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Inorganic nanoparticles as nucleic acid transporters into eukaryotic cells

Rinat N. Amirkhanov, Valentina F. Zarytova, Marina A. Zenkova

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences
prosp. Akademika Lavrentieva 8, 630090 Novosibirsk, Russian Federation

The review is concerned with inorganic nanoparticles (gold, titanium dioxide, silica, iron oxides, calcium phosphate) used as nucleic acid transporters into mammalian cells. Methods for the synthesis of nanoparticles and approaches to surface modification through covalent or noncovalent attachment of low- or high-molecular-weight compounds are considered. The data available from the literature on biological action of nucleic acids delivered into the cells by nanoparticles and on the effect of nanoparticles and their conjugates and complexes on the cell survival are summarized. Pathways of cellular internalization of nanoparticles and the mechanism of their excretion, as well as the ways of release of nucleic acids from their complexes with nanoparticles after the cellular uptake are described.
The bibliography includes 161 references.

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I. Introduction

An extensive search for new drugs and their wide use contribute to the extension of human life and the reduction in mortality from many diseases. Nucleic acids and their synthetic analogues hold promise as drugs.[1–4] The principle of action of these drugs is based on the formation of a complex with cellular RNA or DNA *via* complementary Watson – Crick base pairing. Nucleic acid fragments can be used to block the synthesis of unwanted proteins in an organism or to restore the functions of damaged genes,[2,5,6] thus reducing disease severity and duration of disease signs and symptoms. To act on a new target, it is sufficient just to alter the nucleic acid (NA) sequence. An important advantage of drugs based on nucleic acids or their short fragments over other medicines is their rapid and easy adaptation to various (for example, viral or genetic) diseases. Nucleic acids are employed as drugs in so-called antisense therapy. This approach is based on the arrest of the protein synthesis involved in the development of the disease through the inhibition of the messenger RNA (mRNA) translation by complementary nucleotide sequences (antisense oligonucleotides).

R.N.Amirkhanov. PhD in Chemistry, Junior Researcher of the Laboratory of Biochemistry of Nucleic Acids at the ICBFM SB RAS.
Telephone: +7(383)363 – 5162, e-mail: rinat@niboch.nsc.ru
Current research interests: nanoparticles (in particular, titanium dioxide), antisense oligonucleotides, peptide nucleic acids, influenza virus, flow cytofluorometry, investigation of cell survival.
V.F.Zarytova. Doctor of Chemical Sciences, Professor, Chief Researcher of the Laboratory of Chemistry of Nucleic Acids at the same Institute.
Telephone: +7(383)363 – 5124, e-mail: zarytova@niboch.nsc.ru
Current research interests: delivery of nucleic acids into cells, construction of nanocomposites from nucleic acid fragments, oligonucleotides.
M.A.Zenkova. Doctor of Biological Sciences, Professor, Head of the Laboratory of Biochemistry of Nucleic Acids at the same Institute.
Telephone: +7(383)363 – 5160, e-mail: marzen@niboch.nsc.ru
Current research interests: structures and functions of RNA, siRNA, miRNA; antisense oligonucleotides, regulation of gene expression, antagomirs, targeted delivery of nucleic acids into cells and tissues; tumour progression, metastasis, multiple drug resistance, cell vaccines, antiviral drugs.

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The main obstacle to the wide biomedical application of NAs is their delivery into target cells and to the targets within the cell. Since the efficiency of spontaneous NA transport into the cells is very low,[7] special transport systems are required for their delivery. The transfer of NAs into cells is complicated by negative charges of their molecules, and the efficiency of cellular uptake of NAs is very low. Besides, NAs are susceptible to hydrolysis by cellular enzymes. The use of viral delivery systems having high transfection activity provides an approach to solve this problem.[8] However, viral vectors are potentially harmful to the organism because they can cause unwanted side effects, such as immune responses[9] and inflammatory reactions, and even induce tumour (carcinogenic effect).[10]

Less efficient non-viral systems are currently commonly used for NA delivery into cells.[11,12] An example is the nucleic acid delivery into cells by nanoparticles.[13–15] The wide application of this approach was made possible by the progress in nanotechnology. Nanotechnology deals with objects one dimension of which is <100 nm. Materials with such sizes exhibit new properties different from those observed at large size. Nanoparticles have a number of advantages as NA delivery systems over other known systems. Due to the optical properties or other spectrometric characteristics, nanoparticles can be visualized in the living cell or in an organism by tomography, spectrometry and(or) microscopy.[13] Biodegradability of some types of nanoparticles[16] enables the solution of the problem related to their excretion. Due to photoactivity of nanoparticles,[17,18] they can be activated after the delivery. A high surface area to volume ratio provides a rather high drug loading capacity per surface area of nanoparticles. Besides, nanoparticles can protect the drugs to be delivered, for example nucleic acids, from cellular enzymes,[19] thus increasing the lifetime of the molecules delivered to the target. Another attractive feature of nanoparticles is the simplicity of their synthesis and the feasibility of additional modification.

Many materials that are applied to synthesize nanoparticles are used for many years as food additives (for example, titanium dioxide is the additive E171, gold is E175 and silica is E551), antibacterial agents (gold, silver, titanium dioxide), prosthetic materials (titanium and silicon oxides, gold) and so on.

A great diversity of different types of nanoparticles and their numerous applications as drug carriers are described in the literature.[9,20–25] Nanoparticles and derived nanocomposite can be divided into two classes: organic and inorganic. Inorganic nanoparticles have a number of advantages, such as, for example, the relative simplicity of the synthesis and the feasibility of modification.

In general, nanoparticles are internalized into cells *via* phagocytosis or macropinocytosis depending on the nanoparticle size and are trapped inside lysosomes after cellular uptake. Hence, different strategies for the modification of nanoparticles are required to allow them to escape from lysosomes. An appropriate modification may provide receptor-mediated cellular uptake of nanoparticles, while the uptake of unmodified nanoparticles into cells can occur through certain nonspecific interactions.[26]

The present review focuses on spherical-shaped inorganic nanoparticles, their conjugates with low-molecular-weight compounds and complexes with organic polymers, while organic nanoparticles are only briefly described. Such materials as silicon dioxide (silica), titanium dioxide, iron oxide, calcium phosphate and gold nanoparticles modified with polymers or containing different functional groups on the surface are of considerable interest. The review is also concerned with methods for the synthesis and modifications of nanoparticles and the feasibility of their conjugation and complexation with NAs. Visualization techniques for nanoparticles, their complexes and conjugates are described. Particular attention is given to biological effects of nucleic acids that are delivered by nanoparticles. Their toxicity is analyzed. The review primarily includes the studies published in the past 15 years. Such a long period of time is considered because the drug delivery by nanoparticles has attracted growing interest since the beginning of the 21st century and most of the methods currently used for the synthesis of nanoparticles have been already developed by that time. Recent studies were concerned with covalent and noncovalent surface modification of nanoparticles.

The delivery of nucleic acids by both organic and inorganic nanoparticles is the subject of considerable interest, as evidenced by a large number of reviews,[13,23,24,27–31] which were published in the past decade and which focused on the delivery of small interfering RNA (siRNA),[27,29] short single-stranded oligonucleotides,[24,28,29,31] plasmid DNA (pDNA)[23,29,30] and other compounds by nanoparticles.[13] As opposed to the reviews published earlier, in this review emphasis is placed on biological effects of NAs delivered by nanoparticles. Besides, the results of research on the delivery of different types of NAs using several types of nanoparticles are summarized.

