Achieving successful delivery of oligonucleotides — From physico-chemical characterization to in vivo evaluation

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Abstract

RNA interference is one of the most promising fields in modern medicine to treat several diseases, ranging from cancer to cardiac diseases, passing through viral infections and metabolic pathologies. Since the discovery of the potential therapeutic properties of non-self oligonucleotides, it was clear that it is important to develop delivery systems that are able to increase plasma stability and bestow membrane-crossing abilities to the oligonucleotides in order to reach their cytoplasmic targets. Polymer therapeutics, among other systems, are widely investigated as delivery systems for therapeutic agents, such as oligonucleotides. Physico-chemical characterization of the supramolecular polyplexes obtained upon charge interaction or covalent conjugation between the polymeric carrier and the oligonucleotides is critical. Appropriate characterization is fundamental in order to predict and understand the in vivo silencing efficacy and to avoid undesired side effects and toxicity profile. Shedding light on the physico-chemical and in vitro requirements of a polyplex leads to an efficient in vivo delivery system for RNAi therapeutics. In this review, we will present the most common techniques for characterization of obtained polymer/oligonucleotide polyplexes and conjugates which limit the clinical translation of this promising technology.

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1. Introduction

RNA interference (RNAi) is the term coined in 1998 by Fire and Mello to describe the phenomenon that occurs in Caenorhabditis elegans when double-stranded RNA (dsRNA) introduced into cells binds to endogenous messenger RNA (mRNA) transcript, to manipulate the expression of an endogenous gene (Fire et al., 1998). RNAi was demonstrated to happen also in mammalian cells (Elbashir et al., 2001) and was soon exploited to treat several diseases in animals (Lakka et al., 2004; Omoto et al., 2004; Song et al., 2003; Soutschek et al., 2004). These findings led in 2004 to the first RNAi therapeutic (vascular endothelial growth factor small interfering RNA (VEGF siRNA)) to enter clinical trials for the treatment of macular degeneration (NCT00722384) (Garba and Mousa, 2010).

Two types of endogenous double-stranded RNA molecules are responsible of RNAi, defined as small interfering RNA (siRNA) or microRNA (miRNA). siRNAs are obtained when dsRNA is processed in the cytoplasm by the endonuclease enzyme Dicer that catalyzes the

![Fig. 1. RNAi by miRNA and siRNA. Cytoplasmic long double-stranded RNAs are cleaved to siRNA by Dicer. Pre-miRNAs are transported to the cytoplasm and cleaved by Dicer into mature miRNAs. Both the mature miRNAs and the siRNAs are incorporated into the RISC complex, that eliminates the sense strand and induces Watson-Crick base pairing (full or partial) with target mRNA, consequented by gene silencing either by mRNA degradation or by repression of the translation.](image-url)
formation of 21–23 base-pair fragments (Hannon, 2002). At this point, the siRNA is introduced in the RNA induced silencing complex (RISC), a multiprotein complex that degrades the passenger (or sense) strand and catalyzes the binding of the guide (or antisense) strand included in the RISC complex to the target mRNA. The siRNA antisense strand is perfectly complementary to the mRNA target sequence, and following the binding, the mRNA is cleaved and degraded by cellular nucleases, silencing the target gene expression (Fig. 1).

miRNAs are obtained by a similar mechanism from hairpin microRNA precursors (pre-miRNA) that are substrate for the Dicer to form miRNA duplex of about 22 nucleotides in length. The miRNA is then incorporated in the RISC complex (called miRISC) where the guide sense is maintained and binds to the mRNA. One miRNA sequence can bind hundreds of miRNA target sequences, in some cases with a perfect match, inducing degradation of the miRNA, or as an imprecise match, leading to inhibition of the translation of the mRNA (Fig. 1).

RNAi (siRNA or miRNA) expression and functionality are significantly impaired in several diseases, and therefore, the RNAi pathway is an attractive target for novel therapies.

2. Pathologies treated with RNAi

2.1. Cancer

RNAi mediated mechanisms are involved in tumorigenesis and tumor progression. Therefore, siRNA and miRNA have emerged as promising strategies for anticancer therapy.

VEGF is a key mediator of angiogenesis and it is a well-known target for anti-angiogenic treatment (Ferrara, 2005). RNAi technology has already been tested in Phase I clinical trials as anti-VEGF treatment in solid cancers with lipidic nanoparticles (ALN-VSP02) loaded with siRNA targeting VEGF and kinesin spindle protein (KSP) (Tabernero et al., 2013) (NCT00882180 and NCT01158079). In addition, several formulations loaded with VEGF siRNA have been developed in preclinical studies (Chen et al., 2014), miRNAs are also involved in cancer angiogenesis (Maroo et al., 2014), and therefore, several miRNA-bearing formulations have been proposed as anti-angiogenic therapy (Dai and Tan, 2015; Liu et al., 2013).

Tumor angiogenesis is not the only mechanism treated by RNAi. Several other cancer-related targets have been identified, and siRNA and miRNA therapies have been developed. Polo-like kinase 1 (PLK1), known to be overexpressed in several types of cancer, plays a role in cell cycle progression (Liu and Erikson, 2003). PLK1 siRNA is known to be overexpressed in several types of cancer, plays a role in cell cycle progression (Liu and Erikson, 2003). PLK1 siRNA is

2.2. Metabolic diseases

RNAi can be exploited for the treatment of type 1 diabetes, targeting the pancreas-associated immune cells to downregulate the expression of key proteins. Although lipid-based carriers seem to be the preferential delivery systems to the pancreas and liver, polymeric systems can also deliver RNAi efficiently to these organs when targeted (Leconet et al., 2012). Chitosan-based delivery systems seem promising also in the treatment of type 2 diabetes (Jean et al., 2012). In addition, RNAi can offer a therapeutic option for diabetes-related ulcers. Matrix metalloproteinase (MMP) is overexpressed in diabetic ulcers, and MMP-2 siRNA complexed with linear polyethyleneimine (PEI) can significantly decrease the level of MMP-2, increasing the recovery rates of diabetic ulcers (Kim and Yoo, 2013).

2.3. Cardiovascular diseases

Many cardiovascular diseases have been correlated to miRNA and siRNA dysregulation in cardiomyocytes and in smooth muscle cells. Targeting miRNA involved in RNAi mechanisms has been selected in several cardiopathies, such as cardiac hypertrophy, cardiomyocytes apoptosis, hypertension, coronary diseases and myocarditis (Kwekkeboom et al., 2014; Tang et al., 2007). Although the cardiac tissue is very challenging for the delivery of oligonucleotides due to the unfavorable biodistribution of macromolecules in the heart (Kwekkeboom et al., 2014), interesting results have been obtained with local injection of formulated oligonucleotides in the myocardium (Kim et al., 2013; Somasuntharam et al., 2013). In addition, other cardiovascular diseases can be treated with RNAi therapy. For example, atherosclerosis is characterized by inflammatory responses to accumulation of oxidized low-density lipoproteins (LDLs) that leads to plaque formation in the arterial walls (Gu et al., 2014). It is possible to exploit siRNA therapy to reduce inflammatory response to oxidized LDL (Gu et al., 2014) or to reduce LDL formation in the liver targeting hepatic proteins involved in their formation, such as apoliprotein B (Tachibana et al., 2014).

