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Establishment of magnetofection – a novel method using superparamagnetic nanoparticles and magnetic force to enhance and to target nucleic acid delivery

vorgelegt von

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Erklärung

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Ehrenwörtliche Versicherung

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To my parents

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1 INTRODUCTION

1.1 Nucleic acids as drugs

In all living organisms, deoxyribonucleic acid (DNA) is the carrier of the genetic information and ribonucleic acid (RNA) is responsible for the regulated translation of this information into structural and functional molecules.

Given the distinguished role of nucleic acids in living systems, one can conclude that any cellular process may be influenced to some particular purpose by the introduction of nucleic acids into cells from outside. Already in 1966, Tatum formulated the basic concepts of nucleic acid therapy: gene complementation, modification/regulation of gene activities, and gene repair or replacement (Tatum, 1966).

Today, great efforts are put into the development of nucleic acid drugs which potentially can be used to treat diseases like e.g. Duchenne muscular dystrophy, cystic fibrosis, haemophilia, cancer or angiopathies. Drugs based on nucleic acids include **expressing sequences** (like complementary DNA, genes inclusive noncoding regulatory regions, messenger RNA), **gene silencing molecules** (like triple helix-forming oligonucleotides, antisense, small interfering RNA, long double-stranded RNA, ribozymes, deoxyribozymes, aptamers) and **nucleic acids for gene repair/replacement** (triple helix-forming oligonucleotides, RNA-DNA oligonucleotides or chimeraplasts, small DNA fragments).

Very commonly used is complementary (c)DNA which is cloned into bacterial plasmids or viral vectors and that is e.g. expressed under control of strong viral promoters (like e.g. the cyto-megalo-virus promoter). Successfully delivered cDNA is deposited in the nucleus either extrachromosomally or it is integrated into the host genome which is e.g. a special feature of retroviruses.

1.2 Delivery of nucleic acids

Current nucleic acid drugs are supposed to act either in the cytoplasm or in the nucleus of cells and therefore efficient transport to these sites is the prerequisite for any therapeutic benefit. Nature itself has provided the ideal solution for this delivery problem in the form of viruses. These obligatorily parasitic entities need to cross cellular membranes and ultimately need to shuttle their genetic information into cell nuclei in order to propagate. Consequently, genetically engineered viruses were among the earliest and in many respects are still the most

efficient shuttles (e.g. adenoviruses or retroviruses) used for nucleic acid delivery (Barzon et al., 2005).

In addition to viral vectors also nonviral vectors, composed of synthetic modules, were developed. The nonviral vector engineers try to mimick viruses in terms of nucleic acid compaction, cell specificity, cellular uptake, endosomal escape, nuclear transport, exploitation of cellular functions and stability (Plank et al., 2005). Today, the most commonly used nonviral vectors are cationic lipid-nucleic acid complexes (lipoplexes) which were inspired by viral membrane envelopes and polycation-nucleic acid complexes (polyplexes) which were inspired by viral capsid proteins. Lipofection (transfection with lipoplexes) was developed in 1987 by Felgner et al. who used N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) to prepare small unilamellar liposomes which were able to form cationic lipid-DNA complexes for successful *in vitro* transfection (Felgner et al., 1987). In 1989, Behr et al. prepared lipopolyamine-coated DNA complexes highly efficient in gene transfer through simple addition of excess lipospermine solution (e.g. dioctadecylamidoglycylspermine, abbreviated as DOGS) to DNA (Behr et al., 1989). Examples for popular polyplexes are poly-L-lysine (Wu and Wu, 1987), polyamidoamine dendrimers (Haensler and Szoka, 1993) and polyethylenimine (PEI) (Boussif et al., 1995). Crucial are the positive charges of the lipids and polyelectrolytes as they enable binding and compaction of the negatively charged nucleic acids. This compaction creates vector particles of small (often less than 100 nm) and uniform size and within the complexes the nucleic acids are also protected from degradation by nucleases (Vijayanathan et al., 2002). Further, the positive net charge of lipoplexes and polyplexes enables electrostatic binding of nucleic acid vectors to negatively charged proteoglycans (bearing heparan sulfate) on the cellular surfaces and thus mediates cellular uptake (Belting, 2003). It is generally accepted that endocytosis is the major cellular uptake mechanism for lipoplexes. However, depending on the biophysical properties of lipoplexes, direct fusion with the cytoplasmic membrane can occur as well (Lin et al., 2003; Pedroso de Lima et al., 2001). The endosomal escape of nucleic acids formulated as lipoplexes is thought to be mediated by lipid exchange reactions between the endosomal membrane and the lipoplex; i.e. anionic lipids from the endosomal membrane compete with the nucleic acid for binding to the cationic lipid moieties and thereby release the nucleic acid from the complex. Through this process, the endosomal membrane is destabilized (Xu and Szoka, 1996; Zelphati and Szoka, 1996a; Zelphati and Szoka, 1996b). Polyplexes are internalized by endocytosis as well (Rejman et al., 2005). The endosomal escape of PEI was explained by the “proton sponge hypothesis” (Boussif et al., 1995) which was experimentally

confirmed by Sonawane et al. (Sonawane et al., 2003). At physiological pH, PEI has a great buffering capacity by virtue of its secondary and tertiary amines and it will buffer the acidification process within endosomes. This means that the endosomal proton pump (H+ ATPase) needs to pump way more protons into the endosome until the natural endosomal pH of about 5.5 to 6.5 is reached. Because of H^+/ Cl^- charge coupling, endosomal Cl^- entry is increased as well and consequently osmotic swelling and endosomal leakage/lysis is promoted. An additional mechanic destabilization may be provided through swelling of the internalized polymer itself due to electrostatic repulsion of its protonated amino groups. Similar mechanisms probably account for the activity of polyamidoamine dendrimer whereas polylysine is already fully protonated at physiological pH and has therefore no endosomal buffering capacity (Sonawane et al., 2003). Additionaly, membrane-active agents can enhance the endosomolytic potential of polyplexes. For example (inactivated) adenovirus particles (Cotten et al., 1992; Curiel et al., 1991; Wagner et al., 1992b) or pH-specific fusogenic peptides such as the N-terminus of influenza virus haemagglutinin HA-2 (Plank et al., 1994; Wagner et al., 1992a) and the N-terminus of rhinovirus HR V2 VP-1 protein (Zauner et al., 1995) are able to disrupt the endosomal membrane at acidic pH. Further modules that can be added to nonviral vectors are e.g. nuclear localization signals (Ritter et al., 2003; Rudolph et al., 2004) to enhance nuclear uptake, or molecules like e.g. polyethylene glycol (PEG) that reduce the surface charge of polyplexes which results in decreased plasma protein binding, decreased vector and erythrocyte aggregation, stabilized complex size and prolonged circulation in the blood (Finsinger et al., 2000; Gunther et al., 2005).

The delivery of naked or packaged nucleic acids for therapeutic purposes can either follow the **ex vivo** or the **in vivo** strategy. In the ex vivo approach, the target cells are removed from the patient, get genetically manipulated by nucleic acids *in vitro*, and are transferred back into the organism. In the in vivo approach, the nucleic acid drugs are delivered either systemically or directly to the target organ (in situ).

1.3 Localized drug and nucleic acid delivery

1.3.1 The importance of localized delivery

The general objective in drug delivery is to obtain a drug concentration at the target site that is high enough to show therapeutic effect. Optimum drug delivery would even enable to exploit locally the full dose-response range to the level of saturation of the biologic process the drug

is designed to act on. But *in vivo* it is often difficult to achieve sufficiently high drug concentrations at the target site because clearance of drugs by macrophages of the reticuloendothelial system (reticulum and endothelial cells in liver, spleen, lung and bone marrow) and by mobile neutrophiles, clearance by the kidney, interactions with blood proteins, metabolism (enzymatic degradation), immune responses and complex biodistribution patterns limit the bioavailability of a drug at a target site and therefore high doses have to be applied.

From *in vitro* experiments with plasmid DNA as nucleic acid drug, it has been estimated for polyethylenimine(PEI)-DNA vectors, that of about 700.000 plasmid copies applied per cell in a standard transfection, roughly 50.000 copies per cell will be present in the cell after 7 hours of incubation (Kichler et al., 2001). In another publication, it was estimated that one out of 100 microinjected cytoplasmic pDNA copies in PEI-DNA formulation reaches the nucleus (Pollard et al., 1998). These two estimates together would predict that at least 1.400 plasmid copies in PEI formulation per cell would be required in order to have one copy reach the nucleus. As it can not be assumed that each cell-associated copy is located in the cytoplasm, a more realistic estimate would predict that rather 10.000 or more copies in PEI formulation per cell would be required for this purpose. *In vivo*, as a consequence of the limited bioavailability of drugs at the target site, the required nucleic acid copy number per cell will be much higher than in cell culture. In summary, the threshold of action for nucleic acid delivery in terms of required copy number per target cell can be quite high.

Unfortunately, high doses of many drugs are toxic for organs which are not the desired target site (e.g. kidney or liver toxicity). Sometimes toxicity even sets in before any therapeutic effect is detectable. The art is to apply a dose that results in a concentration with therapeutic effect at the target tissue while systemic toxicity and side effects are bearable for the patient. This therapeutic window is often narrow (Plank et al., 2003a). Therefore, localization (targeting) of drug delivery is an important objective and serves mainly three related purposes: Firstly, to exceed the local threshold of drug action at the target site while remaining below this threshold at non-target sites, secondly, to avoid side effects in this manner, and thirdly, to enlarge the therapeutic window (i.e. to locally exploit the full dose-response range of a drug).

1.3.2 Hierarchies of localization (targeting)

Useful classifications of drug targeting, exemplified by tumor targeting, have been published by Lübbe et al. (Lubbe et al., 2001). Among these, a discrimination between first, second, third order targeting (Lubbe et al., 2001) is useful and in addition a fourth order of targeting is appropriate for nucleic acid delivery. According to Lübbe et al., first order targeting refers to the localization of a drug at the capillary bed of the target site (organ or tissue). Second order targeting refers to the selective passage of the drug to target versus normal cells, and third order targeting involves uptake into cells by processes such as endocytosis. Fourth order targeting for nucleic acids is e.g. site-specific genomic integration versus random integration or extrachromosomal (episomal) localization.

1.3.3 Passive and active targeting

Passive targeting refers to the preferred accumulation of a drug formulation or a nucleic acid vector in a particular tissue based on the biophysical properties, notably size and charge, of the formulation (Nishikawa et al., 2005). Additionally, special characteristics of certain tissues can lead to site specific concentration of a drug. For example the enhanced permeability of tumor vasculature and the inadequate lymphatic drainage of solid tumors (enhanced permeability and retention [EPR] effect) results in an accumulation of long circulating particles (e.g. particles shielded with polyethylene glycol) in tumor tissues (Greish et al., 2003). Therefore passive targeting can be seen as a method exploiting the physical and biological characteristics of the drug and the recipient organism, respectively.

For example, after tail vein injection of cationic nucleic acid formulations in mice, the cationic vectors aggregate due to interactions with serum proteins and blood cells and the large aggregates are trapped in the capillary bed of the lung, the first-pass organ from the tail vein route. Barron et al. injected cationic lipoplexes intravenously into mice and they explained the preferred gene transfer to lung by a particular high density of proteoglycans on lung endothelial cells to which cationic vectors can bind for efficient cellular uptake (Barron et al., 1999). Additionally, Zou et al. (Zou et al., 2000) showed that small positively charged linear PEI-DNA complexes, intravenously injected in a large nonionic glucose bolus, efficiently transfected alveolar cells (including pneumocytes). They assumed that the large nonionic glucose bolus prevents aggregation as well as mixing of the cationic complexes and excess free PEI with blood. This may enable extravasation in the area of lung alveoli and the

cationic vectors can be taken up by the heparan sulphate receptors on alveolar cells. Apart from the lung tropism of cationic vectors, intravenously injected cationic complexes accumulate in the liver where they are efficiently taken up by phagocytes (Takakura et al., 2002). In contrast to cationic vectors, intravenously administered naked plasmid DNA (negatively charged) shows no passive targeting to the lung but to the liver. Polyanionic macromolecules like naked DNA are efficiently taken up by the scavenger receptors of liver nonparenchymal cells, such as Kupffer and endothelial cells. But despite efficient uptake, no significant gene expression was detected in the liver probably because naked plasmid DNA is rapidly degraded by nucleases in the serum and in the cells (Kawabata et al., 1995).
Enhanced gene transfer into the nucleus of dividing cells can also be seen as a kind of passive targeting. For example, Moloney murine leukemia virus based retroviral vectors can only transduce dividing cells, such as tumor cells. This has been applied for specific delivery of a suicide gene to glioblastoma (Rainov and Ren, 2003). Further, transfection with most types of nonviral vectors is strongly enhanced in dividing cells, leading to increased expression levels in tumors (Wagner et al., 2004).

Traditionally, **active targeting** is using some form of molecular recognition which allows a formulation to specifically interact with target cells (e.g. receptor-ligand and antigen-antibody interactions). A more comprehensive definition suggests that any active procedure exerted on a formulation which will lead to localization of a drug (e.g. nuclear localization sequences) is active targeting. Further, there are active techniques for local control of delivery (e.g. release of a drug from its shell by ultrasound) and nucleic acid expression (e.g. transcriptional targeting). All biological and all physical methods of localization and of local control belong to the category of active targeting, the only exception is the biodistribution due to biophysical properties of the drug. Methods of active targeting are available for all four hierarchies of localization.

1.4 Biological methods of targeting applied in research up to now

1.4.1 Receptor-ligand interactions

Wu & Wu first introduced nonviral receptor-mediated gene delivery (Wu and Wu, 1987; Wu and Wu, 1988a; Wu and Wu, 1988b). By coupling asialoorosomucoid, a natural ligand of the asialoglycoprotein receptor on hepatocytes, to the DNA compacting moiety polylysine, they

generated vectors with increased target cell specificity that are taken up into cells by receptor-mediated endocytosis. Following a similar concept, Wagner et al. established "transferrinfection", based on bioconjugates of (iron-loaded) transferrin and polycations that enter cells by transferrin receptor mediated endocytosis (Cotten et al., 1990; Wagner et al., 1990; Zenke et al., 1990). The transferrin receptor is widely expressed on actively dividing cells, such as tumor cells. Further ligands employed are e.g. galactose which binds to the asialoglycoprotein receptor on hepatocytes (Plank et al., 1992), mannose which binds to the mannose-receptor abundantly expressed on antigen-presenting dendritic cells and macrophages (Diebold et al., 2002; Kawakami et al., 2000), folate which binds to the folate-receptor on tumor cells (Reddy et al., 2005), and epidermal growth factor (EGF) which binds to the EGF-receptor on tumor cells (Ogris and Wagner, 2002). In the meantime a multitude of suitable receptor ligands attached to nucleic acid binding moieties have been described. These include synthetic carbohydrates, synthetic peptides and recombinant proteins (Gust and Zenke, 2002; Pardridge, 2001; Varga et al., 2000). Another approach for targeting cell type-specific surface molecules is the use of monoclonal antibodies, antibody fragments that bind the antigen (Fab), and single chain antibody fragments (scFv) consisting of the variable domains of the light and heavy chain (Gust and Zenke, 2002). For example, monoclonal anti-CD3 antibodies coupled to PEI efficiently transfected T lymphocytes and stimulated human peripheral blood mononuclear cells (Buschle et al., 1995; O'Neill et al., 2001). Similarly, immuneglobulin G (IgG) was efficient for Fc receptor (which binds the constant region of the antibody molecule) mediated gene delivery into alveolar macrophages (Rojanasakul et al., 1994). Fab antibody fragments of the antihuman epidermal growth factor receptor (EGFR) antibody conjugated to polylysine efficiently bound DNA and successfully targeted EGFR-hyperproducing tumor cells (Chen et al., 1998). An ErbB2-specific single chain antibody fragment (scFv) coupled to protamine, cationic lipid and vector DNA selectively transfected ErbB2-positive human breast cancer cells (Li et al., 2001).

In conclusion, providing vectors with targeting ligands can greatly improve transfection efficiencies and specificities if at the same time non-specific interactions can be reduced. This has been demonstrated *in vitro* and *in vivo*, particularly in tumor targeting upon intravenous adminstration. Vectors were shielded from non-specific interactions by PEGylation while epidermal growth factor (EGF), transferrin or galactose provided targeting specificity (Frisch et al., 2004; Ogris et al., 2003). Another example is a particular class of lipid-based nanoparticles with bound nucleic acids, provided with an integrin $\alpha_v\beta_3$-targeting ligand which

mediated efficient and therapeutically relevant gene delivery to tumor endothelium (Hood et al., 2002).

1.4.2 Localization sequences

In the field of nucleic acid therapy, especially nuclear localization sequences (NLS) are of great interest because in non-mitotic cells uptake of delivered nucleic acids into the nucleus is a very rare event (Escriou et al., 2003). NLS are short peptide sequences comprising predominantly basic amino acids of endogenous or exogenous proteins such as transcription factors, ribosomal proteins, oncogene products, or the large T antigen of the simian virus (Poon and Jans, 2005) which mediate their transport from the cytoplasm through nuclear pore complexes (NPC) into the cell nucleus by interaction with specific nuclear shuttle proteins called nuclear transport receptors or karyopherins (importin β, transportin, or the adapter importin α). Whereas small molecules such as metabolites pass the NPC through passive diffusion (the channel diameter is limited to 9 nm), larger proteins require a selective (and active) transport mode (the channel diameter opens up to 45 nm) which is based on nuclear localization signals (Peters, 2005). The nuclear transport receptors bind their transport cargo in the cytoplasm through nuclear localization sequences (NLS) and subsequently mediate their translocation via direct interaction with the NPC to the nuclear side, release the cargo and finally return to the cytoplasm to begin with a new shuttling cycle. Directionality of the transport process is accomplished through a RanGTP concentration gradient across the nuclear envelope, i.e. low cytoplasmic and high nuclear RanGTP concentration. RanGTP binds to the dimeric transport complex consisting of the nuclear transport receptor and the cargo in the nucleus thereby dissociating the cargo from the nuclear transport receptor resulting in the release of the cargo in the nucleus. “Classical” NLS motifs (e.g. from the large T antigen of the simian virus) do not bind directly to the nuclear transport receptor (importin β or transportin) but they require the adapter molecule importin α (Pemberton and Paschal, 2005).

Strategies to provide nucleic acids with nuclear localization signals (NLS) are either direct conjugation (covalent binding) of a NLS to the nucleic acid (Zanta et al., 1999) or noncovalent incorporation of NLS peptides into gene vector complexes and NLS-modification of the gene transfer carrier, respectively (Chan et al., 2000; Ritter et al., 2003; Rudolph et al., 2003).

Apart from nuclear localization signals on peptides, Dean et al. have reported that **certain plasmid DNA sequences translocate into the nucleus** after cytoplasmic delivery. Such nuclear translocation has been observed when a 72 bp fragment of the simian virus 40 (SV40) enhancer element is present on the plasmid DNA. The authors postulated the "piggyback" mechanism which is based on the binding of transcription factors (harbouring NLS) to the enhancer element and transport into the nucleus by exploiting the endogenous nucleocytoplasmic transport machinery (Dean et al., 2005).
Finally, it has to be mentioned that Pollard et al. found out that **polymers like PEI or polylysine** promote gene delivery from the cytoplasm into the nucleus (Pollard et al., 1998).

1.4.3 Site-specific genomic integration

The wild-type **adeno-associated virus (AAV)** can insert its genome at a specific site (AAVS1) in human chromosome 19 through the activity of a specific replicase/integrase protein (Rep) binding both the AAVS1 and the viral inverted terminal repeats (ITRs). But due to the very limited packaging size of AAV particles, AAV vectors are deleted of all viral genes and have therefore lost the Rep-mediated, site-specific integration property of the wild-type virus (Ponnazhagan et al., 2001). Recchia et al. generated a hybrid AAV-adenovirus vector carrying a double-reporter gene integration cassette flanked by AAV ITRs and a tightly regulated, drug-inducible Rep expression cassette (Recchia et al., 2004). Rep-dependent integration of ITR-flanked cassettes of intact size and function was obtained in human primary cells, cell lines and in an AAVS1 transgenic mouse model.
Site-specific genomic integration has also been achieved with the **ϕC31 integrase system**. This is a recombinase found in a Streptomyces phage that mediates stable chromosomal integration of genes into host genomes without any additional co-factors. The genomic integration is unidirectional and sequence specific. The ϕC31 integrase mediates the integration of attB attachment sites of the transgenic DNA into attP attachment sites in the host genome, which occur as pseudo-attP attachment sites in mammalian genomes (Groth and Calos, 2004).
Distantly related to the ϕC31 integrase is the Lactococcus lactis cremoris phage **TP901-1 integrase** which carried out efficient site-specific intramolecular integration on a transfected plasmid substrate in the human cell environment without any additional co-factors (Stoll et al., 2002). Recombinases such as Cre, FLP and beta-recombinase perform site-specific integration and excision (Branda and Dymecki, 2004; Canosa et al., 2003). But as the net

integration frequency is low, these recombinases are not an appropriate tool for site-specific gene therapy approaches.

The bacterial **transposon Tn7** is unique among transposons, in that it inserts into a single site in the Escherichia coli chromosome. This site is termed attTn7 and insertion is mediated by the sequence-specific DNA binding of the target selector protein TnsD. Kuduvalli et al. (Kuduvalli et al., 2005) showed that Tn7 can transpose *in vitro* downstream to the glutamine-fructose-6-phosphate-transaminase-1 and 2 gene (gfpt-1 and gfpt-2) in the human genome. The presence of a Tn7 element at a target site prevents multiple insertions and one would expect a single copy of Tn7 to be inserted in a site- and orientation-specific manner.

1.5 Biological methods of local control applied in research up to now

1.5.1 Tissue-specific and inducible promoters ("transcriptional targeting")

Tissue-specific promoters switch on their genes only in certain tissues. Therefore in gene therapy, tissue-specific promoters are combined with reporter and therapeutic genes to achieve local tissue-specific expression. Some examples are the tyrosinase promoter to target melanocytes and melanomas, the prostate-specific antigen (PSA) promoter which is expressed predominantly in the prostate, the albumin-promoter which is active in the liver and hepatocellular carcinomas, the surfactant protein B-promoter for targeting type II alveolar and bronchial cells, the ovarian-specific promoter which is expressed in ovarian tissue and the human vascular smooth muscle cell (VSMC) alpha-actin promoter which is active in vascular smooth muscle, cardiac muscle and skeletal muscle (Robson and Hirst, 2003).

Inducible promoters can be activated or attenuated by exogenous stimuli like e.g **glucose or drugs**. By means of such stimuli the duration and level of gene expression can be regulated. For example, the human insulin promoter and the glucose 6-phosphatase promoter may be useful for local regulated hepatic insulin gene expression in type 1 diabetes (Burkhardt et al., 2003). The most famous drug-responsive promoters are tetracycline controlled (Toniatti et al., 2004). In the tet-off system (Gossen and Bujard, 1992), an engineered minimal promoter is activated in the absence of tetracycline whereas in the tet-on system (Gossen et al., 1995), tetracycline derivatives (like doxycycline or anhydrotetracycline) activate transcription.

1.5.2 Activation of prodrugs

A very elegant method of local control is the conversion of a prodrug into its active form in the target tissue. Two examples for such a strategy are the antibody directed enzyme-prodrug therapy (ADEPT) and the gene directed enzyme prodrug therapy (GDEPT).

The antibody directed enzyme-prodrug therapy or ADEPT includes a two-step process for drug delivery. In the first step, monoclonal antibodies conjugated chemically to enzymes or monoclonal antibody-enzyme fusion proteins were delivered to cell surfaces presenting the corresponding antigens. In the second step, prodrugs were administered and converted into active agents at the sites where monoclonal antibody-enzyme conjugates are located. An example for an enzyme used in ADEPT is the cytosine deaminase, a protein that converts the non-cytotoxic 5-fluorocytosine into the anticancer drug 5-fluorouracil (Sharma et al., 2005).

The gene directed enzyme prodrug therapy (GDEPT) is a therapy for malignant diseases. An exogenous gene coding for an enzyme is delivered to the target tumor cells. The expressed enzyme can then convert a non-toxic prodrug into a cytotoxic drug. One of the most frequently used systems is the Herpes simplex virus thymidine kinase (HSV-tk) combined with ganciclovir (McKeown et al., 2004).

1.5.3 Triggering localized drug delivery

Enzyme-activated targeting of liposomes uses tissue-specific enzymes to locally transform stable liposomes (e.g. composed of N-acetylated alanyl alanine linked to dioleoylphosphatidylethanolamine [N-Ac-AA-DOPE]) into fusogenic lipid structures (hexagonal phase). The tissue-specific enzymes can be located in the vicinity of the target cells, can be presented by target cells or can be located in endolysosomal compartments of target cells. For example the enzyme elastase is abundant in inflammatory tissues (like in cystic fibrosis lungs or rheumatoid arthritis joints), either in a free form or bound to a receptor of human neutrophils (Meers, 2001).

pH-sensitive liposomes, polymers and peptides provide a further strategy for triggered localized drug delivery. In response to lower pH in pathological tissues (e.g. tumor, metastases, inflammation and infection tissues) or in endolysosomal compartments, pH-sensitive liposomes (containing e.g. DOPE) can be locally transformed from a stable lamellar phase into fusogenic lipid structures (hexagonal phase) and their encapsulated drugs are released directly into the cytoplasm of the target cells (Simoes et al., 2004). Analogously, the

incorporation of pH-specific polymers (like e.g. PEI) or pH-specific fusogenic peptides (such as the N-terminus of influenza virus haemagglutinin HA-2) into nucleic acid complexes enables disruption of the endosomal membrane at acidic pH and thus release of the nucleic acid vector into the cytoplasm (Wagner, 2004).

Finally, **biological stimuli-sensitive hydrogels** have the potential for locally acting controlled drug and nucleic acid release systems. Hydrogels can protect an entrapped drug from hostile environments (e.g. the presence of enzymes and low pH in the stomach) and they can control drug release by reversible volume phase transitions or gel-sol phase transitions in response to environmental stimuli. Biological stimuli are e.g. pH, glucose concentration, specific ion concentrations, specific antigens and thrombin (Miyata et al., 2002; Qiu and Park, 2001).

1.6 Physical methods of targeting applied in research up to now

1.6.1 Gravitational force

Methods using gravitational force are only applicable in cell culture, but cell culture serves as an instructive model from which conclusions for in vivo applications can be drawn. From cell culture experiments, Luo and Saltzman concluded that nucleic acid transfection efficiency is limited by the number of vector – cell contacts or in other words by low vector concentration at the cell surface (Gemeinhart et al., 2005). The chance for a vector to get in contact with a cell is mainly by Brownian motion (diffusion). The probability of contact increases with drug concentration, incubation time and temperature (which can not be chosen arbitrarily). The number of contacts between vector and cells is proportional to the number of cellular uptake events unless saturation of cells with vectors is reached. In the following lines three methods exploiting gravitational force to obtain enhanced accumulation of vectors on cells in cell culture (first order targeting) are introduced.

Settlement of nucleic acid vectors under gravity as a method to increase transfection efficiencies in cell culture was used by Luo and Saltzman (Luo et al., 2004; Luo and Saltzman, 2000). They associated vectors with dense silica particles that sedimented vectors on the cell surfaces and consequently the vector concentration on the cell layer and the transfection efficiency was significantly increased.

The precipitate formation method exploits the phenomenon that large and heavy vector precipitates settle in cell culture. Therefore with vector precipitates the number of vector - cell contacts is significantly higher than with small vectors which have only a chance to get in

contact with the cells by Brownian motion (Graham and van der Eb, 1973; Tovell and Colter, 1967). Already in 1973 Graham and Van der Ebb established the calcium phosphate precipitation method (Graham and van der Eb, 1973). Also for PEI-DNA vectors, it has been found that large DNA complexes transfect more efficiently than smaller ones (Ogris et al., 1998).

Centrifugation (acceleration) of nucleic acid vectors down to the cell layer(s) in cell culture results in enhanced vector accumulation on the cell surfaces (Boussif et al., 1996; Bunnell et al., 1995; Huth et al., 2004; O'Doherty et al., 2000).

An example for increased transduction of non-adherent pheripheral blood lymphocytes with retroviral vectors by centrifugation was given by Bunnell et al. (Bunnell et al., 1995). Centrifugation of the retroviral vector and PBLs-containing supernatant was one important step in their optimized transduction protocol.

1.6.2 Local injection

Direct injection of nucleic acids into target tissues was performed by several groups in the 1980ies (Benvenisty and Reshef, 1986; Dubensky et al., 1984; Will et al., 1982). For example Benvenisty and Reshef injected DNA precipitated with calcium phosphate intraperitoneally into newborn rats and observed gene expression in liver and spleen. In 1990, Wolff and coworkers found that direct intramuscular injection of naked DNA and RNA expression vectors leads to high and persistent transfected gene expression (Wolff et al., 1990), (Herweijer and Wolff, 2003).

1.6.3 Intravascular delivery combined with occlusion of the blood outflow from the target organ

Intravascular nucleic acid delivery into the liver combined with occlusion of the blood outflow from the liver was used by Budker et al. in 1996. They found that naked DNA injected in hypertonic solution intraportally in mice with transient occlusion of hepatic veins leads to quite efficient gene delivery to hepatocytes (Budker et al., 1996). Later, the same group injected naked plasmid DNA and siRNA into transiently isolated distal veins of mammalian limbs and obtained efficient nucleic acid delivery to limb skeletal muscle (Hagstrom et al., 2004).

1.6.4 Hydrodynamic force

Hydrodynamic methods of nucleic acid delivery in cell culture are only using the acceleration of vectors towards target cells whereas hydrodynamic methods of nucleic acid delivery *in vivo* are a combination of orthotopic (localized) vector administration and an acceleration of vectors towards target cells concomittant with permeabilization of the target tissue.

Nucleic acid vector flow towards target cells is a method for cell culture experiments. Chuck et al. (Chuck et al., 1996) showed that the short half-lives of retroviral vectors limit the distance that they can effectively travel in cell culture by Brownian motion and that therefore only a relatively small number of vectors can get in contact with the cell layer(s). But net convective flow of vector-containing medium through a layer of target cells increased the vector - cell contacts and consequently enhanced the transduction efficiencies significantly.
Hydroporation is an *in vivo* method which was established in two independent studies in 1999. Rapid injection of large volumes of DNA solutions (volumes equalling or exceeding the animal's blood volume) in tail veins of mice resulted in enormous expression levels in the livers of the animals with up to 40 % of the hepatocytes becoming transfected (Liu et al., 1999; Zhang et al., 1999). The mechanism of this method (in mice) involves a transient irregularity of heart function, but importantly an enlargement of liver fenestrations and a transient permeabilization of hepatocyte membranes (Zhang et al., 2004). Most recently, it was shown that the method may be relevant in therapy, as it can be applied in transiently isolated limbs to achieve highly efficient nucleic acid delivery throughout muscle cells of the isolated limb (Hagstrom et al., 2004).

1.6.5 Aerosolization

Aerosols are solid (Greek: sol) particles and/or liquid drops, that float in the air (Greek: aero). Aerosol particles can be from ca. 1 nm to ca. 100 μm in diameter. The aerosol therapy enables direct deposition of drugs (= localization) in the upper and lower airways. The optimum particle size in drug aerosols is between 1 and 5 μm. Droplets with higher diameters are not delivered to the lower airways and droplets with lower diameter are exhaled to a large extent by the patient.

In nucleic acid delivery, especially PEI-based formulations have proven stable during jet nebulization and in animal models gene expression is restricted to the lung (Densmore, 2003; Rudolph et al., 2004).

1.6.6 Ballistic methods

In the gene gun method, gold particles coated with DNA are shot into target tissues or cells by using a gene gun. This approach allows DNA to penetrate directly through cell membranes into the cytoplasm or even nuclei and to bypass the endosome/lysosome where it would get enzymatically degraded. For example skin, liver and muscle have been successfully transfected (Wells, 2004).

The biojector method is using an instrument, the biojector, which employs compressed carbon dioxide as a power source to eject liquid medication through a tiny orifice that is held against the patient's skin. In this way, an ultra-fine stream of high pressure fluid is created that penetrates the skin without using a needle and intramuscular or subcutaneous injections are possible. Trimble et al. used the biojector technique to deliver DNA vaccines intradermally (Trimble et al., 2003). Also Mumper and Cui described the usage of biojectors to deliver plasmid DNA-coated cationic nanoparticles intradermally for genetic immunization (Mumper and Cui, 2003).

1.6.7 Systems for controlled drug release

Controlled release systems for low-molecular-weight drugs and proteins are well established in industry and recently researchers try to adapt these systems to the delivery of nucleic acids (Pannier and Shea, 2004). Systems for controlled release of plasmid DNA (gene activated matrices) were shown to increase transgene expression and enhance the duration of expression relative to naked plasmid DNA delivery upon injection of aqueous solutions. As an advantage of these systems, naked plasmid DNA or gene vectors are delivered locally which avoids distribution to more distant tissues and reduces both toxicity to nontarget cells and immune response to the gene vector. Generally, there are two classes of controlled release depots: **solid drug carrier systems** (which get implanted) like e.g. collagen or poly(lactide-co-glycolide) (PLG) matrices and **injectable carrier systems** (which solidify to form a depot after injection) like e.g. PEG-(poly-lactic-co-glycolic acid)-PEG (PEG-PLGA-PEG) hydrogels, PLG in glycofurol, fibrin glue or collagen solutions. Another type of injectable

implants for controlled drug release are nanoparticles consisting of PLG, PLGA, gelatin or chitosan and microparticles composed of PLGA or poly-ortho-esters (Plank et al., 2005). In our laboratory, collagen sponges were used as solid nucleic acid carrriers and *in vitro* and *in vivo* experiments revealed that especially the coating with copolymer-protected PEI-DNA vectors leads to sustained gene delivery (Scherer et al., 2002b).

1.6.8 Electric fields

Electroporation is a widely used physical method to introduce polar and charged agents such as dyes, drugs, DNA, RNA, proteins, peptides and amino acids into cells. Traditionally, electroporation is performed with large electrodes in a batch mode to transform bacteria or to transfect eukaryotic cells in suspension. *In vivo* electroporation, also called electrotransfer, is a promising strategy for the local treatment of muscle disorders or of tumors. Special electrodes produce extremely localized electric fields and thus locally enhanced nucleic acid transfer is possible (Andre and Mir, 2004; Bloquel et al., 2004).

The Nucleofector technology, developed by Amaxa biosystems (www.amaxa.de), uses a combination of electric pulses and special solutions to introduce nucleic acids directly into the nucleus of cells in culture. Consequently, transfection of cells is no longer dependent on cell division, i.e. even non-dividing cells such as resting blood cells or neurons can be transfected with high efficiencies (Hamm et al., 2002).

1.6.9 Magnetic drug targeting

The principle of magnetic drug targeting is that drugs bound to magnetic particles are guided by an external magnetic field to target tissues like e.g. tumors. Successful targeting of magnetic particle-drug complexes has been shown in animal models and the magnetic carriers are well tolerated by animals and humans.

A detailed description of the development of magnetic drug targeting and its current state is given in 1.8.

1.7 Physical methods of local control applied in research up to now

1.7.1 Stress-inducible promoters ("transcriptional targeting")

In gene therapy, the local generation of physical stress enables local tissue-specific expression of delivered therapeutic genes which are driven by stress-inducible promoters. For example **hyperthermia** (locally generated through ultrasound, lasers, microwaves or magnetite cationic liposomes in an alternating magnetic field) induces transcription of genes under control of heat-sensitive promoters like the HSP70 or gadd 153 promoter. The HSP70 promoter is further inducible by **low frequency (< 300 Hz) electromagnetic fields (EMFs)**. **Hypoxia** (e.g. in tumors) activates genes under control of hypoxia response element (HRE) which are contained e.g. in the erythropoietin (Epo) and the vascular endothelial growth factor (VEGF) gene. And finally, **ionizing radiation (IR)** activates IR response elements which were found in the early growth response 1 (Egr1) gene promoter (Blank and Goodman, 2004; Robson and Hirst, 2003).

1.7.2 Triggering localized drug delivery

Drugs can be formulated in a manner that makes physical activation necessary to induce drug release. In these systems, release of the drug takes place at the site to which the physical trigger is focussed. Examples for such controlled release formulations are microbubbles, temperature-sensitive liposomes and physical stimuli-sensitive hydrogels.

Microbubbles are gas-filled microspheres (smaller than 5 – 7 μm) or gas emulsions. The shell can consist of renografin, indocyanin green, carbohydrates such as dextrose, proteins, denatured proteins, surfactants, lipids or synthetic polymers such as polylactides. Perfluorocarbons have turned out to be ideal gases for microbubble preparation due to low aqueous solubility and sufficient volatility. More recent compositions are so-called nanoemulsions consisting of a bubble shell filled with a liquid perfluorocarbon. Both low molecular weight drugs and high molecular weight drugs such as nucleic acids can be associated with microbubbles. At the target site ultrasound is used to trigger local drug release from the microbubbles (Bekeredjian et al., 2003; Tsutsui et al., 2004).

Temperature-sensitive liposomes can release their drugs specifically at a target area where heat is applied. For example dipalmitoylphosphatidylcholine (DPPC) membranes undergoe a gel-to-liquid phase transition at 41 degrees Celsius and liposomes made of DPPC release their

contents at this clinically attainable temperature. Inclusion of various colipids, such as distearoylphosphatidylcholine (DSPC) and cholesterol, can further improve the temperature sensitivity of DPPC liposomes. Additionally, thermosensitive polymers like e.g. poly(N-isopropylacrylamide) (PNIPAM) are employed to optimize temperature-sensitive liposomes (Kono and Takagishi, 2004).

Finally, **physical stimuli-sensitive hydrogels** can control drug release by reversible swelling-shrinking phase transitions or gel-sol (solution) phase transitions in response to physical stimuli like e.g. temperature, electric current, light and pressure (Qiu and Park, 2001).

1.8 The development of magnetic drug targeting and its current state

Magnetic drug targeting was already mentioned in 1.6 (physical methods of targeting applied in research up to now) but because of its great importance for this thesis its developement and current state is described here in an extra paragraph.

In 1963, Mayers et al. showed in animal experiments that radioactive and nonradioactive carbonyl iron particles (1 to 3 μm in size) could be held in a specific location in blood and lymphatic vessels by an external permanent magnet. Further they demonstrated magnetically controlled extravasation of radioactive iron particles from arteries. From these results Mayer and coworkers concluded that magnetically directed iron particles could be used as contrast agents for roentgenogramms, for localized radiation therapy or for targeted delivery of chemotherapeutic agents (Meyers et al., 1963). In 1965, Alksne and Fingerhut showed in experiments with dogs that it is possible to induce thrombosis of an artery or of an artificial aneurism by intra-arterial administration of carbonyl iron-albumin spheres (average diameter 3 μm) and attraction of these particles to an external permanent magnet (Alksne and Fingerhut, 1965). In 1975, Turner et al. injected carbonyl iron-silicone microspheres into the arteries of dogs and successfully directed the particles to the kidney by a superconducting electromagnet. This technique should be used for arterial vascular occlusion of hypernephromas and other organs (Turner et al., 1975). **Widder et al.** (Widder et al., 1978) were probably the first who successfully realized the concept of magnetically controlled drug targeting. They prepared biodegradable albumin microspheres with entrapped Fe_3O_4 and adriamycin HCl with a mean particle size of 1 μm. When these magnetic albumin microspheres were infused into the caudal artery of rat tails, approximately 50% of the carriers was retained in the targeted tail segment exposed to a permanent magnetic field of 8000 Oe (oersteds) and the adriamycin concentration at the target segment was comparable to

that achieved by administration of a 100-fold higher dose of the free drug. In a further publication, Widder and coworkers described the application of this drug delivery system to Yoshida sarcoma grown in the rat tail (Widder et al., 1981). Most of the treated animals had complete tumor remission in contrast to progressive tumor growth in the control group. Independently of Widder et al., Kato et al. developed a magnetic control system of microcapsules. They constructed two prototypes of ferromagnetic ethylcellulose microcapsules containing the anticancer drug mitomycin C (FM-MMC-mc): the outer type (approximately 300 μm in diameter) with zinc ferrite on the capsular surface and the inner type (approximately 250 μm in diameter) with zinc ferrite in the core. Both types were magnetically responsive and provided sustained release properties. Animal studies showed that the microcapsules could be magnetically controlled in the artery and urinary bladder and VX2 tumors in the rabbit hind limb and urinary bladder were succesfully treated (Kato et al., 1984). In 1989, Gupta et al. demonstrated by transmission electron microscopy that adriamycin-associated magnetic albumin microspheres (similar preparations as those used by Widder et al.) traverse the vascular endothelium of even healthy tissue and confirmed second-order drug targeting (Gupta et al., 1989). In 1994, Häfeli et al. prepared biodegradable poly(lactic acid) microspheres that incorporate magnetite and the beta-emitter Yttrium-90 (Hafeli et al., 1994). Subsequently, they showed in a murine tumor model that 24 h after intraperitoneal injection, roughly 73% of the radioactivity was found in a subcutaneous tumor exposed to a magnet (Hafeli et al., 1995). A new and much smaller type of magnetic carrier, starch-phosphate coated iron oxide nanoparticles with an average diameter of 100 nm, was used by Lübbe et al. (Lubbe et al., 1996a). The endstanding negatively charged phosphate groups on the surface of these particles allowed reversible electrostatic binding of the positively charged chemotherapeutic agent epirubicin. In animal experiments, epirubicin loaded nanospheres were injected intravenously in tumor-bearing nude mice and rats and directed into the tumor using a permanent magnetic field. The magnetic carrier/epirubicin complex was well tolerated by the animals and tumor remission was achieved. As a second step, Lübbe et al. used this approach for the first clinical experiments in human patients with magnetic drug targeting worldwide (Lubbe et al., 1996b). This phase I clinical trial included 14 patients with advanced solid tumors and for magnetic drug targeting a permanent magnet was arranged at the tumor surface outside of the organism. The studies showed that magnetic drug targeting with epirubicin (4'-epidoxorubicin) was well tolerated and that the ferrofluid could be successfully directed to the tumors in about one-half of the patients. Lubbe et coworkers concluded that magnetic drug targeting seems to be safe but improvements are

necessary to make it more effective. Subsequently Alexiou et al. tried to improve the efficacy of magnetic drug targeting in tumor-bearing rabbits (Alexiou et al., 2000). They used starch-phosphate coated iron oxide particles (100 nm in size) as well and the positively charged chemotherapeutic agent mitoxantrone which was reversibly (electrostatically) bound to the negatively charged phosphate groups. The ferrofluid-mitoxantrone complexes were injected intra-arterially (femoral artery) or intravenously (ear vein) and for magnetic drug targeting an external extremely strong electromagnet (1.7 Tesla) was focused on the experimental VX-2 squamous cell carcinoma in the median portion of the hind limb of rabbits. The intra-arterial application of the complexes plus magnetic field resulted in significant complete and permanent remission of the tumors compared with the control group (no treatment) and the intravenous group. Additionally, no signs of toxicity were detected. A special type of magnetic particles (MTCs, Magnetic Targeted Carriers) was developed and used by FeRx Incorporated, San Diego, USA. MTCs are microparticles (0.5 to 5 μm in size) composed of metallic (elemental) iron and activated carbon and are prepared by a high-energy milling process (Goodwin et al., 1999; Rudge et al., 2000). These particles have a higher magnetic susceptibility than particles made of iron oxides (including magnetite [Fe_3O_4] and hematite [Fe_2O_3]) and are therefore captured more efficiently by an external magnetic field. Many chemotherapeutic agents and also peptides and proteins can be simply adsorbed to the activated carbon component of MTCs and controlled release (desorption) of the drugs is possible (Johnson et al., 2002; Rudge et al., 2001). In a swine model, MTC and MTC-drug suspension was administered intra-arterially by placing a catheter proximal to the selected target site and a magnet was positioned on the body surface above the desired site (Goodwin et al., 1999; Goodwin et al., 2001). Even irreversible binding of radionuclides to MTCs could be achieved by simple chemistry modifications (Hafeli et al., 2003). However, the lead product of FeRx was doxorubicin adsorbed to MTC (MTC-DOX) and it was used in a clinical study where 32 patients with hepatocellular carcinoma have been enrolled. But in April 2004, a phase II/III clinical trial involving this technology has been discontinued as the clinical endpoints could not be met with statistical significance.

In summary, at least in animal models it has been clearly demonstrated that (i) magnetic drug targeting is feasible even if the drug administration site is remote from the target site under magnetic field influence, (ii) that magnetic particles can extravasate under the influence of the magnetic field and that the magnetic carriers are well tolerated.

1.9 Topic of this thesis

As described in 1.1, up to date several approaches for nucleic acid-based therapy are available and once nucleic acid vectors are accumulated at the target cells (like e.g. in cell culture) they show in many cases efficient nucleic acid transfer to the desired cellular compartments (nucleus or cytosol). But despite numerous approaches (see above), efficient concentration of nucleic acid vectors at the target site *in vivo* is still one of the major challenges for clinical successful nucleic acid therapy.

A very promising physical method to localize anti-cancer drugs *in vivo* is provided by magnetic drug targeting (see 1.6.9 and 1.8). In this method, the drug is bound to magnetic particles and an external magnetic field can guide the applied magnetic particle-drug complex to the desired site. At least in animal models it has been clearly demonstrated that magnetic drug targeting is feasible even if the drug administration site is remote from the target site under magnetic field influence, that magnetic particles can extravasate under the influence of the magnetic field and that the magnetic carriers are well tolerated.

Basis of this thesis was the idea to use the principle of magnetic drug targeting for the delivery of nucleic acids. Similar as cytostatics, nucleic acids require third order targeting which involves cellular uptake and localization in the cytoplasm or in the nucleus. Moreover, some nucleic acid approaches require even fourth order targeting when e.g. site-specific genomic integration is desired. Referring to the chemical structure, nucleic acids are quite different from classical cytostatics. While anti-cancer drugs are usually low molecular weight molecules, nucleic acids are high molecular weight macromolecules harbouring a high number of negative charges.

The objective of this thesis was to bind nucleic acid vectors to magnetic particles in a way that allows nucleic acid vectors to be magnetically controlled and additionally at the target site the nucleic acid vectors should still retain all their functionalities like e.g. endosomal escape or accessibility to the transcriptional machinery.

For this thesis superparamagnetic iron oxide nanoparticles with various cationic or anionic coatings were provided by Chemicell GmbH, Berlin, and the first step was to find and to further develop appropriate particles for vector binding and efficient magnetically controlled nucleic acid transfer in cell culture without toxic side effects. Subsequently, the mechanism of magnetic nucleic acid targeting, termed “magnetofection”, was examined by cell culture experiments and through electron microscopy. In further cell culture experiments the magnetofection method was optimized, the nucleic acid transfer efficiency compared to

standard transfection methods without magnet, magnetic-field guided localization of nucleic acid transfer was shown, different cell types were transfected and the applicability of magnetofection in a pig, a rabbit and a rat model was proven. In all experiments bacterial plasmids harbouring the cDNA of reporter genes (either luciferase or β-galactosidase) were chosen as representatives for the large number of different nucleic acid-based drugs and exemplary nonviral vectors were used.

2 MATERIALS AND METHODS

2.1 Abbreviations, reagents and materials

If not otherwise stated, reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Abbreviation	Article	purchased from
ACC	DSMZ number	
aqua dest.	distilled water	
ATP	adenosine-5'-triphosphate	Roche, Mannheim
AVET	adenovirus-enhanced-transfection	
α-^{32}P-dATP	deoxyadenosin-5'-triphosphate which contains radioactive ^{32}P in the α position (phosphorus atom bonded to the ribose)	Hartmann Analytic, Braunschweig
	β-mercaptoethanol	
bPEI	biotinylated polyethylenimine, preparation see in "General methods"	
Bq	becquerel	
Br	bromine	
BSA	bovine serum albumin	Bio-Rad, Munich
°C	degrees Celsius	
CA membrane	cellulose acetate membrane	Peske, Aindling-Pichl, Germany
cDNA	complementary DNA	
	cell culture dishes, 96-well and 24-well and 6-well plates, tissue culture flasks (all articles gamma-sterilized and cell culture-treated)	produced by TPP, Switzerland purchased from Peske, Aindling-Pichl, Germany
	chloroform	Fluka, Neu-Ulm
CHO-K1 cells	Chinese hamster ovary cell line	DSMZ, Braunschweig
Chol	cholesterol	
Ci	curie	
cm	centimeter	
CO_2	carbon dioxide	
COPROG	copolymer-protected gene vector	
cP	centipoise = 1/100 poise	
c.p.m.	counts per minute	
d	diameter	
DMEM-medium	Dulbecco's Modified Eagles medium	Gibco, Eggenstein
DMF	N, N dimethylformamide	
DMSO	dimethyl sulfoxide	
DNA	deoxyribonucleic acid	

DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (transfection reagent)	Avanti Polar Lipds, USA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)	
DTT	dithiothreitol	
e.g.	for example	
Ellman Reagent	dithiobis-2-nitrobenzoic acid	Aldrich, USA
EM	electron microscope	
EtOH	ethanol	
FCS	fetal calf serum	Gibco, Eggenstein
	formaldehyde	
g	gramm	
g	gravitational constant (at centrifugation)	
GP	GenePORTER (transfection reagent)	Gene Therapy Systems (GTS), La Jolla, CA, USA
	glucose	
	glutaraldehyde	
	glycil-glycine	
h	hours	
h	height	
HaCaT cells	cell line derived from human keratinocytes	kindly provided by Dr. Martin Mempel, Dermatology, TU Munich, Germany
HBS	HEPES buffered saline (20 mM HEPES, pH 7.3; 150 mM NaCl)	
HCl	hydrochloric acid	
HeLa cells	human cervix carcinoma cell line	DSMZ, Braunschweig
HEPES	N-2-hydroxyethyl-piperazine-N‘-2-ethanesulphonic acid	Aldrich, USA
HepG2 cells	human hepatic carcinoma cell line	DSMZ, Braunschweig
H_2O	water (aqua)	
i.m.	intramuscular	
inact. adv	inactivated adenovirus: the adenovirus was psoralen-treated (to inactivate the viral DNA) and biotinylated; it was used as endosomolytic agent	kindly provided by Prof. Dr. Ernst Wagner, Vienna University Biocenter, Austria
INF7	synthesized membrane-disrupting peptide derived from the amino-terminal sequence of influenza virus hemagglutinin HA-2	in house synthesis
i.v.	intravenous	
kb	kilobases	
KCl	potassium chloride	
kDa	kilodalton = 1000 dalton	
$K_3Fe(CN)_6$	potassium ferricyanide crystalline	
$K_4Fe(CN)_6x3H_2O$	potassium ferricyanide trihydrate	

kg	kilogramm	
kV	kilovolt	
l	liter	
LDV	laser Doppler velocimetry	
LF	Lipofectamine (transfection reagent)	Life Technologies, Karlsruhe
	luciferase	Roche Mannheim
	luciferin	Roche Mannheim
µA	microampere	
µg	microgramm	
µl	microliter	
µm	micrometer	
µM	micromolar (µmol/l)	
M	molar (mol/l)	
mg	milligramm	
$MgCl_2$	magnesium chloride	
$MgSO_4$	magnesium sulfate	
min	minutes	
ml	milliliter	
mm	millimeter	
mM	millimolar (millimol/l)	
mmol	millimol	
mT	millitesla	
mV	millivolt	
Mw	molecular weight in g/mol or dalton	
NaCl	sodium chloride	
Nd-Fe-B magnet	neodymium-iron-boron magnet (NeoDelta)	IBS Magnet, Berlin
ng	nanogramm	
NHS-LC-Biotin	succinimidyl-6-(biotinamido) hexanoate	Pierce, Rockford, IL, USA
NIH	National Institute of Health	
NIH 3T3 cells	mouse fibroblast cell line (from the NIH)	DSMZ, Braunschweig
nm	nanometer	
nmol	nanomol	
N/P ratio	nitrogen (from the PEI)/phosphate (from the DNA) ratio	
^{32}P	radioactive phosphorus	
PBS	phosphate buffered saline	
pCMV-β-gal	plasmid coding for the β-galactosidase	kindly provided by Dr. Walter Schmidt, Intercell, Vienna, Austria
PCR	polymerase chain reaction	
pDNA	plasmid-DNA (purified by cesium chloride gradient)	
PEG	polyethylene glycol	
PEI	polyethylenimine, if not otherwise stated 25 kD; preparation see in "General methods"	
pg	picogramm	

pH	negative decadic logarithm of the hydrogen ion (H^+) concentration	
pL	polylysine	
p55pCMV-IVS-luc+	plasmid coding for the firefly luciferase	kindly provided by Andrew Baker, Bayer, USA
prep	preparation	
PROCOP	protective copolymer	
P6YE5C	copolymer of polyethylene glycol (6000 Da) and the negatively charged peptide $(YE_5)_2K\varepsilon C$	in house synthesis
RIF-1 cells	mouse radiation-induced fibrosarcoma cell line	kindly provided by Ellen Kolbe, Experimental Oncology, TU Munich, Germany
rpm	rounds per minute	
SDS	sodiumdodecylsulphate	
sec	seconds	
SPDP	succinimidylpyridyldithiopropionate = 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester	
St-pL	streptavidinylated polylysine	kindly provided by Prof. Dr. Ernst Wagner, Vienna University Biocenter, Austria
	streptavidin	Molecular Probes, Leiden, The Netherlands
TEM	transmission electron microscopy (Philips EM 410 or Philips CM 10)	Philips, Eindhoven, The Netherlands
TFA	trifluoroacetic acid	
Tris	trishydroxymethylaminomethan	
trMAG	superparamagnetic iron oxide (mainly magnetite, Fe_3O_4) nanoparticles with various coatings	Chemicell, Berlin
trMAG-ARA	trMAGs coated with arabinic acid, sodium salt, Mw 250 kDa	Chemicell, Berlin
trMAG-DAEA	trMAGs coated with a polymer prepared from dimethylamine, epichlorohydrine and ethylene diamine.	Chemicell, Berlin
trMAG-DEAE	trMAGs with a dextran monolayer coating, introduction of end-standing DEAE groups with 2-diethylamino-ethyl chloride-hydrochloride	Chemicell, Berlin
trMAG-pACRYL	trMAGs coated with polyacrylic acid, sodium salt, Mw 20 kDa	Chemicell, Berlin
trMAG-pACRYL-MAL	trMAGs coated with polyacrylic acid-co-maleic acid, sodium salt, Mw 50 kDa	Chemicell, Berlin
trMAG-pASP or trMAG-pAsp	trMAGs coated with polyaspartic acid, sodium salt, Mw 3000 kDa	Chemicell, Berlin

trMAG-PEI	trMAGs coated with a monolayer of PEI (Mw 800 kDa, Fluka, Neu-Ulm, Germany)	Chemicell, Berlin
trMAG-PEI-C1/1	trMAGs coated with a commercially available PEI, should result in relatively small particles	Chemicell, Berlin
trMAG-PEI-epichlorohydrin	trMAGs with a monolayer coating of PEI 20 kDa (Aldrich, USA) modified with epichlorohydrin	Chemicell, Berlin
trMAG-PEI-ethoxylated	trMAGs with a monolayer coating of PEI 50 kDa (Aldrich, USA) which has been ethoxylated (80 %)	Chemicell, Berlin
trMAG-PEI-lowMW	trMAGs with a monolayer coating of PEI, Mw 1.7 kDa (Aldrich, USA)	Chemicell, Berlin
trMAG-PEI-SDS	trMAGs with a monolayer coating of PEI 800 kDa (Aldrich, USA) modified by a covalent coupling of sodium dodecyl sulfate (SDS) by carbodiimide activation (N-Ethyl-N'-(dimethylaminopropyl)-carbodiimide)	Chemicell, Berlin
trMAG-PEI-SH	thiolated trMAG-PEI	
trMAG-PEI-Sta	streptavidinylated trMAG-PEI, preparation see in "General methods"	
trMAG-PO4	trMAGs coated with starch-phosphate, Mw 20 kDa	Chemicell, Berlin
trMAG-STARCH-PEI	trMAGs with a multilayer coating of starch, Mw 60 kDa (Fluka, Neu-Ulm, Germany) followed by covalent coupling of PEI via amino groups to the periodate-oxidized starch layer	Chemicell, Berlin
trMAG-13/1	trMAGs coated with a monolayer of PEI 2 kDa	Chemicell, Berlin
trMAG-14/1	trMAGs coated with a monolayer of PEI 60 kDa	Chemicell, Berlin
trMAG-15/1	trMAGs coated with a monolayer of PEI 750 kDa (Fluka, Neu-Ulm, Germany)	Chemicell, Berlin
trMAG-16/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany)	Chemicell, Berlin
trMAG-17/1	trMAGs coated with linear PEI (Aldrich, USA)	Chemicell, Berlin
trMAG-18/1	trMAGs coated with a multilayer of PEI 2000 kDa (Aldrich, USA)	Chemicell, Berlin
trMAG-19/1	trMAGs coated with a multilayer of PEI 2000 kDa (Aldrich, USA), but with a different coating procedure than trMAG-18/1	Chemicell, Berlin

trMAG-20/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany), but with a different coating procedure than trMAG-16/1	Chemicell, Berlin
trMAG-21/1	trMAGs ultraloaded with layers of PEI 800 kDa (Fluka, Neu-Ulm, Germany)	Chemicell, Berlin
trMAG-22/1	trMAGs coated with a commercially available polyamine from Merck, Darmstadt, Germany	Chemicell, Berlin
trMAG-23/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany), but with a different coating procedure than trMAG-16/1	Chemicell, Berlin
trMAG-24/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany), but with a different coating procedure than trMAG-16/1	Chemicell, Berlin
trMAG-25/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany), but with 50% less PEI than trMAG-16/1	Chemicell, Berlin
trMAG-26/1	trMAGs coated with poly(bis(2-chlorethyl)ether-alt-1,3 bis(3-dimethyl-amino)propyl)urea, quaternized	Chemicell, Berlin
UV	ultraviolet (light)	
V	Volt	
w/w ratio	weight/weight ratio (e.g. µg trMAG / µg DNA)	
X-Gal	5-bromo-4-chloro-3-indolyl-beta-galactopyranosid	Roche, Mannheim
	Triton X-100	

Table 1 Abbreviations, reagents, materials and source of supply

2.2 General methods

2.2.1 Radioactive (^{32}P) labeling of plasmid DNA by nick translation

Plasmid DNA (of approximately 5 kb size) was labeled with ^{32}P using the Nick Translation Kit from Amersham-Pharmacia with the protocol of the supplier modified such that the incubation time was 15 min at 15 °C instead of 2 h. α-^{32}P-dATP (Hartmann Analytic, Braunschweig, Germany) with a specific activity of 3000 Ci/mmol was used for the labeling reaction. The labeled plasmid was purified using MicroSpin columns (Pharmacia) and the Promega Wizard PCR Preps DNA Purification System (Promega, Mannheim, Germany) for removal of unincorporated nucleotides and enzymes from the reaction mixture. Agarose gel electrophoresis (1 % gel, 35 min running time at 100 V, ethidiumbromide staining) was used to examine the resulting plasmid. A mixture of labeled and unlabeled plasmid was loaded on the gel. After electrophoresis and gel drying the plasmids were monitored in the UV-light, a photo was taken and an autoradiography performed. By comparing the UV-light photo and the autoradiograph the same size of labeled DNA and unlabeled starting DNA could be confirmed.