II. Organic nanoparticles

Organic nanoparticles are fabricated using various polymers of both natural and synthetic origin. One particle can include several polymers that form a composite. The linear size of one polymer molecule does not generally exceed 1000 nm.[13] In the coiled state, the polymer molecule becomes much smaller and corresponds to the nanoparticle size. Among natural polymers, carbohydrates and proteins are widely applied for this purpose. The well-known biopolymers, such as chitosan, dextran, albumin and gelatin, produced from natural sources are referred to as examples. These polymers are biocompatible with cells, almost nontoxic and biodegradable. Some synthetic polymers, for example, the poly(lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) copolymer, are biodegradable in cells or *in vivo*.[13]

Organic nanoparticles can be prepared from synthetic polymers with the use of the finished polymers [poly(methyl methacrylate) (PMMA), poly(ethyl cyanoacrylate) (PECA), polyamidoamine (PAMAM)] by different physicochemical methods, for example, by the precipitation of dissolved materials as nano-sized particles upon mixing with a precipitant solution[32,33] or by the evaporation of a liquid from drops of an emulsion containing a polymer.[13,34] Organic nanoparticles can also be synthesized during monomer polymerization. This relates to polyethylene glycol (PEG), PLA, PLGA and polystyrene (PS). The latter method is most commonly applied. Apart from the synthesis of linear polymers, it is also possible to prepare branched molecules — dendrimers. The dendrimer synthesis is accomplished following either a divergent or convergent strategy, *i.e.*, a polymer molecule grows *via* the attachment of new

chains to the core that was already formed or through the connection of preformed oligomers giving a polymer. Cyclodextrins (cyclic polymeric carbohydrates) are also used for the delivery of DNA. However, since complexes of cyclodextrins with plasmid DNA are insufficiently stable, methods were developed for the synthesis of modified cyclodextrins (for example, amphiphilic),[20,35,36] which are more readily soluble in water and organic solvents.

An attractive feature of organic polymers as well as of their hybrids with inorganic nanoparticles is their ability to assemble in solution through weak electrostatic and hydrophobic interactions. In this case, the drug loading from the medium can occur spontaneously. The spontaneous self-assembly of nanoparticles from dipeptides was described.[37] Self-assembled nanoparticles based on cyclodextrin were fabricated.[38] It was shown[18] that the replacement of a hydrophilic solvent by a hydrophobic one leads to the drug loading, while the reverse replacement of the solvent hinders the release of the drug from the carrier.

III. Inorganic nanoparticles

Inorganic nanoparticles consist of oxides (iron, silicon or titanium oxides), inorganic metal salts (calcium and magnesium phosphates) and metals (gold, silver).[13,15,18,24,29] Apart from homogeneous nanoparticles, there are also combined nanoparticles, for example, core–shell nanoparticles, the core of which is composed of one material, whereas the shell consists of another one (below, they are denoted by the symbol '/'). This construct combines the properties of its constituent materials.[39]

Nanoparticles that are used in practice have a number of attractive properties: biodegradability (calcium phosphate, silica), resulting in the clearance of nanoparticles from the body; the feasibility of using them for magnetofection (iron oxide), which provides magnetic field-guided delivery of drugs to a specific site within the body.[40,41] Since gold and titanium dioxide nanoparticles exhibit photothermal and photoactive properties, respectively, they can be applied for the partial cell membrane destruction,[42,43] thus facilitating the better cellular uptake of the drug, or for the cleavage of the bond with the drug after its delivery into cells.[44]

III.1. Methods for the synthesis of inorganic nanoparticles

Methods for the synthesis of inorganic nanoparticles are described in the literature in detail. Main methods for the preparation of nanoparticles reported in the cited studies are listed below. Silica nanoparticles having a size of 1–100 nm are synthesized by the hydrolysis of tetraethoxysilane (TEOS).[45–50] Nanoparticles with a controlled pore size (2–20 nm)[51–56] can be fabricated by the template synthesis using a core, which can be easily etched away with a special solvent[57,58] or by hydrothermal treatment.[51] The size of these particles may vary from 60 to 300 nm. Porous nanoparticles can protect compounds loaded in the pores and ensure the delivery of two different compounds, one of which is present in the pores, while the other one is on the surface of nanoparticles.[59] Titanium dioxide nanoparticles, like silica nanoparticles, can be synthesized by the hydrolysis of titanium compounds, for example, titanium tetrachloride,[60–65] titanium tetrabutoxide[66] or titanium tetraisopropoxide.[18,67,68] Nanoparticles with different sizes (4 to 25 nm) are produced depending on the nature of the starting compounds.

The commonly used method for the synthesis of gold nanoparticles is based on the reduction of $HAuCl_4$ with sodium borohydride or trisodium citrate.[7,69–82] In the case of reduction with trisodium citrate, the gold surface is protected by citrate anions.[74,81] The size of the fabricated nanoparticles is generally 2–15 nm. An original method for producing gold nanoparticles was proposed by Ziangirova *et al.*[83] Ascorbic acid was employed to reduce gold in $HAuCl_4$. The reduction reaction is similar to those applied in other methods, but the reduction of gold was performed in organogel prefabricated as microspheres, which were surrounded by a thin water film containing $HAuCl_4$. This approach produces small nanoparticles with a narrow size distribution (20–25 or 5–40 nm depending on the conditions of the fabrication of the organogel). The method holds promise for the synthesis of nanoparticles from different materials.

Magnetic iron oxide nanoparticles can be produced by the coprecipitation of different di- or trivalent iron compounds, for example, by coprecipitation of Fe^{II} and Fe^{III} chlorides,[84,85] precipitation of iron(III) salts and fatty acids,[57,86,87] iron(III) tris(acetylacetonate).[88–90] In this case, the size of iron oxide nanoparticles is 4–12 nm.

Calcium phosphate nanoparticles are known to undergo rapid aggregation. Nanoparticles with sizes of 200–300 nm are stable for 3 days, whereas nanoparticles with a size of 90 nm are stable for only 6 h.[91,92] The most common method for the synthesis of such nanoparticles is based on their precipitation from a solution of calcium phosphate containing NA required for the further delivery. The size of nanoparticles fabricated by this technique is 100–300 nm.[93–104] They may contain one calcium phosphate layer and one NA layer or several alternating layers.

As mentioned above, nanoparticles consisting of different materials, primarily core–shell nanoparticles, are often used. These nanoparticles combine properties of both materials. For example, nanoparticles with an iron oxide core are suitable for magnetofection, whereas a silica shell protects these nanoparticles from aggregation and reduces the toxic effect of iron oxide on the cells. In the general case, these nanoparticles are fabricated by the synthesis of a shell or porous nanoparticles in the presence of another type of finished nanoparticles.[14,44,57,86,105–114]

III.2. Surface modification methods for inorganic nanoparticles

The surface modification of nanoparticles can be applied to vary their physicochemical properties, for example, to increase hydrophilicity, change the surface charge or attach groups, which will enable the targeted delivery of the construct into the cell.[115] The surface modification of inorganic nanoparticles is accomplished by attaching different low- and high-molecular-weight compounds (Table 1). The modifications of nanoparticles can be divided into covalent and noncovalent (the former modification is denoted below by the symbol '-'; the latter one, by the symbol '·') depending on the mode of attachment.

The attachment of low-molecular-weight compounds to nanoparticles is commonly used for modification. Such functional groups as amino, epoxy and carboxyl groups, which are suitable for both covalent and noncovalent (through electrostatic interactions) attachment of nucleic acids and other compounds, are employed for the modification.

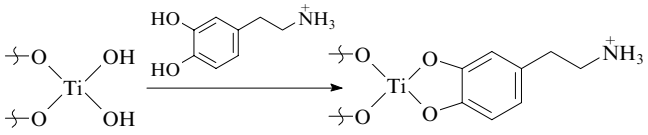
Table 1. Mixed organic and inorganic nanoparticles fabricated by surface modification of inorganic nanoparticles by organic polymers and the type of nucleic acids to be delivered.