2.4. Neurodegenerative diseases

miRNA and siRNA have been investigated as therapeutic agents for several diseases of the central nervous system (CNS), including Alzheimer (Orclicchio et al., 2007), schizophrenia (Feng et al., 2009) and cerebral ischemia (An et al., 2013; Maes et al., 2009). Although the blood brain barrier (BBB) poses a great hurdle to the delivery of oligonucleotides to the brain, intranasal delivery of polyplexes often guarantees brain accumulation (Kanazawa et al., 2013).

3. Delivery of siRNA/miRNA

The clinical use of siRNA and miRNA is limited by several factors:

• They are highly unstable in biological fluids: endo and exonucleases promptly degrade them, resulting in a short half-life and fast kidney absorption.

• The delivery systems used for siRNA and miRNA are not efficient in delivering the molecules to the target cells and tissues.

• The stability of siRNA and miRNA in circulation is limited due to the presence of nucleases.

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3.1. Polymeric delivery systems

Several polymeric carries have been developed for the delivery of oligonucleotides. A systematic and comprehensive presentation of the polymers employed for gene delivery is beyond the scope of this review and can be found elsewhere (Hobel and Aigner, 2013; Lee and Kim, 2014; Tiram et al., 2014; Wu et al., 2012). We hereby briefly present the most common polymeric systems used with focus on the ones investigated in vivo. Table 1 summarizes the chemical structures of the polymeric backbones.

3.1.1. Dendrimers

The term dendrimer derives from the Greek word “dendron”, tree, and the name describes the branched architecture of the structures. Classical dendrimers are typically monodispersed, spherical and symmetric polymers. In virtue of the ease of chemistry, dendrimers have been widely investigated in drug delivery (Gajbhiye et al., 2009). Polyaminated dendrimers guarantee ionic interaction with oligonucleotides, resulting in efficient in vivo transfection agents (Ofek et al., 2010; Watanabe et al., 2009; Yu et al., 2012).

3.1.2. Polyethyleneimine (PEI)

Formed by repeated units of two carbons spacer and one amino group, either linear or branched, PEI was one of the first transfecting agents discovered (Boussif et al., 1995). Despite being an efficient transfecting agent, PEI suffers from high cytotoxicity. Therefore, PEI is often used in combination with other polymeric backbones that bestow lower toxicity to the delivery system (Dahlman et al., 2014; Kaestner et al., 2011).

3.1.3. Chitosan

This natural linear cationic polysaccharide has been widely investigated as carrier for gene delivery due to its low immunogenicity. Chitosan in vivo transfection efficacy depends on several parameters such as molecular weight, degree of deacetylation (DDA) of primary amines along the chitosan chain, method of oligonucleotide association (Tiram et al., 2014). Therefore, it is often used in combination with other transfecting agents to improve its transfecting efficacy (Howard et al., 2009; Ghosn et al., 2010).

3.1.4. Poly(ethylene glycol) (PEG)

PEG is a synthetic neutral polyether compound widely known in the polymer therapeutics field for its biocompatibility, solubility in water and ability to reduce RES recognition. Since it is not positively charged, it is mainly used in combination with other polymers, to reduce recognition by immune system and prolong plasma half-life (Amoozgar and Yeo, 2012). PEG is the only polymer that has yielded a product, presently on the market as Macugen®, that delivers an oligonucleotide, although in this case the oligonucleotide is an aptamer and not a double stranded siRNA (Gragoudas et al., 2004).

3.1.5. Poly(amine acid)

Polymers obtained by repeating units of positively charged amino acids, in particular poly(lysine) (PLL) and poly(arginine) (PLR), are able to deliver nucleic acid into cancer cells (Emi et al., 1997; Wu and Wu, 1988). Due to poor endosomal escape abilities (Varkouhi et al., 2011), they are often used in combination with other polymers to improve their pharmacological properties (Wu et al., 2012).

3.1.6. Poly(acrylates)

Several synthetic copolymers based on acrylates have been developed. The most studied and characterized polymer for drug delivery is N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. HPMA copolymer is a non-immunogenic, non-toxic, water-soluble polymer (Kopecek and Kopeckova, 2010) used for the delivery of oligonucleotides. In order to create an electrostatic complex with the negatively charged oligonucleotides, the neutral HPMA copolymer, it is often used in combination with other cationic polymers (Johnson et al., 2011), or with cationic methacrylates (de Wolf et al., 2008; Lundy et al., 2013; Qin et al., 2011). Alternatively, HPMA copolymer is directly conjugated to the oligonucleotide (York et al., 2010).

3.2. Targeting agents

Besides improving the stability of RNAi and their in vivo silencing properties, the polymeric carriers can bestow targeting abilities to the formulations. Polymeric formulations have the tendency to
accumulate in the liver (Garnett and Kalinleri, 2006; Markovsky et al., 2012), and this feature can be exploited to deliver RNAi to hepatic pathologies (see Section 5.1 below) (Rozema et al., 2007; Watanabe et al., 2009). In addition, nano-sized materials can accumulate in cancer tissues exploiting the enhanced permeability and retention (EPR) effect (Maeda, 2010; Matsumura and Maeda, 1986), making polymer therapeutics effective anticancer drugs and RNAi (Prabhu et al., 2015; Tiram et al., 2014). Nevertheless, the conjugation of targeting ligands to nanocarriers can not only improve the selectivity of the system for the target tissue, but also promote uptake of the genetic material (Yameen et al., 2014). One of the commonly used targeting agents for polymer therapeutics is folic acid (Kim and Song, 2014; T.S. Li et al., 2014; York et al., 2010) to target the folate receptor overexpressed on many cancer cells (Hilgenbrink and Low, 2005). Another well characterized target is the transferrin receptor, expressed in many physiological tissues (including brain) and overexpressed on many cancer cells (Tortorella and Karagiannis, 2014). Therefore, transferrin has been conjugated to several polymer systems to provide receptor-mediated targeting (Davis et al., 2010; Yhee et al., 2013). Integrins, in particular ανβ3 receptor, are also a target for vascular and cancer therapy (Liu et al., 2014; Oe et al., 2014; Wu et al., 2013). Other targeting agents are N-acetyl-galactosamine (NAG), a ligand for hepatocyte targeting (Rozema et al., 2007), and the RVG peptide for brain targeting (Kim et al., 2010).

