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Nucleic Acid Carriers

Inorganic Nanoparticles as Carriers of Nucleic Acids into Cells

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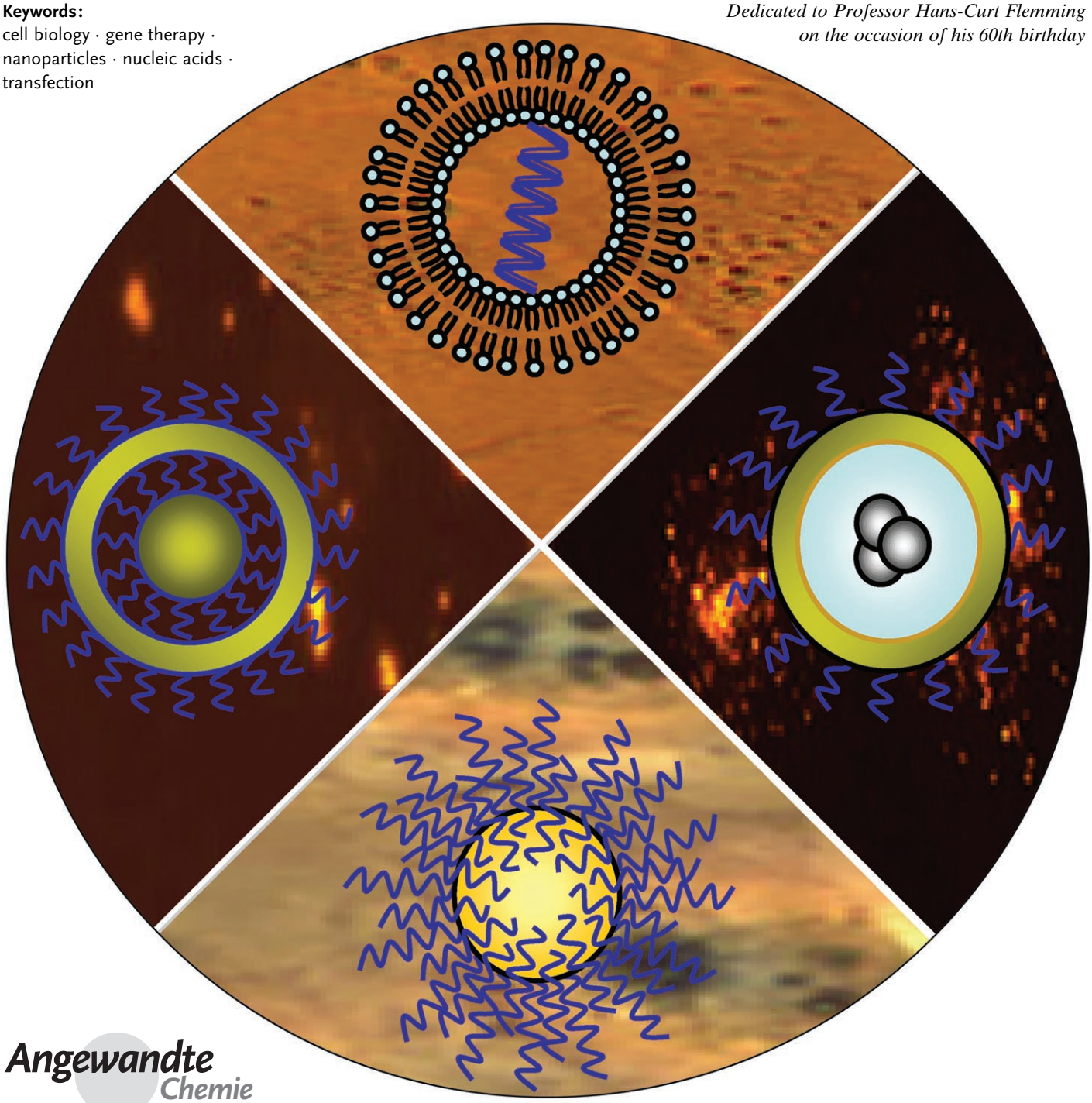
cell biology · gene therapy · nanoparticles · nucleic acids · transfection

Dedicated to Professor Hans-Curt Flemming on the occasion of his 60th birthday



The transfer of nucleic acids (DNA or RNA) into living cells, that is, transfection, is a major technique in current biochemistry and molecular biology. This process permits the selective introduction of genetic material for protein synthesis as well as the selective inhibition of protein synthesis (antisense or gene silencing). As nucleic acids alone are not able to penetrate the cell wall, efficient carriers are needed. Besides viral, polymeric, and liposomal agents, inorganic nanoparticles are especially suitable for this purpose because they can be prepared and surface-functionalized in many different ways. Herein, the current state of the art is discussed from a chemical viewpoint. Advantages and disadvantages of the available methods are compared.

From the Contents

1. Introduction

The application of nanoparticles in medicine is an emerging field of nanobiotechnology.[1] As a result of their small size, nanoparticles can penetrate the cell wall and deliver drugs or biomolecules into living systems, usually for a therapeutic purpose.[2–5] Many different kinds of nanoparticles are known, many have been tested on biosystems, and some approaches have made it into clinical trials. Herein we are summarizing the state of the art of inorganic nanoparticles as carriers for nucleic acids (DNA, RNA, and oligonucleotides) to influence the gene expression of a cell. Because of the huge amount of literature on bioorganic nanoparticulate systems (such as, polycationic and liposomal agents and dendrimers), we will restrict ourselves to inorganic nanoparticles.

2. Transfection

The introduction of DNA, RNA, or oligonucleotides into eukaryotic cells is called transfection.[6] This process involves the uptake of extracellular molecules through the cell membrane into the cytoplasm and also further into the nucleus. If DNA is brought into the nucleus, it can be incorporated into a cell's genetic material and induce the production of specific proteins.[6–8] We distinguish between a transient transfection, where DNA does not integrate into the host chromosome, and a stable transfection, where the foreign DNA is integrated into the chromosome and passed over to the next generation. In contrast, the introduction of small-interfering RNA (siRNA) can selectively turn off the production of specific proteins ("gene silencing" or "antisense technology").[9–15] Such a controlled introduction of genetic sequences into mammalian cells has become an essential tool for analyses of gene structure, function and regulation; it is also the conceptual basis for a medical technique called "gene therapy" that potentially allows the treatment of a wide variety of diseases of both genetic and acquired origin.