Material	Examples of compounds used for surface modification of nanoparticles	Type of NA	Ref.
Titanium dioxide	Enediol ligands (dopamine, alizarin, ascorbic acid, *etc.*), PEG, PEI-FA, lipids, PL	DNA, PNA	15, 18, 66, 116 – 125
Silica	PEI, PL, DOTAP, PAH, mannose, introduction of amino groups by the reaction with APTES and of epoxy groups by the reaction with GPS	siRNA, DNA, pDNA	21, 47 – 49, 52, 53, 57, 86, 105, 110, 111, 126, 127
Iron oxides	PEI-Stearic acid, PEG, PL, PA	DNA, siRNA	84, 85, 89, 128, 129
Gold	PEI, PEG, DODAB, PAH-Cit, chitosan, introduction of carboxyl groups using MUA and of amino groups using cysteamine hydrochloride	DNA, siRNA, PNA	7, 17, 73, 75, 76, 79 – 81, 130, 131
Calcium phosphate	PEI-NA, chitosan, PEI-ALE, PLGA, PEG-PAA, PEG-PMA, PEG-PAsp(DET-Aco)	DNA, siRNA	16, 92 – 94, 100 – 104, 132

Note. ALE is alendronic acid or alendronate, APTES is (3-aminopropyl)triethoxysilane, Cit is citraconic anhydride, DODAB is dimethyldioctadecylammonium bromide, DOTAP is *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate, FA is folic acid, GPS is (3-glycidyloxypropyl)trimethoxysilane, HA is hyaluronic acid, MUA is mercaptoundecanoic acid, PA is poly-L-arginine, PAH is poly(allylamine hydrochloride), PEG-PAA is poly(ethylene glycol)-poly(aspartic acid) block copolymer, PEG-PMA is poly(ethylene glycol)-poly(methacrylic acid) block copolymer, PEG-PAsp(DET-Aco) is poly(ethylene glycol)-poly({*N*-[*N*′-(*N*″-*cis*-aconityl-2-aminoethyl)-2-aminoethyl]aspartamide} block copolymer, PEI is polyethyleneimine, PL is poly-L-lysine, PNA is peptide nucleic acid.

Since the surface of titanium dioxide nanoparticles is quite active, different molecules, in particular enediol ligands (for example, dopamine), can be covalently attached to these nanoparticles (Scheme 1). This property of nanoparticles was employed for the covalent attachment of an oligonucleotide through a dopamine linker.[116, 120]

Scheme 1



Several efficient techniques were developed for non-covalent immobilization of oligonucleotides,[122, 123, 133, 134] deoxyribozymes [135] and peptide nucleic acids [66] onto titanium dioxide nanoparticles to form the corresponding nanocomposites. These methods are based on the adsorption of polylysine-containing oligonucleotides and deoxyribozymes on titanium dioxide nanoparticles, the adsorption of oligonucleotides on nanoparticles in the presence of cetavlon, the adsorption of oligonucleotides or DNA/PNA heteroduplexes on nanoparticles premodified with polylysine (Fig. 1). All these procedures provide sufficiently strong fixation of immobilized NA fragments.

The surface modification of silica nanoparticles is accomplished either by introducing positively charged amino groups under the treatment with silane containing an amino group, for example with aminopropyltriethoxysilane (Scheme 2),[47] or using silane containing the reactive epoxy group, for instance, under the treatment with (3-glycidyloxypropyl)trimethoxysilane (Fig. 2).[52] The former approach can be applied to fabricate positively charged nanoparticles capable of interacting with negatively charged NAs, while the latter approach allows the further covalent attachment of polycations involving an epoxy group. Hartono *et al.*[52] performed the covalent attachment of polylysine through its amino group to the epoxy group of

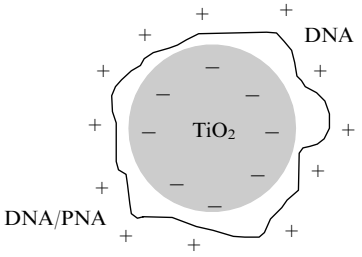
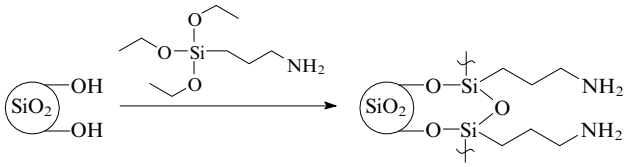


Figure 1. Adsorption of DNA or DNA/PNA heteroduplexes on titanium dioxide nanoparticles coated with polylysine.

GPS, resulting in the modification not only on the surface of nanoparticles but also inside the pores. Amino groups were grafted to the surface of iron oxide (Fe_3O_4) nanoparticles in a similar way using APTES.[136] Then the modification was performed at the amino groups with a derivative of the 3-mer peptide RGD (Arg-Gly-Asp) giving rise to an amide linkage between two components.

Scheme 2



Gold nanoparticles readily react with thiol groups. It should be noted that the compound containing thiol groups can be used directly in the synthesis of gold nanoparticles, resulting in the immediate formation of surface-modified nanoparticles. In particular, the modification can be carried out by the reaction with sulfanylundecanoic acid containing a thiol group at one end and a carboxyl group at the other

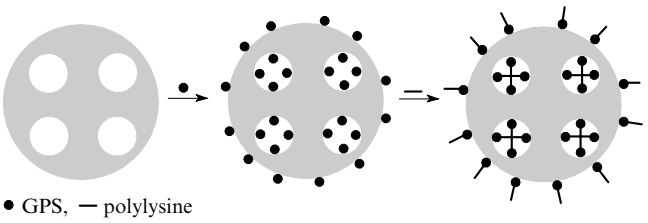


Figure 2. Reaction of silica nanoparticles with (3-glycidyloxypropyl)trimethoxysilane followed by the attachment of polylysine.

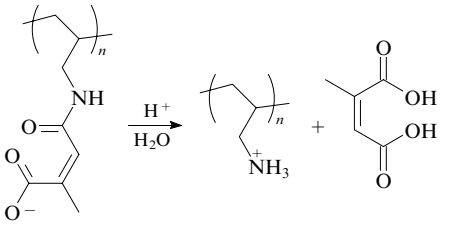
end.[81, 130] The gold nanoparticle conjugates with MUA thus prepared can interact with polycations. The direct attachment of nucleic acids to gold nanoparticles is also feasible. For instance, gold nanoparticle conjugates with PNA containing a cysteine residue at the end of the chain were synthesized in a similar way as the attachment of MUA.[77] Since PNAs have high binding affinity for NAs, these conjugates can serve as nano-sized sensors. Rosi *et al.*[78] used deoxyribooligonucleotides containing one or four terminal thiol groups. As it was expected, there was an inverse correlation between the number of thiol groups in DNA and the number of oligomer chains grafted to nanoparticles, *i.e.*, the larger the number of thiol groups that are attached to nanoparticles, the smaller the number of sites for the subsequent attachment of thiol groups. Thus, for the oligonucleotide containing one SH group, the number of chains on the surface of nanoparticles was 110 – 120; for the oligonucleotide containing four SH groups, the number of chains was 45 – 50. More recently, researchers at this laboratory[75] synthesized small interfering RNA-conjugated gold nanoparticles; however, the modification with oligo(ethylene glycol) thiol at one of siRNA chains was used in the latter case.

The modification of nanoparticles not only provides binding to nucleic acids but also imparts new properties to nanoparticles. For example, the attachment of alizarin to titanium dioxide nanoparticles allows their visualization by fluorescence microscopy,[121, 137] while the starting nanoparticles do not exhibit fluorescence properties. Since alizarin, like dopamine, serves as an enediol ligand, the reaction with titanium dioxide nanoparticles proceeds in a similar way.