4. RNAi delivery systems characterization

Even though the RNAi delivery field has been researched for around a decade, characterization of the supramolecular structures formed by oligonucleotides and delivery vehicle remained a fundamental challenge. A deep understanding of the physico-chemical properties of the polyplexes and their relation with the biological activity allow for a most successful realization of a highly efficient delivery system for oligonucleotides. Common characterization methods are taken from diverse disciplines of nanotechnology, polymer science and biochemistry; several of them are based upon the more established area of plasmid DNA delivery research. Characterization can be divided into two basic approaches: the physico-chemical investigation of shape, size and charge, and the biological properties such as silencing activity and intra-cellular trafficking. We will hereby describe the common methods and the challenges they face (Table 2).

4.1. Size and shape analyses

4.1.1. Microscopy techniques

Gene delivery systems are mostly nanometric in size (Tiram et al., 2014), therefore their imaging demands appropriate resolution microscopy techniques such as atomic force microscopy (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Fig. 2, Table 2).

4.1.1.1. Atomic force microscopy (AFM). Images are taken by measuring the strength of the interactions between a surface and a sharp tip. While maintaining the force constant, the tip follows the surface, moves in three dimensions and obtains a topographic image of the specimen (Binnig et al., 1986). These spatial images make AFM the method of choice to demonstrate polyplex and oligonucleotide morphological changes, such as oligonucleotide condensation along with z-axis elongation upon binding with cationic polymers (Ofek et al., 2010) or the condensation of polyplexes with the addition of a targeting moiety (Patil et al., 2009).

4.1.1.2. Scanning electron microscope (SEM). The sample is being bombarded by primary electron beam that collides with the specimen. Backscattered electrons of the primary beam and secondary electrons that are emitted from the sample can each be collected and manipulated to give a topographic image. The high vacuum required within the sample's chamber raises the need to dehydrate, freeze-dry or fixate biological samples. In addition, insulators should be coated with a metal layer (Donald, 2003). SEM has been widely utilized to characterize the shape of oligonucleotide–polymer complexes, such as sphere (Kim et al., 2012), porous (Lee et al., 2012), rod-shaped (Hasan et al., 2012) and others (Patil et al., 2012).

4.1.1.3. Transmission electron microscopy (TEM). Image is taken by radiating a thin specimen with an electron beam that is sourced from an electron gun and focused by electromagnetic lenses. The transmitted electrons are projected on either a viewing screen or an electronic image device such as charge-coupled device (CCD) camera (Kuntsche et al., 2011). Few sample preparation methods are in use for the imaging of gene delivery systems: The simplest is negative staining, which images a dried-droplet stained with heavy-metal salt (Kuntsche et al., 2011). Based on this technique, some it was possible to visualize the morphology of polyplexes (Hasan et al., 2012; Sizovs et al., 2013; Y. Li et al., 2014) or the loading of hydrogel with siRNA (Patil et al., 2012).

The major drawback in using samples that were prepared by the dry techniques mentioned above is the altered shape resulting from either the strong interactions between the sample and the substrate, crystalization processes, spreading of the droplet, the need for metal coating, piling of salts or the effect of different tips’ geometries (in AFM) (Patil et al., 2009; Sitterberg et al., 2010; Zia et al., 2010). Much more reliable image can be obtained by fluid techniques such as “in situ AFM” and cryo-TEM. Zuckerman et al. have imaged the formation of 60–100 nm polyplexes using cryo-TEM (Zuckerman et al., 2012). In situ AFM has previously allowed the real-time monitoring of various oligonucleotide–vehicle interactions such as complexation or release kinetics (Martin et al., 2000; Wan et al., 2009). In the area of RNAi delivery, Shim et al. have recently studied the release kinetics of siRNA from ketalized linear and branched PEI in conditions that mimic the different cellular compartments (Shim et al., 2010; Zuckerman et al., 2012).

4.1.2. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) measures the hydrodynamic radius of a particle in solution, based upon light scattering of particles moving in Brownian motion, displaying a major advantage compared with the microscopy techniques of sampling a population of particles and converting the measurement into a mean hydrodynamic radius and size distribution (polydispersity). Although DLS is extensively used in RNAi delivery research in order to evaluate the hydrodynamic size and distribution of polyplexes (Lee et al., 2012; Malhotra et al., 2012; Nuhn et al., 2014; Ofek et al., 2010), such a measurement is not always feasible, due to the low tolerability of the DLS technique to heterogeneous solutions (Troiber et al., 2013). RNAi delivery systems face another challenge to the researcher: the formation of a homogenous population. Spontaneous self-assembled complexes of RNAi and delivery vehicle tend to aggregate (Millili et al., 2010; Pozharshki and MacDonald, 2007), and this tendency might increase with the increase of ionic strength of the solution or with the increase of concentration of RNAi/vehicle (Bertin, 2014). The quality of DLS measurement should not be compromised though, even when successful in vitro or in vivo silencing is achieved, since large aggregates will lead to in vivo toxicity. Moreover, high heterogeneity might point out that not all the particles contain RNAi, although they still contribute to the general toxicity (Bertin, 2014).

Since the microscopy techniques will better suit the characterization of a heterogeneous solution (Troiber et al., 2013), a complete size characterization including DLS and at least one microscopy technique will shed light on the nature of the investigated solution including features of size, shape, polydispersity and homogeneity. A well-defined, homogenous population of 10–150 nm particles will probably reflect in prolonged blood circulation with selective tumor accumulation due to the EPR effect (Markovsky et al., 2012).
Table 1
Chemical structures of polymeric backbones.

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Biodegradability</th>
<th>Architecture</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural polymers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>✔</td>
<td>Linear multivalent</td>
<td>Howard et al. (2009), Ghosn et al. (2010), Jean et al. (2012), Han et al. (2013), Plianwong et al. (2013)</td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td>✔</td>
<td>Cyclic</td>
<td>Davis et al. (2010), Zuckerman et al. (2014)</td>
</tr>
<tr>
<td>Synthetic polymers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLL</td>
<td>✔</td>
<td>Linear multivalent</td>
<td>Watanabe et al. (2009), Plianwong et al. (2013), Kim et al. (2010)</td>
</tr>
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<td>PLR</td>
<td>✔</td>
<td>Linear multivalent</td>
<td>Dahlman et al. (2014), Leconet et al. (2012), Tesz et al. (2011), Merkel et al. (2009), Kaestner et al. (2011), Shim et al. (2010), PEI coatings Lee et al. (2012), Merkel et al. (2009)</td>
</tr>
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<td>PEI</td>
<td>✘</td>
<td>Linear or Branched</td>
<td>Waite et al. (2009), Patil et al. (2009), Watanabe et al. (2009), Ofek et al. (2010), Zhou et al. (2011), Yu et al. (2012), Conti et al. (2014)</td>
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<tr>
<td>PG-amine</td>
<td>✔</td>
<td>Dendritic or Hyperrbranched or Star-like</td>
<td>Gragoudas et al. (2004), Dahlman et al. (2014), Davis et al. (2010), Rozema et al. (2007), Zuckerman et al. (2014), Merkel et al. (2009), Ghosn et al. (2010), Merkel et al. (2009), Wooddell et al. (2013), Polach et al. (2012), Davis et al. (2010), Zuckerman et al. (2014)</td>
</tr>
<tr>
<td>PAMAM</td>
<td>✔</td>
<td>Dendritic or Hyperrbranched or Star-like</td>
<td>Gragoudas et al. (2004), Dahlman et al. (2014), Davis et al. (2010), Rozema et al. (2007), Zuckerman et al. (2014), Merkel et al. (2009), Ghosn et al. (2010), Merkel et al. (2009), Wooddell et al. (2013), Polach et al. (2012), Davis et al. (2010), Zuckerman et al. (2014)</td>
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<tr>
<td>PEG</td>
<td>✘</td>
<td>Linear</td>
<td>Hasan et al. (2012)</td>
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<tr>
<td>PLGA</td>
<td>✔</td>
<td>Linear</td>
<td>Hasan et al. (2012)</td>
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<td>HPMA copolymer</td>
<td>✘</td>
<td>Linear multivalent</td>
<td>de Wolf et al. (2008), Johnson et al. (2011), Qin et al. (2011), Lundy et al. (2013), York et al. (2010)</td>
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</table>
4.2. Electrostatic properties: charge and affinity

