface. The viral component on the nanobioconjugate was an

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adenovirus. The adenoviral vectors (Ad) are widely used for gene transfer because of their high transduction efficiency (Kovesdi et al., 1997) and were thought to be key for the development of effective gene transduction technology. However, their application has been limited by the fact that viral vectors generally cannot enter cells that do not express virus-associated receptors (Leopold and Crystal, 2007). The transduction efficiency of Ad is highly dependent upon the expression level of CAR on the target cell surface. Low CAR expression levels result in low concentrations of Ad on the cell surface, thus decreasing the efficiency of gene expression. To overcome this problem, the authors conjugated Ad with GoldMAN and allowed the complex to penetrate target cells in the presence of a magnetic field. The gold nanoparticles were conjugated in an Au–S bond without the need for a linker molecule. Using this nanobioconjugate, which penetrates the plasma membrane directly, an improvement in gene transfer efficiency.

Other approaches using AuNPs are based on the binding of DNA or RNA directly to the surface and their release by light activation. This methodology has been used with nanorods capped with phosphatidylcholine for an ionic interaction with DNA (Takahashi et al., 2005), with 780 nm nanorods bound to thiolated DNA (Chen et al., 2006; Wijaya et al., 2009). Similar approaches have been followed with gold nanoparticles (Ghosh et al., 2008; Giljohann et al., 2009; Han et al., 2006; Rhim et al., 2008).

## CONCLUSIONS

We summarize some of the most important achievements in theranostics, a novel field involving the synergic combination of nanotechnology, gene therapy, and imaging techniques. Researchers working in this area should bear in mind three important points. First, the nanobioconjugate should be fully characterized in terms of its physicochemical features. This characterization would allow for rational design of new probes to improve performance. Second, a detailed explanation of the mechanism by which the new conjugate improves the features of the separate counterparts is also needed. And third, the nanobioconjugate should be carefully monitored once it enters the tissue. Much remains to be done, in terms of chemistry, new advances in nanoparticle synthesis and functionalization are expected and will provide more variety in terms of materials and synthesis of the nanobioconjugates. In gene therapy, the latest findings reveal increased safety through new, more efficient, and safer delivery vectors. Nevertheless, there are still may aspects of gene therapy that need to be addressed and have to be considered case by case, when analyzing new nanobioconjugates including toxic effects of transgenes, immune responses to transgenes and vectors, specific expression of transgenes, and targeted delivery of the vector. The combination of new and improved vectors with nanoparticles, together with the imaging techniques that can be applied, will improve gene therapy. Finally, the combination of imaging techniques such as MRI and PET could provide new options for diagnosis.

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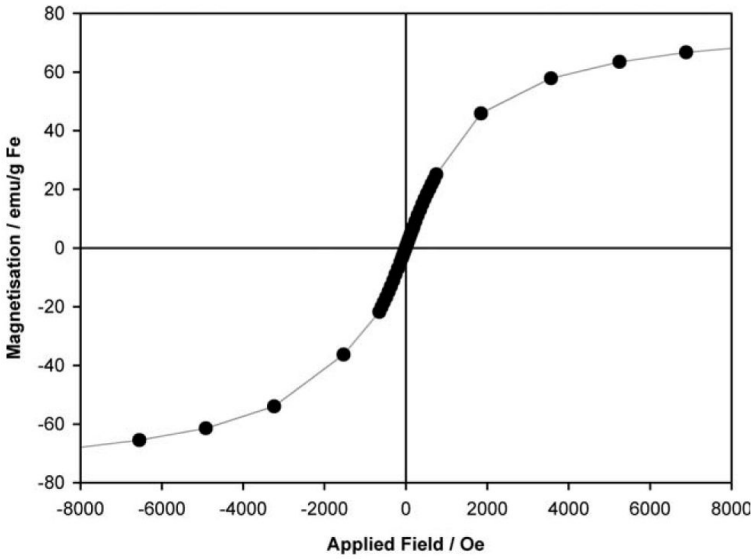



