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Editorial

Delivery of Nucleic Acids

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Nucleic acids have revolutionized biomedical research and have become indispensable research tools. In pharmaceutical development, nucleic acids are at present mostly used as diagnostic tools and for target validation (1–3). Applications of microarrays and PCR, treatment with antisense oligonucleotides or small interfering RNA and breeding of knock-in/knock-out-models can be used for accurate diagnosis and biomarker detection, can improve insight into disease processes and can pinpoint pathways where treatments may interfere. Although the application of nucleic acids as therapeutics promises to be even more exciting, their role as clinically applied drugs is still modest.

At present, two nucleic acid-based drugs (Vitravene™ and Macugen™) are on the market (4). Both drugs are oligonucleotides. Macugen™ is an extracellularly acting aptamer that functions as a growth factor decoy and Vitravene™ is an intracellularly acting antisense molecule that inhibits a viral gene. Both oligonucleotides contain chemically modified backbones and are injected at the site of the pathology in the vitreous of the eye. This exemplifies the difficulties associated with the use of nucleic acids for therapeutic intervention, both regarding their physicochemical as well as their biological properties.

The physicochemical properties of nucleic acids, with molecular weights ranging from 7 kDa for antisense oligonucleotides to over 1 MDa for plasmid DNA, and strong negative charge do not favor membrane passage. Only one class of nucleic acids, aptamers, can act extracellularly, which circumvents the need for cell membrane translocation. Conversely, all other classes need to interact with intracellular targets to be active. The problem is most prominent for plasmid DNA, which has the largest size of all proposed nucleic acid therapeutics and also needs to arrive inside the cell nucleus to be effective. Nuclear localization would in principle require passage through the nuclear pore for which the DNA-molecule is too large (5). These qualities at least partly explain why the marketed drugs are an aptamer and an antisense oligonucleotide.

The biological properties also do not support their application as therapeutics. Nucleic acids are susceptible to the action of nucleases. Therefore the two marked oligonucleotides bear chemically modified backbones. In addition, nucleic acids are rapidly cleared from the body, either via glomerular filtration by the kidneys and excretion into the urine or by (scavenger) receptor uptake and intracellular degradation. Therefore, local injection at the site of the pathology is the preferred administration route for the clinically applied oligonucleotides.

Despite these difficulties, nucleic acids still capture the mind of many pharmaceutical scientists as possible therapeutics. One of the most appealing properties is that a change in a disease target would in principle only require a change in the nucleic acid sequence to obtain a new drug. As the physicochemical properties like size and charge of the molecules remain the same, the same principles can be applied during the drug formulation steps for this new sequence. After successful formulation of the first nucleic acid drug it can be expected that subsequent formulations will follow more easily. In contrast, for small molecular weight drugs, lead compound identification requires high throughput screening, and drug formulation is dependent on the physicochemical and biological characteristics of the compound.

Nevertheless, the difficult biopharmaceutical characteristics of nucleic acids put a lot of demands on the delivery systems that should compensate for these qualities by increasing stability against the action of nucleases, reducing excretion and uptake by non-target tissues and promoting target tissue interaction, target cell association, membrane translocation, and correct intracellular trafficking (6). The articles in this theme issue address this difficult drug formulation process.

The group of Klibanov approached the problem of identifying suitable vectors for plasmid DNA delivery using a high-throughput-synthesis coupled to combinatorial chemistry approach. Their study is based on the cationic polymer poly(ethylene imine) (PEI). Experimental observations of their group and others indicate that PEI molecular weight is positively correlated with degree of transfection but also with severity of toxicity (7, 8). These observations provided the input for synthesizing small molecular weight PEI-derivatives that were cross-linked with oligo-acrylate esters. As many of the factors that contribute to degree of transfection and toxicity as well as the relative contribution of each factor to the overall transfection efficiency are unknown, the high-throughput synthesis approach likely provides a higher chance of finding successful polymers. Indeed, their results show that superior PEI-derivatives could be identified as compared to the presently used 'golden standard' 22 kD PEI both with respect to degree of transfection as well as toxicity both *in vitro* and *in vivo*.

