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New strategies for nucleic acid delivery to conquer cellular and nuclear membranes

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ABSTRACT

After administration to the body, nucleic acid containing nanoparticles (NANs) need to cross several extra- and intracellular barriers to reach the cytoplasm or nucleus of the target cells. In the last decade several groups tried to overcome these barriers by arming non-viral delivery systems with targeting moieties, polyethylene glycol chains, fusogenic peptides and so forth. However, the drawback of this upgrading strategy is that each of the encountered barriers requires a new functionality, leading to very complex multi-component NANs. Moreover, there are currently no components available that can efficiently transport genes or NANs inside the nucleus of non-dividing cells. In this article a new, ultrasound based delivery system that possesses the capacity to simultaneously overcome several key barriers in non-viral nucleic acid delivery is presented. Additionally, a small amphiphilic compound that induces nuclear uptake of plasmid DNA and enhances non-viral gene transfer is presented.

1. Introduction

Progressions in biotechnology have lead to the discovery of the genetic base of different disorders, opening up the possibility to cure these disorders via gene or siRNA therapy. However, the use of nucleic acids as a drug faces major challenges. Indeed, due to their size and their highly negatively charged backbone, they are efficiently kept out of the cells and cell nucleus by several physiological barriers. These include the endothelium of blood vessels, the interstitial matrices, the plasma and nuclear membrane of cells [1]. In this paper we will review our efforts to bypass the latter two membranes by using ultrasound and *trans*-cyclohexane-1,2-diol (TCHD), respectively (Fig. 1).

Although cellular uptake of NANs occurs very efficiently in vitro via endocytosis, the *in vivo* endocytic uptake of NANs remains rather limited, especially when they are coated with non-fouling polymers like polyethylene glycol (PEG) which hamper their cellular adhesion and subsequent endocytic uptake [2]. To overcome the cell and endosomal membrane, ultrasound in combination with microbubbles has recently been introduced as a nucleic acid delivery technique. The basic principle of ultrasound mediated gene delivery is the formation of small perforations in the cell membrane. Microbubbles that are brought into an ultrasonic field start to cavitate, this is the alternate growing and shrinking of the microbubbble. The microbubbles used in ultrasound assisted drug delivery consist of a gas-filled core, covered by an albumin, lipid or polymer shell, that prevents diffusion of the gas out of the core. The use of perfluorocarbons instead of air increases the half-life of microbubbles due to the low solubility of these gases in water based media like blood. When microbubbles are brought into an ultrasonic field they can cause either stable or inertial cavitation. Stable cavitation occurs when the negative pressure phase of the ultrasound causes growth of the microbubbles, while the positive pressure phase shrinks the microbubbles. During stable cavitation the microbubble grows until it reaches its resonant size, whereupon it keeps on cavitating in the ultrasonic field. Stable cavitation is a rather gentle method and creates microstreams in the surrounding cell medium. Inertial cavitation occurs when the microbubble oscillations are so large that the microbubbles finally implode violently producing free radicals, microjets and shockwaves [3]. These microjets and shockwaves can create transient pores in the cell membrane, as evidenced by electron microscopy images [4–6]. In this way, the cell can take up therapeutic nucleic acids by passive diffusion through the created pores [4,5,7,8]. The concept of ultrasound assisted gene/siRNA delivery may rapidly find its way to the clinic as ultrasound radiation combined with microbubbles is already used for several years in the clinic for diagnostic purposes. However, the *in vivo* efficiency of ultrasound assisted gene/siRNA delivery is currently limited by the fact that the microbubbles are injected in the blood stream together with free pDNA [9], which is prone to degradation by circulating nucleases. To compensate this degradation, large amounts of nucleic acids are injected. Furthermore, the concentration of therapeutic DNA/

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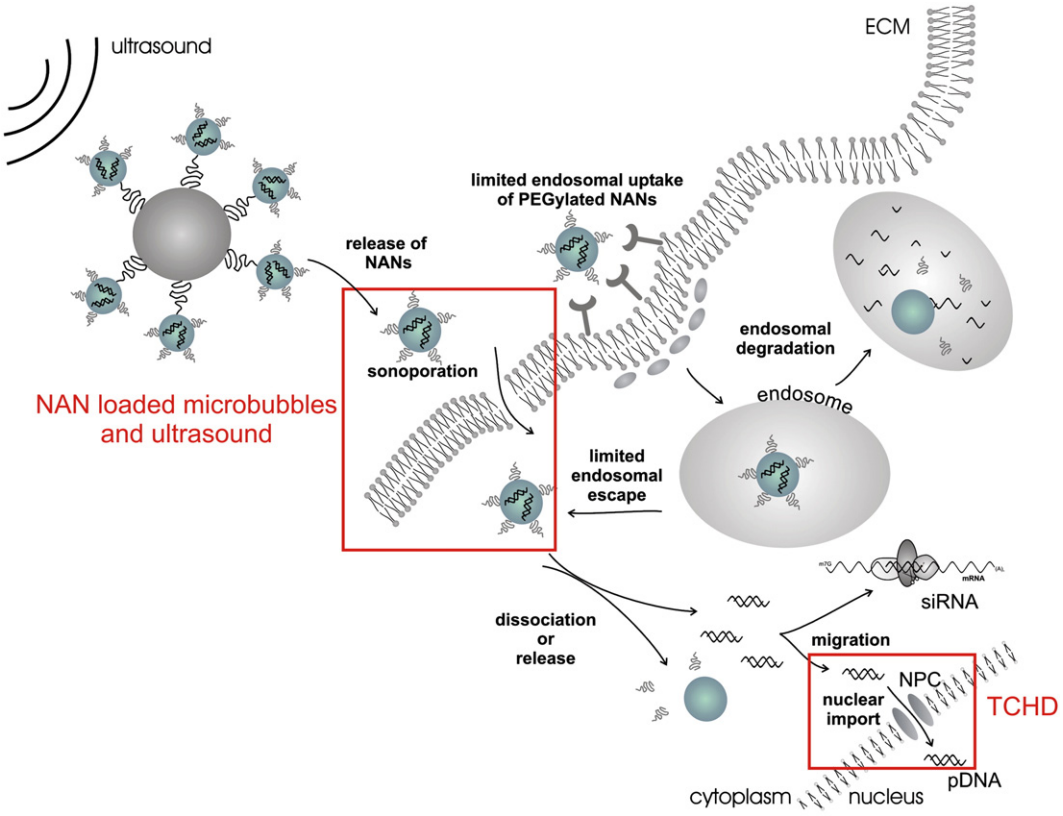