**Fig. 1.**
Example of a magnetization curve for superparamagnetic hydrophilic nanoparticles.

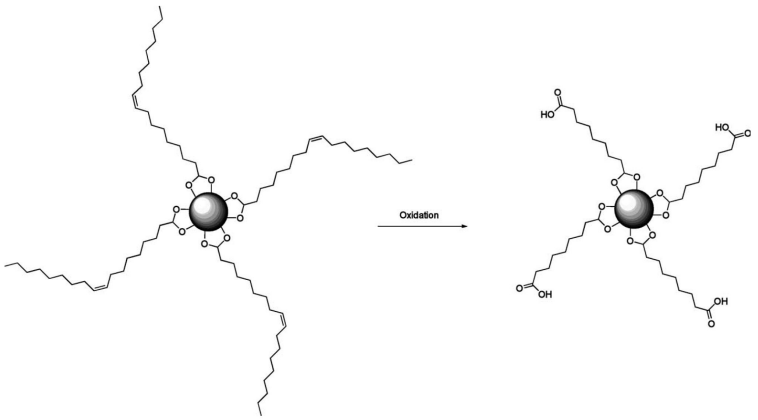



**Fig. 2.**
Chemical modification of surfactant structure rendering hydrophilic nanoparticles.

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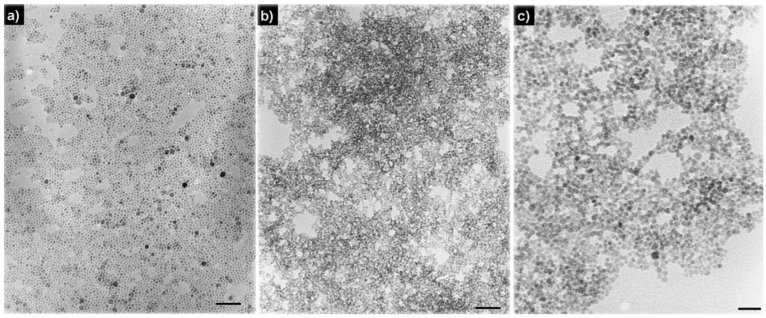



**Fig. 3.**
TEM images of SPIOs: (a) hydrophobic nanoparticle, scale bar is 50 nm; (b) and (c) hydrophilic nanoparticle via oxidation, scale bars are 50 and 20 nm, respectively.

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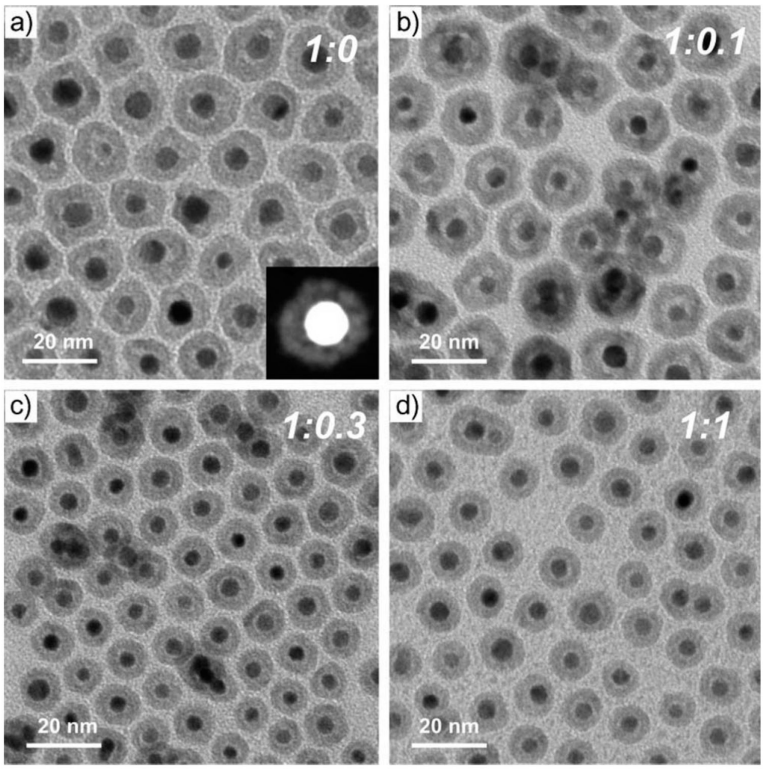



**Fig. 4.**

Transmission electron microscopy (TEM) images of gold/iron oxide (core/hollow-shell) nanoparticles synthesized with different amounts of oleic acid. Molar ratio of oleylamine/oleic acid: (a) 1:0, (b) 1:0.1, (c) 1:0.3, and (d) 1:1. (Reproduced with permission from Shevchenko et al., Adv Mater, 2008, 20, 4323-4329).