Diverse polymers can be used for surface modification of nanoparticles, and there are numerous methods for the attachment of polymers to nanoparticles. The simplest and most common method involves the mixing of charged polymers with nanoparticles. In this case, the complex of nanoparticles with polymers is self-assembled in solution, resulting in the formation of charged nanoparticles capable of interacting with negatively charged nucleic acids or positively charged polymers (see Table 1). Apart from traditional polymers, such as PEI or PEG, their conjugates are also applied. The PEI – stearic acid conjugate, which was used to coat iron oxide nanoparticles, was employed as a proton sponge. This facilitated the endosomal escape of nanoparticles upon varying the pH,[89] the buffer capacity of the conjugate being higher than that of free PEI. The modification of nanoparticles with PEI and folic acid conjugates promotes the cellular uptake of nanoparticles through receptor-mediated endocytosis.[18]

The charge of many polymers changes from negative to positive upon a change in the pH of the medium.[138] One of such polymers was used[7, 81] to coat gold nanoparticles with several successive polymer layers. The first and the last layers contained PEI, the intermediate layer was composed of the negatively charged conjugate, which was formed by the reaction of polyallylamine hydrochloride with citraconic anhydride (PAH-Cit), and the siRNA to be delivered was on the surface of the complex. In a complex with nanoparticles, the proton sponge effect caused by PEI is weaker. However, a change in the pH of the medium from neutral to weakly acidic (pH 5), which is typical of endosomes, causes the hydrolysis of the amide bond in the PAH-Cit conjugate (Scheme 3), resulting in the release of the complex of PEI with siRNA from the carrier, an increase in the buffer capacity of PEI and subsequent endosomal release of NAs.

Scheme 3



The poly(ethylene glycol)-poly(aspartate) block copolymer containing side-chain $NH_2(CH_2)_2NH(CH_2)_2$ moieties, which are modified at the NH_2 group with *cis*-aconitate [PEG-PAsp(DET-Aco)], is another polymer with a variable charge.[102 – 104] The mechanism of hydrolysis of this polymer is similar to that of PAH-Cit. In this case, the amide linkage is hydrolyzed to give positively charged amino groups. This polymer was used to coat biodegradable calcium phosphate or silica nanoparticles. After endosomal uptake and a change in the pH from neutral to weakly acidic, the amide linkage was subjected to hydrolysis, which caused a change in the charge from negative to positive, destabilization of endosomes and drug release.

Apart from direct surface modification, methods were developed for the loading of polymers during the synthesis of nanoparticles. For example, the mixing of hydrogen chloroauric acid with thiolated polyethyleneimine (PEI-SH)[80] or dimethyldioctadecylammonium bromide[79] in the presence of a reducing agent (trisodium citrate or sodium borohydride) gives rise to gold nanoparticles having a polycation-modified surface.

An interesting method for the drug loading (combined with immobilization) was proposed by Ma *et al.*[53] Doxorubicin was loaded in the pores of silica nanoparticles, and DNA was immobilized *via* electrostatic interactions onto the surface of nanoparticles containing amino groups. The fabricated system enables the simultaneous delivery of two drugs. The simultaneous delivery of multiple drugs is a advantage because one drug can enhance the effect of another one. The simultaneous loading of two drugs in the porous silica nanoparticles is widely used for the construction of nanocomposites.[53, 55, 126]

IV. Visualization of nanoparticles, their conjugates and complexes in cells

Visualization is needed to monitor the delivery and localization of nanoparticles and drugs delivered by nanoparticles into cells.[139 – 141] Fluorescently labelled nucleic acid probes are most commonly used for this purpose. Mean-

while, visualization of nanoparticles is also feasible. Thus, free magnetic iron oxide nanoparticles, as well as porous nanoparticles (for example, silica nanoparticles), the pores of which are loaded with iron oxide nanoparticles, can be visualized by magnetic resonance imaging (MRI).[106 – 108, 142] The introduction of gadolinium(III) additives into gold and titanium dioxide nanoparticles also allows the application of MRI.[139, 143, 144] Transmission electron microscopy (TEM) and X-ray microscopy are versatile tools for visualization of nanoparticles and their derivatives.[82, 139, 145] Of particular note is a modern method — X-ray fluorescence microscopy (XFM). The exposure of a sample to X-rays causes the emission of photons, the photon energy being specific for each element. This technique is suitable for the localization of required elements in the cell and was successfully applied to study the transfection of MCF-7/WS8 cancer cells with titanium dioxide nanoparticles.[120]

Nanocomposites can also be visualized by means of surface modification of nanoparticles with low- or high-molecular-weight compounds. Thus, the surface modification of TiO_2 nanoparticles with alizarin [121] or fluorescently labelled polylysine [122] allows their visualization by fluorescence microscopy.

V. Interaction of nanoparticles with eukaryotic cells

Interactions of nanoparticles with eukaryotic cells are studied using both unmodified nanoparticles and nanoparticles with a modified surface immobilized with important biologically active agents, including NAs. Nanoparticles coated with polycations,[111] *e.g.*, polyethyleneimine,[85, 93] polylysine,[52, 86, 123, 133] polyarginine [128] and so on,[7, 79] are widely employed for the construction of nanoparticle-based delivery systems for nucleic acids. A polycation immobilized on the particle surface is either bound to NAs through electrostatic interactions or prevents the release of drugs loaded into the pores of nanoparticles.[52] It should be noted that the polycation can be covalently attached to NAs,[123, 134] which increases the strength of NA binding in nanoconstructs and the biological effect.[146] Bishop *et al.*[82] fabricated nanoconstructs using two or more polymer layers for the simultaneous delivery of several types of NAs (*e.g.*, DNA and siRNA) or using an additional outer layer, which coats the whole construct, protects it from external stimuli and is degradable in the cell medium. Apart from the above method, drugs were designed using nucleic acid – nanoparticle conjugates linked *via* a linker group,[69, 77, 78, 116, 147, 148] but the number of such studies is much smaller.

The efficiency of the interaction of nanoparticles and their composites with cells can be assessed from the accumulation of NAs in the cells, as well as from biological activity exhibited by the NA delivered into the cells. Nanoparticles or their modified forms can also have a significant toxic effect on the living cell.

V.1. Influence of nanoparticles on eukaryotic cell viability

The retention of eukaryotic cell viability is an important parameter of the interaction of nanoparticles with eukaryotic cells. Nanoparticles not only have a potential to enhance positive therapeutic activity but also can exert an unwanted toxic effect on the cell. Hence, the effect of drugs, including nanoparticles, on cell viability is of great importance.

Tetrazole derivatives, for example, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), are used in most studies for the assessment of cell viability. In this method, the application of tetrazole derivatives is based on the reduction of tetrazole to formazan by mitochondrial reductase, which is present only in mitochondria of living cells. Formazan that is produced after the reduction is dissolved in aqueous or organic solutions depending on the tetrazole derivative used. The cell viability is assessed by measuring the absorbance of a formazan solution: the larger the number of viable cells, the higher the absorbance of the solution. This method of cell viability assessment is simple and is suitable for the parallel analysis of a large number of samples.

Most data available in the literature demonstrate that nanoparticles do not exhibit pronounced cell toxicity,[142, 149] and the cell viability is, on the average, $>90\%$ after incubation (for 1 – 3 days) with nanoconstructs based on nanoparticles and NAs in the range of concentrations exhibiting biological activity. It should be noted that the modification of nanoparticles with polycations substantially reduces the toxicity of the latter.[7, 46, 54, 80]

An increase in the concentration of free PEI (molecular mass is 800 or 25 000 Da) leads to a decrease in the cell viability to 0% – 10%, whereas the cell viability in the presence of nanoparticles containing PEI remains at a level $>80\%$.[55, 80] We obtained similar results in studies of titanium dioxide nanoparticles coated with polylysine. It should be emphasized that the cell viability of different cell cultures can be different under the same conditions.[7, 54] The cell viability of HeLa cells in the presence of PEI was assessed at 80%, whereas the cell viability of MCF-7R cell was only 60%.[7] This fact should be taken into account in the drug design, because the replacement of a system for drug testing can greatly influence the final results (both positive and negative).

