Abstract

Advances in biotechnology have led to an accelerated discovery of macromolecular therapeutics such as peptides, proteins and polynucleotides. These macromolecules can be targeted against a variety of diseases, each requiring delivery to a well-defined compartment of the body. As such therapeutics are often prone to degradation before reaching their target site, or do not reach their target site at all, they often require a special formulation. This review focuses on several types of materials that are currently under investigation for the delivery of nucleic acid therapeutics and aims to pinpoint the limitations of these materials with the ultimate goal to identify the material challenges which, in our opinion, will constitute a new generation of ‘intelligent’ materials for nucleic acid delivery. Such ‘intelligent’ materials should be able to sense and respond to environmental changes. The generated response to these environmental changes should give the material new properties that favor the intracellular delivery of their payload. Besides dealing with material properties, we especially aim to focus on the biological barriers such intelligent materials will have to deal with when used for the delivery of nucleic acids. Furthermore, we briefly discuss the advanced light microscopy techniques that are often used to visualize and quantify the steps of the delivery process of nucleic acids.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Gene therapy; Non-viral carriers; Extracellular matrix; Endocytosis; Nuclear uptake; Advanced light microscopy

1. Introduction

Advances in biotechnology have led to an accelerated discovery of macromolecular therapeutics such as peptides, proteins and polynucleotides. These macromolecules can be targeted against a variety of diseases, each requiring delivery to a well-defined compartment of the body. As such therapeutics are often prone to degradation before reaching their target site, or do not reach their target site at all, they often require a special formulation. In addition, tunable release profiles at the target site could be desirable. To optimize their therapeutic effect such types of drugs will require an advanced packaging. This review focuses on several materials that are under investigation for the administration of macromolecular therapeutics. Considering the numerous disease targets, the increasing number of macromolecular drug candidates and the various routes of administration, various types of materials with widely different characteristics are needed for drug delivery. It is beyond the goal of this review to discuss all the types of intelligent materials that are currently under investigation for the delivery of macromolecular therapeutics. Instead, this review aims to pinpoint the limitations of the current generation of materials with the ultimate goal to identify the material challenges that, in our opinion, will constitute a new generation of ‘intelligent’ materials. Such ‘intelligent’ materials should be able to sense and respond to environmental changes. The generated response to these environmental changes should give the material new properties that favor the delivery of nucleic acids. Besides dealing with material properties, we especially aim to focus on the biological barriers such intelligent materials will have to deal with when used for the intracellular delivery of nucleic acids, being the class of macromolecular drugs we prefer.
to discuss in this paper. In our opinion, knowledge of and insights in these biochemical and biological barriers will be of primary importance to material scientists for designing the next generation of advanced materials for drug delivery.

Macromolecular therapeutics such as antisense oligonucleotides (AONs), small interfering RNA (siRNA) and plasmid DNA (pDNA) show potential in the treatment of a wide variety of inherited and acquired genetic disorders, viral infections and cancer. Gene therapy aims to deliver these nucleic acids to cells to introduce novel genes or repair malfunctioning genes. Delivery of genetic material to cells provides multiple challenges for the material scientist as naked nucleic acids are often prone to degradation before reaching their target site, or do not reach their target site at all. Basically, to protect polynucleotides from degradation before reaching their target and to improve cellular uptake, they are often conjugated to synthetic carrier systems. In the second chapter we aim to overview the different categories of materials that are currently investigated in the field of non-viral gene therapy, such as liposomes, cationic polymers and matrix polymeric particles. These materials spontaneously form interpolyelectrolyte complexes with the nucleic acids, which we will further refer to as nucleic acid nanoparticles (NANs). We will briefly introduce the general characteristics of these DNA carrier systems and the resulting NANs, without claiming to overview all the existing materials in each category.

In Section 3, we will discuss in more detail the characteristics of a specific category of NANs, namely biodegradable NANs which slowly release their nucleic acids in the extracellular or intracellular environment. These biodegradable NANs can by micron- or nanosized and have to fulfill some basic requirements. Also the loading and release features of some major classes of biodegradable NANs will be discussed.

Extracellular matrices, like blood, the interstitium between cells, mucus on epithelial surfaces and so on are the first barriers that NANs encounter on their way to their target cells. Extracellular matrices may dramatically alter the surface of NANs, thereby inducing their disassembly or changing their cellular uptake and intracellular processing. Also, the physicochemical entrapment of NANs may prevent them from reaching their target. In blood, NANs should evade the mononuclear phagocyte system and cross the endothelium of blood vessels to accumulate in the target tissue. The fourth chapter aims to teach material scientists on this biophysical matter with a special emphasis on what may happen to NANs when they arrive in blood, mucus and vitreous, as occurs after intravenous injection, inhalation of NANs into the lungs or intravitreal injection of the eye respectively. Subsequently, we will focus on the most recent strategies to overcome these extracellular barriers.

Once NANs have reached their target cells they have to gain access to the intracellular environment and deliver the nucleic acids to the required cell compartment e.g. somewhere in the cytoplasm (in the case of AONs and siRNA) or the nucleus (in the case of pDNA). On their delivery route NANs have to pass the cellular membrane, break through the endosomal membranes as they mostly reside in endosomes after cellular uptake, cross the nuclear membrane and finally release intact nucleic acids at the most appropriate time and place in the cells. Note that the intracellular environment is not a friendly place for foreign nucleic acids, as it is packed with nucleases ready to eliminate the released nucleic acids. The fifth chapter deals with the intracellular processing of NANs. We especially aimed to reveal how material properties influence the intracellular behavior of NANs. Considering the efficient intracellular path as followed by viruses when infecting cells, Section 5 proposes some unexploited ideas to design new types of NANs.

Although we gained an increasing amount of knowledge in the past few years, the intracellular processing of NANs remains rather obscure. At one hand this is due to the limited understanding of the biological complexity of cells and the limited view we have on the physicochemical architecture of the NANs exposed to the cells. Indeed, for many types of NANs we do not know exactly how they look like, which is partly due to the absence of suitable instruments or technical limitations of the existing instruments. On the other hand, however, one should realize that mapping (quantitatively and qualitatively) the intracellular processing of nanomaterials is a huge technical challenge. A detailed knowledge of the transport and dynamics of NANs in living cells, which needs the visualization of even individual NANs in cells, is crucial to feed material scientists with ideas to design more efficient and intelligent NANs. Even after the major progress made in the last decade in advanced light-microscopy, visualizing nanoscopic matter in living cells and tissues remains very challenging. As advanced light-microscopy is currently the only door to get access to a better understanding of the “life” of nanomatter in cells, in Section 6 we discuss our and others experience with advanced light-microscopy techniques used to visualize and quantify the steps of the delivery of nucleic acids in cells by NANs.

In the final chapter of this review, we aim to formulate the conclusions and perspectives that are of importance to the material scientist.
2. Established and emerging materials for nucleic acid delivery

To introduce the different types of materials that are currently under investigation for nucleic acid delivery, this chapter overviews some major characteristics of the various classes of nanomaterials proposed to carry nucleic acids.

2.1. Liposomes as nucleic acid carriers

Cationic liposomes are the most established materials for nucleic acid packaging. When dispersed in an aqueous medium, phospholipids, which consist of a hydrophilic polar head group and a hydrophobic hydrocarbon tail, form spherical bilayered structures, called liposomes, with an internal aqueous phase and also a water phase between the successive bilayers. While multilamellar liposomes consist of a number of lipid phospholipid bilayers and have a size typically ranging from about 200 to 800 nm, unilamellar liposomes consist of a single phospholipid bilayer and are above (in the case of large unilamellar vesicles) or below (in the case of small unilamellar vesicles) 200 nm in size [1]. For DNA delivery, the nucleic acids can be encapsulated into the liposomes’ interior or bound to the liposomes’ surface by electrostatic interactions. A variety of cationic, anionic and synthetically modified lipids and combinations thereof have been used to deliver a wide range of DNA-based therapeutics. The poor encapsulation efficiencies of the negatively charged nucleic acids in anionic liposomes has prevented their widespread use. Cationic liposomes, however, have been successful for achieving biological effects in cell culture [2]. The effectiveness of complexes of nucleic acid with cationic liposomes (which are called lipoplexes) depends on the type and nature of the cationic lipid, the cell type, the type of nucleic acid (e.g. nucleic acids chemistry and length) and the method of formation of the lipoplexes [3,4].

Cationic liposomes generally consist of a mixture of zwitterionic and positively charged lipids. While the positive charge on the polar head group of the cationic lipids is important for binding the negatively charged nucleic acids, the features of the alkyl chains play an important role in the organization of the lipoplexes. As discussed in Section 5, lipoplexes mostly enter the cells by endocytosis. If the lipoplexes remain in the endosomes, they are prone to degradation or secretion from the cell, without delivering the nucleic acids in the cells. To promote the endosomal escape of lipoplexes, a cone-like lipid (with a small head group and expanding alkyl chains) should be incorporated in the liposomes. Cone-like lipids, such as the neutral dioleoylphosphatidylethanolamine (DOPE) can adopt a hexagonal phase that disturbs the endosomal membrane thereby provoking endosomal escape [5,6]. The general structure of liposomes is depicted in Fig. 1, together with the commonly used cationic lipid N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethyl-ammoniumchloride (DOTAP) and the neutral lipid DOPE.

Upon mixing cationic liposomes with negatively charged nucleic acids, so-called lipoplexes are spontaneously formed through electrostatic interactions. Interaction between DNA and cationic lipids is a self-assembly process that is triggered by DNA-mediated fusion of liposomes, resulting in lipid-bilayers with the DNA sandwiched in between [7–9] (Fig. 2A). Due to this mechanism of lipoplex formation, the lipoplexes shield the nucleic acids from degradation by nucleases in the surrounding environment.

The positive surface of cationic lipoplexes enhances their cellular uptake. Upon systemic application, however, the charged particles interact with blood components and non-target tissue, triggering toxic effects, reducing circulation times and targeting ability [10]. Also, large aggregates can be found, leading to clogging of the blood capillaries. A common approach for masking the surface charge of lipoplexes is to coat particles with a hydrophilic polymer such as...
polyethyleneglycol (PEG). This “PEGylation” of lipoplexes prevents their aggregation, lowers toxicity and increases circulation time [11]. On the other hand, as we discuss in Section 5, PEGylation negatively interferes with the delivery process of the nucleic acids from the lipoplexes. Also, we have obtained experimental evidence that when pegylated liposomes are used to prepare the lipoplexes, the nucleic acids are not entrapped between successive lipid bilayers, but remain unprotected at the surface of the pegylated lipoplexes (Fig. 2B) [12,13]. Therefore, post-pegylation of lipoplexes is an attractive strategy, as will be discussed in Section 4.

Besides the PEGylation of the lipoplexes’ surface, also sugars, peptides and proteins are often bound to the surface of lipoplexes in an effort to target them to specific cells. As this review does not cover the targeting of NANs we would like to refer to Bartsch et al. for more information on the targeting of lipoplexes [14].

2.2. Cationic polymers as nucleic acid carriers

Polyplexes are nanosized self-assemblies of cationic polymers and nucleic acids. Tremendous research on the potential of cationic polymers as carriers of nucleic acids has been done in the last decade [15–18]. As for lipoplexes it is believed that the net positive charge of polyplexes improves their adhesion to the negatively charged cell membranes and thus their cellular uptake. While diethylaminoethyl-dextran (DEAE-dextran) can be considered as predecessor of the cationic polymers for gene transfection, poly(ethylene imine) (PEI) is, highly likely, the most widespread polycation investigated for nucleic acids. The major reason for this is that, compared to other kinds of cationic polymers, PEI based polyplexes are the most efficient in intracellular delivery of nucleic acids [19,20]. The repeating unit of PEI consists of two carbon atoms followed by a protonatable nitrogen atom (Fig. 3). PEI is available in a linear and branched conformation and in a wide variety of molecular weights. As discussed in Section 5, it is believed that the buffering effect by the amine groups on PEI keeps the endosomal pH neutral, which results in osmotic bursting of the endosomes and release of the polyplexes in the cytosol of the cells.

Besides PEI many other cationic polymers such as cationic polymethacrylates, cationic polypeptides, cationic celluloses and so on are under investigation as nucleic acids carriers [21–23]. To lower the toxicity of the cationic polymers a recent trend is especially to focus on biodegradable ones, which degrade into less toxic low molecular weight products that are excreted by the kidney. As an example, recently the Langer group introduced poly-β-aminoesters as a novel type of degradable polycations which are synthesized by Michael addition of a bisacrylate and a diamine and are degradable through the ester bounds between the monomeric units (Fig. 4) [24–27]. A large variety of bisacrylates and diamines have been employed resulting in a large library of degradable polycations, which were all in parallel tested for their capability of DNA complexation and transfection ability. This high throughput approach allowed to identify those cationic polymers which are the most promising for nucleic acid delivery.

Fig. 2. Structure of (A) a multilamellar non-pegylated lipoplex with DNA sandwiched in between the lipid bilayers and (B) an unilamellar pegylated liposome with DNA attached to the surface. Reprinted with permission from Sanders et al. [12] © Nature Publishing Group.

Fig. 3. Chemical structure of polyethyleneimine polymers with a linear (A) or a branched (B) backbone.
Although lipoplexes are currently being clinically evaluated further than polyplexes, there remains a substantial scientific interest in polyplexes (especially in those based on biodegradable cationic polymers) with as major aims (a) to improve the delivery efficiency of the nucleic acids and (b) to develop less toxic cationic polymers which are, like many types of polycations, often severely toxic to cells due to all kinds of aspecific (electrostatic) interactions with all kinds of cellular components. Another major challenge is to develop “safe” polyplexes that do not aggregate in serum, as aggregation can lead to immediate clogging of the blood capillaries with lethal consequences. Aggregation of polyplexes mainly occurs through adsorbed serum proteins. Therefore, cationic co-polymers with non-fouling properties are under development. Such co-polymers (see further below) mostly consist of a polycationic segment, which complexes the nucleic acids in the core of the polyplexes, and another segment (like PEG, polysaccharides, etc.) which provides the polyplexes with a non-fouling shield. In this way, so-called core–shell nanoparticles are formed (Fig. 5).

2.3. Block copolymers as nucleic acid carriers

It is well known that in aqueous medium amphiphilic block copolymers, which are constituted from one (or more) hydrophilic and one (or more) hydrophobic segment, may self-assemble into spherical structures such as micelles and polymerosomes. In case of micelles the hydrophobic segments form an inner core surrounded by a corona of hydrophilic segments which are in contact with the aqueous exterior [28,29]. Generally speaking, such block copolymer micelles, which have a size of several tens of nanometers, are due to their hydrophobic interior not suitable for the encapsulation of nucleic acids. An alternative approach that allows the encapsulation of nucleic acids are the so called polycation complex micelles [30–32]. As an example, nucleic acids can be incorporated into polyion complex micelles by mixing DNA (acting as polyanion) with a solution containing a poly(ethylene glycol-block-l-lysine) block copolymer, resulting in water soluble sub 100 nm NANs [33]. It has been shown that polycation complexes micelles protect the nucleic acids from degradation by nucleases and remain stable in the presence of serum proteins [34]. To retain also lower molecular weight nucleic acids, like AONs, polycation complex micelles with a cross-linked core have been developed, e.g. by the formation of disulfide bonds between thiol groups introduced on the poly-L-lysine segments [35]. As such disulfide bonds are cleaved in a reducing environment, encapsulated AONs may be released from the polycation complex micelles intracellularly as the intracellular environment is more reducing than the extracellular space.