2.2.2 Cell culture, transfection and reporter gene assays

2.2.2.1 Cells

NIH 3T3 mouse fibroblasts (DSMZ #ACC 59), chinese hamster ovary (CHO-K1) cells (DSMZ #ACC 110), human hepatic carcinoma (HepG2) cells (DSMZ #ACC 180), human cervix carcinoma (HeLa) cells (DSMZ #ACC 57) and mouse radiation-induced fibrosarcoma (RIF-1) cells (kindly provided by Ellen Kolbe, Experimental Oncology, TU Munich, Germany) were grown at 37 °C in an atmosphere of 5 % CO_2 in Dulbecco's Modified Eagles medium (DMEM, purchased from Gibco, Eggenstein, Germany) supplemented with 10 % fetal calf serum (FCS, purchased from Gibco, Eggenstein, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all three purchased from Gibco, Eggenstein, Germany). HaCaT cells (cell line derived from human keratinocytes, kindly provided by Dr. Martin Mempel, Dermatology, TU Munich, Germany) and primary human foreskin keratinocytes (kindly provided by Dr. Martin Mempel, Dermatology, TU Munich, Germany) were kept under the same conditions but the DMEM medium contained no supplements.

2.2.2.2 Transfection

One day prior to addition of DNA complexes, cells were seeded at a density of 20,000 (NIH 3T3, CHO-K1, HaCaT, RIF-1 and primary human keratinocytes) or 45,000 (HepG2) cells per well of a 96-well plate or 250,000 (NIH 3T3 and HeLa) cells per well of a 6-well plate (gamma-sterilized and cell culture treated plates; produced by TPP, Switzerland; purchased from Peske, Aindling-Pichl, Germany) giving rise to an almost confluent cell layer on the day of complex addition.

Unless otherwise stated, 50 µl and 500 µl of vector formulations were added to cells kept in 150 µl and 1.5 ml fresh medium, respectively (96 well-plate and six-well plate formats).

When the **magnetofection** method was used, cells were incubated with superparamagnetic iron oxide nanoparticles (trMAGs; from Chemicell, Berlin, Germany) containing vectors (magnetofectins) usually for 10 to 20 min. During the incubation times the culture dishes were placed upon sintered Nd-Fe-B magnets (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany). The dimensions of the magnets for six- and 24-well plates were 20 x 10 x 5 mm. For 96-well plates the format was cylindrical (d = 6 mm, h = 5 mm) and the magnets were inserted in an acrylic glass template in 96-well plate format with strictly alternating polarization. The fields of the individual magnets influence each other such that the vector dose becomes concentrated in the centers of individual wells. As control without magnet, cells in a separate plate were incubated for the same time with the same vectors but without application of a magnetic field.

When a **standard transfection method** (without magnetic beads) was used, cells were incubated with e.g. PEI-DNA complexes usually for 2 to 4 h.

After the incubation with DNA vectors (with or without magnet) the cells were washed once with fresh medium, grown usually for 24 h and subjected to the luciferase or β-galactosidase assay as described below.

Preparation of DNA complexes: If not otherwise stated, equal volumes of stock solutions in water containing the various components of the complexes were mixed sequentially by pipetting or gentle vortexing. After each mixing step, complexes were incubated for 15 min. For gene transfer one component had to be plasmid-DNA. Either p55pCMV-IVS-luc+, a plasmid coding for the firefly luciferase (kindly provided by Andrew Baker, Bayer, USA) or pCMV-β-gal, a plasmid coding for the β-galactosidase (kindly provided by Walter Schmidt,

Intercell, Vienna, Austria) was used. Both plasmids were purified by cesium chloride gradient centrifugation by Ursula Putz, TU Munich, Germany. The last component was usually NaCl to adjust the final concentration to 150 mM NaCl and to start salt induced aggregation or glucose to obtain a final concentration of 5 % glucose.

If one component were trMAG particles, the complex was called magnetofectin and could be used for gene transfer with magnetic forces (magnetofection).

If there was no trMAG component in the complex the transfection was called a standard transfection (like e.g. PEI-DNA).

The sequence of writing like e.g. trMAG / DNA / PEI reflects the sequence of mixing the components. In this example, DNA was pipetted to trMAGs, followed by addition of PEI and, if desired, finally NaCl or glucose was added. In this type of writing the NaCl or glucose component is not mentioned.

The final volume of a complex solution was calculated for 50 µl and 500 µl per well in triplicates or quadruples of a 96-well and 6-well plate, respectively.

2.2.2.3 Luciferase assay

Twenty-four hours after transfection, cells were washed once with phosphate buffered saline (PBS) and incubated with 100 µl (96-well plate) or 500 µl (6-well plate) of lysis buffer (0.1 % Triton X-100 in 250 mM Tris pH 7.8). Ten to 50 µl each of the cell lysates were transferred to black 96-well plates, mixed with 100 µl of luciferin buffer (60 mM dithiothreitol, 10 mM magnesium sulfate, 1 mM ATP, 30 µM D(-)-luciferin, in 25 mM glycil-glycine pH 7.8) and assayed for bioluminescence using the Microplate Scintillation & Luminescence counter "TopCount" (Canberra Packard, Groningen, The Netherlands) with a count time of 12 s and a count delay of 10 min.

To obtain a calibration curve 200, 100, 50, 25, 12.6, 6.25, 3.13, 1.57, 0.78, 0.39, 0.2, 0.1, 0.05, 0.025, 0.013 and 0 ng luciferase (Roche, Mannheim, Germany) each in 50 µl lysis buffer (2-fold dilution series) were measured under the same conditions as the samples.

The protein content of the cell lysates was determined using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) adapted for use in a 96-well plate format. Five to 10 µl each of the cell lysates were transferred to transparent 96-well plates (type "flat bottom"; here: from Nunc, Denmark), mixed with 155 to 150 µl aqua dest and 40 µl Bio-Rad Protein Assay dye concentrate. The absorbance (at 630 nm) was measured using the absorbance reader

"Biolumin 690" and the computer programme "Xperiment" (both from Molecular Dynamics, USA).

To obtain a protein standard curve 9.667, 6.445, 4.296, 2.864, 1.910, 1.273, 0.849, 0.566, 0.377, 0.252, 0.168 and 0 µg bovine serum albumine (BSA) / 200 µl were measured (1.5-fold dilution series). Bio-Rad Protein Assay Standard II was bought as BSA.

Specific luciferase activity in picograms or nanograms luciferase per milligram of protein were calculated from the luciferase and protein calibration curves.

2.2.2.4 β-Galactosidase assay

Cells were usually plated in 6-well plates. Twenty-four hours after transfection the cells were washed with phosphate buffered saline (PBS). Subsequently the cells were incubated between 20 min and 12 h with **staining solution** (protocol see below) at 37 °C. After staining cells were washed with PBS, observed macroscopically and microscopically and eventually pictures were taken.

Staining buffer: 10 mM phosphate buffer pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6x3H_2O$, 3.3 mM $K_3Fe(CN)_6$.

For 10 ml of **staining solution** 20 mg of 5-bromo-4-chloro-3-indolyl-beta-galactopyranosid (X-Gal; Roche, Mannheim, Germany) were dissolved in 1 ml N, N Dimethylformamide (DMF; Sigma, Steinheim, Germany), filled up to 10 ml with staining buffer and filtered through a 0.22 µm filter (Milipore, Eschborn, Germany).

2.2.3 Preparation of DOTAP-Cholesterol cationic liposomes

In a silanized 15 ml screw cap glass tube a 5 mM DOTAP/5 mM Cholesterol chloroform solution was prepared. To generate a regular lipid film on the inner surface of the tube, the chloroform was evaporated with a rotary evaporator (Rotavapor-R, Büchi, Switzerland). The evaporator was ventilated with Argon gas (Linde, Germany) to exclude oxygen. The tube was kept in vacuo overnight. The next day, 15 ml of a 5 % glucose solution were added to the tube, vortexed for 30 seconds and exposed for 30 min to ultrasonication (Sonicater: Sonorex RK 510 H, from Bandelin, Germany) to produce a stable liposomal suspension.

2.2.4 Preparation of polyethylenimine (PEI)

PEI (25 kDa) as supplied by the manufacturer (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in water, the pH was adjusted to 7.4 by the addition of HCl (hydrochloric acid) and the concentration was adjusted to 10 mg/ml. The material was dialyzed against water by using dialysis tubes with a pore size of 12-14 kDa (Serva, Heidelberg, Germany) followed by sterile filtration (0.20 µm cellulose acetate CA membrane, Peske, Aindling-Pichl, Germany). The concentration of PEI relative to the original solution was determined using the ninhydrin assay (analogous to the protocol in "2.4.1.1 Ninhydrin assay to determine the amount of PEI in trMAG-PEI particle suspensions").

2.2.5 Biotinylation of PEI (bPEI)

An aliquot of PEI solution (17.2 mg) was lyophilized and redissolved in 0.5 ml 20 mM HEPES pH 7.4. Two equivalents of succinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin; Pierce, Rockford, IL, USA; 68.8 µl of a 20 mM solution in DMSO) were added. After reaction at room temperature for 3 hours, the material was purified via gel filtration (Sephadex G-25 filled in a HR 10/10 column, Pharmacia, Freiburg, Germany. Flow rate 1 ml/min with water as eluent). The PEI concentration of the product fraction was 4.39 mg/ml according to the ninhydrin assay (performed analogous to the protocol in "2.4.1.1 Ninhydrin assay to determine the amount of PEI in trMAG-PEI particle suspensions").

2.2.6 Coupling of streptavidin to trMAG-PEI (trMAG-PEI-Sta)

Streptavidin-SPDP: Five mg streptavidin (Molecular Probes, Leiden, The Netherlands) were dissolved in 500 µl HBS (20 mM HEPES/150 mM sodium chloride pH 7.4) and purified by gel filtration (Sephadex G-25; PD-10 columns, Pharmacia, Sweden) using the same buffer. The pooled product fractions were concentrated to 520 µl containing 3.4 mg (56 nmol) streptavidin using a speed-vac. To this solution, a 3.5-fold excess of succinimidyl-pyridyl-dithiopropionate (SPDP; 32 mM in 100 % ethanol) was added. After reaction at room temperature over night, the material was purified via gel filtration in HBS (Sephadex G-25 filled in a HR 10/10 column; Pharmacia, Sweden; flow rate 0.5 ml/min). The concentration of coupled pyridyl-dithiopropionate was 75 µM, the concentration of streptavidin was 1.6 mg/ml, corresponding to a substitution of approximately 2.8 PDP per streptavidin molecule.

trMAG-PEI-SH: Thiolation of trMAG-PEI was carried out by adding 4 µl SPDP (10 mM in ethanol) to 5 mg trMAG-PEI in 250 µl water, followed by addition of 246 µl 20 mM HEPES pH 7.4. The reaction was carried out in a microcentrifuge tube which was shaken over night at full speed at 37 ˚C in an Eppendorf shaker (Thermomixer 5436). Subsequently, the material was washed exhaustively with 0.1 % TFA. After reduction by addition of β-mercaptoethanol, the total amount of coupled pyridyl-dithiopropionate was determined to be approximately 13 nmol. The material was again washed exhaustively with 0.1 % TFA.

A 3-fold excess of streptavidin-SPDP (thiopyridyl groups over thiol groups) was added to the thiolated magnetic particle pellet. After reaction over night, one third of the available thiopyridyl groups had reacted, indicating a quantitative reaction. The product was washed exhaustively with water.

2.3 Characteristics of magnetic nanoparticles (trMAGs) used in this study

2.3.1 Measurement of particle size by dynamic light scattering

The particles listed in table 2 (section 3 "Results") were diluted to a concentration of 10 µg/ml aqua dest. and their size distributions were determined by dynamic light scattering using the Zetasizer 3000 HS, Malvern Instruments, Herrenberg, Germany.
Approximately 1 ml of each sample was filled into a cuvette and measured with the following specifications: 10 measurements per sample prepared in water, viscosity of water 0.89 cP, temperature 25°C.

2.3.2 Transmission electron microscopy of trMAGs

Preparation of the trMAG particles:
For electron microscopy aqueous solutions with 10 µg trMAG-PEI and 20 µg trMAG-16/1 per ml were prepared.

The transmission electron microscopy (TEM) was performed by Jean-Serge Rémy, Strasbourg, following the protocol of Erbacher et al. (Erbacher et al., 1998).
A carbon film was prepared on cleaved mica, using evaporation of carbon rods under vacuum. The flotation technique was then used to cover the electron microscope copper/rhodium grids (300 Mesh, Touzard and Matignon, Courtaboeuf, France) with carbon film. After drying overnight, the grids were kept on a blotting paper placed in a Petri dish. Just before the samples were added, the grids were glow discharged (110 mV, 25-30 µA, 25 sec).
A 5 µl drop of each solution prepared above was then poured onto a grid. Observations of the samples containing the electron-dense trMAGs were performed at 80 kV with a Philips EM 410 transmission electron microscope (Eindhoven, The Netherlands).

2.4 Binding of DNA to magnetic particles

2.4.1 Examination of trMAG-PEI as representative for positively charged magnetic beads with a monolayer of PEI

2.4.1.1 Ninhydrin assay to determine the amount of PEI in trMAG-PEI particle suspensions

The ninhydrin assay enables the quantitative and qualitative analysis of primary and secondary amines contained in PEI.

Determination of µg PEI / µg trMAG

Sample: 120 µg **trMAG-PEI** particles in 6 µl aqueous suspension were pipetted into an Eppendorf tube.
PEI standard row: 0, 3, 6, 9 and 12 µl of an aqueous 1 mg/ml PEI (800 kDa) stock solution were added each into an Eppendorf tube.
The sample and the standard row were prepared and examined in triplicates.
Ninhydrin assay: 75 µl phenol-ethanol (76 g phenol in 24 g ethanol), 100 µl 1.3 promille Kaliumcyanid in pyridine and 75 µl ninhydrin (2.5 g ninhydrin in 50 ml ethanol) were added to all the Eppendorf tubes prepared above. All reaction vessels were shaken and incubated for 5 min at 95 °C. After addition of 1 ml 60 % ethanol (EtOH) to all tubes the magnetic beads were centrifuged with 14000 rpm for 3 min in a EBA 12 R centrifuge from Hettich, Tuttlingen, Germany. 500 µl of the supernatants and the PEI standard row solutions were used to determine the extinction of the produced ninhydrin derivatives (Ruheman's Purple) photometrically at 570 nm with a DU 640 spectrophotometer from Beckmann, Munich, Germany. By comparing the extinctions of the samples with the extinctions of the PEI standard row the amount of µg PEI per µg trMAG-PEI could be calculated.
But in PEI bound to iron oxide particles not all primary and seconday amines may be accessible for the ninhydrin. Therefore the trMAGs contain probably even more PEI than the assay shows.

Detection of unbound PEI in trMAG suspension

Sample: 160 µg of trMAG-PEI particles in 1 ml aqueous suspension.

Preparation of supernatants: Approximately 1ml the sample was pipetted into a well of a 24-well plate (TPP, Switzerland). The trMAGs were sedimented by placing a sintered 20 x 10 x 5 mm Nd-Fe-B magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) underneath the well for 1h. Three times 120 µl of the supernatant were pipetted into 3 Eppendorf tubes (for examination in triplicates).
The ninhydrin assay should detect PEI which was not bound to trMAGs in the supernatants in the Eppendorf tubes. The assay was carried out as described above but without centrifugation after addition of 60 % EtOH. Further it was not a quantitative but only a qualitative analysis: blue staining indicated unbound PEI in the supernatant.

2.4.1.2 DNA-binding curves

The generally used protocol for radioactive (^{32}P) labeling of plasmid DNA by nick translation is described in "General methods" (2.2.1).
The magnets used were sintered Nd-Fe-B magnets (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany). The magnet format was cylindrical (d = 6 mm, h = 5 mm) and 96 of such magnets were inserted in an acrylic glass template in 96-well plate format with strictly alternating polarization. The fields of the individual magnets influenced each other such that the vector dose becomes concentrated in the centers of individual wells.

Preparation of trMAG-PEI / DNA complexes: In two separate set-ups, 120 µl each of DNA stock solution (124.8 µg cold plasmid plus 1.56 x 10^7 c.p.m. ^{32}P-labeled plasmid in 3120 µl of water) were added to 120 µl each of a dilution series of trMAG-PEI in water. The trMAG-PEI dilution series was calculated to result in 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 µg trMAG-PEI/µg DNA after mixing by pipetting. After 15 min of incubation, either 240 µl each of water or of 300 mM sodium chloride (for salt-induced aggregation) were added to the mixture. After 20 min of incubation the trMAG-PEI / DNA complexes (in water and in 150 mM NaCl) were ready for the binding studies.

Preparation of trMAG-PEI / DNA plus PEI or DOTAP-Chol: In two separate set-ups, 120 µl each of DNA stock solution (124.8 µg cold plasmid plus 1.56 x 10^7 c.p.m. ^{32}P-labeled plasmid in 3120 µl of water) were added to 120 µl each of a dilution series of trMAG-PEI in water. The trMAG-PEI dilution series was calculated to result in 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 4.0 µg trMAG-PEI/µg DNA after mixing by pipetting. After 15 min of incubation, either 120 µl each of PEI stock solutions (41.7 µg/ml in water) or of DOTAP-Cholesterol liposome stock suspensions (121.2 µl 5 mM liposome stock per ml in water) were added to the mixture. This resulted in PEI:DNA N/P ratios of 8 or DOTAP:DNA charge ratios of 5. After further 15-min incubation, 120 µl each of 600 mM sodium chloride (for salt-induced aggregation) were added to the complexes. After a 20-min incubation, the trMAG-PEI / DNA / PEI and trMAG-PEI / DNA / DOTAP-Chol complexes (both in 150 mM NaCl) were ready for studying the DNA association.

Binding studies: 120 µl each of the complexes prepared above were transferred to the wells of a U-bottom 96-well plate in triplicates. The plate was positioned upon the 96-well format magnetic plate. After 30 min of magnetic sedimentation, 80 µl supernatants were removed and mixed with 125 µl each of Microscint 40 (Canberra Packard, Dreieich, Germany) in an opaque 96-well plate. The samples were counted using a Topcount instrument (Canberra Packard, count delay set to 10 min, count time in triplicates, 5 min each).
The binding was calculated as: % bound = 100 x c.p.m. (sample) / c.p.m. (reference).
As reference (100 % of the DNA unbound) the samples with a trMAG/DNA w/w ratio of 0 were taken.

2.4.1.3 Measurement of zeta potential by laser Doppler velocimetry (LDV)

Preparation of samples in aqua dest.:
trMAG-PEI: 15 µg trMAG-PEI/1.5 ml
trMAG-PEI / DNA: 15 µg plasmid DNA in 750 µl water were added to 15 µg trMAG-PEI in the same volume of water while vortexing. After a incubation time of 15 min the complexes were ready for measurement of the zeta potential.

The zetapotentials of the samples were determined by laser Doppler velocimetry (LDV) using the Zetasizer 3000 HS, Malvern Instruments, Herrenberg, Germany. Roughly 50 ml aqua dest were injected into the electrophoresis chamber for rinsing by using a syringe before

each sample injection of approximately 1.5 ml. Measurements were performed with the following specifications: sampling time 5 sec, 10 measurements per sample, viscosity of water 0.89 cP, dielectric constant of water 80.4, temperature 25°C, beam mode F(Ka) = 1.50 (Smoluchowsky equation, for calculation of the zetapotential). After all measurements 50 ml ethanol (EtOH, 70%) were injected to keep the instrument sterile.

2.4.1.4 Particle sizes in 150 mM NaCl

Preparation of trMAG-PEI / DNA / PEI (w/w = 1; N/P = 8) complexes:
10 µg plasmid DNA in 333.3 µl water were pipetted to 10 µg trMAG-PEI in the same volume of water, homogenized and incubated. After 15 min 10.4 µg PEI (25 kDa) in 303.3 µl water were added to the trMAG plus DNA containing solution. After vortex a 15 min incubation time followed. Finally, the ternary complex solution was filled up to 1 ml with 30 µl 5 M NaCl (to adjust the ionic strength to 150 mM) and vortexed gently.
Size measurements were performed with the following specifications: 60 measurements for the ternary complex sample prepared in 150 mM NaCl, viscosity of 150 mM NaCl 1.14 cP, temperature 25°C.

2.4.1.5 Transmission electron microscopy

Formulation of trMAG-PEI plus PEI-DNA (w/w = 1; N/P = 8):
4 µg plasmid DNA in 100 µl water were added to 4.2 µg PEI (25 kDa) in the same volume of water while vortexing (giving rise to an N/P ratio of 8). After 15 min incubation 4 µg trMAG-PEI in 100 µl water were pipetted to the PEI-DNA vectors and vortexed gently. After 15 min of incubation the solution was filled up to a final volume of 400 µl and the ionic strength was adjusted to 150 mM sodium chloride (initialization of salt induced aggregation).

Transmission electron microscopy was performed by Jean-Serge Rémy, Strasbourg, exactly as described in 2.3.2, but additionally trMAG-PEI / PEI-DNA complexes were stained with 30 µl of an aqueous uranyl-acetate solution (1%, w/w) for 20 sec and then excess liquid was removed with blotting paper.

2.4.2 Examination of trMAG-16/1 as representative for positively charged magnetic beads with a multilayer of PEI

2.4.2.1 Ninhydrin assay to determine the amount of PEI in trMAG-16/1 particle suspensions

The assay was performed exactly as described in 2.4.1.1.

2.4.2.2 DNA-binding curve

Preparation of trMAG-16/1 / DNA complexes: 120 µl each of DNA stock solution (124.8 µg cold plasmid plus 1.56 x 10^7 c.p.m. ^{32}P-labeled plasmid in 3120 µl of water) were added to 120 µl each of a dilution series of trMAG-16/1 in water. The trMAG-16/1 dilution series was calculated to result in 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 µg trMAG-16/1 / µg DNA after mixing by pipetting. After 15 min, 240 µl each of water were added to the mixture and the complexes were incubated for further 20 min.

The **binding studies** were performed exactly as previously described in 2.4.1.2.

2.4.2.3 Measurement of zeta potential by laser Doppler velocimetry (LDV)

Preparation of samples in aqua dest.:
trMAG-16/1: 60 µg trMAG-16/1 / 1.5 ml
trMAG-16/1 / DNA: 30 µg plasmid DNA in 750 µl water were added to 60 µg trMAG-16/1 in the same volume of water while vortexing. After a incubation time of 15 min the complexes were ready for measurement of the zeta potential.

The zetapotential was determined exactly as described in 2.4.1.3.

2.4.2.4 Transmission electron microscopy

Formulation of trMAG-16/1 plus DNA: 3 µg plasmid DNA in 100 µl water were added to 6 µg trMAG-16/1 in 100 µl water while vortexing. After 15 min incubation the solution was

filled up to a final volume of 300 µl and the ionic strength was adjusted to 150 mM sodium chloride (initialization of salt induced aggregation).

Transmission electron microscopy was performed by Jean-Serge Rémy, Strasbourg, exactly as described in 2.3.2.

2.4.3 Examination of trMAG-pAsp as representative for negatively charged magnetic beads

2.4.3.1 DNA-binding studies

Preparation of PEI / DNA / trMAG-pAsp: 960 µl of aqueous DNA solution (28.8 µg cold plasmid plus 5.31 x 10^6 c.p.m. ^{32}P-labeled plasmid) were added to 960 µl aqueous PEI solution (with 30 µg PEI, to obtain a N/P ratio of 8). The complexes were mixed by pipetting. After 15 min of incubation, 960 µl aqueous trMAG-pAsp solution (28.8 µg trMAG-pAsp) were added to the mixture, vortexed gently and incubated for further 15 min.
1396.8 µl of this preparation were transferred into a new reaction vessel and either 43.2 µl of water or 43.2 µl of a 5 M NaCl solution were added to obtain PEI / DNA / trMAG-pAsp complexes in water or in 150 mM sodium chloride.
Preparation of DNA / trMAG-pAsp / PEI: 960 µl of aqueous DNA solution (28.8 µg cold plasmid plus 5.31 x 10^6 c.p.m. ^{32}P-labeled plasmid) were added to 960 µl aqueous trMAG-pAsp solution (28.8 µg trMAG-pAsp) and mixed by pipetting. After a 15-min incubation, 960 µl aqueous PEI solution (with 30 µg PEI, to obtain a N/P ratio of 8) were added to the mixture, vortexed gently and incubated for further 15 min.
1396.8 µl of this preparation were transferred into a new reaction vessel and either 43.2 µl of water or 43.2 µl of a 5 M NaCl solution were added to obtain DNA / trMAG-pAsp / PEI complexes in water or in 150 mM sodium chloride.

Binding studies: In two separate set-ups, 200 µl each of the trMAG-pAsp complexes were transferred to the wells of a U-bottom 96-well plate in triplicates. One plate was positioned upon the 96-well format magnetic plate whereas the other plate was not exposed to a magnetic field. All further steps followed the protocol described in 2.4.1.2. As references (with 100 % of the DNA unbound) the corresponding samples in the plate without magnetic sedimentation were taken.

2.5 Magnetofection in cell culture

2.5.1 Transfection with positively charged trMAGs

2.5.1.1 trMAG particles and naked DNA

Cells: NIH 3T3 seeded in 96-well plates.

General settings: 1 µg DNA/well.

trMAG/DNA w/w ratios: 0.5, 1, 2, 4, 8, 16, whereas the ratios 8 and 16 were only examined with trMAG-PEI and trMAG-13/1 to trMAG-17/1.

Each preparation examined in triplicates.

DNA stock: 59.9 µg/ml in water.

trMAG stocks: each 1 mg/ml in water.

trMAGs: trMAG-PEI, trMAG-13/1, trMAG-14/1, trMAG-15/1, trMAG-16/1, trMAG-17/1, trMAG-18/1, trMAG-19/1, trMAG-20/1, trMAG-21/1, trMAG-22/1, trMAG-23/1, trMAG-24/1, trMAG-25/1, trMAG-26/1.

Controls: each preparation without magnet (in triplicates each); naked DNA without trMAGs (in triplicates).

Incubation time: cells were incubated with vectors (with or without magnet) for 20 min.

Reporter gene assay: luciferase.

Vector preparation: trMAG / DNA complexes: 120,3 µl DNA stock each were added to 120.3 µl of each trMAG suspension (see table). Finally 120.3 µl of 15 % glucose or 120.3 µl of 450 mM NaCl, respectively, were added to each vector preparation to obtain final concentrations of 5 % glucose or 150 mM NaCl.

trMAG suspensions:

trMAG/DNA (w/w)	0	0.5	1	2	4	8	16
µg trMAG in 120.3 µl H_2O	0	3.6	7.2	14.4	28.8	57.6	115.2

2.5.1.2 trMAG / DNA complexes and additional PEI

Cells: CHO-K1 seeded in 96-well plates.

General settings: 0.5 µg DNA/well.

trMAG/DNA w/w ratios: 0.4, 0.8, 1, 2, 4, 8.

PEI/DNA N/P ratio = 8.

Each preparation examined in quadruples.

DNA stock: 40 µg/ml in water.

trMAG stocks: each 1 mg/ml in water.

PEI (25 kDa) stock: 41.7 µg/ml in water.

trMAGs: trMAG-DEAE, trMAG-DAEA, trMAG-STARCH-PEI, trMAG-PEI-ethoxylated, trMAG-PEI-epichlorohydrin, trMAG-PEI-lowMW, trMAG-PEI-SDS, trMAG-PEI-C1/1.

Controls: each preparation without magnet (in quadruples each).

Incubation time: cells were incubated with vectors (with or without magnet) for 15 min.

Reporter gene assay: luciferase.

Vector preparation: trMAG / DNA / PEI complexes: 125 µl DNA stock each were added to 125 µl of each trMAG suspension (see table). Subsequently 125 µl PEI stock each were added. Finally 125 µl of 0.6 M NaCl were added to each vector preparation to obtain final concentrations of 150 mM NaCl. After addition of NaCl the complexes were incubated for 45 min (salt induced aggregation).

trMAG suspensions:

trMAG/DNA (w/w)	0.4	0.8	1	2	4	8
µg trMAG in 125 µl H_2O	2	4	5	10	20	30

2.5.2 Transfection with negatively charged trMAGs

2.5.2.1 trMAGs and PEI-DNA complexes

Cells: NIH 3T3 and HepG2 seeded in two 96-well plates each.

General settings: 0.5 µg DNA/well.

trMAG/DNA w/w ratios: 0, 0.5, 1, 2, 4, 8.

PEI/DNA N/P ratio = 8.

Each preparation examined in triplicates.

DNA stock: 240 µg/7.2 ml in water.

trMAG stocks: each 1 mg/ml in water.

PEI (25 kDa) stock: 250.2 µg/7.2 ml in water.

trMAGs: trMAG-ARA, trMAG-pACRYL, trMAG-pACRYL-MAL, trMAG-pASP.
Controls: each preparation without magnet (in triplicates each).
Incubation time: cells were incubated with vectors (with or without magnet) for 10 min.
Reporter gene assay: luciferase.
Vector preparation: trMAG / PEI-DNA complexes: To preform PEI-DNA, 6545 µl of DNA stock were added to 6545 µl of PEI stock.
Three times 144 µl (triplicates) of each trMAG suspension containing 38.4 µg trMAGs were added consecutively to row A of a round-bottom 96-well plate (Nunc, Denmark). All other wells were filled with 72 µl of water. Using a multichannel pipettor, 72 µl each were transferred from row A to row B, from row B to row C, and so on. The dilution series finished at row F. Then, 144 µl PEI-DNA were pipetted to each well. Finally, 24 µl 50 % glucose solution per well were added to obtain a final concentration of 5 % glucose.

2.5.3 Hints to the mechanism of magnetofection

2.5.3.1 Influence of endosomolytic substances in magnetofection

PEI, PEI-bAdv, Lipofectamine and GenePORTER as additives

Cells: NIH 3T3 and CHO-K1 seeded in 96-well plates.
General settings: 0.1 µg DNA/well at GenePORTER and Lipofectamine, 0.5 µg DNA/well at all other formulations.
50 µl transfection volume/well for each preparation.
trMAG/DNA w/w ratio: 2.
PEI/DNA N/P ratio = 8.
Chemically inactivated adenovirus (inact. adv): 7.2×10^8 inact. adv particles/0.5 µg DNA.
5 µl GenePORTER (Gene Therapy Systems, La Jolla, CA, USA)/1 µg DNA or 4 µl Lipofectamine (Life Technologies, Karlsruhe, Germany)/1 µg DNA.
Each preparation examined in triplicates.
DNA stocks: 1.56 µg/0.195 ml in serum-free DMEM for GenePORTER and Lipofectamine, 11.7 µg/0.2925 ml in HBS for all other formulations.
trMAG stocks: 1 mg/ml in serum-free DMEM for GenePORTER and Lipofectamine, 1 mg/ml in HBS for all other formulations.

trMAG suspensions: 1.56 µg/0.0975 ml in serum-free DMEM for GenePORTER and Lipofectamine, 11.7 µg/0.1463 ml in HBS for all other formulations.

PEI (25 kDa) stock: 8.1 µg/0.195 ml in HBS.

Inact. adv stock: 5.59 x 10^9 virus particles per 0.0975 ml in HBS.

GenePORTER stock: 3.9 µl/0.0975 ml in serum-free DMEM.

Lipofectamine stock: 3.1 µl/0.0975 ml in serum-free DMEM.

trMAGs: trMAG-PEI.

Controls: corresponding standard vectors without trMAGs and without magnetic field (each in triplicates).

Incubation times: cells were incubated with vectors containing inact. adv for 20 min, incubation with all other vectors was 10 min.

Reporter gene assay: luciferase.

Vector preparation: trMAG-PEI / DNA complexes: 45 µl DNA stock were added to 45 µl trMAG suspension. Finally, 90 µl HBS were pipetted to the mixture.

trMAG-PEI / DNA / PEI complexes: 45 µl DNA stock were mixed with 45 µl trMAG suspension. Then, 45 µl PEI stock were pipetted to the mixture. Finally, 45 µl HBS were added to the complexes.

PEI-DNA complexes: 45 µl DNA stock were pipetted to 45 µl PEI stock. Finally, 90 µl HBS were added to the complexes.

trMAG-PEI / DNA / PEI / inact. adv complexes (AVET): 45 µl DNA stock were added to 45 µl trMAG suspension. Subsequently 45 µl PEI stock were added. Finally, 45 µl inact. adv stock were pipetted to the complexes and mixed very gently.

PEI-DNA / inact. adv complexes (AVET): 45 µl DNA stock were added to 45 µl PEI stock. Then, 45 µl inact. adv stock were pipetted to the complexes and mixed very gently. Finally, 45 µl HBS were added.

trMAG-PEI / DNA / Lipofectamine complexes: 45 µl DNA stock were mixed with 45 µl trMAG suspension. Subsequently 45 µl Lipofectamine stock were pipetted to the mixture. Finally, 45 µl serum-free DMEM were added to the complexes.

Lipofectamine-DNA complexes: 45 µl DNA stock were pipetted to 45 µl Lipofectamine stock. Finally, 90 µl serum-free DMEM were added to the complexes.

trMAG-PEI / DNA / GenePORTER complexes: 45 µl DNA stock were mixed with 45 µl trMAG suspension. Subsequently 45 µl GenePORTER stock were pipetted to the mixture. Finally, 45 µl serum-free DMEM were added to the complexes.

GenePORTER-DNA complexes: 45 µl DNA stock were pipetted to 45 µl GenePORTER stock. Finally, 90 µl serum-free DMEM were added to the complexes.

Application of vectors: Fifty µl each of the DNA complexes were added to cells kept in 150 µl fresh medium (complete). Only when complexes containing GenePORTER or Lipofectamine were used cells were freshly supplemented with 50 µl serum-free DMEM and 50 µl transfection volume/well were applied.

A synthetic influenza virus peptide (INF7) as additive

Cells: NIH 3T3 seeded in a 96-well plate.

General settings: 1 µg DNA/well.

trMAG/DNA w/w ratios: 0.5, 1, 2, 4, 8, 16.

Influenza virus derived peptide INF7: INF7/DNA -/- ratio = 1.

All formulations prepared in 5 % glucose.

Each preparation examined in quadruples.

DNA stock: 93.6 µg/1.17 ml in water.

trMAG stock: 2 mg/ml in water.

trMAG suspension: 249.6 µl trMAG stock plus 140.4 µl water.

trMAG suspension dilution series: Five tubes were filled with 195 µl water. To the first tube 195 µl of trMAG suspension were added, mixed and 195 µl from the first tube were added to the second tube, mixed and so on.

INF7 stock: 13.6 µl INF7 solution (13.9 mM with regard to negative charges) plus 766.4 µl water.

Glucose stock: 480 µl 50 % glucose plus 720 µl water.

trMAGs: trMAG-16/1

Controls: trMAG-16/1 / DNA without INF7 (each in quadruples).

Incubation time: cells were incubated with vectors and magnet for 10 min.

Reporter gene assay: luciferase.

Vector preparation: DNA / INF7 / trMAG-16/1 complexes: In six tubes, 60 µl DNA stock each were mixed with 60 µl INF7 stock each. Then, 60 µl each of trMAG suspension or of a trMAG suspension dilution were added to the tubes. Finally, 60 µl glucose stock each were pipetted to the complexes to obtain a final concentration of 5 % glucose.

trMAG-16/1 / DNA / INF7 complexes: 60 µl each of the trMAG suspension and of its five dilutions were filled into six tubes. Subsequently 60µl DNA stock were pipetted to each tube.

Then, 60 µl INF7 stock each were added. Finally, 60 µl glucose stock each were pipetted to the complexes.

trMAG-16/1 / DNA complexes: 60 µl DNA stock each were added to six tubes containing 60 µl of trMAG suspension or one of its five dilutions. Then, 60 µl water each were pipetted to the mixtures. Finally, 60 µl glucose stock each were added to the complexes.

2.5.3.2 The fate of magnetic particles during magnetofection (transmission electron microscopy)

Cells and transfections

Approximately 300 000 HeLa cells were seeded per 35 mm dish.

The next day the cells were washed with PBS and 1.5 ml fresh complete medium were added per dish. Then, each dish was incubated with 500 µl of trMAG-16/1 / DNA complexes (2.5 µg DNA/dish, trMAG/DNA w/w ratio = 2, preparation of complexes in 5 % glucose) for 1, 5 and 15 min. During these times a rectangular Nd-Fe-B magnet of 20 x 10 x 5 mm (Neo Delta; remanence Br, 1080-1150 mT; purchased from IBS magnet, Berlin, Germany) was placed underneath each dish. One dish was incubated first for 15 min with vectors and with magnet, then the medium was changed and the cells were incubated for further 24 h without magnet. After the incubation times the cells were fixed immediately.

Preparation of the samples and electron microscopy

Cells were fixed in 1 % glutaraldehyde in Sorensens buffer (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 7.4) for 1 h at room temperature. After fixation the cells were washed once with Sorensens buffer. Then the cells were post fixed in 1 % aqueous osmium tetroxide for 1 h and washed twice with Sorensens buffer followed by a dehydration series in graded ethanols (30 %, 50 % and 70 %). Each step was done two times for 10 min. The samples were stored in 70 % ethanol overnight at 4°C. The next day the cells were further dehydrated in 90 %, 95 %, and three times in 100 % ethanol, all done at 4°C. One additional 100 % ethanol step was done at room temperature. Afterwards a 1:1 mixture of the resin Epon 812 and 100% ethanol was put on the cells for two hours. The solution was taken off and the pure Epon mix was put into the dishes overnight. The next day the Epon solution was replaced by fresh Epon two times and placed in an oven at 50°C for a two day period to polymerize. The sectioning of the polymerised blocks was done by a ultramicrotome. For contrast staining 4 % uranyl acetate and 0,2 % lead citrate were used. A Philips CM 10 transmission electron microscope

(Eindhoven, The Netherlands) located at the Department of Anatomy II, Ludwig-Maximilians-University, Munich, Germany, was applied for microscopy.
The fixation, block preparation, sectioning and electron microscopy was performed mainly by Jim Lausier (Department of Pediatrics, Ludwig-Maximilians-University, Munich, Germany), assisted by Sabine Herzmann (Department of Anatomy II, Ludwig-Maximilians-University, Munich, Germany).

2.5.3.3 Reporter gene expression kinetic with magnetofection and standard transfection

Cells: NIH 3T3 seeded in 96-well plates.
General settings: 1 µg DNA/well.
trMAG/DNA w/w ratio: 4.
PEI/DNA N/P ratio = 8.
Each preparation in 0.9 % (150 mM) NaCl.
Each preparation at each time point examined in triplicates.
DNA stock: 58.8 µg/0.980 ml in 0.9 % NaCl.
trMAG stock: 16 mg/ml in water.
trMAG suspension: 109.2 µg/0.455 ml in 0.9 % NaCl.
PEI (25 kDa) stock: 61.3 µg/0.980 ml in 0.9 % NaCl.
trMAGs: trMAG-16/1.
Controls: PEI-DNA standard transfections without magnet (in triplicates each).
Incubation time: maximum time of incubation with vectors with or without magnet was 8 h.
Reporter gene assay: luciferase.
Vector preparation: trMAG-16/1 / DNA / PEI complexes: 455 µl DNA stock were added to the 455 µl trMAG suspension. Finally, 455 µl PEI stock were pipetted to the mixture.
PEI-DNA complexes: 455 µl DNA stock were mixed with 455 µl PEI stock. Finally, 455 µl 0.9 % NaCl were added to the complexes.
Cells transfected with each preparation in triplicates were lysed after 1, 2, 4, 8, 24 and 48 h.

2.5.3.4 Influence of the magnet on reporter gene expression

Transfections without magnetic particle containing vectors but with application of a magnetic field

Cells: NIH 3T3 seeded in 96-well plates.
General settings: 0.5 µg DNA/well.
PEI/DNA N/P ratio = 8.
Streptavidinylated polylysine: 25 ng St-pL/0.5 µg DNA.
Chemically inactivated adenovirus (inact. adv): 7.2 x 10^8 inact. adv particles/0.5 µg DNA.
Liposomes: DOTAP/Cholesterol mol/mol ratio = 1.
DOTAP-Cholesterol / DNA +/- = 5.
Protective copolymer P6YE5C: P6YE5C/DNA -/- ratio = 2.
Each preparation examined in quadruples.
DNA stock: 28 µg/0.7 ml in HBS.
PEI (25 kDa) stock: 23.4 µg/0.56 ml in HBS.
Streptavidinylated polylysine (St-pL) stock: 0.26 µg/0.132 ml in HBS.
Inact. adv stock: 1.44 x 10^{10} virus particles per 0.25 ml in HBS.
DOTAP-Cholesterol stock: 17.4 µl 5 mM DOTAP-Cholesterol plus 126.6 µl HBS.
P6YE5C stock: 29.6 mM in terms of negative charges (in water).
P6YE5C solution: 1.2 µl P6YE5C stock plus 142.8 µl HBS.
Controls: comparison of the vectors with and without magnet (each in quadruples).
Incubation times: cells were incubated with vectors with or without magnet for 3 h.
Reporter gene assay: luciferase.
Vector preparation: PEI-DNA / bAdv complexes: 120 µl DNA stock were mixed with 120 µl PEI stock. Subsequently 120 µl inact. adv stock were added and mixed very gently. Finally, 120 µl HBS were pipetted to the complexes.
bAdv / St-pL / DNA / PEI complexes: 120 µl inact.adv stock were added to 120 µl St-pL stock and mixed very gently. Then, 120 µl DNA stock were pipetted to the mixture. Finally, 120 µl PEI stock were added to the complexes.
PEI-DNA complexes: 120 µl DNA stock were added to 120 µl PEI stock. Finally, 240 µl HBS were pipetted to the mixture.
DOTAP-Cholesterol / DNA complexes: 120 µl DNA stock were added to 120 µl DOTAP-Cholesterol stock. Finally, 240 µl HBS were pipetted to the mixture.

PEI-DNA / P6YE5C complexes: 120 µl DNA stock were mixed with 120 µl PEI stock. Then, 120 µl P6YE5C solution wer pipetted to the mixture. Finally, 120 µl HBS were added to the complexes.

2.5.4 Critical parameters in optimizing magnetofection

2.5.4.1 Dose-response studies at different trMAG / DNA (w/w) ratios

Magnetofection with trMAG-PEI / DNA / DOTAP-Cholesterol

Cells: CHO-K1 seeded in two 96-well plates.
General settings: starting concentration of 0.5 µg DNA/well.
Liposomes: DOTAP/Cholesterol mol/mol ratio = 1.
DOTAP-Cholesterol / DNA +/- = 5.
trMAG/DNA w/w ratios: 0, 0.2, 0.4, 0.6, 0.8, 1, 2, 4.
Each preparation examined in quadruples.
DNA stock: 92.16 µg/2.304 ml in water.
trMAG stock: 1 mg/ml in water.
DOTAP-Cholesterol stock: 279.3 µl 5 mM DOTAP-Cholesterol liposomes in water plus 2024 µl water.
trMAGs: trMAG-PEI.
Controls: DOTAP-Cholesterol / DNA complexes without trMAGs (each in quadruples).
Incubation time: cells were incubated with vectors and magnet for 10 min.
Reporter gene assay: luciferase.
Vector preparation: trMAG-PEI / DNA / DOTAP-Cholesterol complexes: 250 µl DNA stock each were added to 250 µl of each trMAG suspension (see table below). Subsequently 250 µl DOTAP-Cholesterol each were added to the trMAG / DNA mixtures. Further, 250 µl 600 mM NaCl each were added to the tubes. Then, 4 x 240 µl of each composition (quadruples) were added to positions A1, B1, C1, D1 and E1, F1, G1, H1 and A7, B7, C7, D7 and E7, F7, G7, H7 of two round-bottom 96-well plates (Nunc, Denmark). All other wells were filled with 120 µl 150 mM NaCl. Using a multichannel pipettor, 120 µl each were transferred from row 1 and 7, respectively, to rows 2 and 8, respectively, to rows 3 and 9, and so on. Total handling time was about 20 min.

trMAG suspensions:

trMAG/DNA (w/w)	0	0.2	0.4	0.6	0.8	1	2	4
µg trMAG in 250 µl H_2O	0	2	4	6	8	10	20	40

Application of vectors: Fifty µl each of the DNA complex dilution series were added to cells kept in 150 µl fresh medium (complete).

Magnetofection with trMAG-PEI / DNA plus GenePORTER or plus Lipofectamine

Cells: CHO-K1 seeded in a 96-well plate.
General settings: starting concentration of 0.1 µg DNA/well.
100 µl transfection volume/well in serum-free DMEM.
5 µl GenePORTER (Gene Therapy Systems, La Jolla, CA, USA)/1 µg DNA or 4 µl Lipofectamine (Life Technologies, Karlsruhe, Germany)/1 µg DNA.
trMAG/DNA w/w ratios: 0, 0.5, 1, 2, 4, 6, 8, 10.
Each preparation examined in triplicates.
DNA stock: 6 µg/0.3 ml in DMEM (without supplements).
trMAG stocks: 0.1 mg/ml in DMEM (without supplements) and 1 mg/ml in DMEM (without supplements).
trMAGs: trMAG-PEI.
Controls: GenePORTER-DNA or Lipofectamine-DNA complexes without trMAGs (each in triplicates).
Incubation time: cells were incubated with vectors and magnet for 10 min.
Reporter gene assay: luciferase.
Vector preparation: trMAG-PEI / DNA / GenePORTER or Lipofectamine complexes: 36 µl DNA stock each were added to 36 µl of each trMAG suspension (see table below). Incubation was not longer than the required handling time. Subsequently 3.6 µl of GenePORTER or 2.9 µl of Lipofectamine diluted to 72 µl with DMEM were added to each trMAG / DNA mixture. After 20 min incubation, the DNA complexes were filled up to 720 µl with DMEM. Then, 3 x 230 µl of each composition (triplicates) were added consecutively to rows A and E , respectively, of a round-bottom 96-well plate (Nunc, Denmark). All other rows were filled with 115 µl DMEM. Using a multichannel pipettor, 115 µl each were transferred from row A

and E, respectively, to rows B and F, respectively, to rows C and G, and so on. Total handling time was about 20 min.

trMAG suspensions:

trMAG/DNA (w/w)	0	0.5	1	2	4	6	8	10
µg trMAG in 36 µl DMEM	0	0.36	0.72	1.44	2.88	3.6	5.76	7.2

Application of vectors: Serum-containing medium was removed from the plate and replaced with 100 µl each of the DNA complex dilution series. After a incubation time of 10 min with complexes and magnet cells were washed with complete DMEM.

2.5.4.2 Comparison of positively with negatively charged trMAGs regarding the transfection efficiency

Cells: NIH 3T3 seeded in 96-well plate.
General settings: 0.5 µg DNA/well.
trMAG/DNA w/w ratio: 1.
PEI/DNA N/P ratio = 8.
Influenza virus derived peptide INF7: INF7/DNA -/- ratio = 1.
Each preparation examined in triplicates.
DNA stock: 25 µg/0.624 ml in HBS.
trMAG stocks: each 1 mg/ml in HBS.
PEI (25 kDa) stock: 26 µg/0.624 ml in HBS.
INF7 stock: 28.3 nmol negative charges/0.234 ml in HBS.
trMAGs: trMAG-PEI, trMAG-PO4.
Controls: each preparation without magnet (in triplicates each).
Incubation time: cells were incubated with vectors (with or without magnet) for 10 min.
Reporter gene assay: luciferase.
Vector preparation: Preformation of PEI-DNA (N/P = 8) complexes: 520 µl DNA stock were added to 520 µl PEI stock.
PEI-DNA / INF7 / trMAG-PEI or trMAG-PO4 complexes: 195 µl PEI-DNA were added to 97.5 µl INF7 stock. To this mixture, 97.5 µl trMAG-PEI or trMAG-PO4 suspension (both consisting of 3.9 µg trMAG in 97.5 µl HBS) were added.

PEI-DNA / trMAG-PEI or trMAG-PO4 complexes: 195 µl PEI-DNA were added to 97.5 µl trMAG-PEI or trMAG-PO4 suspension (both consisting of 3.9 µg trMAG in 97.5 µl HBS). Further, 97.5 µl HBS were added.