V.2. Protection of nucleic acids by nanoparticles from nuclease degradation

Nanoparticles not only can reduce toxicity of different polycations immobilized on their surface but also can protect immobilized NAs from degradation by cellular nucleases.[59, 98, 99, 150] Shi *et al.*[111] demonstrated that free plasmid DNA was completely hydrolyzed in the cell culture medium RPMI-1640 in the presence of 10% serum and could not be visualized after incubation for 12 h, whereas the same plasmid DNA in complex with $Fe_3O_4/SiO_2 \cdot$PAH nanoparticles could still be detected by agarose gel electrophoresis within 48 h. These authors also found that free DNA was completely hydrolyzed in the presence of DNase I within 30 min, while it was stable in complex with these nanoparticles for 6 h. The authors hypothesized that the polycation binds to DNA through electrostatic interactions, thus creating a physical shield that protects DNA from enzymatic degradation. Nucleic acid – nanoparticle conjugates have a similar effect. Consider as an example the studies [69, 78, 151] performed by the research team at the Northwestern University (USA). They demonstrated that the lifetime of NAs (both DNA and RNA) increases several times upon the covalent attachment to the surface of gold nanoparticles in the presence of 10% serum. For instance, it was shown that the lifetime of DNA in a

conjugate is 4.5 times longer than that of free DNA.[78] The lifetime of RNA in a conjugate increased sixfold and was 13.5 h.[69] It should be noted that this time is sufficient for the delivery of NAs into cells and the manifestation of their biological effect. It was shown that DNA – TiO_2 nanoparticle conjugates were stable and resistant to nuclease degradation for at least 12 h.[121]

V.3. Transport of nanoparticles loaded with nucleic acids and other agents to eukaryotic cells and their biological effect after delivery

Apart from the successful delivery of NAs with a transporter nanoparticle, the biological effect of the NA immobilized on the nanoparticle surface is an important characteristic of the interaction between nanoparticles and the cells. To exert a biological effect, NA should interact with its target within the cell and arrest target gene expression. However, the efficient NA transfection not necessarily causes a strong biological effect, because the NA that is present in the cell should be accessible to the target. This may be hampered by the entrapment of NA-immobilized nanoparticles in endosomal compartments inside the cell [152, 153] or by steric hindrance. The efficiency of delivery can be improved, for example, by introducing chemical modifications into nanoparticles or by means of different physical stimuli, such as changes in the temperature, the application of a magnetic field and so on.[115]

The nanoparticle delivery into cells can be assessed by X-ray microscopy, TEM, optical microscopy, fluorescence cytometry and so on. Fluorescence microscopy and flow cytofluorometry are universal methods suitable for the evaluation of both the transfection efficiency and the biological effect.[121, 154, 155] In order to estimate the transfection by these methods, NAs and nanoparticles are fluorescently labeled. The biological effect can be assessed by these methods either by investigating pDNA delivery (from the level of transgene expression) or by using cells that express green fluorescent protein (GFP) (from the degree of suppression of its expression). Green fluorescent protein is most commonly applied to study the biological activity of nanoparticles.[47, 85, 73, 130, 156] The GFP and luciferase expression can be monitored by measuring the fluorescence or luminescence intensity directly with a spectrometer. In the case of other targets, different from GFP, the target gene expression can be quantified using the polymerase chain reaction (PCR) by measuring the mRNA level or by means of Western blotting and enzyme immunoassay by measuring the protein level.[57, 105, 108] For example, these methods were applied to study the suppression of the expression of vascular endothelial growth factor (VEGF) [102, 103, 108] and the apoptosis regulator Bcl-2.[92, 93]

Apart from the methods that are employed for direct detection of the biological effect manifested as a decrease or increase (pDNA delivery) in gene expression, the biological effect can be determined indirectly without measuring the changes in the target gene expression. Genes encoding polo-like kinase 1 [(PLK1) and Mirk (minibrain-related kinase] served as targets in the study.[52] Proteins expressed by these genes are essential for KHOS osteosarcoma cell growth. Studies by fluorescence microscopy demonstrated that the siRNA delivery by polylysine-modified silica nanoparticles ($SiO_2 \cdot PL \cdot siRNA$) suppressed the expression of these genes, resulting in a 30% decrease in cell viability, whereas in the absence of siRNA, the cell viability decreased by 10%. It should be noted that the siRNA delivery by nanoparticles modified by attaching quaternary ammonium cations (SiO_2-$NH_3^+ \cdot siRNA$) instead of polycations to the nanoparticle surface led to a 15% decrease in cell viability. This difference between the nanoparticles coated with the polycation ($SiO_2 \cdot PL$) and SiO_2-NH_3^+ nanoparticles can be attributed to the fact that the former particles contain a larger number of sites accessible to NA binding, resulting in an increase in the biological effect of the nanocomplexes. It cannot be ruled out that a decrease in the efficiency of SiO_2-$NH_3^+ \cdot siRNA$ nanoparticles is due to the less efficient NA binding to individual positively charged amino groups on their surface. However, the observed difference between two types of nanocomplexes is insufficiently large (30% and 15%, respectively) to draw a conclusion about the role of the polycation in the increase in the efficiency of NA delivery.

Han *et al.*[7] assessed the biological effect of siRNA targeted to the mRNA of the MDR1 gene encoding P-glycoprotein by measuring the cell viability. The P-glycoprotein is excessively expressed in MCF-7R cells and is responsible for their multiple drug resistance. These cells are resistant to doxorubicin, because the P-glycoprotein acts as a pump effluxing hydrophobic compounds from the cells, thus decreasing the drug concentration inside the cell. The charge-conversional polymer PAH-Cit was employed. The charge of this polymer changes depending on the pH of the medium. After the treatment of the cells with gold nanoparticles loaded with siRNA, the cellular uptake of doxorubicin increased 20-fold compared to the system in the absence of siRNA. The PCR assay demonstrated that the MDR1 gene mRNA level decreased by 80% compared to that achieved with the use of siRNA non-specific towards the MDR1 gene mRNA.

The indirect assessment of the biological effect was reported by Li and co-workers [150] They constructed a delivery system for pDNA, which expresses shRNA (shRNA is small hairpin RNA) targeting VEGF mRNA, based on porous silica nanoparticles [$SiO_2(Fe_3O_4)$(doxorubicin) $\cdot$ PEI-FA $\cdot$ pDNA]. Doxorubicin and magnetic iron oxide nanoparticles were included into the pores of the nanoparticles. It was shown that the viability of cells treated with shRNA and doxorubicin decreased by 10% and 50%, respectively; the simultaneous use of shRNA and doxorubicin in the absence of magnetic field and under magnetic field resulted in a decrease in the cell viability by 70% and 75%, respectively. A decrease in the VEGF mRNA level was confirmed by PCR and was evaluated at 90%, while the VEGF mRNA level in the case of the pDNA delivery using the commercial transfection reagent LipoFilter was 50% relative to the control.

The efficiency of delivery and the biological effect of NA delivered by nanoparticles are often compared with the corresponding characteristics of commercial transfection reagents, *e.g.*, Lipofectamine 2000 (hereinafter, lipofectamine), which form liposomes in aqueous solutions. These systems are considered as the gold standard for transfection and are commonly employed as the control to compare the results obtained by different research teams.

The efficiency of the final biological effect of NAs observed after the transfection with nanoparticles is usually comparable with that observed after the traditional transfection using lipofectamine.[47, 57, 91, 92, 108] For instance, the level of GFP expression in HeLa cells decreased by 70%

after the treatment with oligonucleotide-loaded silica nanoparticles containing amino groups on the surface,[47] while the treatment with lipofectamine led to a 50% decrease in the level of expression. Meanwhile, Chen *et al.*[57] observed an inverse ratio for these values when examining the siRNA delivery by PEI-coated porous silica nanoparticles with iron oxide nanoparticles incorporated into the pores [$SiO_2(Fe_3O_4)\cdot PEI\cdot siRNA$]. The Western blot assay demonstrated that the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression decreased by 64% when nanoparticles were used as a transporter; in the case of lipofectamine, the expression decreased by 80%. As can be seen from the above examples, the difference between the biological effects of NAs delivered as complexes with nanoparticles or with lipofectamine is small.

