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Oration

Formulation and delivery solutions for the next generation biotherapeutics

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ABSTRACT

In 2018 I was appointed full professor of Pharmaceutical Biotechnology & Delivery at the Pharmaceutics division of the department of Pharmaceutical Sciences at Utrecht University, The Netherlands. In this contribution to the *Orations – New Horizons* of the *Journal of Controlled Release* I will introduce my research group (see also www.uu.nl/pharmaceutics) and will highlight my current and future research projects. In coming years the focus of my research will be on the administration of biotherapeutics, aiming to control their fate from the site of injection to the site of action. I will discuss issues related to formulation of biotherapeutics into nanomedicines (NMs), intracellular delivery of nucleic acids as well as protein therapeutics, and targeted delivery of biotherapeutics beyond the liver. In addition, I will provide a forward view on how current developments in the drug delivery and gene therapy field may result in sustainable and cost-effective dosing regimens for biotherapeutics.

1. Setting the boundaries of my research

It gives me great pleasure to be invited by the *Journal of Controlled Release* to introduce my research group and myself as a recently appointed professor in the field of pharmaceutics and pharmaceutical biotechnology. Let me start by providing a short introduction into the research area I am active in. This focuses exclusively on the pharmaceutics of biotherapeutics (Fig. 1). Biotherapeutics have a few common aspects: they are relatively large, hydrophilic molecules and due to their biological origin, are prone to enzymatic degradation in the human body. For the latter reason, most biotherapeutics cannot be administered via the oral route and are restricted to parenteral routes of administration, mostly via needle injections [1]. Even though many research groups aim to develop strategies to deliver biotherapeutics via more patient-friendly routes, such as the oral, intranasal or pulmonary routes, this is not the focus of my research. Instead, my aim is to control the fate of biotherapeutics from the site of injection (intravenously or subcutaneously) to the site of action with the aim to safely and efficiently deliver biotherapeutics to their intended target site.

Another limitation of biotherapeutics is hampered passage over cell membranes. As a consequence, biotherapeutics have difficulties reaching intracellular targets and often rely on advanced delivery systems that can transport them over the cell membrane into subcellular compartments within the cell (e.g. cytosol, nucleus, mitochondria) [2]. A particular research area that completely relies on efficient intracellular delivery of therapeutic cargo is the field of gene therapy. At *Pharmaceutics* we have set ourselves the goal to develop synthetic and biomimetic delivery systems for nucleic acids (oligonucleotides, mRNA, minicircle DNA) and gene editing components such as CRISPR-Cas with the aim to transiently or permanently correct gene disfunction in target tissue or organs [3].

A specific problem related to the use of biotherapeutics is the potential to trigger undesired immune responses [4–6]. Prolonged administration of therapeutic proteins can induce drug-specific antibody formation that could significantly affect efficacy and sometimes cause serious adverse effects. Similarly, delivery of nucleic acids (NA) can trigger innate immune responses that can in turn inhibit the activity of these molecules inside cells or cause allergic reactions [7]. Understanding the mechanisms underlying this immune activation is important to be able to engineer safer biotherapeutics and/or delivery systems. Furthermore, many protein-based biotherapeutics suffer from stability issues during storage and handling. Protein therapeutics are mostly stored in sterile vials in liquid or freeze-dried form or as prefilled syringes ready for parenteral administration, making use of specific excipients and storage conditions to guarantee a prolonged shelf life of the drug product. Nevertheless, both chemical and physical instabilities during storage as well as improper handling before administration can result in degradation and/or aggregation of the protein therapeutic which upon administration can result in reduced activity and increased chance of triggering immune reactions. Getting to grips with the mechanisms underlying protein degradation and aggregation and finding ways to prevent this is important to guarantee safe and effective

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use of biotherapeutics and is also part of my research. In summary, the research of my group centers around the formulation and delivery aspects of biotherapeutics administered locally or parenterally, with a particular focus on:

- Developing methods for intracellular delivery of biotherapeutics
- Development of synthetic vectors for CRISPR-Cas mediated gene editing
- Development of delivery systems for prophylactic and therapeutic vaccines
- Understanding the immune reactions that might be evoked against carrier and therapeutic cargo and developing strategies to prevent this
- Stability of complex formulations of biotherapeutics during storage and handling

Clearly, there are enough challenges ahead to keep a newly appointed professor, such as myself, busy for the rest of his/her career.

2. Nanomedicine formulation

A great part of the research in my group deals with the formulation and characterization of nanoparticulate delivery systems for protein and nucleic acid-based drugs (Fig. 1). To this end, we make use of polymers, peptides and lipids as materials to construct these nanocarriers. We work on the formulation of a diverse set of biotherapeutics, each having their specific requirements for proper formulation. Roughly, these can be divided into 5 categories:

Small nucleic acids: These include antisense oligonucleotides (ASO), splice-switching oligonucleotides (SSO), editing oligonucleotides (EON) and short interfering RNA (siRNA). The preferred mode of

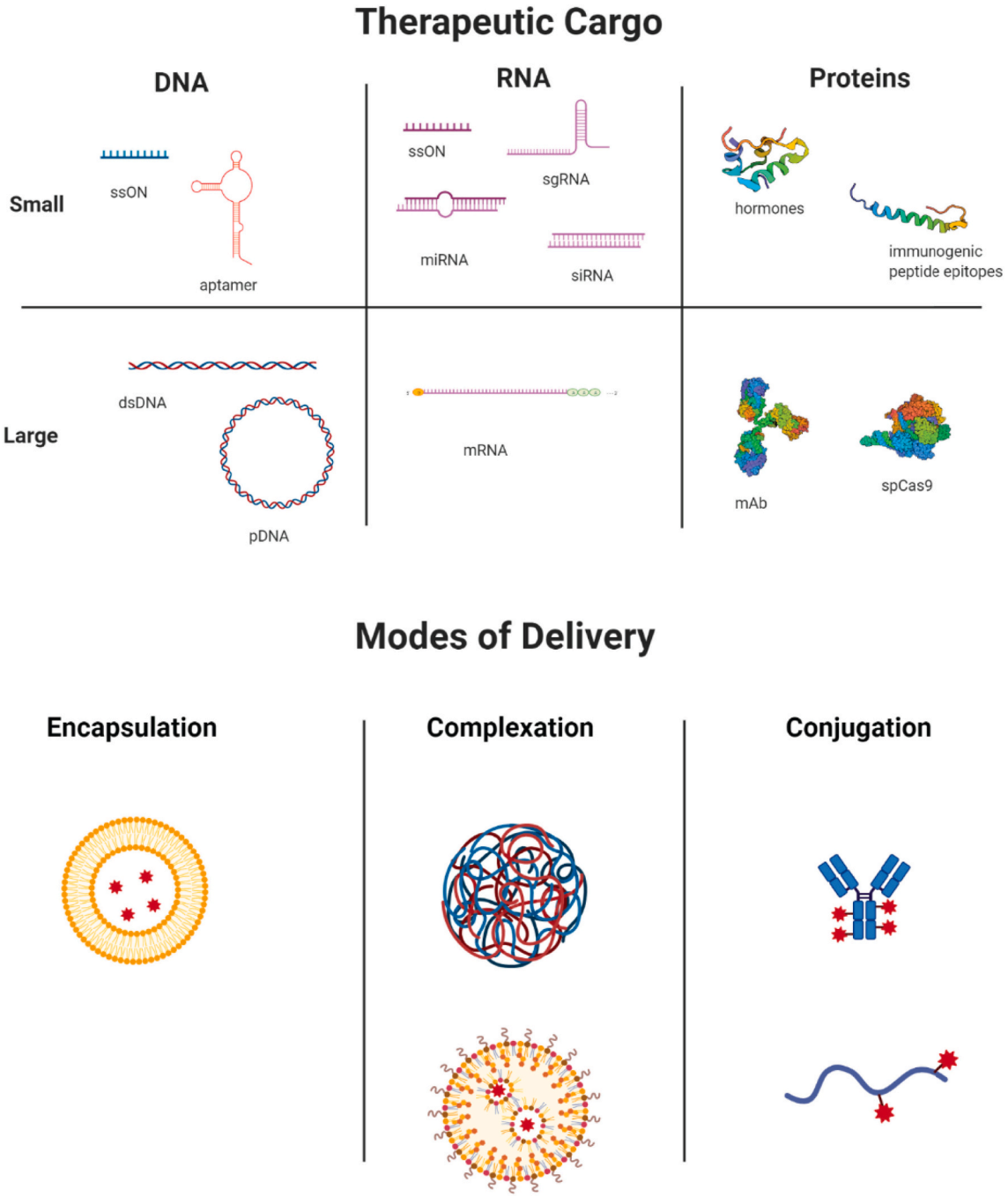

Fig. 1. Overview of the biotherapeutic molecules with their modes of delivery that are being explored in the Pharmaceutics group.

formulation for these molecules is complexation (see also Section 2.2), for which we use both cationic and ionizable lipids and polymers.

Large nucleic acids: This group encompasses NA with sizes of >200 nucleotides. Examples are plasmid DNA, minicircle DNA, PCR products and mRNA. In general, less charge density is needed for stable complexation compared to small NA and thus requires a different design of the nanocarrier.

CRISPR-Cas: These gene editing tools are in particularly challenging to formulate as they consist of 2 (for knock out) and 3 (for gene correction) macromolecular components that need to be delivered into the cells in definite proportions to exert optimal gene editing activity. These components are preferably delivered in pre-assembled state, meaning the Cas protein in complex with the sgRNA (Cas RNP), but can also be provided as gene construct or mRNA that first require conversion by transcription/translation into the active RNA-guided nuclease [3].

Therapeutic proteins: We focus our research on therapeutic proteins that need to be delivered into intracellular compartments in order to exert their activity. Cas-nucleases for gene editing as described above is one example, but one can also think of recombinant proteins to treat a library of different diseases, including recombinant enzymes for lysosomal storage diseases [8], and toxins for oncolytic therapy [9,10].

