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NANOPARTICLES AS A TOOL FOR TRANSFECTION AND TRANSGENESIS – A REVIEW*

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Abstract

Nanoparticles can be an alternative for currently used viral and non-viral systems of transporting exogenous DNA into cells, and furthermore, can be an effective way to produce transgenic animals. The possibility of linking them with proteins, lipids and of adding ligands enables improved transfection by making the crossing of membranes and the breaking of the endosomal barrier more efficient. Additionally, by the addition of magnetic particles it is possible to amend the intracellular kinetics of nanoparticle-DNA complexes. This review considers the use of nanoparticles to transfect cells and embryos and their possible application as a non-viral vector in animal transgenesis.

Key words: transgenesis, nanoparticles, transfection

Nowadays, transgenic animals are used in numerous fields, including as bioreactors of various proteins and other compounds needed in the pharmaceutical industry (Moura et al., 2011; Wang et al., 2013; Lipiński et al., 2012; Houdebine, 2009), as potential donors for xenotransplantation (Ekser et al., 2012), as animal models of different diseases (Song et al., 2011), and for carrying exogenous genes which allow them to feature some new functions (Laible et al., 2015, Marshall et al., 2006), the most important among which are the resistance to common diseases (Perrier et al., 2002; Rothschild et al., 2014), being a source of products with novel, desired traits (Laible, 2009; Ward, 2000; Nam et al., 2013; Keefer, 2004), or having an improved environmental impact (Golovan et al., 2001; Maga and Murray, 2010).

Since the 1980s, pronuclear microinjection has become the most popular method of transgenic animal production. Nevertheless, it still has disadvantages which are

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hard to solve, such as causing a high embryo mortality rate or the costs of equipment. It also demands a skilled operator. Additionally, this method requires some modifications when used for different animal species (Jura et al., 2007; Hammer et al., 1986; Verma et al., 2008). A high efficiency rate of gene expression in transgenesis might be achieved by the use of viral vectors, but they can also cause immune response in cells (Whitelaw et al., 2008). Other methods, such as sperm mediated transfer (Gandolfi, 2000; Smith and Corrado, 2005), electroporation or other physical methods, do not achieve the necessary efficiency (Smorąg et al., 2013). Facing the limitations of currently popular methods for transferring the exogenous DNA into the cells, researchers have continually searched for non-viral vectors which would avoid the most significant problems and at the same time would let them obtain a high efficiency of gene expression. The main purpose of this review is to evaluate the potential of using nanoparticles as a non-viral vector for animal transgenesis in view of current knowledge about their usage in drug and gene delivery.

Transfection with the use of nanoparticles – cellular fates

Nanoparticles are naturally or artificially produced objects with at least one of the three dimensions under 100 nanometres, often showing different characteristics than atoms of solid materials of the same substation. Despite their small sizes, nanoparticles have a larger surface for adhesion than other particles, simultaneously featuring high stability. Thanks to this, they are able to successfully cross the cells membranes, input into the cells and join with naturally occurring intracellular pathways, with significant accuracy of bringing the specific particles to the intended target place. As they have a great potential for the transport and protection of compounds inside the cells allowing them to avoid digestion by enzymes or being stored up in endosomes, nanoparticles have generated huge interest as a tool for cell process imaging, as a part of various systems for carrying drugs into cells, or finally for gene delivery (Barkalina et al., 2014; Svenson et al., 2012). The properties of nanoparticles which allow them to bind with nucleic acids by specific and non-specific bonds covalent between functional groups and non-covalent bonds are similar to those existing naturally between DNA and repressor proteins *in vivo* (An et al., 2012). The efficiency of transporting exogenous DNA inside the cells is restricted by two major factors: endocytosis, the way of crossing the cell membrane, or by proper cell receptor activation and the breaking of the endosomal barrier. Gemeinhart et al. (2005) showed that inside the cells, nanoparticles linked with a fluorescent marker were gathered in lysosomes, closer to the nucleus, but they did not cross the nuclear membrane. In fact, this did not interfere with the expression of protein coded by a given gene construct and gives a proof that nanoparticles can take part in the endosomal pathway and can transport DNA through cytoplasm to the nucleus. There are nanoparticles of different kinds of chemicals which have various traits, chemical properties, physical properties and structure. The main groups of nanoparticles which were already used and proved to be suitable for gene delivery and/or reproductive medicine, their characteristics are shown in Table 1.