2.4. Polyelectrolyte microcapsules as nucleic acid carriers

Polyelectrolyte microcapsules are a novel class of materials fabricated by alternate (so called layer by layer, LbL) adsorption of charged polyelectrolytes onto an oppositely charged (mostly spherical) template tens of nanometers to
tens of microns in size. After dissolution of the template “hollow” capsules are obtained (Fig. 6) [36]. As these capsules have only recently been introduced, by Sukhorukov et al. in the late nineties, they are just being discovered by drug delivery scientists [37,38]. So far only a limited number of reports deal with the encapsulation of nucleic acids in polyelectrolyte capsules [39,40]. In most cases a porous inorganic template (like calcium carbonate or silica) is used which is, before polyelectrolyte coating, impinged with a nucleic acid solution. After polyelectrolyte coating the template is dissolved (e.g. by the use of EDTA in case of calcium carbonate or HF in case of silica) while the nucleic acids remain entrapped within the capsules. Generally speaking, polyelectrolyte microcapsules made with synthetic polyelectrolytes, such as, e.g. polystyrenesulphonate and polyallylamine hydrochloride, are rather stable entities and are not expected to disassemble spontaneously in biological fluids like serum. This could be considered as an advantage as nucleic acids remain encapsulated and well protected. However, once inside cell the polyelectrolyte capsules should open and release the nucleic acids. Recently three approaches have been reported which might be promising for intracellular delivery of nucleic acids from polyelectrolyte capsules. Our group showed that polyelectrolyte capsules made from biodegradable cationic polyelectrolytes like polypeptides (e.g. polyarginine) upon cellular uptake degrade intracellularly and thus release their payload [41]. Another approach was presented by Skirtach et al. using polyelectrolyte capsules functionalised with silver nanoparticles: IR-laser irradiation causes thermal heating and vibration of the silver nanoparticles leading to total destruction of the capsules [42]. It was shown in vitro that such capsules could be efficiently taken up by cancer cells and destroyed once inside the cells. A third approach was presented by Zelikin et al. using capsules made of alternating layers of polyvinylpyrrolidone and polymethacrylic acid held together by hydrogen bonds instead of by electrostatic interactions [43]. Under physiological conditions such capsules are only stable when the successive layers are cross-linked through disulfide linkages. Cleavage of the disulfide linkages in a reducing environment comparable to the one in cells dissolves the capsules and releases the encapsulated material. All these concepts were shown for the encapsulation of fluorescent macromolecules (such as e.g. fluorescently labeled AONs) but no biological effects were shown so far [43]. Nevertheless it is anticipated that these capsules, due to their multifunctionality and high loading capacity, show high potential as intracellular delivery vehicle with possible applications in nucleic acid delivery.

2.5. Polymeric matrices as nucleic acid carriers

Besides the major interest in lipoplexes and polyplexes, which arise through spontaneous self-assembling of nucleic acids and carrier molecules and which have a rather undefined and complex architecture, there is also a major interest in micro- and nanoparticles in which the nucleic acids are “simply” embedded in a biodegradable polymer matrix. Such “matrix particles” should allow a “simple” sustained release of the nucleic acids from the matrix particles into the extracellular space of the target tissue, which should prolong the duration of DNA expression due to a continuous DNA uptake by the target cells. Although naked DNA can transfect muscle cells [44,45], considering the
rapid in vivo degradation and the inefficient cellular uptake of naked DNA one could wonder, however, whether extracellular slow release of naked DNA makes sense. Biodegradable micro- and nanoparticles that slowly release the DNA in the target cells after cellular uptake of the particles are an important challenge and became widely investigated. As there is experimental evidence that microparticles larger than 1 μm are internalized through phagocytosis preferentially by antigen presenting cells (APCs, such as macrophages and dendritic cells), but not by other cells, such biodegradable DNA microparticles are especially investigated for (DNA) vaccination purposes against viruses and tumors as APCs play a pivotal role in initiating of immune responses. These slowly releasing micro- and nanoparticles will be discussed in the following chapter of this review.

3. Slow release of nucleic acids: a simple challenge?

3.1. Extracellular and intracellular “dissolving” DNA particles

Independent on whether the nucleic acid particles have to slowly release the nucleic acids extra- or intracellularly, independent on whether they are micron- or nanosized, to make a chance as slow release carrier of nucleic acids the particles should fulfil some basic requirements. (a) Considering the low transfection efficiency of pDNA, the particles should be able to carry a high amount of intact pDNA. Detrimental conditions during particle formation like polymerization reactions involving free radicals or sonication to form the emulsion should be avoided as they may degrade the nucleic acids. (b) in vivo, the polymer matrix should protect the encapsulated nucleic acids against nucleases, as it is well known that, even within minutes of exposure to serum, nucleic acids begin to break down rapidly. However, generally speaking, most types of biodegradable DNA particles seem to adequately protect nucleic acids in vivo. (c) To avoid the loss of nucleic acids the burst release from the particles should be as low as possible.

The continuation of this chapter especially compares loading and release features of some major classes of biodegradable DNA matrix particles. The first class concerns the poly(lactic-co-glycolic acid) (PLGA, Fig. 7A) micro- and nanoparticles which are the most studied biodegradable DNA matrix particles (see Section 3.2) [46]. PLGA is used in several FDA approved formulations, GMP grade PLGA is commercially available and it has a long history of safe use in both medical applications (like implants, internal sutures) and drug delivery (like peptide and protein delivery). All these features made PLGA attractive to many researchers involved in DNA delivery. However, one could argue that, although PLGA is useful for protein delivery, it was not really designed for the delivery of DNA. PLGA shows, indeed, several drawbacks for DNA delivery. First, because of the large size and hydrophilic character of DNA, encapsulation of plasmid DNA in hydrophobic PLGA microspheres is a challenge. Second, it is well known that the hydrolysis of PLGA may substantially decrease the pH in PLGA microspheres, potentially resulting in DNA degradation [47,48], although basic additives like Mg(OH)₂ and Ca(OH)₂ encapsulated in the PLGA microspheres may overcome this [49]. Third, the rate of DNA release and thus also subsequent antigen production in case of DNA vaccination, is often too slow, which may prevent an optimal immune response. Given the limitations of PLGA microparticles for slow delivery of DNA there was a clear need for developing new types of biodegradable matrix particles for slow release of DNA, discussed in Section 3.3, which should fulfil the following requirements. (a) The

![Fig. 7. (A) PLGA, (B) PBAE and (C) bisacrylamide acetal cross-linker.](image-url)
degradation of the matrix particles should not generate any hostile (such as acidic) internal environment, (b) they should allow both an efficient and high incorporation of plasmid DNA and (c) they should be relatively stable at neutral pH but hydrolyse rapidly around pH 5 being the pH inside the phagosomal compartment of the APCs. This would minimize premature release in the blood and interstitial tissue space while accelerating DNA release inside the cells.

3.2. PLGA micro- and nanoparticles containing plasmid DNA

3.2.1. Preparation of plasmid DNA containing PLGA particles

Approaches for encapsulating plasmid DNA within PLGA microspheres have been the spray-drying [47] and, especially, the double emulsion (water–oil–water) technique [48,50,51]. Originally the encapsulation of plasmid DNA in PLGA microparticles by the double emulsion method seemed problematic as the DNA loading was as low as 1–2 \( \mu g/mg \) PLGA [48]. Tinsley-Bown et al. [52] succeeded in optimizing the double emulsion process by changing the organic solvent from dichloromethane to ethyl acetate and optimizing various process parameters. Also the Alonso group reported on optimized techniques (water–oil–water emulsion/solvent evaporation technique as well as a water–oil emulsion/solvent diffusion technique) to produce (PEGylated) PLGA nanoparticles (smaller than 300 nm) highly loaded with plasmid DNA (up to 10–12 \( \mu g \) plasmid DNA/mg polymer with high encapsulation efficiencies between 60 and 90\% [53]).

Inactivation of the plasmid DNA during encapsulation by the double emulsion method was addressed by Ando et al. [54] who proposed a cryopreparation technique in order to prevent the exposure of the plasmid DNA to shear forces. To overcome plasmid DNA degradation the O’Hagan lab [55] proposed to use PLGA microparticles that showed a positive surface charge through the inclusion of cationic surfactants (like the cationic lipid DOTAP). After preparing the positively charged PLGA microparticles, the plasmid DNA was then adsorbed in an efficient way (loading efficiency between 60 and 90\%) to the surface of the particles. Although in this way the plasmid DNA may indeed be better protected during the preparation of the particles, one should note that in vivo DNA associated to the surface may become more easily degraded and may result in a significant burst release as indeed observed in vitro experiments by Singh et al. [55].

3.2.2. Enhancing the loading of PLGA particles with plasmid DNA

The encapsulation of plasmid DNA in PLGA has been rather problematic, often as low as a few \( \mu g/mg \) polymer [48]. Several attempts have been reported to enhance the loading of PLGA particles with DNA. The Merkle group showed that the hydrophobicity and the molecular weight of the PLGA has a profound influence on the encapsulation efficiency, the hydrophilic polymers showing higher encapsulation efficiency [47]. Another approach made use of PLA grafted with cationic polysaccharides facilitating the adsorption of the negatively charged DNA [56]. Rather similar to the approach of the O’Hagan group (see above) Kusonwiriyawong et al. [57] prepared cationic PLGA microparticles by solving cationic surfactants (like water insoluble stearylamine) in the organic solvent in which the PLGA was solved. Still another strategy was to reduce the negative charge of plasmid DNA by condensing with poly(aminocarids), such as poly-L-lysine, before encapsulation in PLGA microparticles [58,59]. The preliminary step of DNA complexation significantly enhanced the encapsulation efficiency. A similar approach, using polyethyleneimine, was reported by De Rosa et al. for loading PLGA particles with AONs [60].

3.2.3. Accelerating the release of plasmid DNA from PLGA particles

Generally speaking, after an initial burst release, plasmid DNA release from PLGA particles occurs slowly during several days/weeks [50,53,55,57]. It is believed that the degradation of the PLGA particles, through a bulk homogeneous hydrolytic process, determines the release of plasmid DNA. Consequently it can be expected that using more hydrophilic PLGA not only improves the encapsulation efficiency of DNA but also results in a faster release of plasmid DNA, as indeed observed by Kusonwiriyawong et al. [57] and Tinsley-Bown et al. [52], amongst others. Besides the chemical composition of the PLGA (PLA versus PGA content), many other factors, like PLGA molecular weight [57], particle size and morphology, have been reported to influence the release. For example Perez et al. [53] reported that plasmid DNA release kinetics depend on the plasmid incorporation technique: nanoparticles prepared by the water–oil emulsion/diffusion technique released their content rapidly whereas those obtained by the water-oil-water emulsion method showed an initial burst followed by a slow release for at least 28 days.
As discussed under Section 3.1 conventional PLGA particles are not ideal for the development of DNA vaccines as the rate of DNA release from these particles is mostly too slow to induce immune responses as (1) the amount of plasmid DNA immediately available after cellular uptake by APCs is too limited and (2) plasmid DNA released after days/weeks from the particles may be significantly damaged due to the acid microenvironment in PLGA particles. Therefore several groups tried to develop PLGA formulations, which should rapidly release plasmid DNA in a biologically active form. The strategy of the O’Hagan lab [55], which involved the adsorption of plasmid DNA at the surface, rather than entrapping the plasmid DNA in the PLGA particles (see under Section 3.2.1) resulted in a substantial faster release: *in vitro* release experiments 35% was released at day 1, however, it still took 14 days to release up to 75% of the adsorbed plasmid DNA. Very recently the Langer group [61] reported on plasmid DNA encapsulated in hybrid microparticles consisting of a mixture of PLGA and biodegradable poly-β aminoesters (PBAE, Fig. 7B). The PBAE’s they studied, which degrade hydrolytically to yield 1,4-butanediol and β amino acids, showed a half-life of a couple of hours, degrading faster at pH 7 than at pH 5 [24]. The hybrid PLGA/PBAE microparticles were prepared by the double emulsion method as conventional PLGA microparticles. The incorporation of PBAE (15–25%) did not alter the structure nor loading of the PLGA particles but significantly altered the release rate: microparticles prepared from PBAE had the ability to rapidly release their content, also when exposed to acidic endosomal pH [62]. A spectacular increase in *vitro* gene transfection of macrophages (upon 3–5 orders of magnitude) upon adding PBAE to the PLGA microparticle formulations was observed. Besides the fast plasmid DNA release it was also speculated that PBAE should be able to absorb protons in the endosomes during phagosomal acidification and thus may provide a means of phagosomal escape by osmotic membrane disruption using a proton sponge like mechanism.

3.3. **Plasmid DNA containing micro- and nanoparticles which degrade fast in endosomes**

The drawbacks of conventional PLGA particles for plasmid DNA delivery encouraged some groups to search for better alternatives. Most of them especially tried to overcome the too slow release of plasmid DNA from PLGA particles by developing particles which retain the plasmid DNA at neutral pH but which show fast and complete plasmid DNA release at the more acidic lysosomal pH.

3.3.1. **Microparticles having acetal cross-linkers**

The Fréchet group reported on plasmid DNA encapsulated into acrylamide based particles having an acid-cleavable bisacrylamide acetal cross-linker (Fig. 7C; [63]). The microparticles were previously studied for intracellular protein delivery [64]. The plasmid DNA loading equalled 0.88 μg/mg polymer while the encapsulation efficiency was around 40%. Although the microparticles were prepared via radical polymerization in an inverse emulsion system, the plasmid DNA remained unchanged during the polymerization procedure, although the tertiary structure seemed to be affected as most of it turned into the open circular form. Retention of the DNA payload at physiological pH 7.4 (as the pores in the microparticle are small enough to keep the plasmid DNA encapsulated) with complete release in only 2 h at lysosomal pH was indeed demonstrated. The plasmid DNA was physically (not electrostatically) encapsulated in the cross-linked microparticles without the use of polycations, which should be a clear advantage as polycations are often bioincompatible due to interactions with oppositely charged biomolecules such as proteins.

3.3.2. **DNA containing poly(ortho-ester) microparticles**

Wang et al. [65] recently reported on biodegradable poly(ortho-ester) (POE) microparticles loaded with plasmid DNA which were prepared by the double emulsion method. POEs are interesting as the ortho-esters bond in the polymer backbone are relatively stable at physiological neutral pH but hydrolyze rapidly at around pH 5 [66]. POEs, since long time under investigation as biodegradable materials for controlled delivery of (protein) drugs, are synthesized by condensation copolymerization of a diketene acetal and diols, which forms the hydrolyzable ortho-ester backbone of the polymer. In contrast to polyesters of the PLGA family, which undergo bulk erosion, POEs show a predominantly surface-confined erosion process. Therefore, small quantities of acid hydrolysis products of POEs are able to diffuse away, potentially minimizing DNA degradation, especially advantageous when using larger microparticles. At physiologic pH 7.4 an initial burst of DNA release from POE microparticles was observed, with the total amount of DNA released after 1 week being about 30%. At pH 5.0 plasmid DNA release from POE particles accelerated dramatically with all plasmid DNA released in 24 h. In mice plasmid DNA containing POE microparticles...
were able to both generate immune responses (after single vaccination with the β-gal encoding plasmid as a model) as well as to suppress the growth of tumour cells which make them very attractive for further studies.