It should be noted that nanoparticles have other useful properties apart from the delivery of NAs. Thus, the above-described porous silica nanoparticles containing magnetic oxide iron nanoparticles[57] can be visualized by MRI and the delivery can be targeted to specific sites by applying a magnetic field. Besides, further modification of nanoparticles is also feasible, which can improve their biological activity. However, in some cases the efficiency of NA delivery by nanoparticles is much lower compared to that achieved with the use of lipofectamine. For example, Kim *et al.*[157] recently demonstrated that the level of luminescence was 400 arb. units after the delivery of pDNA encoding luciferase by magnetic iron oxide nanoparticles into lung carcinoma NCI-H292 cells, and this level increased twofold (to 800 arb. units) under a magnetic field. However, this result was 3 times worse than that observed in the case of pDNA delivery by lipofectamine (2700 arb. units). Therefore, it is necessary to further improve constructs based on magnetic nanoparticles because the observed biological effect is in some cases lower than that with the use of commercial transporters. Nevertheless, the magnetic field-guided targeted drug delivery to specific sites of the body holds promise.

Like in the previous studies, a comparison of the effect exerted by siRNA on the Bcl-2 gene expression in prostate adenocarcinoma PC-3 cells did not reveal[92] substantial differences between delivery systems based on calcium phosphate nanoparticles and lipofectamine. However, the delivery system based on nanoparticles proved to be more sensitive to the presence of mismatches compared to lipofectamine; therefore, its effect is more specific. For instance, the real-time PCR assay and Western blotting demonstrated that the replacement of 13 out of 21 nucleotides in siRNA led to a 5–6-fold increase in the Bcl-2 gene expression (as follows from the accumulation of mRNA or the protein) using a delivery system based on nanoparticles as compared to a similar system containing wild-type siRNA, whereas a four-fold increase in the gene expression was observed in the case of lipofectamine-mediated delivery. Similar results were obtained in HeLa cells.[91]

Rhim *et al.*[19] demonstrated that the application of a delivery system based on a complex of nanoparticles with lipofectamine resulted in a substantial increase in the biological effect compared to free lipofectamine. The delivery of a lipofectamine-coated complex of gold nanoparticles with pDNA into human lung cancer A549 cells was employed. This transport system allowed an increase in the expression of GFP encoded by pDNA to be delivered by a factor of 1.5–4 (depending on the amount of the lipid) compared to the delivery using free lipofectamine, the toxicity of the construct based on nanoparticles being much lower. Thus, the cell viability after the transfection with the complex of nanoparticles with lipofectamine was 80%–100%; that for free lipofectamine was 40%. These data are in agreement with the results, which are considered in Section V.1 and which demonstrate that the immobilization of the polycation on nanoparticles reduces the toxicity of the former.

The transport system for the delivery of a short DNA fragment (DNA complementary to the 3′-end of influenza A virus RNA [A/Aichi/2/68 (H3N2) strain] encoding the NP protein) into canine kidney MDCK cells by means of titanium dioxide nanoparticles coated with the polylysine–DNA conjugate (PL-DNA) was examined. It was demonstrated that the oligonucleotide delivered by this construct ($TiO_2\cdot$PL-DNA) can decrease the influenza A virus titer by 3.9 $\log(TCID_{50}\ ml^{-1})$ ($TCID_{50}$ is 50% tissue culture infective dose) or 8000-fold compared to the untreated control cell population.[134] This efficiency of the drug was achieved at low concentrations of the DNA fragment (0.1 μmol $litre^{-1}$) and nanoparticles (5 μg ml^{-1}). The efficiency of the nanocomposites is comparable for the delivery before and after the infection of the cells. The use of the DNA complex with lipofectamine led to a decrease in the virus titer by only 2.4 $\log(TCID_{50}\ ml^{-1})$ or by a factor of 250, which is substantially worse than the results obtained using the complex with nanoparticles. An oligonucleotide noncomplementary to influenza virus genes, which was delivered by similar vehicles, did not exert a significant effect on the influenza virus titer. It should be emphasized that a considerable effect was achieved after the delivery of the oligonucleotide included in the $TiO_2\cdot$PL-DNA nanocomposite, whereas the low biological effect was observed with the use of the PL-DNA conjugate, which was not attached to nanoparticles.

In the case of the noncovalent attachment of DNA to polylysine (through electrostatic interactions), a decrease in the influenza A virus titer after the treatment with the $TiO_2\cdot PL\cdot$DNA nanocomposite was 20 times smaller [by 2.7 $\log(TCID_{50}\ ml^{-1})$] and was comparable with the effect of complexes with lipofectamine. A similar effect was observed using the DNA/PNA duplex ($TiO_2\cdot PL\cdot$DNA/PNA), in which the PNA oligonucleotide was targeted to the same region of viral RNA as the above-considered oligonucleotides.[158] It should be noted that the replacement of the DNA sequence by the sequence complementary to the 5′-end of mRNA or cRNA (RNA complementary to viral RNA) led to a decrease in the virus titer by 1.1 $\log(TCID_{50}\ ml^{-1})$.[146]

Levina *et al.*[159] demonstrated that DNA complementary to the 3′-end of viral RNA, which was delivered by the $TiO_2\cdot$PL-DNA conjugate, can suppress the influenza virus replication [decrease the titer by 3.0–3.9 $\log(TCID_{50}\ ml^{-1})$] of different strains [A/Aichi/2/68(H2N2), A/chicken/Kurgan/05/2005(H5N1), A/Salekhard/01/2009(H1N1)]. The system proved to be sensitive to the presence of mismatches: the replacement of one, two or three nucleotides in DNA led to a decrease in the efficiency of the inhibition of the virus replication by a factor of 2, 6, or 30, respectively.

Brief data on other delivery systems based on nanoparticles and their interactions with cells are summarized in Table 2.

Therefore, the above-considered examples demonstrate that NA delivery systems based on nanoparticles are not only as efficient as commercially available transport systems but even surpass them, as evidenced by a strong biological effect, lower toxicity of nanoparticles and sensitivity to mismatches.

Table 2. Delivery and biological effects of nanoparticles, their complexes and conjugates with drugs in eukaryotic cells.

Nanoparticles, their complexes and conjugates	Size /nm	Cells	Biological effects of nanoparticles in cells	Refs
TiO_2	20	HeLa	Cellular uptake of nanoparticles was demonstrated by TEM and TXM	145
TiO_2	21.2	Human colorectal cancer Ls-174-t cells	Photocatalytic properties of nanoparticles under UV irradiation causing the cell death	42
TiO_2-Dopamine-DNA	3 – 5	Human breast cancer MCF-7/WS8 cells; human prostate cancer PC3 cells; rat pheochromocytoma (PC12) cells	TEM and TXM studies demonstrated that nanoconjugates transfected by electroporation are specifically accumulated in mitochondria or nuclei depending on the DNA sequence. A 25-mer oligonucleotide complementary to the antisense chain of the mitochondrial gene for subunit 2 of NADH dehydrogenase and a 15-mer oligonucleotide complementary to the antisense chain of the R18S gene for ribosomal RNA were used	120
$TiO_2 \cdot PL \cdot DNA$	5	MDCK	Cellular uptake was confirmed by confocal laser fluorescence microscopy	122, 123, 133, 134
$TiO_2 \cdot PL \cdot DNA/PNA$	5	HeLa	The same	66
TiO_2-Alizarin	3	*Arabidopsis* seeds	Nanoconjugates enter *Arabidopsis* seeds and are accumulated in the seeds. Nanoconjugates were detected in cell vacuoles and nuclei by microscopy	137
TiO_2-Alizarin	6	Human prostate cancer PC3M cells	Three main endocytic pathways are involved in the cellular uptake of nanoconjugates: clathrin- and caveolin-mediated endocytosis and macropinocytosis. The results were confirmed by flow cytometry using specific inhibitors for each pathway	154
$SiO_2 \cdot PEI \cdot siRNA$	34	Mammalian osteosarcoma U2OS cells	Cells were transfected with pDNA expressing firefly luciferase and sea pansy *Renilla* luciferase. Firefly luciferase served as the target for siRNA; *Renilla* luciferase was used as the control. The expression level of firefly luciferase decreased $\sim$10-fold, whereas the expression level of *Renilla* luciferase remained constant	46
SiO_2-$NH_3^+ \cdot DNA$	47.7	Mouse endothelial C166 cells	The 2.5-fold suppression of the GFP mRNA expression level. The mRNA expression level was estimated by real-time PCR	105
SiO_2(doxorubicin)-$NH_3^+ \cdot DNA$	120 (pores 3.8)	HeLa	Porous nanoparticles coated with DNA were used, the pores were loaded with the anticancer drug doxorubicin. The efficiency of doxorubicin delivery, expressed as the decrease in cell viability, was confirmed; the cell viability decreased to 40% after the treatment with doxorubicin-loaded nanoparticles, to 85% after the treatment with free doxorubicin at the same concentration and to 75% after the treatment with DNA-coated nanoparticles without doxorubicin	53
$SiO_2 \cdot DOTAP \cdot DNA$	8 – 130	Chinese hamster ovary CHO cells	Cellular uptake was confirmed by confocal microscopy. The efficiency of cellular uptake decreased $\sim$10-fold with an increase in the nanoparticle size from 30 to 130 nm	127
$Fe_3O_4/SiO_2 \cdot PAH \cdot pDNA$	50 (nucleus 15)	HeLa	Delivery of pDNA encoding GFP. The GFP expression was observed. Under a magnetic field, the protein expression was concentrated at the center of the magnetic field, which can be attributed to an increase in the number of nanoparticles at the center of the magnetic field and, correspondingly, a decrease at the edges	111