2.5.4.3 Variation of the mixing order during formation of the complexes

DNA complexes including DOTAP-Cholesterol and trMAG-PO4 or trMAG-PEI

Cells: NIH 3T3 seeded in two 96-well plates.
General settings: starting concentration of 0.5 µg DNA/well.
Liposomes: DOTAP/Cholesterol mol/mol ratio = 1.
DOTAP-Cholesterol / DNA +/- = 5.
trMAG/DNA w/w ratio: 1.
Each preparation examined in triplicates.
DNA stock: 30 µg/ml in HBS.
trMAG stocks: 1 mg/ml in HBS.
trMAG suspensions: 30 µg/ml in HBS.
DOTAP-Cholesterol stock: 455 µM in HBS.
trMAGs: trMAG-PO4, trMAG-PEI.
Controls: each preparation without magnet (in triplicates each).
Incubation time: cells were incubated with vectors (with or without magnet) for 10 min.
Reporter gene assay: luciferase.
Vector preparation: DOTAP-Cholesterol / DNA / trMAG complexes: 260 µl DNA stock were added to 260 µl DOTAP-Cholesterol stock followed by mixing with 260 µl trMAG suspension.
trMAG / DNA / DOTAP-Cholesterol complexes: Using the same volumes and reagents, first trMAGs and DNA were mixed and then added to DOTAP-Cholesterol.
Serial dilution series: Three times 240 µl of each composition (triplicates) were added to A1-3, A7-9, E1-3 and E7-9 of a round-bottom 96-well plate (Nunc, Denmark). Positions 1-3 of B, C, D, F, G, H and positions 7-9 of B, C, D, F, G, H were filled with 120 µl HBS. Using a multichannel pipettor, 120 µl each were transferred from row A and E, respectively, to rows B and F, respectively, to rows C and G, and so on. Total handling time was about 20 min.
Application of vectors: Fifty µl each of the DNA complex dilution series were added to cells kept in 150 µl fresh medium (complete).

DNA complexes including PEI and trMAG-PO4

Cells: NIH 3T3 seeded in 96-well plates.
General settings: 0.5 µg DNA/well.
trMAG/DNA w/w ratio: 1.
PEI/DNA N/P ratio = 8.
Each preparation in HBS.
Each preparation examined in quadruples.
DNA stock: 18.7 µg/0.468 ml in HBS.
trMAG stock: 1 mg/ml in HBS.
trMAG suspension: 18.7 µg/0.468 ml in HBS.
PEI (25 kDa) stock: 19.5 µg/0.468 ml in HBS.
t**rMAGs:** trMAG-PO4.
Controls: each preparation without magnet (in quadruples each).
Incubation time: cells were incubated with vectors (with or without magnet) for 10 min.
Reporter gene assay: luciferase.
Vector preparation: PEI-DNA / trMAG-PO4 complexes: 120 µl DNA stock were mixed with 120 µl PEI stock. Subsequently 120 µl trMAG-PO4 suspension were added to the mixture. Finally, 120 µl HBS were pipetted to the complexes.
trMAG-PO4 / PEI / DNA complexes: 120 µl trMAG-PO4 suspension were mixed with 120 µl PEI stock. Subsequently 120 µl DNA stock were added to the mixture. Then, 120 µl HBS were pipetted to the complexes.
trMAG-PO4 / DNA / PEI complexes: 120 µl DNA stock were mixed with 120 µl trMAG-PO4 suspension. Then, 120 µl PEI stock were added. Finally, 120 µl HBS were pipetted to the complexes.

DNA complexes including PEI, trMAG-PO4 and chemically inactivated adenovirus

Cells: NIH 3T3 seeded in 96-well plates.
General settings: 0.5 µg DNA/well.
trMAG/DNA w/w ratio: 1.
PEI/DNA N/P ratio = 8.
Chemically inactivated adenovirus (inact. adv): 7.2×10^8 inact. adv particles/0.5 µg DNA.
Each preparation in HBS.

Each preparation examined in quadruples.

DNA stock: 49.9 µg/1.248 ml in HBS.

trMAG stock: 1 mg/ml in HBS.

trMAG suspension: 49.9 µg/1.248 ml in HBS.

PEI (25 kDa) stock: 52 µg/1.248 ml in HBS.

Inact. adv stock: 7.16 x 10^{10} virus particles per 1.248 ml in HBS.

t**rMAGs:** trMAG-PO4.

Controls: each preparation without magnet (in quadruples each).

Incubation time: cells were incubated with vectors (with or without magnet) for 10 min.

Reporter gene assay: luciferase.

Vector preparation: inact. adv / trMAG-PO4 / DNA / PEI complexes: 120 µl inact. adv stock were added to 120 µl trMAG-PO4 suspension and mixed very gently by pipetting. Subsequently 120 µl of DNA stock were added. Finally, 120 µl PEI stock were pipetted to the mixture.

All other vector preparations were performed with the same components and volumes but with different mixing orders.

2.5.4.4 Kinetics of magnetofection

Cells: NIH 3T3 seeded in 96-well plates.

General settings: 0.1 µg DNA/well.

100 µl transfection volume/well in serum-free DMEM.

4 µl Lipofectamine (Life Technologies, Karlsruhe, Germany)/1 µg DNA or 5 µl GenePORTER (Gene Therapy Systems, La Jolla, CA, USA)/1 µg DNA.

trMAG/DNA w/w ratio: 2.

Each preparation examined in triplicates.

DNA stock: 17.8 µg/0.9 ml in DMEM (without supplements).

trMAG stock: 1 mg/ml in DMEM (without supplements).

Lipofectamine (LF) solution: 41.5 µl LF plus 995.3 µl DMEM.

GenePORTER (GP) solution: 51.8 µl GP plus 985 µl DMEM.

trMAGs: trMAG-PEI.

Controls: Lipofectamine-DNA or GenePORTER-DNA complexes without trMAGs in the presence and absence of a magnet (each in triplicates) and trMAG-PEI / DNA / Lipofectamine or GenePORTER without magnet (each in triplicates).

Incubation time: cells were incubated with vectors for 5, 10, 20, 40 and 240 min and the magnet was applied for 240 min.

Reporter gene assay: luciferase.

Vector preparation: trMAG-PEI / DNA / Lipofectamine (LF) complexes: 216 µl DNA stock were added to 216 µl trMAG-PEI suspension containing 8.64 µg trMAGs. Subsequently 432 µl LF solution were pipetted to this mixture. Finally, the complexes were filled up with DMEM to a final volume of 4320 µl.

trMAG-PEI / DNA / GenePORTER (GP) complexes: 216 µl DNA stock were added to 216 µl trMAG-PEI suspension containing 8.64 µg trMAGs. Then, 432 µl GP solution were pipetted to this mixture. Finally, the complexes were filled up with DMEM to a final volume of 4320 µl.

DNA / Lipofectamine (LF) or DNA / GenePORTER (GP) complexes: exactly the preparation of trMAG-PEI / DNA / LF or GP complexes but instead of 216 µl trMAG-PEI suspension 216 µl DMEM were used.

Application of vectors: Serum-containing medium was removed from the plate and replaced with 100 µl each of the DNA complexes. After incubation with complexes cells were washed with complete DMEM.

2.5.5 Comparison of magnetofection and conventional transfection methods with regard to their gene transfer efficiency

2.5.5.1 Transfection of NIH 3T3 and CHO-K1 cells with different vector formulations

Cells: NIH 3T3 and CHO-K1 seeded in 96-well plates.

General settings: 0.5 µg DNA/well (with PEI), 0.25 µg DNA/well (with AVET-PEI), 0.1 µg DNA/well (with GenePORTER and Lipofectamine).

50 µl transfection volume/well for each preparation.

trMAG/DNA w/w ratios: 1 (with AVET-PEI) and 2 (with PEI, GenePORTER and Lipofectamine).

PEI/DNA N/P ratio = 8.

Chemically inactivated adenovirus (inact. adv): 3.6×10^8 inact. adv particles/0.25 µg DNA.

5 µl GenePORTER (Gene Therapy Systems, La Jolla, CA, USA)/1 µg DNA or 4 µl Lipofectamine (Life Technologies, Karlsruhe, Germany)/1 µg DNA.

Each preparation examined in triplicates.

DNA stocks: 40 µg/ml in water (for PEI), 40 µg/ml in HBS (for AVET-PEI), 20 µg/ml in serum-free DMEM (for GenePORTER and Lipofectamine).
trMAG stocks: 1 mg/ml in water (for PEI), 1 mg/ml in HBS (for AVET-PEI), 1 mg/ml in serum-free DMEM (for GenePORTER and Lipofectamine).
trMAG suspensions: 0 and 80 µg/ml in water (for PEI), 0 and 40 µg/ml in HBS (for AVET-PEI), 0 and 40 µg/ml in serum-free DMEM (for GenePORTER and Lipofectamine).
PEI (25 kDa) stocks: 41.7 µg/ml in water (for PEI) and in HBS (for AVET-PEI).
Inact. adv stock: 5.74 x 10^{10} virus particles per ml in HBS.
GenePorter stock: 50 µl/ml in serum-free DMEM.
Lipofectamine stock: 40 µl/ml in serum-free DMEM.
NaCl stock: 600 mM in water.
trMAGs: trMAG-PEI.
Controls: standard vectors without magnetic field with 10 min and 4 h incubation time (each in triplicates) and magnetofectins without magnetic field with 10 min incubation time (each in triplicates).
Incubation times: cells were incubated with magnetofectins with and without magnet for 10 min and with standard vectors without magnet for 10 min and 4 h.
Reporter gene assay: luciferase.
Vector preparation: DNA / trMAG-PEI / PEI complexes: 195 µl DNA stock were mixed with 195 µl trMAG suspension (80 µg/ml in water). Subsequently 195 µl PEI stock were added to the mixture. Finally, 195 µl NaCl stock were pipetted to the complexes to obtain a concentration of 150 mM NaCl. A final 30-min incubation step was performed for salt-induced aggregation.
PEI-DNA complexes: exactly the preparation of DNA / trMAG-PEI / PEI complexes but instead of 195 µl trMAG-PEI suspension with 80 µg/ml in water, 195 µl trMAG-PEI suspension with 0 µg/ml in water were used.
DNA / PEI / trMAG-PEI / inact. adv complexes (AVET-PEI): 97.5 µl DNA stock were mixed with 97.5 µl PEI stock. Subsequently 97.5 µl trMAG-PEI suspension (40 µg/ml in HBS) were added to the mixture. Then, 97.5 µl inact. adv stock were pipetted to the complexes and mixed very gently. Finally, 390 µl HBS were added for a 1:1 dilution.
DNA / PEI / inact. adv complexes (AVET-PEI): exactly the preparation of DNA / PEI / trMAG-PEI / inact. adv complexes but instead of 97.5 µl trMAG-PEI suspension with 40 µg/ml in HBS, 97.5 µl trMAG-PEI suspension with 0 µg/ml in HBS were used.

DNA / trMAG-PEI / GenePORTER or Lipofectamine complexes: 78 µl DNA stock were mixed with 78 µl trMAG-PEI suspension (40 µg/ml in serum-free DMEM). Subsequently 156 µl GenePORTER or Lipofectamine stock were added. After 20 min incubation, the complexes were diluted 2.5-fold with serum-free DMEM (which means addition of 468 µl medium). Cells were freshly supplemented with 50 µl serum-free DMEM, incubated with 50 µl transfection volume/well, followed by washing and cultivation with serum-containing medium.

DNA / GenePORTER or Lipofectamine complexes: exactly the preparation of DNA / trMAG-PEI / GenePORTER or Lipofectamine complexes but instead of 78 µl trMAG-PEI suspension with 40 µg/ml in serum-free DMEM , 78 µl trMAG-PEI suspension with 0 µg/ml in serum-free DMEM were used.

2.5.5.2 Transfection of NIH 3T3 and CHO-K1 cells with different DNA doses

Lipofectamine as transfection reagent

Cells: NIH 3T3 and CHO-K1 seeded in two 96-well plates each.
General settings: starting concentration of 0.1 µg DNA/well.
50 µl transfection volume/well.
trMAG/DNA w/w ratios: 2.
4 µl Lipofectamine (Life Technologies, Karlsruhe, Germany)/1 µg DNA.
Each preparation examined in triplicates.
DNA stock: 20.7 µg/ 1.296 ml in serum-free DMEM.
trMAG stock: 1 mg/ml in serum-free DMEM.
trMAG suspension: 27.6 µg/0.864 ml in serum-free DMEM.
Lipofectamine stock: 41.4 µl/0.648 ml in serum-free DMEM.
trMAGs: trMAG-PEI.
Controls: standard vectors without magnetic field with 10 min and 4 h incubation time (each in triplicates) and magnetofectins without magnetic field with 10 min and 4h incubation time (each in triplicates).
Incubation times: cells were incubated with magnetofectins with and without magnet for 10 min and 4 h and with standard vectors without magnet for 10 min and 4 h.
Reporter gene assay: luciferase.

Vector preparation: DNA / trMAG-PEI / Lipofectamine complexes: The positions A1-6 of a round-bottom 96-well plate (Nunc, Denmark) were filled with 60 µl trMAG suspension each and then 60 µl DNA stock each were added. Further, 60 µl Lipofectamine stock each were pipetted to the mixtures. Finally, 60 µl serum-free DMEM each were added.
Lipofectamine-DNA complexes: The positions A7-9 were filled with 60 µl Lipofectamine stock each and then 60 µl DNA stock each were added. Finally, 120 µl serum-free DMEM each were pipetted to the complexes.
Serial dilution series: Well 1-9 of row B, C and D were filled with 120 µl serum-free DMEM each. Using a multichannel pipettor, 120 µl each were transferred from row A to row B, to row C, and to row D. The surplus of 120 µl in the wells of row D was dicarded. Then, 120 µl serum-free DMEM were added to the wells of row A-D.
Application of vectors: Cells were freshly supplemented with 50 µl serum-free DMEM and 50 µl transfection volume/well were applied.

2.5.6 Localization of gene transfer using the magnetofection method

Cells: NIH 3T3 seeded in a 6-well plate.
General settings: 6 µg DNA/well.
trMAG/DNA w/w ratio: 1.
Biotinylated PEI (bPEI): bPEI/DNA N/P ratio = 8.
Chemically inactivated and biotinylated adenovirus (inact. bAdv): 8.625×10^9 inact. adv particles/6 µg DNA.
Each preparation in HBS.
DNA stock: 48 µg/ml in HBS.
trMAG stock: 1 mg/ml in HBS.
trMAG suspension: 57.6 µg/ml in HBS.
bPEI (25 kDa) stock: 50 µg/ml in HBS.
Inact. bAdv stock: 6.9×10^{10} virus particles per ml in HBS.
t**rMAGs:** streptavidinylated trMAG-PEI (trMAG-PEI-Sta).
Controls: same complexes but without magnet and complexes lacking trMAGs and no magnet.
Incubation time: cells were incubated with vectors (with or without magnet) for 15 min.
Reporter gene assay: . β-galactosidase.

Vector preparation: bPEI / DNA / inact. bAdv / trMAG-PEI-Sta complexes: 300 µl DNA stock were mixed with 300 µl bPEI stock. Then, 300 µl inact. bAdv stock were added and mixed very gently. Finally, 300 µl trMAG suspension were pipetted to the complexes.
bPEI /DNA / inact. bAdv complexes: 150 µl DNA stock were mixed with 150 µl bPEI stock. Then, 150 µl inact. bAdv stock were added and mixed very gently. Finally, 150 µl HBS were pipetted to the complexes.
X-gal staining: After 24 h, the cells were washed with PBS and subjected to X-gal staining for 45 min.

2.5.7 Magnetofection of other cells

2.5.7.1 HaCaT cells

Cells: HaCaT cells (cell line derived from human keratinocytes, kindly provided by Dr. Martin Mempel, Dermatology, TU Munich, Germany) seeded in 96-well plates. Medium: DMEM without supplements.
General settings: 1 µg DNA/well at PEI containing vectors and 0.1 µg DNA/well at GenePORTER containing vectors.
50 µl transfection volume/well at PEI containing vectors and 100 µl transfection volume/well at GenePORTER containing vectors.
trMAG/DNA w/w ratio: 4.
PEI/DNA N/P ratio = 8.
5 µl GenePORTER (Gene Therapy Systems, La Jolla, CA, USA)/1 µg DNA.
Each preparation examined in triplicates.
DNA stocks: 8.4 µg/0.140 ml in 0.9 % NaCl for PEI containing vectors and 0.84 µg/0.280 ml in serum-free DMEM for GenePORTER containing vectors.
trMAG stocks: 16 mg/ml in 0.9 % NaCl for PEI containing vectors and 1 mg/ml in serum-free DMEM for GenePORTER containing vectors.
trMAG suspensions: 15.6 µg/0.065 ml in 0.9 % NaCl for PEI containing vectors and 1.56 µg/0.130 ml in serum-free DMEM for GenePORTER containing vectors.
PEI (25 kDa) stock: 8.1 µg/0.130 ml in 0.9 % NaCl.
GenePORTER stock: 3.9 µl/0.260 ml in serum-free DMEM.
trMAGs: trMAG-16/1.
Controls: corresponding standard vectors without trMAGs (each in triplicates).

Incubation times: cells were incubated with vectors and magnet for 4h.

Reporter gene assay: luciferase.

Vector preparation: trMAG-16/1 / DNA / PEI complexes: 60 µl DNA stock were mixed with 60 µl trMAG suspension. Finally, 60 µl PEI stock were added to the mixture.

PEI-DNA complexes: 60 µl DNA stock were mixed with 60 µl PEI stock. Finally, 60 µl 0.9 % NaCl were added.

trMAG-16/1 / DNA / GenePORTER complexes: 120 µl DNA stock were mixed with 120 µl trMAG suspension. Finally, 120 µl GenePORTER stock were added to the mixture.

GenePORTER-DNA complexes: 120 µl DNA stock were mixed with 120 µl GenePORTER stock. Finally, 120 µl serum-free DMEM were added to the mixture.

Application of vectors: Fifty µl each of the PEI containing complexes were added to cells kept in 150 µl fresh medium.

Hundred µl each of the GenePORTER containing complexes were added to cells from which the medium was removed before.

2.5.7.2 Primary human keratinocytes

Cells: primary human foreskin keratinocytes (kindly provided by Dr. Martin Mempel, Dermatology, TU Munich, Germany) seeded in 96-well plates. Medium: DMEM without supplements.

General settings: 1 µg DNA/well.

trMAG/DNA w/w ratio: 2.

PEI/DNA N/P ratio = 8.

Preparation in 0.9 % NaCl.

The various incubation times were examined in triplicates each.

DNA stock: 25.7 µg/0.429 ml in 0.9 % NaCl.

trMAG stock: 5 mg/ml in water.

trMAG suspension: 51.5 µg/0.429 ml in 0.9 % NaCl.

PEI (25 kDa) stock: 26.8 µg/0.429 ml in 0.9 % NaCl.

t**rMAGs:** trMAG-16/1.

Controls: 10’ with vectors but no magnet and 4 h with vectors but no magnet (in triplicates each).

Incubation times: 10’ with vectors and 4 h with magnet, 4 h with vectors and 4 h with magnet, 10’ with vectors and 10’ with magnet, 4 h with vectors and only the first 10’ with

magnet. Incubation with vectors and application of a magnetic field always started simultaneously.

Reporter gene assay: luciferase.

Vector preparation: trMAG-16/1 / DNA / PEI complexes: 390 µl DNA stock were mixed with 390 µl trMAG suspension. Finally, 390 µl PEI stock were added to the mixture.

2.5.7.3 RIF-1 cells

Cells: RIF-1 cells (mouse radiation-induced fibrosarcoma cell line, kindly provided by Ellen Kolbe, Experimental Oncology, TU Munich, Germany) seeded in a 96-well plate. Medium: DMEM with supplements.

General settings: 1 µg DNA/well.

trMAG/DNA w/w ratio: 4.

PEI/DNA N/P ratio = 8.

Preparations in 0.9 % NaCl.

Each preparation was examined in triplicates.

DNA stock: 12.6 µg/0.210 ml in 0.9 % NaCl.

trMAG stock: 16 mg/ml in water.

trMAG suspension: 33.6 µg/0.140 ml in 0.9 % NaCl.

PEI (25 kDa) stock: 8.8 µg/0.140 ml in 0.9 % NaCl.

t**rMAGs:** trMAG-16/1.

Control: Incubation with the standard vector PEI-DNA for 2 h (in triplicates).

Incubation times: cells were incubated with magnetofectins for 30 min.

Reporter gene assay: luciferase.

Vector preparation: trMAG-16/1 / DNA / PEI complexes: 65 µl DNA stock were mixed with 65 µl trMAG suspension. Finally, 65 µl PEI stock were added to the mixture.

trMAG-16/1 / DNA complexes: 65 µl DNA stock were mixed with 65 µl trMAG suspension. Finally, 65 µl 0.9 % NaCl were added to the mixture.

PEI-DNA complexes: 65 µl DNA stock were mixed with 65 µl PEI stock. Finally, 65 µl 0.9 % NaCl were added to the complexes.

2.6 Magnetofection in animal experiments

2.6.1 Injection into the ear veins of five pigs

Preparation of vectors: trMAG-PEI / DNA / PEI complexes per animal (2 ears): 1200 µg DNA (plasmid: p55pCMV-IVS-luc+) in 3 ml water were mixed with 1200 µg trMAG-PEI beads in 3 ml water and incubated for 15 min. Subsequently 1250.9 µg PEI (25 kDa) in 3 ml water were added to the mixture, vortexed and incubated for further 15 min. Finally, 360 µl 5 M NaCl in 3 ml water were pipetted to the complexes (to obtain a final concentration of 150 mM), mixed and left for 20 min to allow salt induced aggregation.

Application of vectors: An i.v. cannula (Venflon^{TM}, 22G, Becton Dickinson, Helsingborg, Sweden) was laid as far distal as possible into the Vena auricularis lateralis of each ear. For anesthesia 6 mg Propofol 1 % (Fresenius, Bad Homburg) per kg body weight were injected into one ear. During anesthesia medical oxygen was supplied. Approximately 5 cm downstream of the cannula in the right ear a permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed lengthways above the vein. Subsequently 5 ml of complexes were infused within 3 min. The magnet was attached for 1 h above the vein. Then, 5 ml of complexes were injected within 3 min into the left ear vein but no magnet was applied (control ear without magnet).

Preparation of tissue and blood samples: Twenty-four hours after injection of complexes the animals were sacrificed by intracardiac injection of 1.5 g Pentobarbital (Narcoren, Merial, Hallbergmoos) and 20 ml of KCl (1 M KCl solution, Delta-Pharma, Pfullingen) each. Both ears were removed and the ear veins and 2 samples each of other major organs (heart, lung, liver, spleen and kidney) were isolated. Each ear vein was divided in two samples: first the area of injection and second the area of the vein underneath the magnet (or no magnet). Additionally, blood samples of 5 ml each were taken.
The tissue samples were washed with PBS and added to tubes (conical 2.0 ml screw cap tubes with cap, VWR scientific products, West Chester, USA) which were filled with beads for homogenization (Zirconia beads, 2.5 mm diameter, Biospec Products Inc., Bartlesville, USA) and 500 (for veins) or 750 µl (for organs) lysis buffer (10 ml of 5 x Reporter Lysis Buffer from Promega Corporation, Madison, USA plus 40 ml water plus 1 tablet Complete protease

inhibitor from Boehringer, Mannheim, Germany). Fivehundred µl of blood sample each were pipetted into an empty tube. Each tube was weighed before and after the tissue sample was added. Tubes plus samples were kept on ice all the time. The samples in the tubes were homogenized 3 x 20 seconds by using a Mini-beadbeater (Biospec Products Inc., Bartlesville, USA) and subsequently centrifuged for 10 min at 14000 rpm and 4 °C (centrifuge: EBA 12 R, Hettich, Tuttlingen). Each blood sample was centrifuged for 5 min at 3000 rpm and 4 °C and only the serum was used for luciferase assay.

Luciferase assay: Fifty µl from each prepared sample tube were transferred to a well of a black 96-well plate, mixed with 100 µl of luciferin buffer (Luciferase Assay System, Promega Corporation, Madison, USA) and assayed for bioluminescence using the Microplate Scintillation & Luminescence counter "TopCount" (Canberra Packard, Groningen, The Netherlands) with a count time of 12 s and a count delay of 1 min.
To obtain a calibration curve 200, 100, 50, 25, 12.6, 6.25, 3.13, 1.57, 0.78, 0.39, 0.2, 0.1, 0.05, 0.025, 0.013 and 0 ng luciferase (Roche, Mannheim, Germany) each in 50 µl lysis buffer (2-fold dilution series) were measured under the same conditions as the samples.
After luciferase assay the sample tubes (now lacking 50 µl) were weighed again.
The reporter gene expressions were expressed in pg luciferase/g tissue.

This animal experiment was performed in collaboration with Ulrike Schillinger (veterinarian), Experimental Oncology, TU Munich, Germany.

2.6.2 Injection into the ear arteries of two rabbits

Preparation of vectors: trMAG-16/1 / DNA complexes for two animals (4 ears): 960 µg DNA (plasmid: p55pCMV-IVS-luc+) in 3.6 ml 5 % glucose were mixed with 3840 µg trMAG-16/1 beads in 3.6 ml 5 % glucose and incubated for 10 min.

Application of vectors: An i.v. cannula (Venflon^{TM}, 22G, Becton Dickinson, Helsingborg, Sweden) was laid as far proximal as possible into the ear artery of each ear. For anesthesia 50 mg Ketamin and 4 mg Xylazin per kg body weight were injected intramuscular (i.m.). During anesthesia medical oxygen was supplied. Approximately 5 cm downstream of the cannula in the right ear a permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed

lengthways above the artery. Subsequently 1.5 ml of complexes were injected within 1 min. The magnet was attached for 1 h above the artery. Then, 1.5 ml of complexes were injected within 1 min into the left ear artery but no magnet was applied (control ear without magnet).

Preparation of the artery samples: Approximately 24 hours after injection of complexes the animals were sacrificed by i.v. injection of 120 mg Pentobarbital (Narcoren, Merial, Hallbergmoos) per kg body weight. Both ears were removed and the ear arteries were isolated. Each ear artery was divided in different samples: the area proximal of the magnet, the area underneath the magnet, the area distal of the magnet, and an area remote distal of the magnet; ear arteries without magnet were divided in the corresponding areas.
The different samples were washed with PBS and added to tubes (conical 2.0 ml screw cap tubes with cap, VWR scientific products, West Chester, USA) which were filled with beads for homogenization (Zirconia beads, 2.5 mm diameter, Biospec Products Inc., Bartlesville, USA) and 500 µl lysis buffer (10 ml of 5 x Reporter Lysis Buffer from Promega Corporation, Madison, USA plus 40 ml water plus 1 tablet Complete protease inhibitor from Boehringer, Mannheim, Germany). Each tube was weighed before and after a sample was added. Tubes plus samples were kept on ice all the time. The samples in the tubes were homogenized 3 x 20 seconds by using a Mini-beadbeater (Biospec Products Inc., Bartlesville, USA) and subsequently centrifuged for 10 min at 14000 rpm and 4 °C (centrifuge: EBA 12 R, Hettich, Tuttlingen).

Luciferase assay: followed exactly the protocol described in the animal experiment above "Injection into the ear veins of five pigs".

This animal experiment was performed in collaboration with Ulrike Schillinger (veterinarian), Experimental Oncology, TU Munich, Germany.

2.6.3 Injection into the ilea of rats

Preparation of vectors: trMAG-16/1 / DNA complexes for six animals: 1250 µg DNA (plasmid: pCMV-β-gal) in 3125 µl 5 % glucose were mixed with 2500 µg trMAG-16/1 beads in 3125 µl 5 % glucose and incubated at room temperature.

Application of vectors: After laparatomy of anesthetized Wistar rats in the linea alba region, ileum and caecum were exposed and the guts was clamped off 8 cm in oral direction of the ileo-caecal junction. Ingested material was carefully rinsed towards the caecum by application of 1 ml of isotonic saline. Then, a second clamp was placed 3 cm aborally from the first clamp. One ml of the vector preparation was injected with a 20G needle (Braun, Melsungen, Germany) adjacent to the first clamp. The injection site was closed with surgical suture (Ethilon 3/0 black monofil, from Ethicon, Norderstedt, Germany) while a sterile permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed under the clamped-off section. Five min post injection both clamps were removed. The magnet was left for a total of 20 min. Subsequently, the guts was returned carefully into the abdominal cavity which was closed with surgical suture. Altogether 3 animals with magnet and 3 animals without magnet (controls) were treated.

Preparation of the samples: The animals were sacrificed after 48 hrs. The treated section of the guts and adjacent areas were isolated, rinsed exhaustively with PBS and fixed for 30 min with 2 % formaldehyde (Sigma-Aldrich, Deisenhofen, Germany) and 0.2 % glutaraldehyde (Sigma-Aldrich, Deisenhofen, Germany) in PBS. The tissue was rinsed again with PBS followed by 4 hrs X-Gal staining at 37°C. Subsequently, the tissue was again rinsed exhaustively with PBS and stored over night at 4°C in 2 % formaldehyde/PBS followed by embedding for paraffin and cryosections. Sections were stained with eosin.

This animal experiment was performed in collaboration with Julia Henke and Ulrike Schillinger (veterinarians), Experimental Oncology, TU Munich, Germany.

3 RESULTS

In this chapter the characteristics of the magnetic particles used are shown and the results of experiments which examined the binding of DNA to magnetic beads, transfections with different types of magnetic particles (magnetofections), the mechanism of magnetofection, optimization of magnetofection, the gene transfer efficiency of magnetofections compared to standard transfections, magnetic field-guided localzation of gene transfer, magnetofection of a variety of cells and the applicability of magnetofection *in vivo*, are presented.

3.1 Characteristics of magnetic nanoparticles (trMAGs) used in this study

The beads used in this study were superparamagnetic iron oxide nanoparticles. This kind of particles is usually derived by precipitation from acidic iron(II)/iron(III)-salt solutions upon addition of bases (Schwertmann and Cornell, 1991). To stabilize the particles, they are coated with polymers. Every commercial supplier follows his own special protocol. The particles used in this thesis were synthesized by Chemicell GmbH, Berlin and they differed in their coatings and their sizes. The exact coating procedures were not disclosed by Chemicell. However, the information included in table 2 was provided.

To create paramagnetic gene vectors, the DNA needs to be bound to the magnetic beads. Possible ways of binding are biological binding (e.g. via streptavidin-biotin), chemical-covalent binding and physical binding (via electrostatic or van der Waals interactions). In this thesis physical binding was chosen and Christian Bergemann from Chemicell developed iron oxide nanoparticles coated with cationic or anionic polymers which enable binding of negatively charged DNA or positively charged DNA vectors to the particles via electrostatic interactions or via salt induced aggregation. In the beginning, particles were coated with a monolayer of the cationic polymer polyethylenimine (PEI). PEI is a well known transfection reagent, it is able to compact DNA and it has endosomolytic activities as it acts as a "proton sponge", that means protonation of PEI within endosomes and endosomal Cl^- entry triggers osmotic swelling and destabilization of the endosomal vesicle (Boussif et al., 1995; Sonawane et al., 2003). Based on results in gene delivery (presented in section 3.3.1.1), which showed that a monolayer coating did not promote transfection of naked plasmid DNA to a sufficient extent, the idea of a multilayer coating with PEI arose. Christian Bergemann (Chemicell) synthesized such particles and he also produced iron oxides coated with PEI of different molecular weights, linear PEI (known to be very efficient in gene transfer), chemically

modified PEI, diethylaminoethyl-dextran (DEAE-dextran, forms massive precipitates when added to DNA and leads to enhanced gene transfer) and other cationic and anionic polymers.

3.1.1 Surface coating and size of magnetic particles

In the following table, all magnetic particles used, their surface coatings and the results of size measurements by dynamic light scattering are presented.

Positively charged magnetic particles		
Name of magnetic particle	Coating	average diameter nm +/-
trMAG-PEI	trMAGs coated with a monolayer of polyethylenimine, (PEI, Mw 800 kDa, Fluka, Neu-Ulm, Germany). PEI is a well known transfection reagent.	221.8 +/- 2.3
trMAG-13/1	trMAGs coated with a monolayer of PEI 2 kDa (Sigma-Aldrich, Deisenhofen, Germany).	195.6 +/- 1.5
trMAG-14/1	trMAGs coated with a monolayer of PEI 60 kDa (Sigma-Aldrich, Deisenhofen, Germany).	223.1 +/- 2.9
trMAG-15/1	trMAGs coated with a monolayer of PEI 750 kDa (Fluka, Neu-Ulm, Germany).	327.1 +/- 8.6
trMAG-16/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany). *	236.9 +/- 4.0
trMAG-17/1	trMAGs coated with linear PEI (Aldrich, USA). Linear PEI is an efficient transfection reagent.	n.d.
trMAG-18/1	trMAGs coated with a multilayer of PEI 2000 kDa (Aldrich, USA). *	205.8 +/- 6.4
trMAG-19/1	trMAGs coated with a multilayer of PEI 2000 kDa (Aldrich, USA) but with a different coating procedure than at trMAG-18/1. *	267.8 +/- 18.1
trMAG-20/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany), but with a different coating procedure than at trMAG-16/1. *	356.2 +/- 12.5
trMAG-21/1	trMAGs ultraloaded with layers of PEI 800 kDa (Fluka, Neu-Ulm, Germany). *	390.6 +/- 30.1
trMAG-22/1	trMAGs coated with a commercially available polyamine from Merck, Darmstadt, Germany.	n.d.

trMAG-23/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany) but with a different coating procedure than at trMAG-16/1. *	328.3 +/- 20.0
trMAG-24/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany) but with a different coating procedure than at trMAG-16/1. *	322.2 +/- 13.5
trMAG-25/1	trMAGs coated with a multilayer of PEI 800 kDa (Fluka, Neu-Ulm, Germany) but with 50% less PEI than at trMAG-16/1. *	279.1 +/- 11.1
trMAG-26/1	trMAGs coated with poly(bis(2-chlorethyl)ether-alt-1,3 bis(3-dimethyl-amino)propyl)urea, quaternized. The positive charges should enable DNA binding.	n.d.
trMAG-DEAE	trMAGs with a dextran monolayer coating, introduction of end-standing DEAE groups with 2-diethylamino-ethyl chloride-hydrochloride. DEAE-dextran is known to increase gene transfer.	n.d.
trMAG-DAEA	trMAGs coated with a polymer prepared from dimethylamine, epichlorohydrine and ethylene diamine. The positive charges should enable DNA binding.	175.8 +/- 2.8
trMAG-STARCH-PEI	trMAGs with a multilayer coating of starch, Mw 60 kDa (Fluka, Neu-Ulm, Germany) followed by covalent coupling of PEI via amino groups to the periodate-oxidized starch layer.	197.0 +/- 2.5
trMAG-PEI-ethoxylated	trMAGs with a monolayer coating of PEI 50 kDa (Aldrich, USA) which has been ethoxylated (80 %).	239.4 +/- 6.0
trMAG-PEI-epichlorhydrin	trMAGs with a monolayer coating of PEI 20 kDa (Aldrich, USA) modified with epichlorohydrin.	191.6 +/- 4.1
trMAG-PEI-lowMW	trMAGs with a monolayer coating of PEI, Mw 1.7 kDa (Aldrich, USA).	152.8 +/- 1.2
trMAG-PEI-SDS	trMAGs with a monolayer coating of PEI 800 kDa (Aldrich, USA) modified by a covalent coupling of sodium dodecyl sulfate (SDS) by carbodiimide activation (N-Ethyl-N'-(dimethylaminopropyl)-carbodiimide). SDS should enhance transport through cellular membranes.	n.d.
trMAG-PEI-C1/1	trMAGs coated with a commercially available PEI, should result in relativly small particles.	97.2 +/- 2.4

Negatively charged magnetic particles		
Name of magnetic particle	Coating	average diameter nm +/-
trMAG-ARA	trMAGs coated with arabinic acid, sodium salt, Mw 250 kDa. Negative charges should enable the binding of positively charged gene vectors.	230.2 +/- 2.6
trMAG-pACRYL	trMAGs coated with polyacrylic acid, sodium salt, Mw 20 kDa. Negative charges should enable the binding of positively charged gene vectors.	158.1 +/- 3.0
trMAG-pACRYL-MAL	trMAGs coated with polyacrylic acid-co-maleic acid, sodium salt, Mw 50 kDa. Negative charges should enable the binding of positively charged gene vectors.	221.1 +/- 3.5
trMAG-pASP or trMAG-pAsp	trMAGs coated with polyaspartic acid, sodium salt, Mw 3000 kDa. Negative charges should enable the binding of positively charged gene vectors.	110.1 +/- 1.5
trMAG-PO4	trMAGs coated with starch-phosphate, Mw 20 kDa. Negative charges should enable the binding of positively charged gene vectors.	n.d.

Table 2 Overview of all magnetic particles used, description of their coatings and the results of size measurements in aqua dest. by dynamic light scattering using a Malvern 3000 HS Zetasizer. The different coating procedures and further details are only known by Christian Bergemann, Chemicell, Berlin, Germany.
The coated iron oxides are in this thesis called **"trMAGs"** which is only the short form of **"transMAGs"**, the name usually used in publications. The nomenclature of the various trMAGs was established by Christian Bergemann, Berlin, Germany.
Abbreviations: PEI = polyethylenimine, kDa = kiloDalton, Mw = Molecular weight in g/mol or Dalton, n.d. = not determined because aggregated after long-term storage.

* trMAGs labeled with a star are trMAGs coated with a multilayer of PEI and their suspensions contain **unbound PEI** which is a result of the multilayer coating procedure.

3.1.2 Transmission electron microscopy of trMAGs

To illustrate with examples what such magnetic particles look like one electron micrograph of trMAGs with a monolayer coating of PEI and one electron micrograph of trMAGs with a multilayer coating of PEI (taken by Jean-Serge Rémy, Strasbourg) are presented below.

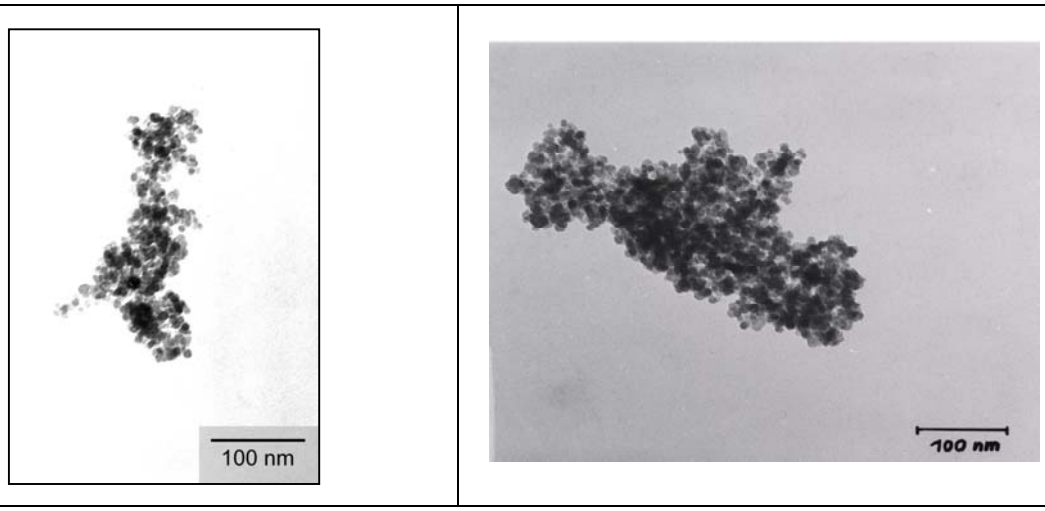


Figure 1 Transmission electron micrographs of magnetic beads coated with a monolayer of PEI (trMAG-PEI, left picture) and a multilayer of PEI (trMAG-16/1, right picture). The samples applied had concentrations of 10 µg trMAG-PEI per ml water and 20 µg trMAG-16/1 per ml water, respectively. No stain was used and therefore only the electron-dense iron oxide crystals but no PEI molecules are visible. The magnetic particles are organized in multi-domain structures of irregular shape.
The electron micrographs were produced by Jean-Serge Rémy, Strasbourg.

The iron oxide beads coated with a monolayer of PEI (trMAG-PEI) and the ones coated with a multilayer of PEI (trMAG-16/1) show both an organization in **multi-domain structures**. Discrepancies in size between light scattering and transmission electron microscopy measurements are probably due to the irregular shape of the structures. Further it has to be considered that with the sample preparation method used here, non-aggregated particles may be washed off through a washing procedure. Additionally, the particles are not studied in suspension but they are allowed to dry on a grid which could change their appearance as well.

3.2 Binding of DNA to magnetic particles

As shown above, all trMAGs are nanoparticles and they have either a positive or a negative surface coating. Therefore, in terms of shape and charge, they have similar characteristics as nonviral gene vectors which can associate with other charged particles via electrostatic interactions or via salt-induced colloid aggregation, a phenomenon well known in colloid chemistry (Hiemenz, 1986).
The question was now if trMAGs could be bound to DNA (vectors) by such physical interactions.
DNA-binding curves were obtained when magnetic particle / DNA (vector) preparations (with less than 1% of the DNA molecules ^{32}P-labeled) were magnetically sedimented as described in "Materials and methods". Non-sedimented radioactivity was determined in the supernatants. From this, the percentage of bound radio-labeled DNA (which is assumed to be directly proportional to the percentage of bound unlabeled DNA) was calculated.

3.2.1 Examination of trMAG-PEI as representative for positively charged magnetic beads with a monolayer of PEI

The trMAG-PEI particles are coated with a monolayer of of PEI 800 kDa. Determination of the PEI content of trMAG-PEI particles by ninhydrin assay revealed that **one µg trMAG-PEI contained 0.07 µg PEI and there was no unbound PEI in suspension**. It has to be considered that when PEI is bound to iron oxides not all primary and secondary amines may be accessible for the ninhydrin reaction and therefore it can be assumed that the actual PEI content is higher than the one determined.

3.2.1.1 DNA-binding curves

Magnetic particle / DNA (vector) associates were prepared at increasing particle / DNA weight ratios. The complexes were either formulated and left in water (**aqua dest.-curves**) or they were formulated in water and then the the ionic strength was adjusted to 150 mM NaCl (**150 mM NaCl-curves**).

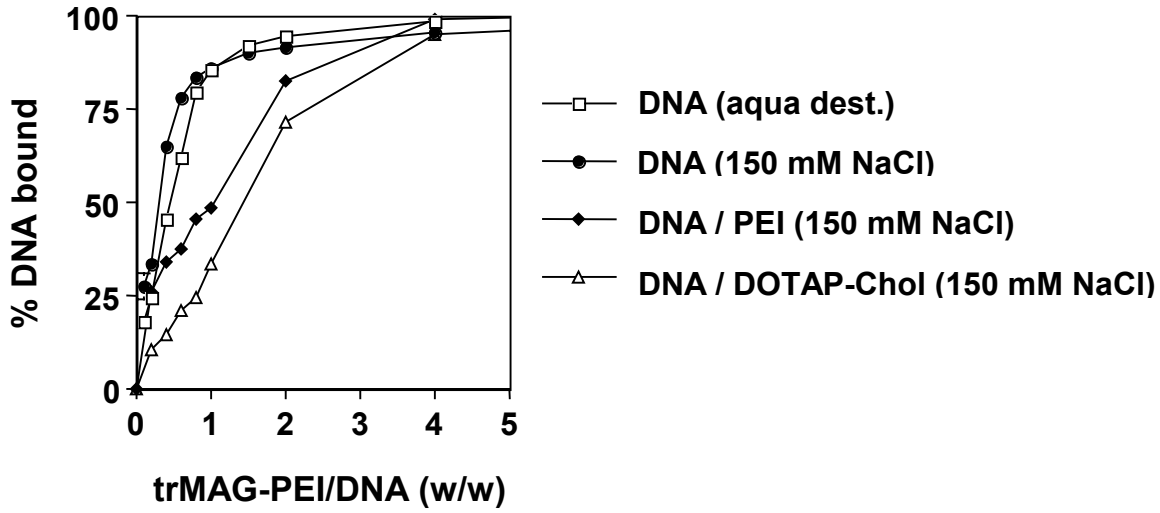


Figure 2 Percentage of radio-labeled DNA which was bound to trMAG-PEI beads in dependence of the particle / DNA weight ratio.
trMAG-PEI / DNA complexes in **aqua dest.** and **150 mM NaCl** [DNA (aqua dest.), DNA (150mM NaCl)]: The resulting DNA binding curves for complexes in water and in 150 mM NaCl were very similar. A saturation with more than 90 % of the DNA bound was achieved at a trMAG-PEI / DNA (w/w) ratio of 2. Approx. 85 % of the DNA dose was associated with beads at a ratio of 1.
trMAG-PEI / DNA / PEI and **trMAG-PEI / DNA / DOTAP-Cholesterol** complexes in **150 mM NaCl** [DNA / PEI (150 mM NaCl), DNA / DOTAP-Chol (150 mM NaCl)]: A saturation with almost 100 % of the DNA bound was achieved at a trMAG / DNA (w/w) ratio of 4 when first DNA and then PEI (N/P = 8) or DOTAP-Cholesterol (+/- = 5) was added to trMAG-PEI. Both binding curves showed a similar shape but the trMAG-PEI / DNA / PEI complexes were more effective in DNA association than the trMAG-PEI / DNA / DOTAP-Cholesterol aggregates.

Measurements with laser Doppler velocimetry (LDV) using a Malvern 3000 HS Zetasizer showed that the average zetapotential of trMAG-PEI in aqua dest. was +38.4 +/- 0.8 mV and of trMAG-PEI / DNA (w/w = 1) in aqua dest. was –35.6 +/- 3.0 mV. This change of the zetapotential from strongly positive to strongly negative when DNA was added to trMAG-PEI beads confirms that DNA is bound to the trMAGs. This binding has to occur via electrostatic interactions.

When trMAG-PEI / DNA / PEI or DOTAP-Chol complexes are formed, first DNA binds electrostatically to trMAG-PEI. But it is assumed that after addition of free PEI or DOTAP-Chol the trMAGs are displaced (free PEI and DOTAP-Chol is assumed to have a higher binding affinity to DNA than PEI bound to iron oxides) and through electrostatic interactions PEI-DNA or DOTAP-Chol-DNA complexes can be formed. A further assumption suggests that these positively charged polyplexes or lipoplexes can aggregate with the positively charged trMAGs upon salt addition.

Compared to using only naked DNA, additional free PEI or DOTAP-Cholesterol moved the point of saturation to higher magnetic beads / DNA (w/w) ratios. At a weight ratio of 2, **92 %**

(naked DNA, saturation), **83 %** (DNA + PEI) and **72 %** (DNA + DOTAP-Chol), respectively, of the DNA dose were associated with the magnetic beads.
Further binding studies in our lab with iodine-125 labeled DNA revealed that the trMAG-PEI particles did not associate with PEI-DNA complexes (N/P = 8) in water but that they did in 150 mM NaCl. (Plank et al., 2003c). This finding demonstrates that for the association of gene vectors with magnetic beads, salt-induced aggregation can be very important.

3.2.1.2 Partcle sizes in 150 mM NaCl

In addition to the DNA-binding curve of trMAG-PEI / DNA / PEI in 150 mM NaCl, the corresponding particle sizes up to 2 hours after adjustment of the ionic strength to 150 mM NaCl were measured by dynamic light scattering.

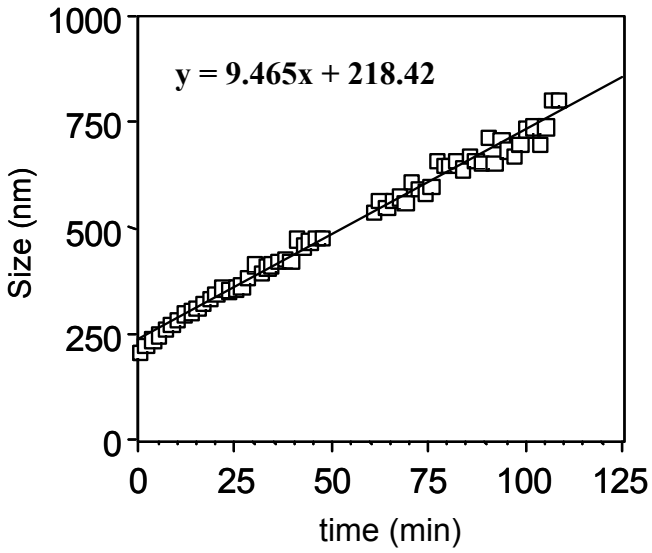


Figure 3 Time-dependent growth of particles resulting from trMAG-PEI beads plus DNA (w/w = 1/1) plus PEI (N/P = 8) in 150 mM NaCl.
This phenomenon can be explained by salt-induced aggregation. The particles aggregated with approximately **linear kinetics** starting at 217 ± 2 and remaining in the sub-micrometer range within two hours.

It is assumed that in the beginning DNA binds electrostatically to trMAG-PEI but after addition of free PEI the trMAGs are displaced and through electrostatic interactions PEI-DNA complexes (positively charged) are formed. After addition of salt, the trMAGs can aggregate with PEI-DNA particles and with increasing time the aggregates get larger and larger. In dynamic light scattering, the particles are assumed to be spherical and therefore the obtained size values of aggregates with irregular shape are only an approximation whereas the time-dependent growth of aggregates can be monitored with reliability.

3.2.1.3 Transmission electron microscopy

To illustrate what such associates produced by salt-induced aggregation look like an electron micrograph of trMAG-PEI particles mixed with PEI-DNA complexes followed by adjustment to 150 mM NaCl was taken by Jean-Serge Rémy, Strasbourg.

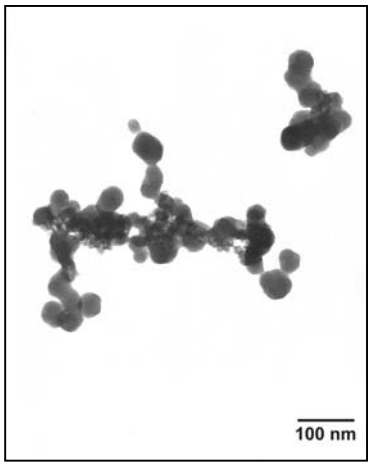


Figure 4 Associates of trMAG-PEI and PEI-DNA (w/w = 1; N/P = 8) vectors produced by salt-induced aggregation.
Staining of the complexes with an aqueous uranyl-acetate solution (1%, w/w) made PEI-DNA complexes electrondense and therefore visible as grey spherical or worm-like structures.
The micrograph shows a tight association of PEI-DNA (more gray spherical or worm-like structures) with iron oxide particles (black crystals).

Salt induced aggregation enables the binding of positively charged PEI-DNA vectors to positively charged trMAG-PEI. This finding is in accordance with the already mentioned results of binding studies with iodine-125.
The electron micrograph gives an impression of the shape of such aggregates although it has to be considered that with the sample preparation method used here, the particles are not in suspension but they are allowed to dry on a grid and this could change their appearance.

3.2.2 Examination of trMAG-16/1 as representative for positively charged magnetic beads with a multilayer coating with PEI

The trMAG-16/1 particles are coated with a multilayer of PEI 800 kDa. Determination of the PEI content of trMAG-16/1 beads by ninhydrin assay revealed that **one µg trMAG-16/1 contained 0.37 µg PEI** (approximately 5.3 times more than trMAG-PEI) **and parts of it are unbound PEI in suspension**. It has to be considered that with PEI bound to iron oxides not all primary and secondary amines may be accessible for the ninhydrin reaction and therefore it can be assumed that the actual PEI content is higher than the one determined.

3.2.2.1 DNA-binding curve

Magnetic particle / DNA associates were prepared at increasing particle / DNA weight ratios in aqua dest. and the binding of DNA to trMAG-16/1 was examined.

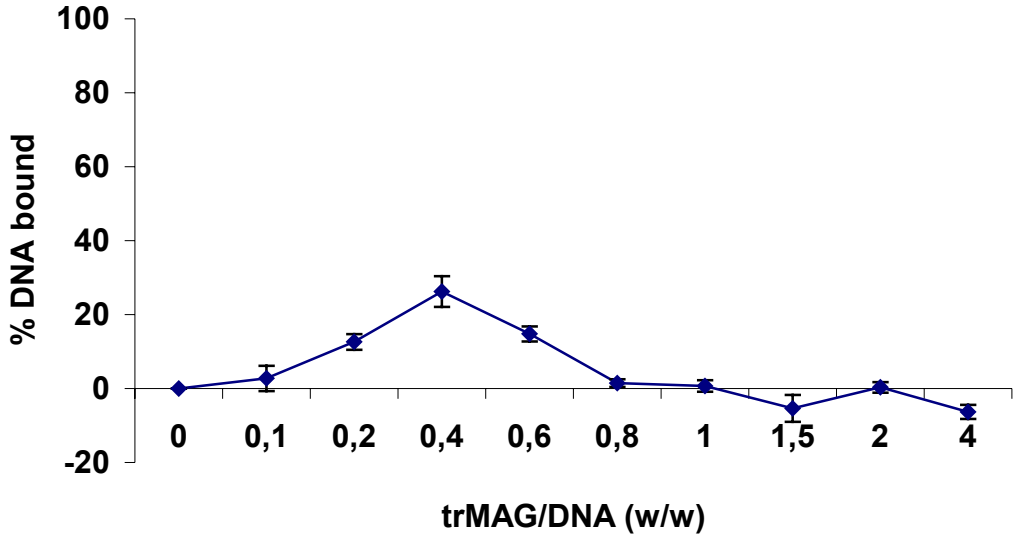


Figure 5 Percentage of radio-labeled DNA which was bound to trMAG-16/1 beads in **aqua dest**. in dependence of the particle / DNA weight ratio.
DNA **binding** was **only possible with** relatively low **trMAG/DNA (w/w) ratios from 0.1 to 0.8. Maximum** binding was achieved **at a trMAG/DNA (w/w) ratio of 0.4 with a relatively low value of 26 %**. The negative values for trMAG/DNA (w/w) ratios of 1.5 and 4 can only be explained by experimental fluctuation.
A possible explanation for this binding curve could comprise two overlaping effects:
First effect: As mentioned before trMAG-16/1 particles contain free unbound PEI in their suspension and it is assumed that DNA binds preferentially to free PEI (to form PEI-DNA complexes) and not to PEI from the trMAG layers as free PEI is better accessible and more flexible. The amount of DNA remains constant but the amount of trMAGs and unbound PEI increases with increasing trMAG/DNA (w/w) ratios. Therefore at low trMAG/DNA ratios where not sufficient unbound PEI is provided to bind all the DNA, the remaining DNA is bound to the trMAGs present and there is still unbound DNA. Thus an increase in trMAGs and unbound PEI leads to an increase of DNA bound to trMAGs until a peak is reached. Further increase of trMAGs and unbound PEI results in less binding of DNA to trMAGs as more and more unbound PEI is provided. At trMAG/DNA (w/w) ratios higher than 0.8 there is so much unbond PEI in solution that it binds all the DNA to form positively charged PEI-DNA complexes and there is no DNA left which could bind to trMAGs. The PEI-DNA complexes formed at trMAG/DNA (w/w) ratios higher than 0.8 have to be positively charged because negatively charged complexes could bind to trMAGs.
Second effect: At low trMAG/DNA (w/w) ratios the PEI-DNA complexes formed are probably negatively charged as there is not so much unbound PEI in suspension. These negatively charged complexes could bind electrostatically to trMAGs. With increasing amounts of trMAGs and unbound PEI more negatively charged PEI-DNA complexes are formed and get bound to the magnetic beads until maximum binding is achieved. If the amount of trMAGs plus unbound PEI is further increased the PEI-DNA complexes get less negatively charged and their binding to trMAGs decreases until even positively charged trMAGs are formed which were not able to bind to trMAG-16/1 particles.