Vaccines: Our ultimate goal is to make therapeutic cancer vaccines whose composition is adjusted to the patient's individual needs. We work with synthetic peptide epitopes, protein antigens as well as antigens encoded by pDNA or mRNA. These entities need to be formulated in such a way that they can be efficiently delivered into professional antigen presenting cells after s.c., i.m. or i.v. administration.

For delivery of these therapeutic cargo, three different modes of drug-nanocarrier design are being explored, which are: 1. Drug encapsulation, defined as the retention of a drug in a confined space by forming an impermeable hydrophobic barrier around it; 2. Drug complexation, which we define as the retention of a drug by electrostatic interaction with the carrier material and 3. Drug conjugation, defined as retention by (reversible) covalent interaction of a drug with a carrier (Fig. 1). There is no predefined preference for either one of these modes and combinations of each are also being explored. For example, core-shell nanoparticles have been constructed in our group which consist of NA (DNA/RNA) complexed to cationic polymers as the core and a lipid bilayer or a dense layer of poly(ethylene glycol) as the shell; this to prevent the polyplex from dissociation through competing polyanions in the surrounding medium [11–13]. For NA-based drugs, complexation is the preferred mode as the polyanionic nature of most NAs enables strong interaction with cationic polymers, peptides or lipids.

2.1. Standardization is key

An important aspect in the construction of nanoparticulate delivery systems is a proper characterization of the nanocarriers and standardization of the methods used to characterize their physicochemical properties and their delivery efficacies. Borne out of frustration of not being able to reproduce published transfection data with lipoplexes and polyplexes we set out to map the various assays and conditions that were being followed to characterize the stability of the used nanocarriers and read out systems to assess transfection efficiency. It became apparent that lack of standardization of the test conditions and absence of critical methodological information and proper benchmarks made it often impossible to reproduce or compare data and to judge the relevance of many of these studies [14]. In fact, most often, rather artificial test conditions were being applied to favour positive transfection outcomes but with limited value for translation towards preclinical animal studies. Fortunately, in recent years the importance of standardization is being recognized and many guidelines and harmonized protocols on nanocarrier characterization and testing can be found [15]. Examples of initiatives for standardized test conditions with the aim to improve reproducibility, quantitative comparisons and facilitate large scale data analysis are the guidelines on the minimum information reporting in bio-nano experimental literature (MIRIBEL) [16–19], the European Nanomedicine Characterization Laboratory (EUNCL.eu), and the Nanotechnology Characterization Unit of the National Cancer Institute (NCL.cancer.gov). This is just the first step, but the most important hurdle still to take is to put this into practice, for which a change in the prevailing publication culture is required, with less emphasis on individual publications as a measure for success and more emphasis on the collective knowledge generation to advance science on nanomedicines (NM) [20].

2.2. But formulation is not universal

There is no such thing as a universal drug delivery system. Each drug cargo has its specific needs and requirements which is related to the physico-chemical features of the drug cargo, the location and accessibility of the molecular target (intravascular/extravascular; intracellular/extracellular) and the route of administration. It is easy to understand that a protein-based drug that acts on a receptor on immune cells within the circulation will require a different DDS than an antisense oligonucleotide that needs to be delivered into the cytosol of myocytes to treat a muscle disease. But even drug molecules with apparently similar physico-chemical characteristics sometimes behave differently when encapsulated into a nanocarrier system. This is what we experienced when encapsulating a splice switching phosphorothioate oligonucleotide with the exact same sequence but with either 2′-O-methoxyethyl (MOE) or 2'-O-Methyl (OMe) sugar modifications into a polymeric nanocarrier consisting of a mixture of poly(lactic glycolic acid) (PLGA) and poly beta-amino ester (PBEA) [21]. Unexpectedly, we found that these seemingly minor chemical modifications resulted in a significant difference in transfection efficiency with the OMe sugar-modified ONs being inactive at all concentrations tested, whereas the MOE sugar-modified ONs showed a notable dose response (Fig. 2). Continuing with the MOE ON polymeric nanoparticles we furthermore demonstrated that the RNA:polymer ratio had an effect on the cellular uptake and functional delivery of the MOE ONs [21]. Whereas at the low RNA:polymer ratios the fluorescently-labelled nanoparticles could be detected as punctuate spots throughout the cell, reminiscent for endocytic uptake, the polyplexes prepared at the highest RNA:polymer ratio appeared to localize at the cell membrane, which translated into a dramatically reduced functional delivery of these exon-skipping ONs (Fig. 2).

This example clearly shows the importance of understanding the interaction of the nanocarrier material and drug at the molecular level. For this, more high-resolution structural information on nanoparticle assembly is needed which has so far been limited. In a project aimed at constructing a peptide nanoparticle as carrier for peptide epitopes for tumor vaccination, we have applied a combination of coarse-grained MD simulations and ssNMR to probe the structure of such peptide nanoparticles at high resolution. We revealed that the utilised amphiphilic peptides did not form a micellar structure as initially hypothesized, but rather formed a sheet of interdigitated peptides that in time folded up to form a closed vesicle [22–24]. Based on this information, we were able to rationally alter the sequence of the peptide to steer its supramolecular assembly towards more rigid interactions or morphologically different assemblies (e.g. fibrils). Similar techniques can be applied to other nanocarriers as well and could help resolving the internal organization of mRNA when formulated in lipid nanoparticles [22,23].

3. Delivery

3.1. Intracellular delivery

Most biotherapeutics have difficulties passing biological membranes, due to their unfavorable size and charge. This poses a serious limitation to the therapeutic applicability of these molecules. With a few exceptions, nearly all protein-based therapeutics on the market act on target

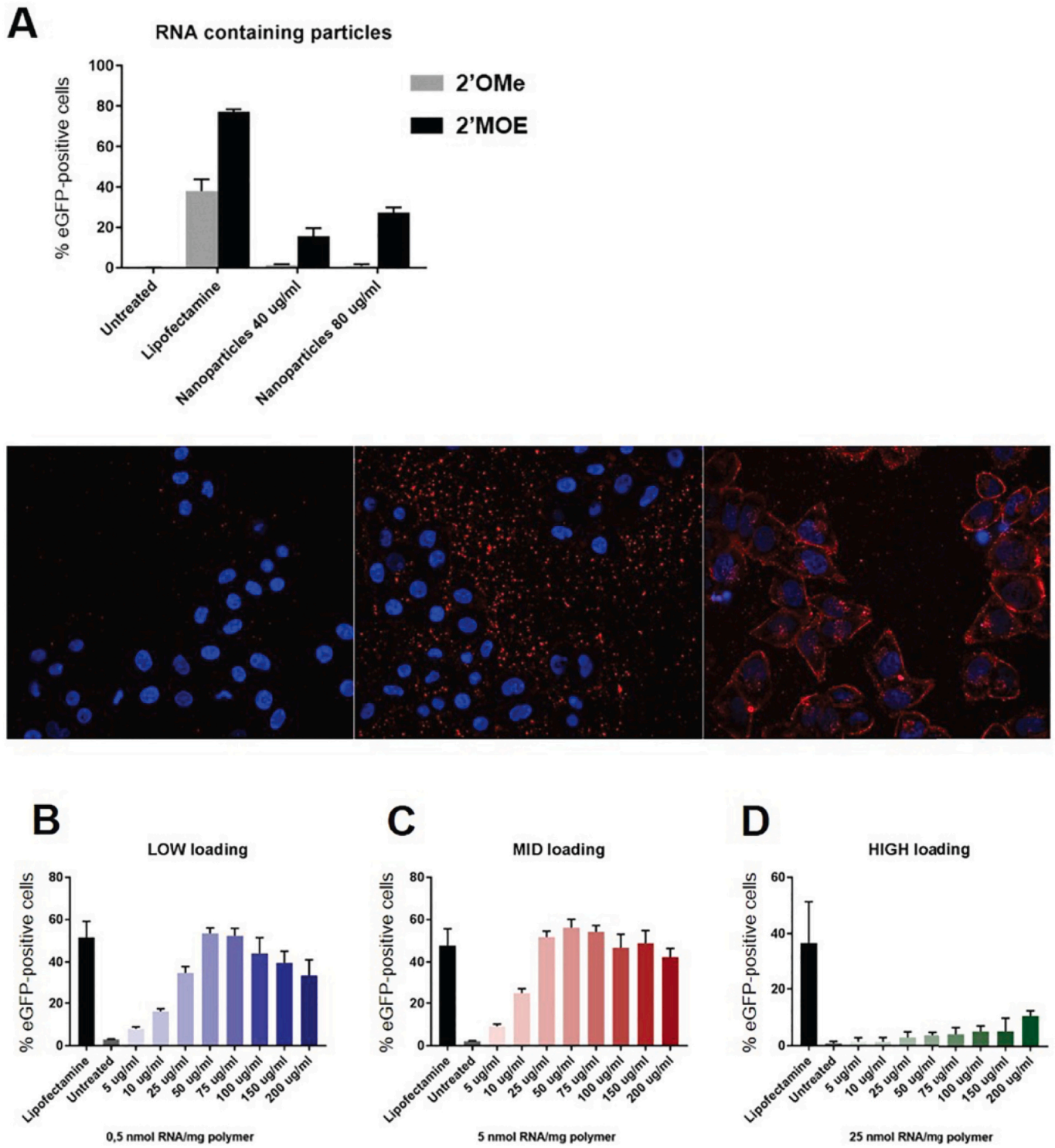

Fig. 2. Panel A: Comparison of the functional delivery of 2'OMe ONs with 2'MOE ONs to HeLa.eGFP-654 cells when formulated in the same PLGA/PBAE nanoparticles. As a positive control for transfection, lipofectamine 3000 was used. Panels B-D: Uptake and transfection efficacy of PLGA/PBAE nanoparticle formulations with varying ON/polymer ratios. High loading seems to impede efficient cellular uptake as most fluorescence can be found at the cell surface. Adapted from Oude Blenke E et al. *J Control Release*. 2019;317:154–165.

molecules that are easily accessible after parenteral delivery (mostly extracellular targets). This is not a coincidence but reflects the technical difficulties getting these biomacromolecules across cell membranes and complex biological barriers such as the blood-brain or blood-retinal barrier. This equally holds true for NA-based drugs. Even though small ON do show some degree of cellular uptake and cytosolic delivery, the efficiency is poor [21]. Larger NA, such as mRNA or DNA often fully rely on delivery systems to get access to the cytosol to exert their therapeutic effect. Finding ways to facilitate intracellular delivery of such biotherapeutics would greatly increase the druggable target space leading to new and improved therapies.