4. Crossing and surviving the extracellular space

The DNA containing microparticles described in Section 3 of this review are designed to release their DNA payload in the extracellular space or to be internalized by migrating dendritic cells after subcutaneous or intramuscular injection. Hence, these DNA microparticles do not have to travel through extracellular matrices to reach their target cells. On the other hand, nucleic acid containing nanoparticles (NANs) have to travel through extracellular barriers such as blood before they can reach their target tissue, which can be situated near as well as far away from the administration site. Additionally, once they reached the tissue of interest these NANs need to be internalized by the target cells. While these cellular barriers for NANs are currently under deep investigation, less attention is given to the influence of extracellular barriers. The nature and number of encountered extracellular barriers depends on the delivery route and location of the target cells. In this part of the review we will focus on (a) how extracellular matrices (ECM) complicate the journey of NANs to their target cells and (b) strategies that can enhance the movement and stability of NANs in extracellular matrices. Many extracellular matrices exist in the human body. Here we will mainly focus on the barrier properties of blood, respiratory mucus and vitreous towards NANs as these three types are important ECMs encountered by NANs and are study objects in our research group. Finally, extravasation of NANs will also be discussed. Gene delivery to tumors is also an important research field. To be effective, NANs must penetrate the tumor interstitial matrix to reach the cancer cells. The tumor interstitium is a complex assembly of collagen, hyaluronan, glycosaminoglycans and proteoglycans [67,68] that, together with the elevated interstitial fluid pressure in solid tumors, is known to limit the mobility of macromolecules and nanoparticles [69]. The transport of nanoparticles in the tumor interstitium has been reviewed in excellent reviews and will therefore not be considered in this paper [70–72].

4.1. The blood components and endothelial cells as a barrier to systemic gene delivery

Systemic application of NANs is attractive as it may allow the distribution of the particles via the bloodstream to tissues that are otherwise difficult to reach via local application. Systemic injection of NANs is especially of interest for the treatment of (malignant) diseases that affect large organs like hepatitis or involve multiple organs like lysosomal storage diseases [73,74]. Finally, in the case of tumor metastasis systemic administration of NANs is the most suitable option to reach all the tumors.

However, the blood forms a major barrier to non-viral gene delivery and during the last decade harmful interactions of several blood components with NANs have been reported [75–77]. Indeed, it is well documented that albumin, the most abundant negatively charged protein in blood, extensively binds to especially cationic NANs causing a neutralization or reversion of their surface charge [78–81]. Neutralization of NANs by albumin or other biomolecules abolishes the electrostatic repulsion that exists between the NANs and hence allows that nanoparticles can come in close proximity during collision. When such close contact happens, attractive Van der Waals forces take place and hold the nanoparticles together resulting in the formation of aggregates. Additionally, in contrast to DNA matrix nanoparticles, the binding of such negatively charged biomacromolecules to self-assembled NANs often de-assembles the NANs. Such a disassembly is due to the competition of the negatively charged albumin with the DNA for binding to the cationic DNA carrier.

The effect of the binding of blood components on the biological activity of the NANs depends on the type of NANs. Indeed, a dramatic inhibitory effect of serum on the transfection capacity of lipoplexes containing polycationic lipids or DOPE has been reported, while the transfection efficiency of complexes containing cholesterol is much less affected [78,79,82–85]. The latter has been attributed to the higher stability of cholesterol containing NANs in biological fluids [86,87]. Many other blood components like fibrinogen, heparin, oleic acid, high density lipoprotein (HDL) and low density lipoprotein (LDL) have been reported to bind to cationic NANs as well and to affect their gene transfer capacity [80,88–90]. However, the lipoproteins LDL and HDL have been identified as the most inhibitory components [90]. Additionally, the opsonization and activation of the complement also triggers a rapid clearance of NANs by the mononuclear phagocyte system [88,91]. Furthermore, it has been shown that the binding of cationic NANs to erythrocytes leads to an aggregation of the erythrocytes [88,92,93]. The predominant accumulation and gene transfer in the lungs after systemic delivery of especially polyplexes has been attributed to a blockage of small lung capillaries
by aggregated polyplexes and/or large erythrocytes/polyplex clusters [81,88,94–96]. Indeed, this blockage of the lung capillaries gives the polyplexes sufficient time to transfect the endothelial cells of the lung capillaries. After 1–2 h these lung-associated NANs partially redistribute to the liver and spleen, where they also cause gene transfer [94,95]. Besides macrophages, other blood cells (e.g. lymphocytes) and endothelial cells also take up systemically delivered NANs [82,97]. If these cells are not the target, this non-specific uptake will further reduce the amount of NANs that can reach their target cells.

The second barriers that NANs encounter after systemic delivery are the endothelial cells and basement membranes. Indeed, NANs have to extravasate before they can reach tissues localized outside the bloodstream. The ability of NANs to extravasate is mainly determined by their size and the permeability of the capillary walls that greatly varies between tissues [98]. Based on the morphology of the endothelial and basement membrane one can divide capillary endothelium into continuous, fenestrated and discontinuous endothelium [99]. The continuous endothelium, which is found in all types of muscular tissues, lung, skin and subcutaneous tissues, is the tightest one and prevents the passage of materials greater than 2 nm. The brain endothelium offers an even stronger barrier: only small hydrophobic molecules can cross the blood-brain barrier. Fenestrated endothelia, which occur in the intestinal mucosa, the kidney, the endocrine and exocrine glands, contain openings of 40–60 nm in diameter. However, the continuous basement membrane surrounding these capillaries prevents the passage of macromolecules larger than 11 nm. Discontinuous capillaries or sinusoidal capillaries are found in the liver, spleen and bone marrow. These capillaries have endothelial junctions of about 150 nm or even up to 500 nm following certain reports and contain either no (liver) or a discontinuous basement membrane (spleen and bone marrow). Leaky capillaries are also found at sites of inflammation and in tumors [100]. Extravasation of particles with a diameter of up to 400 nm in certain tumors has been reported [101]. However, other reports found no extravasation of particles larger than 100 nm in tumors [102].

Based on the size-dependent permeability of the endothelia discussed above one can conclude that NANs can only access the liver, spleen and bone marrow after systemic application provided that they are smaller than 150 nm. The smallest particles are the adeno-associated viruses and the non-viral monomolecular DNA complexes reported by the group of Behr et al., which have a diameter of about 20–30 nm [103,104]. However, it has recently been demonstrated that viral vectors can penetrate the capillary walls also via transcytosis, an active transport mechanism of material through cells [105,106]. Transcytosis of macromolecules has also been obtained after conjugation to antibodies that trigger caveolae mediated transcytosis in lung endothelia [107].

4.2. Respiratory mucus as a barrier to respiratory gene delivery

Gene delivery to the respiratory epithelium holds therapeutic potential for pulmonary disorders like lung cancer, alpha-1 antitrypsin deficiency, asthma and cystic fibrosis (CF) [108–111]. The latter disease was one of the first targets in gene therapy. However, clinical trials, using both viral and non-viral NANs, have revealed that the delivery of therapeutic DNA to the target cells in the lungs of especially CF patients is a very difficult task [110,112]. The presence of a thick and viscoelastic layer of lung mucus on top of the respiratory epithelium is considered as one of the main causes of the failure of CF gene therapy [113]. CF mucus can be considered as a biopolymer network with meshes that are filled with free biopolymers (Fig. 8). CF mucus may decrease gene transfer to the underlying epithelium in different ways: (a) it may sterically restrict the transport of the NANs to the target cells, and (b) CF mucus components may interact with NANs thereby changing their structural properties. We and others have demonstrated that the transport of nanoparticles with a diameter above 100 nm and 500 nm are respectively obstructed and blocked by CF mucus [113–115]. Additionally, exposure of cationic NANs to CF mucus components drastically reduced their gene transfer capacity [116]. This decrease was primary due to binding of negatively charged mucus components, like DNA, albumin, alveolar surfactants and mucin to the NANs, causing their aggregation. In line with these observations, Kitson et al. showed that application of CF mucus on top of sheep lung tissue strongly decreased the extent of gene transfer of both NANs and viral NANs [117]. Additionally, adenoviral mediated gene transfer to the lungs is also impeded by neutralizing antibodies present in the mucus [118].

4.3. Vitreous as a barrier to ocular gene delivery

Ocular gene therapy may offer new hope for severe eye diseases like retinitis pigmentosa and age-related macular degeneration (AMD) [119,120]. Many of these ocular diseases are due to a gene defect in the retina, a multi-layered
sensory tissue that lines the back of the eye (Fig. 9A). The blood-retinal barrier and the sclera prevent that large molecules like NANs can access the retina after systemic or topical applications, respectively [121]. Therefore, intravitreal injection, which is less invasive than subretinal injection, may become a clinically acceptable route for the administration of NANs [122]. However, before NANs can reach the retina they have to travel through the vitreous. Vitreous is a gel-like material built up from collagen fibrils bridged by proteoglycan filaments that contain negatively charged glycosaminoglycans (GAGs) [123]. Similar to CF mucus, one can expect that this biopolymer network may immobilize NANs and that GAGs may bind to the NANs. Recent studies have confirmed this assumption. The group of Urtti et al. showed that a thin layer of vitreous on top of retinal cells almost completely blocks the gene expression of cationic polyplexes and lipoplexes [124]. Moreover, we recently showed that cationic lipoplexes severely aggregate when mixed with vitreous (Fig. 9B) [125]. This aggregation is most likely due to the binding of negatively charged biopolymers in the vitreous, such as GAGs, to the cationic lipoplexes, which neutralizes their surface charge thus leading to aggregation. These aggregated lipoplexes become completely immobilized in the vitreous gel and have hence little chance to reach the retinal cells. On top, binding of GAGs to lipo- and polyplexes may also impede the intracellular processes which lead to successful gene expression [126].

4.4. Strategies aimed at increasing the resistance of NANs towards the ‘hostile’ extracellular environment

The findings above strongly emphasize the need for NANs that (1) are small enough to penetrate through capillary walls and extracellular matrices, (2) do not aggregate in extracellular matrices or cause aggregation of erythrocytes, (3) do not disassemble in extracellular matrices and (4) do not become covered by complement proteins and other serum
proteins that trigger a rapid clearance of the NANs by the mononuclear phagocyte system. The strategies to overcome these extracellular matrices in gene delivery have focussed both on modification of the NANs as well as modification and/or manipulation of the extracellular barriers themselves.

4.4.1. Surface coating of NANs to avoid non-specific interactions

Coating of nanoparticles with polyethylene glycol (PEG) is since decades considered as the golden standard to avoid rapid uptake of nanoparticles by the macrophages, interaction of extracellular components with nanoparticles and aggregation of nanoparticles in buffer and extracellular matrices [127]. Consequently, this strategy has also been evaluated for NANs. Several studies have demonstrated that shielding of the surface of NANs with PEG prevents their interaction with ECM components, prevents aggregation, reduces toxicity, prevents uptake by the mononuclear phagocytic system, enhances the circulation time in the bloodstream and improves their journey through extracellular matrices like serum, sputum and vitreous [12,88,116,125,128–131]. Additionally, PEGylation of viral NANs has also attained much attention to prevent their recognition by the immune system and to enable a retargeting of these viral NANs to the desired tissues [132–134].

NANs can be shielded with polymers via two approaches. In the first method, which can only be used for NANs made via self-assembly, the cationic DNA carrier is covalently coupled to the shielding polymer and subsequently mixed with the DNA [135–140]. During the self-assembling of the NANs, the DNA and the cationic carrier interact with each other creating a slightly hydrophobic core that is surrounded by a shield of hydrophilic polymers (see Fig. 5). This method has as disadvantage that the shielding polymers can hinder the self-assembling process between the cationic carrier and the anionic DNA, especially when high amounts of shielding polymer are used. Therefore, post-shielding has been considered, involving the physical incorporation or (covalent) attachment of the shielding PEGylated polymer or PEGylated lipids to pre-formed NANs [141–145]. The cationic surface of pre-assembled NANs also allows ionic coating by negatively charged polymers. The latter strategy was used by Finsinger et al., who synthesized protective copolymers (PROCOPs) by linking PEG to negatively charged peptides that subsequently bind to the cationic surface of the NANs by electrostatic interactions [146]. Interestingly, one of the peptides they used was INF-7, a fusogenic peptide derived from the influenza virus. Additionally, Cheung et al. [147] used the anionic polymer poly(propylacrylic acid) (PPAA), a polymer also known to disrupt endosomal membranes, to coat cationic NANs, while Nicolazzi et al. [148] proved that anionic PEG-lipids enhanced the colloidal stability of liposome based NANs. A further advantage of using ionic coating is that the shielding polymers can dissociate from the NANs in the acidic environment of the endosomes, allowing as discussed further, an efficient endosomal escape. A drawback of this strategy is, however, that the stability of the ionic coating in ECM such as full serum or blood is questionable and has not yet been demonstrated.

The presence of hydrophilic polymers on the surface of NANs prevents aggregation by avoiding that NANs can come in close proximity to each other during collision. Further, when present in sufficient amounts, these dangling polymers protruding on the surface of NANs also avoid that macromolecules can reach the charged core of the NANs. Unfortunately, they can also prevent close interactions between the NANs and cell membranes and prevent endosomal escape, which has a negative effect on the biological efficiency of such shielded NANs [128,149–152]. As discussed further in Section 5 this has been overcome by reversible shielding of NANs with polyethylene glycol (PEG), which implies that the NANs loose their protective shield at or in the target cells. Additionally, one should also realize that there are gaps between the polymer chains that can allow small charged molecules to reach the surface of the NANs. Indeed, we have for example observed (unpublished data) that albumin is able to penetrate between the PEG of lipoplexes containing 5 mol% PEG-lipids and to compete with nucleic acids for binding to the positive charges. The size of these gaps depends on the degree of shielding and the chain length of the polymers. However, one should realize that long PEG chains (\(\sim >10 \text{kDa}\)) may entangle in the biopolymer network of biogels [153]. Therefore, particles containing such long PEG chains may become immobilized in mucus or vitreous.

Another drawback of putting PEG on the surface NANs is that a second injection of PEGylated NANs (e.g., lipoplexes) results, in contrast to the first injection, in a rapid clearance of the NANs from the circulation [154]. The formation of antibodies against the PEGs on the lipoplexes would be responsible for this rapid clearance. In contrast, the half-life of empty PEG liposomes did not change after repeated administration [155]. Therefore, it has been proposed that the CpG motifs in DNA, which are known to be immunogenetic, act as an adjuvant for the production of antibodies to the PEG chains [156]. However, several groups have demonstrated that empty liposomes also induce an antibody response [157,158]. Interestingly, the use of rapidly exchangeable PEG-lipids (like PEG-CerC14; see
Section 5) in liposomes and lipoplexes strongly reduced the induction of antibodies and their subsequent rapid elimination [156]. In light of this immune reaction against PEGylated lipoplexes, several groups have proposed to use e.g. synthetic polypeptides, poly(propylacrylic acid) or polysaccharides instead of PEG as shielding polymer [144,147,159,160].

The dangling ends of the shielding polymers have often been used to provide the NANs with targeting moieties. As recent excellent reviews on targeting of NANs exist we will not consider this topic here [161–163].

4.4.2. Strategies aimed at modifying and/or manipulating the barrier

Both biochemical and physical strategies have been evaluated to enhance the extravasation of macromolecules and particles. A first component that has been evaluated is the vascular endothelial growth factor (VEGF). VEGF is known to stimulate angiogenesis in pathological conditions like cancer and age-related macular degeneration, an intraocular disorder [164,165]. However, VEGF also enhances the permeability of blood vessels [166]. This property of VEGF has been exploited to enhance the extravasation of macromolecules [167]. Further, Rosenecker et al. reported that substance P can promote extravasation of liposomes in endothelia that contain receptors for substance P [168]. Additionally, migration of liposomes through the endothelial cell monolayer has also been reported in the presence of migrating polymorphonuclear neutrophils [169]. Other components like sodium decanoate and vasoactive amines (e.g. histamine, serotonin, and bradykinin) that are produced during inflammation have also been shown to enhance extravasation of adenoviral vectors and liposomes, respectively [169,170].