When trMAG-16/1 / DNA mixtures were prepared in **water** maximum binding of DNA to magnetic beads was obtained at a trMAG/DNA (w/w) ratio of 0.4 with only 26 % which indicates **inefficient binding**. **With trMAG/DNA (w/w) ratios higher than 0.8 there is**

even no binding of DNA to trMAGs possible. Measurements with laser Doppler velocimetry (LDV) using a Malvern 3000 HS Zetasizer showed that the average zetapotential of trMAG-16/1 in aqua dest. was +64.2 +/- 6.3 mV and of trMAG-16/1 / DNA (w/w = 2) in aqua dest. was +56.6 +/- 1.4 mV which means that there was no significant change of the zetapotential when DNA was added to trMAG-16/1 beads. This result confirms that at higher trMAG/DNA (w/w) ratios no DNA can be bound to trMAG-16/1 particles.

Recent binding studies in our lab with trMAG-16/1 / DNA complexes (including iodine-125 labeled DNA) prepared in **150 mM NaCl** showed that more than 90% of the DNA is associated with trMAG-16/1 particles at trMAG/DNA w/w ratios from 0.5 to 1.1. It is assumed that DNA binds preferentially to free PEI and the resulting PEI-DNA complexes aggregate with trMAG-16/1 particles by salt-induction. But in the same experiment with trMAG/DNA w/w ratios higher than 1.1 a dramatic decrease in DNA binding was obtained and at a ratio of 2 and 4, only 20% and no DNA binding, respectively, was monitored. An explanation for the strong decrease in DNA binding at higher trMAG/DNA w/w ratios could be that higher amounts of free PEI inhibit salt-induced aggregation of PEI-DNA and trMAG-16/1 particles.

3.2.2.2 Transmission electron microscopy

TrMAG-16/1 particles and DNA (trMAG/DNA w/w = 2) were mixed in water, the ionic strength was adjusted to 150 mM NaCl and for illustration an electron micrograph was taken by Jean-Serge Rémy, Strasbourg.

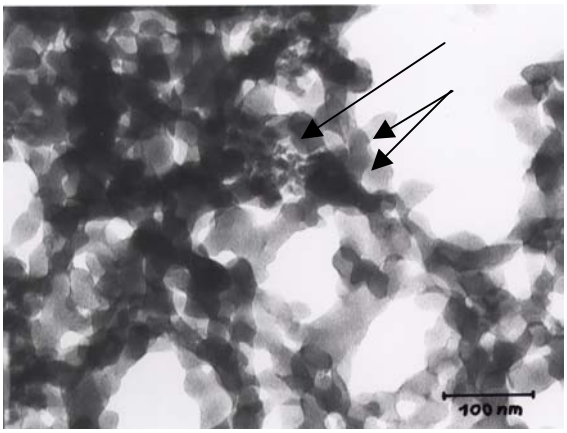


Figure 6 Associates of trMAG-16/1 particles and PEI-DNA complexes (both positively charged) produced by salt-induced aggregation (trMAG/DNA w/w = 2). The aggregate shown has a size of several hundred nanometers (see scale bar in the right lower corner).
The PEI forming the PEI-DNA complexes was originally free unbound PEI which was contained in trMAG-16/1 suspension.
Staining of the complexes with an aqueous uranyl-acetate solution (1%, w/w) made PEI-DNA complexes electron-dense and therefore visible as grey and black structures (see the two lower arrows).
The micrograph shows a tight association of PEI-DNA (grey and black structures indicated by the two lower arrows) with iron oxide particles (much smaller black crystals indicated by the longer upper arrow).

Despite recent binding studies in our lab which revealed that in salt with a trMAG/DNA w/w ratio of 2 only 20% of the DNA is bound to trMAG-16/1 particles, this electron micrograph shows efficient salt-induced aggregation of trMAG-16/1 particles and PEI-DNA complexes at this ratio of 2. An explanation for more efficient aggregation in electron microscopy experiments could be that through a washing step in the sample preparation free PEI (which could inhibit aggregation) is removed. Further, the addition of uranyl-acetate for staining could promote salt-induced aggregation and also drying of the samples on a grid could enhance aggregation.

3.2.3 Examination of trMAG-pAsp as representative for negatively charged magnetic beads

The trMAG-pAsp particles are coated with polyaspartic acid (sodium salt, 3000 kDa).

3.2.3.1 DNA-binding studies

The binding of DNA complexes to negatively charged poly aspartic acid coated particles (trMAG-pAsp) in water and after adjustment of the ionic strength to 150 mM NaCl was examined.

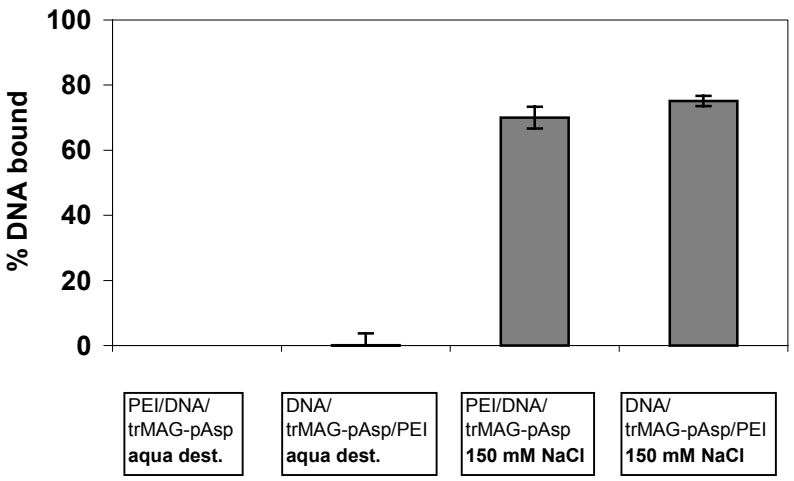


Figure 7 Percentage of DNA bound to negatively charged trMAG-pAsp particles (w/w = 1) in water and after adjustment of the ionic strength to 150 mM NaCl.
Additionally the mixing order of the complex components was varied:
Firstly PEI-DNA (N/P = 8) complexes with a positive net charge were preformed and subsequently trMAG-pAsp beads (negatively charged) were added.
Secondly the PEI (N/P = 8) was added to a solution containing DNA and trMAG-pAsp particles.
Independent of the mixing sequence **in water there was no binding of DNA to the magnetic beads possible but in 150 mM NaCl approximately 70 % of the DNA complexes were associated with magnetic particles** via salt-induced aggregation.

Mixing negativly charged DNA and trMAG-pAsp and subsequent addition of positively charged PEI did not lead to electrostatic binding of DNA to the magnetic beads in water. Surprisingly, even positively charged PEI-DNA complexes (N/P = 8) could not bind electrostatically to negatively charged poly aspartic acid coated beads in aqua dest. An explanation could be that with a N/P ratio of 8 there is an excess of PEI that is not complexed to DNA (Boeckle et al., 2004) but could bind to trMAG-pAsp instead of PEI-DNA.

In contrast, after adjustment of the ionic strength to 150 mM NaCl with any mixing order, more than 70 % of the DNA dose is bound to trMAG-pAsp particles. Similarly as positively charged trMAG-PEI or trMAG-16/1 particles and positively charged PEI-DNA complexes (see figure 2, 4 and 6) can aggregate in 150 mM NaCl, the trMAG-pAsp beads with electrostatically bound PEI (resulting in net positively charged particles) can bind to positively charged PEI-DNA complexes via **salt induced colloid aggregation**.

3.3 Magnetofection in cell culture

In cell culture it was examined if gene transfer with magnetic beads (positively or negatively charged) plus DNA and eventually PEI is possible, which type of magnetic particle is the most efficient in transfections, if a magnetic field would enhance gene transfer, what the mechanism of magnetofection is, what the critical parameters for magnetofection are (e.g. DNA dose-response profiles or the kinetics of magnetofection), the comparison of magnetofection with standard transfection methods, if a distinct localization of paramagnetic gene vectors via magnetic field is possible and if magnetofection is applicable to different cell types.

If not otherwise stated, all transfections were performed in 96-well plates in triplicates. **Sintered Nd-Fe-B magnets (Neo Delta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) were used**. For 96-well plates, the magnet shape was cylindrical (d = 6 mm, h = 5 mm) and the magnets were inserted in an acrylic glass template in 96-well plate format with strictly alternating polarization. The fields of the individual magnets influence each other such that the vector dose becomes concentrated in the centers of individual wells. If not otherwise stated, all **luciferase and β-galactosidase assays** were performed **24 hours after transfection** of the cells.

3.3.1 Transfection efficiency with positively charged trMAGs

In the following experiments, it was examined if gene transfer with magnetic particles is possible, if a permanent magnet placed underneath the cell culture plate could enhance transfections with magnetic particles, which type of magnetic beads is the most successful in gene transfer, which trMAG/DNA (w/w) ratios show maximum transfection efficiencies and if smaller complexes (prepared in salt-free medium) or larger complexes (which aggregate in 150 mM NaCl) lead to higher reporter gene expression. Complexes formed in salt-free medium (e.g. water or 5% glucose) are small and their size is stable for hours. Originally the idea has been that small particles are taken up by cells more easily (e.g. through endocytosis) and therefore lead to more efficient gene transfer. But Ogris et al. showed that larger gene vectors resulting from salt-induced aggregation are more efficient in transfections (Ogris et al., 1998).

First, positively charged trMAGs, most of them loaded with PEI in mono- or multilayers, were examined.

3.3.1.1 trMAG particles and naked DNA

trMAG-PEI and trMAG-13/1 – trMAG-26/1 are all superparamagnetic iron oxide beads coated with cationic polymers. Most of these particles are coated with PEI and they differ in the type of coating (monolayer or multilayer) and in the molecular weight of PEI (see table 2). The beads were mixed with naked plasmid DNA coding for the firefly luciferase at increasing trMAG/DNA (w/w) ratios. The efficiency of the complexes in luciferase gene transfer into NIH3T3 mouse fibroblasts (using 1 µg DNA / well) with and without application of a magnetic field was determined.

Preparation in water and subsequent adjustment to 5% glucose

The particles were mixed with DNA in water and subsequently glucose was added to obtain a final concentration of 5% glucose. The glucose does not alter the electrostatic binding behaviour but in contrast to aqua dest.; the 5% glucose solution can be added to the cells without causing osmotic stress. In the previously shown binding studies with trMAG-16/1 particles in water (figure 5) at trMAG/DNA (w/w) ratios higher than 0.8; there was no electrostatic binding of DNA to PEI multilayer coated trMAGs possible as the entire DNA dose associated with unbound PEI which is contained in the suspensions of all multilayer beads. Therefore it was assumed that transfections with PEI multilayer coated particles formulated in water and adjusted to 5% glucose would only lead to reporter gene expression at low trMAG/DNA (w/w) ratios. The cells (kept in serum-containing medium) were incubated with complexes for 20 minutes and during this time either a magnet was applied or not (control). After incubation the cells were washed and fresh medium was added.

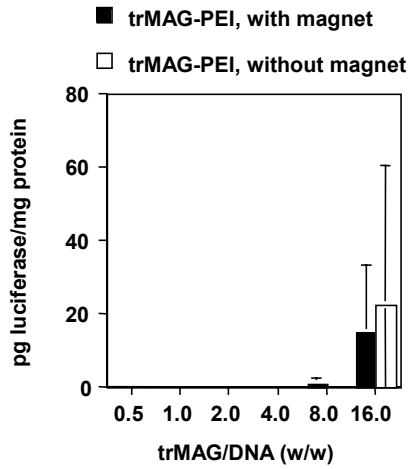


Figure 8 Transfection with trMAG-PEI. The particles are coated with a monolayer of PEI 800kDa (Fluka).

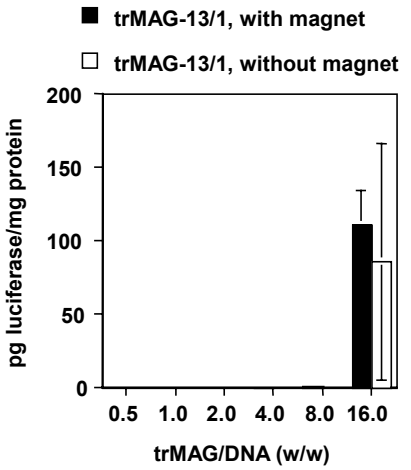


Figure 9 Transfection with trMAG-13/1. The particles are coated with a monolayer of PEI 2 kDa (Sigma-Aldrich).

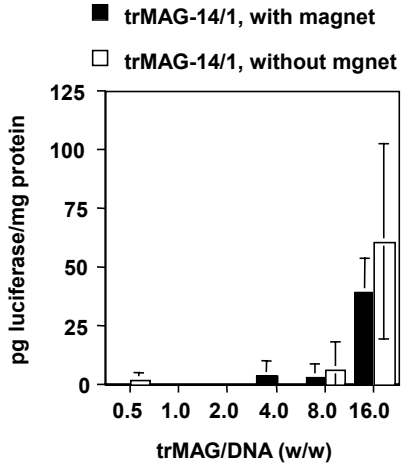


Figure 10 Transfection with trMAG-14/1. The particles are coated with a monolayer of PEI 60 kDa (Sigma-Aldrich).

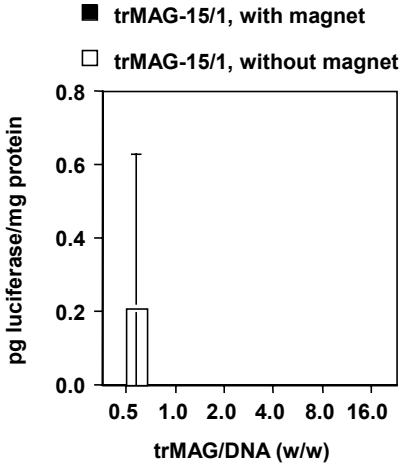


Figure 11 Transfection with trMAG-15/1. The particles are coated with a monolayer of PEI 750 kDa (Fluka).

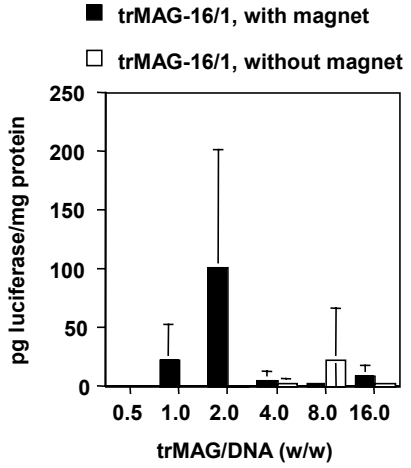


Figure 12 Transfection with trMAG-16/1. The particles are coated with a multilayer of PEI 800 kDa (Fluka).

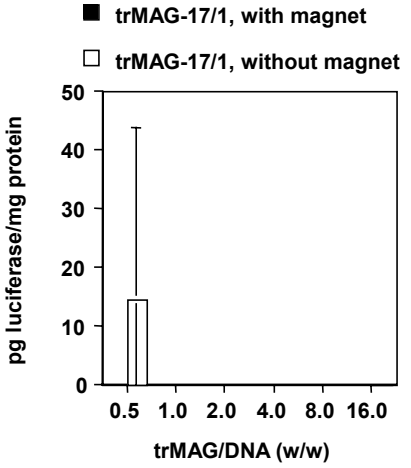


Figure 13 Transfection with trMAG-17/1. The particles are coated with linear PEI (Aldrich).

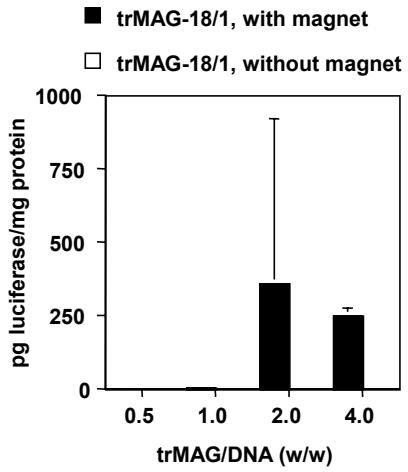


Figure 14 Transfection with trMAG-18/1. The particles are coated with a multilayer of PEI 2000 kDa (Aldrich).

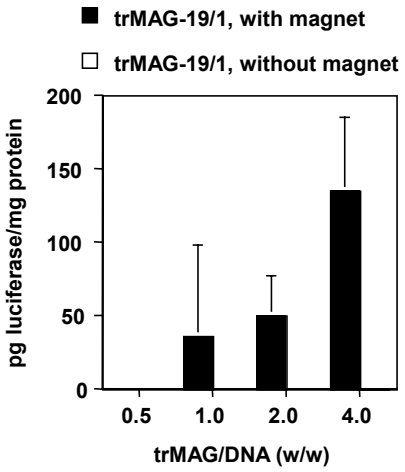


Figure 15 Transfection with trMAG-19/1. The difference between trMAG-19/1 and trMAG-18/1 (fig. 12) is only the coating procedure.

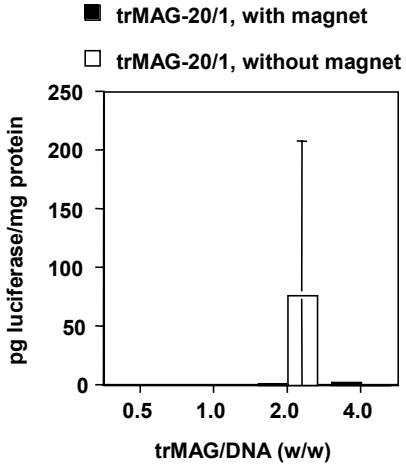


Figure 16 Transfection with trMAG-20/1. The difference between trMAG-20/1 and trMAG-16/1 (fig. 10) is only the coating procedure.

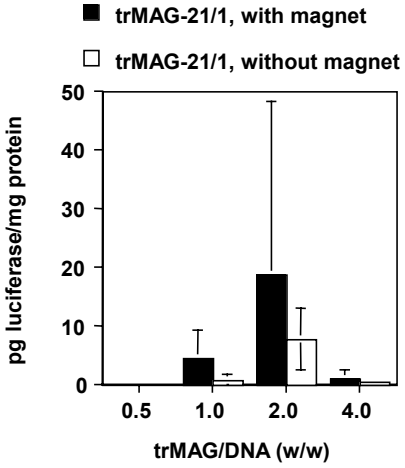


Figure 17 Transfection with trMAG-21/1. The difference between trMAG-21/1 and trMAG-16/1 (fig. 10) is that trMAG-21/1 is ultraloaded with PEI.

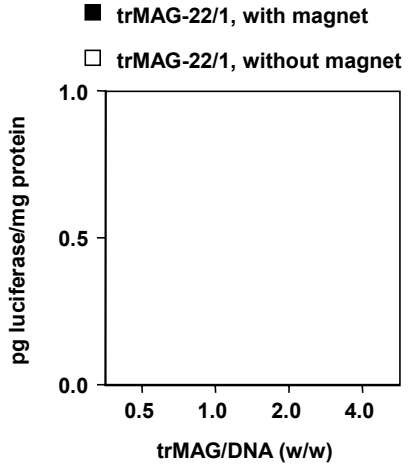


Figure 18 Transfection with trMAG-22/1. The particles are coated with a commercially available polyamine from Merck, Darmstadt.

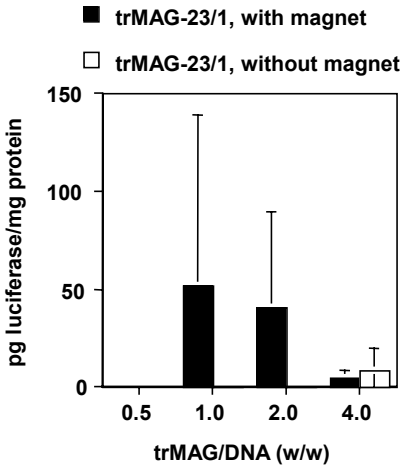


Figure 19 Transfection with trMAG-23/1. The difference between trMAG-23/1 and trMAG-16/1 (fig. 10) is only the coating procedure.

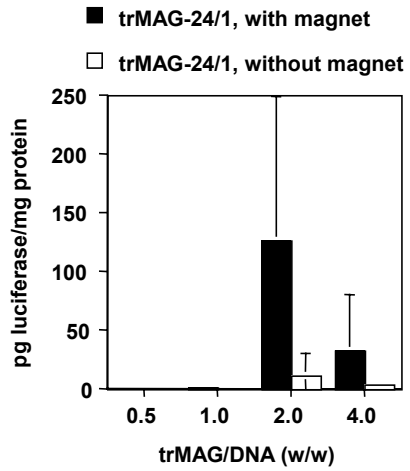


Figure 20 Transfection with trMAG-24/1. The difference between trMAG-24/1 and trMAG-16/1 (fig. 10) is only the coating procedure.

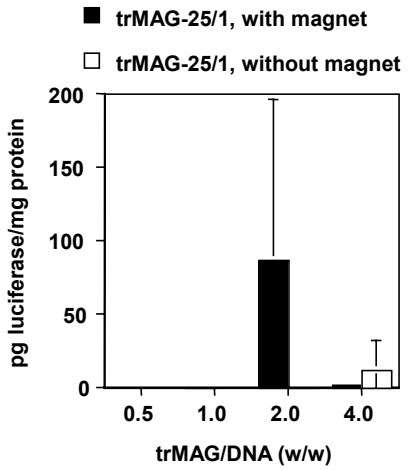


Figure 21 Transfection with trMAG-25/1 which is an analogue to trMAG-16/1 (fig. 10) but is coated with 50 % less PEI.

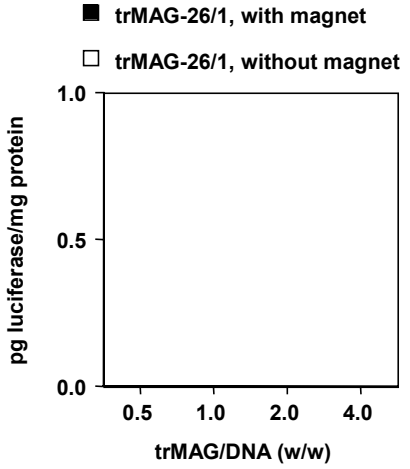


Figure 22 Transfection with trMAG-26/1. The particles are coated with poly(bis(2-chlorethyl)ether-alt-1,3 bis(3-dimethylamino)propyl)urea, quaternized.

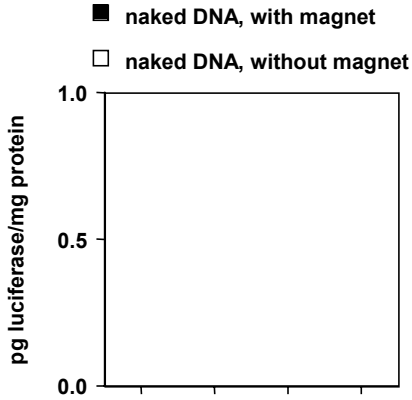


Figure 23 Transfection with naked DNA (1 µg/well) without trMAG particles (control).

The **highest efficiency** in gene transfer (approx. 370 pg luciferase/mg protein) was observed at a **trMAG/DNA (w/w) ratio of 2 with trMAG-18/1** particles (fig.14) which are coated with a multilayer of PEI 2000 kDa (Aldrich) and as is the case with all multilayer coated particles their suspension contains unbound PEI. The trMAG-19/1 particles (fig.15) differ from trMAG-18/1 only in the coating procedure and showed with more than 130 pg luciferase/mg protein the second highest value. Transfection efficiencies roughly around 100 pg luciferase/mg protein were reached by using trMAG-16/1 (fig. 12), trMAG-24/1 (fig. 20) and trMAG-25/1 (fig. 21) beads, all with a multilayer of PEI 800 kDa (Fluka) and unbound PEI in suspension. These relatively high transfection efficiencies of trMAGs coated with multilayers of PEI and unbound PEI in suspension at trMAG/DNA ratios of 2 or higher may seem surprising at first glance. From binding studies (figure 5) we know that unbound PEI in

suspension inhibits electrostatic binding of DNA to the trMAGs at higher trMAG/DNA (w/w) ratios. Yet trMAG-16/1 particles showed their peak transfection at a trMAG/DNA (w/w) ratio of 2 although the binding studies revealed no association of DNA with magnetic particles when complexes were prepared in water. In contrast, with a trMAG/DNA (w/w) ratio of 0.5 where DNA is bound electrostatically to trMAG-16/1 beads no gene transfer was detected. A simple explanation for this apparent contradiction is that association of DNA, PEI and magnetic particles is initiated as soon as the complexes are added to the serum-containing cell culture medium. At least a fraction of the DNA dose would then be associated with magnetic particles by the time these are sedimented on the cells. The relatively sharp optimum in dependence of magnetic particle to DNA ratio can be explained by the fact that a minimum amount of PEI (free or in complexes) per endosome may be required to exert the proton sponge effect. This would explain the low transfection at weight ratios below 2. On the other hand, ratios above 2 may lead to a situation where DNA is entirely bound to free PEI. These pre-formed complexes would be opsonized with serum in the culture medium and would in this manner be inhibited from interacting with magnetic particles. In addition, the binding studies in 150 mM NaCl demonstrate that there is no binding between magnetic particles and DNA at weight ratios of 4 or higher and thus also in serum-containing medium magnetic particle/DNA association may be strongly reduced at this ratios. Further, higher amounts of trMAGs may be cell-toxic and therefore reduce the transfection efficiency.

Under conditions where an association of DNA with magnetic particles can be assumed, strong enhancements by applying a magnetic field were observed.

The trMAG-PEI (fig. 8), trMAG-13/1 (fig. 9) and trMAG-14/1 (fig. 10) beads (all with a monolayer of PEI) showed only increased transfection values (trMAG-13/1 even more than 100 pg luciferase/mg protein) at a trMAG/DNA (w/w) ratio of 16. At this ratio the trMAGs settled already during complex preparation in water, probably because such a high amount of trMAGs could not be suspended in the given volume. Application of a magnetic field during transfection did not play a major role anymore because the visible large heavy aggregates which were formed in cell culture medium sedimented very quickly anyway.

Transfection with trMAG-20/1 (fig. 16), coated with a multilayer of PEI 800 kDa (Fluka) and unbound PEI in suspension, resulted in nearly no gene transfer. The difference to trMAG-16/1 particles which showed relatively high transfection efficiencies was only the coating procedure. This result indicates the importance of the type of coating procedure on gene transfer efficiency. Unfortunately, the coating procedure was not disclosed by the manufacturer.

The trMAG-17/1 particles (fig. 13), coated with linear PEI (Aldrich), showed no significant gene expression which means that linear PEI (a successful transfection reagent in transfections without magnetic beads) is not as suitable as branched PEI for coating iron oxide crystals.

The high standard deviations observed in transfections with the iron oxide beads are a phenomenon that occurs generally in experiments with relatively low transfection efficiencies (compared to standard polyfections or lipofections).

In transfections with trMAG-22/1 (fig. 18), coated with a commercially available polyamine, and trMAG-26/1 (fig. 22), coated with poly(bis(2-chlorethyl)ether-alt-1,3 bis(3-dimethylamino)propyl)urea quaternized, absolutely no gene expression was detectable.

Transfection without any iron oxide beads only with naked DNA (fig. 23) resulted in no gene transfer, no matter if a magnetic field was applied or not. This was a control to show that the presence of trMAGs enhances gene transfer.

Preparation in water and subsequent adjustment of the ionic strength to 150 mM NaCl

The particles were mixed with DNA and the ionic strength was adjusted to 150 mM NaCl. The cells (kept in serum-containing medium) were incubated with complexes for 20 minutes and during this time either a magnet was applied or not (control). After incubation, the cells were washed and fresh medium was added.

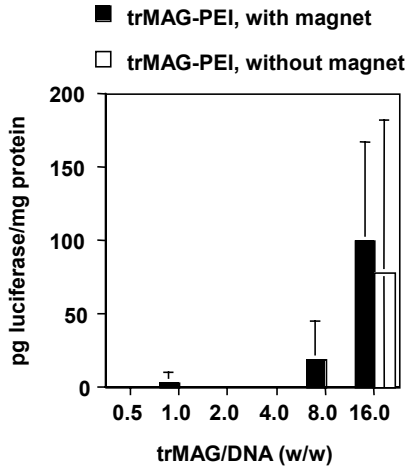


Figure 24 Transfection with trMAG-PEI. The particles are coated with a monolayer of PEI 800 kDa (Fluka).

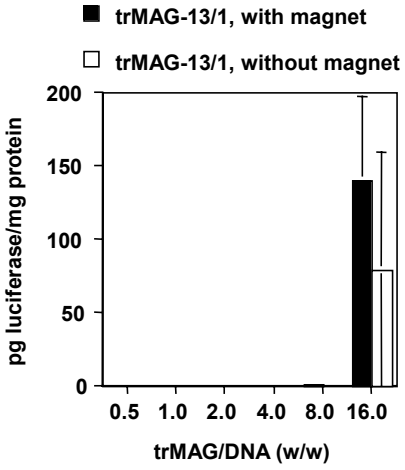


Figure 25 Transfection with trMAG-13/1. The particles are coated with a monolayer of PEI 2 kDa (Sigma-Aldrich).

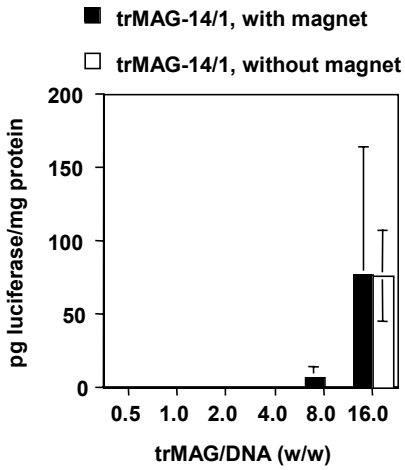


Figure 26 Transfection with trMAG-14/1. The particles are coated with a monolayer of PEI 60 kDa (Sigma-Aldrich).

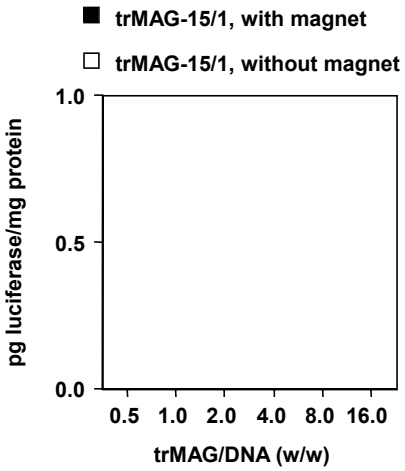


Figure 27 Transfection with trMAG-15/1. The particles are coated with a monolayer of PEI 750 kDa (Fluka).

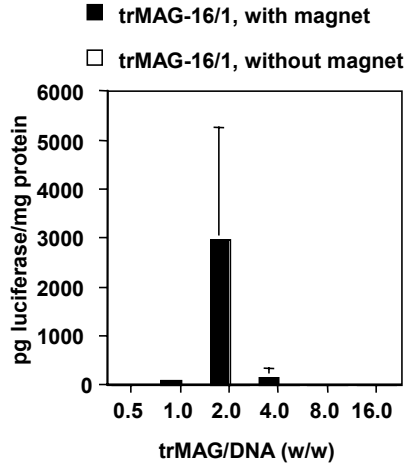


Figure 28 Transfection with trMAG-16/1. The particles are coated with a multilayer of PEI 800 kDa (Fluka).

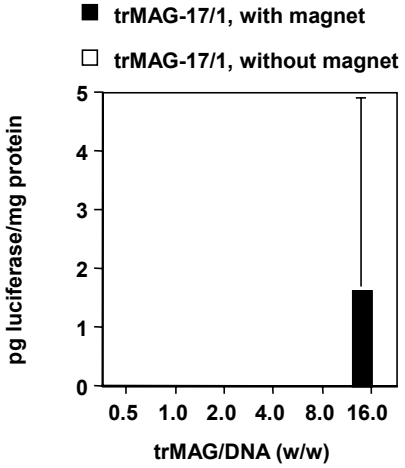


Figure 29 Transfection with trMAG-17/1. The particles are coated with linear PEI (Aldrich).

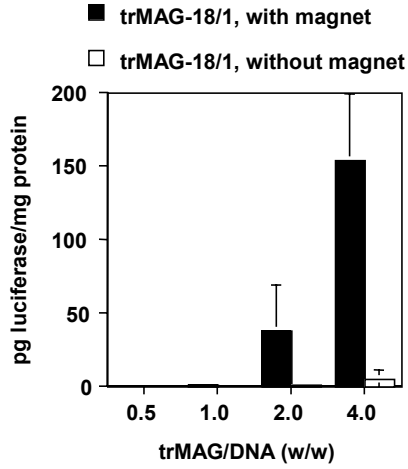


Figure 30 Transfection with trMAG-18/1. The particles are coated with a multilayer of PEI 2000 kDa (Aldrich).

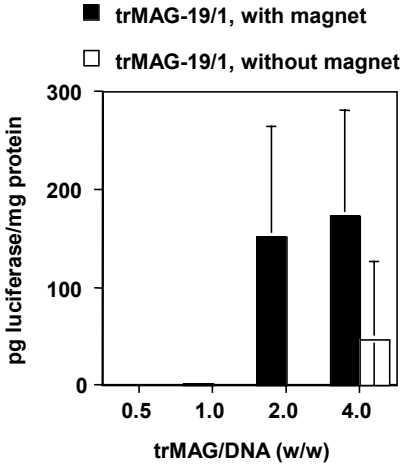


Figure 31 Transfection with trMAG-19/1. The difference between trMAG-19/1 and trMAG-18/1 (fig. 28) is only the coating procedure.

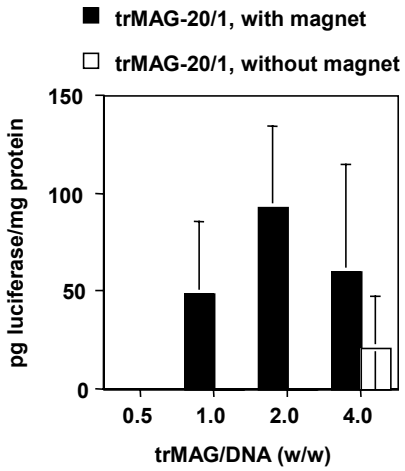


Figure 32 Transfection with trMAG-20/1. The difference between trMAG-20/1 and trMAG-16/1 (fig. 26) is only the coating procedure.

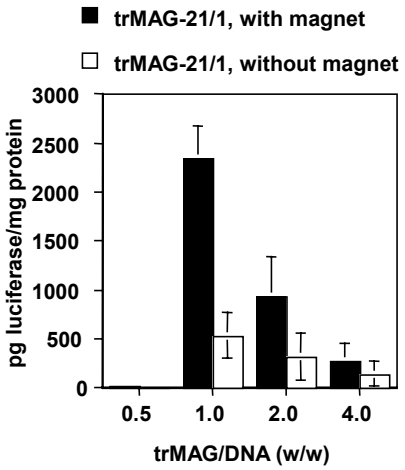


Figure 33 Transfection with trMAG-21/1. The difference between trMAG-21/1 and trMAG-16/1 (fig. 26) is that trMAG-21/1 is ultraloaded with PEI.

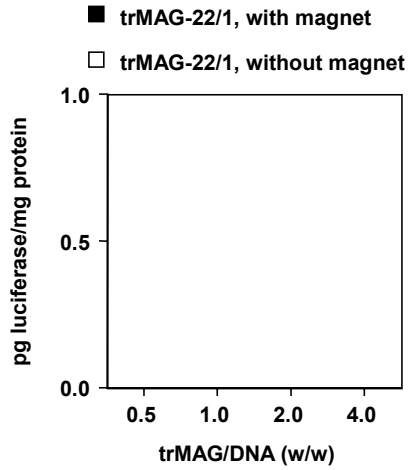


Figure 34 Transfection with trMAG-22/1. The particles are coated with a commercially available polyamine from Merck, Darmstadt.

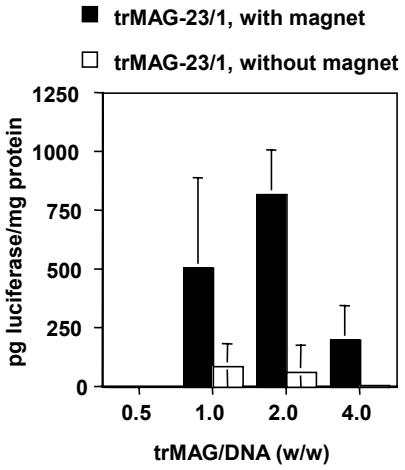


Figure 35 Transfection with trMAG-23/1. The difference between trMAG-23/1 and trMAG-16/1 (fig. 26) is only the coating procedure.

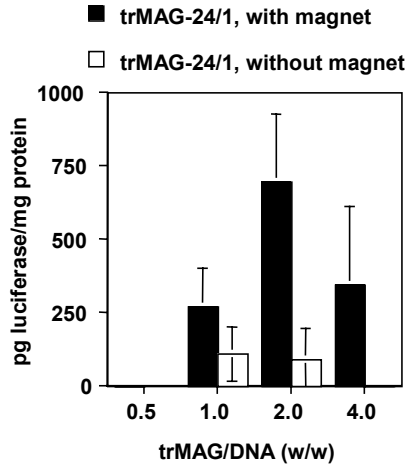


Figure 36 Transfection with trMAG-24/1. The difference between trMAG-24/1 and trMAG-16/1 (fig. 26) is only the coating procedure.

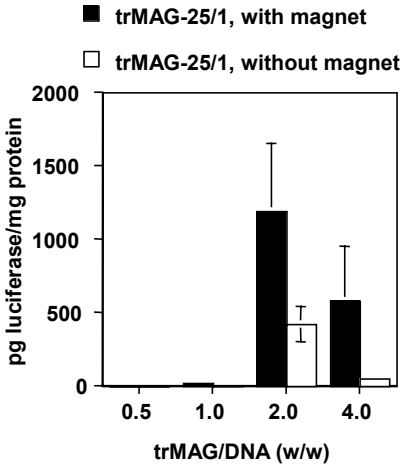


Figure 37 Transfection with trMAG-25/1 which is an analogue to trMAG-16/1 (fig. 26) but is coated with 50 % less PEI.

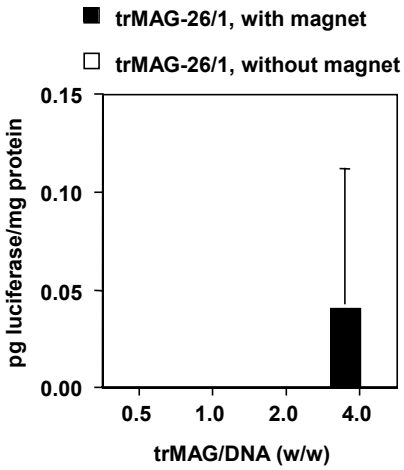


Figure 38 Transfection with trMAG-26/1. The particles are coated with poly(bis(2-chlorethyl)ether-alt-1,3 bis(3-dimethylamino)propyl)urea, quaternized.

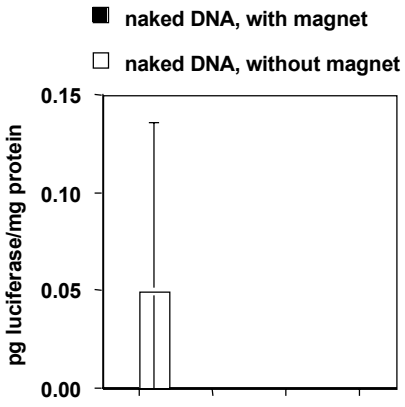


Figure 39 Transfection with naked DNA (1 µg/well) without trMAG particles (control).

When the complexes were aggregated in 150 mM NaCl the **highest efficiency** in gene transfer (approx. 3000 pg luciferase/mg protein) was observed at a **trMAG/DNA (w/w) ratio of 2 with trMAG-16/1** particles (fig.28) which are coated with a multilayer of PEI 800 kDa (Fluka) and unbound PEI in suspension (for explanation of the sharp transfection optimum at trMAG/DNA w/w = 2, see the same arguments as used previously for the preparation in 5% glucose). Similar to the results with complexes in 5% glucose, particles covered with a multilayer of high molecular weight PEI (here 800 kDa) and unbound PEI in suspension (trMAG-16/1 [fig. 28], trMAG-21/1 [fig. 33], trMAG-23/1 [fig. 35], trMAG-24/1 [fig. 36] and trMAG-25/1 [fig. 37]) at trMAG/DNA (w/w) ratios of 1 or 2 were the most successful in gene transfer.

When the **preparations were allowed to aggregate in salt solution their efficiency in transfecting cells was strongly increased.** An explanation could be that the addition of salt directly after mixing the components gives the trMAGs and PEI-DNA complexes more time to aggregate compared to preparations in 5% glucose. The latter can only aggregate during the short period of time in salt and serum-containing cell culture medium before the magnet is placed underneath the well and when trMAGs get in contact with PEI-DNA during magnetic sedimentation. In the larger aggregates more DNA is bound to magnetic beads, larger aggregates are sedimented by magnetic force more efficiently and in previous work Ogris et al. showed that larger vectors are more efficient in gene transfer (Ogris et al., 1998).

trMAG-18/1 (fig. 30) and trMAG-19/1 (fig. 31), with their 2000 kDa PEI multilayer the most successful particles in the 5 % glucose series, showed after aggregation in NaCl solution with peak values of little more than 150 pg luciferase/mg protein much poorer transfection

efficiencies than 800 kDa PEI multilayer beads like trMAG-16/1. This finding indicates that one has to consider the medium for complex preparation before choosing the type of particle.

From the results with preparations **in glucose and** with **salt-induced aggregates** it can be concluded that the **most efficient particles in gene transfer were iron oxides coated with a multilayer of high molecular weight branched PEI plus free PEI** in suspension at trMAG/DNA (w/w) ratios of 1 or 2 (eventually 4, but higher amounts of trMAGs may already be cell-toxic and therefore reduce the transfection efficiency).

Especially when the most successful particles (e.g. trMAG-18/1 and trMAG-19/1 in 5% glucose or trMAG-16/1 prepared in NaCl) were used gene transfer was increased strongly by applying a magnetic field. This indicates that **magnetic sedimentation of paramagnetic vectors is possible** and that the **association of DNA with magnetic particles is compatible with gene delivery**.

The trMAG-PEI (fig. 24), trMAG-13/1 (fig. 25) and trMAG-14/1 (fig. 26) beads, coated with a monolayer of 800, 2 and 60 kDa PEI, respectively, showed only transfection values around 75 pg luciferase/mg protein at a trMAG/DNA (w/w) ratio of 16. As already observed in the 5 % glucose experiments, there were visible precipitates at this high ratio, probably because such a high amount of trMAGs could not be suspended in the given volume. The aggregation may even be enhanced by salt. Application of a magnetic field during transfection did not play a major role anymore because the visible heavy aggregates sedimented very quickly anyway and enhanced transfection.

All experiments revealed that for each type of particle a optimum w/w ratio of beads/DNA can be determined. But the optimum ratio can differ for the same particles in 5 % glucose or in 150 mM NaCl, as seen e.g. with trMAG-18/1 beads in figure 14 and figure 30.

The trMAG-17/1 particles (fig. 29), coated with linear PEI (Aldrich), showed only poor gene expression which means that linear PEI (a successful transfection reagent in transfections without magnetic beads) is not as suitable as branched PEI for coating iron oxide crystals.

In transfections with trMAG-15/1 (fig. 27), coated with a monolayer of 750 kDa PEI, and trMAG-22/1 (fig. 34), coated with a commercially available polyamine, absolutely no gene expression was detectable when the complexes were formed in salt solution.

In all gene transfer experiments with the superparamagnetic particles, the NIH3T3 mouse fibroblasts appeared to be healthy and were proliferating as usual.

In the control experiment without magnetic particles (naked DNA dissolved in 150 mM NaCl, fig. 39), a very low value of 0.05 pg luciferase/mg protein could be detected when no magnetic field was applied.

In summary, particles were found that lead to transfection only with naked DNA and their transfection efficiency was even enhanced by application of a magnetic field. This means that the proof of principle for magnetofection is shown, but compared to standard transfections with polyplexes or lipoplexes, the gene transfer efficiency obtained by magnetofection was low. As iron oxide beads coated with multilayers of high molecular weight branched PEI and unbound PEI in suspension were the most successful particles, it can be speculated that addition of free PEI (or other endosomolytic standard transfection reagents) to the beads may improve their transfection efficiency. Addition of salt to vector preparations increases the gene transfer efficiency as well.

3.3.1.2 trMAG / DNA complexes and additional PEI

The eight different types of trMAGs presented below are iron oxides coated with cationic polymers and **incubation** of CHO-K1 cells **with these beads associated with naked plasmid DNA in 5 % glucose led to no gene transfer.** As seen in the experiments before, iron oxide beads with unbound high molecular weight PEI in suspension were the most successful in transfections and therefore PEI 25 kDa (N/P = 8) was added to the trMAG / DNA complexes in the hope to enable gene transfer. The ionic strength of all vector solutions was adjusted to 150 mM NaCl to allow salt-induced aggregation which had turned out essential in the previous experiments.

The efficiency of these aggregates with increasing bead / DNA (w/w) ratio in luciferase gene transfer into CHO-K1 cells (using 0.5 µg DNA / well) with and without application of a magnetic field was determined. The incubation time of the cells (kept in serum-containing medium) with aggregates in the presence or absence of a magnetic field was 15 min. Then the cells were washed and fresh medium applied.

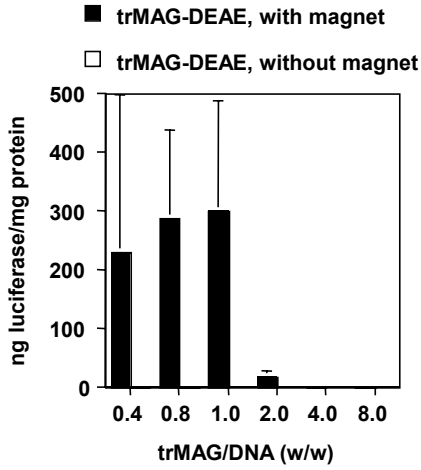


Figure 40 Transfection with trMAG-DEAE. The particles are coated with a monolayer of dextran. Endstanding DEAE groups were introduced with 2-diethylamino-ethyl chloride-hydrochloride.

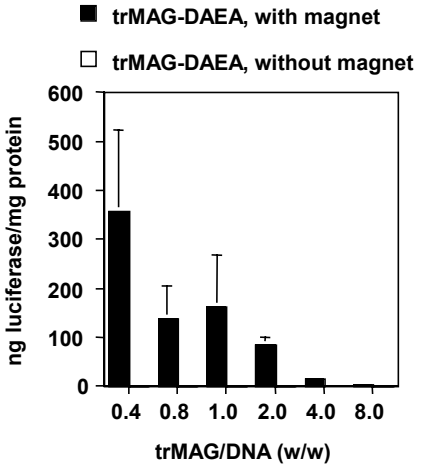


Figure 41 Transfection with trMAG-DAEA. The particles are coated with a polymer prepared from dimethylamine, epichlorohydrine and ethylene diamine.

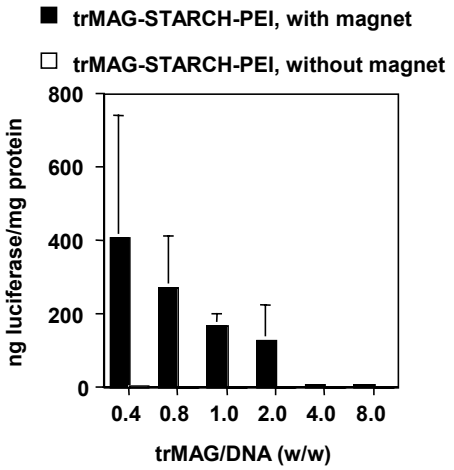


Figure 42 Transfection with trMAG-STARCH-PEI. The particles were coated with a multilayer of starch, followed by covalent coupling of PEI via amino groups to the periodate-oxidized starch layer.

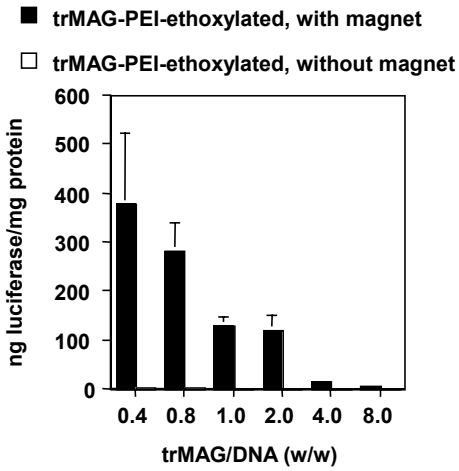


Figure 43 Transfection with trMAG-PEI-ethoxylated. Monolayer coating of the particles with PEI 50 kDa (Aldrich) which has been ethoxylated (80 %).

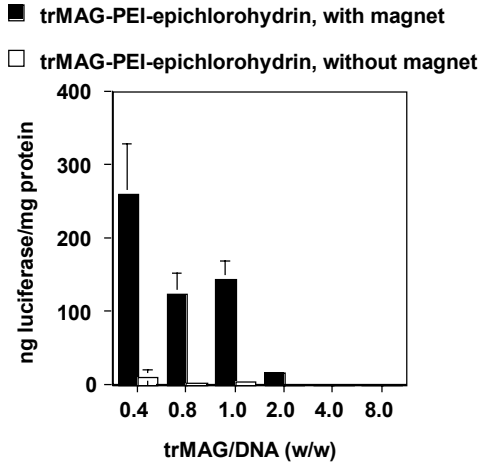


Figure 44 Transfection with trMAG-PEI-epichlorohydrin. Monolayer coating of particles with PEI 20 kDa (Aldrich) modified with epichlorohydrin.

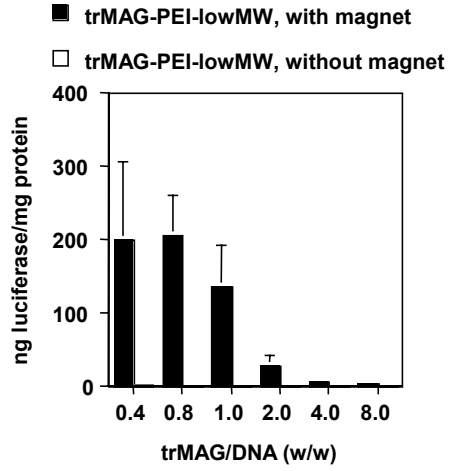


Figure 45 Transfection with trMAG-PEI-lowMW. Monolayer coating of the particles with PEI 1.7 kDa (Aldrich).

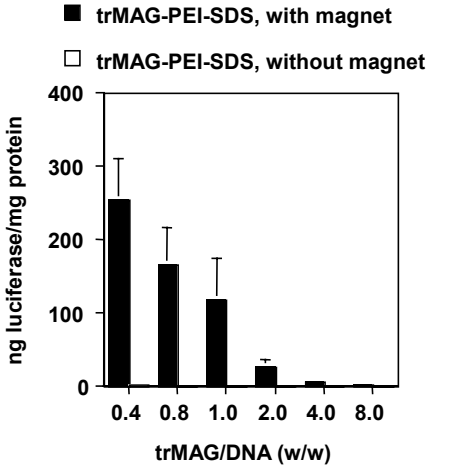


Figure 46 Transfection with trMAG-PEI-SDS. Monolayer coating with PEI 800 kDa modified by a covalent coupling of sodium dodecyl sulfate (SDS).

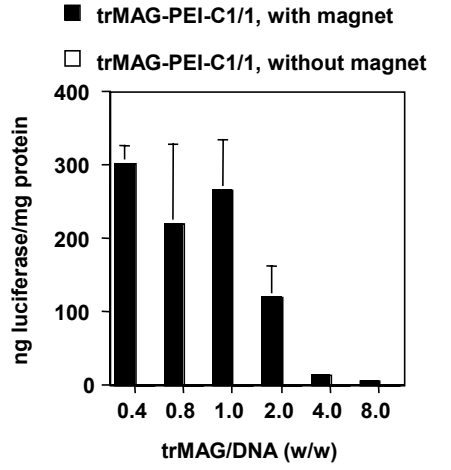


Figure 47 Transfection with trMAG-PEI-C1/1. Particles coated with a commercially available PEI.

No transfection was detected when CHO-K1 cells were incubated with these beads associated with naked plasmid DNA in 5 % glucose. But **with additional PEI** 25 kDa (N/P = 8) **and adjusting ionic strength 150 mM NaCl,** all particles showed **efficient gene transfer** into CHO-K1 cells.

The **highest transfection** (on the average 400 ng luciferase/mg protein) was monitored with **trMAG-STARCH-PEI** (fig. 42) at a beads/DNA (w/w) ratio of 0.4, although this was not significantly higher than with the other particles.

Transfection with all other particles at their optimum w/w ratio including smaller trMAG-PEI-C1/1 (fig. 47) resulted in values between approx. 200-380 ng luciferase/mg protein.

It has to be emphasized that here the gene expression values are given in ng luciferase/mg protein and not as in 3.3.1.1 in pg luciferase/mg protein. This means that with trMAG / DNA complexes plus PEI 25 kDa (N/P = 8) plus salt-induced aggregation 100 to 1000-fold higher efficiencies were achieved than with the best transfections of section 3.3.1.1 with only naked DNA plus salt-induced aggregation. This indicates the **strong enhancing effect of additional free PEI** (probably due to **endosomolytic** activities) in magnetofection and that with PEI, transfection efficiencies comparable to those usually obtained with standard nonviral transfection reagents are possible.

The optimum beads/DNA (w/w) ratio for all beads was relatively low ranging from 0.4-1.