Naked DNA itself cannot successfully enter cells; it requires the assistance of a suitable vector.[16] There are many reports about the direct injection of naked DNA into different tissues, for example, skeletal muscle,[17] liver,[18] thyroid,[19] heart muscle,[20] brain,[21] and urological organs.[22] The cellular uptake of plasmid DNA by injection is very inefficient, for example, in muscle cells less than 1% of the injected dose is taken up.[17] For instance, a tail-vein injection of naked DNA into mice did not result in gene expression in major organs[23] because of its rapid degradation by nucleases in the blood.[24,25]

The cell membrane is a permeable lipid bilayer which constitutes the outer border of a cell. The amphiphilic membrane lipid molecules (mostly phospholipids) have a polar hydrophilic head and two hydrophobic hydrocarbon tails.[26,27] In the cell membrane, there are also receptor proteins, recognition proteins, and transport proteins. The transport of small molecules across the cell membrane can occur by diffusion through channels (so-called passive transport) or with the help of transport proteins (so-called active transport).[28–30] Active transport requires energy, usually in the form of adenosine triphosphate (ATP). For the uptake of macromolecules or nanoparticles most cells use endocytosis, that is, the penetration of the cell membrane and the incorporation into an intracellular vesicle.[31,32] Vonarbourg et al. recently reviewed the factors which influence the uptake of nanoparticles of different nature by the mononuclear phagocyte system (monocytes and phagocytes). This process is the typical mechanism by which nanoparticles are eliminated from the blood.[33]

Figure 1 shows the DNA delivery pathway. First, nanoparticles are adsorbed on the cell membrane. Then, by endocytosis, nanoparticles are taken up by cells.[34,35] Some intracellular processes can prevent the transport of DNA across the cell to the nucleus. Endosomal degradation of DNA can occur during endocytosis inside an endosome if DNA does not escape from the endosome before the fusion

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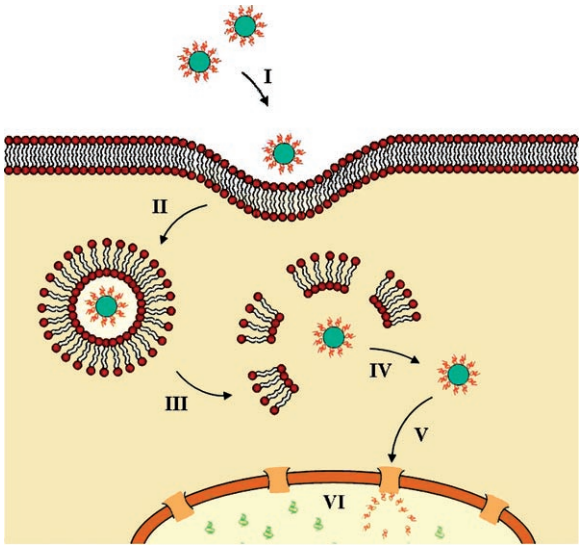

Figure 1. The transfer mechanism of nanoparticles (green circles) into a cell and into its nucleus. **I** Adsorption on the cell membrane. **II** Uptake by endocytosis. **III–IV** Escape from endosomes and intracellular release. **V** Nuclear targeting. **VI** Nuclear entry and gene expression, see text for details. Red foreign DNA, brown lipids, orange nuclear membrane, green cell DNA.

with lysosomes (in which the pH value is under 5).[36] After a successful release of DNA into the cytoplasm, degradation of DNA by specific enzymes (nucleases) can occur. For an efficient introduction of DNA into the nucleus, it has to overcome such obstacles and must be protected from nucleases. The next step is the introduction of DNA into the nucleus. In general, the transfer of molecules into the nucleus occurs through nuclear pore complexes (NPCs), that is, large proteins (nucleoporins) that are inserted into the double nuclear membrane that consists of two lipid bilayers.[37,38] NPCs are highly permeable to small molecules, but they restrict the movement of larger molecules across the nuclear envelope. To overcome this barrier, macromolecules that carry a nuclear localization sequence (NLS) can be recognized by importins and then be actively transported through the pore into the nucleus.[39,40]

Despite extensive studies on nuclear targeting,[41–43] it is still not clear how DNA is transported into the nucleus, that is, alone or incorporated into nanoparticles. One of the possibilities is that nanoparticles are slowly dissolved by acid in the endosomal vesicle and/or in the cytoplasm. Another possibility is that DNA-loaded nanoparticles go to the surface of the nucleus where the import of DNA can occur. In this case, it is advantageous if the DNA is protected by a nanoparticle until its uptake into the nucleus.

3. Methods for Gene Transfer into Living Cells

Gene therapy is the treatment of genetically caused diseases by manipulation of the genetic material of an organism. For such therapy an efficient method for the introduction of a therapeutic gene into cells is required.[44] Gene-delivery systems are generally divided into two categories: viral and nonviral systems. In Table 1, the current transfection methods are summarized and their advantages and disadvantages are shown. Viral carriers (which work by the same mechanisms as natural viruses that cause infectious diseases) are a most effective but rather dangerous method because of the risk of recombination, leading to the generation of viruses capable of replication. Electroporation is a safe, easy, and rather efficient method, but it needs a large amount of DNA and has to be optimized for every cell type. Microinjection only allows one cell at a time to be transfected and is therefore not feasible for a whole organism. Using the gene-gun technique, a shallow penetration of DNA into the tissue is accomplished. Cationic compounds and recombinant proteins were used in clinical trials; however, cationic compounds are usually toxic and recombinant proteins are expensive to prepare.

3.1. Viral Gene-Delivery Systems

Viral gene-delivery systems are based on the ability of viruses to infect cells. Part of the original gene segment of the viral carrier is eliminated and the reporter gene is inserted. This is the oldest method for gene transfer, first demonstrated on *Salmonella* in 1952.[45] Later, for gene transfer into cells, different viral vectors based on retroviruses,[46,47] adenoviruses,[48] adeno-associated viruses,[49] herpes simplex virus,[50] and other viruses were introduced. It is a most efficient method with which to introduce DNA into cells, but it carries

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Table 1: Comparison of different gene-delivery systems.