Ultrasound alone or in combination with diagnostic microbubbles form an attractive method for locally enhancing the extravasation and the mobility of NANs in ECM [171–173]. Microbubbles in combination with ultrasounds are used in the clinic for diagnostic purposes [174]. Therefore, the technique is considered as a safe and very promising tool to enhance the efficacy of (non-)viral vectors. The proposed mechanism by which this technique mediates extravasation has been ascribed as cavitation, which is the ultrasound mediated growing and shrinking of gas-filled microbubbles. After several growing-shrinking cycles, these “cavitating” microbubbles implode, causing shock waves and microjets that perforate the walls of small capillaries leading to the extravasation of particles and even erythrocytes [175–177]. Additionally, the generated microjets enhance the migration depth of particles in tumors and other tissues [178].

Since decades hyperthermia has been used to promote extravasation of e.g. liposomes [179,180]. Currently, the group of Mark Dewhirst is especially renowned for their work on hyperthermia and drug delivery [181]. In particular, they demonstrated that local heating of a tumor till 42 °C increased the pore cut off size of the endothelia to >400 nm allowing an efficient delivery of liposomes to the tumor interstitium [102]. Also the effect of the duration and extent of heating on the extravasation of particles was studied in detail [182] (Fig. 10). Interestingly, hyperthermia does not cause extravasation of particles in normal tissues, potentially allowing tumor-specific delivery of anti-cancer drugs. Recently, the same group showed that hyperthermia combined with Doxil® , a clinically used liposomal formulation of doxorubicin, led to promising results in patients with ovarian cancer [183]. Several reports have demonstrated that hyperthermia also enhances non-viral gene delivery to tumors [184,185].

As discussed above extravasation is not enough to guarantee an efficient delivery of macromolecular drugs or nanoscopic drug carriers to tumor cells. Indeed, these materials still have to overcome the tumor interstitium. As already mentioned above, ultrasounds combined with microbubbles has been shown to enhance the transport of particles into tissues [178]. Another strategy involves the use of electric fields to drag charged macromolecules like DNA through tissues such as the tumor interstitium [186,187].

To reduce the barrier properties of respiratory mucus towards NANs two approaches can be envisaged, namely a cleavage of the biopolymer network and a reduction in mucus production. With regard to the former we showed that rhDNase I, a clinically used enzyme that degrades CF mucus by cleaving DNA chains, caused a threefold increase of the transport of both nanospheres and lipoplexes with a diameter of ~300 nm [113]. Furthermore, Ferrari et al. demonstrated that the efficacy of non-viral gene transfer was both in vitro and in vivo significantly enhanced when the mucus was pre-treated with nacystelyn or N-acetylcysteine [188]. Other groups made similar observations. Iiboshi et al. and Khan et al. found that the intestinal permeability of FITC-dextran (70,000 g/mol) and fluorescent polystyrene microspheres (3.2 μm), orally administered to rats, was improved in the presence of N-acetylcysteine [189,190]. Recently, a two- and threefold enhancement of the bioavailability of nasal delivered dextran and calcitonin was observed in the presence of N-acetylcysteine [191]. Nacystelyn and N-acetylcysteine are clinically used agents that are known to cleave the major gel-forming biopolymer in mucus, i.e. mucin [192]. Finally, Ferrari et al. also showed that
pre-treatment of mice with glycopyrrolate, which decreases mucus production, enhanced the reporter gene expression in the mouse lung [188].

5. The intracellular journey of NANs: finding its way in cells

Once NANs have reached their target cells, they have to gain access to the intracellular environment. Furthermore, they have to deliver the nucleic acids to the required compartment of the cell, i.e. in the cytoplasm in the case of AONs and siRNA and in the nucleus in the case of pDNA. On their delivery route, NANs encounter different barriers such as cellular uptake, escape from the endosomes, trafficking the cytoplasm and crossing the nuclear membrane. On top they should release the nucleic acids at the most appropriate time and location in the cells. Which of the barriers limits the delivery process by NANs the most is currently not well understood and, highly likely, depends strongly on the composition of the NANs. In this chapter we focus on the intracellular barriers NANs meet on their trafficking path and how to overcome them. We discuss the nature of these barriers, the intracellular processes involved and the routinely used strategies to circumvent them. Also, having the efficient intracellular path of viruses in mind, we would like to propose some unexploited material challenges to come up with more advanced nanoparticulate matter which better fulfills the need of efficient nucleic acid delivery.

5.1. Endocytosis: the major uptake route

The cell is the smallest unit of life in our body. It can be seen as a complex organization of multiple intracellular structures that constantly receives and interprets signals from the environment in order to function and stay alive. The cytosol, in which the nucleus and a variety of organelles are embedded, is protected from the outside environment by a bilayer lipid plasmamembrane. The cytosol together with the different organelles is called the cytoplasm. Due to the complex organization of mammalian cells, it comes as no surprise that the income and output of molecules is highly organized. The traffic of newly synthesized molecules to their final destination (e.g. the lysosomes, the golgi apparatus, the extracellular space, etc.) is organized by different biosynthetic pathways. Likewise, the entry of extracellular molecules is organized by endocytic pathways. Modern drug delivery frequently requires delivery to a specific intracellular target. Therefore, increasing knowledge of endocytosis and agents that manipulate endocytic pathways are opening new avenues for more efficient intracellular drug delivery.
The plasmamembrane consists of a lipid bilayer that poses the first barrier in reaching the intracellular environment. Due to their negative charge, naked DNA molecules do not efficiently cross the plasmamembrane. Therefore, they rely on the use of a gene delivery system to gain access to the intracellular environment. Most of the time, NANs enter the cells by endocytosis (Fig. 11, 1a and 1b). Sometimes, they can enter cells via direct fusion with the plasmamembrane with direct delivery of the NANs (Fig. 11, 1c) or naked DNA (Fig. 11, 1d) into the cytoplasm, thus avoiding the endolysosomal compartment. This fusion can occur due to the intrinsic properties of the NANs (e.g. a lipid bilayer around the NANs may facilitate their fusion with the plasmamembrane), or by the incorporation of cell penetrating peptides, such as TAT, into the NANs [193]. The term endocytosis encloses multiple methods of internalization such as clathrin-dependent endocytosis, clathrin-independent endocytosis, macropinocytosis and phagocytosis. The endocytic pathways control the transport of incoming molecules and determine their fate, such as recycling to the plasmamembrane, secretion in the extracellular space or delivery to specific compartments in the cytoplasm like the golgi apparatus, the endoplasmatic reticulum and the lysosomes. Endocytosis is essentially the invagination of the plasmamembrane with the formation of endocytic vesicles. This invagination occurs at specific domains in the plasmamembrane, characterised by a specific lipid and/or protein composition for the different endocytic pathways.

In clathrin-dependent endocytosis, this specific domain is characterized by the presence of clathrin and is called a ‘coated pit’ [194]. Invagination at these coated pits, which cover some 0.5–2% of the plasmamembrane surface, results in the formation of clathrin-coated vesicles. These vesicles loose their clathrin coat upon internalization and fuse with each other or with pre-existing endosomal compartments to form ‘early endosomes’. Early endosomes can either recycle their cargo to the extracellular environment or mature to late endosomes, accompanied by a decrease in pH from neutral to pH 5.9–6.0. Late endosomes on their turn deliver their cargo to the lysosomes (via a fusion-mediated mechanism), accompanied by an additional pH drop from pH 6.0 to 5.0. The average time to mature from early to late endosomes is reported to be 5 min, while late endosomes progress to lysosomes in the range of 30 min [195].

Clathrin-independent endocytosis can be subdivided into lipid-raft mediated endocytosis and caveolae, both characterized by cholesterol and sphingolipids enriched domains in the plasmamembrane. These lipid domains are additionally associated with caveolin-1 in the case of caveolae. Caveolar vesicles are continuously docking on and fusing with at least two endocytic compartments, caveosomes and early endosomes. Caveosomes differ from early endosomes by their neutral pH and deliver their cargo to different intracellular organelles, namely the golgi apparatus or the endoplasmatic reticulum. Due to the potential fusion with early endosomes, however, cargo taken up by the clathrin-independent pathway can switch to the clathrin-dependent pathway, resulting in recycling to the extracellular space or eventual delivery to the lysosomes [196–198].

So far there are only few studies that connect the entry mechanism of lipoplexes and/or polyplexes with the observed transfection efficiencies. It has been suggested that the clathrin-independent pathway leads to more efficient
transfection by NANs since it limits delivery to the lysosomes. Zuhorn et al. found that lipoplex-mediated transfection occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis, rather than through simple fusion with the plasmamembrane [199]. These observations were confirmed by Rejman et al. who found that DOTAP lipoplexes were internalized by cells solely by means of clathrin-mediated endocytosis [200,201]. Lipid-based NANs will thus be delivered to the lysosomes, unless they are able to escape the endosomal vesicles. In contrast to lipoplexes, polyethyleneimine (PEI) polyplexes were found to be internalized both by clathrin-mediated endocytosis and by caveolae. However, only the caveolae-dependent route resulted in effective transfection [200,201]. Von Gersdorff et al., however, found that the internalization route resulting in successful gene expression depended on both the cell line and the PEI polyplex type used [202]. In Huh-7 cells, linear PEI resulted in gene expression through clathrin-dependent endocytosis, while branched PEI was successful by both clathrin-dependent and clathrin-independent endocytosis. In Cos-7 cells, mainly clathrin-dependent endocytosis was involved, while in Hela cells transfection efficiency resulted from clathrin-independent endocytosis. It should be noted that the most successful route for gene expression is not necessarily the most utilized internalization route of NANs. Indeed, linear PEI polyplexes were internalized for 70% by clathrin-independent endocytosis and for 30% by clathrin-dependent endocytosis, while only the latter resulted in gene expression. To study the different endocytic pathways, typically a combination of specific inhibitors and markers for each endocytic pathway is used. Both the absence of internalized NANs in the presence of a specific inhibitor and the colocalization of NANs with a specific endocytic marker then points out the uptake of the NANs via this specific endocytic pathway. Examples of inhibitors and markers, respectively are chlorpromazine and transferrin for clathrin-dependent endocytosis and filipine III and cholera toxin subunit B for clathrin-independent endocytosis.

Assuming information would be available about the most successful internalization pathway for a specific NAN and cell type, gene transfer efficiency could be enlarged by targeting the NANs to the appropriate pathway of endocytosis. One way to target a specific endocytic pathway is by controlling the size of the NANs. Rejman et al. has found that latex beads of <200 nm are taken up via the clathrin-dependent pathway, whereas larger beads (200–500 nm) enter the cells through caveolae/lipid-raft mediated endocytosis [203]. Particles larger than 500 nm would then preferably enter the cells via macropinocytosis, a mechanism of endocytosis in which large droplets of fluid are trapped underneath extensions (ruffles) of the cell surface. Phagocytosis is restricted to specialized cells, such as macrophages, that function to clear large pathogens or cell debris from the circulation. Another approach is to target NANs to the specific lipid domains of the plasmamembrane by coupling the appropriate ligands at the NANs’ surface. Targeting to specific receptors, which are mostly internalized by clathrin-dependent endocytosis, should enhance uptake via this specific endocytic pathway. Receptor-mediated endocytosis is a general mechanism by which eukaryotic cells internalize peptide hormones, growth factors, cytokines, plasma glycoproteins, lysosomal enzymes and toxins [204]. Examples of ligands that target to specific receptors are transferrin [205], asialoglycoproteins (targeting to hepatocytes) [206], epidermal growth factor [207] and folic acid [208] (targeting to cancer cells), low density lipoprotein [209] and so on. Targeting to caveolae/raft lipid domains (e.g. enriched in cholesterol and sphingolipids) on its turn could enhance the clathrin-independent endocytosis. Anderson et al. postulated that lipid shells could play an important role in targeting these specific lipid domains [210].

5.2. Escaping the endosome and release of the nucleic acids

After internalization by a specific endocytic pathway, NANs are present in endosomal vesicles that they should escape in order to exert a therapeutic effect. The mechanisms of endosomal escape differ between lipid-based and polymer-based NANs and are discussed below.

5.2.1. Lipid-based NANs

A possible mechanism for endosomal escape of lipid-based NANs has been proposed by Zelphati and Szoka [211–214]. According to these authors, lipid contact occurs between cationic lipids from the lipoplex and anionic lipids from the inner face of the endosomal membrane resulting in flip-flop of anionic lipids from the cytoplasmic face of the endosomal membrane. These anionic lipids laterally diffuse into the lipoplex and form a charge neutralized ion-pair with the cationic lipids from the lipoplexes, resulting in destabilization of the lipoplexes and displacement of the negatively charged nucleic acids with their release into the cytoplasm of the cells. Recently, Gordon et al. demonstrated, however, that anionic lipids in the endosomal membrane are not a primary prerequisite to trigger
nucleic acids release [215]. On the contrary, neutral helper lipids in the delivery system, such as DOPE, seemed to play a crucial role in improving the endosomal escape by promoting the transition of the lipoplexes from the lamellar phase to the inverted hexagonal phase and destabilizing the endosomal membrane [5,216]. Therefore, the presence of DOPE in a liposome formulation can be considered an important trigger for endosomal escape with release of the nucleic acids in the cytoplasm of the transfected cells.

As discussed in Section 4, PEGylation of the liposomes, by incorporating polyethyleneglycol at the surface, ‘guides’ lipoplexes in a more effective way through the extracellular compartments of the body. PEGylation, however, seems to lower the transfection efficiency of lipoplexes on the intracellular level. This decrease in transfection has been attributed to a worse interaction of PEGylated lipoplexes with cellular membranes and, subsequently, lower uptake by the cells [151,152]. However, other groups showed that the cellular uptake of PEGylated lipoplexes was unaltered and suggested that PEGylation interferes at the step of endosomal escape of the lipoplexes [217,218]. As for non-PEGylated lipoplexes, PEGylated lipoplexes have to escape from the endosomal compartment and release their nucleic acids in the cytoplasm of the cells. At this stage, however, the PEG-chains prevent close contact between the lipids from the PEGylated lipoplexes and the endosomal membrane. Also, the incorporation of PEG-lipids stabilizes the lamellar phase of the lipoplexes so that DOPE is not able to destabilize the endosomal membrane. The nucleic acids thus remain entrapped in the endosomal compartment, precluding their delivery to the nucleus.

Lipid mixing thus seems to be an important parameter to allow for endosomal escape. To overcome the inhibitory effect of pegylation, reversible pegylation has been considered by several groups, in which the PEG-lipids are removed in the endosomal compartment in order to expose the endosomal escape properties of the lipoplexes. Guo et al. calculated that the inhibitory properties of PEGylation could be overcome if the amount of PEG-lipid in the lipoplexes is less than 2.3 \pm 0.6\text{ mol}\% [219]. Therefore, lowering the amount of PEG-lipid on the liposomes’ surface could improve the endosomal escape and hence the biological activity of the nucleic acids delivered by PEGylated liposomes. To be able to remove the PEG-chains on the liposomes’ surface, two strategies are commonly used. One approach makes use of PEG-lipids where a pH sensitive or degradable group links the PEG to the lipid (e.g. PEG-diothoesters, Fig. 12A). The rational behind these approaches is that the linker becomes hydrolyzed upon acidification of the endosomes or cleaved by lysosomal enzymes [220–222]. Another approach is the use of exchangeable PEG-lipids such as PEG-ceramides that simply diffuse out of the lipoplexes in function of time (Fig. 12B) [217]. The rate of PEG-ceramide removal from the lipoplexes depends on the size of the PEG moiety as well as on the acyl chain length, with shorter acyl chains resulting in more rapid deshielding. The loss of the PEG-ceramides seems to facilitate the transition of the lipoplexes into the inverted hexagonal phase, a requirement to enable

---

Fig. 12. Chemical structures of polymers and lipids as discussed in Section 5. (A) PEG-diothoester, (B) PEG-ceramide, (C) PAM, (D) PDMAEMA, (E) PLL, (F) PPAAc, (G) PEAAc and (H) PHPMA-DMAE.
an efficient endosomal release. Thus lipid mixing between the cationic liposomes and the endosomal membrane can occur, resulting in endosomal escape and release of the nucleic acids in the cytoplasm of the cells. An important aspect of cationic lipid-mediated delivery is that the nucleic acids are released in the cytoplasm as such, without the additional need to dissociate from their carrier (Fig. 11, 3b and 3d). As a result, the naked DNA molecules become susceptible to cytoplasmic nucleases as soon as endosomal escape has occurred (Fig. 11, 6a and 6b).