Without application of a magnetic field, the gene expression remained below 10 ng luciferase/mg protein with all particles tested (most of these low values cannot be seen at the scales used in the graphs). The increase in transfection by applying a magnetic field ranged from 3 to 1292148-fold.

In all transfections with these superparamagnetic particles, the cells appeared to be healthy and were proliferating as usual.

3.3.2 Transfection efficiency with negatively charged trMAGs

3.3.2.1 trMAGs and PEI-DNA complexes

In the previously shown binding studies (figure 7), there was no electrostatic binding of negatively charged trMAG-pAsp particles to PEI-DNA complexes in water. But it might be possible that the PEI-DNA complexes and trMAGs with electrostatically bound surplus PEI (resulting in net positively charged particles) could aggregate when they meet each other by Brownian motion during the short period of time in salt-containing cell culture medium before

the magnet is placed underneath the well and when trMAGs get in contact with PEI-DNA during magnetic sedimentation. This would be the same effect as already monitored with trMAGs coated with a multilayer of PEI and unbound PEI in suspension.

The four different trMAGs used are iron oxide beads coated with anionic polyelectrolytes. The negatively charged trMAGs were mixed with net positively charged PEI-DNA (N/P = 8) complexes in water at increasing trMAG / DNA (w/w) ratios and subsequently the solution was adjusted to 5% glucose. The efficiency of the complexes in luciferase gene transfer into NIH3T3 and HepG2 cells (using 0.5 µg DNA / well) with and without application of a magnetic field was determined.

Transfection of NIH3T3 cells with complexes in 5% glucose

NIH3T3 mouse fibroblasts (kept in serum-containing medium) were incubated with complexes for 10 minutes and during this time either a magnet was applied or not (control). After incubation the cells were washed and fresh medium was added.

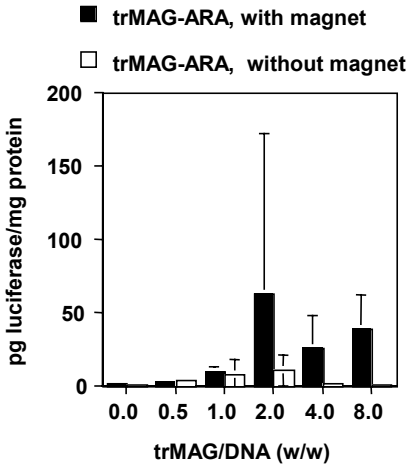


Figure 48 Transfection with trMAG-ARA. The particles are coated with arabinic acid, sodium salt, 250 kDa.

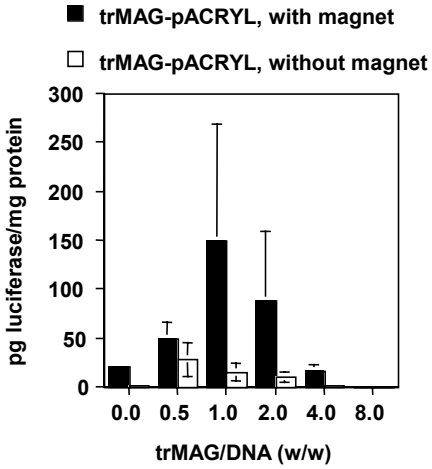


Figure 49 Transfection with trMAG-pACRYL. The particles are coated with polyacrylic acid, sodium salt, 20 kDa.

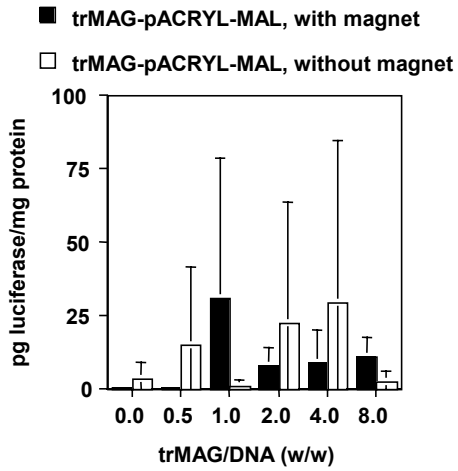


Figure 50 Transfection with trMAG-pACRYL-MAL. The particles are coated with polyacrylic acid-co-maleic acid, sodium salt, 50 kDa.

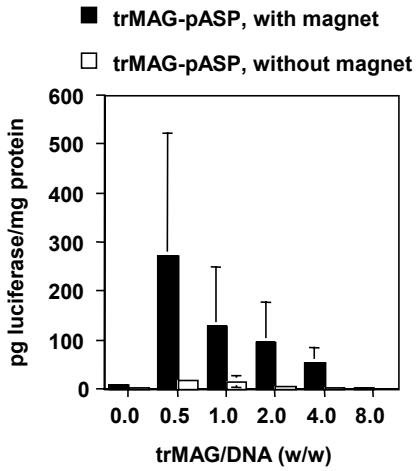


Figure 51 Transfection with trMAG-pASP. The particles are coated with polyaspartic acid, sodium salt, 3000 kDa.

All four types of particles tested showed **gene expression in NIH 3T3** cells although in figure 7 there is no electrostatic binding of PEI-DNA to trMAG-pAsp particles in water. An explanation may be that PEI-DNA complexes and negatively charged trMAGs with electrostatically bound surplus PEI (resulting in net positively charged particles) could aggregate in salt and serum-containing cell culture medium.

The **highest transfection** efficiency (approx. 270 pg luciferase/mg protein) was observed at a trMAG/DNA (w/w) ratio of 0.5 with **trMAG-pASP** particles (fig. 51).

In general, **gene transfer** into NIH3T3 cells **with negatively charged beads (plus PEI-DNA) was roughly as effective as with positively charged particles (plus naked DNA) in 5 % glucose** (see 3.3.1.1, preparation in water and subsequent adjustment to 5 % glucose).

The optimum trMAG/DNA (w/w) ratios for all the negatively charged particles ranged from 0.5-2.

Application of a magnetic field enhanced gene transfer with all particles except with trMAG-pACRYL-MAL (fig.50) indicating that with most negatively charged particles the principle of magnetic sedimentation works. The polyacrylic acid-co-maleic acid covered beads showed the poorest transfection efficiency (approx. 30 pg luciferase/mg protein) and the magnetic force did not play any role in transfecting cells.

In all transfections with the negatively charged particles, the NIH3T3 cells appeared to be healthy and were proliferating as usual.

Transfection of HepG2 cells with complexes prepared in 5% glucose

HepG2 cells (kept in serum-containing medium) were incubated with complexes for 10 minutes and during this time either a magnet was applied or not (control). After incubation the cells were washed and fresh medium was added.

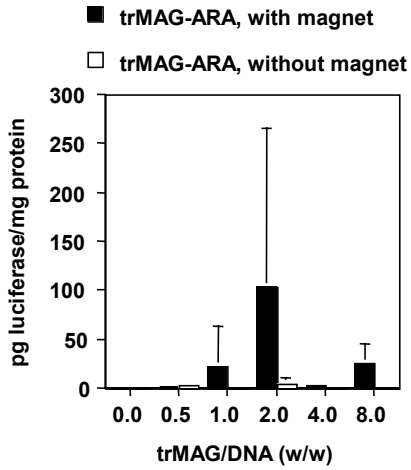


Figure 52 Transfection with trMAG-ARA. The particles are coated with arabinic acid, sodium salt, 250 kDa.

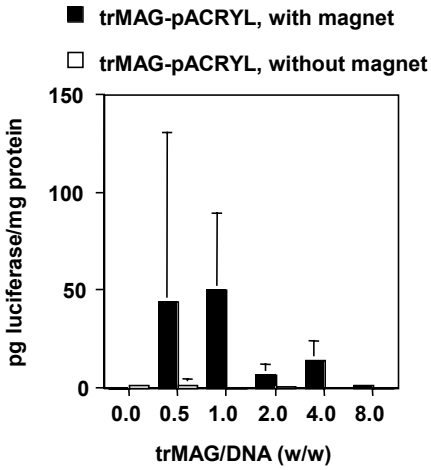


Figure 53 Transfection with trMAG-pACRYL. The particles are coated with polyacrylic acid, sodium salt, 20 kDa.

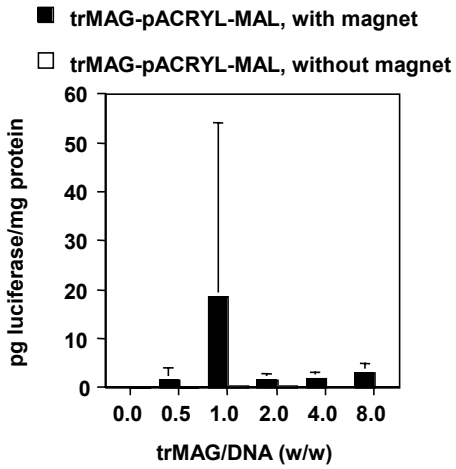


Figure 54 Transfection with trMAG-pACRYL-MAL. The particles are coated with polyacrylic acid-co-maleic acid, sodium salt, 50 kDa.

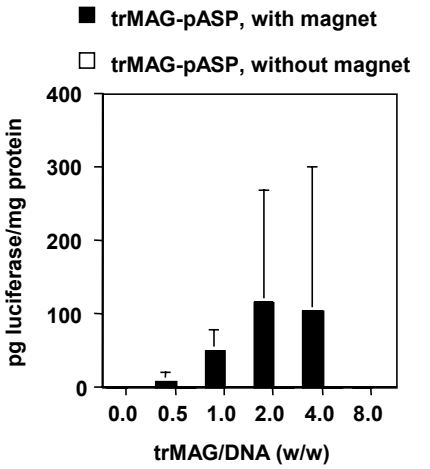


Figure 55 Transfection with trMAG-pASP. The particles are coated with polyaspartic acid, sodium salt, 3000 kDa.

All four types of particles tested showed **gene expression in HepG2** cells. The explanation is again aggregation in the salt and serum-containing cell culture medium.

The **highest efficiency** in transfecting HepG2 cells (approx. 110 pg luciferase/mg protein) was obtained at a trMAG/DNA (w/w) ratio of 2 with **trMAG-pASP** particles (fig. 55) which

were already the most efficient negatively charged beads for gene transfer into NIH3T3 cells (fig. 51).

The lowest transfection efficiency in HepG2 cells (peak transfection: 19 pg luciferase/mg protein) was achieved with trMAG-pACRYL-MAL particles (fig. 54) similarly as in NIH3T3 (fig. 50). But in HepG2 cells, the application of a magnetic field strongly enhanced gene transfer with trMAG-pASP-MAL, whereas in NIH3T3 cells the magnet did not play an important role (fig. 50).

Application of a magnetic field increased the gene transfer into HepG2 cells with all negatively charged particles indicating one more time that magnetic sedimentation is possible.

Gene transfer in HepG2 was usually slightly less efficient than in NIH3T3 cells except with trMAG-ARA beads which showed an average peak value of 100 pg luciferase/mg protein (fig.52) in HepG2 but only 60 pg luciferase/mg protein in NIH3T3 cells (fig.48).

The optimum trMAG/DNA (w/w) ratios for transfecting HepG2 and NIH3T3 cells were identical, only trMAG-pASP particles had a optimum ratio of 2 and 0.5 for HepG2 (fig. 55) and NIH3T3 (fig. 51), respectively.

In all transfections with the negatively charged particles, the HepG2 cells appeared to be healthy and were proliferating as usual.

In summary, **negatively charged trMAGs were found (especially trMAG-pAsp) which showed similar gene expression values as positively charged particles** when both are in 5% glucose. Additionaly, their transfection efficiencies were usually enhanced by application of a magnetic field which means that the **principle of magnetic sedimentation is applicable to negatively charged beads** as well**.**

Further it is assumed that an adjustment of the ionic strength to 150 mM NaCl directly after the mixing of components would significantly improve the transfection efficiencies of negatively charged trMAGs (as already shown for positively charged trMAGs).

3.3.3 Hints to the mechanism of magnetofection

The experiments with positively and negatively charged trMAGs showed that gene transfer with magnetic particles is possible and that application of a magnetic field improved the transfection efficiencies. Further, the addition of free PEI, an endosomolytic polymer commonly used as transfection reagent, enhanced gene expression. All these findings show that magnetofection works but now the question arises what the mechanism of magnetofection is. Are the paramagnetic vectors pulled into the cell (or even into the nucleus) by magnetic force? Does endocytosis play any role? Is it possible that the permanent magnet alone (without magnetic beads) has an enhancing effect on the reporter gene expression?

3.3.3.1 Influence of endosomolytic substances in magnetofection

The transfections with positively charged trMAGs showed that free PEI, which harbours endosomolytic activities, improved the gene transfer efficiencies. This could mean that release of gene vectors from endosomes is an important step in magnetofection. The uptake mechanism which captures vectors in endosomes is called endocytosis. Therefore in the following experiments it was examined if magnetofectins containing substances which enhance endosomal relase are generally more efficient in gene transfer than magnetofectins without endosomolytic additives. The influence of endosomolytic substances on magnetofection gives a hint if endocytosis plays a role in magnetofection.

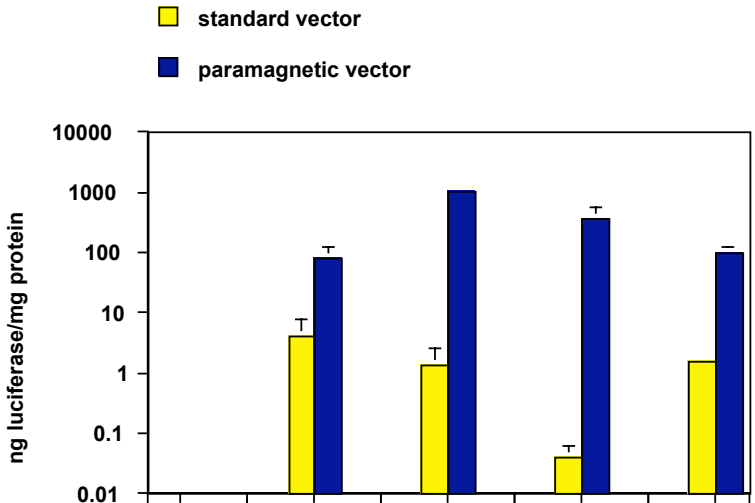
PEI, PEI-bAdv, Lipofectamine and GenePORTER as additives

In the following experiments, trMAG-PEI were chosen as magnetic particles because they are only coated with a monolayer of PEI (800 kDa) and their suspension does not harbour unbound free PEI. As endosomolytic additives PEI, PEI plus biotinylated chemically inactivated adenoviruses (PEI-bAdv), Lipofectamine (LF) and GenePORTER (GP) were taken. PEI (25 kDa, from Sigma-Aldrich, Deisenhofen, Germany) acts as a “proton sponge”, that means protonation of PEI within endosomes and endosomal Cl^- entry triggers osmotic swelling and destabilization of the endosomal vesicle (Boussif et al., 1995; Sonawane et al., 2003). Biotinylated chemically inactivated adenoviruses (b-Adv, kindly provided by Ernst Wagner, Vienna University Biocenter, Austria) expose in acidified endosomes hydrophobic domains of the adenoviral capsid proteins and these domains interact with the endosomal

membrane in a fashion that leads to vesicle rupture (Curiel et al., 1991). Lipofectamine (transfection reagent from Life Technologies, Karlsruhe, Germany) is a cationic lipid and cationic lipid-DNA complexes destabilize endosomal membranes facilitating the release of DNA into the cytoplasm (Xu and Szoka, 1996). GenePORTER (transfection reagnet from Gene Therapy Systems, La Jolla, CA, USA) is a formulation of a cationic lipid and the neutral "helper" lipid DOPE (dioleoylphosphatidylethanolamine) which supports cationic lipids in the formation of an inverted hexagonal phase and thus facilitates endosomal membrane destabilization (Hafez et al., 2001).

PEI, PEI plus b-Adv (PEI-bAdv), Lipofectamine (LF) and GenePORTER (GP) were added to trMAG-PEI / DNA (w/w ratio = 2) complexes and their transfection efficiency was compared to trMAG-PEI / DNA particles without additives. All single components were dissolved in 150 mM NaCl and the complexes were prepared in **150 mM NaCl** to allow salt-induced aggregation. During incubation with trMAG containing vectors, a permanent magnet was applied. Further, the gene transfer efficiency of the corresponding standard vectors (DNA-PEI, DNA / PEI / bAdv, DNA-LF and DNA-GP) without magnetic beads and without magnetic field was determined.

NIH 3T3 or CHO-K1 cells were incubated with vectors in the presence or absence of a magnetic field for 10 or 20 min, followed by a medium change and the gene expression (in ng luciferase/mg protein) was determined after 24 hours.



Additives	No	PEI	PEI-bAdv	Lipofect -amine	Gene PORTER
Cell line	NIH 3T3 CHO-K1	NIH 3T3	NIH 3T3	CHO-K1	CHO-K1
DNA dose (µg/well)	0.5	0.5	0.5	0.1	0.1
Incubation time (min)	10	10	20	10	10

Figure 56 Facilitating magnetic field-guided gene transfer by addition of endosomolytic substances to trMAG-PEI / DNA (w/w ratio = 2) complexes.
The complexes were prepared in **150 mM NaCl** to allow salt-induced aggregation.
Transfection with trMAG-PEI / DNA (w/w ratio = 2) complexes in the presence of a magnetic field resulted in no gene expression in NIH 3T3 or CHO-K1 cells (see column "Additives No"). But **addition of membrane destabilizing substances** (see columns PEI, PEI-bAdv, Lipofectamine and GenePORTER) **enabled magnetic field-guided gene transfer into NIH 3T3 or CHO-K1 cells (blue bars, paramagnetic vectors).**
Standard vectors with the same endosomolytic additives but without magnetic beads and without magnetic field (yellow bars) showed reporter gene expression as well but significantly lower than the corresponding paramagnetic vectors.

The magnetofectin additive PEI enabled gene transfer into NIH 3T3 and Lipofectamine or GenePORTER into CHO-K1 cells with a short incubation time of 10 min with complexes and magnet. PEI plus an inactivated biotinylated adenovirus (bAdv) facilitated magnetofection of NIH 3T3 cells as well but the complexes were left for 20 min on the cells. One might argue that an incubation time of 20 min is not comparable with a incubation time of 10 min which was used for trMAG-PEI / DNA without additives. But nevertheless it can be concluded that only the endosomolytic substances made gene transfer possible, as in figure 24 trMAG-PEI / DNA complexes with a trMAG/DNA w/w ratio of 2 and a 20 min incubation with complexes and magnet showed no gene expression.

The **importance of membrane-destabilizing additives for successful magnetofection** indicates that magnetofectins are captured in endosomes and that paramagnetic vectors are taken up by the cell via **endocytosis**.

Another result of these experiments was that vectors including magnetic particles (paramagnetic vectors or magnetofectins) and an applied magnetic field increased gene

expression values up to 9400-fold (Lipofectamine) compared to standard vectors without beads and without magnet.
A further result was that not only the adjustment of the ionic strength to 150 mM NaCl after mixing of the components results in salt-induced aggregation which associates e.g. trMAG-PEI and PEI-DNA particles, but that mixing of the components from the beginning in 150 mM NaCl forms similar aggregates as they show enhanced transfection efficiencies compared to trMAG-PEI / DNA alone or to corresponding standard vectors.

A synthetic influenza virus peptide (INF7) as additive

In the following experiments, trMAG-16/1 were chosen as magnetic particles. They are coated with a multilayer of PEI 800 kDa and unbound PEI is in suspension. As already shown in fig. 12, trMAG-16/1 alone (prepared in 5% glucose) showed gene transfer. Now it should be examined if addition of a further endosomolytic substance could increase the transfection efficiency. As membrane-destabilizing additive the influenza virus hemagglutinin HA-2 N-terminal fusogenic peptide **INF7** was used. Protonation of the acidic residues of INF7 promotes its transition to a α-helical structure concomittant with endosomal membrane binding and destabilization (Wagner et al., 1992a).
INF7 was added to complexes containing trMAG-16/1 and DNA and their efficiency in gene transfer was compared to trMAG-16/1 / DNA particles without INF7. For the vector formulations in water**,** 1 µg DNA/well, an increasing trMAG/DNA (w/w) ratio, a constant DNA/INF7 charge/charge ratio (-/- = 1) and two different sequences of mixing (DNA / INF7 / trMAG-16/1 and trMAG-16/1 / DNA / INF7) were used. After preparation of the complexes, glucose was added to obtain a final concentration of **5% glucose**.
NIH 3T3 cells (kept in serum-containing medium) were incubated with these vectors for 10 min in the presence of a magnetic field, followed by a medium change and determination of gene expression (in pg luciferase/mg protein) after 24 hours.

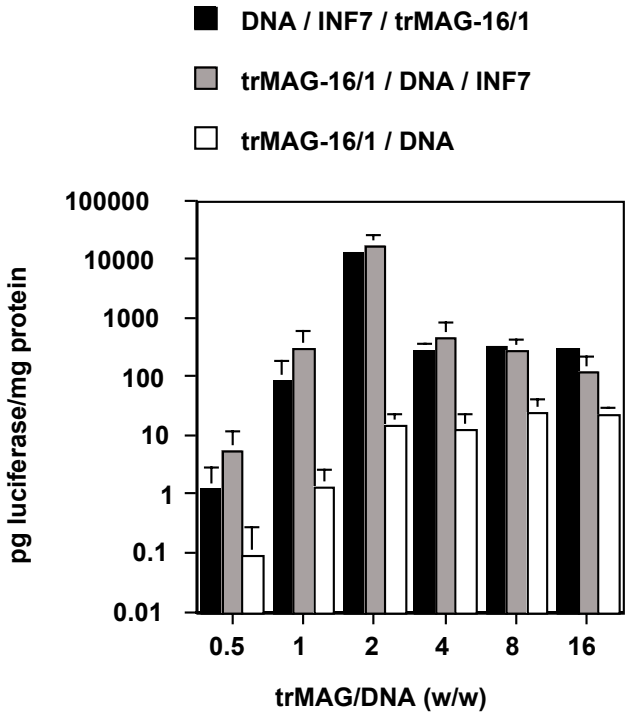


Figure 57 Enhancement of magnetofection efficiency by adding the endosomolytic peptide INF7 to vectors containing trMAG-16/1 and DNA with increasing trMAG/DNA (w/w) ratios.
The complex solutions were adjusted to **5% glucose**.
At all trMAG/DNA (w/w) ratios, **vectors harbouring INF7 were more efficient in gene transfer** than particles lacking this peptide. With a w/w ratio of 2, the enhancement by INF7 was up to 1129-fold and the maximum efficiency in magnetofection was obtained.
The sequence of mixing the components for INF7 containing vectors did not influence the transfection efficiencies very strongly (maximum difference between two mixing sequences was about 5-fold at a trMAG/DNA w/w ratio of 0.5) but up to a w/w ratio of 4 the mixing sequence trMAG-16/1 / DNA / INF7 was more succesful and at higher ratios the mixing sequence DNA / INF7 / trMAG-16/1.
3.3.3.1.1.1

Addition of the synthetic **endosome disruptive peptide INF7** (from Influenza virus) to vectors containing trMAG-16/1 and DNA **increased the magnetofection efficiency up to 1129-fold.**

In 5% glucose, electrostatic interactions are responsible for complex formations. It is assumed that DNA binds preferentially to free PEI and not to PEI from the trMAG layers, as free PEI is better accessible and more flexible. Therefore, in both mixing sequences, unbound PEI from the trMAG-16/1 suspension could form PEI-DNA associates (see trMAG-16/1 binding studies in figure 5). The negatively charged INF7 could act as glue between PEI-DNA (positively charged at trMAG/DNA w/w ratios higher than 0.8) and the positively charged trMAG-16/1 particles. The preferential formation of PEI-DNA complexes and the function of INF7 as glue would also explain why the mixing sequence does not play an important role for the transfection efficiency.

Without INF7, electrostatic binding of DNA to trMAG-16/1 is only possible at the trMAG/DNA (w/w) ratio of 0.5 (see trMAG-16/1 binding studies in figure 5). The mixing of trMAG-16/1 and DNA at trMAG/DNA (w/w) ratios of 1 or higher without INF7 generates positively charged PEI-DNA and separate trMAG-16/1 particles which are not able to bind to each other. But as trMAG-16/1 plus DNA mixed at trMAG/DNA (w/w) ratios higher than 0.8 resulted in gene transfer after magnetic sedimentation, it is assumed that trMAGs and PEI-DNA aggregate when they get in contact with the salt and serum-containing cell culture

medium. Probably salt-induced aggregation plays an important role for all INF7 containing vectors as well.
INF7 containing complexes with a trMAG/DNA (w/w) ratio of 2 (maximum transfection) formed visible large aggregates when pipetted into the wells harbouring salt and serum-containing medium. An explanation for this phenomenon could be that in salt plus serum, complexes turned to electroneutrality. Large and heavy aggregates often increase transfection efficiencies (Ogris et al., 1998) and the magnetic force acts stronger on paramagnetic associates with a larger volume (Zborowski et al., 1995). Nevertheless the NIH 3T3 cells appeared to be healthy and were proliferating as usual.

All the endosomolytic additives used enabled or enhanced gene transfer via magnetofection. This was shown for vectors in **150 mM NaCl** (with PEI, PEI-bAdv, LF and GP) and for vectors in **5% glucose** (with INF7). The results indicate that the magnetofectins are captured in endosomes which is typical for the cellular uptake mechanism of endocytosis. Endosomolytic substances facilitate the escape of gene vectors from endosomal vesicles which is necessary for reporter gene expression. The enhancement of gene expression in magnetofection by endosome disruptive peptides makes it more likely that **paramagnetic vectors are transferred into the cell by endocytosis** than that they are pulled by magnetic force through the cellular membrane.

3.3.3.2 The fate of magnetic particles during magnetofection

A further important question about the mechanism of magnetofection was if trMAGs would be taken up by the cell (like DNA) or if they would be left outside. If the magnetic beads would enter the cell, is the uptake mechanism endocytosis? Is there a magnetic field-guided rapid concentration of magnetofectins on the surface of the cell? Or is the magnet even able to pull trMAGs directly into the cells circumventing endocytosis?
To find answers to these questions, HeLa cells (kept in serum-containing medium) were incubated with trMAG-16/1 / DNA complexes (2.5 µg DNA/35 mm dish, trMAG/DNA w/w = 2, preparation in 5% glucose) and a rectangular Nd-Fe-B magnet of 20 x 10 x 5 mm (Neo Delta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) was placed underneath each dish. After exposure to vectors and magnet for 1 min, 5 min, 15 min and 15 min with further 24 h incubation in fresh complete medium without magnet, the cells were fixed and electron micrographs were taken by Jim Lausier, LMU Munich.

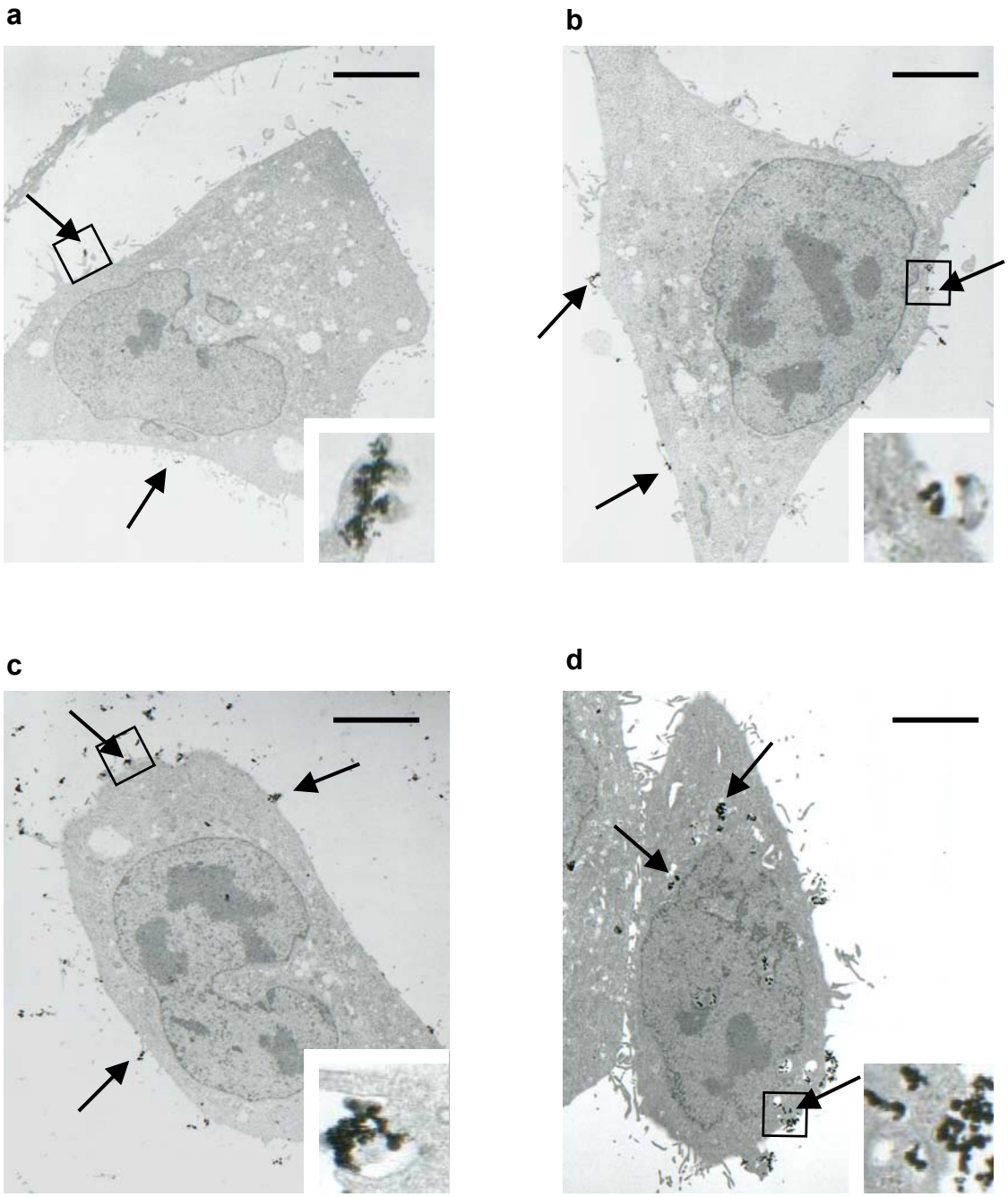


Figure 58 Electron microscopy of HeLa cells transfected with trMAG-16/1 / DNA vectors. Exposure to complexes and magnetic field for (a) 1 min, (b) 5 min, (c) 15 min and (d) 15 min with further 24 h incubation without magnet in fresh complete medium. Arrows indicate the electron dense trMAG-16/1 beads. The DNA is not electron dense and is not visible in these micrographs. The scale bars indicate 5 µm. The insets show a higher magnification of the labeled areas.
(a) After 1 min one can see a few particles around the cell. (b) After 5 min trMAGs start to accumulate on the cell surface. (c) Already after 15 min there is a strong accumulation of magnetic particles on the cell surface and beads are found within the cell. (d) After 15 min plus medium change and 24 h magnet-free incubation, a dramatic increase of beads within the cell, mainly in cytoplasmic endosomal structures and eventually some in the nucleus, was observed.
The magnifications of a-d (insets) show magnetic particles during their internalization process and d (inset) particles captured in endocytotic vesicles. Sometimes there are small finger shaped extensions or narrow lamellae in areas where trMAGs are attached to the cellular surface. These cellular protrusions could indicate a special uptake mechanism of HeLa cells or the protrusions could be artifacts from sectioning.

Accumulation of trMAGs (mixed with DNA in 5% glucose) **around the cell** increased significantly from 1 to 5 min and from 5 to 15 min. It might be that complexes prepared in 150 mM NaCl would accumulate on the cellular surface even more rapid as they form larger and heavier aggregates than trMAGs plus DNA prepared in 5% glucose. Larger trMAG-containing aggregates are more susceptible to magnetic forces and heavier aggregates generally sediment more rapidly.

Efficient uptake of magnetic beads into cells was only obtained when the cells were incubated for further 24 hours without magnetic field in fresh complete medium. Micrograph d shows that **magnetic particles** were definitely **taken up by the cell**.

The accumulation of trMAGs in **endosomal structures** suggests **endocytosis** as cellular uptake mechanism and not a direct traction of complexes into the cell by the magnetic field. These results were confirmed by Huth et al (Huth et al., 2004) and they showed by electron microscopy with trMAG-PEI / gold-labeled DNA complexes (prepared in HBS) that the magnetic particles and the DNA are co-internalized into the cell. Further experiments of this group lead to the assumption that clathrin-dependent and caveolae-mediated endocytosis are involved in magnetofection but their extent of involvement is cell line-dependent. Rejman et al found out that particles smaller than 200 nm are taken up by the clathrin-dependent pathway, but with increasing particle size there is a shift to the caveolae-mediated internalization which becomes the predominant pathway of entry for particles of 500 nm in size (Rejman et al., 2004).

3.3.3.3 Reporter gene expression kinetic with magnetofection and standard transfection

The aim was to find out if the rapid sedimentation of gene vectors with the magnetofection method leads to a different time course of reporter gene expression than in standard transfection.

NIH 3T3 cells were incubated with trMAG-16/1 / DNA / PEI (1 µg DNA/well, trMAG/DNA w/w = 4, N/P = 8, preparation in 150 mM NaCl) or PEI-DNA (1 µg DNA/well, N/P = 8, preparation in 150 mM NaCl) complexes for maximum 8 hours and during this time a magnet was placed underneath the trMAG-vector containing wells. Cells in triplicate wells each were lysed after 1, 2, 4, 8, 12, 24 and 48 hours. After 8 hours the remaining cells were washed and fresh complete medium was added. The reporter gene expressions (in ng luciferase/mg

protein) of magnetofected and standard transfected cells at the different time points were determined and the two kinetics compared.

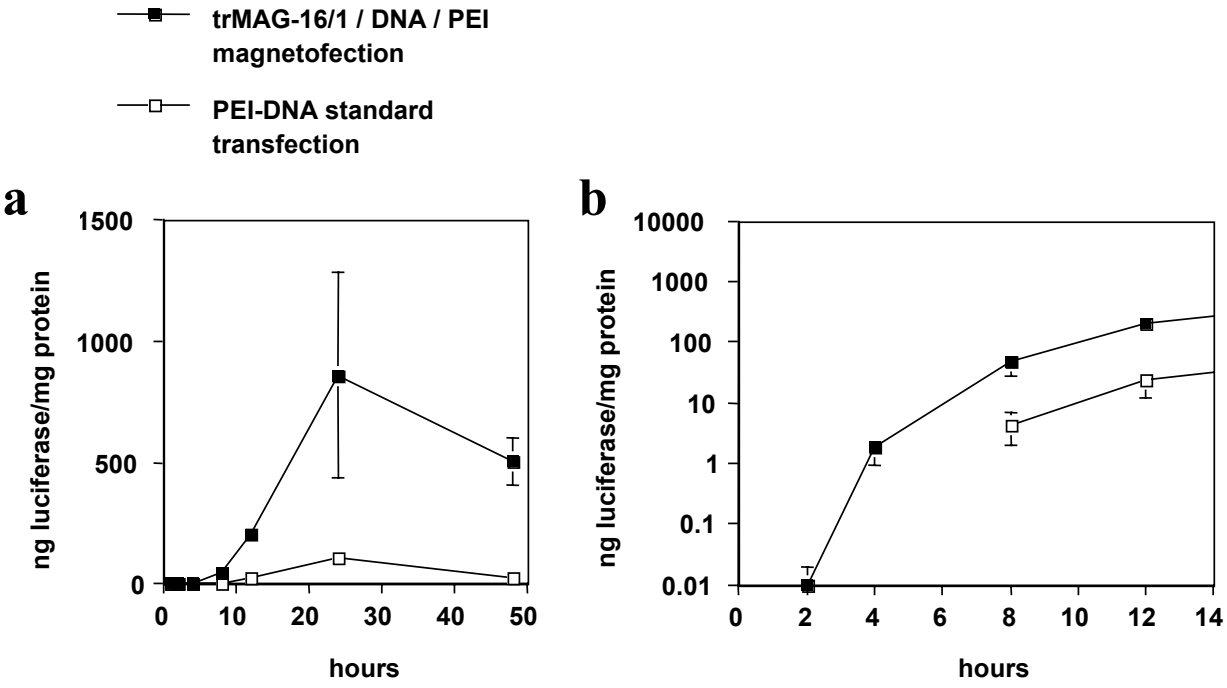


Figure 59 Luciferase gene expression kinetics of magnetofected or standard PEI-transfected NIH 3T3 cells. The different time points indicate hours after addition of gene vectors to the cells.
(a) Complete time course over 50 hours. Maximum gene expression with both transfection methods was detected after 24 hours. Magnetofection consistently leads to higher expression than the standard transfection (enhancement of 9 to 18-fold).
(b) Initial gene expression during the first 14 hours. With magnetofection, gene expression was already detected after 2 hours, with standard transfection only after 8 hours.

Reporter gene expression in **magnetofected cells** started earlier than in standard transfected cells. An explanation could be that the magnetic field leads to a more rapid and synchronized concentration of paramagnetic gene vectors on the cell surface, an earlier and synchronized (and eventually even accelerated) uptake of complexes into the cell and subsequently into the nucleus and finally an earlier and synchronized onset of reporter gene expression. In the course of time, the number of transcription and translation events increases and thus a continuous increase of luciferase protein was monitored until after 24 hours the maximum was detected. The real maximum could also be a bit earlier or later than after 24 hours but with the measurement time intervals chosen peak amounts of luciferase were detected after 24 hours. The following decrease may be due to intracellular plasmid degradation and simultaneous breakdown of the luciferase protein.

An explanation for the later start of gene expression in **standard transfected** cells could be that there is no synchronized sedimentation of gene vectors but in the beginning standard

complexes only get in contact with cells by Brownian motion (later sedimentation of aggregates may occur as well). Thus it may take between 4 and 8 hours until enough PEI per endosome is accumulated to exert the proton sponge effect. Further, it is possible that the minute amounts of luciferase protein produced after 4 hours could not be detected by the luciferase assay used. Unfortunately, in this time course the beginning of detectable gene expression can not be shown as there was no time point for measurement between 4 and 8 hours. The peak amount of luciferase in standard transfected cells could in reality be achieved later than in magnetofected cells as the reporter gene expression processes had a delay compared to the ones in magnetofected cells. But in the graph shown, the maximum amount of luciferase in standard transfected cells appears to be after 24 hours as well probably because the next time point for measurement was not until 48 hours where plasmids and luciferase protein were already strongly degraded. Further, the maximum amount of luciferase in standard transfected cells was not as high as the one in magnetofected cells. An explanation for this phenomenon could be that the sedimentation of trMAG-containing complexes via magnetic force results in more vector-cell contacts than than Brownian motion or sedimentation of standard complexes. Additionally, the on the average longer time span that standard vectors remain in serum-containing cell culture medium could lead to increased vector inactivation and therefore a decrease in gene expression.

3.3.3.4 Influence of the magnet on reporter gene expression

Transfections with non-magnetic standard vectors and simultaneous application of a magnetic field

The experiments with positively and negatively charged trMAGs showed that application of a magnetic field improved the transfection efficiencies of paramagnetic vectors. The explanation so far is that the magnet enables or accelerates the sedimentation of trMAG containing complexes, consequently more cells get in contact with the complexes, their cellular uptake is increased and the result is enhanced reporter gene expression.
Another explanation would be that not (alone) the magnetic sedimentation is the cause for increased gene expression but that the permanent static neodymium-iron-boron (Nd-Fe-B) magnet (1080-1150 mT) used could influence cell physiology in a manner that enhances transfection and/or reporter gene expression.

To examine if the permanent static Nd-Fe-B magnet alone (without magnetic beads) influences the measured reporter gene expression, NIH 3T3 cells were incubated for **3 hours** with various complexes lacking trMAGs (0.5 µg DNA/well) and meanwhile either the permanent magnet or no magnet was applied. Subsequently the cells were washed and fresh complete medium was added. As gene vectors, PEI-DNA / biotinylated inactivated adenovirus (bAdv), bAdv / streptavidinylated polylysine (St-pL) / DNA / PEI, PEI-DNA, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-Cholesterol / DNA and PEI-DNA / P6YE5C (a protective copolymer) were used. Polylysine is a cationic polymer commonly used in transfections (here streptavidinylated to enable biological linkage to the biotinylated inactivated adenovirus), out of DOTAP-Cholesterol solution cationic liposomes are formed with a molar ratio of 1/1 (commonly used in transfections) and P6YE5C is a protective copolymer of polyethylene glycol 6000Da and the negatively charged peptide YE5C ([Ac-YE_5]$_2$K-ε-C). Polylysine (pL) is a high molecular weight cationic polymer which is very effective in DNA condensation and nuclease protection but which is lacking endosomolytic activities (Wagner et al., 1992a). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) is a cationic lipid which is able to condense DNA and to protect DNA from nucleases (Leventis and Silvius, 1990) whereas the neutral lipid cholesterol (Felgner et al., 1994) is assumed to enhance endosomal release. P6YE5C stabilizes polycation / DNA complexes in their minimal size, prevents salt- and serum albumin-induced aggregation, and strongly reduces complement activation and the interaction with serum proteins (Finsinger et al., 2000). To show all these features, P6YE5C has to be added to polycation / DNA complexes in salt-free solution whereas in the following experiment PEI-DNA / P6YE5C was prepared in salt-containing solution. All vectors were prepared in HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid) buffered saline (20 mM HEPES, pH 7.3, 150 mM NaCl), called HBS. The reporter gene expressions (in ng luciferase/mg protein) with and without magnet were compared.

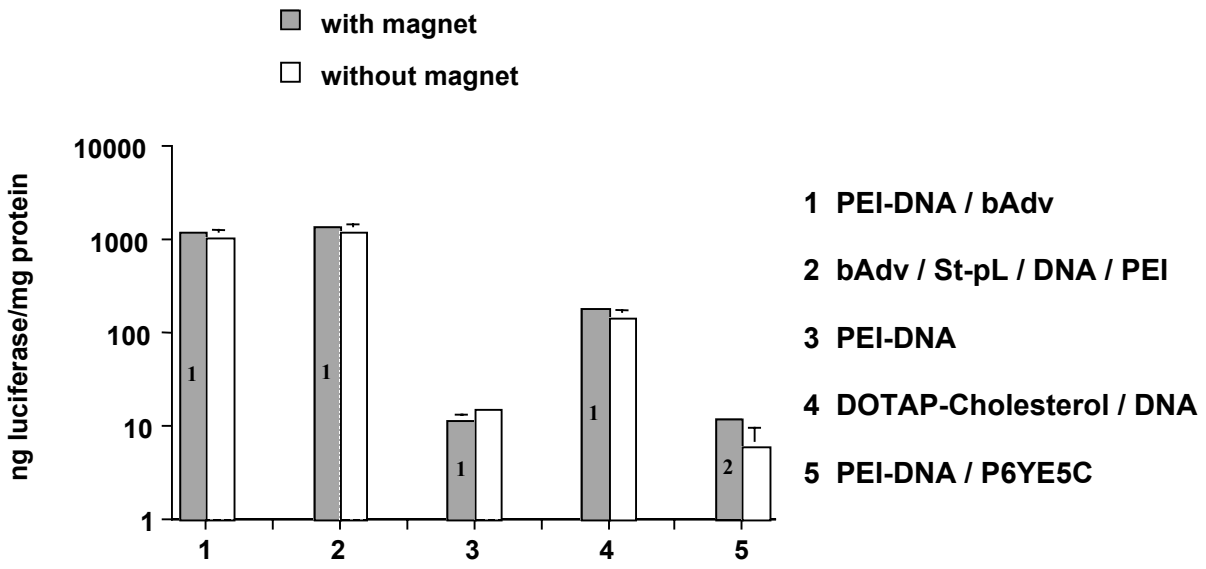


Figure 60 Luciferase gene expressions after transfections with various gene vectors lacking trMAGs with and without magnetic field.
The numbers in the gray bars indicate the fold enhancement when a magnet was applied. The magnet did not influence significantly the reporter gene expression in transfections with vector 1-4. Only with vector 5 the magnetic field lead to a two-fold increase.

Only transfection with vector 5 (PEI-DNA / P6YE5C) was enhanced two-fold by a magnetic field. But a two-fold increase is small, so an inaccuracy in the experiment can be the explanation for the difference.

In summary, **application of a permanent static magnetic field did not affect the measured reporter gene expression in transfections with trMAG-free complexes**.

It can be concluded that the enhancement of transfection by a permanent static neodymium-iron-boron (Nd-Fe-B) magnet (1080-1150 mT) shown in previous experiments is not a result of magnetic influence on cell physiology or activation of gene expression, but is the consequence of magnetic sedimentation of trMAG containing gene vectors.

In summary, all experiments to identify the mechanism of magnetofection justify the following conclusion:

The paramagnetic gene vectors are concentrated efficiently by magnetic force on the cell surface within minutes and immediately the endocytotic uptake of complexes into cells starts. Further steps leading to gene expression probably proceed similar as with standard vectors (lacking trMAGs and a magnetic field) but earlier. Higher efficiency in gene transfer with magnetofection is probably a result of more efficient sedimentation and therefore higher availability of gene vectors on the cellular surface for endocytosis.

3.3.4 Critical parameters in optimizing magnetofection

As shown in previous experiments, various types of positively and negatively charged magnetic particles are useful for magnetic field-guided gene transfer. It turned out that adjustment of the ionic strength to 150 mM NaCl after mixing of the vector components (or alternatively already the mixing in 150 mM NaCl) results in higher transfection efficiencies than the preparations in salt-free solution. The cellular uptake mechanism is assumed to be endocytosis and the addition of endosomolytic substances (like e.g. PEI, bAdv, cationic lipids, INF7) to trMAG-containing gene vectors leads to an increase in gene expression.
To optimize magnetofection, dose-response studies were carried out at different magnetic particle to DNA ratios, positively and negatively charged particles were used, mixing orders were varied systematically and transfection kinetics (optimum incubation time of cells with vectors plus magnet) was examined. Two types of magnetic particles were chosen: trMAG-PEI beads (coated with a monolayer of PEI 800 kDa and therefore positively charged) which showed efficient gene transfer in all previous experiments when an endosomolytic substance was added and trMAG-PO4 beads (coated with starch-phosphate Mw 20 kDa and therefore negatively charged). Haim et al. (Haim et al., 2005) recently used trMAG-PO4 beads (in the reference called TransMAG-PD particles) to form complexes between lentivirus (negatively charged) and magnetic particle by colloidal clustering (facilitated by positively charged ions) and with an applied magnetic field the complexes could efficiently infect cells. In previous work, Lübbe et al. (Lubbe et al., 1996a; Lubbe et al., 1996b) and Alexiou et al. (Alexiou et al., 2000) used the same magnetic particles (in the references called magnetic fluids or ferrofluids) to bind electrostatically positively charged chemotherapeutic agents and the complexes were directed to tumors by application of a magnetic field.

3.3.4.1 Dose-response studies at different trMAG / DNA (w/w) ratios

DNA complexes containing increasing trMAG-PEI / DNA (w/w) ratios and a constant amount of the cationic lipids DOTAP-Cholesterol, GenePorter or Lipofectamine were serially diluted in order to obtain various doses of DNA. The cationic lipids are frequently used commercially available transfection reagents. To obtain dose-response profiles at different trMAG / DNA (w/w) ratios, CHO-K1 cells were incubated with these vector formulations for 10 min in the presence of a magnetic field, washed and fresh serum-containing medium was added. The luciferase gene expression was determined as usual after 24 hours.

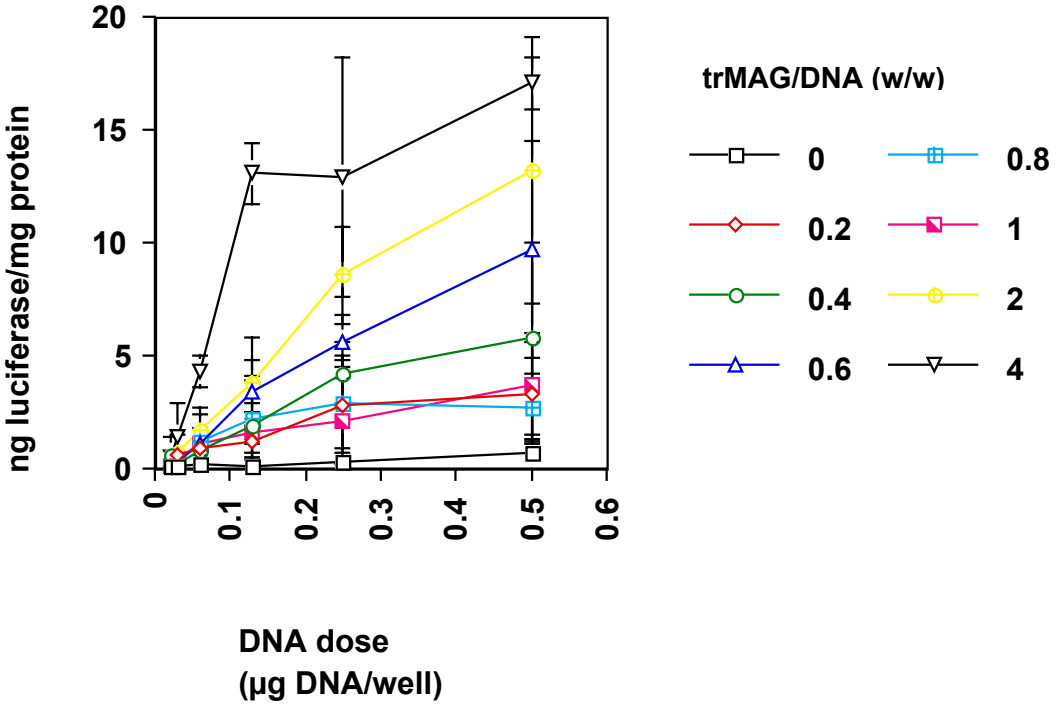


Figure 61 Magnetofection of CHO-K1 cells with trMAG-PEI / DNA / DOTAP-Cholesterol complexes. Vectors were prepared in aqua dest., subsequently the ionic strength was adjusted to 150 mM NaCl and they were added to the cells kept in serum-containing medium. The gene expression (in ng luciferase/mg protein) was examined in dependence of the DNA dose and the trMAG / DNA (w/w) ratio.
The data points represent the averages of quadruples ± standard deviations.
To obtain high gene expression (up to 17 ng luciferase/mg protein) the optimum trMAG / DNA (w/w) ratio was 4 and the optimum DNA dose was 0.5 µg DNA / well. With decreasing amounts of DNA, the gene transfer values decreased at all w/w ratios. Only at the ratio of 4, already with 0.1 µg DNA / well a relatively high transfection efficiency (13 ng luciferase/mg protein) was monitored. The lowest efficiency in gene transfer was obtained without trMAG-PEI particles (w/w = 0).

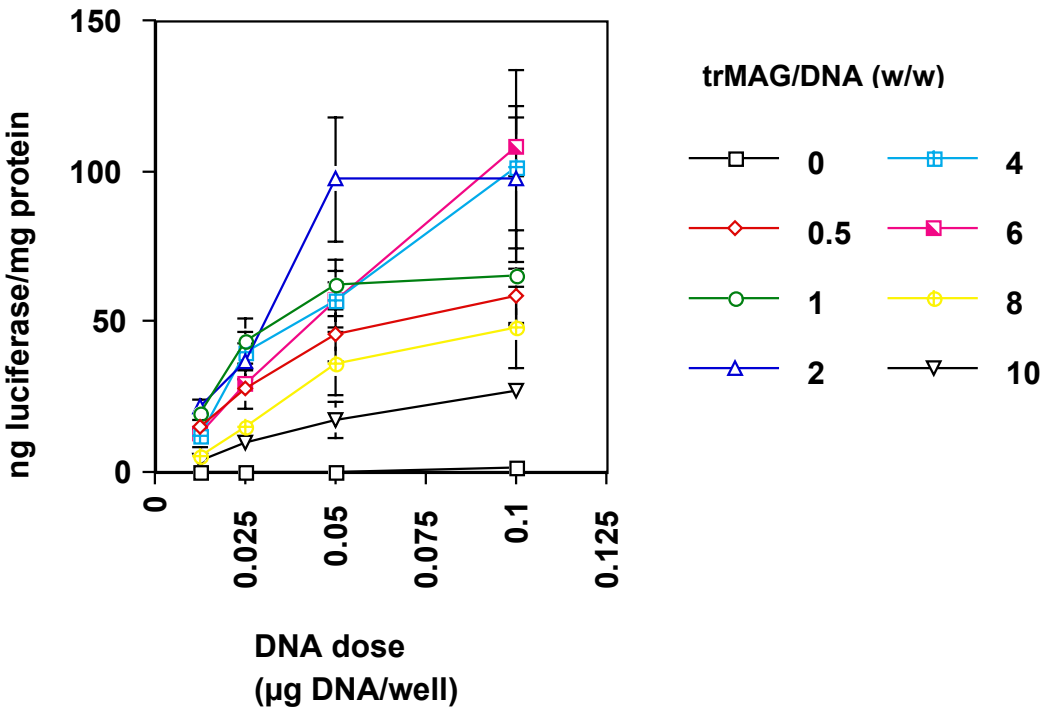


Figure 62 Magnetofection of CHO-K1 cells with trMAG-PEI / DNA / GenePorter complexes. Vectors were prepared in serum-free medium, serum-containig medium was removed from the cells and the complexes were added. The gene expression (in ng luciferase/mg protein) was examined in dependence of the DNA dose and the trMAG / DNA (w/w) ratio.
The data points represent the averages of triplicates ± standard deviations.
The highest efficiency in gene transfer (up to approx. 100 ng luciferase/mg protein) was obtained with trMAG / DNA (w/w) ratios of 2, 4 and 6. The lower ratios 1 and 0.5 showed lower transfection efficiencies and the higher ratios 8 and 10 were even less efficient than the lower ratios probably due to cell toxicity. The lowest efficiency in gene transfer was monitored without trMAG-PEI particles (w/w = 0).
With increasing amounts of DNA, the transfection efficiency increased as well. Up to more than 100 ng luciferase/mg protein were obtained with the highest DNA dose used (0.1 µg DNA / well). Only with a w/w ratio of 2 and 1 at 0.05 µg DNA / well there was a saturation point. The w/w ratio 2 enables even a peak transfection of nearly 100 ng luciferase/mg protein with 0.05 µg DNA / well.
In comparison to transfections with DOTAP-Cholesterol complexes (fig. 61), higher reporter gene expression was obtained throughout the dose range.