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| VIRAL AND INTEGRATIVE |
| --- |
| GAMMA-RETROVIRUSES |
| LENTIVIRUSES |
| FOAMY VIRUSES |
| ADENOASSOCIATED VIRUSES (AAV) |
| **VIRAL AND NON- INTEGRATIVE** |
| ADENOVIRUSES |
| HERPEX VIRUSES |
| POXVIRUSES |
| VACCINIA VIRUSES |
| **NON VIRAL AND NON-INTEGRATIVE** |
| LIPOSOMES |
| PLASMIDS |

**Fig. 5.**
Delivery vectors used in gene therapy strategies.

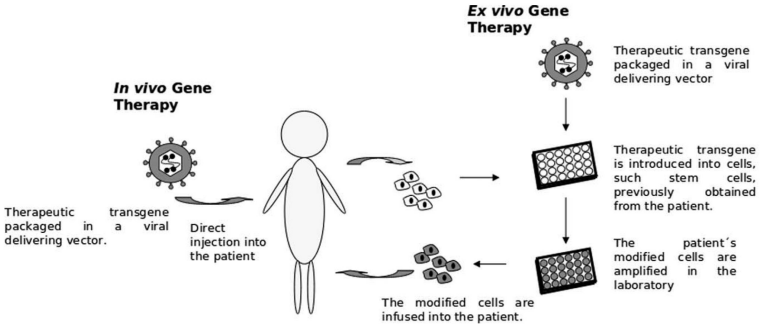



**Fig. 6.**
Two different gene therapy strategies.



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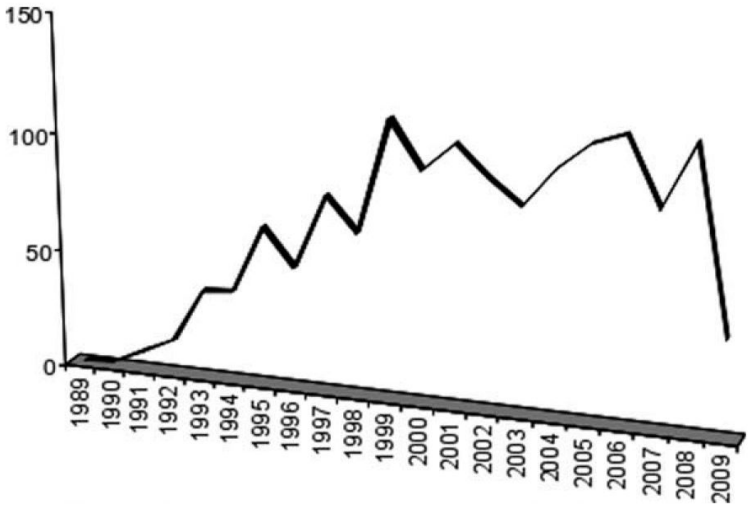



**Fig. 7.**
Clinical trials assessing gene therapy worldwide. Data obtained from http://www.wiley.co.uk/genmed/clinical/.

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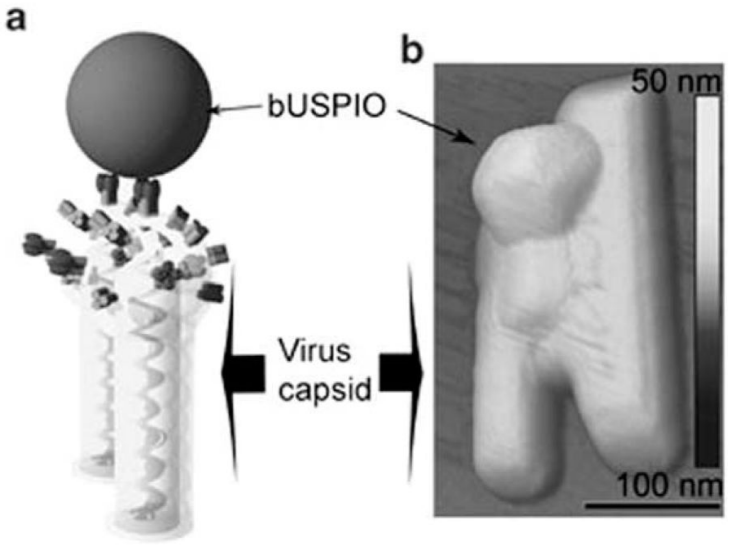



**Fig. 8.**

Presentation of biotinilated USPIO-conjugated avidindisplaying baculovirus (Baavi). The nanobioconjugate structure is shown in the figure (a). Nanoparticle attachment to viral particles was confirmed by AFM (b). In-plane scale bar is shown at the bottom; out-of-plane scale (height) is demonstrated by the sliding color scale. (Reproduced with permission from Räty et al., Gene Therapy, 2006, 1440-1446).