Fig. 1. Schematic representation of cellular barriers in non-viral gene therapy. The barriers that are overcome using NAN loaded microbubbles combined with ultrasound treatment and TCHD are framed.

siRNA in the blood should be high enough to stimulate the passive diffusion of these nucleic acids through the membrane perforations. For this reason it would be very advantageous to have the nucleic acids associated with the microbubbles. We hypothesize that nucleic acids bound to the microbubble surface will be released upon ultrasound radiation and dragged along with the microjets and shockwave inside the irradiated cells [9,10]. It has been reported that albumin based microbubbles can bind small amounts of oligonucleotides or pDNA [11–13]. However, it is unlikely that the pDNA will still be associated to these microbubbles after injection in the blood, which contains huge amounts of albumin. Furthermore, albumin based microbubbles can only bind a very small amount of plasmid DNA. Alternatively, the pDNA can be attached onto the microbubble via electrostatic interactions. Vannan et al. and Christansen et al. coupled the DNA electrostatically to cationic lipid microbubbles [14,15]. We previously succeeded in loading pDNA on an albumin based microbubble that was coated with a positively charged polymer. In this way, a much higher loading of the DNA on the microbubble and a better protection of the DNA against nuclease degradation was obtained [16]. However, sonication of these DNA coated microbubbles results in the release of DNA–polymer aggregates that are too large to be taken up by target cells (unpublished results). Another disadvantage of this strategy is that the charged microbubbles are not PEGylated. This makes them vulnerable to interaction with oppositely charged blood components, which may lead to a release of pDNA from these positively charged microbubbles. Therefore, we tried to overcome the limitations of these nucleic acid bearing microbubbles by attaching pre-formed PEGylated DNA–liposome complexes (PEG-lipoplexes) to neutral lipid based microbubbles via a biotin–avidin binding.

A major barrier for non-viral pDNA delivery, besides cellular uptake and endosomal release, is the trafficking of pDNA into the nucleus. As most target cells are not or very slowly dividing, the pDNA cannot enter the nucleus by accident during disassembly of the nuclear envelope (NE). Nucleocytoplasmic transport proceeds through the nuclear pore complexes (NPCs) which form channels in the NE with a diameter of ~40 nm [17,18]. Vertebrate NPCs have a mass of ~125 MDa and contain 30–50 different proteins, which are called nucleoporins (Nups). Water, ions, small macromolecules (<20–40 kDa) [19] and small neutral particles (<5 nm) can diffuse freely across the NPC [20], while larger macromolecules generally need a particular transport signal sequence, such as a nuclear localization signal (NLS), to be transported efficiently into the nucleus. NLS containing proteins are recognized by nuclear transport receptors (NTRs) that mediate their active translocation through the NE. The majority of NTRs belong to the family of karyopherin β proteins, also called importin β-like proteins [21,22]. The molecular mass of pDNA can be estimated between 2 and 10 MDa making it unlikely that pDNA can enter the nucleus via passive diffusion. To promote the nuclear import of pDNA, one possible strategy is to hijack the nuclear import machinery by attaching NLS peptides, NLS containing proteins or even importin β to the pDNA. However, all these attempts have achieved only limited success [23–27]. Alternatively, it has been shown that the nuclear uptake of macromolecules can be enhanced significantly by addition of the amphiphilic molecule *trans*-cyclohexane-1,2-diol (TCHD) [28]. The mechanism by which TCHD causes nuclear localization of macromolecules can be explained based on the recent finding that the inner channel of the NPCs is filled with a hydrogel. This hydrogel is build-up by cross-linked nucleoporins. The cross-links are formed between hydrophobic phenylalanine–glycine (FG) repeats present in the nucleoporins. We believe that TCHD causes a temporary, non-selective gating of the pore by inducing a disruption of the hydrophobic interactions between the hydrophobic repeats of the nucleoporins. Such a disruption is expected to increase the mesh-size of the hydrogel present in the NPC and hence nuclear entry of