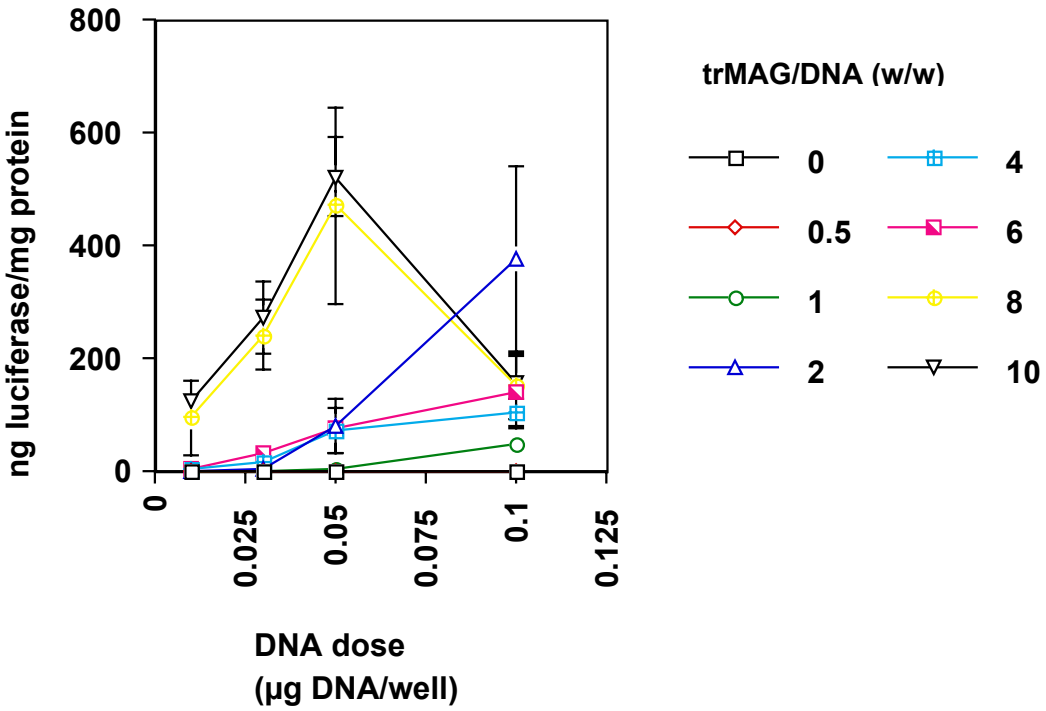


Figure 63 Magnetofection of CHO-K1 cells with trMAG-PEI / DNA / Lipofectamine complexes. Vectors were prepared in serum-free medium, serum-containig medium was removed from the cells and the complexes were added. The gene expression (in ng luciferase/mg protein) was examined in dependence of the DNA dose and the trMAG / DNA (w/w) ratio.
The data points represent the averages of triplicates ± standard deviations.
The highest efficiency in gene transfer (more than 500 ng luciferase/mg protein) was obtained with the highest trMAG / DNA (w/w) ratio of 10. With decreasing w/w ratio the peak transfection values decreased. The only exception from this tendency was the w/w ratio of 2 showed the third highest transfection efficiency (approx. 370 ng luciferase/mg protein). The lowest gene transfer efficiency was monitored with a ratio of 0.5 (data not seen in this scale) and 0 (without trMAG-PEI).
With increasing amounts of DNA, generally gene expression was increasing. But at a w/w ratio of 10 and 8 the optimum transfection efficiency (approx. 520 and 480 ng luciferase/mg protein) was obtained with only 0.05 µg DNA / well and using more DNA resulted in a strong decrease in gene transfer probably due to toxic effects.
In general, Lipofectamine complexes were more efficient in transfecting CHO-K1 cells than GenePorter (fig. 62) or DOTAP-Cholesterol (fig. 61).

The three graphs (fig. 61, 62, 63) revealed that **each type of complex** (with DOTAP-Cholesterol, GenePorter or Lipofectamine) used for magnetofection has its **individual optimum trMAG-PEI / DNA (w/w) ratio and DNA dose** to obtain maximum transfection efficiency. But on the average, a magnetic particle to DNA ratio of 2 and higher DNA-doses appeared to be useful.

3.3.4.2 Comparison of positively with negatively charged trMAGs regarding the transfection efficiency

To answer the question if positively or negatively charged magnetic beads are more efficient in magnetofection, gene transfer experiments with trMAG-PEI particles as representative for positively charged trMAGs and with trMAG-PO4 particles as representative for negatively charged trMAGs were performed under identical conditions (mixing order, buffers, cell line). The complexes were formed either of PEI plus DNA plus trMAGs or of PEI plus DNA plus a synthetic endosome-disruptive Influenza peptide (INF7) plus trMAGs. The cells (kept in serum-containing medium) were incubated with these aggregates for 10 min. with or without application of a magnetic field, subsequently medium was changed and as usual after 24 hours the gene transfer efficiency (in ng luciferase/mg protein) was determined.

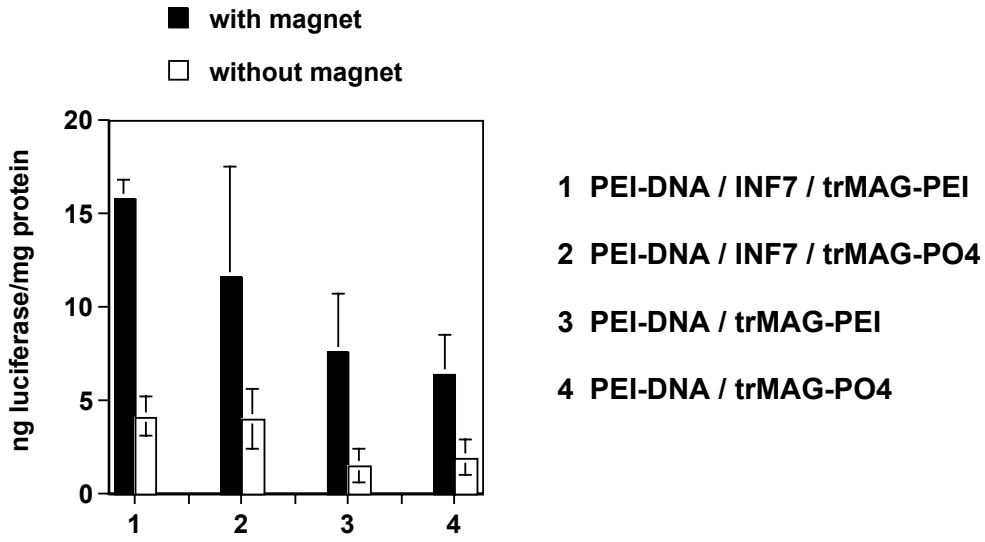


Figure 64 Transfection of NIH3T3 cells with positively charged trMAG-PEI (columns **1** and **3**) and negatively charged trMAG-PO4 (columns **2** and **4**).
Comparing columns of **1** and **2** (PEI-DNA, INF7 and trMAGs) reveals that with magnet the trMAG-PEI complexes (**1**) were slightly superior in transfecting cells whereas without magnet there was no difference.
Comparing column **3** and **4** (PEI-DNA and trMAGs) shows that with magnet the trMAG-PEI complexes (**3**) were slightly more effective whereas without magnet the values of trMAG-PO4 aggregates (**4**) were slightly higher.
In summary, there were **no significant differences between trMAG-PEI and trMAG-PO4 particles regarding the transfection efficiency**.
With all complexes harbouring the INF7 peptide (**1** and **2**) a slightly higher transfection efficiency was obtained than with complexes lacking this peptide. The magnetic field led to significantly enhanced gene transfer in all four cases.

The graph reveals that there is **no significant difference** in gene transfer (maximum 1.5-fold between **1** and **2**, with magnet) when **positively** charged trMAG-PEI **or negatively charged** trMAG-PO4 **particles** were used for transfection. trMAG-PEI beads are known to be very efficient in magnetofection when combined with endosomolytic substances (like e.g. PEI or INF7) and here it is shown that the negatively charged trMAG-PO4 particles are equally efficient.
The influenza peptide added to trMAGs enhanced the transfection efficiency at least 2-fold (between **1** and **3** and between **4** and **2**, with magnet).

3.3.4.3 Variation of the mixing order of vector components during formation of the complexes

DNA-complexes including DOTAP-Cholesterol and trMAG-PO4 or trMAG-PEI

To examine if the sequence of mixing influences the magnetofection efficiency, complexes were either prepared in the mixing order DOTAP-Cholesterol plus DNA plus trMAGs (fig. 65 and 67) or DNA plus trMAGs plus DOTAP-Cholesterol (fig. 66 and 68). The trMAGs used were trMAG-PO4 (coated with starch-phosphate, fig. 65 and 66) or trMAG-PEI (coated with a monolayer of PEI, fig. 67 and 68). DOTAP-Cholesterol and DNA was formulated with a charge ratio of +/- = 5 and the w/w ratio of trMAGs / DNA was 1. All preparations were mixed in HBS. A serial dilution series was carried out to obtain a DNA dose-response profile. NIH 3T3 cells (kept in serum-containing medium) were incubated for 10 min with complexes in the presence or absence of a magnetic field, followed by a medium change and as usual after 24 hours the gene expression (in ng luciferase/mg protein) was determined.

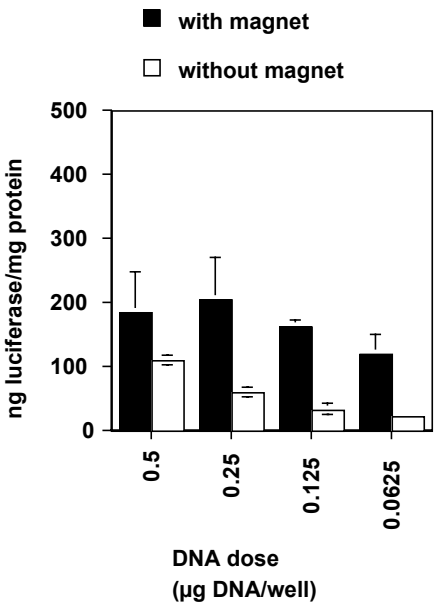


Figure 65 Transfection of NIH3T3 cells with **trMAG-PO4** containing complexes prepared in the mixing order: **DOTAP-Cholesterol** plus **DNA** plus **trMAG-PO4**. The highest magnetofection efficiencies were obtained with 0.25 (maximum) and 0.5 µg DNA/well.

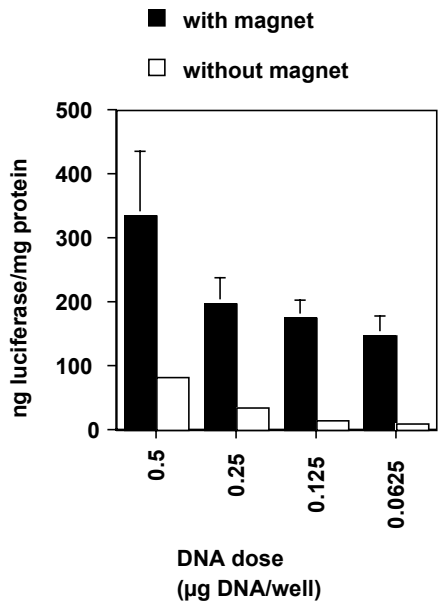


Figure 66 Transfection of NIH3T3 cells with **trMAG-PO4** containing complexes prepared in the mixing order: **DNA** plus **trMAG-PO4** plus **DOTAP-Cholesterol**. The resulting peak magnetofection (at a dose of 0.5 µg DNA/well) was approx. 1.5-fold higher than the maximum value obtained with the mixing sequence used in fig. 65.

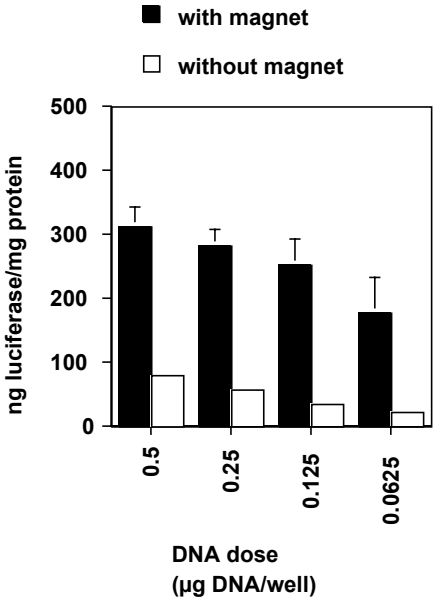


Figure 67 Transfection of NIH3T3 cells with **trMAG-PEI** containing complexes prepared in the mixing order: **DOTAP-Cholesterol** plus **DNA** plus **trMAG-PEI**. The highest magnetofection efficiency was obtained with the highest DNA dose used (0.5 µg DNA/well).

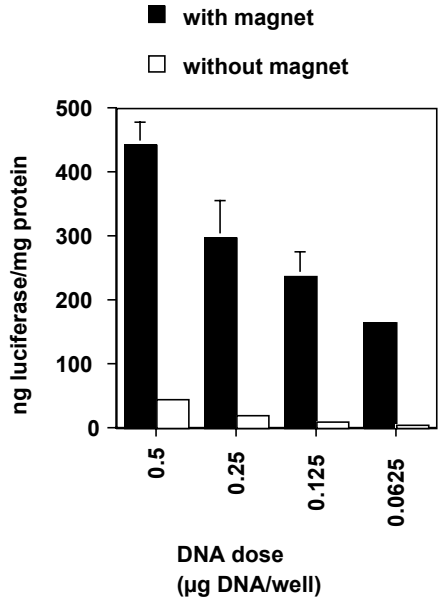


Figure 68 Transfection of NIH3T3 cells with **trMAG-PEI** containing complexes prepared in the mixing order: **DNA** plus **trMAG-PEI** plus **DOTAP-Cholesterol**. Peak magnetofection (at 0.5 µg DNA/well) was approx. 1.5-fold higher than the peak value observed with the mixing order used in fig. 67.

Gene transfer with trMAG-PO4 and with trMAG-PEI particles in **both mixing orders** resulted in similar magnetofection efficiencies and there were **no significant differences**.
Only the peak transfection values with trMAG-PO4 (fig. 66) and trMAG-PEI (fig. 68) were approximately 1.5-fold higher with the mixing order DNA plus trMAGs plus DOTAP-Cholesterol when a magnetic field was applied (magnetofection). But a 1.5-fold enhancement is not very significant. trMAG-PO4 beads with the mixing sequence DOTAP-Cholesterol plus DNA plus trMAG-PO4 showed their maximum transfection efficiency in the presence of a magnet with 0.25 µg DNA/well (fig. 65), but with the highest DNA dose of 0.5 µg DNA/well (where peak transfection is obtained in the other 3 formulations) almost the same efficiency was obtained and the difference between the two is not significant.
With increasing amounts of DNA, reporter gene expression (in ng luciferase/mg protein) increased (only exception: 0.5 µg DNA/well with magnet in fig. 65). But it has to be mentioned that for the absolute amount of luciferase produced per well there was no pronounced DNA dose-response relationship in the dose range tested. The higher DNA doses tended to be toxic by visual inspection.
Generally, transfections with magnet resulted in significantly higher gene transfer efficiencies than transfections without magnet.
Magnetofections with trMAG-PEI particles were slightly more efficient than with trMAG-PO4 beads, but the difference was not significant.

DNA-complexes including PEI and trMAG-PO4

Complexes were prepared in HBS with the mixing order PEI-DNA plus trMAG-PO4 or trMAG-PO4 plus PEI plus DNA or trMAG-PO4 plus DNA plus PEI to examine if the sequence of mixing influences the magnetofection efficiency. For the formulation, 0.5 µg DNA/well, a w/w ratio trMAG/DNA of 1 and PEI/DNA with a N/P ratio of 8 were used. NIH 3T3 cells (kept in serum-containing medium) were incubated with the aggregates for 10 min with or without application of a magnetic field. Subsequently the medium was changed and after 24 hours reporter gene expression was determined. All transfections were performed in quadruples.

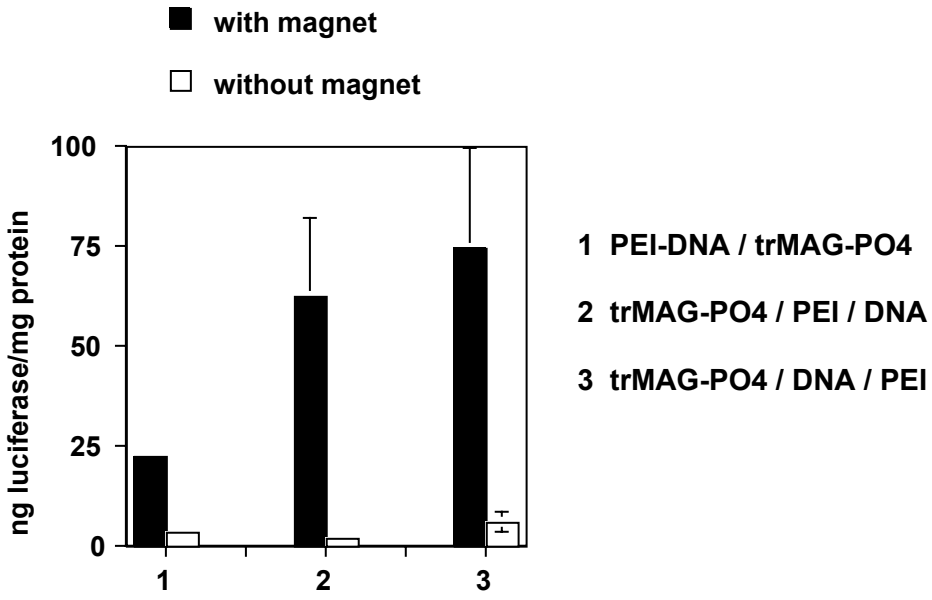


Figure 69 DNA-complexes including PEI and trMAG-PO4 particles were prepared in three different mixing sequences and their efficiency in transfecting NIH 3T3 cells was determined. In the presence of a magnet, mixing order 3 (trMAG-PO4 / DNA / PEI) showed the highest gene transfer efficiency followed by mixing order 2 (trMAG-PO4 / PEI / DNA). In general, application of a magnetic field during incubation with complexes strongly enhanced the transfection efficiency.

The sequence of mixing influenced the efficiency of gene transfer with magnet (magnetofection). **Mixing order 3** was 1.2 and 3.4-fold more efficient than mixing order 2 and 1, respectively, but only the 3.4-fold enhancement is assumed to be significant. Mixing order 2 showed significantly (2.8-fold) higher gene expression than mixing order 1 as well. An explanation for the reduced magnetofection efficiency with mixing order 1 could be that PEI in complexes with preformed PEI-DNA and negatively charged trMAGs might be less efficient in its endosomolytic activity.

DNA-complexes including PEI, trMAG-PO4 and chemically inactivated adenovirus

The complexes for magnetofection were prepared in HBS in eight different mixing orders (fig. 70). 0.5 µg DNA/well, a w/w ratio trMAG/DNA of 1, PEI/DNA with a N/P ratio of 8 and 7.2 x 10^8 chemically inactivated adenovirus particles/0.5 µg DNA were used to formulate the aggregates. NIH 3T3 cells (kept in serum-containing medium) were incubated with these complexes for 10 min in the presence or absence of a magnetic field. Each transfection was carried out in quadruples. After a medium change and after further 24 hours the gene expression (in ng luciferase/mg protein) was determined.

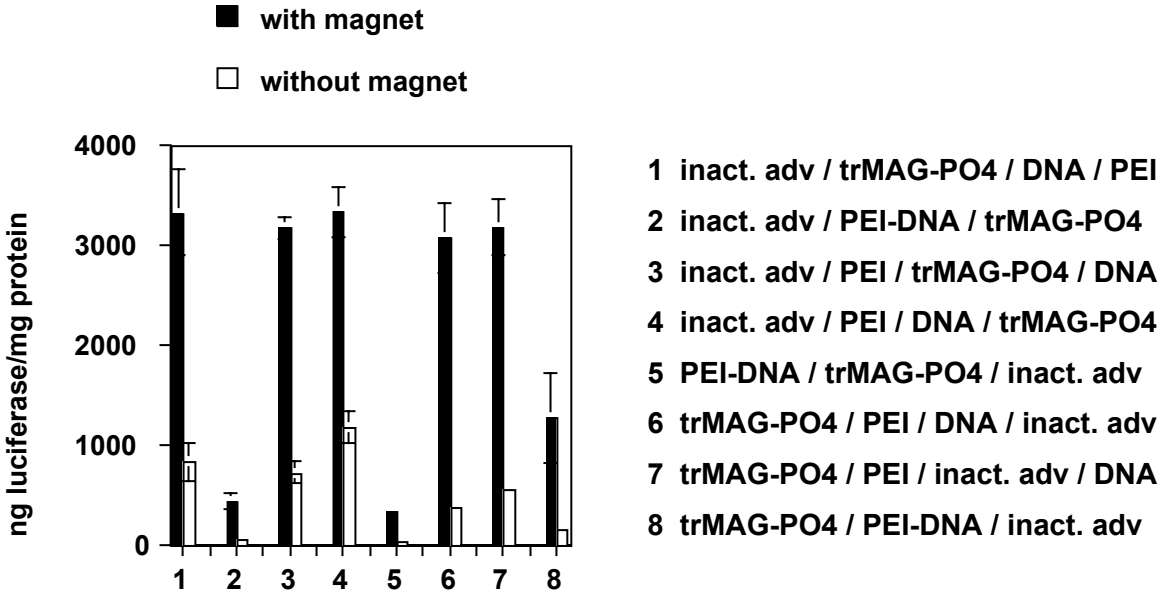


Figure 70 DNA-complexes including PEI, trMAG-PO4 and psoralen-treated adenovirus particles were prepared in eight different mixing sequences and their efficiency in transfecting NIH 3T3 cells was determined. Mixing order 4 showed the highest gene transfer efficiency whereas with mixing order 5 the lowest value was obtained. Mixing sequences 2, 5 and 8 (all with preformed PEI-DNA complexes) resulted in significantly lower transfection efficiencies than the mixing sequences 1, 3, 4, 6 and 7 which showed no significant efficiency differences among each other. Generally, the transfections with magnet resulted in much higher gene expression than the transfections without magnet.

Similar as in fig. 69 **aggregates harbouring preformed PEI-DNA** complexes (**mixing order 2, 5 and 8**) showed **significantly lower magnetofection efficiencies** than the other formulations. E.g. mixing order 5 was 11 times less efficient in magnetofection than mixing order 4. And even with application of a magnetic field mixing order 5 showed a 4-fold lower transfection efficiency than mixing order 4 without magnet.

The sequence of mixing had a minor impact on the gene transfer efficiency with magnet when **mixing order 1, 3, 4, 6 and 7** were used. These formulations **showed all similarly high gene expression** values of more than 3000 ng luciferase/mg protein.

Addition of chemically inactivated adenovirus particles to the complexes strongly enhanced the gene transfer efficiency. E.g. the peak magnetofection value in fig. 70 (with inact. adenovirus) was 45-fold higher than the peak magnetofection value in fig. 69 (same components but without inact. adenovirus).

These transfections with inactivated adenoviruses may provide valuable hints for the use of active adenoviral gene vectors in magnetofection. At least it was shown that adenoviral particles, PEI and negatively charged trMAGs can be successfully combined in a complex and

also the amounts of each component used could serve as a guide line for magnetofections with active adenoviruses.

In summary, **in most cases the mixing order does not influence the magnetofection efficiency significantly when the complexes are prepared in salt-containing solution** (here HBS). An explanation could be that in salt-solution aggregates are formed which usually harbour all components added. But the **preformation of PEI-DNA complexes in combination with negatively charged particles** (like e.g. in fig. 69 and 70) can lead to **reduced magnetofection efficiencies,** eventually because PEI might be less efficient in its endosomolytic activity in such associates.

3.3.4.4 Kinetics of magnetofection

In the magnetofection experiments presented before, cells were usually incubated with vectors for 10-20 min. The aim of the following two experiments was to find out the **optimum time of incubation** of NIH 3T3 cells with DNA-complexes including cationic lipids in magnetofection and the comparison to usual standard transfections.
As vectors in the first experiment (fig. 71) trMAG-PEI / DNA / Lipofectamine (LF) and DNA / LF and in the second experiment (fig. 72) trMAG-PEI / DNA / GenePorter (GP) and DNA / GP were chosen. For the formulations 0.1 µg DNA/well, a trMAG/DNA (w/w) ratio of 2 and 4 µl of Lipofectamine (LF) or 5 µl of GenePorter (GP) per µg of DNA were used. The complexes were prepared in serum-free medium, serum-containig medium was removed from the cells and the complexes were added. After 5, 10, 20, 40 and 240 minutes of incubation, the vector formulations were removed from the cells and cells were washed. The cells were positioned upon a magnetic plate for 240 min or were kept without magnet (standard conditions) during incubation with complexes. Luciferase gene expression was determined 20 hours after the start of the experiment in ng luciferase/mg protein.

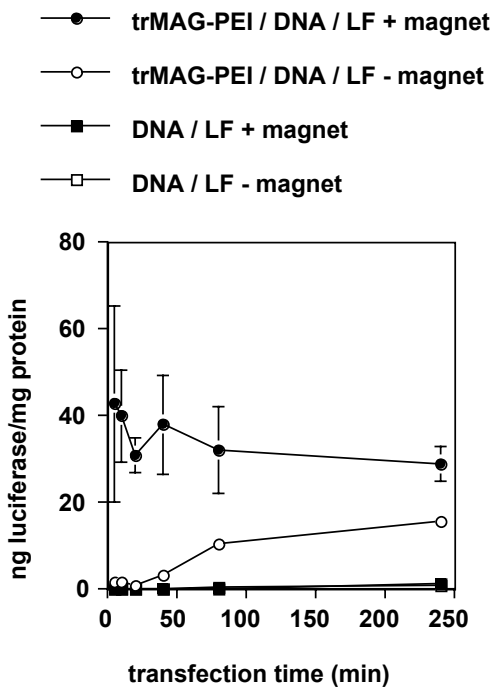


Figure 71 Kinetics of transfections in NIH 3T3 cells using the cationic lipid Lipofectamine (LF) with or without trMAG particles in the presence or absence of a magnetic field.
Maximum gene expression was found already after 5 min of incubation with trMAG-PEI / DNA / LF complexes and an applied magnetic field (magnetofection). The following decrease in this curve is probably not significant.

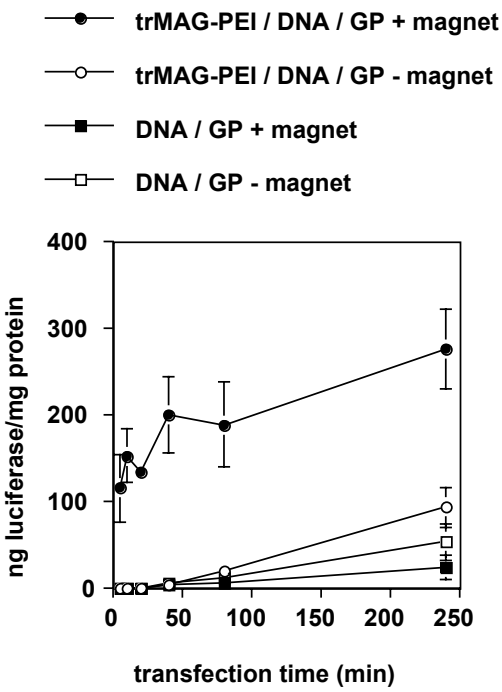


Figure 72 Kinetics of transfections in NIH 3T3 cells using the cationic lipid GenePorter (GP) with or without trMAG particles in the presence or absence of a magnetic field.
The highest efficiency in gene transfer was obtained with trMAG-PEI / DNA / GP complexes and an applied magnetic field (magnetofection). Approx. 42 % of the final reporter gene expression level was already achieved after 5 min. The transfection efficiency increased over time with a moderate slope.

In **fig. 71** with trMAG-PEI / DNA / LF complexes and an applied magnetic field (magnetofection) maximum gene expression was found after 5 min of incubation. Longer incubation times led even to a slight but not significant decrease in transfection efficiency. An explanation for this curve could be that already after 5 min of incubation the main proportion of gene vectors was magnetically sedimented and was in tight contact with the cells so that it was not removed by the washing step. The proportion which was sedimented only with longer incubation times could be not significant for the gene transfer efficiency. Another possible explanation for this curve could be that with the proportion of complexes sedimented after 5 min the cells are saturated and longer times of incubation and more sedimented aggregates have no enhancing effect on transfection efficiency or they could even be toxic.

In **fig. 72** with trMAG-PEI / DNA / GP complexes and an applied magnetic field (magnetofection), 42 % of the final reporter gene expression level was achieved after 5 min of incubation. With longer incubation, the transfection efficiency increased over time with a moderate slope and maximum gene expression after 4 h of incubation was only 2.4-fold

higher than gene expression after 5 min of incubation. If it is assumed that this 2.4-fold increase is significant, then an explanation for this curve in comparison with fig. 71 could be that the proportion of gene vectors which was magnetically sedimented after 5 min was not as high as for trMAG-PEI / DNA / LF complexes plus magnet in fig 71. It might be possible that GP leads to slower salt-induced aggregation than LF, thus paramagnetic GP aggregates could grow slowlier, consequently their magnetic susceptibility might increase slowlier (Voltairas et al., 2002) and therefore with increasing incubation time in salt-containing culture medium the proportion of gene vectors which is magnetically sedimented still increases.

From these two experiments it can be concluded that **with magnetofection already 5 min of incubation with complexes prepared in salt-containing solution can lead to optimum gene transfer efficiency or to a transfection efficiency close to the optimum.** But still, optimum incubation time has to be found out individually for each vector and cell type.

Magnetic sedimentation was also examined in the electron microscopy studies in fig. 58. Cells were incubated with complexes for 1, 5 and 15 min and an incubation time of 15 min was necessary for tight association of complexes with cells. It has to be considered that the complexes for electron microscopy were prepared in 5% glucose and therefore smaller associates were formed which need more time to magnetically sediment than larger aggregates of salt-preparations. Additionally, in the experiment in fig. 57 complexes prepared in 5% glucose showed efficient magnetofection after a 10 min-incubation. From these results it can be assumed that with complexes prepared in salt-free solution an incubation time of 10-15 min is necessary for efficient magnetofection.

A further result of the two kinetics experiments (fig. 71 and 72) was that the the highest gene expression was obtained with the magnetofection method (complexes including trMAG-PEI particles and a magnetic field applied).

But also adding trMAGs to the complexes without application of a magnetic field enhanced the transfection efficiency compared to using the standard vector formulations, especially when longer incubation times were used. An explanation for this phenomenon could be that the trMAG containing aggregates are heavier due to the iron oxide component and therefore settle more efficiently. Consequently there were more contacts between cells and gene vectors and therefore the cellular uptake and the transfection efficiency was increased. This interpretation is supported by the results of magnetofection experiments shown later in figure 75.

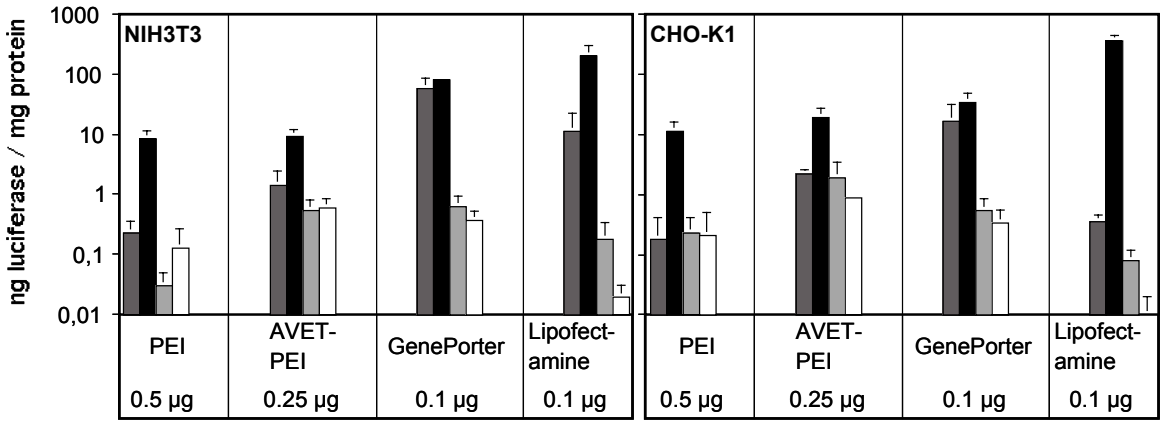
At any time point, GenePorter formulations (fig. 72) were more efficient than Lipofectamine formulations (fig. 71).

3.3.5 Comparison of magnetofection and conventional transfection methods with regard to their gene transfer efficiency

As shown in the experiments before, the principle of magnetofection works and magnetic particles combined with standard transfection reagents lead to high magnetofection efficiencies. Gene transfer efficiencies of optimized magnetofections and of standard transfection protocols were compared in the following experiments.

3.3.5.1 Transfection of NIH 3T3 and CHO-K1 cells with different vector formulations

Aim of the following experiments was to compare the gene transfer efficiency of magnetofection and the corresponding standard transfection methods (without trMAG particles / without magnetic field / long incubation times). In this context, four different vector types and two different cell lines were examined. As standard vectors PEI-DNA, DNA / PEI / inactivated adenovirus (adenovirus enhanced transfection with PEI or **AVET-PEI**), GenePorter-DNA and Lipofectamine-DNA complexes were used. The corresponding vectors for magnetofection included additional trMAG-PEI particles (trMAG/DNA w/w ratio = 2). The formulations were prepared in HBS (AVET-PEI), in serum-free but salt-containing cell culture medium (GenePorter-DNA and Lipofectamine-DNA complexes) or in water with subsequent adjustment of the ionic strength to 150 mM NaCl (PEI-DNA). NIH 3T3 and CHO-K1 cells were incubated with vectors for 10 min or 4 hours in the presence or absence of a magnetic field. Subsequently the cells were washed and fresh complete medium was added. The gene expression (in ng luciferase/mg protein) was determined in triplicates as usually after 24 hours.



Enhancement over	PEI		AVET-PEI		Gene Porter		Lipofect-amine	
	NIH	CHO	NIH	CHO	NIH	CHO	NIH	CHO
magnetofectin 10 min w\o mag.	325	51	17	10	133	63	1176	4389
std. vector 10 min w\o mag.	65	54	16	21	222	104	9655	34506
std. vector 4 hrs w\o mag.	38	64	7	8	1.4	2	18	971

Figure 73 Efficacy of magnetofectins (magnetic particle containing vectors) in NIH 3T3 and CHO-K1 cells upon short-time (10 min) incubation in the presence (black bars) and in the absence (light gray bars) of a magnetic field compared with standard transfections with the parent vectors (dark gray bars, 4-h incubation; white bars, 10-min incubation). The table below the graph specifies the enhancements that were achieved upon the influence of the magnetic field on paramagnetic vectors compared with transfections in the absence of the magnetic field with paramagnetic vectors or the parent standard vectors which did not contain trMAG-PEI. The data demonstrate that magnetofection can strongly enhance transfection efficiencies over standard transfection protocols. The relative enhancements are dependent on vector type, cell line and incubation time.

With all vector types except GenePorter, **the magnetofection method showed significantly higher gene expression in both cell lines than the corresponding transfections without magnet**. As explanation for this phenomenon it is assumed that due to magnetic sedimentation more gene vectors get in tight contact with cells (even within a relatively short period of time) and consequently the cellular uptake and the transfection efficiency are enhanced. Without magnetic field, the chance for gene vectors to get in contact with cells is limited by Brownian motion and only larger aggregates efficiently sediment within the incubation time. But it has to be mentioned that with GenePorter containing vectors magnetofection in NIH 3T3 and CHO-K1 cells was only 1.4 and 2-fold, respectively, more efficient than the standard transfection with a 4 h incubation time. As a 1.4 and 2-fold enhancement is not assumed to be significant it can be concluded that **in dependence on the parental vector, standard transfections with longer incubation times (4 h) can result in equally high transfection efficiencies than magnetofections with 10 min incubation time.** As explanation it is assumed that high concentrations of some gene vectors (like e.g.

GenePorter) on cellular surfaces can lead to saturation of uptake processes or even toxicity and therefore the higher number of vector-cell contacts achieved with magnetic sedimentation does not lead to a further improvement. The results do vary between experiments. E.g. in fig. 72 the same 10 min-magnetofection of NIH 3T3 cells with GenePorter was up to 5 times more efficient in gene transfer than the 4 h incubation with standard GenePorter-DNA. This may be explained by slight variations in transfection parameters such as incubation times during vector preparation, cell density and passage number at the time of transfection.

Maximum enhancement by magnetofection was obtained with Lipofectamine containing complexes in CHO-K1 cells: **10 min incubation** with magnetofectins and a simultaneously applied magnetic field resulted in a **971-fold higher gene transfer efficiency than 4 h incubation with standard vectors.**

The magnetofection with Lipofectamine complexes (with only 0.1 µg DNA/well) was the most efficient in NIH 3T3 and CHO-K1 cells followed by magnetofections with GenePorter (also 0.1 µg DNA/well).

Independent of variability between experiments, magnetofection was always at least as efficient as the parent vector, in most cases substantially more efficient.

3.3.5.2 Transfection of NIH 3T3 and CHO-K1 cells with different DNA doses

In fig. 73 magnetofection with Lipofectamine (LF) showed strong enhancement of gene transfer efficiency over 4 h-incubation with standard LF-DNA vectors. In the following experiments the LF-magnetofection was examined more in detail by establishing a dose-response profile.

Vectors for magnetofection included trMAG-PEI particles with a trMAG/DNA (w/w) ratio of 2. The formulations were prepared in serum-free cell culture medium. In addition to magnetofection and standard transfection the efficiency of gene transfer with complexes containing trMAGs but without application of a magnetic field was examined. All transfections were performed in triplicates.

NIH 3T3 and CHO-K1 cells were incubated with vectors for 10 min or 4 hours, followed by a medium change, and the next day gene expression was determined.

Transfection of NIH 3T3 cells

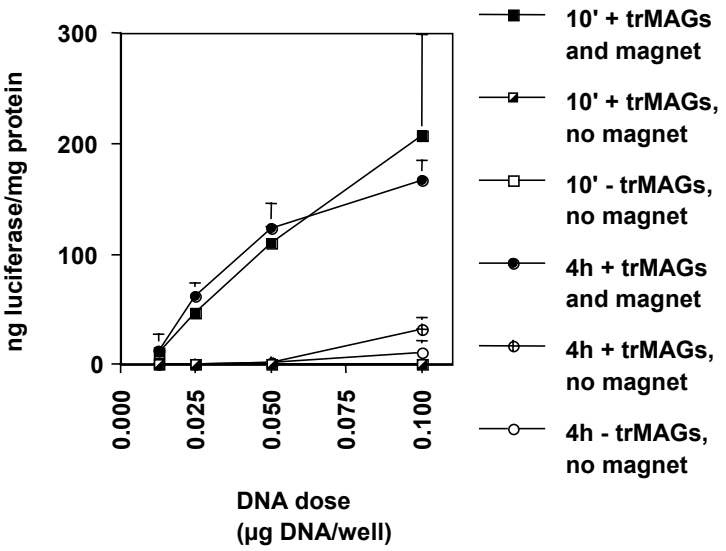


Figure 74 Transfection with **Lipofectamine (LF)-DNA** complexes and increasing amounts of DNA. Magnetofections (10' or 4h + trMAGs and magnet) showed higher gene expression than standard LF-DNA transfections (4h – trMAGs, no magnet) at all doses of DNA.

Transfection of CHO-K1 cells

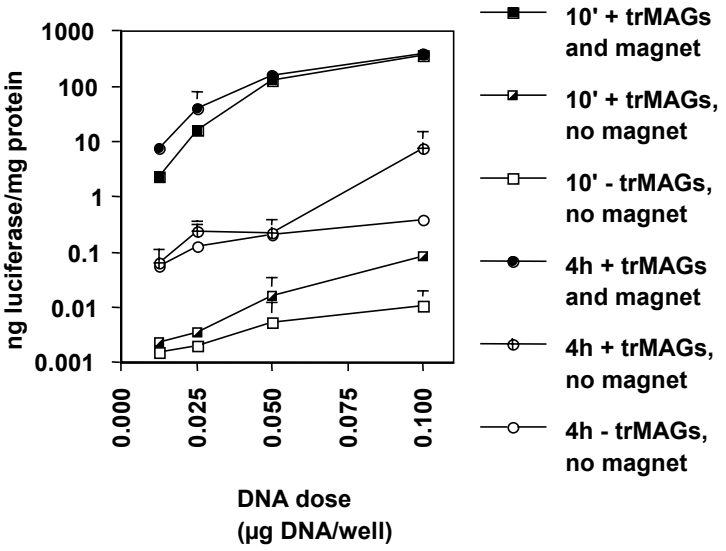


Figure 75 Transfection with **Lipofectamine (LF)-DNA** complexes and increasing amounts of DNA. Magnetofections (10' or 4h + trMAGs and magnet) showed higher gene expression than standard LF-DNA (4h – trMAGs, no magnet) at all DNA doses. At 0.05 µg DNA/well magnetofections and standard LF-DNA approached saturation.

Using NIH 3T3 and CHO-K1 cells, **the magnetofection method** with Lipofectamine (LF)-DNA was **more efficient under all settings than the corresponding standard method** (4h – trMAGs, no magnet). A maximum enhancement of **180-fold** was achieved in NIH 3T3 cells with 4h LF-DNA magnetofection at 0.025 µg DNA (fig. 74) and a maximum enhancement of **970.9** and **1037.2-fold** in CHO-K1 cells with 10' and 4h LF-DNA magnetofection,

respectively, at 0.1 µg DNA (fig. 75). As explanation it is proposed that magnetic sedimentation increases the number of vector-cell contacts at all DNA-doses used.

In NIH 3T3 cells (fig. 74), the enhancements of LF-magnetofection over the corresponding standard transfection did not correlate with the DNA-dose. But Plank et al. found out that in NIH 3T3 cells the enhancements of 4 h GenePorter-magnetofection over 4 h standard GenePorter transfection increased with decreasing DNA-doses (unpublished data). In contrast, in CHO-K1 cells (fig. 75), the enhancements of the 10 min and 4 h LF-magnetofection over the corresponding standard transfection increased with higher amounts of DNA/well. From these results it can be concluded that correlations between enhancement and DNA-dose can be obtained in some cases but they depend on the type of vector and the cell line used.

Figure 74 shows an example where the 10' LF-DNA magnetofection is with the comparably low DNA doses of 0.025 and 0.050 µg DNA/well **4.1** and **9.6-fold**, respectively, more efficient in transfecting NIH 3T3 cells than the standard LF-DNA method with 0.1 µg DNA/well (the highest dose used) and a 4h incubation time with complexes. Figure 75 gives an example with CHO-K1 cells where the 10' LF-DNA magnetofection achieved with 0.0125, 0.025 and 0.05 µg DNA/well **6.3**, **43.2** and **336.8-fold** higher gene expression than the standard LF-transfection using the much higher dose of 0.1 µg DNA/well and the much longer incubation time of 4 hours. Obviously, even if higher DNA-doses and longer incubation times significantly increase the number of vector-cell contacts by Brownian motion and by sedimentation in standard transfections, in some cases with magnetic sedimentation with low DNA-doses and shorter incubation times still more vectors get in contact with the cells. In contrast to the results in fig. 74 and 75, Plank et al. (Plank et al., 2003c) showed (in fig 6 D) that 10 min GenePorter-magnetofection of NIH 3T3 cells was not significantly more efficient in gene transfer than 4 h standard GenePorter transfection due to toxicity at higher DNA doses (0.05 and 0.1 µg DNA/well of a 96-well plate).

In all transfection methods used, gene transfer efficiency increased with DNA dose. Only in figure 75 at 0.05 µg DNA/well, LF-DNA magnetofections and the standard LF-DNA transfection seemed to approach a point of saturation.

The lowest efficiencies were observed in the absence of a magnetic field. Efficiency improved with incubation time, explained by the correlation of incubation time and the number of vector-cell contacts.

In summary, from the experiments with different DNA-doses it can be concluded that **in dependence on the vector type and probably other factors** (like e.g. the cell line used and the confluency of cells during experimentation) **in many cases magnetofection is much**

more efficient than standard transfection and DNA-doses and incubation times can be reduced significantly. In other cases, especially at higher DNA-doses, standard transfection can be as efficient in gene transfer as magnetofection, but only with longer incubation times.

3.3.6 Localization of gene transfer using the magnetofection method

As shown in the previous experiments, magnetofection is a very efficient method for transfections in cell culture. A further advantage of magnetofection, especially *in vivo*, could be the localization of gene transfer to the site of magnetic field influence.

As a model for *in vivo* gene delivery, it was tested whether gene vectors can be targeted to a selected area of a target tissue by magnetic force. Therefore, transfections of NIH 3T3 cells (kept in serum-containing medium) were carried out with the LacZ reporter gene in a six-well plate. Two wells were incubated for 15 min with biotinylated (b)PEI / DNA / biotinylated inactivated adenovirus (bAdv) / streptavidinylated trMAG-PEI (trMAG-PEI-Sta) complexes and meanwhile a permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed underneath one of these two wells. As an additional control, the third well was incubated for 15 min with AVET complexes (bPEI / DNA / bAdv, without trMAGs) and no magnetic field was applied. A DNA dose of 6 µg DNA/well, bPEI-DNA (N/P = 8) and a trMAG / DNA (w/w) ratio of 1 was used. The complexes were prepared in HBS. After incubation with complexes, the cells were washed and fresh complete medium was added. After 24 hours the cells were subjected to X-gal staining for 45 min.

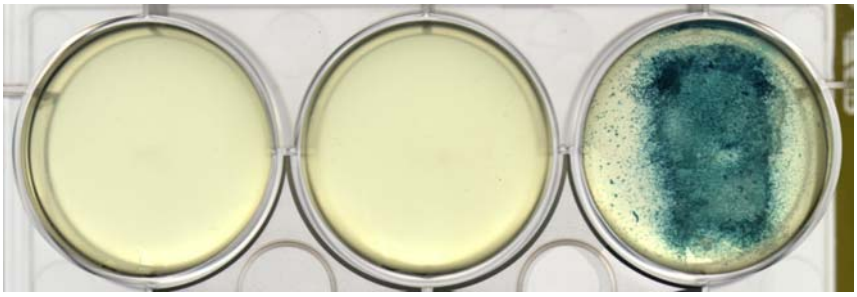


Figure 76 Localization of Lac Z gene delivery by a magnetic field. The NIH 3T3 cells in the right well were incubated for 15 min with bPEI / DNA / bAdv / trMAG-PEI-Sta complexes and a rectangular magnet was applied. As controls without magnet, cells in the well in middle were incubated with the same vector formulation and cells in the left well with AVET (bPEI / DNA bAdv) complexes. Cells stained in blue indicate reporter gene expression. Macroscopically, only in the right well blue cells were visible and gene delivery was confined to an area defined by the shape of the applied magnet and its gradient field.

This experiment showed that the magnetofection method (i.e. using trMAG containing complexes and a magnetic field) enables the **targeting of gene transfer** to a selected area within the well. The explanation is that the magnetic gradient field induces a movement of the

paramagnetic vectors towards the highest density of magnetic field lines. Therefore when a permanent magnet is placed underneath the cell culture dish, the vectors accumulate on cells reflecting the shape of the applied magnet and its gradient field.

3.3.7 Magnetofection of other cells

In all magnetofection experiments so far, either the mouse fibroblast cell line NIH 3T3 or the chinese hamster ovarian cell line CHO-K1 (both see e.g. in fig. 73) or the human hepatic carcinoma cell line HepG2 (see e.g. figure 52-55) was used. Therefore it was interesting to find out if magnetofection would be also successful in transfecting other types of cells like e.g. the human keratinocyte cell line HaCaT, primary human keratinocytes or the mouse radiation-induced fibrosarcoma cell line RIF-1.

3.3.7.1 HaCaT cells

HaCaT cells (cell line derived from human keratinocytes), kept in serum-free medium, were incubated for 4 hours with the magnetofectins trMAG-16/1 / DNA / PEI and trMAG-16/1 / DNA / GenePORTER (GP) and the corresponding standard vectors PEI-DNA and GP-DNA. During the time of incubation, a magnetic field was applied. The vectors were formulated with 0.1 µg DNA/well, a trMAG/DNA w/w ratio of 4, a N/P ratio of 8 and 5 µl GP/µg DNA. Complexes containing PEI were prepared in 150 mM NaCl solution and complexes containing GP in serum-free cell culture medium.
After incubation with complexes, the cells were washed and fresh serum-free medium was added. The next day, the reporter gene expressions of magnetofected and standard transfected cells were determined.

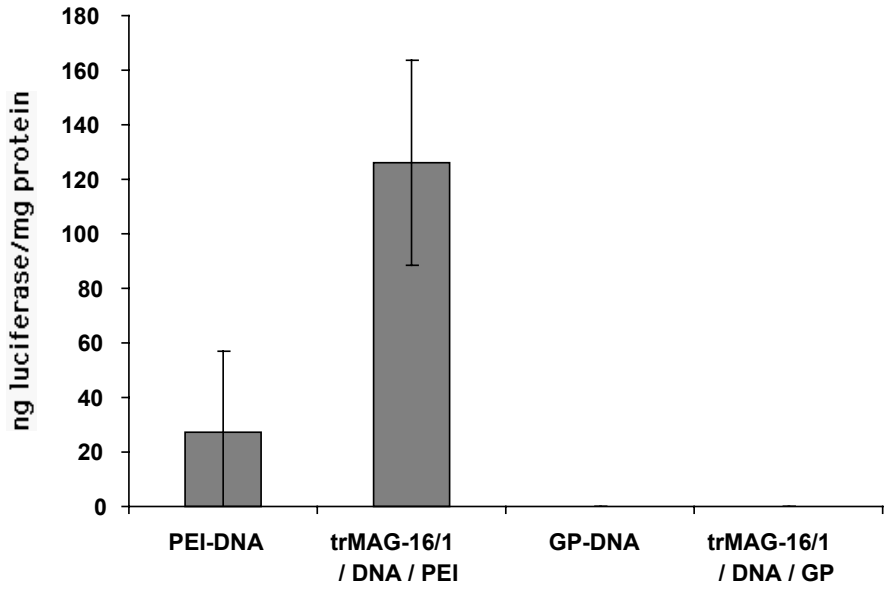


Figure 77 Transfection of HaCaT cells in the presence of a magnetic field. Each transfection was carried out in triplicates.
Only the PEI based vectors lead to detectable luciferase gene expression whereas the formulation containing magnetic beads (trMAG-16/1) was the most efficient.

The experiment showed that the **magnetofection** method was **successful in transfecting HaCaT** cells but only with PEI as endosomolytic additive and not with the cationic lipid GenePORTER. Standard PEI-DNA complexes lead to luciferase gene expression as well but trMAG-16/1 / DNA / PEI vectors in the presence of a magnetic field were nearly 5 times more efficient. It appears that **magnetofection leads to an increase in transfection efficiency only if already the parental vector is able to successfully transfect the target cells**. It can be concluded that the efficiency of magnetofection is strongly dependent on the parental vector.

3.3.7.2 Primary human keratinocytes

As an example for primary cells (which are usually harder to transfect than cell lines) primary human foreskin keratinocytes, kept in serum-free medium, were chosen. These cells were incubated with trMAG-16/1 / DNA / PEI (1 µg DNA/well, trMAG/DNA w/w ratio = 2, N/P = 8, preparation in 150 mM NaCl) complexes. Different times of incubation with vectors (10’ or 4 hours) and variations in the time of magnetic field exposure (10’, 4 hours or no magnet) were tested to approach the optimum conditions. Each transfection was carried out in triplicates. After incubation with gene vectors, the cells were washed and fresh serum-free medium was added. The next day, reporter gene expression of the transfected cells was determined.

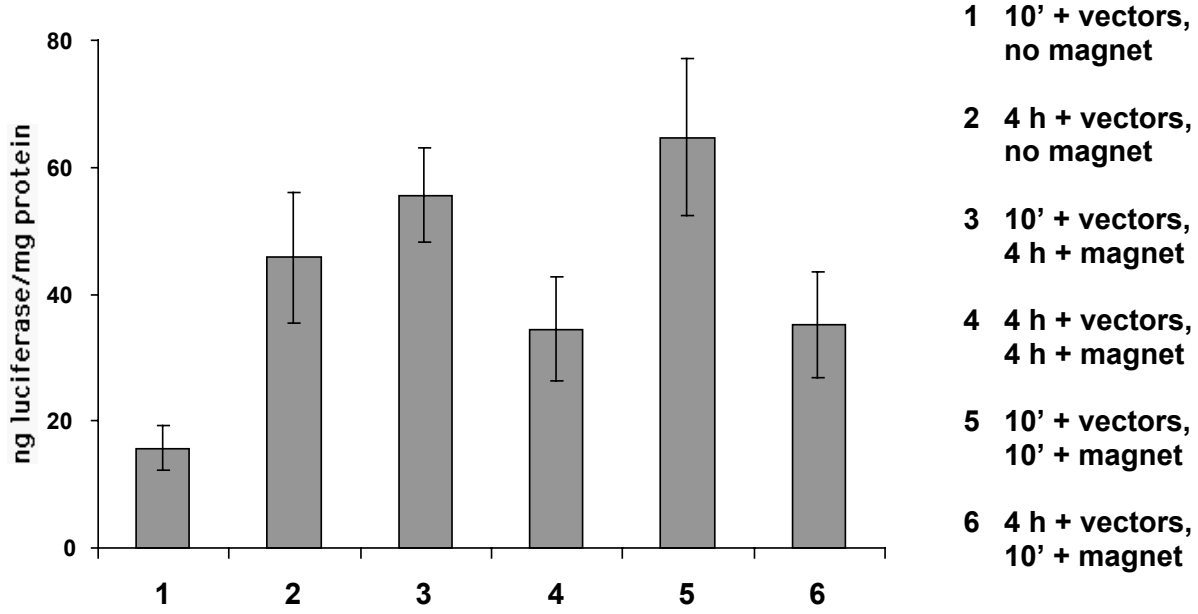


Figure 78 Transfection of primary human keratinocytes with trMAG-16/1 / DNA / PEI as vector. The time of incubation with these complexes and the time a magnetic field was applied is indicated in the graph.
The most important result was that the magnetofection method is able to transfect these primary cells. But also transfections with magnetofectins without magnetic field (1 and 2) showed luciferase gene expression whereas longer incubation (4 h) with vectors led to a significant enhancement because the number of vector-cell contacts by Brownian motion and by sedimentation increased. With magnetofection method 5 (10' + vectors, 10' + magnet) and 3 (10' + vectors, 4 h + magnet) the highest values for gene expression were achieved but there was no significant enhancement compared to 4 h incubation with vectors and no magnet (2). The longer exposure to a magnetic field in method 3 compared to method 5 did not significantly change reporter gene expression indicating that the magnet has no additional effect on transfection apart from magnetic sedimentation. The lower values with magnetofection method 6 and 4 indicate that a 4 h incubation with complexes and the additional influence of a magnet for 10' or 4 h may reduce reporter gene expression due to toxicity effects.