Table 1. The types of nanoparticles which were successfully used for gene delivery and/or reproductive medicine and their characteristics

Types of nanoparticles				
Mesoporous silica	Polymers	lipids	carbon-based	metals
The mesoporous silica nanoparticles formulations are built of honeycomb-like structures. The channels enable encapsulating molecules and their intracellular delivery. These kinds of nanoparticles are also stable and biocompatible. They could be used for creating delivery platforms together with magnetic nanoparticles, micelles and polymers (Slowing et al., 2008). It is possible to obtain mesoporous silica nanoparticles with different pores size and morphology. Both these features affect the process of loading molecules into the pores (Wang et al., 2015).	There are numerous polymers which are now used as drug and gene agents in reproductive medicine, e.g. poly-L-lactide-coglycolide (PLGA), poly-L-lactic acid (PLA), chitosan, gelatine and polyamidoamine (PAMAM). They form different shapes of nanoparticles, dendrimers (Barkalina et al., 2014). It is possible to link them with functional groups, use their natural shapes or mix natural and synthetic polymers to obtain effective and precisely targeted delivery platforms (Nitta et al., 2013).	Lipid nanoparticles are another form of the biomimetic molecules. Nanoparticles consisting of phospholipids form mono- or bi-layered structures, while solid lipid nanoparticles create nanospheres whose lipid core is stabilised by polymers or surfactants (Barkalina et al., 2014). Cationic lipids bind negatively charged nucleic acids by ionic reactions. The inside part of the nanosphere is hydrophobic, which enables encapsulation and improvement of water solubility of substations carried by them (Carmona-Ribeiro, 2010). However, the nanocarriers of this type are less stable than those formed with polymers (Kang et al., 2015).	The forms of carbon-based nanoparticles which have been successfully used for gene delivery are graphene oxide and carbon nanotubes (CNT). They are not dispersable in water, so they need to be functionalised to be used with water solvents to avoid precipitation and agglomeration. Carbon nanotubes cause different cytotoxicity dependent on their structure, functional groups added or concentration. CNT are also claimed to interfere with some kinds of cytotoxicity dyes (Zanin et al., 2014).	The nanoparticles of noble metals were of particular interest as non-viral delivery systems, due to their being possibly less reactive than other types (Austin et al., 2014). The semiconductors nanoparticles (Cd, Se, Te) are rather used for cell imaging (Barkalina et al., 2014). The gold nanoparticles are successful in delivering drugs and genes to cells, their core is inert, but could be functionalised. Molecules may be bound with them by covalent and non-covalent conjugation (Ghosh et al., 2008). Silver nanoparticles are known for their antibacterial effect, but also higher toxicity (Austin et al., 2014).

Modification of nanoparticle-DNA complexes to improve crossing of the membranes

The main characteristics of nanoparticles enable their usage in cells transfection, but it also seems that it is crucial to work out the optimal technique which will improve gene expression, without affecting the cells and without causing damage to them.

The ability of the nanoparticles to take part in endothelial transport makes it possible to elaborate the most precise way of targeting the gene construct to an exact location (Nitta et al., 2013). Nanoparticles from different chemical compounds and elements act the same way as non-viral vectors for transfection, which enables them to transport DNA across cell membranes through endocytosis. The DNA stays wrapped and is easily released from the endosomes and is also protected from digestion by nucleases. As there are many different kinds of nanoparticles it is crucial to find those which would be the most suitable for transfection of mammal cells.