5.2.2. Polymer-based NAnNs

As outlined in Section 2, there is a wide variety of cationic polymers under investigation for the delivery of nucleic acids. Some polycations have an intrinsic endosomolytic activity, while others need the co-addition or covalent coupling of ‘helper molecules’ to induce the endosomal escape [223]. Polymers with an intrinsic endosomolytic activity, such as polyamidoamine dendrimers (PAM, Fig. 12C), poly dimethylaminoethylmethacrylate (pDMAEMA, Fig. 12D) and polyethyleneimine (PEI, Fig. 3), mostly have a high charge density and a highly branched structure, with the potential to be protonated. At physiological pH 7.4, not all of the protonatable units of such polymers are protonated. Upon acidification of the endosomal compartment, however, the polymers become extensively protonated, inducing their endosomolytic activity. In general, these polymers are believed to escape the endosomes via the proton sponge mechanism [20,224]. The buffering capacity of the polymers prevents the acidification of the endosomes, which on its turn causes more and more protons to be pumped in the endosomes, accompanied with an influx of Cl\(^-\) ions. This builds up the osmotic pressure with the accumulation of water in the endosomes. Also, because of repulsion between the protonated amine groups, swelling of the polymer can occur. This swelling of the polymer in combination with the osmotic swelling eventually causes the endosomes to rupture, with release of the polyplexes in the cytosol of the cells as such (Fig. 11, 3a and 3c). Clearly, this is in contrast to lipoplexes where the nucleic acids are believed to be released at the step of endosomal escape (see above). In theory, all polyplexes based on polymers containing basic groups with buffering capacity between pH 7 and 5 (in other words with a p\(K_a\) at or below physiological pH) should be able to escape the endosomes via the proton sponge mechanism. However, Funhoff et al. demonstrated that endosomal escape is not always enhanced by polymers buffering at low pH [225]. Also, the proton sponge theory has been doubted by Godbey et al. who found that lysosomes were not buffered during transfection with polyplexes formed with PEI [226]. Forrest et al. also found that acidification of endosomes still occurred when transfection experiments with PEI polyplexes were conducted. He concluded, however, that the proton sponge theory is still valid, but that acidification is a part of this process [227]. On the other hand, Sonawane et al. clearly demonstrated the enhanced Cl\(^-\) accumulation and swelling of the endosomes with the strongly buffering PEI and PAM, but not with the non-buffering poly-L-lysine (PLL) [228]. It was also reported that PLGA shows intrinsic endosomal escape properties as it undergoes a selective reversal from anionic to cationic surface charge in the acidic endosomal compartment, which allows it to interact with the endosomal membrane and escape into the cytosol [229].

Cationic polymers without intrinsic endosomal escape properties, such as PLL (Fig. 12E), are devoid of a hydrophobic domain and cannot fuse and/or destabilize the endosomal membrane. Therefore, they require the co-addition or covalent coupling of fusogenic or endosome-disruptive molecules to induce their endosomal escape. These endosomotropic agents, which can be synthetic or virus-derived, mostly depend on the lowering of the endosomal pH for their activity, exposing more hydrophobic regions for interaction with the lipid bilayers from the endosomal membrane. Molecules with endosomal escape properties include polyethylacrylic acid (Fig. 12G) as well as virus-derived fusogenic peptides such as hemagglutinin HA-2 from the influenza virus [223,230]. Other synthetic amphiphatic peptides that exert a pH-dependent membrane destabilization that were synthesized to mimic the endosomal escape properties of the viral fusogenic peptides, are GALA (glutamic acid-alanine-leucine-alanine) and KALA (lysine–alanine–leucine–alanine). GALA contains 30 amino acids with a major repeat sequence of glutamic acid–alanine–leucine–alanine with a N-terminal tryptophan and undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0. The hydrophobic regions of the amphipathic α-helix may then interact with the lipid bilayers of the endosomal membrane to destabilize them. KALA also undergoes a pH dependent conformational change from random coil to α-helix, but in contrast to GALA, it does so when the pH is increased from pH 5.0 to 7.5, thereby exposing the hydrophobic regions that seem to be important to destabilize the endosomal membrane.

Shielding of the polyplexes with PEG-chains also lowers the endosomal escape [231]. Therefore, the use of removable PEG-chains is also recommended in the case of PEGylated polyplexes [231]. Acid-triggered deshielding of
polylexes can be achieved via cleavage of hydrazone-linked PEG or acetal-linked PEG [128]. Other chemical linkages that may possess pH-dependent hydrolysis include vinyl ethers and orthoesters [128].

Depending on the endosomal escape mechanism, the nucleic acids reach the cytosol in “naked” form or while still encapsulated in a nanoparticle (Fig. 11, 3a–d). In the latter case, the protection of the DNA against enzymatic degradation is, highly likely, maintained in the cytoplasm. On the other hand, most likely, an additional step of dissociation of the DNA from its carrier is needed to be able to establish a therapeutic effect (Fig. 11, 4a and b). It has been suggested that PEI/DNA complexes can enter the nucleus as an associated complex [232]. Also, the dissociation of plasmid DNA from PEI seemed to be unnecessary to establish a therapeutic effect [233,234]. Itaka et al., however, found that linear PEI, which released its plasmid DNA more easily, showed a higher transfection efficiency than branched PEI that formed more stable complexes [235]. Also Bertschinger et al. demonstrated that branched PEIs have higher affinity for DNA than linear PEIs [236]. For PLL/DNA polylexes, again low-molecular-weight PLL polymers that released DNA more easily yielded better transfection efficiencies [237]. These examples demonstrate that vector unpacking is a potential barrier for polyplex-mediated nucleic acid delivery. In general, low-molecular-weight cationic polymers are expected to release the therapeutic DNA more easily as there are less points of interaction between the DNA and a single polymer chain. However, the reduced affinity of low-molecular-weight polycations for DNA also implies that the resulting polylexes are less stable in physiological conditions. Therefore, high-molecular-weight polycations were designed that can be cleaved by intracellular conditions into their low-molecular-weight building blocks, thereby facilitating the release of nucleic acids. Read et al., for example, evaluated reducible polycations (RPCs) that were prepared by the oxidative polycondensation of Cys-Lys10-Cys [238]. These RPCs efficiently condensed nucleic acids and cleavage of the disulfide bonds by intracellular reduction indeed facilitated the delivery of nucleic acids when compared to non-reducible polycations. Also Mok et al. produced PEG/NANs that were crosslinked through the formation of disulfide bonds and degraded in a reductive environment, thereby releasing the plasmid DNA [239]. Funhoff et al. synthesized a cationic polymer that subsequently degrades at physiological pH, thereby releasing the DNA in the cytosol of the cell [21]. The polymer consists of a monomer carbonic acid 2-dimethylaminooethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester (HPMA-DMAE) and degrades through hydrolysis of the carbonate ester at pH 7.4 but not at pH 5.0 (Fig. 12H).

5.3. Traveling through the cytosol

5.3.1. The intracellular transport machinery

The cytoplasm is a viscous compartment containing a scaffold of filaments termed the cytoskeleton. The filaments divide into three types: actin filaments (7 nm), microtubules (25 nm) and intermediate filaments (12 nm). These filaments are dynamic and can assemble and disassemble according to molecular cues. It appears that the composition of the cytoskeleton makes free diffusion of macromolecules limited [240,241]. Therefore, most likely, NANs will have to make use of the efficient intracellular transport mechanisms to reach their target site. The transport system in cells is composed of two main components, namely the actin filaments and the microtubules [242].

Actin filaments are built up from actin monomers that have polymerized after binding ATP. Actin monomers bind to the plus, barbed end of the growing filaments and dissociate from the minus, pointed end. Additional crosslinking and branching of actin filaments allows cells to form specialized structures such as microvilli, stress fibers, ruffles, cortical actin, lamellipodia and filopodia. Underlying the plasmamembrane, we find the cortical actin, a dense layer of branched actin filaments, which can serve as a barrier for incoming viral and non-viral particles. For trafficking, cargo can travel along the actin filaments using the myosin family of motor proteins, which generally move towards the plus end of the actin filaments, while hydrolyzing ATP [243]. Alternatively, cargo can attach to the growing end of a polymerizing actin filament, which generates directional propulsion. Actin filaments are more involved in short, non-directed movement.

Microtubules are assembled of α- and β-tubulin dimers that organize into hollow, cylindrical filaments while hydrolyzing GTP. The plus ends of microtubules are pointed towards the plasmamembrane, while the minus ends are anchored to the microtubule organising center adjacent to the nucleus. Microtubules play an important role in cell division and the intracellular trafficking of organelles and other cargo, using the dynein and kinesin families of motor proteins for movement. Dyneins move cargo towards the minus end of the microtubules (e.g. towards the nucleus), while kinesins move cargo towards the plus end of the microtubules (e.g. towards the plasmamembrane) [244,245].
Motors that move toward both minus- and plus-ends of microtubules have not been identified in nature. In contrast to actin filaments, microtubules are associated with long, directed movement in cells.

5.3.2. Intracellular transport of NANs

Microtubules and the kinesin and dynein motor proteins are responsible for the trafficking of endosomal vesicles [244]. Therefore, incoming nanoparticles can traffic the cytoplasm while still present in the endosomal vesicles (Fig. 11, 2a). The direction in which the “endosomal nanoparticles” move is dependent on which motor proteins are working on the endosomes, while the distance of cytoplasmic traveling depends on the time during which the motor proteins remain attached to the endosomes. Endosomes can also move bidirectionally, switching between the kinesin and dynein motor proteins on a time scale of once a minute [244]. Of course, the distance traveled is also dependent on the time of endosomal escape, making it more likely to reach the nucleus when the nanoparticles do not escape from the endosomes too soon after entry. On the other hand, since endosomal vesicles are trafficked to the lysosomes, endosomal escape of the cargo at the appropriate time (e.g. traveled to the perinuclear region but not yet fused with lysosomes), remains important for cargo that is not resistant to the destructive lysosomal environment.

Apart from in endosomal vesicles, NANs can diffuse to the perinuclear region as such (Fig. 11, 2b). Passive diffusion through the cytoplasm, crowded with cell organelles and the actin and microtubules network, is expected to be limited. Directed transport of NANs, by hijacking the dynein motors, could be an interesting strategy to traffic from the cell periphery to the cell nucleus [206]. Dynein has a head group, which binds the microtubules, and a tail for binding of cargo. Which parameters determine recognition of cargo, such as NANs, by dynein remains unclear. Wang et al. demonstrated that coupling of cytoplasmic dynein to anionic latex beads stimulated their microtubules-assisted transport to the nucleus, demonstrating that dynein is indeed an interesting target for directed transport of NANs [246].

Recently, real-time multiple particle tracking using video microscopy has gained interest to elucidate the intracellular transport of non-viral gene delivery systems [247]. Bausinger et al. found that PEI/DNA polyplexes exhibit three different kinds of intracellular transport: random diffusion, active diffusion using the actin cytoskeleton and, most importantly, active diffusion to the cell nucleus within the endosomal vesicles using the microtubules [248]. Also, Suh et al. observed that PEI/DNA polyplexes achieved rapid perinuclear accumulation due to their active transport along the microtubules [249]. In the perinuclear region, however, most of the particles were immobile, with only limited access to the nuclear interior.

5.3.3. Intracellular transport of naked nucleic acids

Free DNA that is present in the cytoplasm can either passively diffuse or be actively transported to the nuclear (Fig. 11, 2c). Lukacs et al. found that the diffusion of small nucleic acid fragments (<250 bp) in the cytoplasm was only modestly slowed down when compared with that in saline but was greatly reduced when larger DNA fragments such as pDNA were used [241]. Later on Dauty et al. found that the actin cytoskeleton, which has a mesh size of about 150 nm, was the principal determinant of this size-dependent DNA mobility in the cytoplasm [240]. Small nucleic acid fragments like AONs and siRNA have no difficulties in reaching and entering the nucleus [250–252]. Free plasmids, however, show only limited diffusion from the injection site. It has recently been shown that, apart from passive diffusion, free pDNA can utilize the microtubules network to traffic to the nucleus, probably using the dynein motor proteins [253]. Also, molecules bearing nuclear localisation signals (NLSs) arrive at the nuclear membrane by active transport along the microtubules, evoked by NLS recognition of the dynein motor proteins [254]. This demonstrates that NLSs have not only an important function in nuclear import, but can also help to concentrate the NLS-bearing cargo at the perinuclear region before nuclear import takes place.

5.4. Breaking through the nuclear membrane. Or not?

The nucleus is separated from the cytoplasm by two lipid bilayer membranes. The outer membrane is continuous with the endoplasmatic reticulum, while the inner membrane lies within the nucleus. On several locations throughout the nuclear envelope, the outer and inner membrane fuse, forming channels that connect the cytoplasm and the nucleoplasm. These channels are called the nuclear pore complexes (NPCs) and play an important role in transport into and out of the cell nucleus. The NPCs contain an aqueous channel of about 9 nm that allows passive diffusion of small molecules up to 40 kDa. For larger molecules, an energy and signal-dependent mechanism is required, enlarging the NPCs to about 40 nm [255]. This active transport is mediated by nuclear localization signals (NLSs) and specific
carrier molecules such as members from the importine-β family. Molecules destined for nuclear import either bear their own NLSs or are recognized by endogenous NLSs. NLS containing cargo binds in the cytoplasm to the importine-α subunit of an importine-α/β dimer. Alternatively, the NLS containing cargo can directly bind the importine-β, without the need for the importine-α adaptor molecule. Then, the construct docks via the importine-β subunit to the cytoplasmic face of the NPCs, followed by translocation through the NPCs into the nucleus. Binding of the nuclear RanGTP to the importine-β subunit releases the importine-α/NLS-cargo in the nuclear environment, where the NLS-cargo is released from the importine-α subunit. The importine-α and β subunits are then recycled back to the cytoplasm to mediate a new round of nuclear import [256–258].

The precise mechanism by which the carrier-cargo complexes are translocated through the NPCs remains controversial [259]. The NPCs contain different nucleoporins that are characterized by the presence of phenyl-glycine (FG) repeat motifs. Nucleoporin FG repeats are found in the central channels as well as the nucleoplasmic and cytoplasmic face of the NPCs and appear to be directly involved in nuclear trafficking. In the affinity gradient model, it is suggested that material is translocated through the NPCs by hopping between different FG repeats by increased affinity for nucleoporins closer to the nucleus [255]. In the Brownian affinity-gate model, transport across the NPCs occurs through diffusion stimulated by the accumulation of macromolecules at the cytoplasmic face of the NPCs [255]. Alternatively, in the selected phase model, Ribbeck and Gorlich suggested that the FG repeats of the nucleoporins could interact with each other to generate a tightly crosslinked gel. This gel then acts as a selective phase which prevents the passage of other macromolecules, but allows passage of macromolecules that can interact with the FG repeats and break the crosslinks between them [260]. We recently demonstrated that non-selective gating of the nuclear pores by trans-cyclohexane-1,2-diol, an amphipathic alcohol that can break the hydrophobic interactions between the FG-repeats, drastically increased the nuclear entry and gene transfer of plasmid DNA [261].