Magnetofection was **successful in transfecting primary keratinocytes**. Additional exposure to a magnetic field after incubation with vectors (3) did not significantly change reporter gene expression, indicating that the magnet has no additional effect on transfection apart from magnetic sedimentation. This finding was confirmed by experiments of Huth et al. (Huth et al., 2004).

But longer incubation times (4 h) with magnetofectins plus application of a magnetic field (6 and 4) lowered the luciferase gene expression probably due to toxic effects to the primary cells which are usually more sensitive than cell lines. On the one hand, this gene expression reducing effect is obvious from the graph but on the other hand the reduction of gene expression due to longer incubation times plus the influence of a magnet was always lower than 2-fold which is not very significant.

Further, incubation with magnetofectins for 4 h and no magnet (2) led to a gene transfer efficiency which was not significantly lower than the one achieved with the most successful

magnetofection methods (5 and 3). An explanation could be that the high concentrations of gene vectors on cells, obtained by magnetic sedimentation, are toxic and therefore with magnet there is no significant enhancement. But with magnetofection it is possible to achieve the same gene expression levels with only 10 min incubation (5) whereas without magnetic field (2) a 4 h-incubation time with complexes is necessary.

3.3.7.3 RIF-1 cells

Mouse radiation-induced fibrosarcoma (RIF-1) cells (kept in serum-containing medium) were incubated for 30 min with the magnetofectins trMAG-16/1 / DNA and trMAG-16/1 / DNA / PEI (1 µg DNA/well, trMAG/DNA w/w = 4, N/P = 8) and meanwhile a magnetic field was applied. Further RIF-1 cells were incubated with the standard vector PEI-DNA (1 µg DNA/well, N/P = 8) for 2 hours. All complexes were prepared in 150 mM NaCl.
After incubation with vectors, the cells were washed and fresh serum-containing medium was added. The next day, luciferase gene expression was determined.

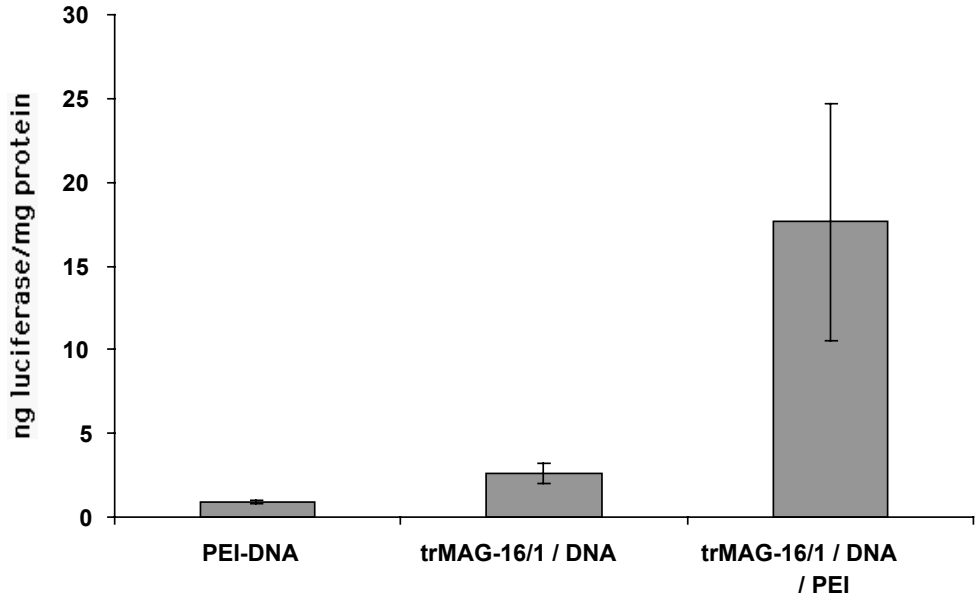


Figure 79 Transfection of RIF-1 cells with trMAG containing vectors in the presence of a magnetic field and for comparison with PEI-DNA. Each transfection was carried out in triplicates.
Magnetofection with trMAG-16/1 / DNA / PEI and trMAG-16/1 / DNA was approximately 20 and 3 times more efficient in luciferase gene transfer than the standard PEI-DNA transfection.

The **magnetofection was successful in transfecting RIF-1** cells and a 30 min incubation time with magnetofectins and an applied magnet resulted in clearly higher reporter gene expression than a 2 hour incubation time with the standard PEI-DNA gene vectors.
The addition of free PEI to trMAG-16/1 / DNA complexes enhanced the gene transfer efficiency of the magnetofection method roughly 7-fold.

In summary, the results showed that **magnetofection is useful for a variety of different cell lines and even for primary cells**. Magnetofections with shorter incubation times can be more efficient in gene transfer than standard transfections with longer incubation times. But magnetofection leads only to an increase in transfection efficiency if already the parental vector is able to successfully transfect the target cells.
Meanwhile, the applicability of magnetofection to further cells types was shown by several groups: e.g. in HeLa (human cervix carcinoma) and BEAS-2B (bronchial epithelial) cells (Huth et al., 2004), in 16HBE (human bronchial epithelial) and human or porcine primary airway epithelial cells and an ex vivo porcine airway epithelium organ model (Gersting et al., 2004), in primary HUVEC (human umbilical vein endothelial) and primary porcine aortic endothelial cells (Krotz et al., 2003b), in HT 1080 (human fibrosarcoma) cells (Plank et al., 2003a), in CT 26 (colon carcinoma) cells (Plank et al., 2003c), and in B16F10 (murine melanoma) and primary rabbit chondrocytes and nasal epithelial cells and peripheral blood lymphocytes (Plank et al, unpublished data).

3.4 Magnetofection in animal experiments

From the experiments shown previously it can be concluded that magnetofection is a method which enables efficient and localized gene transfer in cell culture. Even primary cells were transfected successfully by magnetofection. Now, it was interesting if magnetofection is also efficient in animal models and if gene transfer can be localized *in vivo* as well.

3.4.1 Injection into the ear veins of pigs

For proof-of-principle that with magnetofection *in vivo* localized gene transfer is possible and also as a model for gene delivery to endothelial cells, trMAG-PEI / DNA / PEI complexes (prepared in water and subsequently the ionic strength was adjusted to 150 mM NaCl) were infused into the right and left ear vein of 5 pigs and a permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was was attached for 1 hour above the right veins proximal to the injection site. The left ear veins served as controls without magnet. An injection volume was 5 ml, containing a DNA dose of 500 µg, a trMAG/DNA w/w ratio of 1, and a N/P ratio of 8. The dose was injected over a time span of 3 min.
24 hours after injection, the ear veins and other major organs (heart, lung, liver, spleen and kidney) were isolated, blood samples were taken and their reporter gene expressions (in pg luciferase/g tissue) were determined.

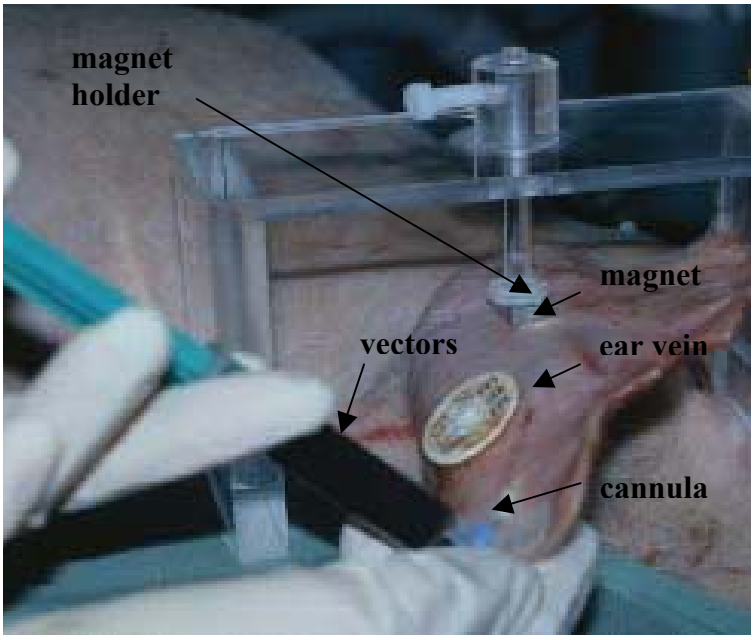
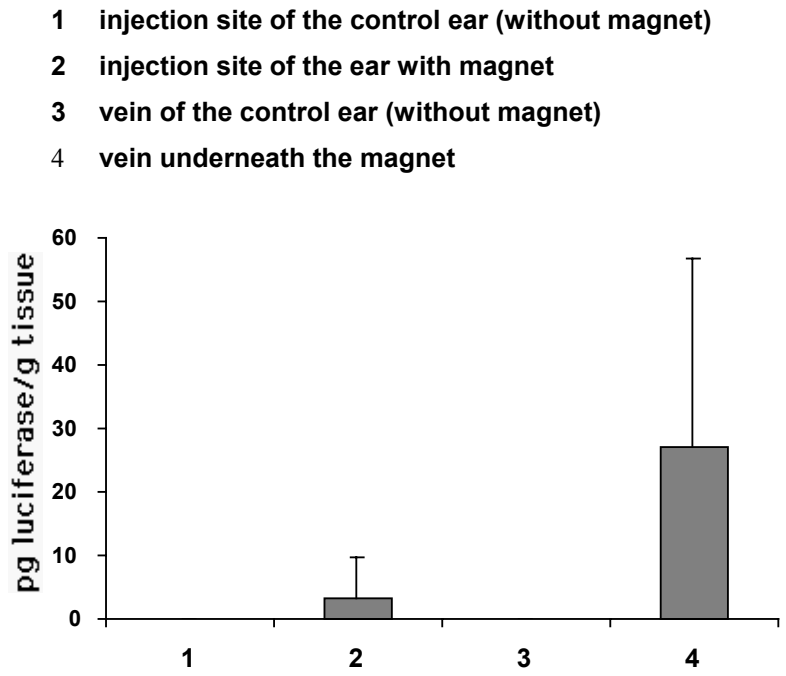


Figure 80 Experimental set-up:
A permanent magnet, attached to a plunger adjustable for height, was placed above the ear vein of a pig without causing any pressure on the blood vessel. Subsequently, paramagnetic gene vectors (brown suspension in the syringe) were injected via a cannula into the ear vein upstream of the magnet.



animal no.	site 1	site 2	site 3	site 4
1	0	14.6	0	77.8
2	0	0	0.2	11.9
3	0	0	0	7.2
4	0	0.1	0	8.7
5	0	1.3	0	29.3
av	0	3.2	0.0	27
stdev	0	6.4	0.1	29.8

Figure 81 Luciferase gene expression in the ear veins of pigs after injection of magnetofectins with or without a magnet placed downstream. The graph shows that on the average without magnet (1 and 3) no significant gene transfer was monitored whereas with magnet, maximum values were obtained underneath the magnetic field (4). Single values (in pg luciferase/g tissue) of each pig are given in the table. Only the area of the ear vein which was under direct influence of the magnetic field (4) showed reporter gene expression in all 5 pigs.

This animal experiment was performed in collaboration with Ulrike Schillinger, TU Munich (Schillinger, 2002).

No reporter gene expression was observed in the control blood vessels (except in site 3 of pig 2) and in the samples of any major organ or blood, while reproducible, though variable luciferase expression was found in all vein samples which were lying underneath the magnet.

These results indicate that the magnetofection method (including magnetofectins and an applied magnet) enables **localized gene transfer in *in vivo***. It is assumed that the magnetic field holds back the paramagnetic gene vectors and thus enables localized transfection.

An explanation for gene expression in the injection site with magnet in three animals could be that in these cases the cannula tubes were pointing towards the blood vessel wall and therefore gene transfer was enhanced at this site.

3.4.2 Injection into the ear artery of rabbits

As further proof-of-principle for localized gene transfer *in vivo* via magnetofection, trMAG-16/1 / DNA complexes (prepared in 5% glucose) were infused into the right and left ear artery of 2 rabbits and a permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed for 1 hour above the right arteries downstream to the injection site. As controls without magnet, the left ear arteries were used. An injection volume was 1.5 ml, containing a DNA dose of 200 µg and a trMAG/DNA w/w ratio of 4. The dose was injected over a time span of 1 min.

24 hours after injection, the ear arteries were isolated and their reporter gene expressions (in pg luciferase/g tissue) were determined.

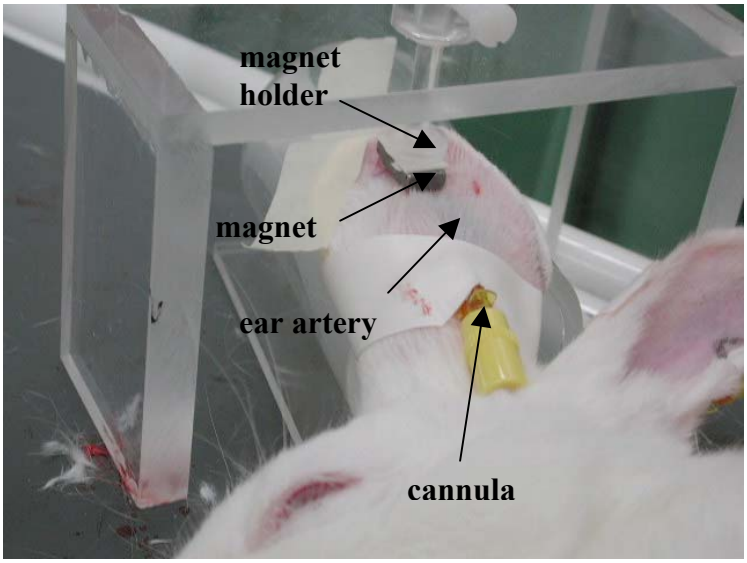
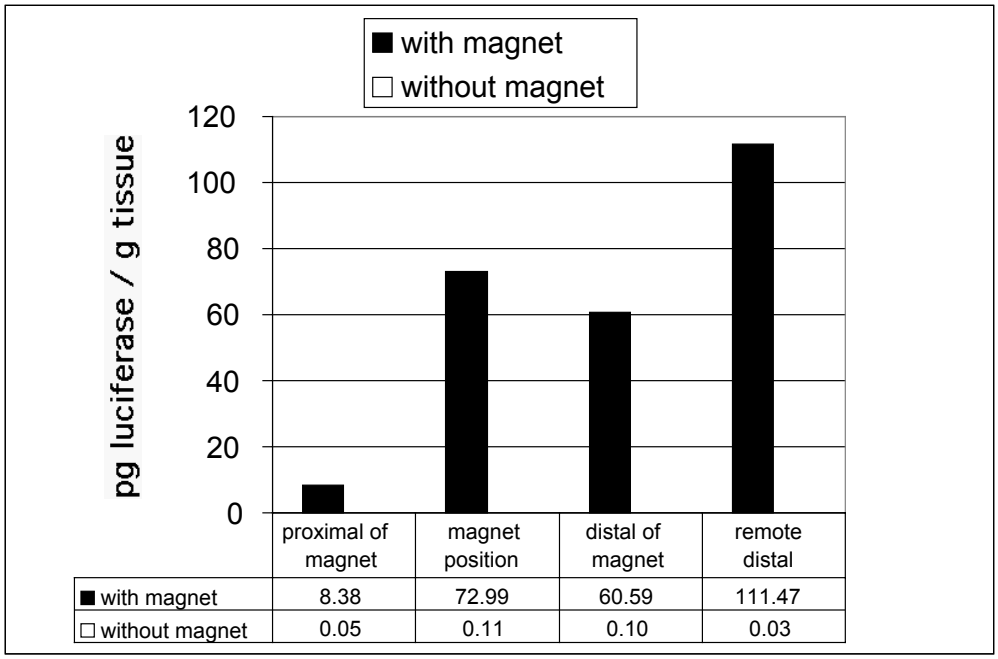


Figure 82 Experimental set-up:
A permanent magnet, attached to a plunger adjustable for height, was placed above the ear artery of a rabbit without causing any pressure on the blood vessel. Subsequently, paramagnetic gene vectors were injected via a cannula into the ear artery upstream of the magnet.



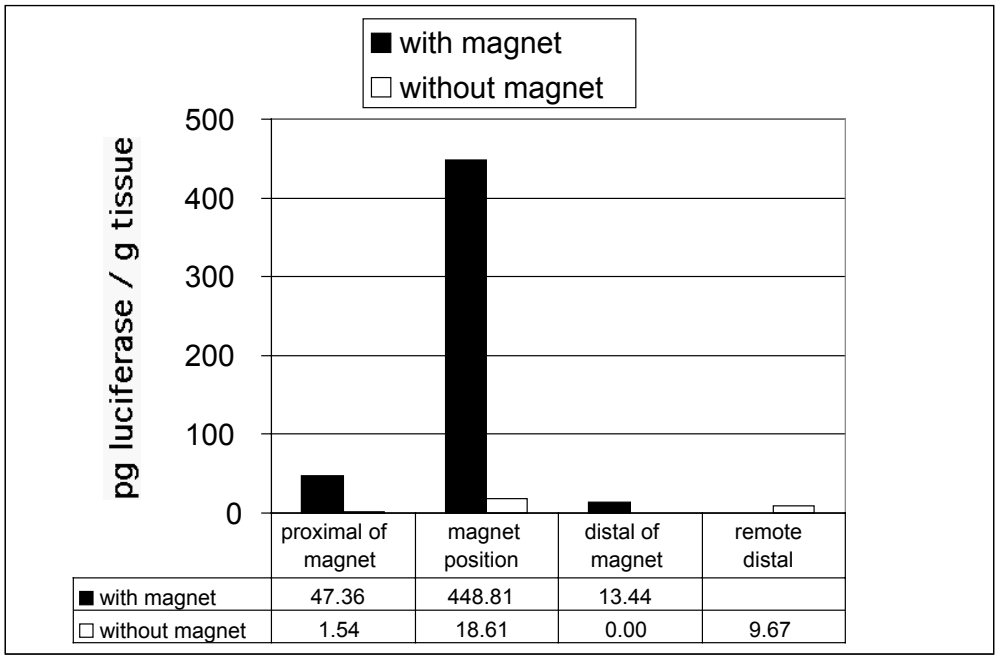


Figure 83 Luciferase gene expression in the ear arteries of rabbits after injection of magnetofectins with or without a magnet placed downstream. Two single experiments are shown, one in the upper and one in the lower graph.

Upper graph: With magnet, gene expression was higher in all positions than without magnetic field. With magnet, maximum expression was obtained remote distal of the magnet, the second highest gene transfer efficiency was achieved in magnet position and distal the transfection efficiency was also comparatively high. An explanation for these results could be that with magnet, gene vectors were accumulated in the magnet position, but after removal of the magnet aggregated gene vectors were washed away and were trapped in arterioles (distal) and the capillary bed (remote distal). Further, with magnet, the proximal value was higher than without magnet, probably because the proximal position is still influenced by the magnet.

Lower graph: The highest efficiency in gene transfer was detected in the magnet position. In this area the magnetic field enhanced the transfection efficiency 24.1-fold compared to without magnet. Obviously, in this experiment with magnet gene vectors were accumulated in the magnet position, but possibly due to anatomic characteristics of the artery, removal of the magnet did not lead to massive release of gene vector aggregates from the magnet position. Further, with magnet, gene expression was clearly higher than without magnet in the proximal position. In the set-up with magnet, the remote distal sample was added to the distal sample and therefore the expression “remote distal” is included in the value “distal”.

These animal experiments were performed in collaboration with Ulrike Schillinger, TU Munich.

In both experiments, the application of a magnet (magnetofection method) led to strongly enhanced gene expression in magnet position. An explanation could be rapid aggregation of trMAGs and PEI-DNA particles after injection into the salt- and protein-containing blood and subsequent attraction to the site where the magnetic field lines have the highest density (namely in the magnet position). But also proximal, an applied magnet led to significant enhancements. An explanation for this phenomenon could be that even if proximal the density of magnetic field lines is much lower, some paramagnetic gene vectors can still be trapped. The maximum gene expression remote distal of the magnet and the high value distal of the magnet in the upper graph could be caused through vector aggregates which were washed away from the magnet position by the blood stream after removal of the magnet. These aggregates could then be trapped downstream in arterioles (distal) and the capillary bed (remote distal).

In summary, in these two experiments with magnetofection, **efficient gene transfer** and a **tendency to localized gene expression** was observed even in arteries which are under higher pressure than veins.

3.4.3 Injection into the ilea of rats

For proof-of-principle that magnetofection *in vivo* enables localized gene transfer even in organs with harsh conditions for transfections, magnetofectins were injected into the ileum lumens of rats where degradative enzymes, degraded nutrition and bacteria are located. The high frequency of malignancies in the gut makes it an important target for gene therapy.
After laparatomy of rats, the ilea were exposed and a section of 3 cm was rinsed with isotonic saline, clamped off and trMAG-16/1 / DNA vectors in 1 ml 5% glucose (DNA dose: 200 µg DNA/ml, LacZ reporter gene, trMAG/DNA w/w ratio: 2) were injected. A permanent Neodymium-Iron-Boron magnet (NeoDelta; remanence Br, 1080-1150 mT; purchased from IBS Magnet, Berlin, Germany) with 20 x 10 x 5 mm was placed under the clamped-off region. Five minutes after injection, both clamps were removed and the magnet was left for a total of 20 min. As control, the same procedure was performed but without magnet. Subsequently, the guts were returned into the abdominal cavity. After 48 h the treated region

of the gut and adjacent areas were isolated, fixed in formaldehyde/glutaraldehyde/PBS, X-gal stained, paraffin embedded and histological sections were stained with eosin.

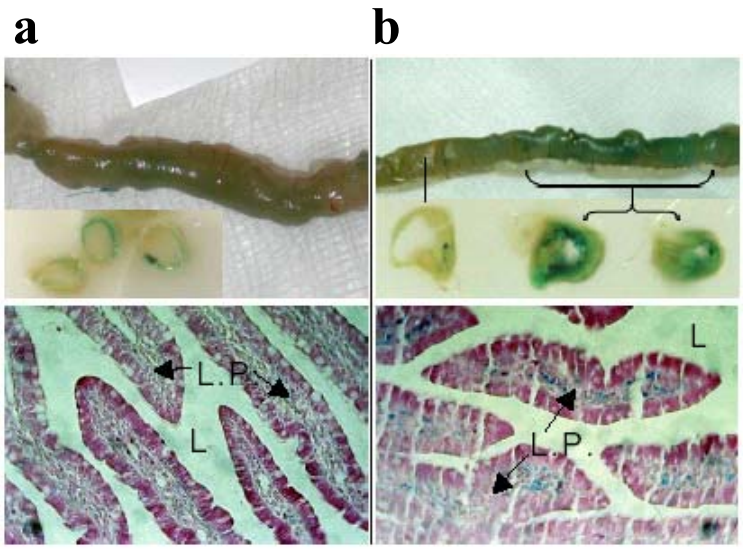


Figure 84 X-gal staining performed 48 h after trMAG-16/1 / DNA vectors were applied to the ilea of rats in the absence (a) and under the influence of a magnetic field for 20 min (b).
Blue staining reveals efficient gene delivery only in the presence of magnet (b), both on the macroscopic level (upper panel) and on the microscopic level (lower panel). Upper panel: intestinal tubes after X-gal stain. Inserts: cross-sections of tubes embedded in paraffin. Lower panel: Paraffin sections counter-stained with eosin, 400x magnification. X-gal staining is found in the lamina propria.
L, lumen; LP, lamina propria.

This animal experiment was performed in collaboration with Julia Henke and Ulrike Schillinger, both TU Munich.

Strong and consistent X-gal staining was found in the area of the tissue which was under influence of the **magnetic field** whereas in the absence of a magnet (untreated control tissue) much weaker staining was observed. The efficient transfection with magnet was confined to the ileum lamina propria.

This result indicates that **magnetofection enables localized gene transfer even in organs with harsh conditions for transfections like e.g. the gut**.

Like in the experiment before (injection into the ear artery of rabbits), the trMAG-16/1 / DNA formulations were prepared in 5% glucose. But as shown previously in the binding studies, trMAG-16/1 particles were not able to bind DNA in salt-free solution with a trMAG/DNA w/w ratio of 2. But nevertheless, gene transfer via magnetofection worked. An explanation could be that from previous rinsing with isotonic saline, salt remained in the ileum and thus trMAG-16/1 particles and PEI-DNA complexes could aggregate before the magnet was applied. Actually, in the clamped-off region there was a similar scenario as in cell culture magnetofections with vectors prepared in glucose. Further, degradative enzymes (e.g. nucleases) in the ileum were not able to fully inactivate the paramagnetic gene vectors.

From all the animal experiments it can be concluded that efficient and localized gene transfer *in vivo* is possible.

4 DISCUSSION

4.1 Background and objective of the thesis

A major barrier to clinical application of nucleic acid therapy is that **only a fraction of the applied vector dose gets in contact with the target cells**. As nucleic acid delivery is a mass action process (Zabner et al., 1995), an increase in vector concentration at the target site would lead automatically to enhanced transfection efficiencies. This phenomenon can be shown in cell culture experiments. *In vitro* transfection is at least partly a diffusion-controlled process and acceleration of vectors towards the target cells leads to a local increase in vector concentration and the result is a great enhancement of nucleic acid delivery. For example by adding dense silica nanoparticles to nucleic acid vectors, gravitational force is used to sediment particles onto cells and the transfection efficiency is increased significantly (Luo and Saltzman, 2000). Analogously, the enhanced sedimentation of larger vector particles (Ogris et al., 1998), the formation of precipitates (Jordan and Wurm, 2004) and the use of centrifugal force (Bunnell et al., 1995) lead to an increased number of vector-cell contacts and contribute to enhanced nucleic acid transfer efficiencies. Additionally, convective flow of transfection medium towards target cells enhances the transfection efficiency (Chuck et al., 1996).

A further critical point in nucleic acid vector delivery is that **systemic distribution can cause toxicity in nontarget organs**. For example the overexpression of suicide genes can result in undesired cell death in tissues which are not the therapeutic target (van der Eb et al., 2004).

Generally, it is difficult to achieve an effective local dose at the target site without causing systemic toxicity. This problem exists not only for nucleic acid vectors but also for classical low molecular weight drugs like e.g. cytostatics. A very promising physical method to target anti-cancer drugs *in vivo* is provided by **magnetic drug targeting** (see 1.6.9 and 1.8). In this method, the anti-cancer drugs are bound to magnetic particles and guided by an external magnetic field to the target tissue.

The major objective of this thesis was to **apply the principle of magnetic drug targeting for the delivery of nucleic acids** which are high molecular weight molecules with a high number of negative charges.

4.2 Binding of nucleic acids to magnetic particles

A fundamental prerequisite for combination of magnetic drug targeting with nucleic acid therapy is the binding of nucleic acid vectors to magnetic particles in a way that enables targeting plus functionality of the nucleic acid drug. In principle, possible ways of binding are biological binding (e.g. via streptavidin-biotin or antigen-antibody bridges), chemical-covalent binding and physical binding (via electrostatic or van der Waals interactions). In this thesis we concentrated on physical binding as it is reversible which may be advantageous for intracellular processing of the nucleic acid vector. For this purpose Christian Bergemann from Chemicell (Berlin) developed superparamagnetic iron oxide nanoparticles coated with cationic or anionic polymers (called trMAGs). Especially the coating with PEI seemed promising because it is an excellent transfection reagent (Demeneix et al., 1998).

Binding studies using magnetic sedimentation of radioactive labelled DNA revealed that naked plasmid DNA can be bound efficiently to positively charged magnetic particles by **electrostatic interactions** (fig. 2). But as soon as additional components like e.g. PEI or DOTAP-Cholesterol are present, efficient electrostatic binding of DNA (vectors) to positively and negatively charged magnetic particles in pure water is not possible (chapter 3.2). Efficient electrostatic binding of DNA to charged magnetic beads in the presence of a third component is only possible via **salt-induced colloid aggregation** (chapter 3.2), a natural process where charged particles (like e.g. charged magnetic beads and charged PEI-DNA or DOTAP-Cholesterol-DNA particles) associate with each other at physiological salt concentration (150 mM NaCl) to form supramolecular aggregates (Hiemenz, 1986). Salt-induced aggregation provides a simple and efficient method for the binding of nucleic acids and additional components to charged magnetic particles. A problem might be that injection of larger aggregates into blood vessels could lead to embolism or that e.g. serum proteins could cause dissociation of small aggregates. But as size measurements showed (fig. 3), the (appropriate) size of aggregates can be chosen by their incubation time in 150 mM NaCl.

Contemporaneous to our work, Hughes et al. found three different biological strategies to bind retroviral vectors to magnetic particles (Hughes et al., 2001). They conjugated streptavidinylated magnetic particles to (i) a biotinylated antibody directed against the retroviral vectors (ii) biotinylated lectin which binds to retroviral vectors and (iii) biotinylated retroviruses. Later, also Pandori et al. used streptavidin-biotin bridges to couple their magnetic particles to adenoviral vectors (Pandori et al., 2002) and Mah et al. used (magnetic) avidin-microspheres to bind biotinylated heparan sulfate which was reversibly bound to

adeno-associated viruses (Mah et al., 2002). A further physical strategy was employed by Haim et al. who formed complexes between lentiviral vectors and negatively charged magnetic beads by colloidal clustering which was facilitated by positively charged ions (Haim et al., 2005). In general, more stable conjugations like biotin-streptavidin or antigen-antibody bridges are assumed to be advantageous for efficient targeting whereas reversible binding e.g. via physical interactions may facilitate intracellular processing of nucleic acid vectors. Further, molecules like streptavidin could stimulate immune responses when applied *in vivo*. But at least *in vitro*, all these binding methods enable magnetic targeting plus functionality of the nucleic acid vectors and future studies (under *in vitro* and *in vivo* conditions) will reveal which way of binding is the most appropriate for applications in research and therapy. In any event, the way of magnetic vector assembly chosen and optimized in this thesis, self assembly by physical interaction was sufficient to achieve magnetic nucleic acid targeting *in vitro* and *in vivo*.

4.3 Transfections with magnetic particle/DNA associates

Having established this simple way of magnetic vector assembly, the question was if nucleic acid transfer with magnetic particles is possible and if magnets placed underneath cell culture dishes could improve transfection efficiencies.

For this purpose transfections in cell culture with various positively and negatively charged magnetic particles were carried out in the presence and absence of a magnetic field (3.3.1 and 3.3.2) and the results showed that **nucleic acid transfer via magnetic particle/DNA associates (magnetofectins) is possible and that an applied magnetic field enhances gene expression**. Transfection with magnetofectins in the presence of a magnetic field is named "**magnetofection**".

Among the positively charged magnetic beads, particles coated with multilayers of PEI and unbound PEI in suspension show the highest transfection efficiencies (3.3.1.1). An appropriate magnetic particle/DNA (w/w) ratio is between 1 and 4. But not only the preparation in 150 mM NaCl which allows association of magnetic particles, DNA and PEI by salt-induced aggregation but also the preparation in 5 % glucose leads to gene transfer. The explanation is that despite inefficient electrostatic binding of DNA to charged magnetic beads in the presence of free PEI, the charged particles associate with emerged charged PEI-DNA particles when the vectors are added to the salt- and serum-containing cell culture medium for transfection. But complexes prepared in 150 mM NaCl are more efficient in gene transfer than

vectors prepared in 5 % glucose, presumably because they have more time to aggregate (not only in the salt- and serum-containing medium but also during incubation in 150 mM NaCl) and larger aggregates are shown to be more efficient in cell culture transfections (Ogris et al., 1998). The **enhancing effect of free PEI and preparation in 150 mM NaCl on transfection efficiency** could be confirmed by transfections with positively charged magnetic particles which show gene transfer only in the presence of free PEI plus preparation in salt solution (3.3.1.2).

In addition, transfections with negatively charged magnetic particles (e.g. coated with polyaspartic acid) plus preformed PEI-DNA complexes (N/P = 8) led to similar efficiencies in gene transfer as positively charged magnetic beads (3.3.2), which means that both types of magnetic particles are useful for magnetic field guided nucleic acid delivery (magnetofection).

4.4 Mechanism of magnetofection

Transfections in cell culture revealed that nucleic acid transfer via magnetic particle/DNA associates (magnetofectins) into cells is possible and that a magnetic field improves gene expression (3.3.1 and 3.3.2). But what is the mechanism of magnetic field-guided nucleic acid delivery (magnetofection)? Are the paramagnetic vectors pulled into the cell by the applied magnetic force? Does endocytosis play any role? Is it possible that the permanent magnet alone (without magnetic beads) has an enhancing effect on the reporter gene expression?

A first interesting finding was that free PEI enhances the efficiency of magnetofectins in magnetofections (3.3.1.1 and 3.3.1.2). A possible explanation for this enhancement was that via the proton sponge effect PEI promotes the release of DNA which is captured in endosomes after its cellular uptake by endocytosis (Sonawane et al., 2003). But to find out if endosomal escape and consequently endocytosis do really play a role in magnetofection, it was examined if magnetofectins containing endosomolytic additives are generally more efficient in magnetofection than magnetofectins lacking these components. Apart from PEI, a chemically inactivated adenovirus, the cationic lipid Lipofectamine, the cationic lipid GenePORTER or the synthetic influenza virus peptide INF7 were used as endosomolytic additives (3.3.3.1) and all of them had an enhancing effect on gene transfer via magnetofection. These results indicate that in magnetofection the cellular uptake mechanism for nucleic acid vectors is endocytosis. But what is the fate of the magnetic particles during magnetofection? Are they taken up by the cell like the nucleic acids or are they left outside?

To answer this question, transmission electron microscopy pictures of magnetofected cells were taken (3.3.3.2). These images showed that the magnetic particles were concentrated around the cell within minutes and immediately their cellular uptake starts. The accumulation of magnetic particles in endosomal structures suggests endocytosis as uptake mechanism and not the traction of paramagnetic vectors through the cellular membrane by magnetic forces. These results were later confirmed by Huth et al (Huth et al., 2004). With gold-labeled DNA they could even show that magnetic particles and DNA are co-internalized into the cell. From transfections with endocytosis inhibitors and fluorescence microscopy, this group came to the conclusion that the uptake mechanism for magnetofectins containing free PEI is similar as for PEI-DNA complexes where clathrin-dependent and caveolae-mediated endocytosis are involved and the extent of involvement is cell line-dependent. The size of magnetofectins may influence the uptake mechanism as well because by using fluorescence-activated cell sorting and fluorescent/confocal microscopy Rejman et al. found that fluorescent latex beads smaller than 200 nm are taken up by the clathrin-dependent pathway, but with increasing particle size there is a shift to the caveolae-mediated internalization which becomes the predominant pathway of entry for particles of 500 nm in size (Rejman et al., 2005; Rejman et al., 2004).

From the enhancing effect of a magnetic field in transfections with relatively short incubation times (10-20 min) with paramagnetic vectors (3.3.1 and 3.3.2) and from the electron micrographs (3.3.3.2) which showed that magnetic particles under influence of a magnet are concentrated around the cell within minutes, it is assumed that through magnetic sedimentation gene vectors get in contact with cells much faster than through diffusion or non-magnetic sedimentation. Now it was interesting to find out if this accelerated sedimentation in the magnetofection method leads to a different time course of reporter gene expression compared to a standard polyplex transfection. Therefore magnetofections with magnetofectins containing free PEI and transfections with PEI-DNA complexes were performed and in magnetofected cells reporter gene expression could be detected much earlier (already after 2 hours) than in standard PEI-transfected cells (after 8 hours). Additionally, magnetofection consistently leads to higher gene expression (3.3.3.3). Presumably, in magnetofection all steps from cellular contact with gene vectors to gene expression proceed similar as with standard polyfection but they proceed earlier and in a synchronized manner (Haim et al., 2005). The higher gene expressions in magnetofection experiments could be explained by higher vector concentrations on the cellular surface.

Finally, the question arose if the enhancing effect of a magnetic field on transfections with paramagnetic vectors is (only) the result of accelerated sedimentation or if the neodymium-

iron-boron (Nd-Fe-B) magnet used in all our experiments influences cell physiology in a manner that enhances transfection and/or reporter gene expression. It is well documented that low frequency electromagnetic fields (EMFs) and static magnetic fields can have biological effects on cells and tissues. It is assumed that their primary site of action is the plasma membrane (Pagliara et al., 2005; Rosen, 2003) and e.g. phagocytosis (Flipo et al., 1998; Mykhaylyk et al., 2005) or cellular metabolic activity (Sabo et al., 2002) can be reduced by magnetic fields. Further, it is known that EMFs activate genes under control of EMF-sensitive promoters (Goodman and Blank, 2002), that static electromagnets induce the expression of oncogenes (Hiraoka et al., 1992; Hirose et al., 2003) and that a permanent static neodymium magnet (300 mT) changes the expression of some genes in Escherichia coli (Potenza et al., 2004). Therefore we performed transfections with various non-magnetic standard vectors and our permanent static Nd-Fe-B magnet (1080-1150 mT) was applied simultaneously (3.3.3.4). The results showed that application of the permanent static magnet (which was used in all experiments in this thesis) does not influence the measured reporter gene expression. Further, Huth et al. used magnetic particle containing vectors to examine the same question (Huth et al., 2004). The magnetofectins were spun down onto the cellular surface and incubated with and without application of a permanent static magnet. But also these experiments with magnetic particle containing vectors showed no detectable enhancement (or decrease) in reporter gene expression when a magnetic field was applied. Thus it can be concluded that the enhancing effect of a magnetic field is mainly the result of accelerated vector sedimentation and not of changes in cell physiology, enhancement of cellular uptake or activation of luciferase gene expression driven by the hCMV promoter.

In summary, from our experiments and the experiments of Huth et al (Huth et al., 2004), the following mechanism is proposed for magnetofection: The **paramagnetic nucleic acid vectors are concentrated efficiently by magnetic force on the cell surface** within minutes and immediately their **endocytotic uptake** starts. Further steps leading to gene expression proceed similar as with standard polyplexes but earlier. Higher efficiency in nucleic acid transfer with magnetofection is probably mainly a result of more efficient sedimentation and therefore higher availability of nucleic acid vectors on the cellular surface for endocytosis.

4.5 Critical parameters in optimizing magnetofection

As it was shown that the principle of magnetofection works (3.3.1 and 3.3.2) and the mechanism was elucidated (3.3.3), the next challenge was to optimize the magnetofection

method in cell culture. From various transfection experiments it is known that the addition of endosomolytic substances (3.3.3.1) and the preparation of vectors in 150 mM NaCl (3.3.1) improve the efficiency of magnetofection. Additionally, mechanistic studies (3.3.3) revealed that in magnetofection cellular uptake of vectors and further steps leading to gene expression proceed similar as in standard transfections, therefore it can be assumed that all components which improve the efficiency of standard vectors (like e.g. nuclear localization signals) lead to an improvement of magnetofection vectors as well.

To further optimize magnetofection, dose-response studies were carried out at different magnetic particle to DNA ratios, the magnetofection efficiency of positively and negatively charged magnetic beads was compared, different sequences of mixing the components of magnetofectins were examined and the incubation time of cells with paramagnetic vectors was varied.

Magnetofections with increasing magnetic particle / DNA (w/w) ratios and various doses of DNA (3.3.4.1) revealed that for each type of vector (here magnetofectins including either the cationic lipid DOTAP-Cholesterol, GenePorter or Lipofectamine) the individual **optimum magnetic particle / DNA (w/w) ratio and DNA dose** has to be found. But a magnetic particle / DNA (w/w) ratio of 2 turned out to be very efficient in all the examined types of paramagnetic vectors. Within the ranges of DNA doses tested (which comprised relatively low doses) increasing amounts of DNA lead to increasing magnetofection efficiency in most cases. In general, increasing amounts of magnetic particles or (and) DNA enhance the availability of vectors for cells until saturation or even toxicity sets in.

To answer the question if **positively or negatively charged magnetic beads** are more efficient in magnetofection, gene transfer experiments with magnetic particles coated with a monolayer of PEI (trMAG-PEI) and magnetic particles coated with starch-phosphate (trMAG-PO4) were performed under identical conditions (3.3.4.2). But no significant differences in magnetofection efficiency were detected and thus it can be concluded that in regard to efficiency it does not play any role if positively or negatively charged magnetic beads are chosen.

To find out if the **sequence of mixing the components of magnetofectins** influences the magnetofection efficiency, complexes consisting of (i) DOTAP-Cholesterol, DNA and positively or negatively charged magnetic beads, (ii) PEI, DNA and negatively charged magnetic beads and (iii) PEI, DNA, chemically inactivated adenovirus and negatively charged magnetic beads, were examined (3.3.4.3). But when the magnetofectins were prepared in salt-containing solution, no significant differences in magnetofection efficiency were obtained

except for preformed PEI-DNA particles which significantly reduced the efficiency of magnetofection. It is assumed that in 150 mM NaCl aggregates are formed which harbour all components added and therefore the mixing sequence is usually not crucial. The lower magnetofection efficiencies with preformed PEI-DNA particles are difficult to understand and require further examination.

In magnetofection experiments, cells are usually incubated with vectors for 10-20 minutes. To find out the **optimum incubation time**, cells in culture were incubated with cationic lipid (Lipofectamine or GenePorter) containing magnetofectins for 5, 10, 20, 40 and 240 minutes in the presence of a magnetic field (3.3.4.4). The results revealed that with magnetofection already 5 minutes of incubation with vectors prepared in salt-containing solution lead to optimum gene transfer efficiency or to an efficiency close to the optimum. But still, optimum incubation time has to be found out individually for each vector type and presumbly also for each cell type.

4.6 Comparison of magnetofection and conventional transfection methods with regard to their gene transfer efficiency

As already observed in experiments to optimize the incubation time of cells with complexes for magnetofection (figure 71 and 72), five minutes incubation of cells with magnetic particle containing lipoplexes in the presence of a magnetic field (magnetofection) resulted in significantly higher transgene expression than 4 hours incubation with the same lipoplexes without magnetic particles (corresponding conventional or standard transfection). But is magnetofection (in which cells are usually exposed to paramagnetic vectors plus a magnetic field for 30 min or less) always more efficient than the corresponding standard transfection (in which cells are usually incubated with nonmagnetic vectors for 2 to 4 hours)?

To compare the gene transfer efficiency of standard transfections and magnetofections comprehensively (fig. 73), two different cell types (NIH 3T3 and CHO-K1) were incubated for 4 hours with four different standard vectors (PEI-DNA, DNA /PEI / inactivated adenovirus, GenePorter-DNA and Lipofectamine-DNA) and for comparison for 10 minutes with the same vectors but plus magnetic particles and in the presence of a magnetic field (magnetofections). The results showed that with all vector types except GenePorter, the magnetofection method leads to significantly higher gene expression (up to 971-fold) in both cell lines than the corresponding standard transfection. The explanation for the enhanced gene transfer efficiencies with magnetofection is that magnetic sedimentation enables in a short

period of time (here 10 min) more vector-cell contacts than the standard transfection with relatively long incubation times (here 4 hours). This assumption was proven to be true by Gersting et al. (Gersting et al., 2004) who compared magnetofection and standard transfection in regard to adhesion patterns of fluorescently labeled gene transfer complexes on airway epithelial (16HBE) cells by fluorescence microscopy. In contrast to the 3 to 5-fold enhancement in figure 72, magnetofection with GenePorter complexes in figure 73 did not lead to a significant enhancement. This difference may be due to slight variations in transfection parameters such as incubation times during vector preparation, cell density and passage number at the time of transfection. In general, it is assumed that high concentrations of GenePorter-DNA vectors on cellular surfaces lead to saturation of uptake processes or even toxicity and therefore the higher number of vector-cell contacts achieved through magnetic sedimentation do not enhance gene transfer dramatically.

In the experiments mentioned above, the nucleic acid transfer efficiencies of magnetofection and the corresponding standard transfection were only compared at one DNA dose each. The next interesting question is how these two methods compare at different DNA doses. Therefore dose-response profiles of magnetofection and standard transfection with Lipofectamine-DNA complexes in two cell lines (NIH 3T3 and CHO-K1) were established and compared (figure 74 and 75). Over the range of DNA doses tested (from 0.0125 to 0.1 µg DNA/well), with equal DNA doses magnetofection showed always significantly higher gene expression than the corresponding standard transfection. Additionally, magnetofections with lower DNA doses can be more efficient than standard lipofection with much higher doses. For example in CHO-K1 cells (figure 75), magnetofection with an incubation time of 10 minutes achieved with 0.0125, 0.025 and 0.05 µg DNA/well 6.3, 43.2 and 336.8-fold higher gene expression than the standard transfection with 0.1 µg DNA/well and an incubation time of 4 hours. Obviously, even if higher DNA doses and longer incubation times significantly increase the the number of vector-cell contacts by Brownian motion and sedimentation in standard transfections, in some cases with magnetic sedimentation with low DNA doses and shorter incubation times still more vectors get in contact with the cells.

In addition to the experiments presented in this thesis, a number of colleagues from our institute and from other groups compared the efficiency of magnetofection and standard transfection as well. Gersting et al. found that in airway epithelial cells (16HBE cell line and primary cells) magnetofection was, with an incubation time of 15 min, more or at least equally efficient in gene transfer than standard PEI-polyfection with a 4 h incubation time. Further, magnetofection improved the DNA dose-response relationship significantly (Gersting

et al., 2004). Improved transfection efficiencies and DNA dose-response profiles through magnetofection were also observed with the lipofection reagent Metafectene in NIH 3T3 cells (Plank et al., 2003a), with various cationic lipids and PEI in primary human umbilical vein endothelial cells (HUVECs) (Krotz et al., 2003b) and with the lipofection reagent DMRIE in CT26 cells (Plank et al., 2003c). In the latter experiment, Plank et al. further demonstrated that not only the overall transgene expression but also the percentage of transfected cells can be enhanced by magnetofection. In a further publication of Krötz et al. (Krotz et al., 2003a), it was shown that magnetofection with various lipid vectors and PEI does not only improve the transfection efficiencies and dose-response relationships with plasmid DNA but also with antisense-ODNs in HUVEC cells. In these experiments Krötz and coworkers also found that magnetofection with its shorter incubation time is less toxic and therefore a useful tool for physiological examinations in sensitive primary cells. But it has to be mentioned that in contrast to magnetofection with FUGENE plus plasmid DNA (Krotz et al., 2003b), magnetofection with FUGENE plus antisense-ODNs was less efficient than the standard FUGENE transfection. Among the many comparisons performed, this was the only case where magnetofection led to a decrease in transfection efficiency. Additionally to DNA, magnetofection also increased the efficiency of transfections of siRNA. Plank et al. (Plank et al., 2003a) demonstrated that efficient knock down of stable eGFP expression in HT1080 cells with linear PEI and synthetic siRNA was only achieved through magnetofection. This result also indicates the potential of magnetofection for nucleic acid transfer into cells which are difficult to transfect with standard methods. The experiments described so far concerned only nonviral nucleic acid vectors, but magnetofection also improved the transduction efficiencies of adenoviruses (Scherer et al., 2002a), retroviruses (Haim et al., 2005; Scherer et al., 2002a) and measles viruses (Kadota et al., 2005).

All the experiments discussed in this chapter compared magnetofection with the corresponding standard transfection or transduction but it would also be interesting to perform side by side comparisons with other physical methods like e.g. centrifugation, convective flow towards the target cells, biolistic methods or electroporation.

In summary, **usually magnetofection is significantly more efficient than standard transfection or transduction**, but there are rare cases in which magnetofection is only equally or even less efficient. The **often improved nucleic acid dose-response profiles and reduced incubation times** with vectors make magnetofection a less material and time consuming method which could be especially useful for automated high throughput screening of genes and of therapeutically useful sequences.

4.7 Localization of nucleic acid transfer using the magnetofection method

The magnetic field-guided sedimentation of paramagnetic vectors enhances significantly the efficiency of nucleic acid transfer into cells in culture. But is a distinct localization of nucleic acid transfer to a certain target area via magnetofection possible as well? As a model, it was tested whether gene vectors (consisting of PEI, plasmid DNA harbouring the LacZ reporter gene, a chemically inactivated adenovirus and magnetic particles) can be targeted to a selected area within a well of a six-well plate. The results (fig. 76) showed that LacZ gene expression was confined to an rectangular area defined by the shape of the permanent magnet placed underneath the well. The same result was obtained by Martina Anton with adenoviruses (Scherer et al., 2002a). Using the same principle as in magnetofection, also Hughes et al. (Hughes et al., 2001), Pandori et al. (Pandori et al., 2002) and Mah et al. (Mah et al., 2002) illustrated impressively the magnetic field-guided localization of reporter gene delivery with retroviruses, adenoviruses and adeno-associated viruses, respectively. Obviously, the magnetic field gradient induces a movement of the paramagnetic vectors towards the highest density of magnetic field lines and therefore the vectors accumulate on cells reflecting the shape of the applied magnet. Therefore magnetic field-guided nucleic acid vector delivery offers e.g. the possibility to evaluate transfected cells compared to the untransfected control cells within the same well or it enables the examination of the influence of secreted transgene-encoded factors on neighbouring untransfected cells.

4.8 Applicability of magnetofection to different cell types

To establish and to examine the magnetofection method, the mouse fibroblast cell line NIH 3T3, the chinese hamster ovarian cell line CHO-K1 (both e.g. in fig 73) and the human hepatic carcinoma cell line HepG2 (e.g. in fig. 52-55) were used. But is magnetofection also succesful in transfecting other types of cells? To answer this question, the human keratinocyte cell line HaCaT, primary human keratinocytes and the mouse radiation-induced fibrosarcoma cell line RIF-1 were transfected via magnetofection. The results (fig. 77-79) showed that magnetofection enables successful transfection of all three types of cells (including also the primary cells, which are usually harder to transfect) but in some cases vector components or exposure times with vectors and magnet have to be optimized to yield satisfying results. Meanwhile, the applicability of magnetofection to further cell types was shown in several publications: for example HeLa (human cervix carcinoma) and BEAS-2B (bronchial

epithelial) cells (Huth et al., 2004), 16HBE (human bronchial epithelial) and human or porcine primary airway epithelial cells and an ex vivo porcine airway epithelium organ model (Gersting et al., 2004), primary HUVEC (human umbilical vein endothelial) and primary porcine aortic endothelial cells (Krotz et al., 2003a; Krotz et al., 2003b), primary rabbit articular chondrocytes (Schillinger et al., 2005), HT 1080 (human fibrosarcoma) cells (Plank et al., 2003a), CT 26 (colon carcinoma) cells (Plank et al., 2003c), K562 (human myeloid leukemia) cells and primary human peripheral blood lymphocytes (Scherer et al., 2002a), B95a (adherent marmoset β-cervical carcinoma) and Vero (African green monkey kidney) and L929 (mouse fibrosarcoma) cells (Kadota et al., 2005), and RAE (primary rat aortic endothelial) cells (Haim et al., 2005) were accessible to magnetofection. Additionally, all cells which were ever tested for magnetofection (more than 90 different cell lines and more than 25 different primary cells) are listed on the homepage of OZ BIOSCIENCES (www.ozbiosciences.com) and the list is regularly updated.

In summary, it can be concluded that magnetofection is an efficient transfection / transduction method for a huge number of different cells, including primary and other hard-to-transfect / transduce cells.

4.9 Magnetofection *in vivo*

As described in the previous chapters, magnetofection is highly efficient in nucleic acid delivery in cell culture and it enables localization of nucleic acid transfer within a cell culture dish (fig. 76). These features make magnetofection attractive for *in vitro* research and for *ex vivo* nucleic acid therapy. But is magnetofection also efficient in localized nucleic acid delivery *in vivo*?

To answer this question, three proof-of-principle experiments were performed. Firstly, magnetofectins (consisting of magnetic particles, DNA and PEI) were injected into ear veins of pigs (fig.81) and a permanent magnet was placed above the blood vessel downstream of the injection site. Secondly, magnetofectins (magnetic particles with free PEI in suspension and DNA) were injected into ear arteries of rabbits (fig. 83) and a permanent magnet was attached on the artery downstream of the injection site. Thirdly, after laparatomy magnetofectins (magnetic particles with free PEI in suspension and DNA) were injected into the ileum lumen of rats and a permanent magnet was placed downstream of the administration site (fig. 84). The results of these three experiments revealed that reporter gene transfer was strongly enhanced in the area under influence of a magnetic field whereas without application of a

magnet (controls) either no or only poor transfection was achieved. Similar results were obtained by two other groups which work in close collaboration with us. Martina Anton and coworkers injected magnetic particle-adenovirus associates into the stomachs (which were exposed after laparatomy) of mice while a permanent magnet was positioned to the outside of the stomach wall. Despite the harsh conditions in the stomach (low pH and degradative enzymes), effective localized reporter gene delivery was obtained, while a control experiment without magnet hardly yielded any gene transfer (Scherer et al., 2002a). Further, Krötz et al. injected fluorescence-labeled antisense oligodeoxynucleotides (ODNs) into the femoral artery of mice and during injection a permanent magnet was applied to one testicle exposed by surgery. Confocal fluorescence microscopy revealed that specific uptake of ODNs was only observed in cremaster muscle blood vessels of testicles which were exposed to a magnetic field (Krotz et al., 2003a). This experiment demonstrated that magnetic-field-targeted nucleic acid delivery is even possible if the the magnetic field-exposed target site is not in the direct vicinity of the administration site.