Linking nanoparticles with other compounds into multifunctional, complex transport units increases the efficacy of crossing cell membranes and intracellular transport (Jiang et al., 2012; Ahmad et al., 2015). Bonding proteins or peptides to the nanoparticles improved transfection efficiency from five- to ten-fold, depending on the cell type (Pozzi et al., 2014; Li et al., 2014), mostly by activating integrin receptors, which makes this pathway similar to the entry pathway used by adenoviruses and some bacteria such as *Salmonella* sp. or *Yersinia tuberculosis*. On the other hand, the addition of lipid particles caused increased release of endosomal DNA (Hart, 2010). Delgado et al. (2011) managed to improve transfection efficiency with the use of solid lipid nanoparticles by adding protamine, up to six-fold in kidney cells, but the same multifunctional unit, DNA/protamine/SLN (Solid Lipid Nanoparticles), lowered transfection efficiency in HEK 293 line cells (human embryonic kidney cells) in comparison to the control group without protamine. This gives us hope that by the possibility of joining the necessary ligands, transfection with the use of nanoparticles might be adjusted to the given cell types. Bahrami et al. (2014) have shown that different kinds of nanoparticles bond with the cell membrane in different ways. The differences in creating these bonds depend on their spherical or non-spherical shape, and also on the various adhesion potentials shown by different nanoparticles and the membrane shape changes caused by their entry. Experiments made by Prabha et al. (2002) showed that the size of nanoparticles significantly affected the transfection efficiency: 27- and 4-fold when smaller nanoparticles were used in comparison to larger ones for COS-1 (African green monkey kidney cells) and HEK 293 cell lines, respectively. The cellular uptake, surface charges and DNA release were the same in both dispersions, of small and larger nanoparticles, which shows that the efficiency of using nanoparticles for gene delivery is affected by numerous factors and that their usage should be specifically adjusted, depending on the cell type and conditions of application. Moreover, different substances used as a dispersive agent for nanoparticles, such as porcine lung surfactant and bovine serum albumin for sixteen different types of nanoparticles, have shown a significant influence on their agglomeration in the solution (Sauer et al., 2015). Additionally, research conducted by Wang et al. (2014 a) proves that the cooperative entry of nanoparticles through

membranes is beneficial for the nanoparticles of different shapes, for both oblate ellipsoidal nanoparticles and spherical ones. Together, these studies show the possibility of various modifications of nanoparticles for both *in vitro* and *in vivo* application.

The transfection of the cell lines *in vitro* performed with use of various kinds of nanoparticles resulted in different efficiency, toxicity and was tissue specific. These numerous tests showed that nanoparticles as carriers have efficiency comparable to common non-viral transfection methods. Research done by Tabatt et al. (2004) compares transfection achieved by the use of liposomes, cationic solid lipid nanoparticles and two commercial transfectants for COS-1 line cells (African green monkey kidney fibroblast-like cells), using four different transfection media. The efficiency of luciferase gene expression in groups transfected by solid lipid nanoparticles and by lysosome (both consisting of DOTAP – N-(1-(2,3-Dioleoyloxy) propyl)-N,N,N-trimethylammonium methyl-sulfate) have shown no statistically important differences, remaining at the same level in each of the transfection media. However, the obtained transfection efficiencies were lower than efficiency with the use of the commercial transfectant EscortTM (Sigma, France), consisting of DOPE (1,2-di-(cis-9-octadecenoyl)-sn-glycero-3-phosphoethanolamine) (Tabatt et al., 2004). Researchers achieved the same expression level of green fluorescent protein and luciferase protein by using solid lipid nanoparticles on HepG2 cells (human liver hepatocellular carcinoma cell line) as with the commercially available Lipofectamine (Cortesi et al., 2014). Research conducted by Severino et al. (2015) also points out the potential toxicity of cationic lipids used as nanocarriers for gene delivery. They managed to attain expression of human dynein, but also proved that the higher concentrations of SLN remain cytotoxic and cause a decrease in the number of living cells. Another group compared the transfection efficiency of DNA/DOTAP complexes and nanoparticles consisting of protamine, DNA and a lipid layer on different cell lines: CHO (Chinese hamster ovary cells), HEK293 (human embryonic kidney cells), NIH 3T3 (mouse embryonic fibroblasts), and A17 (murine cancer cells) (Caracciolo et al., 2011). The protamine/DNA/lipid nanoparticles were more effective at achieving expression of green and red fluorescent proteins in each of the cell lines, even in spite of different cell lines susceptibility to transfection. The toxic effect of cationic lipids necessitates the search for another compound which would be able to replace them. Different polymers of β-aminoesters as nanocarriers managed to improve the transfection efficiency of hESC (human embryonic stem cells) up to four-fold, while maintaining low cytotoxicity (Green et al., 2008). This gives us hope that nanoparticles are able to form, together with the necessary addition of other compounds which have the needed functional groups, widespread effective platforms for nucleic acid delivery.