Transfection method		Advantages	Disadvantages
Viral methods[44,45,202]		highly efficient[46–50]	immunogenicity,[48,51,52] carcinogenicity,[48,51,52] inflammation[53]
Physical methods	electroporation[59–63,203–205]	easy to perform; efficient	optimization for every cell line required; a large amount of DNA is necessary
	microinjection[17–24,64]	exact direction of nucleic acid into a single cell	one cell after the other, that is, a slow, sequential method
	gene gun[65–69]	useful for genetic vaccination	shallow penetration of DNA into the tissue
Chemical methods	cationic compounds[76]	easy preparation	toxicity[72,75,82]
	recombinant proteins[56–58]	high biocompatibility	expensive
	polymeric nanoparticles, for example, polylactide[206,207]	easy preparation; size controllable; easy functionalization	limited efficiency; some are toxic
	inorganic nanoparticles[98,99]	easy preparation; size-controllable; easy functionalization	limited efficiency; some are toxic

serious risks, such as the possibility of recombination, strong immunogenicity, inflammatory response, and carcinogenicity.[48,51–53]

At present, there are no viral-based methods which would allow a safe and efficient gene delivery for clinical treatment.[54] Therefore, nonviral delivery systems have advantages for gene transfer even though they show a lower efficiency than viral systems. Helm et al. reviewed their applicability for spinal fusion through the induction of the production of bone-growth-stimulating proteins (such as, bone morphogenic proteins (BMPs)).[55]

3.2. Recombinant Proteins

Recombinant proteins, so-called TAT proteins (TAT = trans-activating transcriptional activator), are a special type of DNA vector which contain a nuclear localization sequence. Like a virus, they have the capability to penetrate a cell membrane and especially to overcome the nuclear-membrane barrier to deliver their genetic material. Such proteins may include polylysine segments,[56] protamine,[57,58] or histones to bind DNA and to form stable complexes which help to protect DNA from intracellular degradation by nucleases.[58]

3.3. Electroporation

Electroporation is a popular in vitro technique for introducing plasmid DNA into living cells. It was introduced in 1982 for the transfection of mammalian cells.[59] The application of electric pulses opens pores in the cell membrane through which DNA can pass and directly enter into the cytoplasm. Then, the pores close again and the DNA is trapped within the cell. This technique was applied to introduce plasmid DNA into tissues, such as muscles,[60] melanoma,[61] and liver.[62] Its efficiency varies greatly with cell types.[60,63]

3.4. Microinjection

Conceptually, the microinjection of naked plasmid DNA into a cell is the easiest method for DNA delivery. Its drawback is its sequential character, that is, the fact that only one cell at a time can be treated with DNA. It is, therefore, not applicable for research with large numbers of cells and for in vivo DNA delivery.[17–24,64]

3.5. Gene Gun

The gene gun ("biolistic particle delivery") is the most novel physical transfection method.[65] This technique is based on gold nanoparticles which are coated with DNA and then shot into target tissues or cells.[66] This approach allows DNA to penetrate directly through cell membranes into the cytoplasm or even the nucleus, and to bypass the endosomes, thus avoiding enzymatic degradation. The major limitation is the shallow penetration of the particles into the tissue. The depth of the particle penetration in the skeletal muscle of mouse did not exceed 0.5 mm.[67] Skin, liver, and muscle were all transfected by the gene-gun technique, but the efficiency depended on the tissue, for example, 10–20% of skin epidermal cells were transfected, whereas only 1–5% of muscle cells.[66–68] In vivo gene-gun application typically results in short-term and low-level gene expression. Nevertheless, it might be suitable for genetic vaccination.[69]

4. Chemical Methods Based On Nanoparticles

The chemical methods are generally based on nanoparticles, liposomes, or micelles which form a complex with DNA or incorporate DNA and serve as carriers. These methods can be divided into three groups: Cationic compounds, recombinant proteins, and inorganic nanoparticles. The different types of nanoparticles are shown in Figure 2.

4.1. Cationic Organic Molecules and Polymers

This approach uses the electrostatic attraction between negatively charged nucleic acids and cationic carriers, typically cationic polyelectrolytes (e.g. polylysine[70,71] or polyethyleneimine[72–75]) or liposomes/micelles from cationic surfactants (usually lipids).[76] These nanoparticle assemblies are taken up by cells.[77] In 1987, Felgner and co-workers were the

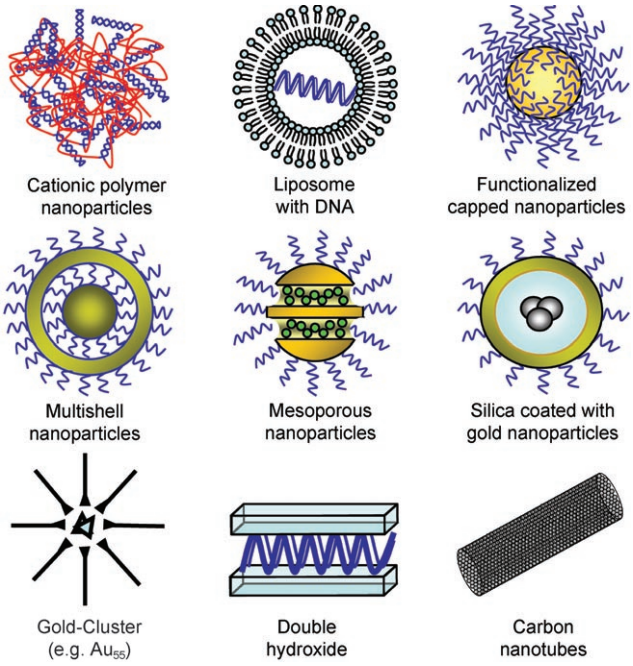

Figure 2. Different types of nanoparticles which can be used for the transfer of nucleic acids into living cells.

first to use the cationic lipid dioleoyltrimethylammonium chloride (DOTMA) in a 1:1 molar ratio with the neutral lipid dioleoylphosphatidylethanolamine (DOPE) to condense DNA for transfection.[78] Since then, a variety of cationic lipids was developed for gene transfection; liposomes also play a major role.[79,80]

One of the first polymers in nonviral gene delivery was poly(L-lysine) (PLL).[70,71] PLL particles with a size around 100 nm were easily taken up by cells, although the transfection efficiency remained low.[70] The reporter-gene expression was improved by the inclusion of targeting moieties, such as chloroquine[81] or fusogenic peptides. However, poly(L-lysine) is toxic and not approved for clinical use.[82] Another polymer which is widely used for transfection is poly(ethylenimine) (PEI). DNA-loaded PEI particles were delivered into liver[73] and lung tissue.[74] Again, the major drawback of this polymer is its toxicity.[72,75] Two frequently used commercial transfection agents are Polyfect and Lipofectamin. Polyfect consists of dendrimer molecules that radially branch from a central core. Amino groups at the end of the branches are positively charged and therefore strongly interact with the negatively charged phosphate groups of nucleic acids, forming compact structures.[83] Lipofectamine is a cationic-lipid transfection agent used for the introduction of DNA into eukaryotic cells. It was efficiently applied to many cell lines, for example, NIH 3T3, COS-1, and fibroblasts.[84]