4.2.1. Zeta potential

Zeta potential is the potential between a particle and its surrounding solution, measured at the boundary of the particle's hydrodynamic radius. The zeta potential is directly related to the surface charge of a particle. The surface charge of RNAi delivery systems has great importance to the success of a systemic delivery, its stability, the interaction with the negatively charged cell membrane proteins, and the subsequent release of its content to the cytoplasm (Boussif et al., 1995; Yezhelyev et al., 2008). On the other hand, excess charge will cause aggregation with blood components and will activate the immune system (Lee et al., 2008). An overall mild positive charge raises another difficulty of reduced solution stability and aggregation inclination (Honary and Zahir, 2013). Such a low positive charge of heterogeneous solution will not necessarily reflect the surface charge of the RNAi-loaded particles, hence will not guarantee their cellular internalization (Bertin, 2014).

4.2.2. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay, also known as gel shift or gel retardation assay, was originally developed for the evaluation of protein–DNA binding affinity (Fried and Crothers, 1981). In the context of RNAi delivery, it is a common method to illustrate the formation of polyplexes' characterization. The common use of the zeta potential analysis is to show alterations in charge corresponding to the polyplex contents, such as N/P ratio (the ratio between nitrogen atoms of the cationic polymers and the negative phosphate atoms of the oligonucleotides) (Ge et al., 2014), increased ratio of lipid/polymer (Hasan et al., 2012), PEI coatings (Lee et al., 2012), or others (Ofek et al., 2010; Waite et al., 2009). The charge requirements from RNAi delivery systems are complex, sometimes even contradicted: on the one hand, dense positive charge is crucial to the interactions with the negatively charged membrane proteins that further assist cellular internalization via endocytosis (Xiang et al., 2012). In addition, polymers bearing multiple amine groups (characterized by positive zeta potential) were demonstrated before to function as proton sponges: by absorbing protons inside acidic organelles, the osmotic pressure raises, what leads to rupture of the compartment's membrane and the subsequent release of its content to the cytoplasm (Boussif et al., 1995; Yezhelyev et al., 2008). On the other hand, excess charge will cause aggregation with blood components and will activate the immune system (Lee et al., 2008). An overall mild positive charge raises another difficulty of reduced solution stability and aggregation inclination (Honary and Zahir, 2013). Such a low positive charge of heterogeneous solution will not necessarily reflect the surface charge of the RNAi-loaded particles, hence will not guarantee their cellular internalization (Bertin, 2014).

Table 2

Analytical techniques and characterization methods.

<table>
<thead>
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<th>Characterization aspect</th>
<th>Method</th>
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<td>AFM</td>
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<td></td>
<td>DLS</td>
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<td>Charge and affinity</td>
<td>Zeta potential</td>
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<td>Ethidium bromide exclusion</td>
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<td>Cellular uptake</td>
<td>Lysate’s fluorescence</td>
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shape and charge (Fried, 1989). The extent of polymer-induced migration inhibition compared to naked RNAi is visualized by ethidium bromide under UV light (Chang Kang and Bae, 2011; Geoghegan et al., 2012; Ofek et al., 2010).

The appearance of several bands at the same N/P ratio might indicate either the presence of free RNA, heterogeneous population of complexes (Fried, 1989) or the degradation of the complex that is induced by the electrophoresis conditions (Ohsaki et al., 2002).

4.2.3. Ethidium bromide exclusion assay

Intercalation of some dyes (such as ethidium bromide or PicoGreen) between oligonucleotide base pairs is accompanied by emitted fluorescence. Cationic polymers or lipids condense DNA in electrical attraction-dependent manner resulting in exclusion of ethidium bromide from its binding sites within the oligonucleotides. This exclusion leads to reduction in fluorescence which is correlated to the affinity of complexation (Geall and Blagbrough, 2000). The basic method includes titration of cationic polymer or lipid particle solution into oligonucleotide solution supplemented with ethidium bromide. Fluorescence of the oligonucleotide solution is measured by spectrophotometer before, during and after the titration. Fluorescence is then plotted against N/P ratio (Waite et al., 2009). Alternatively, samples of various N/P ratios can be loaded on acrylamide/agarose gel to form a combined EMSA and ethidium bromide exclusion figure, which presents both fluorescence intensity as well as migration retardation (Patil et al., 2009). The importance of delivery vehicle:RNAi binding affinity was demonstrated by Han et al., that developed a series of galactose modified trimethyl chitosan-cysteine (GTC) polyplexes with increased affinity to siRNA, although maintaining equal sizes and zeta potentials. Weak siRNA affinity resulted in reduced stability towards serum, while much stronger affinity has inhibited the release of the siRNA in the cytoplasm. The selection of the proper ratio between the vehicle and the RNAi, reflecting in an adequate affinity between the two components, led to efficient in vitro silencing activity and in vivo tumor shrinkage (Han et al., 2013).