Most cationic polymers exhibit a molecular weight distribution. De Wolf *et al.* investigated the effects of fractionation of the biodegradable polymer poly(2-dimethylamino ethylamino)phosphazene (p(DMAEA)-ppz) into four different molecular weight fractions on *in vitro*/*in vivo* transfection of plasmid DNA and polymer-DNA-complex-

induced toxicity. Marked differences in transfection and toxicity profile were observed for the various molecular weights. Clear correlation between molecular weight and toxicity was seen, whereas degree of transfection was dependent on toxicity and colloidal stability of the complexes. These results underline that there is a strong need to use polymers with defined molecular weights and narrow distributions to be able to accurately determine the value of a specific polymer as nucleic acid carrier.

Dendritic α,ε-poly(L-lysine)s were the subject of investigation as possible oligonucleotide vectors in the study of Eom *et al.* In this study they compared a panel of dendrimers varying in generation and number of poly-L-lysine units in the core with respect to their effectiveness regarding delivery of an antisense oligonucleotide that modifies splicing in the nucleus. The advantage of studying this with dendrimers is the monodisperse character and specified architecture of the polymer, circumventing the difficulties in interpreting results identified by de Wolf *et al.* In addition, amino-acid derivatives can be easily prepared which allows gaining insight on the rate-limiting steps of the transfection process in relation to polymer character. It appeared that both charge-density of dendrimers and molecular weight are positively correlated to antisense activity. The most successful hyperbranched, high generation dendrimers were shown to form compact particles of approximately 70 nm with the antisense oligonucleotide, which showed colloidal stability in the presence of serum which is an important quality for *in vivo* application. In addition, the particles enhanced antisense accumulation in the nucleus. Importantly, the optimal dendrimer did not show toxicity when complexed to the antisense oligonucleotide *in vitro.* Similar dendrimeric structures have also been complexed to plasmid DNA which yielded much larger structures, that still effectuated efficient gene transfection and limited toxicity (9).

The various strategies described above to identify and characterize efficient vectors for oligonucleotides or plasmid DNA largely treat transfection and toxicity as a black box, where the net transfection efficiency is the overall result of different processes inside the cell. The first step towards gene transfection is cell association and uptake of the gene carriers. Depending on their surface characteristics and size, gene carriers can be taken up via different endocytic routes, including clathrin- or caveolae-dependent endocytosis, macropinocytosis and phagocytosis. However, not all routes of uptake may lead to effective release of nonviral carriers and its associated DNA required for transgene expression. Van der Aa *et al.* have looked into the cellular uptake mechanisms of two well established cationic polymers used for DNA transfection, namely polyethyleneimine (PEI) and poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA). They showed by spectral bio-imaging that both DNA carriers were internalized in COS-7 cells via clathin- and caveolae-dependent pathways. Blocking either routes of uptake with specific uptake inhibitors resulted in only marginal decrease in the total amount of polyplexes that were internalized, suggesting that uptake routes may be interchangeable. However, blocking the caveolae-dependent uptake route resulted in complete loss of gene transfection, whereas blocking the clathrin-dependent uptake route did not show a significant reduction in gene expression. This shows that the caveolae-dependent uptake pathway is the preferred route for obtaining gene transfection in COS-7 cells. These findings have implications for the development of new or improvement of existing gene carriers. Increased gene transfection efficiencies may be obtained by specifically steering gene carriers towards preferred uptake routes. Although it is at present still unclear which factors determine the uptake pathway that is being followed, the size of the gene carriers seems to play an important role (10). However, cellular factors and surface characteristics of the gene carriers also have an effect on the way these carriers are being internalized. Nevertheless, the study by van der Aa *et al.* shows that by focusing in on single steps of the multistep process leading to gene transfection, valuable information can be obtained that can be used to further optimize nucleic acid delivery vectors.

The majority of nucleic acid therapeutics are to a higher degree dependent on delivery systems for successful therapeutic intervention than conventional drugs. The transfection efficiency of currently used delivery systems is still too low for many clinical applications. Therefore, new delivery agents are needed that combine a high degree of transfection with acceptable toxicity profiles. Combinatorial chemistry could provide important input to identify such compounds. These new delivery agents should be well characterized regarding their physicochemical and structural characteristics and should ideally be adapted to the nucleic acid payload (e.g. plasmid or oligonucleotide) to optimally tailor delivery needs. Important information on the delivery needs and rate limiting steps of the transfection process can be obtained by in depth analysis of the intracellular trafficking of nucleic acids. Ultimately all these aspects of nucleic acid carrier design should come together to develop safe and clinically effective nucleic acid therapeutics.

Enrico Mastrobattista, Wim E. Hennink, Raymond M. Schiffelers
Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, P.O. Box 80.082, 3508 TB, Utrecht, The Netherlands

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