The practical problems which are encountered when a synthetic compound is brought from the laboratory to a clinical application were outlined by McNeil and Perrie for cationic liposomes.[8] There are problems with the toxicity of cationic polymers[72,75] and liposomes,[82] and in general, the efficiency of nonviral systems is smaller than that of viral systems.[7] However, some cationic-lipid–DNA complexes were used in clinical trials.[85,86] They were successfully applied to deliver plasmid DNA to the lung,[87] the brain,[88] tumors,[89,90] and the skin.[91]

4.2. Inorganic Nanoparticles as Carriers of Nucleic Acids

The fact that cells take up nanoparticles can be used to bring nucleic acids into a living cell.[92] The chemistry of inorganic nanoparticles is highly advanced,[93–96] therefore many classes of inorganic nanoparticles have been used as carriers.[97–99] The inorganic materials used for DNA delivery comprise calcium phosphate, carbon nanotubes, silica, gold, magnetite, quantum dots, strontium phosphate, magnesium phosphate, manganese phosphate, and double hydroxides (anionic clays).

Although inorganic nanoparticles show only moderate transfection efficiencies, they possess some advantages over organic nanoparticles: They are not subject to microbial attack, they can be easily prepared, they often have a low toxicity, and they exhibit a good storage stability. It must be emphasized that DNA must be protected from intracellular attack by suitable "packaging". DNA that is only adsorbed on the surface of a nanoparticle is easily degraded by nucleases (see Ref. [64] for a review on the requirements for a successful transfection). Table 2 summarizes some features of inorganic nanoparticles for biological application.

4.2.1. Metallic Nanoparticles

The chemistry of metallic nanoparticles is well explored, particularly with respect to nanoparticles of the noble metals, gold, silver, palladium, platinum.[94] Usually, they are prepared by reduction of the corresponding metal salts in the presence of a suitable protecting group which prevents further aggregation (e.g., Au_{55} clusters[100]).

Gold nanoparticles (typical sizes: 10–20 nm) are easily taken up by cells.[101–104] It was recently shown by Schmid et al. that Au_{55} clusters effectively interact with DNA[105] and can be used as anticancer agent.[105] This interaction appears to be a matter of particle size (1.4 nm for Au_{55} clusters), that is, these small gold clusters are intercalated into DNA strands. The surface of gold can be conveniently covalently functionalized using thiols (as in self-assembled monolayers (SAMs)), and oligonucleotides can be attached to the particle surface.[106] Oishi et al. reported polymer nanoparticles which were assembled with gold nanoparticles and functionalized by thiol-oligonucleotide conjugates.[107] Oligonucleotide-loaded gold nanoparticles were also used for gene-silencing experiments by Mirkin et al.[108] Salem et al. reported bimetallic nanorods consisting of gold and nickel as a nonviral gene-delivery system.[109] The gold and nickel segments in these nanorods can selectively bind plasmid DNA and target ligands. The pathway of gold–peptide nanoparticles inside cells was studied by Tkachenko et al.[110]

Silver has been used for a long time as bactericide,[111] for example, to prevent biofilms. This research has now been extended to silver nanoparticles[112] which can be prepared in many different sizes and shapes[113] which is important

Table 2: Some key properties of inorganic nanoparticles which are used for transfection in cell biology.[a]

Kind of nanoparticle	Chemical composition[b]	Typical size range	Solubility in $\mu g\,L^{-1}$[c]	Comments
Cadmium sulfide	CdS	2–5 nm	0.69 $ng\,L^{-1}$	toxic, fluorescent, semiconducting
Calcium phosphate	$Ca_5(PO_4)_3OH$ (hydroxyapatite)	10–100 nm	6.1 $mg\,L^{-1}$[d]	biodegradable, biocompatible; may be made fluorescent by incorporation of lanthanides; cations and anions may be substituted
Carbon nanotubes	C_n	diameter of a few nm and length of a few μm	0	Not biodegradable, hollow; may be covalently functionalized to improve solubility and may be loaded with molecules
Cobalt-platinum	$CoPt_3$	3–10 nm	≈0	ferromagnetic or superparamagnetic; toxic in uncoated form
Gold	Au	1–50 nm	≈0	easily covalently functionalized, for example, with thiols
Iron oxide (magnetite)	Fe_3O_4	5–20 nm	≈0	ferromagnetic or superparamagnetic; harmful for cells in uncoated form; solubility increases with falling pH
Layered double hydroxides	$Mg_6Al_2(CO_3)(OH)_{16}\cdot 4H_2O$ (hydrotalcite)	50–200 nm	moderate, increases below pH 5–6	high selective anion exchange capacity; biodegradable in slightly acidic environment; cations can be substituted
Nickel	Ni	5–100 nm	≈0	immunogenic, toxic
Silica	$SiO_2\cdot n\,H_2O$	3–100 nm	ca. 120 mg $SiO_2\,L^{-1}$ (for silica particles)	Biodegradable; available also in micro- or mesoporous form (e.g., zeolites); easily functionalizable, for example, by chlorosilanes
Silver	Ag	5–100 nm	≈0	Bactericidal; dissolution product (Ag^+) potentially harmful for cells
Zinc oxide	ZnO	3–60 nm	1.6 to 5 $mg\,L^{-1}$	fluorescent, semiconducting
Zinc sulfide	ZnS	3–50 nm	67 $ng\,L^{-1}$	fluorescing, semiconducting

[a] Note that in general it must be distinguished between the solubility in ionic form (which is given here) and the solubility in the form of a nanoparticulate dispersion (i.e. as intact nanoparticles). [b] Sometimes idealized. [c] The solubility was computed for standard solids in pure water (pH 7) at 25 °C, using the solubility products of CdS (1.40×10^{-29} M²), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; $10^{-116.8}$ M[18]), and ZnS (2.91×10^{-25} M²). The other solubilities are taken from the literature. The solubility of the other compounds cannot be computed because it depends on the chemical species present on their surface. In any case, nanoscopic systems have a higher solubility than macroscopic phases owing to their higher specific surface area, and an appropriate surface functionalization can strongly enhance the solubility. For metals and alloys, the solubility also depends on the composition of the surrounding solution (e.g. its oxidative potential). [d] Computed for stoichiometric hydroxyapatite.

because the biocidal action appears to be size-dependent.[114] However, there are still many open questions, for example, the dosage dependence and the risk of bacterial resistance.[115]

4.2.2. ***Iron Oxides***

The magnetic properties of iron oxide nanoparticles (such as, magnetite, Fe_3O_4) can be used, for example, for cell sorting, for magnetic guidance in the body, and for tumor thermotherapy.[116–119] If the particles are subjected to a rapidly changing magnetic field, they can destroy the tissue of a tumor by hyperthermia.[117,120] Another approach is the magnetic guidance to a selected part of the body, for example, into a tumor.[121–124]