Table 2 (continued).

Nanoparticles, their complexes and conjugates	Size /nm	Cells	Biological effects of nanoparticles in cells	Refs
CaP · siRNA · (PEG-PAA or PEG-PMA)	Overall 180 – 280	HEK293	Cells contained the plasmid pGL3 and pRL-TK DNAs expressing luciferase. The expression level of firefly luciferase from *Photinus pyralis* GL3 decreased by 60% with respect to the unaltered expression level of *Renilla reniformis* RL-TK luciferase	100, 101
CaP · PEG-PAsp(DET-Aco) · siRNA	Overall 42, 100	Pancreatic PANC-1 cancer cells	A charge-conversional polymer, which facilitates endosomal escape of nanoparticles upon changes in the pH value of the medium, was used. A 82% decrease in the level of VEGF mRNA gene expression was confirmed by real-time PCR	102, 103
Fe_3O_4 · PEI-SA · mcDNA	10.4 (overall 64)	Human breast cancer MCF-7 cells	Accumulation of nanoparticles in the cytoplasm was confirmed by Berlin blue staining	89
Fe_3O_4 · Polycation · pDNA	Overall 13 – 34	Human liver cancer Hep cells	Delivery of pDNA encoding β-galactosidase. The transfection efficiency was tested *via* assay of β-galactosidase activity. The magnetic field was shown to enhance transfection efficiency	84
Fe_3O_4 · PEG · (PA, or PL, or PEI) · siRNA	12	Brain tumour C6 cells; breast cancer MCF-7 cells; prostate cancer TC2 cells	The efficiency of GFP expression decreased by 30% – 80% depending on the type of cells, which was manifested in the decrease in the fluorescence intensity normalized to the total number of viable cells	128
Au-siRNA	13	HeLa	In the presence of nanoparticles, the firefly luciferase activity decreased by 73%; in the presence of free siRNA, by 33%. The level of *Renilla* luciferase as the control remained unchanged	69, 78
Au-NH_3^+ · pDNA	1.4 (overall 200 – 600)	Mouse myoblast C2C12 cells	Delivery of pDNA encoding mouse interleukin 2. The real-time PCR assay demonstrated that the use of nanoparticles for the delivery led to a 6.4 times higher mRNA level compared to that in the case of delivery by PEI (25 kDa); the enzyme immunoassay demonstrated that the expression of the protein interleukin 2 is 3.2 times higher. The concentration dependence was observed: the level of interleukin-2 mRNA expression increased with an increase in the amount of siRNA immobilized on nanoparticles	17
Au · DODAB · pDNA	14	HEK 293	The pDNA encoding GL3 luciferase was used. Cellular uptake was confirmed by SEM. The biological effect was assessed from the luminescence intensity normalized to the amount of the protein luciferase. The trasnfection efficiency decreased ~10-fold compared to DODAB · DNA	79
Au · PEI · siRNA · PEI	15.5	Chinese hamster ovary CHO-K1 cells	The concentration dependence for the decrease in the GFP expression depending on the amount of siRNA was found. An increase in the concentration of siRNA from 0.15 to 0.37 nmol litre^{-1} led to a 80% decrease in the GFP expression compared to the control (noncomplementary siRNA)	130
Au-LNA-NLS · siRNA	16.4	$CD4^+CD25^+$ Regulatory T cells isolated from Foxp3/eGFP reporter mice	Gold nanoparticle – LNA – transport peptide NLS conjugate. LNA was introduced in order to achieve better stabilization of nanoparticles; it does not exhibit biological effects. The degree of uptake was 10% in the presence of 8.1×10^6 nanoparticles in the cell, as estimated by flow cytometry (the fluorescent label AF-488 was introduced into LNA). The uptake was confirmed by confocal microscopy. After 48 h, the eGFP level decreased by 10% and 36% in the presence of 1.6×10^6 and 3.2×10^6 nanoparticles in the cell, respectively. After 72 h, the eGFP level decreased by 20% and 49% at the same doses of nanoparticles, respectively	156

Note. AF-488 is AlexaFluor 488, CaP is calcium phosphate, LNA is locked nucleic acid, mcDNA is minicircle DNA (gene vector) that forms hairpins, NADH is the reduced form of nicotinamide adenine dinucleotide, NLS is the nuclear localization signal, PEI-SA is stearic acid-modified PEI, SEM is scanning electron microscopy, TXM is transmission X-ray microscopy.

VI. Problem of efficient endosomal release of nucleic acids delivered into cells in complexes with nanoparticles

Efficient endosomal escape of NAs is an important issue to be considered in developing systems for NA delivery into cells.[59] After the cellular uptake, NAs attached to nanoparticles are entrapped inside endosomes and are not accessible to their targets. Different approaches to the solution of this problem were proposed. For instance, there is evidence that polycations can be fused with both the cell membrane and the endosomal membrane. This leads, first, to the improvement of cellular uptake of nanoparticles and, second, to destabilization of the endosomal membrane and the successful escape of nanoparticles from endosomes to the cytosol.[52]

Other ways of increasing the efficiency of endosomal escape of nanoparticles were also described in the literature. One of approaches is based on the application of polymers which can change a charge in response to changes in the pH of the medium. Complexes based on polymer-coated gold nanoparticles were investigated.[7,81] The complex was composed of several layers: a PEI layer directly coating gold nanoparticles, an outer layer containing a PEI complex with siRNA and an intermediate layer consisting of polyallylamine–citraconic anhydride conjugates. After internalization of these complexes into endosomes and a change in the pH from neutral to weakly acidic, the bond between PAH and Cit was hydrolytically cleaved. This led to the release of the PEI·siRNA complex from nanoparticles and the improvement of the endosomal escape of siRNA, which is most likely attributed to the enhancement of the proton sponge effect of PEI after the release. The use of a charge-conversional polymer resulted in a 3–4-fold increase in the transfection efficiency in HeLa cells compared to free PEI or lipofectamine, as it was shown by fluorescence microscopy. The feasibility of this approach was confirmed in HEK 293T cells.

A similar experiment, but with the employment of a traditional polyanion (polystyrenesulfonate, PSS) instead of PAH-Cit, demonstrated that the transfection efficiency of this complex is low.[81] This is evidence in favour of the proton sponge effect after the amide bond cleavage in PAH-Cit at low pH values, resulting in the release of the PEI·siRNA complex from the conjugate with nanoparticles and a more efficient disruption of the endosomal membrane, while an acid-liable bond is absent in PSS and, consequently, PEI·siRNA is not released from the conjugate with nanoparticles.