One of the main research lines of the *Pharmaceutics* group is therefore to design drug delivery systems that facilitate intracellular (*i.e.* cytosolic) delivery of biotherapeutics, in particularly nucleic acid-based

drugs [3,13,25–27]. In principle, two approaches can be followed to gain access to the cytosol. The first approach is through direct fusion of a vesicular nanocarrier such as a liposome with the cell membrane. This route could be in particularly interesting in case cell-specific targeting does not lead to cellular internalization of the drug carrier. Membrane fusion can be accomplished by making use of specific lipid compositions that trigger destabilization upon close contact with the cell membrane. Such systems have been described and despite promising *in vitro* data, their intrinsic instability does not allow *in vivo* application as it leads to nanocarrier aggregation and dissociation. Alternatively, viral fusion proteins can be inserted into the drug carrier. We have followed a biomimetic approach based on the fusion of intracellular transport vesicles induced by SNARE proteins [28]. These proteins fold up into SNAREpins, whose interaction is primarily driven by coiled-coil formation. Synthetic coiled coil peptides, derived from these SNARE proteins have been used to trigger plasma membrane fusion between liposomes. We have shown that this approach can also trigger specific binding and membrane destabilization between a cell membrane and a liposome when the cell membrane and liposomes are equipped with complementary coil peptides that can specifically interact to form a coiled-coiled pair (Fig. 3). Binding by coil formation was very specific and only happened when cells displayed the coil peptide and not with adjacent cells lacking the peptide. Furthermore, it resulted in intracellular release of a splice-switching ON that was encapsulated into these liposomes. We are currently investigating how this system can be adapted to apply for specific in vivo delivery of cargo to circulating cells (both immune and tumor cells).

Another approach to gain access to the cytosol is through endosomal escape. Many cells have the capacity to internalize nanoparticles when bound to their plasma membrane by specific or adsorptive interactions. Once inside the endosomes, pH induced membrane destabilization can facilitate release of the entire nanocarriers or their cargo into the cytosol. To this end, we have used cell penetrating peptides (CPPs) that typically have an amino acid sequence with a high abundance of positively charged amino acids such as lysine and arginine (polycationic) or have an amino acid sequence that provide the peptide in its 3D conformation with a hydrophobic part and a hydrophilic part (amphipathic), enabling such peptides to adsorb or insert into cell membranes. CPPs have been extensively explored and many reports exists on how these peptides facilitate intracellular delivery, but lack of standardization made it difficult to compare these published data. We performed a head-to-head comparison of >90 different CPPs described in literature to be able to deliver pDNA into various cells in culture, using standardized test conditions in a high content screening platform that enabled us to screen for cytotoxicity, cell uptake and transfection efficiency at the same time. Strikingly, from those 96 peptides only a few were able to show significant transfection, again stressing the importance of standardization to improve reproducibility in nanoparticle research [29]. These selected CPPs have been used to deliver pDNA as well as CRISPR-Cas9 ribonucleoprotein complexes efficiently into cells in culture with a good toxicity profile (Fig. 4). Although further research is needed, this approach might become a feasible alternative for electroporation to transfect immune cells.

For direct *in vivo* application of CPPs, these peptides need to be either encapsulated or conjugated to the nanocarriers. For conjugation, the type of chemical linkage to the carrier as well as the orientation of the CPP when displayed on the nanoparticle surface is important as most of these peptides are only membrane-active when free in solution or with a free N-terminus to enable insertion and membrane pore formation. In a recent study, we have coupled an acid-sensitive variant of melittin, which has demonstrated to be endosomolytic [27] to the surface of liposomes with the use of an acid sensitive hydrazone linker. When conjugating the acid melittin peptide with its N-terminus to the liposomal surface, complete inactivation of the peptide was accomplished. Exposure of the liposomes to low pH resulted in release and activation of the melittin-derived peptides, leading to lysis of liposomes and release of their content.

Similarly, we have coupled the membrane-active melittin and GALA peptides to the surface of a core-shell polymeric nanoparticle using click chemistry [30]. The surface exposed peptides facilitated binding, internalization and endosomal escape of the mRNA encoding eGFP that was complexed inside the core of these nanoparticles, resulting in efficient transfection of several cell types, including dendritic cells that are known to be difficult to transfect.

3.2. Triggered release systems

In the above examples, the low pH within the endosomes is causing the trigger for membrane disruption, leading to cytosolic release. Other external triggers, such as exposure to elevated temperature, magnetic fields, ultrasound or light can also be applied to facilitate extracellular or intracellular drug release from nanocarriers, in particularly liposomes.

To make liposomes sensitive to near infrared light, which in general has a relatively good depth of tissue penetration, we incorporated indocyanine green (ICG) into thermosensitive liposomes [26]. ICG can be excited at a NIR wavelength of 800 nm, leading to local heating of the probe. When incorporated into the bilayer of thermosensitive liposomes, this local heating will lead to fluidization of the bilayer and content release. We demonstrated that light-triggered release was fast, leading to complete release of an antisense ON inside liposomes in a matter of seconds, whereas the non-exposed liposomes remain stable [26]. Giving the speed of release and the excellent spatio-temporal control, such

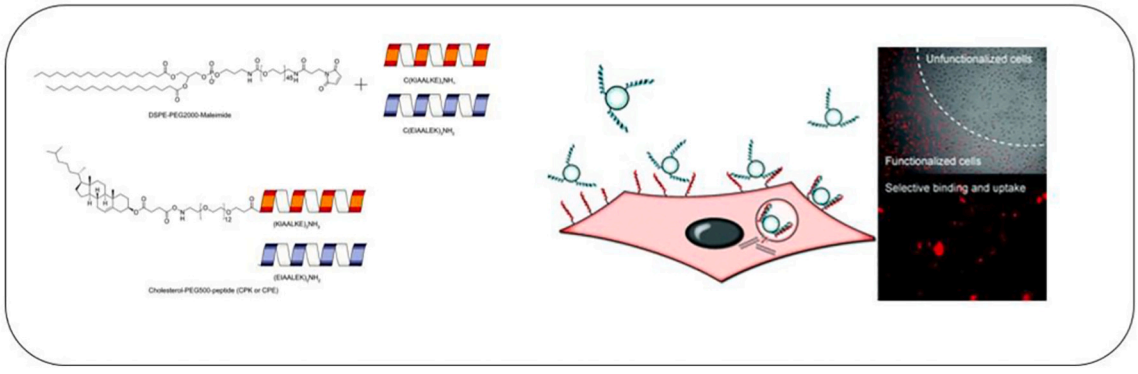

Fig. 3. Highly specific coiled-coil mediated spatial binding and uptake of liposomes into cells. Liposomes functionalized with one of the pairs of a synthetically derived heterologous coiled coil were incubated with HeLa pLUC705 cells of which a specific region was labelled with the complementary coiled coil peptide. Liposome binding was highly specific and only took place in the presence of both complementary coil peptides. Binding let to internalization and functional delivery of exon-skipping ONs entrapped inside these liposomes. Adapted from Oude Blenke E et al. *Nanoscale*. 2016;8(16):8955–8965.