Gould et al. reported iron oxide particles with diameters ranging from less than 10 nm to 300 nm that can serve as a carrier for DNA.[125] Cheng et al. prepared magnetite nanoparticles with a diameter of 9 nm from Fe^{2+}, Fe^{3+}, and tetramethylammonium hydroxide. The nanoparticles were tested on Cos-7 monkey kidney cells, and they showed no cytotoxic effect at various doses of magnetite.[126] We note that magnetic iron oxides are often applied together with a suitable coating to improve their biocompatibility and functionalizability. Silica-coated magnetite nanoparticles were prepared by Bruce et al. and functionalized with amine groups to which oligonucleotides were covalently bound (Figure 3).[127,128]

A new approach was presented by Farle et al. where magnetite was incorporated into silica and then coated with gold. These magnetic particles can then be surface-functionalized and subsequently directed within the body, for example, to tumor cells.[129] Landfester and Ramirez showed how magnetite nanoparticles can be encapsulated in polymers by microemulsions.[130] Plank et al. presented the concept of magnetofection and showed a strongly enhanced uptake of DNA by cells after treatment with transfection agents, superparamagnetic particles (magnetite or neodymium-iron-boron), and application of an external magnetic field (Figure 4).[131–133]

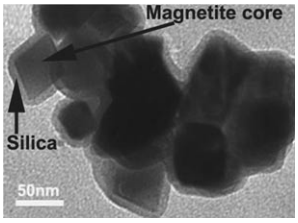

Figure 3. TEM micrograph of silica-coated magnetite nanoparticles used for transfection. The silica layer can in turn be covalently functionalized by organic molecules, using the silanol groups in the surface. Reprinted from Ref. [128], Copyright 2005, with permission from Elsevier.

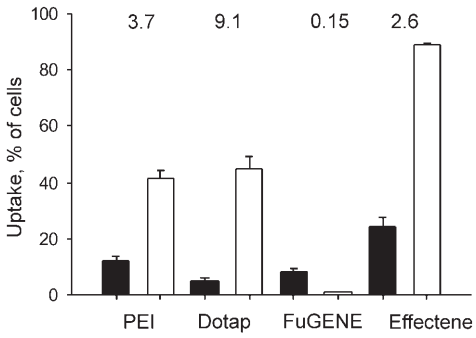

Figure 4. Efficiency of antisense-oligodesoxyribonucleotide (ODN) uptake by magnetofection (addition of superparamagnetic particles and application of an external magnetic field) using different transfection reagents. Comparison of the uptake of fluorescence-labeled (Cy3) antisense-ODN 4 h after 15 min of standard transfection (black bars) or magnetofection (white bars) using different transfection reagents (PEI/DOTAP-cholesterol, FuGENE, Effectene), followed by intense washing and addition of new medium. The numbers above the bars show the *n*-fold increase achieved by magnetofection. Reprinted by permission from Macmillan Publishers Ltd., Ref. [133], Copyright 2003.

Morishita et al. also showed that it is possible to increase the transfection efficiency of viral vectors with superparamagnetic iron oxide nanoparticles (SPION).[134] The interaction of surface-modified superparamagnetic iron oxides nanoparticles with cells was investigated by Gupta et al.[135–137] The unfunctionalized iron oxide nanoparticles alone were cytotoxic (disruption of the cytoskeleton organization) whereas the same nanoparticles functionalized with pullulan (a polysaccharide obtained from yeast) did not show such adverse effects. This study emphasizes the importance of the particle surface for the biological performance. Zhang et al. showed that polyethylene glycol-functionalized magnetite nanoparticles were taken up by macrophages (RAW 264.7) to a much lower extent than unfunctionalized magnetite nanoparticles, whereas for breast cancer cells (BT20), the opposite effect was observed. Clearly, different cell lines show a different selectivity towards the hydrophilicity of the particle's surface when it comes to the uptake of nanoparticles.[138] Berry et al. investigated the effect of pure and functionalized (either with dextran or albumin) iron oxide nanoparticles (diameters of 8–10 nm) on fibroblasts. They found that all three kinds of nanoparticles were well taken up by the cells, and that both unfunctionalized and dextran-functionalized nanoparticles induced cell death whereas albumin-coated nanoparticles did not hinder cell proliferation. Again, the nanoparticle surface appears to be more important than the composition of its core.[139]

4.2.3. ***Carbon Nanotubes***

Following the discovery of carbon nanotubes (CNT) by Iijima in 1991,[140] they were the subject of many investigations because of their special structural, mechanical, electrical, and chemical properties. Two different types are known: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs),[141] with diameters of a few nanometers and lengths up to 1 mm.[142,143] Their main characteristic property is their high ratio of length to diameter. Carbon nanotubes can be prepared on the gram-scale. They have found application as efficient biosensors,[144] as substrates for directed cell growth,[145] as supports for the adhesion of liposaccharides to mimic the cell membrane,[146] for transfection,[147] and for controlled drug release.[148] Carbon nanotubes are practically insoluble in biological (aqueous) environment and only a surface functionalization, for example, with polymers, can increase their solubility. Their chemical inertness, together with the option to functionalize them or to load the inside of the tube with biomolecules,[149] makes them attractive as carriers.[141,148,150] However, as carbon nanotubes are not biodegradable, their fate inside a cell is unclear. They must be excreted by suitable mechanisms without degradation. Carbon nanotubes were found to be cytotoxic in vitro to various mammalian cell lines.[148] Interestingly, the cytotoxicity of carbon nanotubes towards macrophages strongly depends on their structure. Jia et al. found a decrease in cytotoxicity in the row of SWNTs > MWNT (with diameters ranging from 10 to 20 nm) > quartz > C_{60}.[151] Major efforts were therefore directed to increase the solubility and to reduce the toxicity of the carbon nanotubes to obtain a better delivery system.