Endosomes can be destabilized by porous nanoparticles, the pores of which are loaded with low-molecular-weight compounds, which facilitate the disruption of membranes. Bhattarai *et al.*[55] employed the known lysosomotropic agent chloroquine, which inhibits endosomal acidification, as such a compound. In order to deliver chloroquine into B16F10 murine melanoma cells, this agent was loaded into the pores of silica nanoparticles, the particles were coated with several polymer layers, and pDNA encoding luciferase was immobilized on the surface of the resulting complex. The efficiency of luciferase expression achieved with the use of chloroquine-loaded nanoparticles and nanoparticles that do not contain this agent differs by no more than ~5-fold depending on the nature of the polycation used to coat the nanoparticles. It should be noted that the immobilization of pDNA reduced the escape of chloroquine from the pores by a factor of 2–4. This is quite predictable since pDNA immobilized on the nanoparticle surface additionally hinders the drug transport.

VII. Release of nucleic acids and other compounds from complexes with nanoparticles

The possibility of drug release from complexes with nanoparticles is no less important than their delivery and endosomal escape. Gold nanoparticles were found to have photothermal properties,[44,113] which are manifested in a local increase in the temperature of the nanoparticle surface induced by infrared laser radiation. Infrared radiation triggers the dissociation of the DNA/DNA or RNA/RNA duplex from their conjugates with gold nanoparticles. This resulted in the release of the oligonucleotide, which is not covalently attached to nanoparticles, thus enhancing the efficiency of the interaction with the target. The illumination by a laser with a wavelength of 800 nm led to a 50% decrease in the GFP expression in lung cancer H1299 cells, whereas only a 25% decrease in the level of expression was observed in the absence of laser irradiation.[113]

The photoactive properties of TiO_2 nanoparticles[40,160] can also be employed for the release of drugs from complexes with nanoparticles. For example, the anti-cancer drug paclitaxel was loaded into the pores of TiO_2 nanoparticles coated with the PEI-FA conjugate.[18] After the delivery into KB nasopharyngeal cancer cells, nanoparticles were illuminated by UV light, resulting in the cleavage of the bond between TiO_2 and PEI and the subsequent drug release. This effect was confirmed by the data on a decrease in KB cell viability (a 81% decrease in the presence of the drug and only a 27% decrease in its absence). Therefore, the photoactive properties of nanoparticles can be employed for the efficient drug release from the surface of carrier nanoparticles.

The dissociation of DNA/PNA duplexes[124,125] can occur similarly to the dissociation of DNA/DNA or RNA/RNA duplexes described above. The DNA/PNA duplex is linked to the TiO_2·PL complex through electrostatic interactions between the positively charged amino groups of polylysine and the negatively charged phosphate groups of DNA. Since the PNA molecule does not have a charge, the duplex is connected to TiO_2·PL through DNA.[124,125] The dissociation of the duplex immobilized on TiO_2·PL depends on its thermal stability and, consequently, the rate of release of PNA from the carrier can be controlled.

Besides, nucleic acids can be released from nanoparticles due to the cleavage of a particular bond in a multilayer complex, as for example, in the case of the PAH-Cit polymer described above.[7,81]

The application of polymers or nanoparticles that are hydrolyzable or soluble in the cell medium, holds promise. The use of such nanoparticles and polymers can temporarily protect the drugs to be delivered, which are incorporated in the complex, and provide their release with time. Bishop *et al.*[82] employed a complex of gold nanoparticles coated with a layer of polymers hydrolyzable in the cell. This complex protected NA during the delivery and then degraded in the cell to release NA, thus ensuring biological activity. The latter was manifested in either suppression of target gene expression or the expression of the delivered

pDNA. In human glioblastoma GBM319 cells, a ~10-fold decrease in GFP expression in GFP-positive cells (effect of siRNA) and a ~10-fold increase in GFP expression in GFP-negative cells (effect of pDNA) were observed.

Ito *et al.*[93] employed the pDNA·PEI·HA complex coated with calcium phosphate. Following the cellular uptake (mouse melanoma B16 cells were used), nanoparticles are trapped in endosomes, where calcium phosphate is gradually dissolved in an acidic environment to release the pDNA·PEI·HA complex. This complex possesses the proton sponge effect and can escape from endosomes, after which the pDNA expression can occur and the amount of GFP can grow. The maximum amount of the latter was observed by confocal microscopy on the fourth day after transfection; then the GFP level decreased due to the hydrolysis of pDNA.

VIII. Problem of the targeting of nanoparticles with nucleic acids to target cells

Apart from the issue associated with the drug release, which facilitates the enhancement of biological effects, the targeted drug delivery into specific cell compartments or cells in the body is a challenge.[161] The solution of this problem will improve the efficiency of drug delivery to target cells and, consequently, enhance biological effects. Nanoparticles containing ligands for specific cell receptors are commonly used for the targeted delivery. In the above-considered study,[18] the authors applied TiO_2 nanoparticles coated with the PEI-FA conjugate, in which folic acid served as the ligand. The delivery of this agent into KB nasopharyngeal cancer cells, which are characterized by overexpression of folic acid receptors, led to a five-fold increase in the degree of transfection compared to lung cancer A549 cells containing a smaller number of folic acid receptors. The same PEI-FA conjugate was used in the complex with silica nanoparticles.[150]

Park and co-workers[54] employed SiO_2 nanoparticles coated with the PEI-mannose conjugate. Upon the transfection of Raw 264.7 cells (mouse macrophages) containing a large number of mannose receptors, the efficiency of cellular uptake of mannose-loaded nanoparticles increased 20-fold with respect to the carrier in the absence of mannose. Similar efficiency of uptake into HeLa cells, which do not exhibit a pronounced mannose receptor expression, was observed in both cases.

As can be seen from these examples, the efficiency of drug delivery into ligand-specific cells can be substantially increased and, consequently, the selectivity of drug action on specific cells can be enhanced by including ligands for cell receptors into nanoparticles.

IX. Conclusion

The data considered in the present review demonstrate that nanoparticles are widely used for the delivery of nucleic acids. Diverse methods were developed for the synthesis of various nanoparticles and their modification with polymers and nucleic acids, in particular, their short fragments, oligonucleotides and large plasmid DNA. It should be noted that NAs are mainly bound *via* electrostatic interactions with the nanoparticle surface modified by introducing positively charged groups or with a polycation immobilized on the nanoparticle surface. This method is commonly applied due to the simplicity of the assembly of constructs since it is sufficient to mix the required components for the preparation of the final complex. It should be emphasized that the toxicity of polycations in such complexes is substantially lower that that of free polycations.

However, this approach is not suitable for the delivery of uncharged NAs, for example peptide nucleic acids. Nanoparticle-PNA conjugates were synthesized;[77, 147] however, these studies have not gone forward. Interesting delivery systems for PNA in the form of DNA/PNA duplexes immobilized on the polycation-coated nanoparticle surface were developed.[103, 158] This transport system allows the delivery of PNA into cells, resulting in biological activity, namely, the suppression of influenza virus replication.

Systems for the targeted drug delivery based on nanoparticles containing ligands for specific cell receptors have attracted considerable attention. Some studies are focused on the enhancement of the cellular uptake of drugs. For instance, transport systems containing polymers or low-molecular-weight compounds (*e.g.*, chloroquine) capable of destabilizing endosomes were proposed.

The release of drugs from conjugates employed for the drug delivery is still a challenge. This process can be activated by external stimuli, for example, by light or laser irradiation, as in the case of titanium dioxide nanoparticles[18] or gold nanoparticles.[44, 113] This leads to the release of the delivered compounds from complexes with the carriers. In the case of nucleic acid duplexes, the latter can dissociate from a complex or a conjugate with the carrier. Apart from the complete escape of the drug, the partial release from the carrier is also possible. For example, in the case of calcium phosphate nanoparticles, the dissolution of calcium phosphate is feasible.[93]

Therefore, nanoparticles are promising delivery systems for nucleic acids and other drugs. Main areas for further investigation aimed at the development and improvement of this delivery strategy were laid down.

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