For small nucleic acid fragments like AONs, the nuclear envelope poses no barrier. Upon microinjection in the cytoplasm, free ONs can indeed rapidly accumulate in the nuclear environment [250,251]. When delivered by NANs, most likely dissociation of the ONs from the carrier is needed. In the case of cationic lipids, as discussed above, this dissociation occurs at the step of endosomal escape [262,263]. For cationic polymers, an additional dissociation step in the cytoplasm will be required. It has indeed been shown that PEI improves the antisense activity of a phosphodiester but not of a phosphothioate ON, because the latter was not able to dissociate from the PEI polymers due to the stronger binding [264]. When the NANs are small enough (<40 nm), they can gain access to the nuclear environment via the NPCs, postponing the need for dissociation of the nucleic acids from the nanoparticles (Fig. 11, 5a). It should be noted that phosphodiester ONs suffer from a very rapid intracellular degradation (in the order of minutes), making it difficult to reach the nucleus intact [263,265,266]. siRNA, which is double-stranded, appears more resistant towards enzymatic degradation, probably partially explaining the increased biological activity when compared to AONs. In general, antisense therapy is most likely to be only successful with chemically modified ONs that have an increased resistance towards enzymatic degradation.

Several studies have pointed out that the nuclear membrane is an important barrier for larger DNA fragments like plasmid DNA [267–269]. Plasmid DNA can reach the nuclear envelope in a free form or while being complexed in NANs, requiring an additional dissociation step before nuclear accumulation can occur. As discussed above, both plasmid DNA and NANs most likely reach the perinuclear region via active transport along the MT. Microinjection experiments have demonstrated that only 0.1% of the cytoplasmatic plasmids is able to reach the nuclear interior in non-dividing cells [270]. Nuclear accumulation of plasmid DNA is, however, greatly enhanced during cell division when the nuclear envelope is temporarily broken down [271,272]. In non-dividing cells, plasmid DNA needs a NLS to gain access to the nuclear interior via the NPCs [273]. Therefore, coupling of NLSs to plasmid DNA has been an attractive, although not so successful, strategy to increase nuclear transport [274]. The most commonly used NLS peptide is the SV40 large T-antigen-derived NLS (PKKKRKV). This cationic NLS can bind randomly to DNA via electrostatic interactions. To prevent binding to transcriptionally active regions, sequence specific binding via peptide nucleic acids (PNA)-clamps has been proposed by Branden et al. [275]. NLSs peptides can also be coupled to NANs instead of to the DNA. In this case, however, the benefit from the NLS is lost when release of the nucleic acids from the NANs occurs. Apart from the need for a NLS, DNA size also plays an important role in nuclear delivery, making it easier for smaller DNA fragments [276,277]. DNA with a size up to 1 kb can enter the nucleus rather efficiently after coupling of a NLS peptide [278]. For larger DNA fragments, coupling of NLSs has not always been successful [279]. Also, the plasmid DNA topology influences the gene expression efficiency, with the more compact supercoiled plasmid DNA topology being more efficient when compared to its open circular or linear analogue [269,280]. Since we showed that once in the
nucleus only the amount and not the topology of the plasmid DNA determines the gene expression, this indicates that, highly likely, SC plasmid DNA is more efficient in reaching the nuclear interior from the cytoplasmic compartment [269].

The amount of plasmid DNA that can reach the cells nucleus is influenced by the amount of plasmid DNA that is present in the cytoplasm of the cells. Therefore, intracellular degradation of the plasmid DNA also significantly limits the gene transfer efficiency [281–283]. Lechardeur et al. estimated the apparent half-life of double-stranded DNA in the cytosol of HeLa cells between 50 and 90 min while Pollard et al. showed that the delivery of pDNA in COS-7 and A549 cells was prevented by Ca²⁺-sensitive nuclease [284,285]. Clearly, when using NANs to deliver pDNA, the time and place of release of the nucleic acids will play an important role in generating a therapeutic outcome. Ideally, the delivery system should release the pDNA close to the nuclear membrane. Since the translocation of the pDNA to the cell nucleus is greatly enhanced upon cell division, the time between release of the pDNA and cell division should be as short as possible to avoid degradation of the pDNA in the waiting period. Therefore, delivery systems where the release of pDNA is triggered by cell division could greatly enhance the transfection efficiency. Another approach could be to develop delivery systems that become entrapped in the cell nucleus during cell division and release their pDNA directly in the cell nucleus.

5.5. Efficient gene delivery: how do viruses do it?

A viral particle is a small and simple life-form, composed of a protein shell, called capsid, wrapped around the RNA or DNA genome. Enveloped viruses have, in addition, a bilayer lipid membrane that protects the capsid and the genome and can function as a ‘transport vesicle’. Due to their simple structure, viruses cannot grow or reproduce apart from a living cell. Instead, a virus invades living cells and uses their chemical machinery to keep itself alive and to replicate. Viruses have adopted many different strategies to infect a host cell, including hijacking the different endocytic routes. Most viruses bind to receptors at the cell surface and internalize via clathrin-dependent endocytosis. Subsequently, they escape the endosomal compartment somehow assisted by the lowering of the pH in the early and/or the late endosomes. Enveloped viruses can gain access to the cytoplasm by a fusion reaction of the viral lipid bilayer membrane with the plasma membrane. They can, however, also be endocytosed and fuse with the endosomal membrane upon lowering of the pH. Following fusion of the viral membrane with either the plasmamembrane or the membrane from the endosomal vesicles, the non-enveloped capsid is present in the cytoplasm of the cell. Depending on the entry route, the viral capsid can traffic to the nucleus via direct binding to the microtubules, or take a ride in the endosomal vesicles. During cytoplasmic trafficking and uncoating, structural changes can occur, exposing new layers of the viral particles like NLSs. These structural changes can occur in response to changes in pH, reductive environment, Ca²⁺ concentration or enzymatic activity like phosphorylation. Nuclear entry is often the final step in the complex transport and uncoating program of viruses [286]. Typically it involves recognition by importins, transport to the nucleus and docking to the NPC. Viruses can undergo extensive disassembly before nuclear entering, release their genome into the nucleus without immediate capsid disassembly or enter the nucleus as a whole. Depending on the virus involved, the genome can be transiently transcribed or permanently integrate in the cells genome. Also, some viruses are dependent on cell division for nuclear entry, limiting their infectious behavior to dividing cells.

After binding of viruses to their primary receptor, additional recruitment of co-receptors can be necessary to initiate internalisation. Co-receptors typically bind particles with a lower affinity than the primary receptors and therefore act after viral binding to the primary receptors. This is the case, for example, for adenoviral entry. Adenovirus is a non-enveloped DNA virus (60–90 nm) with projecting fibers on the capsid. After binding to the CAR receptor, it recruits integrin for internalisation and is internalised by receptor-mediated endocytosis. Subsequently, the virus escapes in a fiber-free form into the cytosol after a pH induced membrane lysis that ruptures the endosomal membrane. This lytic event is caused by the viral penton protein and the adenovirus-integrin complex [287]. In the cytosol the viral particle undergoes a series of further disassembly events, such as the digestion of a protein that connects DNA to the capsids wall. The viral capsids are transported to the nucleus via MT using the dynein motor protein [288,289] and bind to the cytoplasmic face of the NPC, followed by transfer of the DNA in the nucleus. The receptors and ligands involved in docking of the virus to the NPC and the mechanisms of capsid disassembly and DNA transfer into the nucleus remain unclear. The transfection pathway of the enveloped DNA virus Herpes Simplex Virus-1 (HSV-1), is very similar to that of the non-enveloped adenovirus, apart from the entry pathway. Indeed, binding of the enveloped HSV-1 induces a fusion reaction of the viral lipid bilayer membrane with the plasma membrane, releasing the capsid in the cytoplasm of
the cells. HSV-1 then takes a ride along the MT to the perinuclear region, through direct interaction between the viral UL34 protein and the cytoplasmic dynein complex [287].

Retroviruses are enveloped RNA viruses that can stably integrate their genome into the host genome [243]. They bind via an envelope glycoprotein to a plasmamembrane receptor that results in fusion of the envelope with the plasmamembrane. Some retroviruses contain pH-dependent envelope proteins and enter the cells via clathrin-dependent endocytosis. In this case, the enveloped viruses fuse with the endosomal membrane due to the decreased pH in the early and/or late endosomes. Following fusion of the viral membrane with either the plasmamembrane or the membrane from the endosomal vesicles, the capsid is present in the cytoplasm of the cell. The capsid can bind dynein motors and traffic along the microtubules towards the nucleus. During this trafficking, uncoating of the virus particles occurs and RNA is transcribed into cDNA using the viral enzyme, reverse transcriptase. At the nucleus, viral cDNA awaits cell division or traffics through the NPC and integrates into the host genome, using multiple cellular and viral factors. All retroviruses express the structural group-specific antigen (Gag), polymerase (Pol) and envelope (Env) proteins. Simple retroviruses, such as murine leukaemia virus (MLV) just contain the three characteristic structural genes. Complex retroviruses, however, such as HIV-1 and simian immunodeficiency virus, contain additional regulatory and accessory proteins. The HIV-1 accessory protein Nef is thought to facilitate HIV-1 trafficking through the cortical actin barrier by an actin depolymerising activity [290]. Viruses entering the cell via endosomes bypass this cortical actin barrier by using the endogenous trafficking of endosomes.

Papovaviruses (55–60 nm) and parvoviruses (18–24 nm) are examples of non-enveloped DNA viruses that can enter the nucleus intact [286]. Papovaviruses, from which the best known is simian virus 40 (SV40), enter the cell by caveolae and are transported in the endosomal vesicles to the endoplasmatic reticulum. The capsid structural proteins contain NLSs, which are only exposed upon changes in the capsid structure induced by a reductive environment or changes in the Ca2+ concentration. There is evidence that viruses pass from the cytosol to the nucleus, and that they do so in intact form despite of their large size. However, other routes, like direct penetration from the lumen of the endoplasmatic reticulum through the nuclear membrane, cannot be excluded. Parvoviruses enter via clathrin-dependent endocytosis and require low pH for penetration into the cytoplasm [291]. They also depend on the MT for transport to the nucleus. Due to their small size, they can pass through the NPC without the need for capsid disassembly or deformation, although some structural capsid rearrangements are needed to expose the NLSs [286,287,291].

5.6. Material lessons to learn from the viral infection pathway

Fig. 11 gives an overview of the different intracellular routes viruses and NANs can follow, as discussed above. The actual route that NANs will follow, can be guided by carefully choosing their material properties. With the efficient infectious pathway of viruses in mind it should be possible to make material recommendations to create safe NANs which are much more efficient in nucleic acid delivery than the current NAN generation.

The first step in the nucleic acid delivery process is the entry of the NANs in the cytoplasm. To avoid the cortical actin barrier underlying the plasmamembrane, NANs entering via endocytosis (Fig. 11, 1a and 1b) should be a better choice than NANs that fuse with the plasmamembrane (Fig. 11, 1c). Targeting clathrin-dependent endocytosis can be achieved by coupling of specific ligands to NANs, such as transferrin. Also, the size of NANs should preferably be kept smaller than 200 nm to promote clathrin-dependent endocytosis. Following endocytosis, NANs are present in endosomal vesicles that will most likely traffic to the perinuclear region via the microtubules (Fig. 11, 2a). Endosomal escape in the early stage of transfection should be avoided since the transport of NANs (not entrapped in endosomes) to the perinuclear region (either via MT or via passive diffusion) is believed to be less efficient than endosomal trafficking (Fig. 11, 2b). Furthermore, if the nucleic acids are released from the NANs close to the plasmamembrane they have to travel unprotected to the perinuclear region via the MT or passive diffusion (Fig. 11, 2c) and thus become fully exposed to nucleases (Fig. 11, 6a). To enhance the transport of NANs or nucleic acids that do escape close to the plasmamembrane, the NANs or nucleic acids could be equipped with ligands that are recognized by the MT motor protein dynein. This, however, would require an extra material challenge that can be avoided by taking a ride in the endosomal vesicles. In certain cases, one of the dynein light chains (LC8) is involved in the recognition of the cargo.

Two different protein motifs can be recognized by LC8 in its target proteins: a (K/R)XTQT and a G(IV)QVD motif [292,293]. Martinez-Moreno et al. identified viral sequences which associate with the dynein light chain LC8; such sequences may thus be suitable to enhance perinuclear transport [294]. Also, the 25 N-terminal amino acids of the p53 protein have been reported to trigger dynein binding [242]. The aforementioned proteins can be coupled both to NANs
or plasmid DNA to enhance their cytoplasmic trafficking. In the case of plasmid DNA, however, the coupling of NLSs could be more appropriate to enhance both the translocation to the nucleus as the nuclear import itself. It has indeed been shown that NLSs, such as NFκB p50, not only mediate nuclear import but also enhance the transport of the DNA towards the nucleus along MT in a dynein-dependent manner [295].

To avoid rapid endosomal escape, it could be an advantage to make use of NANs that only expose their endosomolytic properties upon lowering of the endosomal pH. GALA is such an amphipatic peptide that undergoes a pH-induced transition from a random coil to a α-helix, exposing the hydrophobic regions that are responsible for destabilizing the endosomal membrane. Also, NANs could be equipped with fusogenic peptides, such as INF-7 from influenza, and shielded from the environment by PEGylation of the NANs. Acidification of the endosomal compartment then exposes the fusogenic properties of the NANs, after the PEG-chains were removed from the NAN by a pH-induced deshielding. Also mellitin is a strong fusogenic peptide, which unfortunately also displays high lytic activity at neutral pH. However, the lytic activity at neutral pH can be masked by acetylation of the mellitin with a dimethylmaleic anhydride derivate. Following acidification, the maleamate shield is cleaved and the lytic activity of mellitin is restored [296].

If the NANs are unable to escape the endosomes, eventually, degradation of the NANs and the nucleic acids they are carrying will take place in the lysosomes (Fig. 11, 7a, 7b, 8a and 8b). Preferably, endosomal escape occurs close to the nuclear membrane and results in the delivery of intact NANs in the cytoplasm (Fig. 11, 3c) rather than free nucleic acids (Fig. 11, 3d). Most of the polymer-based NANs escape in the cytoplasm as such, without release of the nucleic acids they are carrying, but are not small enough to reach the nuclear interior of a non-dividing cell through the NPC. In dividing cells, however, the nuclear membrane is temporarily broken down during cell division and NANs are expected to gain access to the nuclear interior more easily (Fig. 11, 5a). In the nuclear interior, the NANs should then, in most cases, release the nucleic acids before a therapeutic effect can be obtained (Fig. 11, 4c). To enhance the release of nucleic acids from NANs in the nucleus, we propose to use biodegradable NANs: gradual degradation of the NANs in the nucleus should result in a gradual release of the nucleic acids. Biodegradable NANs frequently contain hydrolysable ester linkages or reducible disulfide bonds. With reducible disulfide bonds the NANs will disassemble in the reductive environment of the cytoplasm, after endosomal escape has occurred, rather than in the acidic endosomal compartment. When the NANs release their nucleic acids in the cytoplasm of the cell (Fig. 11, 4b), the nucleic acids still have to overcome the nuclear membrane (Fig. 11, 5b). As the nuclear accumulation of plasmid DNA is greatly enhanced when cell division occurs, enough intact plasmid DNA should be available in the perinuclear region at the time of cell division. When the plasmid DNA has been released from the NANs, the amount of available intact plasmid DNA is expected to depend on the time scale between plasmid DNA release and cell division. Indeed, the longer it takes before cell division occurs, the larger the fraction of the released plasmid DNA that is degraded in the cytoplasm of the cell (Fig. 11, 6b). We propose it could be beneficial to develop NANs which are either incorporated in the cell nucleus during cell division, or from which the release of nucleic acids is triggered by cell division. An interesting protein from this point of view could be C-myc, which remains attached to the MT when the cells are in rest but dissociates from the MT upon triggers received during cell division [242].