Harrison and Atala have reviewed the use of carbon nanotubes for tissue engineering, and conclude with the following sentences that bring the present state and the possible problems to the point:[150] "*While new uses of carbon nanotubes for biomedical applications are being developed, concerns about cytotoxicity may be mitigated by chemical functionalization. However, there will be some limitations to this nanomaterial since it is not biodegradable. Yet, it has been shown to be excreted* in vivo *and so could be cleared from the body once it is no longer needed.*"

Recently, Liu et al. have demonstrated that carbon nanotubes functionalized with covalently bound siRNA can lead to an efficient delivery of these nucleic acids into human T-cells and primary cells (Figure 5).[147]

4.2.4. ***Double Hydroxides/clays***

Layered double hydroxides (LDHs; also known as anionic clays or hydrotalcites) constitute a class of clays which contain positively charged layers. They have the general formula $M^{II}_{1-x}M^{III}_{x}(OH)_2(A^-)_x \cdot n\,H_2O$ with the archetype hydrotalcite, $Mg_6Al_2(OH)_{16}CO_3 \cdot 4\,H_2O$.[152] Interlayer anions and water molecules are present in the interlayer space and can be exchanged by other molecules.[153–155] LDHs with high anion-exchange capacity have attracted particular attention in the field of bio-hybrid nanomaterials owing to their high biocompatibility, high chemical stability, and controlled release rate. LDHs have a high potential to exchange intercalated anions by a variety of negatively charged biomolecules such as DNA, vitamins, drugs, or sugars.[153] Organic molecules can be released from LDHs at a rate that depends on the pH value and the ionic strength of the surrounding medium.[92,156] Choy et al. reported a biomolecular–inorganic hybrid, a class of anionic exchanging clays, incorporating DNA.[157] Because of its negative charge, DNA can be

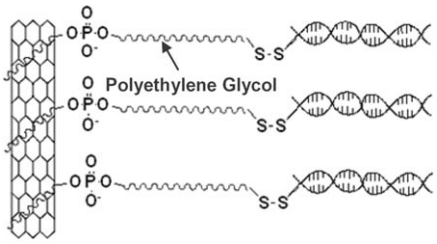

Figure 5. Carbon nanotubes for siRNA delivery into human T-cells. The nanotubes are functionalized with PL-PEG2000-NH_2 (PL = phospholipid, noncovalent bond; PEG = poly(ethylene glycol, M_w 2000) followed by the covalent attachment of thiol-siRNA through disulfide linkages. Reprinted with permission from Ref. [147].

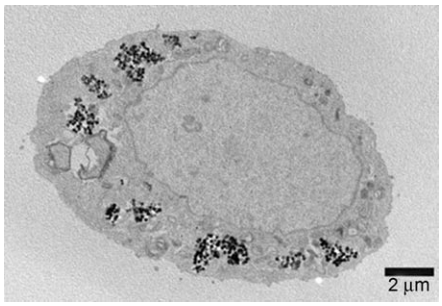

Figure 6. Dye-loaded mesoporous silicate particles (black) which were functionalized with DNA and endocytosed by a Chinese hamster ovarian cell (TEM picture). Reprinted with permission from Ref. [164]. Copyright 2004 American Chemical Society.

strongly incorporated into such a layered double hydroxide. If the material is prepared in nanoparticulate form with incorporated DNA, the particles can be used for transfection with high efficiency.[157–159] The solubility of such particulate LDHs strongly depends on the composition and properties of the solvent and strongly increases at lower pH values (see Refs. [160, 161] for solubility data). It is likely that these compounds can be dissolved (for example, by lysosomes which have an acidic internal environment) and removed from the cells in ionic form.

4.2.5. ***Silica***

The preparation of silica nanoparticles by suitable sol–gel processing routes is well established.[162] The presence of silanol groups on the surface allows an easy functionalization, for example, by attaching functionalized chlorosilanes. This property together with the high biocompatibility of silica has inspired many researchers to use them as carriers for drug release or transfection. A successful transfer of DNA into living cells was reported by Chen et al. Sodium chloride-modified silica nanoparticles had diameters of 10–100 nm and showed a transfection efficiency of about 70% without cytotoxicity. The administration of such silica nanoparticles to mice showed no pathological cell changes.[163] Radu et al. reported a novel gene-delivery system, where polyamidoamine dendrimers were covalently attached to the surface of mesoporous silica nanoparticles. These nanoparticles, with a size of 250 nm, formed a complex with plasmid DNA. A successful introduction of these nanoparticles into neural glia cells, human cervical cancer cells, and Chinese hamster ovarian (CHO) cells was observed with a higher transfection efficiency than that obtained with commercial transfection agents.[164] This concept is promising because the mesoporous particles can be used as carriers for nucleic acids, and in addition, dye molecules can be brought into the mesopores to allow the tracing of the nanoparticles in the cell by, for example, fluorescence microscopy. However, the particles were found in the cytoplasm but not in the nucleus, a fact which underscores the barrier action of the nuclear membrane (Figure 6).[164]

Luo et al.[165] noticed that unfunctionalized silica nanoparticles can serve as mediators for the uptake of DNA into cells by adsorbing on the cell surface.[166] This observation was developed into a modular system where silica nanoparticles (diameter about 225 nm) increased the concentration of DNA in the presence of a transfection reagent on the cell surface (simply by sedimentation of the nanoparticles on the cells), thereby increasing the transfection efficiency by a factor of ten. The silica nanoparticles alone were not active for cell transfection.[167] The co-precipitation of other inorganic or polymeric particles together with DNA on cell surfaces also led to a good transfection efficiency, comparable with commercial transfection agents. The increase in transfection efficiency could be directly related to the rate of sedimentation, for example, very small or low-density nanoparticles did not show an effect. The chemical composition of the nanoparticles was not of any influence, that is, this enhanced uptake of DNA is a kind of "mechanical" effect where the nanoparticles appear to exert some pressure upon the cell surface.[168]

4.2.6. ***Calcium Phosphate***

Calcium phosphates are the inorganic component of biological hard tissues, for example, bone, teeth, and tendons, where they occur as carbonated hydroxyapatite. With the exception of enamel, they are always found as nanoparticles.[169–171] Because of their biocompatibility there are no concerns about an inherent cell toxicity. However, they may increase the (usually very low) intracellular level of calcium after biodegradation which could be harmful to cells. Therefore, these particles have to be excreted from the cell before they dissolve in the cytoplasm and cause a harmful increase in the intracellular concentration of calcium.

The standard calcium phosphate transfection method, originally discovered by Graham and van der Eb in 1973, is very easy and straightforward.[172] The preparation of the calcium phosphate carrier for transfection consists of only a few steps: Mixing of calcium chloride solution with DNA and a subsequent addition of phosphate-buffered saline solution results in the formation of fine precipitates (nano- and microparticles) of calcium phosphate with DNA. This dispersion is added to a cell suspension, and the nanoparticles are taken up by the cells. The affinity of calcium phosphate to the phosphate groups in nucleic acids is probably the reason for the good adherence of the DNA to calcium phosphate (Figure 7).