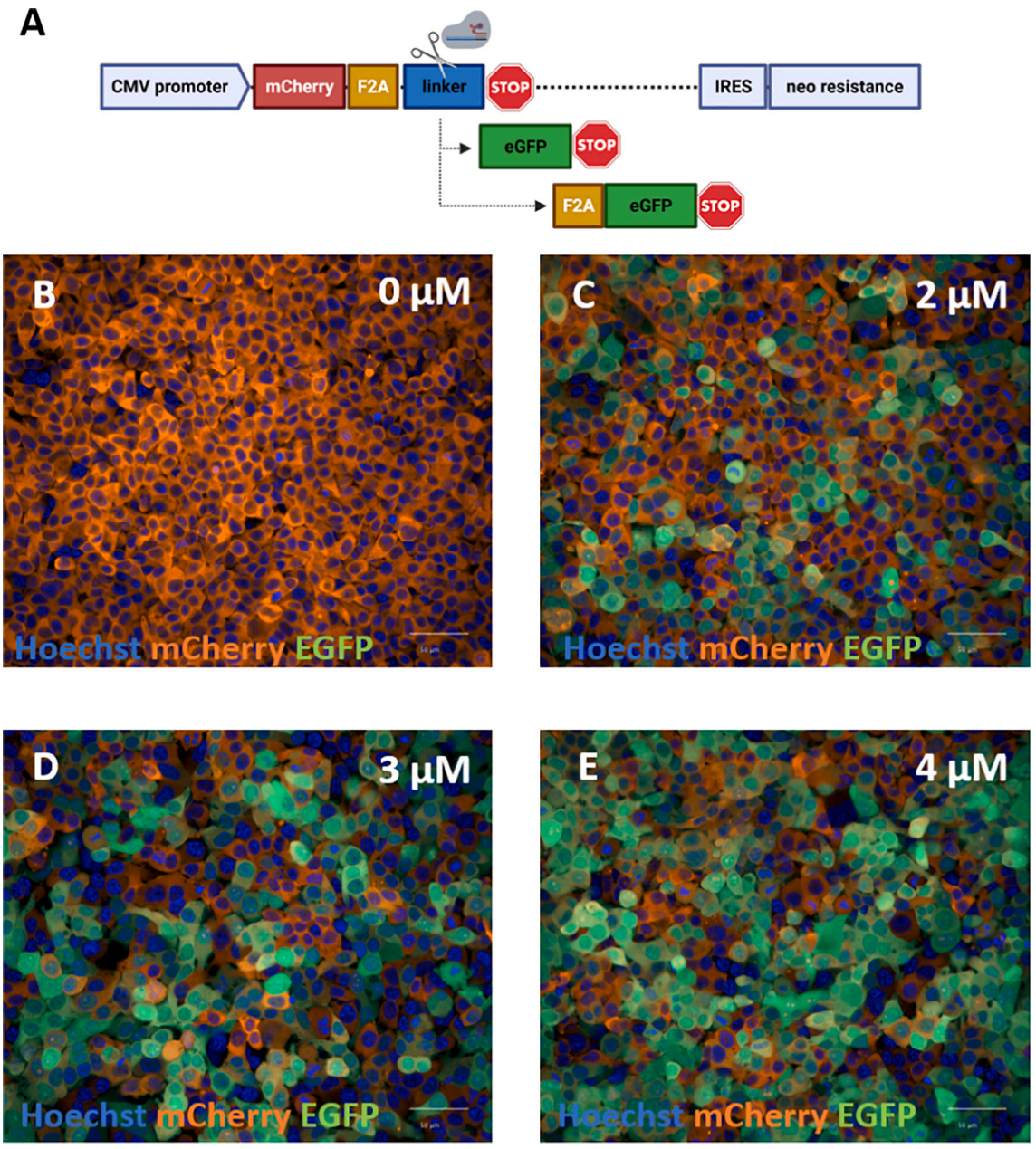

Fig. 4. Cell-penetrating peptide LAH5-mediated functional delivery of CRISPR-Cas RNPs into HEK293T stoplight cells. These cells constitutively express mCherry, but upon the introduction of spCas9 mediated n + 1 and n + 2 frameshifts in the linker sequence, co-expression of eGFP will occur (panel A) [87]. Cells were incubated with 10 nM Cas9 protein and 20 nM sgRNA in the presence of 0, 2, 3 and 4 uM of LAH5 peptide for 24 h, medium was refreshed and cells were incubated for another 24 h before analysis by confocal microscopy (panel B,C,D and E, respectively). The appearance of eGFP fluorescence as a measure of Cas9 RNP activity is clearly visible and LAH5 dose dependent. Courtesy of Mert Öktem, unpublished results.

light-triggered liposomes are ideal for on-demand drug delivery. For example, immobilization of these liposomes in an injectable hydrogel that is administered intradermally or subcutaneously would enable light-triggered control over the release of liposome-entrapped drug molecules without the need for bulky drug infusion pumps.

Besides extracellular drug release, we also demonstrated that such light-triggered liposomes could facilitate intracellular delivery of ON cargo [26]. By allowing cells to internalize the liposomes before exposing them to NIR light, the liposomes that reside inside endocytic compartments are being disrupted. This presumably leads to exchange of lysolipids with the endosomal membrane and subsequent destabilization, although further research is needed to clarify the exact mechanism of cytosolic drug release.

High-intensity focused ultrasound (HIFU) is also being explored by our group to facilitate intracellular drug release as well as to promote passage of nanoparticles over the blood-retinal barrier. It is a non-invasive technique that enables the transfer of energy using ultrasonic waves to induce local thermal or mechanical damage. The latter can be obtained by inertial cavitation, in which the presence of gas-filled microbubbles start to oscillate in the ultrasonic field that can eventually implode. This implosion is associated with the formation of yet streams that can mechanically damage tissues and cells in close proximity. We are currently exploring this technique to enable cell entry of large macromolecules such as proteins and nucleic acids by transiently permeabilizing cells. In addition, we are investigating the possibility to enhance the permeability of the blood-retinal barrier for passage of drug molecules or entire nanocarriers for drug delivery to the retina.

4. Specific applications

4.1. CRISPR-Cas delivery

The CRISPR-Cas technology has revolutionized the field of gene therapy as it enables the introduction of precise edits within the human genome. This would offer potential therapies for genetic diseases of which over 10,000 are known in humans. CRISPR-Cas stands for Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated protein (Cas) that together form the adaptive immune system in certain prokaryotes that confers resistance to foreign genetic elements such as bacteriophages and plasmid DNA. This system has been adapted to introduce double strand DNA cuts at precise locations in the genome of mammalian cells by guiding the Cas endonuclease to a specific sequence by virtue of an associated RNA (guide RNA or gRNA). This DNA damage leads to activation of the endogenous DNA repair pathway, which can either randomly anneal the DNA ends, which often leads to insertions or deletions at the cut site or can activate a process of homology-directed repair, in which the damaged DNA is corrected by making use of a DNA template. Providing such a template *in trans* gives control over the editing process and thereby provides the option to correct, insert or delate small or larger sequences within the genome in a precise manner.

For therapeutic applications, the various CRISPR-Cas components need to be delivered into the nucleus of target cells. This requires the delivery of three different components (i.e. Cas protein, single guide RNA and DNA template for HDR) at optimal ratios into the nucleus of target cells, preferably during the right phase of the cell cycle (G2/S phase) so that HDR will be active. Unfortunately, delivery is severely hampered by the presence of membrane barriers (plasma and nuclear envelope) that virtually block entry of such large molecules. Furthermore, most cells, in particularly immune cells, are equipped with cytosolic DNA pattern recognition receptors (PRRs), that can sense the entry of foreign DNA/RNA and activate signal transduction cascades that leads to production of type 1 interferons, which virtually shuts down the cell. For effective delivery both membrane passage as well as prevention of immune activation by PRRs should be addressed.

We set ourselves the goal to develop a CRISPR-Cas based gene therapy for the treatment of progressive familial intrahepatic cholestasis type 3 (PFIC-3), an autosomal recessive genetic disease caused by mutations in the *ABCB4* gene encoding multidrug resistance protein 3 (MDR3) (Fig. 5). This protein shuttles phosphatidylcholine from hepatocytes into the bile canaliculi. Normally, phosphatidylcholine neutralizes the toxic effects of bile acids, but in its absence causes cholangitis. If not treated, this will lead to fulminant liver failure and death in childhood. An effective gene therapy for PFIC-3 should correct the particular disease-causing mutation in the *ABCB4* gene in a large number of hepatocytes. Since lipid nanoparticles have a proven track record for

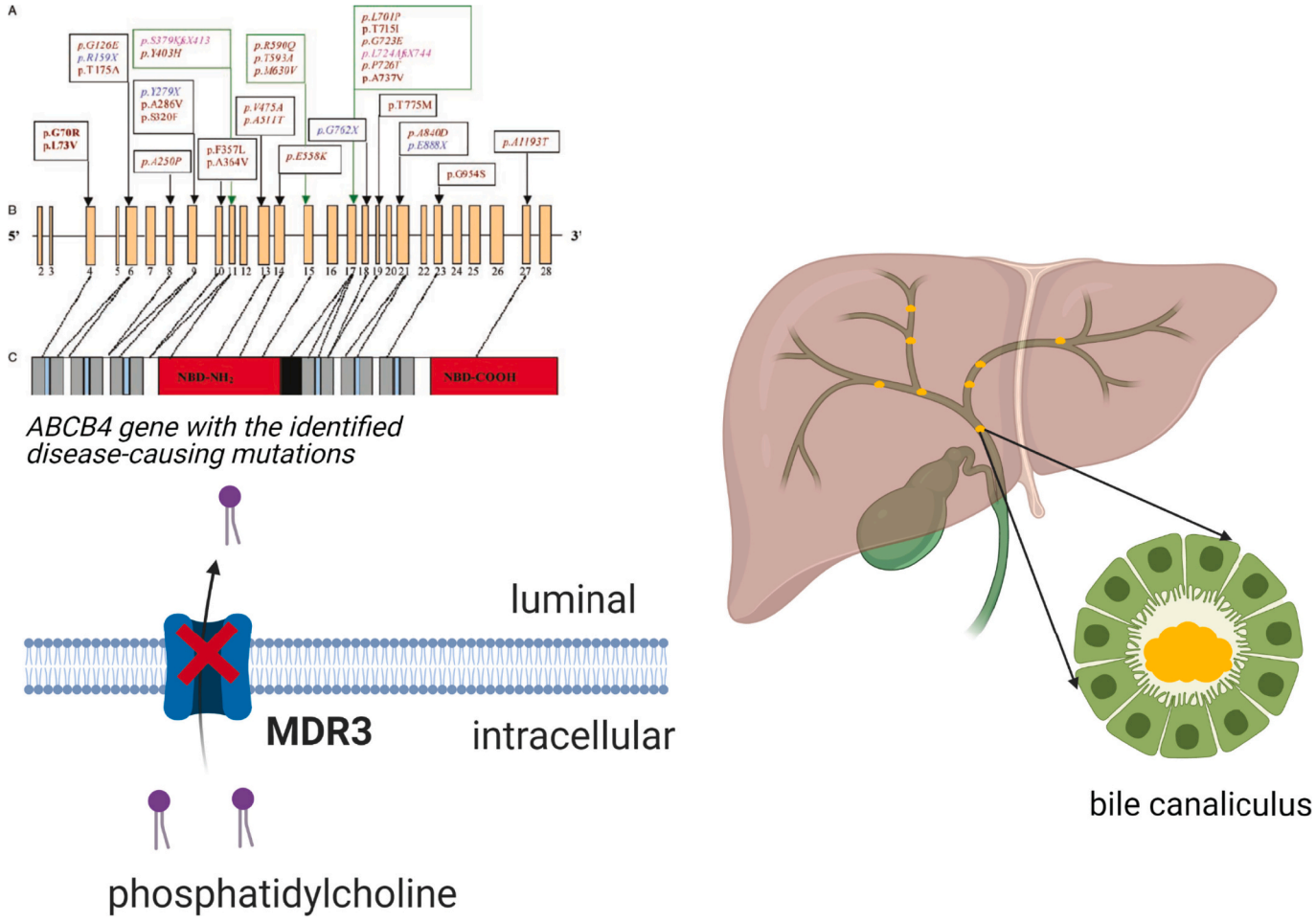

Fig. 5. Proposal for CRISPR-Cas gene therapy of progressive familiar intrahepatic cholestasis type 3 (PFIC-3). This disease is characterized by a diverse set of mutations in the *ABCB4* gene encoding the multidrug resistance protein 3 (MDR3), whose function is to transport phosphatidylcholine from the interior of hepatocytes into bile canaliculi to neutralize the cytotoxic effects of bile acids. Without this neutralization, liver damage will occur leading to liver cirrhosis and eventually liver transplantation is required. By correcting the individual mutations in the *ABCB4* gene using CRISPR-Cas, it is anticipated that part of the MDR3 function can be restored. This requires delivery of all three CRISPR-Cas components (Cas nuclease, sgRNA and HDR DNA template) to be delivered into affected hepatocytes. The disease causing mutations as listed here were copied from Colombo et al. [88].