6. Understanding the extracellular and intracellular journey of NANs by advanced light microscopy

As discussed in the previous chapters, several barriers need to be overcome for a successful delivery of nucleic acids from nanoparticles. The transport and dynamics of NANs in living cells and extracellular matrices are crucial factors which, amongst other factors, determine the place in tissues and cells where NANs will release their nucleic acid payload. We believe that a better understanding of the dynamics of NANs in tissues and cells will contribute to a more intelligent optimization of the architecture of NANs. Currently several advanced light microscopy techniques are explored to visualize the movement and to get a better insight in the biophysical behavior of NANs in cells and tissues during the various stages of their delivery process. This chapter reviews the most important advanced light-microscopy techniques that are employed to this end and reports on the progress in this field.

6.1. Epi-fluorescence and confocal microscopy

In epi-fluorescence and confocal microscopy, NANs are visualized by tagging them with fluorescent molecules. One can use fluorescent dyes of different colors simultaneously to differently stain the nucleic acids, their carriers,
cellular compartments etc. Multi-color epi-fluorescence microscopy is indeed frequently used to study the uptake and processing of NANs in living cells [250,262,284,297–300]. For example, Huth et al. [300] have used epi-fluorescence microscopy to elucidate the internalization pathways of pH sensitive liposomes in cells. They used fluorescent tracers that can specifically label a particular internalization pathway (e.g. AlexaFluor-Transferrin to label the clathrin containing endosomes). By labeling the liposomes with a fluorophore of a different color, images were analyzed for regions where both colors were present, i.e. where the colors were 'co-localized'. By performing co-localization analysis, the authors have found that clathrin-dependent endocytosis was indeed an internalization pathway for the liposomes used in their study.

A notorious drawback of epi-fluorescence microscopy is the out-of-focus fluorescence in thick samples, which degrades the contrast of the in-focus objects. This is, however, effectively remedied by confocal microscopes, such as the confocal laser scanning microscope (CLSM), which has become very popular since its commercial emergence in 1987 (see [301] for a recent review). Because of the ability to record thin optical sections, images from CLSMs have much better contrast and are much more reliable for colocalization analysis. For a detailed overview and discussion covering many aspects of confocal microscopy and its use in a biological context, we refer the reader to ‘The Handbook’ [302]. Because of its clear advantages over epi-fluorescence microscopy, the CLSM has become an increasingly popular tool in the last decade to study the intracellular processing of NANs [199,203,228,232,295,303–310]. In our own work, for example, we have used the CLSM to study the uptake and distribution in living cells of cationic polyplexes [311] and the uptake and dissociation of lipoplexes [263].

The accuracy of co-localization studies is, however, limited by the resolution of the microscope setup, which is about ~170 nm with high-quality objective lenses on a CLSM. This means that two small molecules that are closer together than 170 nm cannot be resolved anymore and appear as a single entity in the microscope image. In other words, it could be difficult to distinguish nucleic acids that are still present in the NANs or released nucleic acids that remain close to the NANs. This is, however, important information when one wishes to study the release of the nucleic acids from the NANs. In some instances, this fundamental limit can be overcome by using fluorescence resonance energy transfer (FRET) [312]. FRET can occur between two fluorescent molecules, termed a donor and acceptor that are in very close proximity (typically <10 nm) of each other. The acceptor fluorophore must have an excitation spectrum that is (partly) overlapping with the emission spectrum of the donor fluorophore. When the donor molecule is excited, it can transfer a part of its energy non-radiatively to the acceptor molecule [313]. This leads to quenching of the donor fluorescence and, if the acceptor is a fluorophore, the emission of acceptor fluorescence. Since the FRET efficiency is inversely proportional to the sixth power of the intermolecular distance, it is a very sensitive marker to probe molecular interactions in the 1–10 nm range. FRET can be detected with (confocal) fluorescence microscopy by measuring the decrease in donor fluorescence and/or the increase in acceptor fluorescence [314]. In the context of nucleic acid delivery, FRET (confocal) microscopy has been used to study the intracellular dissociation of lipoplexes [212] and polyplexes [235]. Zelphati et al. labeled AONs with a fluorescein (donor) fluorophore and the liposomes with a rhodamine (acceptor) fluorophore, while Itaka et al. used plasmid DNA double-labeled with a fluorescein (donor) and a Cy3 (acceptor) fluorophore. In these studies, FRET occurred as long as the nucleic acids were compacted in the NANs, while it disappeared when the nucleic acids were released. FRET microscopy has also been used to study the degradation of AONs. In this case, the AONs were double-labeled with a donor and an acceptor fluorophore. As long as the AONs are intact, FRET will occur, i.e. the donor fluorescence will be quenched in favour of the emission of acceptor fluorescence. When the AONs are degraded by nucleases, the donor and acceptor are no longer in close proximity and FRET can no longer occur causing an increase in donor fluorescence and a decrease in acceptor fluorescence [266]. We have also combined FRET with FCS as a sensitive method to study the intracellular degradation of double-labeled AONs [263,265] and siRNA. By following the ratio of acceptor versus donor fluorescence over time with the sensitive detectors of the FCS instrument (see below), it was possible to determine the relative resistance against nucleases both in buffer and in living cells.

Fluorescence (confocal) microscopy is very well suited for studying the uptake and distribution of NANs. Although more challenging than uptake studies, the release of nucleic acids from NANs in cells can be well investigated by FRET (confocal) microscopy if the appropriate choice of fluorophores is made. However, to study the dynamics of NANs in cells and tissues complementary methods that are based on (confocal) epi-fluorescence microscopy are needed: FRAP, FCS and SPT. We will review each of them below. A recent review covering some basic principles of each method has been published recently [315].
6.2. Fluorescence recovery after photobleaching (FRAP)

FRAP is a (confocal) fluorescence microscopy method for studying the mobility of fluorescently labeled molecules and nanoparticles on a microscopic scale. Since its conception in the 1970s [316,317] it has found widespread application in biophysical, biomedical and pharmaceutical sciences [318–321]. In a FRAP experiment, one makes use of a fluorescence phenomenon called photobleaching. As shown in Fig. 13, in a typical FRAP experiment a high intensity light beam is used to quickly photobleach a fraction of the fluorescent molecules/nanoparticles in a particular micron-sized area. Since the molecules are in general constantly undergoing a diffusional motion, the photobleached molecules will gradually be replaced by intact fluorophores from the surrounding unbleached areas. The rate of fluorescence recovery reflects the mobility of the fluorescent molecules in the sample and can be quantified by an appropriate model to obtain the effective translational diffusion coefficient. Additionally, the fraction of immobile molecules can be calculated from the recovery curve as well. In relation to FCS and SPT, which will be discussed in the following sections, it is important to note that the diffusion coefficient as calculated from a FRAP experiment results from the movement of a very large number of molecules/nanoparticles over a micron-sized area. As a consequence, in case there are two or more subpopulations with different mobility characteristics, it is generally very difficult to distinguish between them [322]. In that case, FCS and especially SPT may be more appropriate.

In relation to nucleic acid delivery, FRAP has been used to study the mobility of NANs in extracellular matrices, such as mucus [323–325], (tumor) cell interstitium [69,326–329] and vitreous [125,325]. For example our group could show by FRAP that attaching hydrophilic polyethylene glycol (PEG) chains at the surface of nanoparticles can circumvent their binding to fibrillar structures in the vitreous and increases their mobility (see also Section 4). FRAP has also been used to study the mobility of nucleic acids in living cells [241,308,330]. As already mentioned above, the diffusion of small nucleic acid fragments (<250 bp) in the cytoplasm was only modestly slowed down when compared with that in saline but was greatly reduced when larger DNA fragments such as pDNA were used.

---

**Fig. 13.** Schematic representation of a FRAP experiment. Just before bleaching, the fluorescence is measured in a specific area (circular in this example) of the sample. The initial fluorescence before bleaching is indicated on the fluorescence recovery curve as $F_i$. At $t = 0$ a high intensity light beam instantly bleaches the molecules in the observed area, resulting in a drop in fluorescence to $F(0)$. Due to diffusion, the bleached molecules will be gradually replaced in the bleached area by non-bleached fluorescent molecules from the surroundings. This results in a recovery of the observed fluorescence within the observed area. At the end of the experiment ($t \to \infty$), the fluorescence will recover to a level $F(\infty)$, which is equal to $F_i$ if all fluorescent molecules in the observed area are mobile or less than $F_i$ (as in this example) when a part of the fluorescent molecules are immobile in the observed area. The rate of fluorescence recovery is a measure for the diffusion coefficient of the fluorescently labelled molecules, which can be calculated by fitting of an appropriate physical model to the experimental recovery curve.
6.3. Fluorescence correlation spectroscopy (FCS)

FCS is a complementary technique to FRAP that was developed during the same period [331–335]. FCS measurements are typically being performed on CLSM based instruments. Here, however, the laser beam is held stationary at a fixed location in the sample (see Fig. 14A). By combination of the focused laser beam and the confocal pinhole, the fluorescence coming from only a small volume will be detected, which is usually referred to as the ‘confocal detection volume’. When a fluorescent molecule/nanoparticle diffuses into the confocal detection volume, a slight increase in fluorescence intensity will occur. Similarly, when a molecule/nanoparticle diffuses out of the volume, there will be a decrease in fluorescence intensity. The continuous movement of molecules in and out of the detection volume gives rise to a fluorescence fluctuation profile (Fig. 14B). A prerequisite for confocal FCS is to bring the number of molecules/nanoparticles low enough, so that each of them contributes substantially to the measured fluorescence signal. From many individual fluorescence fluctuations, an auto-correlation curve can be calculated, from which the average number of molecules/nanoparticles in the detection volume and their dynamic properties can be calculated by fitting of the auto-correlation curve to an appropriate model. We refer the reader to some of the many reviews on FCS for more details on the theoretical aspects [336–341].

It is possible to distinguish between two (or more) diffusing subpopulations by auto-correlation analysis only if there is a substantial difference in magnitude of the respective diffusion coefficients. It has been reported that the diffusion coefficient of two subpopulations should differ by at least a factor 1.6 under optimal conditions [342]. This means that the molecular weight of both types of molecules/nanoparticles should be different by at least a factor $\sim 8$ [343]. For subpopulations of similar size, two-color cross-correlation fluctuation spectroscopy (FCCS) is an alternative way to detect molecular interactions independent on a change in the diffusion coefficient of the reaction partners. Here, the interacting molecules are labeled with fluorophores of different colors whose fluorescence can be detected in separate channels. When both types of molecules are moving independently, there will be no correlation

![Fig. 14. (A) Schematic setup of the FCS instrument. The excitation light from the laser passes through a beam expander (BE) and is reflected by a triple chroic mirror (TM) into the objective lens of the microscope. The objective lens focuses the laser light to a small diffraction limited volume in the sample with fluorescently labeled molecules/particles. The light emitted by the fluorescent molecules in the excitation volume goes back through the objective lens and the triple chroic mirror, and is split into a red and a green component by a second dichroic mirror (DM). The fluorescence light in each channel first passes a fluorescence filter (F) and is subsequently focused through a confocal pinhole (P) onto the detector. (B) Diffusion of molecules in and out of the confocal volume causes fluorescence intensity fluctuations. (C) From these fluorescence intensity fluctuations an auto-correlation curve can be derived from which the diffusion coefficient can be calculated by fitting of an appropriate physical model.](image-url)
between the fluctuations of each color. However, when they interact and are moving together through the detection volume, (a fraction of) the fluctuations will occur synchronized in both channels. Quantitative information on association and dissociation can be obtained by performing cross-correlation analysis on both signals [343–346].

Since FCS became a commercially available technique in the late 1990s, FCS has been used to explore the barriers in the delivery process of nucleic acids [321]. (Single color) FCS and auto-correlation analysis was used to examine the molecular movement of AONs in the nuclei of living cells [330], the self-assembling process of lipoplexes [347,348], the hydrolysis of DNA by several enzymes [349] and the intracellular fate of PEI and PEI/DNA complexes [350]. Furthermore, (dual color) FCS and cross-correlation analysis has been used to study the degradation of AONs [345] in buffer and the release of DNA from cationic lipid vesicles in buffer [309]. However, studying the complexation and dissociation of self-assembling NANs by auto- or cross-correlation analysis is usually not possible [351]. As NANs consist of a large number of nucleic acid molecules and a large number of carrier molecules, each molecule being fluorescently labeled, the NANs contain many fluorophores. Consequently, passage of the NANs through the detection volume of the FCS instrument causes very bright intensity peaks in the fluorescence fluctuation profiles that complicates classical analysis. A statistical method was developed to study the occurrence of such peaks objectively and providing an alternative means to examine complexation and dissociation [352,353]. In this context, since correlation analysis is not performed anymore, the term FCS is usually replaced by FFS (fluorescence fluctuation spectroscopy). Our group has used FFS analysis to study the association and dissociation of AONs and cationic polymers in buffer [354–358] and of AONs and cationic liposomes in living cells [359].

FCS has proven to be a valuable tool for studying the association and dissociation of self-assembling NANs like lipo- and polyplexes and the stability of nucleic acids, both in buffer and in living cells [351]. Theoretically it could be also used to study the movement of nucleic acids and NANs in living cells. Clearly, to characterize the movement of NANs by FCS, one should be able to auto- or cross-correlate the fluorescence fluctuation profiles. However, it is our experience that there remain many difficulties to auto- or cross-correlate the fluorescence fluctuation profiles as obtained from intracellularly moving NANs. Experimental difficulties are, amongst others, photobleaching, autofluorescence, movement of the cell, light absorption and scattering, low signal to noise ratio, etc. [339,343,346,360]. Also, one should realize that a batch of self-assembled NANs, like lipo- and polyplexes, does not contain NANs of all the same size and composition. On top, the NANs are present at different locations in the cell: some of them may reside in endosomes while other ones are in cytosol. Subsequently, one can expect that NANs in cells show a large distribution of mobilities which makes the auto- and cross-correlation analysis much more difficult. In addition, a single FCS measurement only provides time-averaged information on the molecules/nanoparticles that have passed a single location in the cell as the FCS detection volume originates from a focused laser beam that only illuminates that specific place in the cell (e.g. typically some μm dimension). The laser beam thus has to be focused on each cellular compartment of interest, requiring numerous FCS measurements to obtain information on the diffusion of nucleic acids or NANs in different cell compartments (e.g. somewhere in the cytoplasm or the nucleus). This is another reason why FCS is less suited to study the intracellular processing of NANs. Indeed, one should have an overview of the different cell compartments to study all steps of the intracellular transport of NANs simultaneously.

For this purpose single particle tracking seems to be the method of choice, as will be explained below.

6.4. Single particle tracking (SPT)

As the name implies, SPT is a fluorescence microscopy based method where the movement of individual fluorescently labeled nanoparticles or molecules can be visualized in time and space [247,361]. The nanoparticles and molecules in this context are usually smaller than the resolution of the microscope, in which case they are observed in the image plane as diffraction limited spots of light whose size depends on the resolution of the objective lens being used. Imaging of single nanoparticles is possible if the concentration is low enough so that there is less than one particle per diffraction limited spot. The microscope set-up is typically an epi-fluorescence microscope adjusted for wide-field laser illumination with a fast and sensitive CCD camera [248]. This CCD camera generates ‘movies’ from the diffusing nucleic acids or NANs in the sample (Fig. 15). Therefore, in contrast to FCS measurements that monitor the events at a single location in the cell (typically some μm dimensions), SPT monitors an area covering one or more cells simultaneously (typically some 100 μm dimensions).