4.3. Cellular uptake

Positively-charged polyplexes can internalize into cells via endocytosis or non-endocytotic pathways (Xiang et al., 2012). Once inside the cells, the RNAi should reach the cytoplasm which is their site of activity (J. Wang et al., 2010). Characterizing cellular uptake and intracellular trafficking of the RNAi polyplexes is, therefore, important in order to study their activity outcomes. One approach to characterize internalization is based upon fluorescence of cell-lystes. The cells are first transfected by polyplexes containing RNAi conjugated to fluorescent probe. Fluorescence of the lysates at the relevant wavelength is read and normalized to the protein content (Mo et al., 2012). Another method is based on flow cytometry analysis of cells that were previously transfected with polyplexes containing intercalated fluorescent probe-RNAi (Chang Kang and Bae, 2011). In order to follow the membrane crossing as well as the intracellular trafficking, confocal microscopy is usually the method of choice. Polyplexes based on fluorescent-probe conjugated RNAi combined with actin filaments and nucleus stains can demonstrate intracellular localization of RNAi (Ofek et al., 2010). Fluorescent probe labeling of both RNAi and polymer assists the real-time evaluation of polyplexes intracellular trafficking (Sizovs et al., 2013), or the colocalization of dendrimer–RNAi polyplexes during their internalization pathway (Patil et al., 2009). Confocal microscopy feature of collecting light from a thin focal plane, combined with the ability to scan over sequential focal planes to form a Z-stack, assists locating the RNAi intracellularly rather than adsorbed to the membrane or outside the cells (Hasan et al., 2012; Kang et al., 2013). Intracellular trafficking of polyplexes by the vesicular pathway can be visualized by staining the endosome and lysosome acidic compartments and measuring the extent of RNAi colocalized with them (Kim et al., 2012). Alternatively, specific markers for early endosomes and lysosomes such as anti-EEA1 and LAMP1, respectively, can be used to track a complete intracellular pathway (Gilleron et al., 2013). In addition, some interesting work was done to track intracellular pathways of polyplexes and lipoplexes utilizing TEM (Alhaddad et al., 2012; Gilleron et al., 2013).

4.4. In vitro silencing activity

Each delivery system is measured first by its ability to specifically silence a gene of choice. In general, the extent of silencing is measured relatively to non-treated cells (control) while specificity is ensured by using non-targeting RNAi as negative control. Commercial transfection reagent such as Lipofectamine® 2000 can be used as a potent silencing efficiency positive control (Hasan et al., 2012). Analysis of the extent of silencing can be done by using RNAi against a reporter gene such as luciferase (Chang Kang and Bae, 2011) or EGFp (Mo et al., 2012). Culture cells can either stably express the reporter gene (Mo et al., 2012) or be transfected first with a plasmid containing the reporter gene and then with the polyplex containing the relevant RNAi (Chang Kang and Bae, 2011). In any of these methods, the expression of reporter gene should be also normalized to number of living cells, measured by any protein content or viability assay (Chang Kang and Bae, 2011). Alternatively, commercial kits of dual luciferase reporter assay have the benefits of two reporter genes on the same plasmid: one that is subjected to the specific RNAi regulation tested at the experiment, and the other that is expressed constitutively and used for normalization (Kim et al., 2012). Other methods to evaluate the RNAi’s silencing activity include the analysis of mRNA products such as relative quantity of the relevant RNA by RT-PCR (Patil et al., 2009) or the expression of the encoded protein as measured by western blot (WB) (Hasan et al., 2012). Most researchers will consider more than 50% silencing to be a successful silencing activity (Alabi et al., 2013).

4.4.1. Stability

The stability of the oligonucleotide–polymer complex is important from a few aspects: (i) increased stability against the blood’s nucleases is one of the core requirements from an RNAi delivery system (Tiram et al., 2014) and (ii) storage and route of administration conditions are important from a pharmaceutical and research point of view. In order to achieve controlled release, the conditions of complex degradation should be fully realized. The basic stability assay includes incubation of polyplex solution in the examined surroundings, such as buffer (Hasan et al., 2012; Mo et al., 2012), RNase (Plianwong et al., 2013) or serum (Biswa et al., 2013), followed by electrophoretic migration. Deterioration of the complex is indicated by rapid migration of the released siRNA. To follow the release kinetics, the remaining RNAi can be extracted from the polyplexes after the incubation and evaluated separately on gel (Hasan et al., 2012). Few other methods were suggested to evaluate the integrity of the polyplexes, such as, time course DLS (Hasan et al., 2012), or TEM images of polyplexes before and after lyophilization (Sizovs et al., 2013). Generally, particles are considered stable if they maintain RNAi encapsulation for up to 24 h in the evaluated media (Biswa et al., 2013; Hasan et al., 2012).

5. From physico-chemical characterization to in vivo successful delivery

The overall aim of a delivery system for oligonucleotide is to enable safe and efficient RNAi activity in vivo. Polymer-based carriers have been successfully used to deliver oligonucleotides to different compartments of the body, both via systemic and local routes of administration (see Table 3). High molecular weight polymeric systems possess several features that make them good candidates for
oligonucleotide delivery. They can undergo size-based non-selective accumulation through the EPR effect in cancer tissues (Maeda, 2013), as well as preferential accumulation in the liver if required (Markovsky et al., 2012). They can be chemically modified to introduce properties that facilitate local delivery of oligonucleotides in particular tissues.

Here we attempt to define the structure-activity relationship of the polyplexes dissecting all the different components (polymeric nanocarriers, characterization techniques, targeted tissue and targeting agents) (Fig 2).

5.1. Systemic administration — hepatic delivery

Amino acid–based dendrimers for extravasation dependent delivery of siRNA to the liver were used for the treatment of hypercholesterolemia in statin-non-responders or in patients with homozygous familial disease (Watanabe et al., 2009). Good tolerability was seen upon intravenous injection of 2.5 mg/kg of unmodified siApoBII complexed with poly(l-lysine) dendritic vehicle together with 50% reduction in ApoB mRNA levels as compared with siLucerase incorporated in the same system. Decrease in blood levels of low density lipoproteins was measured in Apo-E-deficient mice treated with a single dose of 50 μg ApoB, human and murine RNAi.

A promising system targeted to the liver was developed by Rozema et al. The polymer–based system called Dynamic PolyConjugates (DPC) is functionalized with N-acetyl-galactosamine (NAG) ligand for hepatocyte targeting and linked to siRNA with a disulfide bond for reductive release (Rozema et al., 2007). The basis of the system is an endosomolytic backbone, amphipathic poly(vinyl ether), that is reversibly masked by PEG and the targeting moiety, but once in the endosome, goes through selective activation at the acidic environment, to release its cargo to the cytoplasm. Single intravenous injection of DPC carrying ApoB siRNA at a dose of 2.5 mg/kg reduced mRNA levels in hepatocytes by 80–90% maintaining the effect for more than 7 days. No liver toxicity was observed on the basis of serum liver enzyme analyses, cytokine assay or liver histology. While at the same time, fatty liver phenotype was discovered by hepatic section staining, probably due to accumulation of untransported triglycerides resulting from ApoB depletion (Chen et al., 2000). PET CT screening with radioactively-labeled siRNA in the DPC showed that 70% of the injected dose accumulated in the liver 1 h post-intravenous injection. However, radiolabeled-DPC not conjugated with siRNA showed that 98% of the injected dose accumulated in the liver. In addition, 10% of siRNA-labeled DPC accumulated in the bladder, but this was not the case for backbone-labeled DPC. These different distribution patterns may indicate premature siRNA release in the bloodstream before it reaches its target site (Mudd et al., 2010). Efforts are made to improve this system by controlled polymerization of the backbone, modification of masking groups to allow prolonged plasma circulation time and conjugation of different targeting moieties to enable other organ localization. In addition, two different approaches using DPC were further developed.