delivering nucleic acids into hepatocytes and are also under investigation for CRISPR-Cas delivery [3,31–33], we focus our efforts on these delivery systems. Our approach is different in the sense that we aim to deliver the ready-made Cas9 RNP into the cells as this modality is instantaneously but transiently active, thereby reducing the chances of potential off target effects. Initial testing on reporter cells has demonstrated that such LNPs can indeed deliver the Cas9 RNP into cells, leading to gene knock out. We are currently investigating the possibility to co-deliver an HDR template to enable gene correction. Initial results look promising with approximately 1–8% of gene correction after a single administration (unpublished data).

Most likely, given the relative low yields of gene correction, multiple rounds of gene editing will be required to reach a therapeutic threshold in clinical settings. Unfortunately, repeated injections with the bacterial Cas9 RNPs formulated in LNPs will undoubtedly lead to Cas-specific immune responses. In fact, pre-existing antibodies against several variants of the Cas protein have been reported in serum of humans and high levels of anti-Cas9 antibodies were detected after adenoviral delivery of the DNA encoding Cas9 [34–36]. Even though encapsulation of Cas9 RNP inside LNPs would provide some protection against the neutralizing effects of antibodies, they cannot protect against the observed cellular (T cell-mediated) responses. These could in principle lead to CTL-mediated killing of gene-edited cells in case the Cas9 protein is being expressed for some time after gene editing has occurred.

In the next section, I will describe our efforts to prevent such detrimental immune responses with the aid of nanoparticles.

4.2. Nanoparticles for modulating immune responses

An active research line in my group is the development of prophylactic and therapeutic vaccines for the induction of antigen-specific immune responses. Depending on the vaccine composition these can either activate or tolerize the immune system (Fig. 7). Nanoparticulate delivery systems have long been used as antigen carriers for vaccination as they have some unique intrinsic properties that make them ideally suitable for this purpose. First, they can protect the associated antigen from degradation or prevent rapid clearance from the injection site. Second, they can direct the delivery of these antigens to antigen presenting cells. Many cells of the innate immune system express pattern-recognition receptors, including scavenger receptors that can efficiently bind nanoparticulate material. This guarantees that the antigens are adequately delivered to those innate immune cells for processing and subsequent antigen presentation. The conditions under which the innate immune cells sense the nanoparticles and the cues it receives determines the subsequent immune response, which can have a pro-inflammatory or tolerogenic nature.

For vaccination against infectious diseases optimal activation of the innate immune system is required that leads to effective antigen-presentation and activation of the adaptive immune system to build up immunological memory. A good way to achieve this is through biomimicry: by copying specific structural as well as functional features of microorganisms in the vaccine delivery system, optimal immune activation in a safe and controlled fashion can be achieved. Recently, Lou et al. constructed such a biomimetic modular core-shell polymeric nanoparticle of which the core consisted of synthetic ssRNA to resemble viral RNA (Fig. 6) [11]. This core serves as a pathogen-associated molecular pattern (PAMP) and triggers innate immune activation through TLR-7/8 and to a lesser extent RIG-I. To this core a flexible layer of poly (ethylene glycol) can be conjugated to which antigens as well as targeting ligands can be attached to facilitate uptake by antigen presenting cells. It was demonstrated that such virus-mimicking particles, loaded with OVA antigen and functionalized with mannose for specific uptake by dendritic cells resulted in DC activation and maturation and elicited strong OVA-specific cellular and humoral immune responses in mice. The ease at which the shell composition of these virus-mimicking nanoparticles can be altered make this vaccine platform ideally suited for rapid response vaccines to fight rapidly evolving pathogens, as we are currently experiencing with the COVID-19 pandemic.

4.3. Induction of immunological tolerance to biological drugs

Nanoparticles can also be applied to modulate the immune system in such a way that it attenuates undesired immune responses, such as those that occur in auto immune diseases, allergy and immune responses directed against biological drugs that can occur after prolonged administration (Fig. 7). Instead of systemically suppressing the immune system, one could render the immune system tolerogenic for specific antigens, without compromising immune reactions against invading pathogens. Key to the induction of such a tolerogenic response are dendritic cells (DCs). These, located throughout the body continually sample their surroundings by phagocytosing nearby materials. In case the DC detects potentially dangerous material via a wide spectrum of pattern recognition receptors that recognize conserved structures found on many microorganisms, the DC matures and presents the antigenic

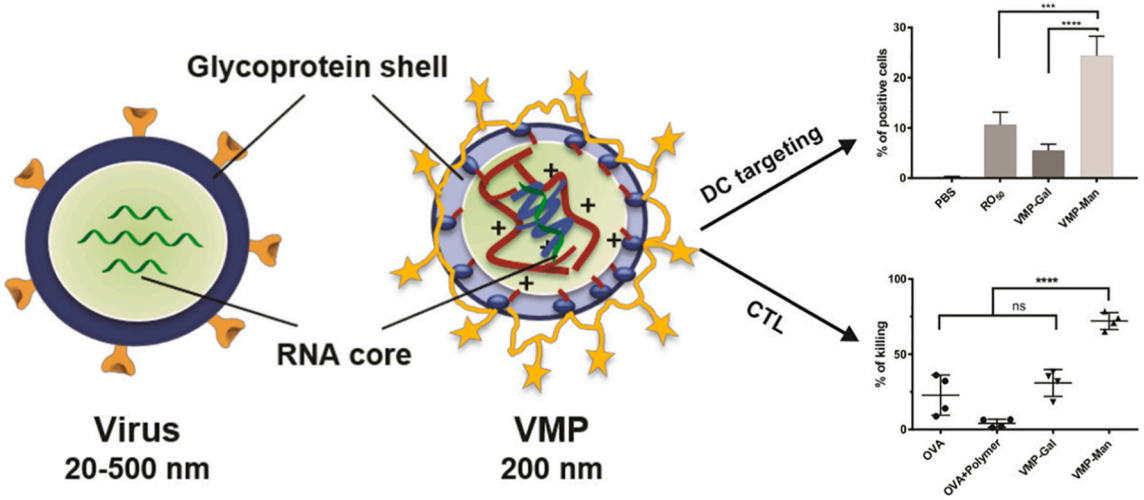

Fig. 6. Virus-mimicking nanoparticles (VMP) have been designed to resemble the structural organization of many viruses. They consist of a polymeric core complexed with RNA- and an outer shell layer containing protein antigens. The VMP particles specifically target to dendritic cells, promote antigen cross-presentation, and induce DC maturation, resulting in elicitation of robust antigen-specific cytotoxic T lymphocytes responses and superior antibody production. Figure copied from Lou et al. [11].

epitopes of the engulfed material on major histocompatibility complex class II (MHC-II) together with costimulatory receptors to signal T cells that they have found potential danger. This combination of antigen and T-cell co-receptor stimulation is the basis of peripheral tolerance. If the handshake between DCs and T cells occurs in the absence of co-stimulation or in an environment where other signals overrule co-stimulatory signals, T cells can lose their effector function and become anergized. These T cells are either converted into regulatory T cells (Treg) that play a role in regulation or suppression of other immune cells, or are deleted via apoptosis. Antigen-specific tolerogenic DCs can be generated by co-administering with antigen immunosuppressive pharmacological agents such as rapamycin or dexamethasone [37–39]. Alternatively, specific cytokines that have a tolerogenic effects, such as interleukin-10 (IL-10) or transforming growth factor β (TNF-β) can be co-administered. The use of nanoparticles to stimulate targeted delivery of such tolerogenic antigen mixtures have recently gained increasing attention [37–39]. Research in my group focuses on the induction of immunological tolerance against biological drugs with the use of tolerogenic NPs [40]. Many patients that are treated with biological drugs for prolonged periods of time develop anti-drug antibodies (ADA) that can have an effect on its therapeutic activity and in some cases cause life threatening reactions. Similarly, viral vectors or other components (e.g.