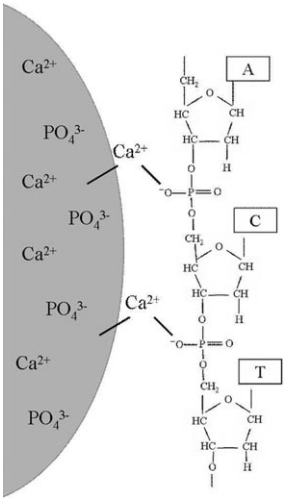

Figure 7. Model of the interaction between the surface of a calcium phosphate nanoparticle and a nucleic acid.

The precipitation conditions of the standard calcium phosphate method are decisive for the cell transfection efficiency. The main parameters are pH value, concentrations of $CaCl_2$ and DNA, temperature, and the time between precipitation and transfection.[172] The transfection efficiency also strongly depends on the kind of cells.[172] Quite often, the reproducibility is poor. Orrantia and Chang followed the pathway of ^{32}P-marked DNA inside the cell and concluded that the morphology of the colloids (mainly the particle size) and the protection from enzymes that degrade nucleic acid played a major role for the transfection efficiency.[173] Loyter et al. also stressed the importance of the nanoparticle size after studies with ^{3}H-marked DNA.[34] From a chemical point of view, it is understandable that this process depends on many variables that all influence the nucleation and subsequent crystal growth of calcium phosphate. With time, insufficiently protected nanocrystals will grow to microcrystals by Ostwald-ripening, and their ability for transfection will be lost.

Following these pathways, custom-made calcium phosphate nanoparticles were prepared for transfection by different groups. This activity was also inspired by the observation that calcium phosphate nanoparticles in general have a high biocompatibility and a good biodegradability compared to other types of nanoparticles. Maitra, even denoted them as "*second-generation nonviral vectors in gene therapy*".[174] A successful transfection was reported with DNA-loaded calcium phosphate nanoparticles functionalized with bovine serum albumin (BSA; particle diameter 23.5–34.5 nm).[175] Block-copolymer/calcium phosphate nanoparticle assemblies were prepared by Kakizawa et al. and used for cell transfection. The high biocompatibility of this system was emphasized.[176–178] Olton et al. prepared monodisperse calcium phosphate nanoparticles (with an unusually high Ca:P ratios of 110:1 to 300:1 and a typical diameter of 25–50 nm) by precipitation in the presence of DNA and found a most efficient transfection.[179] The Ca:P ratio in crystalline calcium phosphates is typically around 1.5:1,[170] thus, it is not clear from which chemical compound these particles were formed, although X-ray diffraction indicated hydroxyapatite. Other earth-alkaline phosphate nanoparticles showed a similar behavior. Bhakta et al. prepared magnesium phosphate and manganese phosphate nanoparticles with a particle size of 100–130 nm functionalized with DNA.[180] Brash et al. reported the preparation and characterization of strontium phosphate nanoparticles and their application for both transient and stable transfection.[181]

Concerning the biocompatibility of calcium phosphate nanoparticles, Liu et al. reported an apoptotic action of unfunctionalized calcium phosphate nanoparticles of about 50 nm diameter on a hepatoma cell line in the concentration range of 50–200 $mg\,L^{-1}$.[182] However, questions remain about the actual size of the nanoparticles investigated because the crystal growth was not inhibited (no surface functionalization). The adverse effect on the cells may be due to a harmful increase in the intracellular calcium concentration. Europium-doped calcium phosphate nanoparticles showed fluorescence, and the pathway of the nanoparticles could be followed inside pancreatic cells.[183–185] It was also possible to prepare terbium-doped (green fluorescence) and europium-doped (red fluorescence) calcium phosphate nanoparticles, colloidally stabilized by DNA, which were easily taken up by cells, and showed a sufficiently high internal crystallinity to give a reasonable fluorescence signal.[186] The accumulation of DNA-loaded calcium phosphate nanoparticles which also contained red-fluorescing tetramenthylrhodamin isothiocyanate (TRITC) BSA inside a cell and its nucleus was observed by fluorescence microscopy (Figure 8).[187]

Calcium phosphate nanoparticles can be prepared by rapid precipitation, followed by an immediate surface functionalization with DNA[188] or oligonucleotides.[189] These particles typically have a size of 80 nm and form stable colloidal solutions. As discussed in Section 2, a major problem

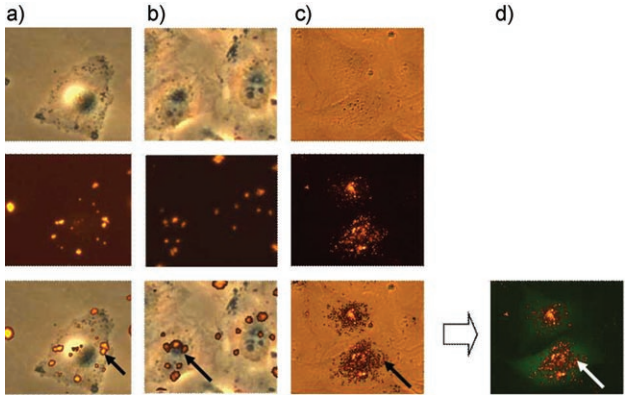

Figure 8. Transmission light microscopy (top row), fluorescence microscopy (center row), and overlay of both pictures (bottom row) of transfection experiments with human T-HUVEC cells. In light microscopy (top), the cells and their nuclei are visible. In the central row, the calcium phosphate/DNA/TRITC-BSA nanoparticles appear as bright red dots. In the bottom row, arrows indicate binding of nanoparticles to the cell surface after 2 h (a), penetration into the cytoplasm after 8 h (b), and accumulation on the nuclear membrane after 48 h (c). After 48 h, the transfected cells appear green as a result of the expression of enhanced green-fluorescent protein (EGFP). The incorporated red-fluorescing nanoparticles are also clearly visible (d).

is the intracellular degradation of the DNA-loaded nanoparticles on their pathway towards the nucleus. Some studies were carried out to elucidate the pathway of the calcium phosphate/DNA conjugates into the cell. Strain and Wyllie found less than 7% of the added DNA inside the cytoplasm and less than 4% in the nucleus. Only 0.5% of the DNA was still undegraded and active.[190] We have prepared multishell nanoparticles (Figure 9) in which DNA is incorporated both