The first approach addresses tolerability concerns, attributed to the polymer’s excretion and chronic toxicity since the vinyl ether polymer is non-degradable. Parmar et al. used bioreducible poly(amido amine) polymers containing disulfide linkages in the backbone to produce a biodegradable, pH-responsive endosomolytic polymer conjugate (Parmar et al., 2013). Screening of potential candidates was performed by RBC lysis assay in vitro. Next, imidazole-based modification relying on histamine incorporation was tested further to show 80% ApoB knockdown (KD) efficacy 48 h following single 3 mg/kg intravenous injection. Although promising, this approach still suffers from complicated chemistry, polydisperse polymeric backbone and should be investigated in depth for efficacy in vivo.

The second approach is to split the DPC in two co-injected formulations (Wong et al., 2012). Administering a mixture of cholesterol-Factor IV (F7) siRNA together with PEGylated NAG-targeted endosomolytic polymer resulted in 500-fold increase in chol-siRNA efficacy and over 90% reduction of target gene in mice (Wooddell et al., 2013). In a series of in vivo studies, it was shown that both cholesterol conjugation of siRNA and the hepatoto-targeted NAG-polymer are required for target gene KD. This co-delivery approach was further evaluated in rhesus monkeys at 2 mg/kg dose of chol-siApoB co-injected with NAG-polymer. Reduction of 78% in serum ApoB protein was observed 14 days after single intravenous injection and maintained until day 30, full protein recovery was observed on day 50. This was accompanied by reduction in serum cholesterol and triglyceride levels.

5.2. Systemic administration — non-hepatic delivery

5.2.1. Blood compartment

Most in vivo delivery systems discussed in this review that bear true clinical potential or undergoing clinical testing is restricted to liver delivery or to local delivery. Thus, it is not surprising that many research groups are looking for smart and elegant solutions for other than liver targeting carriers. Dahlman et al. have made an attempt to target the endothelial compartment with siRNA bypassing hepatocytes (Dahlman et al., 2014). In their work, they developed a hybrid system between PEI and lipids (C15) and further mixed it with PEGylated (2 kDa) lipids (C14) which resulted in 7C1 multimamellar vesicles. In vitro studies showed reduction of 85% in target mRNA expression in HeLa cells, human dermal microvascular endothelial cells (HMVEC) and mouse brain endothelial cells (bEnd.3) at concentration of 30 nM. High renal accumulation was observed at 4 and 24 h after IV injection of 7C1. Hence, it was not surprising to find that the concentration of 7C1 decreased by 50% within 20 min of IV injection indicating that the formulation was rapidly cleared or endocytosed. The hypothesis that 7C1 reaches endothelial cells preferentially was tested by silencing ICAM-2 gene that is expressed exclusively in endothelial cells. It was found that expression of ICAM-2 mRNA in pulmonary, cardiovascular and renal endothelium was reduced by 92% and 70%, respectively. Interestingly, gene depletion duration was tissue specific, pulmonary endothelial cells showed ICAM-2 depletion for more than 21 days, whereas in cardiovascular and renal endothelium, the effect gradually diminished reaching 50% of initial expression after 10 days. Phenotypic changes were observed due to depletion of VECad gene, which resulted in vascular permeability as shown by 2.5-fold extravasation of Evans Blue Dye of the pulmonary vasculature. To assess the ability of 7C1 to avoid hepatic accumulation, hepatocyte specific gene factor 7 was targeted by siF7 7C1 formulation, but only modest reduction in F7 blood levels was measured, while the same siRNA delivered by liver specific vehicle showed 95% decrease in F7 blood levels. Moreover, when targeted together with Tie2 siRNA to the liver, only decrease in Tie2 mRNA was observed. Next, using LLC model inhibition of primary tumor growth was assessed by KD of VEGFR-1 and DI4 receptors. 40% and 70%, respectively, accompanied by increased tumor necrosis. To investigate the effect of polyplexes on tumor metastasis, LLC tumors were treated with siVEGFR-1. This siRNA reduced surface metastasis by 52% while siDI4 reduced 63% compared with control. It became evident that many nucleic acids can stimulate innate cytokine responses, which may be amplified by efficient delivery into the cell by carriers (Judge and MacLachlan, 2008). Thus, when evaluating a carrier for siRNA delivery, it is crucial to assess its immunogenic potential. In the case of 7C1 system, no serum cytokine elevation or mRNA expression was found after IV administration.

Endothelial dysfunction is related to various diseases such as diabetes, renal chronic disease, stroke, peripheral vascular disease, cancer and chronic inflammation (Rajendran et al., 2013). Thus, 7C1 nano-sized formulation is aimed to serve as research tool to manipulate gene expression in vivo and to explore gene interactions in the endothelium.

An additional example of a bloodstream delivery is a system targeted to macrophages and dendritic cells. They represent potentially important targets for RNAi therapeutics on the basis of their role in mediating...
inflammation and immune responses. Moreover, they promote pathogenic responses in such diseases as rheumatoid arthritis, atherosclerosis and inflammatory bowel disease (Duffield, 2003). Delivery of siRNA to such cells has been achieved by its encapsulation within GErPs (β-1,3-α-glucan-encapsulated siRNA particles), due to glucan-specific receptors on the phagocytic cells. In addition to a glucan layer, an amphiphatic histidine-rich Endo-Perter (EP) peptide was added (Tesz et al., 2011). It was shown that EP is crucial for gene silencing, KD was observed in different cell types (macrophages and fibroblasts) when this peptide was complexed with siRNA without having PEI in the formulation, but further shielding with glucan layer caused gene depletion in macrophages only.

More specific delivery can be achieved when targeting macrophages in the spleen and microglia in the brain while overcoming the blood brain barrier. For this purpose, Kim et al. developed an RVG-targeted poly arginine-based carrier for targeting macrophages and microglia upon intravenous delivery (Kim et al., 2010). RVG peptide binds specifically to neuronal cells expressing AchR (acetylcholine receptor) α7 subunit. Conjugation of nona-arginine (9dR) residues to its carboxy-terminal residues in RVG-9dR targeting moiety, which enables the attached siRNA to internalize into neuronal cells. TNF-α is an attractive target for siRNA as macrophages and microglial cells are believed to be the major source of TNF-α in the central nervous system (CNS) and TNF-α plays a major role in neuronal apoptosis. Therefore, RVG-9dR targeted TNF-α siRNA complex was used for treatment of tumor-bearing mice resulting in nearly 90% and 65% reduction in TNF-α mRNA levels compared to that of siLuc-treated-mice macrophages and microglia, respectively. The addition of a targeting moiety to this system enabled specific delivery to a preferential population of cells.