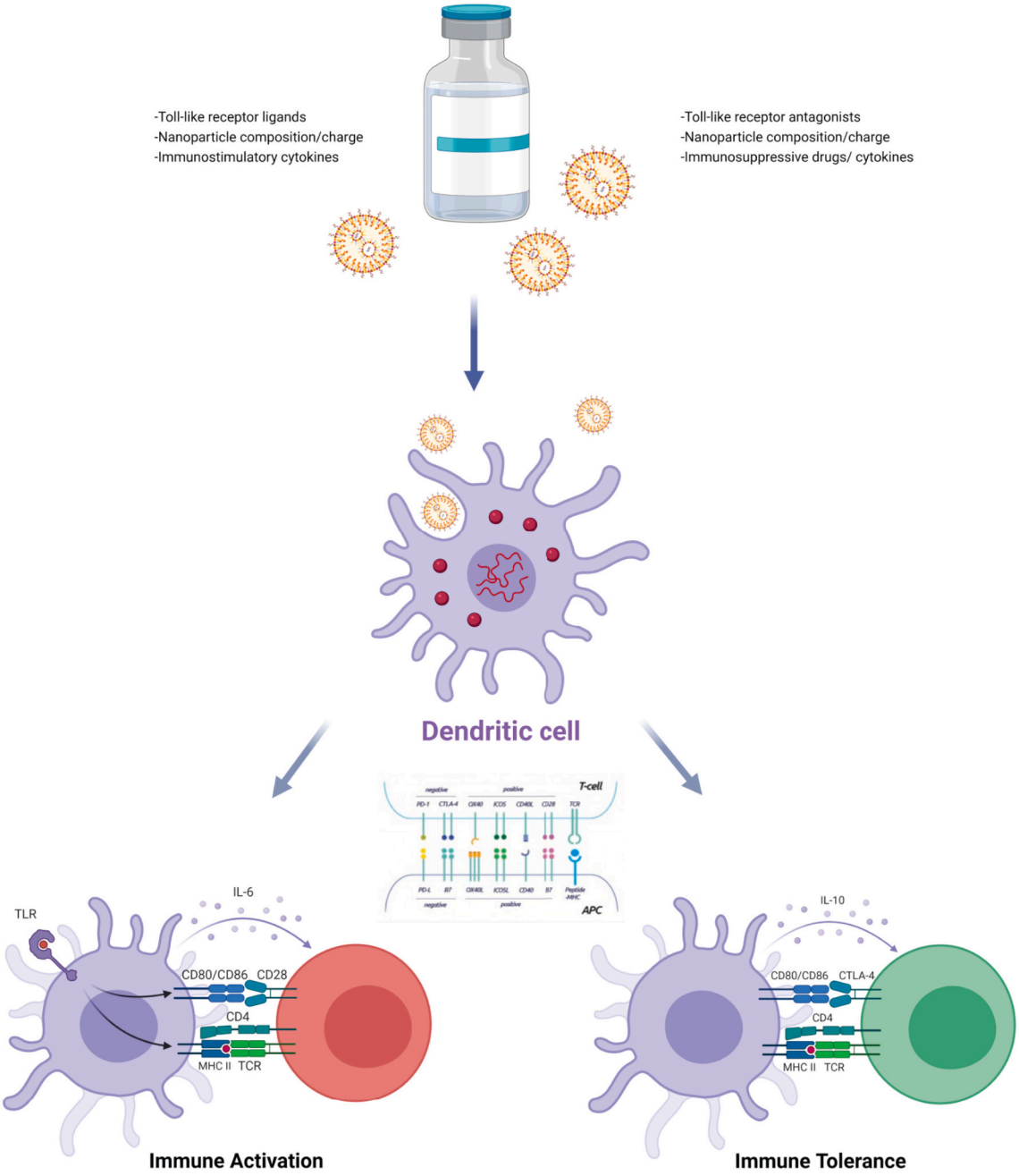

Fig. 7. Immune activating or tolerizing vaccines. Vaccine antigens delivered to antigen presenting cells can lead to immune activation but can also dampen undesired immune responses or tolerize the immune system in an antigen-specific fashion. The size, shape, charge and composition of the vaccine carrier, the presence or absence of co-stimulatory signals, the type of antigen presenting cells involved in antigen processing and the environment in which this takes place all contribute to the type of immune response that will be triggered. Antigen-specific tolerogenic nanoparticles can be made by co-encapsulating with the antigen an immunosuppressive drug that will lead to prolonged antigen-specific immune tolerance. This can be useful for the prevention of undesired immune responses against biological drugs or carriers.

TALENs, CRISPR-Cas) used for gene therapy are often immunogenic, thereby preventing repetitive use and in the case of pre-existing antibodies the therapeutic approach might fail. Tolerizing the patient's immune systems to these components prior to starting the therapy could be an effective strategy to prevent such detrimental immune reactions. Our research aims to gain a better understanding of the immunological mechanisms underlying tolerance induction as well as the specific features tolNPs should have to mount a durable tolerogenic response. Despite some promising proof-of-concept publications showing feasibility to induce antigen-specific immune tolerance in mice and humans, it is at present still unclear which subsets of antigen presenting cells are responsible for tolerance induction and which lymphoid organs (e.g. liver, spleen, lymph nodes) need to be targeted. Also, it is unclear how specific tolerance induction actually is and if tolerance might be inadvertently activated against pathogens that are present during the tolerizing regimen. In the coming years we hope to shed light on these matters.

5. Stability

Proper storage and handling of biopharmaceutical products as well as complex biological formulations such as vaccines or NM are of utmost importance to guarantee their quality and activity upon use. Most of these complex drug products require cold chain storage that can sometimes be a complicating factor for world-wide distribution as has recently become apparent for some of the COVID-19 vaccines [41]. Understanding the cause of potential stability issues is key to be able to find appropriate solutions that can prevent degradation or aggregation of the drug substance or drug carrier and requires in-depth research that is often neglected or started too late by pharmaceutical companies in the development process of a drug product. But also strict compliance to the instructions on how the drug product should be handled or prepared for use is an important aspect that is often ignored. Several accounts point to the notion that biological drugs are in many cases not stored or handled properly by patients and physicians [42–44]. Improper handling can easily cause aggregation or degradation of the drug substance that might lead to loss of activity or induction of immune responses. Further research on the impact of specific procedures during the storage or handling of biological drugs is therefore important to guarantee finished product stability. For example, many biological drugs used in hospitals need to be reconstituted and prepared for use in the hospital pharmacy. Quite often, these medications are being transported to the patients with a pneumatic tube system (PTS) which can lead to exposure of the medication to vigorous shaking and shocks. Most hospitals are aware of this and have validated their PTS and keep a list of “do-not-tube” medications, but these lists have been compiled based on suspected vulnerability but empirical research is often lacking. Together with Prof. Jiskoot at Leiden University and dr. Mirjam Crul at Amsterdam Medical Hospital, we have started to monitor the effects of PTS transportation on the structural integrity of specific monoclonal antibody products and its effect on the formation of protein aggregates. By using an in-tube logging device we monitor exposure to light, temperature, humidity and g-forces in 3 dimensions during the transport phase and perform in-depth structural analysis of the mAbs before and after transportation. Although this research is still ongoing, our preliminary data show large variations in shock exposure depending on the type of PTS used with acceleration g-forces sometimes reaching 70–80 xg (Fig. 8). Such shocks led to an increase in the subvisible particle count in the infusion bags for some mAb products.

In line with this example, future research will be directed to identifying most common stress conditions of finished drug products and the effects of combinations of such stress conditions on the integrity of the drug substance. The ultimate aim would be to map changes in drug substance during storage and handling, but also after administration to the patient and relate those to the occurrence of immune reactions in patients.

6. Future directions

If we would fast-forward to the year 2040, how would the drug delivery landscape look like and what would have been the contribution of my group to this? As we cannot predict the paradigm shifts and breakthrough discoveries of the near future our predictions are rather speculative, but by extrapolating the developments that are currently ongoing in the drug delivery field we can roughly sketch the contours of future applications of drug delivery. I have highlighted some of my thoughts on this below, some of these ideas are currently being developed in my research group.

6.1. *Targeting beyond the liver*

Despite extensive research in this area, systemic drug delivery with NMs still suffers from major impediments that limit its applicability. One of them is predominant uptake of NMs by cells of the mononuclear phagocyte system (MPS). Modification of NMs with poly(ethylene glycol) can greatly retard MPS uptake, leading to a significant increase in blood circulation times, which in turn favours passive accumulation in tissues and organs beyond liver and spleen. Nevertheless, the amount of NMs that can be delivered to targets beyond liver and spleen remains limited and eventually the majority of NMs still end up in cells of the MPS. Future research in my group will focus its attention to the development of strategies that would enable improved MPS avoidance in

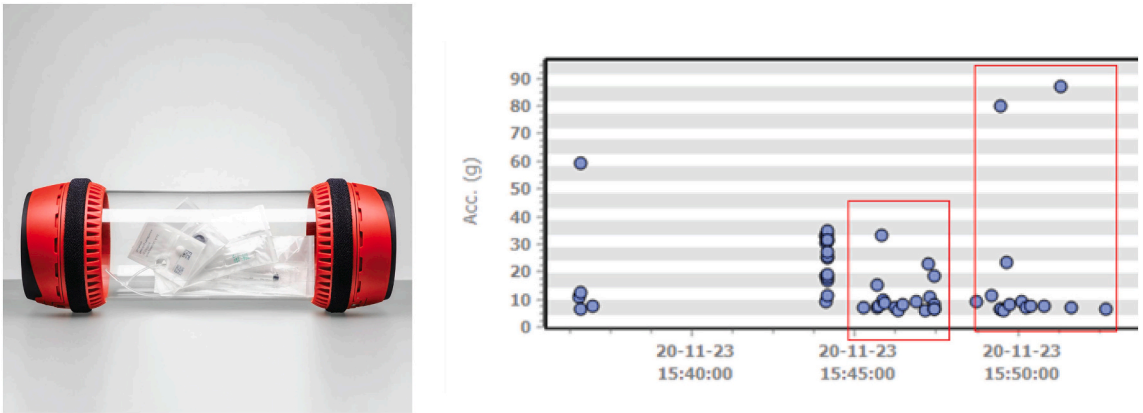

Fig. 8. Shock exposure of medicines in a pneumatic tube system using in-line loggers. Measured acceleration forces can sometimes reach peak values as high as 80 ×g. The impact of such shock exposures on the induction of protein aggregation are at present unknown and are subject of investigation in our group. Courtesy of Roderick van den Berg.

addition to enhanced retention to organs and tissues beyond liver and spleen.