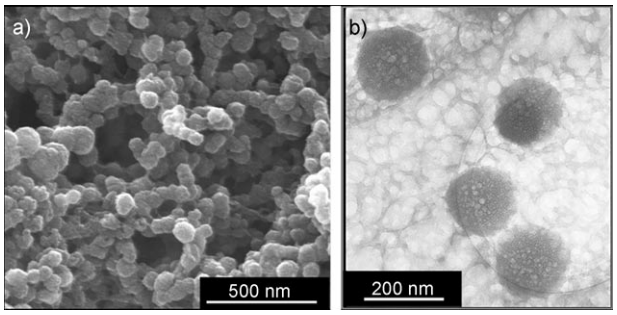

Figure 9. Scanning electron micrograph of calcium phosphate/DNA/BSA nanoparticles (left). Transmission electron micrograph of calcium phosphate/oligonucleotide nanoparticles (right).

inside the particle, where it is protected from degradations, and outside, where it serves as a protecting layer against aggregation and precipitation.[191] The transfection efficiency was considerably increased by this process.[192] The same concept worked well for gene silencing/antisense experiments with HeLa-EGFP cells where the green fluorescence was effectively inhibited by siRNA-functionalized calcium phosphate nanoparticles.[189]

4.2.7. ***Quantum Dots***

Quantum dots are small nanoparticles with typical diameters of a few nanometers (typically < 10 nm) which consist of II–VI or III–V semiconductors (e.g. CdS, CdSe, ZnS, ZnSe, ZnO, GaAs, InAs; sometimes in a core–shell structure).[193] They are protected against aggregation by suitable capping agents which can also be functionalized. They show favorable optical properties (highly efficient fluorescence owing to quantum confinement effects and a good resistance towards photobleaching) which are exploited, for example, for biomedical imaging.[194,195] Although their major application lies in the field of imaging, they were also employed for transfection. Tan et al. showed the preparation of self-tracking chitosan nanoparticles (diameter about 40 nm) with encapsulated CdSe/ZnS quantum dots and their application for siRNA interference. A high efficiency in gene silencing occurred after functionalization of the particle surface with suitable antibodies (HER2) that target specific receptors on the cell surface (Figure 10).[196]

Akerman et al. showed how ZnS/CdSe quantum dots coated with specific peptides can be used to target different cells and organs both in vitro and in vivo.[197] Srinivasan et al. encapsulated CdSe/ZnS quantum dots in a functionalized block-copolymer and attached DNA to the particle surface.

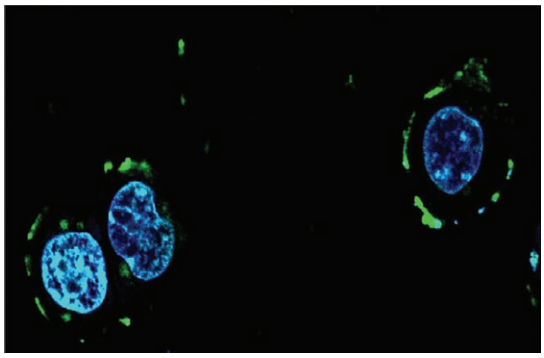

Figure 10. Green-fluorescing chitosan/CdSe/ZnS nanoparticles which were functionalized on their surface with antibodies that recognize the cell wall (HER2 antibody surface labeling). The nuclei of the SKBR3 cells were stained blue with 4′,6-diamino-2-phenylindol (DAPI). Magnification = 40×. Reprinted from Ref. [196], Copyright 2007, with permission from Elsevier.

The quantum dots served as fluorescence marker to visualize the transport of DNA into living cells during transfection.[198] Nikolic et al. showed how different nanoparticles (CdSe/CdS, Fe_3O_4, and $CoPt_3$) could be coated with amine-functionalized polyethylene oxide. In this way, their solubility in water was greatly increased.[199]

The inherent toxicity of II–VI and III–V semiconductor quantum dots (such as, CdSe, CdTe) is a serious issue for biological applications. As shown by Aryal et al., there are two possible reasons for the toxicity of quantum dots: The presence of surface cations (such as Cd^{2+}) and the formation of photoinitiated radicals.[200] Metallothioneins, that is, cysteine-rich proteins which are present in a cell, are able to mobilize cadmium from the nanoparticle surface by complexation, which leads to an enhanced rate of dissolution and higher toxicity. It was proposed that capping of the surface, either by silica or by compounds which form stronger complexes with cadmium than metallothioneins, might diminish this effect.[200] However, it may be argued that the long-term fate of such toxic quantum dots inside a cell is not clear, even if the surface is kinetically stabilized. Nevertheless, as the quantity of material in such nanoparticles is very small, the toxic effect may not be very serious.

A method to increase the biocompatability was demonstrated by Zhang et al. who showed, in a very comprehensive analysis, how the gene expression of fibroblasts changed when they were exposed to silica-coated quantum dots. The surface of CdSe/ZnS core–shell quantum dots was first silanized and then coated with polyethylene glycol (PEG). These surface-modified nanoparticles were not harmful to the cells, genes which are upregulated by heavy-metal exposure were not effected by the presence of these nanoparticles.[201]

5. Summary

Many different kinds of nanoparticles can be loaded with nucleic acids (DNA or RNA) and cells appear to be quite indifferent to the chemical nature of these nanoparticles when it comes to an uptake by endocytosis. Regarding their size, the

upper limit for an efficient uptake through the cell membrane appears to be around 100 nm. The surface functionalization of the nanoparticle is important for uptake and short-term cellular interaction, whereas the chemical composition of the interior (the "bulk") is important for long-term biodegradability and biocompatibility. For transfection, the nucleic acids must be protected from premature degradation, for example, by nucleases, inside the cell, so that they can transfer their genetic information. Both magnetic and mechanical factors can be beneficial for the cellular uptake of nanoparticles. The transfer of nanoparticles into the cell nucleus is necessary for transfection with DNA, whereas for antisense strategies with siRNA (gene silencing), it is sufficient to deliver siRNA into the cytoplasm. Therefore, the optimal carriers may be different for these two applications.

For a clinical application, such as in gene therapy, there is of course the requirement for a high transfection efficiency, but the aspects of biocompatibility, long-term biodegradation, and site-selective application have to be addressed as well. Inorganic nanoparticles offer many ways to prepare systems with a defined particle size, surface functionalization, nucleic acid protection, and biocompatibility. As it is possible to fine-tune their nanostructure, for example, by coating them with different layers or by loading internal nanopores, their use as carriers can be extended. For example, such coatings allow the shielding of internal, toxic ions (such as Cd^{2+}), the protection of internal nucleic acids from degradation, and the fine-tuning of the hydrophobic/hydrophilic surface properties.

Finally, we believe that a better understanding of the fate of the nanoparticles inside of the cell, and of the interactions between the organic and inorganic parts of the particles will lead to a delivery system suitable for clinical use.

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