The single nanoparticle movies need to be analyzed in order to find the positions of the individual particles in each frame of the movie. Although the resolution of the microscope is typically around 250 nm, the position of a nanoparticle...
can be determined with subresolution accuracy by finding the centre of the diffraction limited spot. Various methods have been reported for finding the position of nanoparticles, including cross-correlation, sum absolute difference, centroid identification and a Gaussian fit \[362\]. Reported localization accuracies are usually in the order of tens of nanometers \[248,363–369\], although the spatial resolution for a mobile nanoparticle will be inherently worse depending on how fast the nanoparticle is moving with respect to the camera integration time. The process of finding nanoparticle positions in images can be automated with suitable image analysis algorithms \[370–372\], thus allowing to find the positions of many nanoparticles simultaneously. To emphasize the principle of tracking many individual nanoparticles simultaneously, this technique is sometimes also explicitly referred to as ‘multiple particle tracking’ \[247,249,373\].

Having found the positions of the nanoparticles in the movie, individual trajectories can be calculated by pairing corresponding features between images. A nearest neighbour algorithm is typically used to construct the tracks \[372\], although similarity in fluorescence intensity has been used as well to increase the tracking accuracy \[369,374\]. Next, the tracks can be analyzed and sorted according to several modes of motion, such as free diffusion, directed transport, anomalous diffusion and corralled diffusion, which have been reviewed elsewhere \[247,361\]. Furthermore, a distribution of quantities characterizing the motion (diffusion coefficient, velocity, corral size, etc.) can be obtained by analyzing many individual tracks \[248\].

An entirely different kind of analysis, based on correlation analysis, is sometimes applied to SPT movies as well. The method is referred to as image correlation spectroscopy (ICS) and is in fact a hybrid technique of SPT and FCS. By performing correlation analysis on individual image frames, an estimate of the number of nanoparticles can be obtained \[375\]. Correlation analysis on the entire movie sequence allows calculating mobility parameters and is similar to the standard method of auto-correlation in FCS \[376–378\]. Just as is the case for FCS, auto- and cross-correlation are data reduction techniques averaging information from many individual nanoparticles. Despite the inherent loss of detailed information, correlation analysis is useful to describe the overall behavior of a large collection of particles. Moreover it has the advantage over SPT that it does not require extensive image analysis for tracking individual particles. Although originally mainly applied to the study of membrane diffusion \[361,363–365,368,374,379–382\], in the last few years SPT has also been used for studying the intracellular transfection pathway of viruses \[299,367,369,383,384\].
and the delivery of nucleic acids from NANs in biological environments [115,247–249,305,309,378,385]. For example, Suh et al. [249] have found by SPT that polyplexes undergo fast active transport towards the perinuclear region where a large fraction of the nanoparticles become immobile over time. Therefore, they suggest that the bottleneck in the delivery of nucleic acids from NANs is not the cytoplasmic transport of the NANs, but rather the nuclear entry of the NANs or (released) nucleic acids. By fluorescent labeling of the actin and tubulin networks of living cells, Bausinger et al. [248] have shown by SPT directly the interaction of polyplexes with both cytoskeletons and the different modes of motion in the various stages of the intracellular processing of NANs. It is expected that in the coming years SPT will play an increasingly important role in the elucidation of NAN transport in living cells.

6.5. Is there a method of choice to explore the extra- and intracellular fate of NANs?

While FRAP, FCS and SPT are complementary techniques, it depends on the type of information wanted which method is more suitable. If one is interested in the overall behavior of NANs in extracellular matrices, FRAP would be the method of choice as it requires a nanoparticle concentration of >100 nM and thus gives an idea on the movement of many thousands of nanoparticles in a micron-sized area, which is often too large to allow intracellular measurements. FRAP can typically measure the diffusion coefficient in the range of 0.1–100 μm²/s. By using a stationary focused laser beam, FCS can probe the mobility in a submicron area. FCS requires a relatively low concentration of fluorescent nanoparticles typically in the range of 0.1–100 nM. The diffusion coefficient as calculated from correlation analysis, however, is still an average value obtained from the movement of many hundreds or thousands of nanoparticles. FCS is ideally suited to measure diffusion coefficients >100 μm²/s. Slower diffusion can in principle be measured as well at the expense of a longer measurement time and, consequently, an increased risk of artefacts such as photobleaching and cell damage. Dual color FCS, as discussed above, is suited to study the association and dissociation of NANs in various environments, although some difficulties in the interpretation of the data may arise. Our experience with the use of dual-color FCS to study the association and dissociation of NANs has been reviewed recently [351]. SPT, finally, currently gives the most detailed information on the dynamics of NANs as it allows one to follow and analyze the movement of single nanoparticles or molecules. While FRAP and FCS are able to assess the average dynamics of a large ensemble of nanoparticles in a particular region of the sample, SPT can tell you the diffusion dynamics of each individual nanoparticle. For SPT measurements there is no lower limit concentration range for the nanoparticles while the maximum concentration is in the order of 1 nM as the nanoparticles should be sufficiently separated from each other to be visible in the images as individual spots of light. The smallest measurable diffusion coefficient is determined by the localization accuracy of a single particle, while the maximum diffusion coefficient mainly depends on the maximum achievable frame rate. SPT can typically measure diffusion coefficients in a range of 0.0001–10 μm²/s.

7. Conclusions: from a bottom-to-top approach to a top-to-bottom approach

Since more than a decade now, pharmacists, material scientists and biophysicists are intensively studying the design, preparation and cell biological and systemical behavior of nanosized particles carrying nucleic acids. In the 1990s, the focus was especially on plasmid DNA delivery and the delivery of antisense oligonucleotides (AONs). The relatively slow progress and frustrations on in vivo gene therapy with non-viral carriers as well as the negative outcome of some major clinical trials (mostly administrating naked AONs with intravenous injection) has somewhat tempered the enthusiasm of the believers of non-viral gene therapy [386]. The discovery of small interfering RNA (siRNA) in 1998 by Fire and Mellow [387], however, has brought the search for suitable materials for the delivery of nucleic acids back on track. Indeed, the first promising in vivo studies with siRNA, which resulted in the efficient knock-down of the targeted proteins, has caused a revival in the research interest in the field of non-viral gene delivery.

Undoubtedly, the extensive research in the past 10–20 years of AONs and plasmid DNA delivery has revealed some general findings that should be taken into account when developing new gene delivery systems. As AONs, siRNA and plasmid DNA are three different kinds of molecules, the delivery bottlenecks differ somewhat depending on the type of nucleic acids one wishes to deliver. While plasmid DNA is a large (>2000 bp) double-stranded polynucleotide which should reach the nucleus for its action, AONs and siRNA are small (about 20 bp) oligonucleotides which are respectively single-stranded or double-stranded and can act in the cytoplasm of the cells. In general, the ideal delivery system should guide the nucleic acids to their target cells, increase the cellular uptake of the nucleic acids and help the
nucleic acids to escape from the endosomal compartment into the cytoplasm of the cells. Once in the cytoplasm, siRNA acts through endoribonuclease-containing protein complexes known as RNA-induced silencing complexes (RISCs) that unwind the duplex structure. One strand (the “passenger strand”) is discarded and degraded by intracellular nucleases. The other strand (the “guide strand” or antisense strand) guides the activated RISC complex to the complementary RNA molecules that are cleaved and destroyed. Due to this very efficient intracellular process, delivery of siRNA can be considered successful once the siRNA molecules have reached the cytoplasm of the cells. As siRNA molecules are double-stranded, they are more resistant towards intracellular degradation. Therefore, not the intracellular stability but mainly the dilution of siRNA due to cell division limits how long gene silencing can occur. AONs results in blocking or degradation of the target sequence through a sequence specific binding of the AONs to their complementary target mRNA. Upon their delivery in the cytoplasm, AONs should reach their target mRNA (as they are not actively guided such as siRNA molecules) and should remain intact in the intracellular environment (as they are much more susceptible towards degradation when compared to double-stranded siRNA). Therefore, delivery of AONs to the cytoplasm of the cells is no guarantee for successful knock-down of the targeted proteins. The future of unmodified phosphodiester AONs in antisense therapy can be seriously doubted, even if the delivery systems protect the AONs against enzymatic degradation. Indeed, phosphodiester AONs are the subject of rapid intracellular degradation once delivery of naked AONs in the cytoplasm is achieved. Backbone modifications of AONs with increased resistance towards nucleases are still being explored to increase the knock-down efficiency of AONs, although we believe that currently mainly siRNA delivery is expected to make it to the therapeutic market.

A substantial difference in plasmid DNA delivery is the need to gain access to the nuclear interior of the cells. Also, as backbone modifications of the plasmid DNA are currently not available, the delivery systems should deal with the possible intracellular degradation of the plasmid DNA in the intracellular environment. Another major difference with small oligonucleotides such as siRNA and AONs is the limited intracellular mobility of free plasmid DNA molecules. Apart from the common characteristics expected from each delivery system (e.g. targeting the nucleic acids to the desired part of the body, increase the cellular uptake of the nucleic acids and help the nucleic acids to escape from the endosomal compartment) the delivery system should thus additionally protect the plasmid DNA from intracellular degradation, increase the mobility of plasmid DNA through the cytoplasm of the cells and promote the nuclear entry of the plasmid DNA. The material challenges to achieve, in our opinion, the most efficient way to cross the intracellular environment, as deduced from viral examples, were discussed above under Section 5.6.

The ‘first generation research’ in the field of non-viral gene delivery systems is mainly characterized by a bottom-to-top approach: one starts with the synthesis of a new material which is tested for its physicochemical properties in buffer and subsequently for its biophysical behavior in cell culture. This bottom-to-top approach has made us aware of the delivery problems at the top-phase (e.g. on the in vivo level) and how these correlate to the physicochemical properties of the developed materials. In our opinion, the ‘next generation research’ should be guided by a top-to-bottom approach: one should first identify the top-phase delivery bottlenecks to be able to equip the bottom-phase materials with the appropriate characteristics. Actually, this top-to-bottom approach has been used for years by the pharmaceutical industry in the development of suitable delivery systems for low molecular weight pharmaca. Indeed, the discovery of a potential low molecular weight molecule is routinely followed by the evaluation of the pharmacokinetics and body distribution upon intravenous administration of the molecules. Subsequently, this information is applied to develop, e.g. tablets with the desired release profiles.

In general, it is believed that the administration of nucleic acids requires the use of appropriate delivery systems such as nanoparticles. These nanoparticles typically consist of a core (the nanoparticles interior) and a surface that can be equipped with different functional groups. Also the size and shape of the nanoparticles will influence their in vivo behavior. The top-to-bottom approach in the design of nanoparticles implies that, for a specific disease target, we first evaluate the distribution profile of the nanoparticles in the body. Indeed, the nanoparticles should reach the required compartments upon in vivo administration before proceeding to the delivery of the nucleic acids they are carrying. This can be considered as the ‘distribution phase’. Clearly, the challenges in the distribution phase will depend on the chosen disease target. Some target tissues and target cells (e.g. the eye, the lungs, the skin, etc.) are readily accessible by for example injection, inhalation or local application of the nanoparticles. Upon intravenous administration, however, the distribution phase plays a crucial role in the success rate of the developed nanoparticles. In order to make rapid progress in the distribution field, it is crucial that the knowledge of material scientist, pharmacists and biologists is combined in an interdisciplinary approach. Also, in vitro and in vivo research should be performed in the appropriate cell culture models and animal models, respectively, as a slight adaptation in the environment of the nanoparticles can
significantly alter the distribution profile. It should be noted that, most likely, mainly the nanoparticles size, shape (mostly spherical) and surface properties will determine the distribution profile of the nanoparticles in vivo. Therefore, in order to guide the nanoparticles to the desired compartments of the body, one should optimize these characteristics of the nanoparticles.

Once the nanoparticles succeed in reaching the desired compartments of the body, they should proceed to the so-called ‘delivery phase’. In this phase, mainly the characteristics of the nanoparticles’ interior should be optimized, as the nucleic acids mostly reside in the core of the nanoparticles rather than at the surface. Basic requirements for the materials is that they can be loaded with a sufficient amount of nucleic acids and that they protect the nucleic acids against enzymatic degradation. Furthermore, they should deliver the nucleic acids at the appropriate time and place (e.g. the bloodstream, the cytoplasm, the nucleus, etc.) with the desired release profile (e.g. sustained or pulsed, etc.). In order to optimize the nanoparticles’ characteristics, in our opinion, it is important to perform in vitro measurements in proper biological samples such as serum, vitreous, cystic fibrosis sputum and so on. The development of advanced microscopy techniques as discussed in Section 6, allows us to really observe and learn from the extra- and intracellular behavior (e.g. aggregation, disassembly, diffusion, release profile, etc.) of nanoparticles during the different steps of the delivery process, especially if we are able to correlate these characteristics with the absence of presence of the desired biological effect.

One could wonder whether it is advisable to study the delivery process of viruses, which can be considered as naturally occurring nucleic acid delivery systems. The answer is surely yes. Should we, however, try to mimic the viral particles in order to optimize the therapeutic efficiency of non-viral gene delivery systems? The answer to this question is not so straightforward. First of all, we are currently mainly capable of building ‘simple’ nanoparticles, while naturally occurring viruses have evolved to complex constructs. Second, as we incorporate more and more viral features in our ‘simple’ nanoparticles, we increase the risk of not only evoking the desired features of our viral examples, but also the undesired properties such as possible severe immune response. Therefore, one could wonder whether or not it is more appropriate to stick to ‘simple’ non-viral nanoparticles, which release the nucleic acids in response to simple triggers or concepts such as pH, temperature and so on. In essence, the extra- and intracellular behavior of the current generation of non-viral nanoparticles (and even most viral nanoparticles) results from basic physicochemical principles such as charge interactions (e.g. the aggregation of nanoparticles due to charge neutralization), hydrophobic/hydrophilic interactions (e.g. the destabilization of the endosomal membrane), osmotic pressure (e.g. the swelling of buffering nanoparticles in the acidifying endosomal compartment), hydrolysis (e.g. surface erosion of nanoparticles), redox reactions (e.g. the degradation of S–S bonds in the reductive intracellular environment) and so on. Consequently, unwanted behavior can be avoided by interfering with these basic mechanisms (e.g. the prevention of charge neutralization and thus aggregation by PEGylation of the nanoparticles). From this point of view, our main focus could be to supply FDA approved materials, which are known to be non-toxic, with simple features based on the basic principles as described above. This ‘simple’ approach is also encouraged by the observation that the formulations that have made it to the therapeutic market also consist of ‘simple’ materials such as liposomes and PLGA microspheres that deliver their (low molecular weight) pharmaca via simple processes such as hydrolysis of the particles’ core.

Apart from the ‘distribution phase’ and the ‘delivery phase’, attention should be given to the final ‘production phase’ of the developed nanoparticles. Quality control, upscaling and reproducibility of the production processes are important factors that determine the applicability of a certain nanoparticle for the pharmaceutical industry. Indeed, apart from a fundamental research point of view, there is little use in developing high-tech materials if they cannot be produced at a reasonable amount and cost that matches the global need of the chosen disease targets. Clearly, there is a strong need to prepare NANs in larger quantities and in sufficient quality suitable for clinical trials and commercialization. While initial labscale experiments are frequently performed in simple beaker/stirrer setups, clinical trials and market introduction require more sophisticated technologies, allowing for economic, robust, well controllable and aseptic production of microspheres. To this aim, various technologies have been examined for microsphere preparation. Excellent reviews on this subject have been published recently by Ali et al. [388], Euliss et al. [389] and Zhang et al. [390].

References


The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer, Gene Ther. 11 (2004) 1523–1531.
B. Gupta, T.S. Levchenko, V. P. Torchilin, Intracellular delivery of large molecules and small particles by cell-penetrating proteins and


Katrien Remaut
Bruno G. De Geest
Kevin Braeckmans
Jo Demeester
Stefaan C. De Smedt*

Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

*Corresponding author. Tel.: +32 9 2648076; fax: +32 9 2648189

E-mail address: stefaan.desmedt@ugent.be

1These authors contributed equally to this work.

Received 24 April 2007; accepted 21 June 2007
Available online 6 September 2007