An alternative route of administration was chosen as a result of serum instability and rapid liver accumulation of chitosan nanoparticles following IV administration. TNF-α dicer substrate siRNA (DsiRNA) was complexed with chitosan nanoparticles and injected into a blood-free macrophage-rich environment of the peritoneal cavity to regulate systemic immunity (Howard et al., 2009). Modest reduction in the TNF-α level in the supernatants of LPS-stimulated peritoneal macrophages was revealed. Nonetheless, joint and cartilage integrity was maintained in sodium acetate-induced arthritic mice treated with siTNF-α containing nanoparticles intraperitoneally, albeit arthritic score of this treatment group was higher than that of dexamethasone control. In addition, mice treated with chitosan/TNF-α siRNA nanoparticles showed 100% survival rate. Prophylactic treatment with siTNF-α bearing chitosan nanoparticles, at the inductive phase of the disease and during its onset, attenuated disease progression was assessed by arthritic score. This novel approach successfully reduced systemic inflammation in arthritic joints while targeted to the peritoneal macrophages.

HIV-1 infection can be well treated via RNAi mechanisms. A cocktail of different siRNAs targeting both cellular and viral transcripts in HIV infected cells has been successfully used to inhibit HIV-1 titers in vivo using a PAMAM-based dendrimeric polyplex. Weekly injections (at 0.15 mg/kg siRNA-equivalent dose) of dendrimers/cocktailed siRNAs (equal amount of tat/rev siRNA, TNFPO3 siRNA, and CD4 siRNA) resulted in suppression of the viral load up to three weeks following treatment withdrawal. In addition, viral suppression can be achieved also...
following retreatment several weeks after the last administration. This carrier seems to be an efficient delivery system to the hematopoietic cells, such as CD4+ T cells and macrophages (Zhou et al., 2011).

5.2.2. Distant organs

Systemic administration of oligonucleotides is an effective way to achieve extensive distribution in the body but at the same time, it is challenging. Polach et al. developed a polyaminated lipidic backbone (Staramine) for in vivo delivery of siRNA. Staramine can undergo covalent modification in order to allow the conjugation of several pending groups to improve the efficiency of siRNA delivery in vivo (Polach et al., 2012). Staramine formulation for lung endothelium delivery following systemic administration was modified with methoxypolyethylene glycol (mPEG): Star-mPEG550, a polydisperse mPEG or Star-mPEG515, a monodisperse mPEG. mPEG-conjugation to Staramine improved stability and safety maintaining the gene silencing activity in the target tissue. siCav-1 was efficiently delivered by either non-PEGylated Staramine and mPEG550 to the lung, so that almost 100% siCav-1 was detected in the lungs 50 h following IV injection. Treatment with Staramine/siCav-1 nanocomplexes formulated in monodisperse mPEG515 resulted in a consistently slow clearance rate of the siRNA from the lung, compared to those composed of Star-Star-mPEG550, which showed variable clearance rates over multiple experiments.

This hypothesis that Star:Star-mPEG550 delivers siRNA selectively to the endothelial cells of the lung was supported by studies comparing knockdown of GFP transcripts expressed in the lung and CD31 transcripts expressed predominantly in endothelial cells. IV administration of polydisperse mPEG550 modified Staramine/siCD31 (2 mg/kg, two injections) led to ~75% depletion of the CD31 mRNA (as opposed to 50% Cav-1 gene KD after single IV injection of Star-Star-mPEG515), while similar administration of siGFP nanocomplexes led to ~20% knockdown of GFP transcript.

RNAi-based therapies can target the mitotic phase, a hallmark of cancer development and progression. MAD2 targeting siRNA can efficiently inhibit the spindle assembly check point (SAC), which plays a key role in the mitotic pathway and is essential for cells viability. Polyethylenimine (PEI) complexed with MAD2 siRNA is an efficient delivery system for in vivo gene knockdown. Mice bearing human colon cancer HCT116 subcutaneous tumor were repeatedly treated with PEI/MAD2 siRNA complexes via IP injections (2.8 mg/kg cumulative dose of siRNA), MAD2 expression in the tumor tissue decreased by 31% compared to the non-treated group. The inhibition of SAC in vivo resulted in reduction of tumor growth of about 30% compared to non-treated control (Kaestner et al., 2011).

Xiong and Lavasanifar set up a multicomponent carrier for co-delivery of doxorubicin and siRNA based on poly(ethylene oxide) and poly(ε-caprolactone) copolymer. The polymeric backbone was decorated with the c(ε153) targeting RGD4C peptide, Tat peptide to guarantee endosomal escape and polyamine ( spermine) for electrostatic interaction with oligonucleotides. The in vivo trafficking of the supramolecular assemblies has been followed by a near infrared fluorescent probe conjugated to the siRNA backbone. The targeted system demonstrated high intratumoral fluorescence up to 24 h post-IV injection, improved stability and long circulation time of the oligonucleotides (Xiong and Lavasanifar, 2011).

Nanoparticle-siRNA delivery relies on the particles being large enough to be able to circulate for long periods in the bloodstream and at the same time being small enough to non-selectively extravasate through the tumor leaky vessels and accumulate in solid tumors as these lack an effective way of draining them through the impaired lymphatics (EPR effect). RONDEL is a nano-sized cyclodextrin based siRNA-delivery system consisting of a mixture with siRNA and adamantane-coupled PEG stabilizers, some of which carry a transferrin ligand, so as to create 60–80 nm particles (Heidel and Schluep, 2012). To RONDEL delivery system was added siRNA targeting RRM2, a gene involved in DNA replication, and named CALAA-01. CALAA-01 is the first polymer-based system for siRNA delivery that was tested in humans (Davis et al., 2010). This study employed small sample groups, nonetheless it showed that 1) RONDEL-siRNAs accumulated in the tumor tissues in a dose-dependent manner; 2) consistent with this, an RNAi mechanism of action was unambiguously confirmed by 5’ RACE in tumor samples from the patients that received the highest dose; 3) mRNA knockdown lasted for more than 4 weeks and was accompanied by disease stabilization; and 4) RONDEL was safe and well-tolerated in a study that involved repeated injections while dose-limiting toxicity (DLT) has not yet been displayed (Davis et al., 2010). Recently, this system was tested in a larger cohort in humans and additional parameters were evaluated (Zuckerman et al., 2014). The interaction of CALAA-01 with the renal filtration barrier resulted in rapid blood clearance, within 30 min, predominantly by the kidneys exhibiting the same pattern at each given dose. Although preclinical data raised major concerns regarding liver and kidney toxicities, premedication and efficient hydration of patients enabled to avoid these toxicities in humans. The majority of adverse effects resulting from CALAA-01 in humans were primarily hypersensitivity and consequently acute immune responses (rushing, fever, fatigue, etc.). As it was shown in dogs and rats, administration of the delivery system components but not CALAA-01 (delivery formulated with siRNA) resulted in acute hypersensitivity reactions. The study in humans was conducted in 2 parts: the first part of the study included dose escalation (3–30 mg/m²) and was carried out in 19 patients. Some patients that were retreated with higher dose of CALAA-01 experienced DLTs (ischemic colitis, lymphopenia, severe hypersensitivity reaction, and hypotension). It was hypothesized that previous exposure to the drug had led to immunostimulatory effects. Thus, in the second part of the study, Phase Ib study, 5 patients were treated with previously well tolerated high doses of the formulation. Surprisingly, these patients suffered from DLTs that can be attributed to batch-to-batch inconsistency or the stochastic nature of adverse effects. The latest data on CALAA-01 therapy showed disappointing results; suffering from limited sample size, dose limiting toxicities in all patients and poor anti-cancer effects, therefore the study was ended.