Magnetofection used in order to improve transport kinetics of nanoparticles

Joining the nanoparticles used for transfection with magnetic particles which under specific conditions of the magnetic field are able to reach precise target locations inside the cell would help to increase transfection efficiency (Pfeifer et al., 2012; McBain et al., 2008; Grześkowiak et al., 2015). Magnetite crystals have been previously found in living organisms, for example inside the nervous systems of fish,

where they play a major role in sensing direction by the use of the magnetic field. Magnetofection, bonding gene constructs with particles which move in the magnetic field, allows an improvement in the targeting of the nuclear area by the exogenous DNA particles, without the disruption of endocytosis and without breaking the endosomal barrier at the same time. Experiments made by Plank et al. (2003) compared the transfection of CHO-K1l cell lines (Chinese hamster ovary cells) by lipofection and by linking lipotransfectants with different kinds of magnetic particles, also using various DNA concentrations, to judge the cellular uptake of DNA which was luciferase. The influence of magnetic particles was the highest during the first part of transfection, measured after ten minutes from the beginning of the process, when their presence significantly improved the amount of gene construct brought inside the cells. However, after four hours from the beginning of transfection, the levels of their efficiency reached the same point, and the improving impact of magnetic particles was shown only in the group transfected with lower DNA concentration. The next study conducted by the same researchers resulted in achieving different transfection efficiency of NIH 3T3 cells (mouse embryonic fibroblasts) by using nanoparticles consisting of PEI (polyethylenimine) and DOTAP (N-(1-(2,3-Dioleoyloxy) propyl)-N,N,N-trimethylammonium methyl-sulfate) linked with magnetic particles in the conditions of the magnetic field; the transfection was improved but in different ways, depending on the polyanionic or polycationic surface covering of the magnetic particles. Further studies, delivering green fluorescent protein coding gene to cells from lines CT26 (mouse colon fibroblasts) and HUVEC (human vascular endothelium cells), have proved that the magnetofection streamlined the transfection by shortening its duration and enabled the use of a lower concentration of gene construct. Magnetofection improves the first part of transfection, mostly by increasing its kinetics, but the parameters of the application should be adjusted to the type of the cells and the DNA construct, its concentration, as well as to the time of cell incubation. This was also confirmed by research conducted by Wang et al. (2014 a), who obtained the expression of GFP (green fluorescent protein) and DsRed (red fluorescent protein) and later the co-expression of both these proteins in PK-15 cells (porcine kidney cells) by using complexes of Fe3O4 magnetic particles with polyethylenimine, which increased the bonding of fluorescent proteins coding DNA. Plasmids containing DNADsRed (red fluorescent protein) made less stable complexes with nanoparticles than those containing DNAGFP (green fluorescent protein), which later resulted in much lower expression of DsRed than GFP. This also proves the influence of the gene construct on transfection efficiency when it is achieved by these kinds of particles. Despite that, Wang et al. (2014 b) were also able to later enable the co-transfection of these cells, using two plasmid coding DsRed and GFP, obtaining the expression of both these fluorescent proteins in 6.85% of the transfected cells, which was assessed by flow cytometry. Other researchers used magnetic particles surfaced by deacylated polyethylenimine for the transfection, which was previously proved by them to be less toxic than PEI (polyethylenimine) and resulted in efficient expression of green fluorescent protein in P19CL6 cells (mouse embryonal carcinoma cell-line), causing at the same time a low cytotoxicity (Kami et al., 2011). Further research proved that these kinds of magnetic particles, deacylated polyethylenimine

complexes, were able to transfect TIG-1 cells (human fibroblast-like cell-line) using various conditions of the magnetic field, achieving two to four times greater expression of GFP in comparison to the control group transfected without the influence of the magnetic field. The last test made with the same magnetic nanoparticles led to simultaneous co-expression of three fluorescent proteins: green, cyan and yellow in transfected TIG-1 cells. These results suggest that there is a chance for magnetic nanoparticles to become useful platforms for multiple gene delivery in the future.