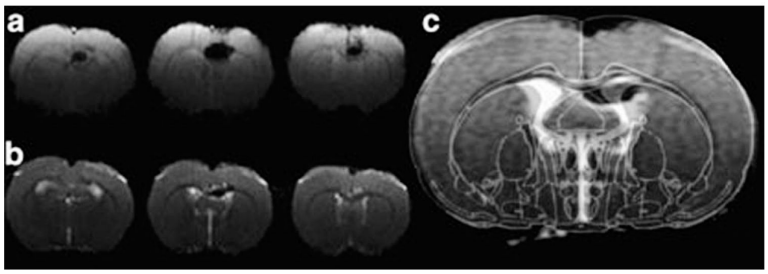



**Fig. 9.**

MRI of intraventricular biotinilated USPIO1Baavi delivery. Representative sequential transverse $T_2$ images are (a) compared to sequential adiabatic $T_2$*-weighted gradient echo MRI-weighted spinecho images and (b) 2 h after biotinilated USPIO-coated Baavi injection. Bright areas represent high signal intensity (such as cerebrospinal fluid in ventricles) and dark areas represent low signal intensity owing to the presence of the contrast agent. A marked signal loss is detectable in $T_2$*-weighted MRI images (a), showing that gradient echo imaging provides improved sensitivity. However, sensitivity appears sufficient and anatomical features are better preserved and delineated in (b). A superimposed anatomical reference map, together with spin echo image (c) of rat brain after Baavi1biotinilated USPIO, is also shown from one animal. In this composite, the ventricles are of high signal intensity (white) and brain regions are delineated by gray lines. (Reproduced with permission from Räty et al., Gene Therapy, 2006, 1440-1446).



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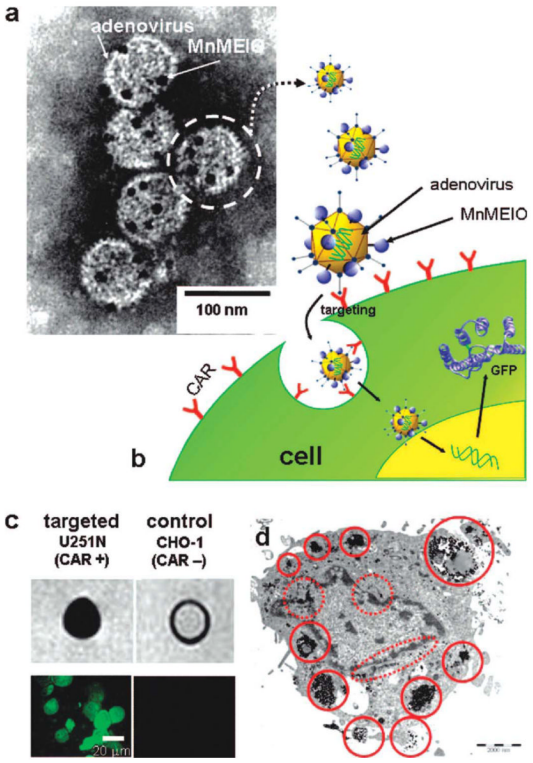



**Fig. 10.**

Adenovirus-MnMEIO nanobioconjugates for targeted MRI and gene delivery: (a) TEM image of the nanobioconjugate; (b) Targeting and gene delivery processes; (c) Such events are imaged only in the CAR-positive cells by a dark contrast in MRI and the GFP expression; (d) TEM image of the nanoprobe-treated U251N cells. Solid circles indicate nanoprobes either under endocytosis or trapped inside endosomes. Dashed circles indicate some nanoprobes found near the nuclear membrane. (Reproduced with permission from Huh et al., Adv Mater, 2007, 19, 3109-3112).