6.2. Mononuclear phagocyte system (MPS) blockade

To prevent uptake of NM by cells of the MPS, there are several strategies that can be followed. PEGylation of NPs does not prevent, but rather slows down uptake by phagocytic cells. Recent work has demonstrated that pre-saturation of the MPS with an injection of empty nanoparticles can lead to temporal MPS blockade, which in turn leads to dramatically increased circulation times of the drug-loaded nanoparticles [45–49]. Pre-saturation can be achieved with empty nanocarriers but for prolonged down-regulation of MPS uptake, more drastic measures have been taken by specifically ablating macrophages with e.g. clodronate liposomes [45,50]. For proof-of-concept studies this approach is perfectly fine, but may not be the ideal solution in the clinical setting. Another approach is based on the CD47 membrane protein ubiquitously found on human cells, which acts amongst others as a "don't eat me" signal to macrophages. Decorating NMs with CD47 or peptides derived from CD47 prevents uptake and degradation by macrophages, but not binding to their plasma membranes. As a consequence, low doses of CD47-decorated NPs can efficiently shield the surface of macrophages and keep them out of business for some time without being toxic [46,51].

6.3. Mechanisms of extravasation

Next to avoiding clearance by cells of the MPS, NM should be able to reach their intended target cell population including those outside the blood compartment. Given that the greatest part of our vasculature consist of endothelial cells with a continuous lining, which in addition display a dense glycocalyx on their luminal side, extravasation of NM is severely hampered if not absent in healthy tissue. Scientists, including my group, have tried for decades to find solutions to this vascular barrier, especially for delivery into the brain [52,53]. Receptors with known transcytotic capacity such as the transferrin receptor and insulin receptor have been exploited to get drugs and NM across the blood-brain barrier (BBB) but with limited success, mostly because of the limited capacity. Future research should address this pitfall and focus on the identification of new receptors on endothelial cells with high transcytotic capacity or for strategies that favour passive, paracellular transport [54,55].

One way of doing this is to take example from nature. Specific serotypes of Adeno-Associated Virus (AAV), including AAV9, have the capacity to efficiently cross the BBB and infect neurons in the CNS [56,57]. The trans-BBB properties are likely dependent on the use of glycans with terminal N-linked galactose as primary receptor, but the actual mechanism of transcytosis remains largely unknown. In-depth analysis and understanding of this transcytosis mechanism could lead to the identification of the main receptor(s) involved, which has recently been done for the engineered AAV-PHP.B vectors that efficiently cross the BBB in mice [58]. This increased understanding of the transcytosis mechanisms of natural viruses will, in turn allow us to develop engineering strategies to get NM across vascular barriers and reach tissues beyond the blood compartment. This requires close, interdisciplinary collaboration between cell biologists, chemists, biological engineers and formulation scientists.

Another strategy to get NM across the vascular barrier and to penetrated into tissue is by hitchhiking along with immune cells [59,60]. By equipping NM with ligand that specifically interact with circulating blood cells such as T cells, these nanoparticles can take advantage of the capacity of these cells to extravasate via diapedesis and reach specific tissues. Although first proof-of-concept for this hitchhike mechanism can be found in the literature, extensive research is needed to test the feasibility of such an approach for improved drug delivery to sites of inflammation.

6.4. Using endothelial cells for polarized secretion

Endothelial cells form a tight, active barrier between blood and underlying tissue. The endothelium is also a major secretory organ, releasing proteins in a polarized fashion in either the circulation or in the vascular matrix. This polarized secretion can be exploited to get protein therapeutics across the vascular barrier [61,62]. By transfecting endothelial cells with mRNA from the apical side using mRNA-loaded lipid NPs and by forcing these endothelial cells to secrete these proteins on the basolateral side with the aid of specific signal peptides, a substantial amount of therapeutic protein could in principle be delivered across the vascular barrier. For this to work, more research is needed to understand the polarized secretion of endothelial cells of different organs in addition to efficient targeting mechanisms to subsets of endothelial cells [63].

6.5. Physical methods for enhanced extravasation

Another strategy to get NM across vascular barriers is by locally weakening the endothelial lining. This could potentially be achieved with pharmacological disruption of the endothelial lining as discussed above or by triggering natural weakening of the barrier via intervention of specific signaling pathways. For example, the second messenger cAMP is involved in keeping the endothelial barrier function intact and strategies to specifically down regulate cAMP, e.g. by locally delivering cAMP degrading enzymes, such as phosphodiesterase 4 might lead to local and transient disruption of this barrier function [64].

Recent unpublished work from our group on local disruption of the blood-retina barrier demonstrated that the application of high intensity focused ultrasound (HIFU) in combination with gas-filled microbubbles can lead to local disruption of this barrier to enable passage of macromolecules and even NM from the blood into the retina. Similarly, HIFU-mediated sonoporation has been applied to temporarily weaken the BBB [53,65]. The transient, localized nature of vascular barrier disruption with HIFU offers opportunities to enhance the extravasation of NM at specific sites in the body, but local damage of the endothelial lining may also lead to activation of the coagulation pathway and therefore should be handled with caution.

In conclusion, future research should not only focus on strategies that allow systemically administered NM to evade MPS clearance but also find ways to enable their efficient and local extravasation, of which several suggestions have been given above.

6.6. The mRNA revolution

Over the past decade we have seen rapid progress in the development of mRNA therapeutics. Chemical engineering of mRNA has led to more stable and less immunogenic mRNAs and with a great push from the COVID-19 pandemic, mRNA-based vaccines have reached the market in a very short timeframe. The flexibility at which mRNA can be adjusted and the relative ease of manufacturing, together with the availability of efficient nanocarrier systems that can deliver these macromolecules into the cytosol of cells make these molecules very suitable for rapid-response interventions. Several different companies have jumped on the bandwagon to develop mRNA-based vaccines against a plethora of infectious diseases, with influenza and HIV as most popular target; and now COVID-19.

But potential applications of mRNA go far beyond vaccines for infectious diseases. Companies like BioNTech and Moderna were primarily focusing on the development of mRNA therapeutics in the immuno-oncology field before COVID-19 arrived. With its intrinsic capacity to induce strong cellular responses, mRNA is ideally suited to induce expression of unique protein antigens and immunostimulatory cytokines in autologous cells, which, via a mechanisms of cross-presentation, can lead to antigen-specific immune activation. Co-expression of pro-inflammatory cytokines or mRNAs encoding checkpoint inhibitors can

be applied to overcome local immunosuppression within the tumor microenvironment [66]. Clinical trials are ongoing (see for example: NCT03739931; NCT04528719;NCT03313778;NCT04163094;NCT03788083) and results are expected to be released soon.

Most of these early studies on cancer mRNA vaccines have focused on shared tumor antigens but mRNA vaccination is also very suitable for personalized cancer vaccines in which a set of unique neoantigens, identified by next generation sequencing on tumor biopsies, can be readily expressed in a short timeframe [67–69]. If we extrapolate the current developments on neoantigen vaccination, it is my prediction that such personalized vaccines will become standard practice for which mRNA is the preferred drug modality over synthetic peptide epitopes because of its ease of manufacturing and formulation and intrinsic capacity to evoke strong cellular immune responses. This, however, requires the development of a robust "universal" delivery system that can be prepared at the bedside, by mixing mRNAs encoding patient-specific poly-neoepitopes with a pre-made, empty nanocarrier prior to administration. Recent insights that LNP-nucleic acid preparations do not necessary require the presence of mRNA during LNP formation, but can be added after empty LNPs have been formed show feasibility of such a mix-and-match approach [70]. Reproducibility as well as GMP-compliant production of such nanoformulations is key and would require full automation and in-process quality checks. Such devices based on microfluidic mixing are currently being developed by several companies.

Other applications of mRNA that will most likely reach the limelight in the near future are in the protein replacement therapy space. This approach would circumvent the problems of complex production and purification processes that are associated with many protein therapeutics and would allow for the *in situ* production of biotherapeutics in a cost-effective manner. Furthermore, it would circumvent the issue of delivering substantial amounts of proteins inside cells and enable direct intracellular or subcellular expression of therapeutic proteins. Several examples of protein-replacement therapies with mRNA exist, most of them are currently at the preclinical stage of development [71–73]. Longevity of expression is often key and may require solutions to prolong the intracellular survival of such therapeutic mRNAs. Self-replication RNAs could be an option, but being from viral origin, these are currently still too immunogenic to be applied for this application [74].

6.7. Gene therapy

Gene therapy applications with synthetic vectors have become a viable and safe alternative to viral vectors. Initially, synthetic vectors suffered from poor delivery efficiencies and short duration of transgene expression, but with the recent developments in improved nanocarrier design and engineering transposable elements these limitations do not longer exist [75]. Furthermore, synthetic vectors have the added benefit of making the delivery of mixed modality therapeutics possible (i.e. combinations of DNA and RNA, DNA and protein or RNA and protein). An example is the development of lipid nanoparticles for the delivery of Cas9 mRNA and sgRNA for the treatment of transthyretin amyloidosis, which is currently in phase I clinical trial [33]. Similarly, lipid nanoparticles have been used for the delivery of tripartite CRISPR-Cas systems, i.e. the Cas nuclease, a sgRNA and a single stranded or double stranded DNA template to mediate HDR-directed repair [76].

In the early days of nonviral delivery of DNA, longevity of transgene expression was an issue as the delivered DNA (mostly pDNA) was not co-replicated during cell division, leading to rapid dilution and loss of transgene expression over time. With the current availability of transposable elements such as Sleeping Beauty, piggyBac, or Tol2 this is no longer an issue and transgenes can be safely integrated into the host cell's genome. Even though integration of such transposable elements is random and may theoretically lead to insertional mutagenesis, such events have so far not been reported in ongoing clinical trials [77]. Furthermore, targeted genomic integration of linear gene constructs into "safe harbor" locations in the genome can be achieved with CRISPR-Cas mediated homology-independent transgene insertion (HITI) [78]. The same targeted integration can be used to place a transgene under the control of a tissue specific promoter [79].