Nanoparticles in transgenic animal production

The main setback for using non-viral transfection reagents is that they rarely combine high efficiency with a low toxicity (Breunig et al., 2007). Hopefully, nanoparticles may become a successful tool for transgenesis – different kinds of nanoparticles and the possibility of modifying them by the addition of proteins or lipids could enable avoidance of their retention in the gametes and embryos (Barkalina et al., 2015). Despite being successful in *in vitro* cell culture transfection, nanoparticles have not been yet widely used for gene delivery in transgenic animal production. Both the evaluation of the nanoparticles' influence on embryos and gametes as well as the assessment for the transfection efficiency seem to be crucial. The research conducted by Yoisungnern et al. (2015) showed that incubation of the mouse spermatozoa with silver nanoparticles lowered sperm viability and inhibited acrosome reaction. It also caused higher mortality and the morphological changes of the spermatozoa were shown more often. Furthermore, the sperm incubated with Ag nanoparticles caused a lower fertility rate when used for *in vitro* fertilisation. The blastocysts obtained with it were characterised with lower expression of the marker genes. On the other hand, Bosman et al. (2005) proved that the addition of the nanoparticles to the culture medium had no effect on the development of the mouse embryos, cultured from the 2-cell stadium to blastocysts. Taylor et al. (2014) used gold and silver nanoparticles for microinjection of murine embryos which have shown normal development. The nanoparticles were injected into one of the blastomeres from 2-cell embryos. During further culture there were no differences in embryo development in the groups of embryos injected with nanoparticles, embryos sham injected or non-injected. These results have led us to hope that nanoparticles might be used for transfecting embryos without causing changes in their development. It is also still necessary to judge not only the viability, but also the cell functions (Taylor et al., 2015). Although nanoparticles have been proven to be successful transfection reagents *in vitro*, there have been few attempts to obtain transgenic animals with their usage. The use of cationized gelatine/calcium phosphate particles with surface modification by cholaminchloride hydrochloride as a nanocarrier caused the expression of the green fluorescent protein in chicken embryos and caused production of green fluorescent protein on the fourth day after transfection (Huang et al., 2012). These nanocomplexes were previously tested by the same group of researchers and they were able to successfully transfect HeLa cells (human cervical adenocarcinoma cells). The expression of the GFP stayed at the same level as in the control group transfected by Lipofectamine, but resulted in lower cytotoxicity than the commercial reagent. Ultra-small graphene oxide nanocarriers with PEI (polyethylenimine) sur-

face modification were used first to transfect *in vitro* cells from lines H293T (human embryonic kidney cell lines) and U2Os (human osteosarcoma cells), where the efficiency of transfection reached about 95% with a low cytotoxicity rate reaching about 10%, and later to produce transgenic embryos of zebrafish (Zhou et al., 2012). The GFP gene constructs were microinjected inside the embryo interlayer of 200 one-cell embryos. 90% of the embryos expressed fluorescent protein and the survival rate was 100% (Zhou et al., 2012). Nanopolymer and hallosite clay nanotubules were the nanotransfectants used to transfect sperm in bovine NanoSMGT (sperm mediated gene transfer using nanoparticles) (Campos et al., 2011). These nanocarriers did not affect sperm mobility and viability and resulted in 40–45% embryos with exogenous DNA expression, compared to respectively 8–10% embryos produced by incubating sperm with the naked DNA and liposomes as the control groups. Nonetheless, the expression of GFP coded by exogenous DNA was not found in any of the produced embryos, which could be a result of low sperm mediated transfer efficiency (Eghbalsaied et al., 2013).

The toxicity of nanoparticles

When considering nanoparticles as a novel system for gene delivery, we cannot ignore their influence on living organisms and their potential toxicity. The evaluation of their toxic and teratogenic effect will be necessary also for their possible biomedical applications. Insufficient data about nanoparticle toxicity and agglomeration, especially with regard to oxidative stress, genotoxic responses and cell organelle damage which may be caused by them, are a potential threat to their biomedical and biotechnological use (Nel et al., 2006). Research conducted by Ema et al. (2010) proved the significant impact of various nanoparticles such as fullerenes, metallic and metal oxide-based nanoparticles on reproductive functions. They strongly affected differentiation of cells, pre- and post-implantation development of embryos, sperm motility and Leydig cell activity in mice and rats. These tests show that the assessment of toxicity is also crucial for establishing nanoparticles as a non-viral gene delivery system which could be not only effective, but also safe in use.

Conclusions

Nanoparticles, due to their ability to protect DNA during its transport into the cells, could be used as non-viral vectors in transgenesis in the not very distant future. Modification of nanoparticles by linking them with many different ligands and compounds helps to improve their intracellular transportation.

Targeting the nanoparticles to specific locations inside the cell, gametes and embryos, could be achieved by magnetofection. Although transfection with the use of nanoparticles causes comparable efficiency and lower cytotoxicity in comparison to commercially available transfection reagents used for cells cultured *in vitro*, it still needs to be proven that the delivery of DNA in this way leads to a similar level of gene expression *in vivo*, especially considering that this technology can cause side effects.

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