These proof-of-principle experiments with reporter genes or fluorescence-labeled ODNs demonstrate the feasibility of magnetic-field targeted and –enhanced nucleic acid delivery in blood vessels (endothelial cells) and in the gastrointestinal tract. Hopefully, further experiments will reveal that magnetofection is applicable to many more organs and tissues and especially to tumors. Magnetofection may be useful as an *in vivo* research tool for studying locally the function of genes or expressed proteins (which could e.g. contribute in endothelial cells of blood vessels to the development of atherosclerosis or hypertension) either through introduction of plasmid DNA or through knock down of genes by antisense ODNs or siRNAs. Additionally, magnetofection could have potential for clinical applications through targeted delivery of nucleic acids encoding therapeutic sequences. For example in a tumor therapy, paramagnetic vectors harbouring tumor suppressor genes (e.g. P53), cytokine genes (e.g. GM-CSF) or suicide genes (e.g. HSV thymidine kinase) could be injected either into tumor-feeding blood vessels or directly into the tumor tissue and an applied magnetic field could hold the vectors in the tumor. This retention could enable efficient local transfection or transduction and only vectors which are not retained at the the target tissue (in the ideal case all vectors are retained) can be spread systemically and contribute to systemic side effects, respectively. It remains to be shown that similarly as for magnetic drug targeting with classical anti-cancer drugs (Alexiou et al., 2000), magnetofection is able to enhance therapeutic effects and to reduce undesired side effects.

4.10 The place of magnetofection in the field of nucleic acid transfer and targeting

In vitro, magnetofection enables rapid contact of nucleic acid vectors with cells and it increases the number of vector-cell contacts. In this way, **magnetofection improves conventional (standard) nucleic acid transfer methods like polyfection, lipofection or viral transduction** with regard to efficiency, nucleic acid dose-response profile and reduced incubation time in many cases. Through its enhanced efficiency, magnetofection is able to compensate for low viral titers as shown e.g. for retroviruses (Scherer et al., 2002a) and measles viruses (Kadota et al., 2005). It is assumed that **magnetofection can be applied to any type of vector** and different vector components can be combined in a way that results in an optimum vehicle for a desired application. Additionally, magnetofection is not restricted to one type of nucleic acid but is **applicable e.g. to cDNAs, oligodeoxynucleotides and RNA molecules**. Magnetofection can also enable nucleic acid delivery to otherwise nonpermissive cells (Plank et al., 2003b) like e.g. shown for adenoviral transduction of cells lacking the coxsackie and adenovirus receptor (CAR) (Scherer et al., 2002a) or for measles viral transduction of cells lacking the signaling lymphocytic activation molecule (SLAM) receptor (Kadota et al., 2005). Further, **magnetofection is an ideal tool for the delivery of nucleic acids to difficult-to-transfect / transduce cells** (including primary and eventually also stem cells) and its toxicity is low enough to enable the examination of sensitive cellular gene / protein functions (Krotz et al., 2003a; Krotz et al., 2003b). Considering the shorter incubation times (reduced from hours to minutes) and the improved nucleic acid dose-response relationships, magnetofection may be a method of choice for automated high throughput screening of genes and of therapeutically useful sequences. An interesting application of magnetofection could also be the **ex vivo** delivery of genes encoding immunomodulatory and graft-protecting molecules to organs determined for transplantation. The tight association of vectors to the target cells could improve the gene transfer efficiency and the reduced incubation times may be advantageous as the grafts should be implanted as fast as possible to avoid serious damage to the organ. Despite all these positive aspects of magnetofection, one has to remember that **there are cases in which magnetofection is only equally or even less efficient than conventional transfection** with the corresponding non-magnetic standard vector. In these cases, the increased number of vector-cell contacts may lead to saturation of uptake processes or toxicity and it is also conceivable that the magnetic particles disturb (inhibit) the function of some vector types. Up to date, no experimental side by side

comparison of magnetofection and other physical methods enhancing nucleic acid delivery, such as centrifugation, convective flow towards the target cells, biolistic methods or electroporation, has been carried out. But each of these methods has its merits. Centrifugation and convective flow is simple and not expensive, biolistic methods enable exact localization of delivery and electroporation is highly efficient. Magnetofection is probably less efficient than electroporation, but it is still a very efficient method and it does not require such an expensive equipment. It also does not require tedious handling steps such as in centrifugation or convective flow and is simpler than biolistic methods (Plank et al., 2003a). When compared to all the other physical methods, **the major advantage of magnetofection is that it is able to combine simplicity, non-expensiveness, localization of delivery, enhanced efficiency and reduction of incubation time and of vector doses**.

In vivo, not only the nucleic acid transfer efficiency but especially the targeting of vectors is of great importance. Magnetofection is assumed to be a very promising targeting method. In the ideal scenario, paramagnetic vectors would be injected either into blood vessels or directly into the target tissue and they would accumulate in a magnetic gradient field. The magnetic field enables even extravasation and tissue penetration at the target site for cytostatics (Widder et al., 1978) and also for gene vectors (unpublished observations by Eissner B and Schillinger S, TU Munich, Germany). The localized vector accumulation would provide a high dose of vectors in the target tissue, an increased number of vector-cell contacts and consequently nucleic acid transfer would be locally strongly enhanced. In the ideal case, the paramagnetic vectors would not distribute systemically and toxic side effects could be prevented. In theory, the magnetic field can be applied to any organ or tissue and thus magnetic drug targeting is not limited e.g. to special types of cells harbouring special receptors, like it is the case for targeting via receptor-ligand interactions. In practice, **proof-of-principle experiments revealed that via magnetofection targeted and efficient nucleic acid transfer is possible**. But unfortunately, in practice up to date it is not possible to apply a strong magnetic gradient field to any desired organ or tissue. Additionally, **the magnetic field can be too weak to attract paramagnetic vectors efficiently** in blood vessels with high flow rate or to enable extravasation or tissue penetration. Further possible limitations are e.g. dissociation of the nucleic acids from the magnetic particles before the target site is reached or reduced functionality of vectors irreversibly bound to the magnetic particles. Magnetofection may also not be the first choice for all *in vivo* applications. Only experimental side by side comparisons of magnetofection and other methods of nucleic acid targeting (like e.g. receptor-ligand interactions, local injection of non-magnetic vectors, hydroporation,

aerosolization, ballistic methods, occlusion of the blood outflow from the target organ, transcriptional targeting and even passive targeting) will reveal the value of magnetofection for certain applications. **The ultimate delivery system might even combine the principle of magnetic targeting with methods of passive and active targeting**. Magnetofection could e.g. assist the target accumulation of a delivery system whose biophysical properties alone favour a passive target tropism and which in addition is equipped with active targeting modules such as receptor ligands or nuclear localization signals (Plank et al., 2003a). One could even think of combinations between magnetofection and e.g. transcriptional targeting, local injection, hydroporation or aerosolization.

4.11 Conclusions and outlook

A very exciting **aim of magnetofection is to use it therapeutically**, like e.g. in tumor targeting or local neo-vessel formation. Therefore, recently a veterinary clinical study of immuno gene therapy of feline fibrosarcoma has been started (Schillinger et al., 2005). The gene coding for human GM-CSF (granulocyte macrophage colony stimulating factor) in magnetic formulation was administered twice in a 1 week interval prior to surgery into the biologically active margins of the fibrosarcoma and a permanent magnet was fixed on the tumor adjacent to the injection site during one hour after vector injection. The immunohistochemistry showed that the GM-CSF gene was expressed in the tumor and some tissue penetration by the vector could be observed. The preliminary clinical outcome after a phase II study with more than 20 patients is a significant increase in tumor-free survival of the cats from only 23% at the 1 year time point in the case of standard therapy (surgery only) to 52% with pre-surgical magnetofection of the human GM-CSF gene (Schillinger et al., 2005). The long-term follow-up will be very interesting as it will reveal the true benefits of this treatment.

Apart from direct injection into the target tissue, the **injection into blood vessels which are rather distant to the target site is assumed to become the most important form of vector administration for therapeutic magnetofection**. But in blood vessels, hydrodynamic forces like the viscous drag force (according to Stoke's law in the blood stream) counteract the magnetic retention and at blood flow rates around 20 cm/s (like in the human aorta) magnetic drug targeting appears even impossible (Plank et al., 2005; Voltairas et al., 2002). To overcome the **barrier of hydrodynamic forces in blood vessels**, several approaches are

conceivable. For example the magnetic fields could be improved and the magnetic particles and their delivery systems could be optimized.

Improvement of the magnetic fields:

The force **F** exerted on a magnetic particle is determined by the formula

$$\vec{F} = \frac{V \cdot \chi}{\mu_O} (\vec{B} \cdot \vec{\nabla}) \vec{B}$$

where **V** is the volume and χ the susceptibility of the magnetic particle, μ_O the magnetic permeability in vacuum, **B** the magnetic induction (= magnetic flux density) and ∇ **B** the field gradient (Babincova et al., 2001). From this expression it is clear that the movement of magnetic particles in a magnetic field is proportional to the magnetic induction (measured in Tesla [T]) and the magnetic field gradient (measured in T/m). But this relationship is only valid when the particles are not magnetically saturated. In the case of saturation magnetization (measured in A•m^2/kg), an increase in particle movement is not possible through higher magnetic induction but only through an increase in the field gradient. In all magnetofections so far, permanent neodymium-iron-boron magnets (according to the supplier, IBS Magnet Berlin, with magnetic inductions of 1.08-1.15 T) were used. The magnetic field gradient of these magnets is dependent on the size and the shape of the magnet and on an eventual placement next to other magnets. For magnetofections in 96-well plates, cylindrical (d = 6 mm, h = 5 mm) magnets inserted in an acrylic glass template in 96-well plate format with strictly alternating polarization were used. Measurements with a Tesla-meter revealed that in the the center of the ground of each well (approximately 2 mm distant from the surface of the magnet) the magnetic induction was approximately between 0.13 and 0.24 T and the magnetic field gradient approximately between 67 and 123 T/m. This magnetic induction leads to approximately 80% saturation of particles consisting of almost pure magnetite (unpublished data, Mykhaylyk O, TU Munich) and the field gradient is comparatively high. Although induction and gradient decrease rapidly with increasing distance from the surface of the magnet, for magnetic sedimentation in cell culture this magnetic device is relatively efficient. Single rectangular (dimensions 20 x 10 x 5 mm) magnets chosen for experiments in 6-well plates and in animal experiments are also not assumed to have higher inductions and field gradients than the 96-well plate magnetic device. In cell culture experiments these magnetic properties are sufficient, but for *in vivo* applications, where e.g. the viscous drag force is counteracting, more improved magnetic fields are desired. Improvements are possible with regard to magnetic induction (an induction of 0.5-0.6 T is necessary to achieve saturation magnetization of magnetite particles) and magnetic field gradient. The strongest magnetic

field ever applied in magnetic drug targeting (here with an anti-cancer drug) was used by Alexiou et al. (Alexiou et al., 2000). It was an electromagnet with a maximum magnetic induction of 1.7 T on the tip of the pole shoe and 10 mm distant to the pole shoe there was still an induction of 1.0 T. Additionaly, the magnet was constructed to achieve high magnetic field gradients. This advanced magnet exerts a strong attractive force on magnetic particles even in regions more distant to the pole shoe. However, all the magnetic fields described above are not assumed to be sufficient for capturing magnetic particles in vessels with very high blood flow rates. A general problem is the rapidly decreasing magnetic induction and field gradient with increasing distance from the pole and also that gradients can not be generated arbitrarily in space. Thus magnetic drug targeting with the magnets mentioned above is only feasible if they can be applied directly to the target site which is often only possible by surgery. A new approach to generate high magnetic field gradients for magnetic drug targeting was proposed by Babincova et al. (Babincova et al., 2001). In an experimental set-up, they placed a ferromagnetic wire alongside a tube (used as a model for blood vessels) which was positioned in a magnetic field and strong gradients were induced which were sufficient to locally capture magnetic (nano)particles under constant flow rate. The authors suggested the use of strong static homogenous magnetic fields (1.5 T or more) generated by magnetic resonance imaging machines plus the placement of ferromagnetic wires near the target site to induce locally very high field gradients. They concluded that with such a construction magnetic particles could be targeted with an efficiency 3-5 magnitudes higher than using permanent magnets. Additionally, the study of Nagel suggests that the magnetic particles themselves generate local field gradients that facilitate the targeting of further particles (Nagel, 2004). If static magnetic fields are harmful, neutral or even positive for the human health is still unknown, but it is documented that they can e.g. have effects on the cellular plasma membrane (Rosen, 2003), cellular metabolic activity (Sabo et al., 2002), trans-membrane flux of calcium ions, apoptosis, phagocytic activity (Flipo et al., 1998), cell differentiation (Pagliara et al., 2005) and oncogene expression (Hirose et al., 2003). In the future, the success of magnetic drug targeting is largely dependent on the developments in magnetic field technology. Perhaps, one day there are even magnets which are able to capture magnetic nanoparticles in blood vessels with high flow rates, the magnetic field can be applied to any region of the body without the need of any surgical intervention and the applied magnetic fields are proofed to be totally harmless or even healthy for the human body.

Improvement of the magnetic particle chemistry and physics:

In all magnetofection experiments so far, iron oxide nanoparticles were used. These particles are "superparamagnetic", meaning that they are strongly attracted to a magnetic field but they do not retain residual magnetism after the field is removed. Therefore they can not agglomerate (like ferromagnetic particles) after removal of the magnetic field. Further, Weissleder et al. found that iron oxide particles used as contrast agents in magnetic resonance imaging (MRI) are fully biocompatible (Weissleder et al., 1989). After intravenous application in rats, the particles were cleared by macrophages in liver and spleen, the iron oxides were degraded in lysosomes via hydrolytic enzymes, and the resulting elemental iron was integrated into the natural iron metabolism (e.g. incorporation into hemoglobin). Additionally, Weissleder and coworkers showed that in rats and beagle dogs a relatively high dose of 167 mg iron/kg body mass still had no toxic effects on the liver or other organs and they mentioned that for clinical MRI a dose of approximately 1 mg iron/kg is proposed. Thus, the 76.9 µg iron oxide particles/kg applied intravenously in pigs for magnetic nucleic acid targeting experiments (3.4.1) can be assumed to be totally safe. Despite the advanced magnetic properties and the biocompatibility of iron oxides, for improvement of magnetic nucleic acid targeting materials with even higher magnetic susceptibility (see χ in the formula above) would be desirable. For example, the ferromagnetic material elementary iron has a higher magnetic susceptibility and a higher saturation magnetization as iron oxide (magnetite) and composite microparticles made from elementary iron and activated carbon were already used for magnetic drug targeting with chemotherapeutic agents in human clinical trials (Johnson et al., 2002; Rudge et al., 2001). Therefore in future it might be interesting to work on the efficient and functional binding of nucleic acids to elementary iron particles and to test these associates in magnetofection.

Further, as shown in the formula above, the magnetic force acting on a particle is not only proportional to the magnetic induction and the field gradient but also to the volume of the particle. Although the viscous drag force in the blood stream is proportional to the particle size as well, it is only proportional to the first power of the particle radius whereas the magnetic force is proportional to the third power of the radius (= volume of the particle) and therefore magnetic particles should be as large as possible to achieve optimum magnetic retentability. But for future optimization of particle sizes it must be considered that the particles should be as large as possible but also that they should not exceed a size which harbours the danger of inducing embolisms in capillaries which only have diameters of approximately 5 µm (Plank et al., 2003a; Plank et al., 2005). For extravasation, it has to be taken into account that the capillary bed is generally permeable to particles smaller than 2 nm,

that the fenestrations in bone marrow, liver and spleen are up to 150 nm, and that in certain tumors there are endothelial gaps or transcellular holes of up to 500 nm (Mykhaylyk et al., 2005).

Improvement of the delivery system:

An advantageous delivery system could e.g. use a multitude of magnetic nanoparticles which are coupled to each other physically, so that the attractive magnetic forces which act on each single nanoparticle could be multiplied and also counteracting physical events like Brownian relaxation could be reduced in this way. Recently, colleagues in our laboratory developed such a system. They incorporated a multitude of paramagnetic nucleic acid vectors into the lipid shell of gas-filled microbubbles (Hellwig et al., 2005). These microbubbles are stable in the blood stream and even if they can adapt their shape to the environment they are constructed smaller than 5 μm in order not to obstruct blood capillaries. The magnetic microbubbles are e.g. injected into the blood stream, held back at the target site by magnetic force and by localized ultrasound release of a multitude of paramagnetic nucleic acid vectors is achieved. Thus using this approach two physical methods of targeting and their benefits are combined: magnetic drug targeting and the use of ultrasound to trigger localized nucleic acid delivery.

Further possibilities to improve magnetic nucleic acid targeting in vessels with higher blood flow rates are e.g. the **local reduction of the flow rate and localized application of magnetofectins through catheters**.

With all the efforts in magnetic field physics, in magnetic particle physics and chemistry, in pharmaceutical formulations and in medical application, hopefully once magnetofection will become an efficient clinical standard therapy for many diseases.

5 SUMMARY

Among the physical methods of drug localization, especially magnetic drug targeting promises great potential. In this method, an anti-cancer drug is bound to magnetic particles and an external magnetic field can guide the administered magnetic particle-drug complex to the desired site.

The objective of this thesis was to apply the principle of magnetic drug targeting to the delivery of nucleic acids in cell culture and *in vivo*.

To establish the method of magnetic nucleic acid targeting (magnetofection), the characteristics (sizes and organization) of different superparamagnetic iron oxide nanoparticles coated with cationic or anionic polymers (termed "trMAGs", synthesized by Chemicell GmbH Berlin), the binding of DNA to the magnetic beads, transfections with the different types of magnetic particles, the mechanism of magnetofection, optimization of magnetofection, the gene transfer efficiency of magnetofections compared to standard (conventional) transfections, magnetic field-guided localization of gene transfer, magnetofection of a variety of cells and the applicability of magnetofection *in vivo*, were examined.

In binding studies, it turned out that efficient binding of charged DNA vectors to charged magnetic particles could be achieved by salt-induced colloid aggregation. Incubation of cells with magnetic particle/DNA associates (magnetofectins) resulted in gene transfer and application of a magnetic field significantly increased gene expression. Additionally, polyethylenimine (PEI) had an enhancing effect on magnetofection efficiency. Mechanistic studies revealed that the paramagnetic vectors are concentrated efficiently by magnetic force on the cell surface within minutes and the predominant uptake mechanism is endocytosis. Comparison of magnetofections and the corresponding standard transfections (same vectors but without magnetic particles and no applied magnetic field) showed that with magnetofection the gene transfer efficiency was usually significantly enhanced (up to 971-fold), the nucleic acid dose-response profile could be improved and the incubation times (of cells with vectors) could be reduced from hours to minutes. Finally, in animal experiments (injection into ear veins of pigs, into ear arteries of rabbits and into ilea of rats) it was demonstrated that magnetofection enables targeted and efficient gene transfer *in vivo*.

6 REFERENCES

Alexiou, C., Arnold, W., Klein, R. J., Parak, F. G., Hulin, P., Bergemann, C., Erhardt, W., Wagenpfeil, S., and Lubbe, A. S. (2000). Locoregional cancer treatment with magnetic drug targeting. Cancer Res *60*, 6641-6648.

Alksne, J. F., and Fingerhut, A. G. (1965). Magnetically controlled metallic thrombosis of intracranial aneurysms. A preliminary report. Bull Los Angeles Neurol Soc *30*, 153-155.

Andre, F., and Mir, L. M. (2004). DNA electrotransfer: its principles and an updated review of its therapeutic applications. Gene Ther *11 Suppl 1*, S33-42.

Babincova, M., Babinec, P., and Bergemann, C. (2001). High-gradient magnetic capture of ferrofluids: implications for drug targeting and tumor embolization. Z Naturforsch [C] *56*, 909-911.

Barron, L. G., Gagne, L., and Szoka, F. C., Jr. (1999). Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration. Hum Gene Ther *10*, 1683-1694.

Barzon, L., Stefani, A., Pacenti, M., and Palu, G. (2005). Versatility of gene therapy vectors through viruses. Expert Opin Biol Ther *5*, 639-662.

Behr, J. P., Demeneix, B., Loeffler, J. P., and Perez-Mutul, J. (1989). Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proc Natl Acad Sci U S A *86*, 6982-6986.

Bekeredjian, R., Chen, S., Frenkel, P. A., Grayburn, P. A., and Shohet, R. V. (2003). Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. Circulation *108*, 1022-1026.

Belting, M. (2003). Heparan sulfate proteoglycan as a plasma membrane carrier. Trends Biochem Sci *28*, 145-151.

Benvenisty, N., and Reshef, L. (1986). Direct introduction of genes into rats and expression of the genes. In Proc Natl Acad Sci U S A, pp. 9551-9555.

Blank, M., and Goodman, R. (2004). Initial interactions in electromagnetic field-induced biosynthesis. J Cell Physiol *199*, 359-363.

Bloquel, C., Fabre, E., Bureau, M. F., and Scherman, D. (2004). Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications. J Gene Med *6 Suppl 1*, S11-23.

Boeckle, S., von Gersdorff, K., van der Piepen, S., Culmsee, C., Wagner, E., and Ogris, M. (2004). Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. J Gene Med *6*, 1102-1111.

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A *92*, 7297-7301.

Boussif, O., Zanta, M. A., and Behr, J. P. (1996). Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther *3*, 1074-1080.

Branda, C. S., and Dymecki, S. M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev Cell *6*, 7-28.

Budker, V., Zhang, G., Knechtle, S., and Wolff, J. A. (1996). Naked DNA delivered intraportally expresses efficiently in hepatocytes. In Gene Ther, pp. 593-598.

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M., and Morgan, R. A. (1995). High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. Proc Natl Acad Sci U S A *92*, 7739-7743.

Burkhardt, B. R., Loiler, S. A., Anderson, J. A., Kilberg, M. S., Crawford, J. M., Flotte, T. R., Goudy, K. S., Ellis, T. M., and Atkinson, M. (2003). Glucose-responsive expression of the human insulin promoter in HepG2 human hepatoma cells. Ann N Y Acad Sci *1005*, 237-241.

Buschle, M., Cotten, M., Kirlappos, H., Mechtler, K., Schaffner, G., Zauner, W., Birnstiel, M. L., and Wagner, E. (1995). Receptor-mediated gene transfer into human T lymphocytes via binding of DNA/CD3 antibody particles to the CD3 T cell receptor complex. Hum Gene Ther *6*, 753-761.

Canosa, I., Lopez, G., Rojo, F., Boocock, M. R., and Alonso, J. C. (2003). Synapsis and strand exchange in the resolution and DNA inversion reactions catalysed by the beta recombinase. Nucleic Acids Res *31*, 1038-1044.

Chan, C. K., Senden, T., and Jans, D. A. (2000). Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines. Gene Ther *7*, 1690-1697.

Chen, J., Gamou, S., Takayanagi, A., Ohtake, Y., Ohtsubo, M., and Shimizu, N. (1998). Receptor-mediated gene delivery using the Fab fragments of anti-epidermal growth factor receptor antibodies: improved immunogene approach. Cancer Gene Ther *5*, 357-364.

Chuck, A. S., Clarke, M. F., and Palsson, B. O. (1996). Retroviral infection is limited by Brownian motion. Hum Gene Ther *7*, 1527-1534.

Cotten, M., Langlerouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., and Birnstiel, M. L. (1990). Transferrin Polycation-Mediated Introduction Of DNA Into Human Leukemic Cells - Stimulation By Agents That Affect The Survival Of Transfected DNA Or Modulate Transferrin Receptor Levels. In Proceedings Of The National Academy Of Sciences Of The United States Of America, pp. 4033-4037.

Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D. T., and Birnstiel, M. L. (1992). High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. Proc Natl Acad Sci U S A *89*, 6094-6098.

Curiel, D. T., Agarwal, S., Wagner, E., and Cotten, M. (1991). Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. Proc Natl Acad Sci U S A *88*, 8850-8854.

Dean, D. A., Strong, D. D., and Zimmer, W. E. (2005). Nuclear entry of nonviral vectors. Gene Ther *12*, 881-890.

Demeneix, B., Behr, J., Boussif, O., Zanta, M. A., Abdallah, B., and Remy, J. (1998). Gene transfer with lipospermines and polyethylenimines. Adv Drug Deliv Rev *30*, 85-95.

Densmore, C. L. (2003). The re-emergence of aerosol gene delivery: a viable approach to lung cancer therapy. Curr Cancer Drug Targets *3*, 275-286.

Diebold, S. S., Plank, C., Cotten, M., Wagner, E., and Zenke, M. (2002). Mannose receptor-mediated gene delivery into antigen presenting dendritic cells. Somat Cell Mol Genet *27*, 65-74.

Dubensky, T. W., Campbell, B. A., and Villarreal, L. P. (1984). Direct transfection of viral and plasmid DNA into the liver or spleen of mice. In Proc Natl Acad Sci U S A, pp. 7529-7533.

Erbacher, P., Zou, S., Bettinger, T., Steffan, A. M., and Remy, J. S. (1998). Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. Pharm Res *15*, 1332-1339.

Escriou, V., Carriere, M., Scherman, D., and Wils, P. (2003). NLS bioconjugates for targeting therapeutic genes to the nucleus. Adv Drug Deliv Rev *55*, 295-306.

Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994). Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem *269*, 2550-2561.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A *84*, 7413-7417.

Finsinger, D., Remy, J. S., Erbacher, P., Koch, C., and Plank, C. (2000). Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. Gene Ther *7*, 1183-1192.

Flipo, D., Fournier, M., Benquet, C., Roux, P., Le Boulaire, C., Pinsky, C., LaBella, F. S., and Krzystyniak, K. (1998). Increased apoptosis, changes in intracellular Ca2+, and functional alterations in lymphocytes and macrophages after in vitro exposure to static magnetic field. J Toxicol Environ Health A *54*, 63-76.

Frisch, B., Carriere, M., Largeau, C., Mathey, F., Masson, C., Schuber, F., Scherman, D., and Escriou, V. (2004). A new triantennary galactose-targeted PEGylated gene carrier, characterization of its complex with DNA, and transfection of hepatoma cells. Bioconjug Chem *15*, 754-764.

Gemeinhart, R. A., Luo, D., and Saltzman, W. M. (2005). Cellular fate of a modular DNA delivery system mediated by silica nanoparticles. Biotechnol Prog *21*, 532-537.

Gersting, S. W., Schillinger, U., Lausier, J., Nicklaus, P., Rudolph, C., Plank, C., Reinhardt, D., and Rosenecker, J. (2004). Gene delivery to respiratory epithelial cells by magnetofection. J Gene Med *6*, 913-922.

Goodman, R., and Blank, M. (2002). Insights into electromagnetic interaction mechanisms. J Cell Physiol *192*, 16-22.

Goodwin, S., Peterson, C., Hoh, C., and Bittner, C. (1999). Targeting and retention of magnetic targeted carriers (MTCs) enhancing intra-arterial chemotherapy. J Magn Magn Mat *194*, 132-139.

Goodwin, S. C., Bittner, C. A., Peterson, C. L., and Wong, G. (2001). Single-dose toxicity study of hepatic intra-arterial infusion of doxorubicin coupled to a novel magnetically targeted drug carrier. Toxicol Sci *60*, 177-183.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A *89*, 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766-1769.

Graham, F. L., and van der Eb, A. J. (1973). Transformation of rat cells by DNA of human adenovirus 5. Virology *54*, 536-539.

Greish, K., Fang, J., Inutsuka, T., Nagamitsu, A., and Maeda, H. (2003). Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting. Clin Pharmacokinet *42*, 1089-1105.

Groth, A. C., and Calos, M. P. (2004). Phage integrases: biology and applications. J Mol Biol *335*, 667-678.

Gunther, M., Wagner, E., and Ogris, M. (2005). Specific targets in tumor tissue for the delivery of therapeutic genes. Curr Med Chem Anti-Canc Agents *5*, 157-171.

Gupta, P. K., Hung, C. T., and Rao, N. S. (1989). Ultrastructural disposition of adriamycin-associated magnetic albumin microspheres in rats. J Pharm Sci *78*, 290-294.

Gust, T. C., and Zenke, M. (2002). Receptor-mediated gene delivery. In ScientificWorldJournal, pp. 224-229.

Haensler, J., and Szoka, F. C., Jr. (1993). Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug Chem *4*, 372-379.

Hafeli, U. O., Sweeney, S. M., Beresford, B. A., Humm, J. L., and Macklis, R. M. (1995). Effective targeting of magnetic radioactive 90Y-microspheres to tumor cells by an externally applied magnetic field. Preliminary in vitro and in vivo results. Nucl Med Biol *22*, 147-155.

Hafeli, U. O., Sweeney, S. M., Beresford, B. A., Sim, E. H., and Macklis, R. M. (1994). Magnetically directed poly(lactic acid) 90Y-microspheres: novel agents for targeted intracavitary radiotherapy. J Biomed Mater Res *28*, 901-908.

Hafeli, U. O., Yu, J., Farudi, F., Li, Y., and Tapolsky, G. (2003). Radiolabeling of magnetic targeted carriers (MTC) with indium-111. Nucl Med Biol *30*, 761-769.

Hafez, I. M., Maurer, N., and Cullis, P. R. (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther *8*, 1188-1196.

Hagstrom, J. E., Hegge, J., Zhang, G., Noble, M., Budker, V., Lewis, D. L., Herweijer, H., and Wolff, J. A. (2004). A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. Mol Ther *10*, 386-398.

Haim, H., Steiner, I., and Panet, A. (2005). Synchronized infection of cell cultures by magnetically controlled virus. J Virol *79*, 622-625.

Hamm, A., Krott, N., Breibach, I., Blindt, R., and Bosserhoff, A. K. (2002). Efficient transfection method for primary cells. Tissue Eng *8*, 235-245.

Hellwig, N., Plank, C., Vlaskou, D., Bridell, H., Sohn, H. Y., Pohl, U., and Krotz, F. (2005). Ultrasound-enhanced microbubble-magnetofection: a new approach for targeted delivery of nucleotides in vivo. Paper presented at: 3rd European Meeting on Vascular Biology and Medicine (Hamburg, J Vasc Biol, S. Karger AG, Basel).

Herweijer, H., and Wolff, J. A. (2003). Progress and prospects: naked DNA gene transfer and therapy. In Gene Ther, pp. 453-458.

Hiemenz, P. (1986). Principles of colloid and surface chemistry, 2nd edn (New York, Marcel Dekker, Inc.).

Hiraoka, M., Miyakoshi, J., Li, Y. P., Shung, B., Takebe, H., and Abe, M. (1992). Induction of c-fos gene expression by exposure to a static magnetic field in HeLaS3 cells. Cancer Res *52*, 6522-6524.

Hirose, H., Nakahara, T., Zhang, Q. M., Yonei, S., and Miyakoshi, J. (2003). Static magnetic field with a strong magnetic field gradient (41.7 T/m) induces c-Jun expression in HL-60 cells. In Vitro Cell Dev Biol Anim *39*, 348-352.

Hood, J. D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R. A., Xiang, R., and Cheresh, D. A. (2002). Tumor regression by targeted gene delivery to the neovasculature. In Science, pp. 2404-2407.

Hughes, C., Galea-Lauri, J., Farzaneh, F., and Darling, D. (2001). Streptavidin paramagnetic particles provide a choice of three affinity-based capture and magnetic concentration strategies for retroviral vectors. Mol Ther *3*, 623-630.

Huth, S., Lausier, J., Gersting, S. W., Rudolph, C., Plank, C., Welsch, U., and Rosenecker, J. (2004). Insights into the mechanism of magnetofection using PEI-based magnetofectins for gene transfer. J Gene Med *6*, 923-936.

Johnson, J., Kent, T., Koda, J., Peterson, C., Rudge, S., and Tapolsky, G. (2002). The MTC technology: a platform technology for the site-specific delivery of pharmaceutical agents. Eur Cells and Mat *3*, 12-15.

Jordan, M., and Wurm, F. (2004). Transfection of adherent and suspended cells by calcium phosphate. Methods *33*, 136-143.

Kadota, S., Kanayama, T., Miyajima, N., Takeuchi, K., and Nagata, K. (2005). Enhancing of measles virus infection by magnetofection. J Virol Methods *128*, 61-66.

Kato, T., Nemoto, R., Mori, H., Abe, R., Unno, K., Goto, A., Murota, H., Harada, M., and Homma, M. (1984). Magnetic microcapsules for targeted delivery of anticancer drugs. Appl Biochem Biotechnol *10*, 199-211.

Kawabata, K., Takakura, Y., and Hashida, M. (1995). The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. Pharm Res *12*, 825-830.

Kawakami, S., Sato, A., Nishikawa, M., Yamashita, F., and Hashida, M. (2000). Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. Gene Ther *7*, 292-299.

Kichler, A., Leborgne, C., Coeytaux, E., and Danos, O. (2001). Polyethylenimine-mediated gene delivery: a mechanistic study. In J Gene Med, pp. 135-144.

Kono, K., and Takagishi, T. (2004). Temperature-sensitive liposomes. Methods Enzymol *387*, 73-82.

Krotz, F., de Wit, C., Sohn, H. Y., Zahler, S., Gloe, T., Pohl, U., and Plank, C. (2003a). Magnetofection--a highly efficient tool for antisense oligonucleotide delivery in vitro and in vivo. Mol Ther *7*, 700-710.

Krotz, F., Sohn, H. Y., Gloe, T., Plank, C., and Pohl, U. (2003b). Magnetofection potentiates gene delivery to cultured endothelial cells. J Vasc Res *40*, 425-434.

Kuduvalli, P. N., Mitra, R., and Craig, N. L. (2005). Site-specific Tn7 transposition into the human genome. Nucleic Acids Res *33*, 857-863.

Leventis, R., and Silvius, J. R. (1990). Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. Biochim Biophys Acta *1023*, 124-132.

Li, X., Stuckert, P., Bosch, I., Marks, J. D., and Marasco, W. A. (2001). Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells. Cancer Gene Ther *8*, 555-565.

Lin, A. J., Slack, N. L., Ahmad, A., George, C. X., Samuel, C. E., and Safinya, C. R. (2003). Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes. In Biophys J, pp. 3307-3316.

Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther *6*, 1258-1266.

Lubbe, A. S., Alexiou, C., and Bergemann, C. (2001). Clinical applications of magnetic drug targeting. In J Surg Res, pp. 200-206.

Lubbe, A. S., Bergemann, C., Huhnt, W., Fricke, T., Riess, H., Brock, J. W., and Huhn, D. (1996a). Preclinical experiences with magnetic drug targeting: tolerance and efficacy. Cancer Res *56*, 4694-4701.

Lubbe, A. S., Bergemann, C., Riess, H., Schriever, F., Reichardt, P., Possinger, K., Matthias, M., Dorken, B., Herrmann, F., Gurtler, R.*, et al.* (1996b). Clinical experiences with magnetic drug targeting: a phase I study with 4'-epidoxorubicin in 14 patients with advanced solid tumors. Cancer Res *56*, 4686-4693.

Luo, D., Han, E., Belcheva, N., and Saltzman, W. M. (2004). A self-assembled, modular DNA delivery system mediated by silica nanoparticles. J Control Release *95*, 333-341.

Luo, D., and Saltzman, W. M. (2000). Enhancement of transfection by physical concentration of DNA at the cell surface. Nat Biotechnol *18*, 893-895.

Mah, C., Fraites, T. J., Jr., Zolotukhin, I., Song, S., Flotte, T. R., Dobson, J., Batich, C., and Byrne, B. J. (2002). Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. Mol Ther *6*, 106-112.

McKeown, S. R., Ward, C., and Robson, T. (2004). Gene-directed enzyme prodrug therapy: a current assessment. Curr Opin Mol Ther *6*, 421-435.

Meers, P. (2001). Enzyme-activated targeting of liposomes. Adv Drug Deliv Rev *53*, 265-272.

Meyers, P. H., Cronic, F., and Nice, C. M., Jr. (1963). Experimental Approach in the Use and Magnetic Control of Metallic Iron Particles in the Lymphatic and Vascular System of Dogs as a Contrast and Isotopic Agent. Am J Roentgenol Radium Ther Nucl Med *90*, 1068-1077.

Miyata, T., Uragami, T., and Nakamae, K. (2002). Biomolecule-sensitive hydrogels. Adv Drug Deliv Rev *54*, 79-98.

Mumper, R. J., and Cui, Z. (2003). Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. Methods *31*, 255-262.

Mykhaylyk, O., Dudchenko, N., and Dudchenko, A. (2005). Doxorubicin magnetic conjugate targeting upon intravenous injection into mice: High gradient magnetic field inhibits the clearance of nanoparticles from the blood. J Magn Magn Mat *293*, 473-482.

Nagel, S. (2004) Theoretische und experimentelle Untersuchungen zum Magnetischen Drug Targeting, Ernst-Moritz-Arndt-Universität, Greifswald.

Nishikawa, M., Takakura, Y., and Hashida, M. (2005). Theoretical considerations involving the pharmacokinetics of plasmid DNA. Adv Drug Deliv Rev *57*, 675-688.

O'Doherty, U., Swiggard, W. J., and Malim, M. H. (2000). Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J Virol *74*, 10074-10080.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., and Wagner, E. (1998). The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther *5*, 1425-1433.

Ogris, M., and Wagner, E. (2002). Tumor-targeted gene transfer with DNA polyplexes. Somat Cell Mol Genet *27*, 85-95.

Ogris, M., Walker, G., Blessing, T., Kircheis, R., Wolschek, M., and Wagner, E. (2003). Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. J Control Release *91*, 173-181.

O'Neill, M. M., Kennedy, C. A., Barton, R. W., and Tatake, R. J. (2001). Receptor-mediated gene delivery to human peripheral blood mononuclear cells using anti-CD3 antibody coupled to polyethylenimine. Gene Ther *8*, 362-368.

Pagliara, P., Lanubile, R., Dwikat, M., Abbro, L., and Dini, L. (2005). Differentiation of monocytic U937 cells under static magnetic field exposure. Eur J Histochem *49*, 75-86.

Pandori, M., Hobson, D., and Sano, T. (2002). Adenovirus-microbead conjugates possess enhanced infectivity: a new strategy for localized gene delivery. Virology *299*, 204-212.

Pannier, A. K., and Shea, L. D. (2004). Controlled release systems for DNA delivery. In Mol Ther, pp. 19-26.

Pardridge, W. M. (2001). Brain drug targeting and gene technologies. In Jpn J Pharmacol, pp. 97-103.

Pedroso de Lima, M. C., Simoes, S., Pires, P., Faneca, H., and Duzgunes, N. (2001). Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. In Adv Drug Deliv Rev, pp. 277-294.

Pemberton, L. F., and Paschal, B. M. (2005). Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic *6*, 187-198.

Peters, R. (2005). Translocation through the nuclear pore complex: selectivity and speed by reduction-of-dimensionality. Traffic *6*, 421-427.

Plank, C., Anton, M., Rudolph, C., Rosenecker, J., and Krotz, F. (2003a). Enhancing and targeting nucleic acid delivery by magnetic force. Expert Opin Biol Ther *3*, 745-758.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994). The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J Biol Chem *269*, 12918-12924.

Plank, C., Scherer, F., and Rudolph, C. (2005). Localized Nucleic Acid Delivery: A Discussion of Selected Methods. In DNA Pharmaceuticals, M. Schleef, ed. (Weinheim, Wiley-VCH Verlag GmbH & Co. KGaA), pp. 55-116.

Plank, C., Scherer, F., Schillinger, U., Bergemann, C., and Anton, M. (2003b). Magnetofection: enhancing and targeting gene delivery with superparamagnetic nanoparticles and magnetic fields. J Liposome Res *13*, 29-32.

Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J., and Rosenecker, J. (2003c). The magnetofection method: using magnetic force to enhance gene delivery. Biol Chem *384*, 737-747.

Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., and Wagner, E. (1992). Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. Bioconjug Chem *3*, 533-539.

Pollard, H., Remy, J. S., Loussouarn, G., Demolombe, S., Behr, J. P., and Escande, D. (1998). Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. In J Biol Chem, pp. 7507-7511.

Ponnazhagan, S., Curiel, D. T., Shaw, D. R., Alvarez, R. D., and Siegal, G. P. (2001). Adeno-associated virus for cancer gene therapy. Cancer Res *61*, 6313-6321.

Poon, I. K., and Jans, D. A. (2005). Regulation of nuclear transport: central role in development and transformation? Traffic *6*, 173-186.

Potenza, L., Ubaldi, L., De Sanctis, R., De Bellis, R., Cucchiarini, L., and Dacha, M. (2004). Effects of a static magnetic field on cell growth and gene expression in Escherichia coli. Mutat Res *561*, 53-62.

Qiu, Y., and Park, K. (2001). Environment-sensitive hydrogels for drug delivery. Adv Drug Deliv Rev *53*, 321-339.

Rainov, N. G., and Ren, H. (2003). Gene therapy for human malignant brain tumors. Cancer J *9*, 180-188.

Recchia, A., Perani, L., Sartori, D., Olgiati, C., and Mavilio, F. (2004). Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. Mol Ther *10*, 660-670.

Reddy, J. A., Allagadda, V. M., and Leamon, C. P. (2005). Targeting therapeutic and imaging agents to folate receptor positive tumors. Curr Pharm Biotechnol *6*, 131-150.

Rejman, J., Bragonzi, A., and Conese, M. (2005). Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther *12*, 468-474.

Rejman, J., Oberle, V., Zuhorn, I. S., and Hoekstra, D. (2004). Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J *377*, 159-169.

Ritter, W., Plank, C., Lausier, J., Rudolph, C., Zink, D., Reinhardt, D., and Rosenecker, J. (2003). A novel transfecting peptide comprising a tetrameric nuclear localization sequence. J Mol Med *81*, 708-717.

Robson, T., and Hirst, D. G. (2003). Transcriptional Targeting in Cancer Gene Therapy. J Biomed Biotechnol *2003*, 110-137.

Rojanasakul, Y., Wang, L. Y., Malanga, C. J., Ma, J. K., and Liaw, J. (1994). Targeted gene delivery to alveolar macrophages via Fc receptor-mediated endocytosis. Pharm Res *11*, 1731-1736.

Rosen, A. D. (2003). Mechanism of action of moderate-intensity static magnetic fields on biological systems. Cell Biochem Biophys *39*, 163-173.

Rudge, S., Peterson, C., Vessely, C., Koda, J., Stevens, S., and Catterall, L. (2001). Adsorption and desorption of chemotherapeutic drugs from a magnetically targeted carrier (MTC). J Control Release *74*, 335-340.

Rudge, S. R., Kurtz, T. L., Vessely, C. R., Catterall, L. G., and Williamson, D. L. (2000). Preparation, characterization, and performance of magnetic iron-carbon composite microparticles for chemotherapy. Biomaterials *21*, 1411-1420.

Rudolph, C., Plank, C., Lausier, J., Schillinger, U., Muller, R. H., and Rosenecker, J. (2003). Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. J Biol Chem *278*, 11411-11418.

Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Muller, R. H., and Rosenecker, J. (2004). Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. Pharm Res *21*, 1662-1669.

Sabo, J., Mirossay, L., Horovcak, L., Sarissky, M., Mirossay, A., and Mojzis, J. (2002). Effects of static magnetic field on human leukemic cell line HL-60. Bioelectrochemistry *56*, 227-231.

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., and Plank, C. (2002a). Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther *9*, 102-109.

Scherer, F., Schillinger, U., Putz, U., Stemberger, A., and Plank, C. (2002b). Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. J Gene Med *4*, 634-643.

Schillinger, U. (2002) Verschiedene Applikationsformen nicht-viraler Genvektoren im Tiermodell unter besonderer Berücksichtigung des lokalen Gentransfers, Inaugural-Dissertation, Ludwig-Maximilians-Universität, München.

Schillinger, U., Brill, T., Rudolph, C., Huth, S., Gersting, S., Krotz, F., Hirschberger, J., Bergemann, C., and Plank, C. (2005). Advances in magnetofection - magnetically guided nucleic acid delivery. J Magn Magn Mat *293*, 501-508.

Schwertmann, U., and Cornell, R. (1991). Iron oxides in the laboratory. Preparation and characterization (Weinheim, Wiley-VCH Verlag GmbH & Co. KGaA).

Sharma, S. K., Bagshawe, K. D., and Begent, R. H. (2005). Advances in antibody-directed enzyme prodrug therapy. Curr Opin Investig Drugs *6*, 611-615.

Simoes, S., Moreira, J. N., Fonseca, C., Duzgunes, N., and de Lima, M. C. (2004). On the formulation of pH-sensitive liposomes with long circulation times. Adv Drug Deliv Rev *56*, 947-965.

Sonawane, N. D., Szoka, F. C., Jr., and Verkman, A. S. (2003). Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem *278*, 44826-44831.

Stoll, S. M., Ginsburg, D. S., and Calos, M. P. (2002). Phage TP901-1 site-specific integrase functions in human cells. J Bacteriol *184*, 3657-3663.

Takakura, Y., Nishikawa, M., Yamashita, F., and Hashida, M. (2002). Influence of physicochemical properties on pharmacokinetics of non-viral vectors for gene delivery. J Drug Target *10*, 99-104.

Tatum, E. L. (1966). Molecular biology, nucleic acids, and the future of medicine. Perspect Biol Med *10*, 19-32.

Toniatti, C., Bujard, H., Cortese, R., and Ciliberto, G. (2004). Gene therapy progress and prospects: transcription regulatory systems. Gene Ther *11*, 649-657.

Tovell, D. R., and Colter, J. S. (1967). Observations on the assay of infectious viral ribonucleic acid: effects of DMSO and DEAE-dextran. Virology *32*, 84-92.

Trimble, C., Lin, C. T., Hung, C. F., Pai, S., Juang, J., He, L., Gillison, M., Pardoll, D., Wu, L., and Wu, T. C. (2003). Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine *21*, 4036-4042.

Tsutsui, J. M., Xie, F., and Porter, R. T. (2004). The use of microbubbles to target drug delivery. Cardiovasc Ultrasound *2*, 23.

Turner, R. D., Rand, R. W., Bentson, J. R., and Mosso, J. A. (1975). Ferromagnetic silicone necrosis of hypernephromas by selective vascular occlusion to the tumor: a new technique. J Urol *113*, 455-459.

van der Eb, M. M., de Leeuw, B., van der Eb, A. J., and Hoeben, R. C. (2004). Side effects of suicide gene therapy. Methods Mol Med *90*, 479-490.

Varga, C. M., Wickham, T. J., and Lauffenburger, D. A. (2000). Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. In Biotechnol Bioeng, pp. 593-605.

Vijayanathan, V., Thomas, T., and Thomas, T. J. (2002). DNA nanoparticles and development of DNA delivery vehicles for gene therapy. Biochemistry *41*, 14085-14094.

Voltairas, P. A., Fotiadis, D. I., and Michalis, L. K. (2002). Hydrodynamics of magnetic drug targeting. J Biomech *35*, 813-821.

Wagner, E. (2004). Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? Pharm Res *21*, 8-14.

Wagner, E., Kircheis, R., and Walker, G. F. (2004). Targeted nucleic acid delivery into tumors: new avenues for cancer therapy. Biomed Pharmacother *58*, 152-161.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992a). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci U S A *89*, 7934-7938.

Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D. T., and Birnstiel, M. L. (1992b). Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. Proc Natl Acad Sci U S A *89*, 6099-6103.

Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990). Transferrin-Polycation Conjugates As Carriers For DNA Uptake Into Cells. In Proceedings Of The National Academy Of Sciences Of The United States Of America, pp. 3410-3414.

Weissleder, R., Stark, D. D., Engelstad, B. L., Bacon, B. R., Compton, C. C., White, D. L., Jacobs, P., and Lewis, J. (1989). Superparamagnetic iron oxide: pharmacokinetics and toxicity. AJR Am J Roentgenol *152*, 167-173.

Wells, D. J. (2004). Gene therapy progress and prospects: electroporation and other physical methods. Gene Ther *11*, 1363-1369.

Widder, K. J., Morris, R. M., Poore, G., Howard, D. P., Jr., and Senyei, A. E. (1981). Tumor remission in Yoshida sarcoma-bearing rts by selective targeting of magnetic albumin microspheres containing doxorubicin. Proc Natl Acad Sci U S A *78*, 579-581.

Widder, K. J., Senyel, A. E., and Scarpelli, G. D. (1978). Magnetic microspheres: a model system of site specific drug delivery in vivo. Proc Soc Exp Biol Med *158*, 141-146.

Will, H., Cattaneo, R., Koch, H. G., Darai, G., Schaller, H., Schellekens, H., van Eerd, P. M., and Deinhardt, F. (1982). Cloned HBV DNA causes hepatitis in chimpanzees. In Nature, pp. 740-742.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Direct Gene Transfer Into Mouse Muscle In vivo. In Science, pp. 1465-1468.

Wu, G. Y., and Wu, C. H. (1987). Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J Biol Chem *262*, 4429-4432.

Wu, G. Y., and Wu, C. H. (1988a). Evidence for targeted gene delivery to HepG2 hepatoma cells in vitro. In Biochemistry, pp. 887-892.

Wu, G. Y., and Wu, C. H. (1988b). Receptor-mediated gene delivery and expression in vivo. In Journal of Biological Chemistry, pp. 14621-14624.

Xu, Y., and Szoka, F. C., Jr. (1996). Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry *35*, 5616-5623.

Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., and Welsh, M. J. (1995). Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem *270*, 18997-19007.

Zanta, M. A., Belguise-Valladier, P., and Behr, J. P. (1999). Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc Natl Acad Sci U S A *96*, 91-96.

Zauner, W., Blaas, D., Kuechler, E., and Wagner, E. (1995). Rhinovirus-mediated endosomal release of transfection complexes. J Virol *69*, 1085-1092.

Zborowski, M., Fuh, C. B., Green, R., Sun, L., and Chalmers, J. J. (1995). Analytical magnetapheresis of ferritin-labeled lymphocytes. Anal Chem *67*, 3702-3712.

Zelphati, O., and Szoka, F. C., Jr. (1996a). Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. In Pharm Res, pp. 1367-1372.

Zelphati, O., and Szoka, F. C., Jr. (1996b). Mechanism of oligonucleotide release from cationic liposomes. In Proc Natl Acad Sci U S A, pp. 11493-11498.

Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990). Receptor-Mediated Endocytosis Of Transferrin Polycation Conjugates - An Efficient Way To Introduce DNA Into Hematopoietic Cells. In Proceedings Of The National Academy Of Sciences Of The United States Of America, pp. 3655-3659.

Zhang, G., Budker, V., and Wolff, J. A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther *10*, 1735-1737.

Zhang, G., Gao, X., Song, Y. K., Vollmer, R., Stolz, D. B., Gasiorowski, J. Z., Dean, D. A., and Liu, D. (2004). Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther *11*, 675-682.

Zou, S. M., Erbacher, P., Remy, J. S., and Behr, J. P. (2000). Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. J Gene Med *2*, 128-134.

7 PUBLICATIONS

Original Papers

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., and Plank, C. (2002a). Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther *9*, 102-109.

Scherer, F., Schillinger, U., Putz, U., Stemberger, A., and Plank, C. (2002b). Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. J Gene Med *4*, 634-643.

Reviews and Book chapters

Plank, C., Scherer, F., Schillinger, U., Bergemann, C., and Anton, M. (2003a). Magnetofection: enhancing and targeting gene delivery with superparamagnetic nanoparticles and magnetic fields. J Liposome Res *13*, 29-32.

Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J., and Rosenecker, J. (2003b). The magnetofection method: using magnetic force to enhance gene delivery. Biol Chem *384*, 737-747.

Plank, C., Scherer, F., and Rudolph, C. (2005). Localized Nucleic Acid Delivery: A Discussion of Selected Methods. In DNA Pharmaceuticals, M. Schleef, ed. (Weinheim, Wiley-VCH Verlag GmbH & Co. KGaA), pp. 55-116.

Poster presentations

Scherer, F., Anton, M., Schillinger, U., Gänsbacher, B., Krüger, A., and Plank, C. (2001). Magnetofection: enhancing and targeting gene delivery by magnetic force. 4th Annual Meeting of the American Society of Gene Therapy, Seattle, Washington, USA.

Scherer, F., Schillinger, U., Lausier, J., Remy, J., and Plank, C. (2001). Optimizing magnetofection. 4th Annual Meeting of the American Society of Gene Therapy, Seattle, Washington, USA.

Scherer, F., Scholdra, D., Stemberger, A., and Plank, C. (1999). Collagen sponges as carriers for synthetic gene vectors. 7th Annual Meeting of the European Society of Gene Therapy, Munich, Germany.

Oral presentation

Scherer, F., and Plank, C. (2001). Magnetofection: enhancement and localization of gene delivery with magnetic particles under the influence of a magnetic field. Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, USA.

Patent

Plank, C., Stemberger, A., and Scherer, F. (2004). Combinations for introducing nucleic acids into cells. European Patent No. 1198489.

8 CURRICULUM VITAE

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Education

1999 to present	Ph.D. work under supervision of Prof. Dr. Ernst Wagner. 1999–2001 at the Institute of Biochemistry, University of Vienna. Since 2001 at the Department of Pharmacy, Chair of Pharmaceutical Biology-Biotechnology, Ludwig-Maximilians-Universität, Munich. Collaboration with PD Dr. Christian Plank, leader of the group "nonviral gene vectors" in the Institute of Experimental Oncology and Therapy Research, Klinikum Rechts der Isar, TU Munich.
1997 – 1998	Diploma work at the Institute of Experimental Oncology and Therapy Research, Klinikum Rechts der Isar, TU Munich, in the group "nonviral gene vectors" of PD Dr. Christian Plank. Title: "Einsatz von Kollagen als Träger zur kontinuierlichen Freisetzung von Genvektoren – Grundlagen für eine neue Gentransfer-Technologie". Supervisor: Prof. Dr. Jürgen Markl, Institut für Zoologie, Johannes Gutenberg Universität, Mainz.
1991 – 1998	Study of biology (diploma), Universität Regensburg 1991–1994, Johannes Gutenberg Universität Mainz 1994–1998. Study of molecular biology, King's College London (ERASMUS programme) 1995–1996.
1982 – 1991	Gymnasium Bad Tölz