In the coming years I expect to see increasing attention in the use of nonviral vectors for gene therapy applications, in particularly for those applications that require delivery of multiple different components. In my lab the focus will be on direct *in vivo* modification of endothelial and liver cells with the purpose of obtaining prolonged and controllable secretion of therapeutic proteins into the circulation to replace lifelong injections of such biotherapeutics (see below).

6.8. Sustainable dosing

Next to treating or curing genetic diseases, gene therapy can also be applied to obtain *in situ* production of therapeutic proteins. Monoclonal antibody therapeutics such as Remicade, Humira, and Enbrel have proven to be effective for the treatment of multifactorial auto-immune diseases such as rheumatoid arthritis, Crohn's disease or multiple sclerosis but are costly and often require lifelong, frequent injections or infusions. Implantation of genetically modified allogeneic cells expressing the therapeutic mAb or even better, direct genetic modification of the patient's own cells would enable the body to produce its own medicine.

At *Pharmaceutics* the long term goal is to construct such "apothecary" cells that should serve as the body's own medicine cabinet from which specific biological drugs can be released into the blood at will and in a controllable fashion (Fig. 9). For this to become reality several challenges lay ahead. Firstly, synthetic nanocarriers should be developed that can be targeted to specific subsets of cells that have a sufficiently long lifespan to provide enduring expression of the therapeutic protein of choice and are preferably in direct or close contact with the vasculature so that the secreted therapeutic proteins can reach the circulation. Examples of such cells are hepatocytes, liver sinusoidal endothelial cells, vascular endothelial cells, smooth muscle cells and potentially adipocytes. Cell selectivity can be reached by the use of cell-type specific targeting ligands or by screening libraries of synthetic nanocarriers of different composition as has been demonstrated for lipid nanoparticles [32,80,81]. Secondly, delivery should lead to safe and stable integration of the transgene. Targeted integration using a recombinase or CRISPR-Cas seems by far the best option as it will reduce the chances of insertional mutagenesis. Furthermore, with targeted integration, the transgene can be placed under the control of an endogenous, tissue specific promoter thereby further increasing cell type specificity. For example, the albumin locus has been used for this purpose to obtain selective expression of factor IX in hepatocytes [79]. Thirdly, in the case where control over the dose of the therapeutic protein being secreted is required, inducible expression systems should be incorporated. Such pharmacologically-controlled expression systems already exist, but are mostly based on the use of drugs such as rapamycin or tetracyclin to trigger dimerization of engineered transcription factors or the use of potentially immunogenic transactivators [82,83]. For long term use, gene regulation systems that work with more inert substances and consist of engineered transcription factors of human origin are to be preferred. Similarly, riboswitches have been engineered to regulate protein expression at the mRNA level and might provide another level of control over gene expression [84]. Finally, a safety switch should be incorporated that allow control over the fate of genetically modified cells in case unforeseen side effects occur. In recent years, much progress has been made with such safety switches, especially in the field of T cell therapy. A system that is based on a split caspase-9, whose complementation is driven by dimerization of the FKBP12 domains in the presence of the chemical dimerizer rimiducid, has shown promising clinical results [85,86].

The above-mentioned requirements clearly show that on demand-

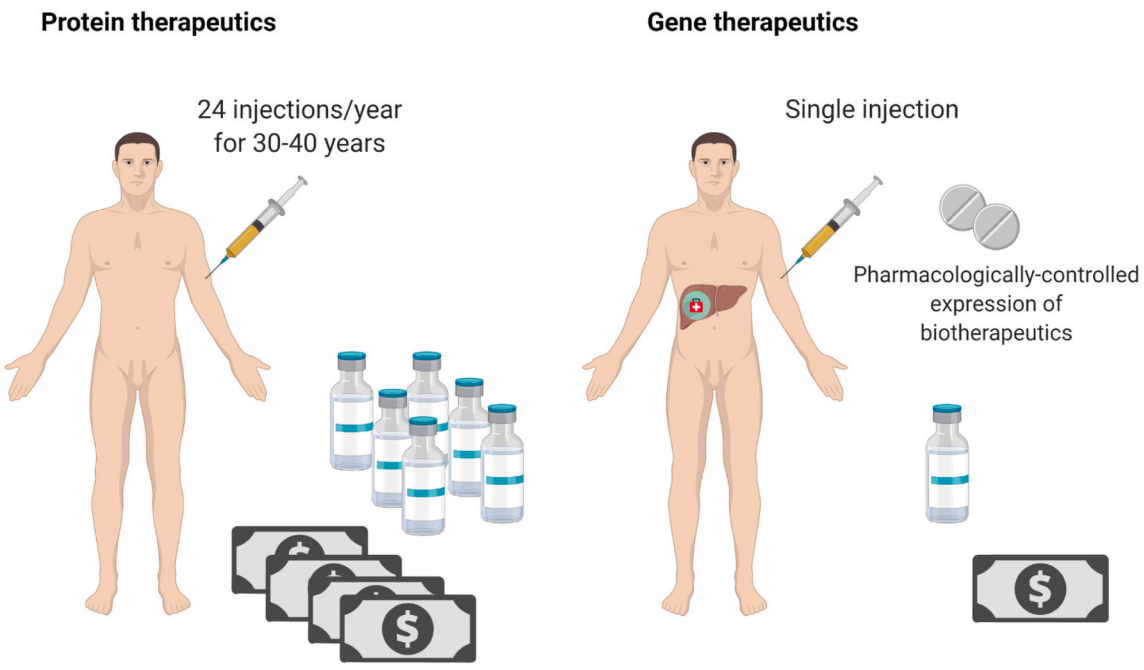

Fig. 9. Gene therapy could in the long run become a sustainable and more economic alternative to lifelong injections with protein therapeutics. By targeted integration of gene constructs that enable the controlled expression and secretion of a recombinant therapeutic protein, such as antibody, enzyme or hormone, the patient's own cells can serve as medicine cabinets to produce a drug at will. Since this intervention is only required once, it is expected to be more patient friendly and economical than the current treatment options.

dosing of therapeutic proteins from *in situ* modified autologous cells has still a long way to go, but at least each individual component needed for this to become reality have been developed. Future research should be directed towards combining these in a most efficient way with of course safety as being one of the highest priorities.

7. Acknowledgments and conclusions

With this overview, I have painted a picture of my group's current and future research. My group is known for its expertise on delivery of biotherapeutics (both proteins and nucleic acids) with applications in gene & cell therapy, vaccination and immunotherapy. With the advent of new gene correction techniques and mRNA as a new class of drugs, there are ample opportunities for developing new medicines for which delivery will be a key aspect. Exciting times and opportunities lie ahead. I therefore feel very privileged to be able to work in this research field at this very time.

My appointment as professor would not have been possible without the help of a large number of people to whom I owe a great deal. First of all, I would like to thank my two supervisors, Prof. Daan Crommelin and Prof. Gert Storm for their trust in me over the years. Without their mentorship my scientific career would have been different. I also would like to thank prof. Wim Hennink for his leadership and guiding the *Pharmaceutics* groups successfully for such a long time. But most importantly, I would like to thank all my talented PhD students and postdocs with whom I had the privilege to work over the past 15 years. I would in particularly like to thank Bo Lou, Leena Kontturi, Erik Oude Blenke, Roderick van den Berg, Charis Rousou, Olivier de Jong, Mert Öktem, Danny Wilbie and Johanna Walther for providing some examples of their research for this report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.06.029.

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Enrico Mastrobattista studied medical biology at Utrecht University, The Netherlands from 1991 to 1996 and obtained his PhD degree at the same university in 2001 on a thesis entitled "Targeted liposomes for cytosolic drug delivery to tumor cells" under the supervision of Prof. Daan Crommelin and Prof. Gert Storm. At that time, drug delivery was primarily focused on small molecule drugs, but with the increasing number of biotherapeutics reaching the market, there was a clear need for dedicated delivery systems for proteins and nucleic acids. Simultaneously, protein engineering was being applied to improve the therapeutic activity of various protein drugs, in particularly monoclonal antibodies and therapeutic enzymes. Dr. Mastrobattista understood the importance of such protein engineering techniques and their potential applications to improve the delivery aspects of protein therapeutics and he therefore applied for a Marie Curie postdoctoral fellowship and was accepted in the lab of Dan Tawfik and Andrew Griffiths at the Centre for Protein Engineering at the MRC Laboratory of Molecular Biology, Cambridge, UK. Being a novice in protein engineering and with only basic molecular biology skills at hand, he experienced a steep learning curve amid nobel laureates and other top scientists in the field of molecular biology. It was the perfect atmosphere to fully dedicate to science in a wonderful setting of the city of Cambridge of which he has fond memories. He worked on a cell-free directed evolution platform based on liposomes and double emulsions for the selection of genetic libraries encoding enzyme variants. This technology would eventually form the basis of digital PCR. With this newly gained knowledge on directed evolution techniques, dr. Mastrobattista was invited to return to the *Pharmaceutics* group in Utrecht in 2004 to setup his own research group combining molecular biology and protein engineering techniques with drug delivery. His research group steadily expanded with diverse projects on protein and nucleic acid delivery using nanoparticulate delivery systems. In 2012 he was promoted to associate professor and was heading a large international consortium (https://www.imi.europa.eu/projects-results/project-factsheets/compact) sponsored by the European Innovative Medicine Initiative in which academic groups, biotech companies and pharmaceutical industry joined forces to collectively work on the delivery issues associated with many biotherapeutics. This is where dr. Mastrobattista experienced the strength of cross-border and cross-institutional collaboration. In 2013 he was awarded the prestigious Dutch Prix Galien for his contribution to advancing the pharmaceutics research in the Netherlands. Since 2019 he is heading the Pharmaceutics division at Utrecht University (www.uu.nl/pharmaceutics) with a great team of international scientists with an open mindset and collective aim to make advanced delivery systems to improve medicines for future generations. An overview of PhD theses written under his guidance with links to the full text online versions can be found in the supplementary information.