5.3. Local administration

Some delivery systems can be used systemically but will mostly arrive at the liver without significant distribution to other organs. Those systems are more attractive to be used for local delivery. Such an example is the PE Gyalted imidazole modified chitosan nanoparticles that showed reduction in GAPDH expression of 49% in the lungs and 30% in the liver following single IV injection of 1 mg/kg siGAPDH (Ghosn et al., 2010). Liver specific ApoB gene was depleted by dose dependent manner after 2 sequential IV injections. In order to bypass the hepatic accumulation and systemic side effects, non-PEGylated imidazole modified chitosan siGAPDH nanoparticles were administered locally to the lungs and upper airways. For intranasal delivery non-PEGylated imidazole chitosan siGAPDH nanoparticles showed 50% decrease in GAPDH gene after 3 sequential low dose administrations, although it did not improve the silencing efficacy achieved by non-modified chitosan. In addition, no increase in gene KD was noted from 1 to 2 mg/kg dose escalation of siRNA formulated with imidazole-modified chitosan. This might be attributed to high viscosity of the formulation thus lower penetration in the lungs. To combine the advantages of lipid-based delivery systems, i.e. high transfection efficacy, together with the benefits of dendrimer-based systems, i.e. controlled synthesis and well-defined molecular structure, a hybrid system was developed (Yu et al., 2012). The amphiphilic system composed of low generation hydrophilic PAMAM dendrimer bearing long alkyl chain showed good transfection efficacy in vitro as was detected by silencing Hsp27 at both the mRNA level and the protein level. Interestingly, neither lipid chain nor the dendrimer alone led to any gene silencing, but only the conjugated system. When administered into subcutaneous tumors at a dose of 3 mg/kg, the amphiphilic dendrplex inhibited cell proliferation as a result of 50% gene knockdown and decreased protein expression. This system will be further studied in.
Polymeric nanocarriers used to deliver oligonucleotides in vivo.

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7. Conclusions

Less than ten years since the Nobel Prize was awarded to Fire and Mello “For their discovery of RNA interference — Gene silencing by double-stranded RNA” (Nobelprize.org., 2014), research in the RNAi field led to (i) the discovery of several target genes for therapeutic purposes, (ii) the development of double stranded oligonucleotides sequences for RNAi on those target genes, and (iii) the improvement of nanocarriers already in use for other therapeutic molecules (low molecular weight drugs, proteins and plasmids) for the delivery of double stranded oligonucleotides. The use of a safe and efficient delivery system is crucial to achieve an effect in vivo. An enlightened design of the carrier, considering physico-chemical properties of the materials and the physio-pathological features of the target tissue and cells seems necessary to the realization of an efficient delivery system. Extensive in vitro characterization of the supramolecular polymer–oligonucleotide assemblies allows to predict whether the system will present adequate pharmacokinetic and biodistribution profiles, low toxicity and transfection efficacy. Finally, the choice of a representative in vivo model for preclinical studies is a necessary step to test the transfection efficacy of the developed delivery system, distinguish from specific silencing properties and possible off-target effects and immune system stimulation. A deeper understanding of the characteristics of the new supramolecular entity will hopefully lead to the development of a successful therapeutic nanomedicine, able to meet the regulatory requirement for a safe and efficient in vivo RNAi.

terms of structure–activity relationship and should be tested for prolonged anti–cancer activity as well as for different administration routes (e.g. intravenous or intraperitoneal).

Inhaled siRNA delivery holds great potential for the treatment of pulmonary diseases as lung cancer (Conti et al., 2014), viral and microbial respiratory diseases (Bitko et al., 2005; Kwok et al., 2009; Zhang and Tripp, 2008), chronic pulmonary diseases (Merkel et al., 2009) and more.

To investigate the fate of inhaled PEI-based polyplexes, PEI 25 kDa and PEI–PEG with different PEGylation patterns (single 20 kDa PEG chain versus ten 2 kDa PEG chains) were designed (Merkel et al., 2009). The study mainly focused on stability in biological fluids relevant to the lung such as mucin and surfactants. Despite sufficient gene depletion results after introduction of naked siRNA into the lungs, formulated delivery of siRNA allowed lower administered dose with enhanced intracellular accumulation and activity. Unmodified PEI, but not PEGylated one, showed the best stability in natural fluid-based conditions in vitro. In addition, residence time in the trachea decreased with PEGylation and was only detectable for the carrier but not for siRNA, as was evaluated by radioactive labeling of the carrier and siRNA, but each was complexed with non-labeled counterpart (labeled polymer with non-labeled siRNA and the other way around). Unmodified PEI remained in the lungs to the greatest extent of all compounds labeled, while the two PEGylated polymers showed only half the dose remaining 48 h post administration. However, PEI–PEI/siRNA complexes are more suitable for lung applications than PEI/siRNA complexes concerning protection of siRNA and residence times in the lung. Although unmodified PEI complex had the most stable pattern in vitro, it seems that in vivo, this polymer strongly interacts with lung surfaces and loses its stability so that great amount of siRNA is excreted in urine following absorption to the bloodstream.

6. From proof of concept to bedside products

The final scope of the drug delivery scientists is to achieve an efficient delivery of oligonucleotide that allows to exploit the RNAi mechanism to successfully treat RNAi-mediated pathologies. In order to fulfill this mission and obtain a new molecular entity approved on the market, several steps must be completed (see Flowchart). Once the adequate material has been selected and the formulation obtained, it is fundamental to proceed with an extensive characterization of the product through reproducible and well controlled experiments. For the pharmaceutical industry, it is in fact desirable to achieve an early proof of concept (POC), in order to proceed with successful clinical trials and possibly to commercialize the product (Paul et al., 2010). In order to pursue the POC, several parameters must be accomplished and extensive characterization achieved. The chemical composition of the polymeric backbone must be defined, and the polyplex must assemble in a controlled and reproducible manner, with known RNAi loading, size, zeta potential and morphology. Chemical and physical stability must be defined, in aequous solutions and in the presence of serum. Cellular uptake and biological activity must be evaluated on multiple cell lines, evaluating the mRNA reductions relative to controls (including non-targeting RNAi). Moreover, an in vitro dose response must be achieved and the silencing at non-toxic concentrations must be obtained.

Once the physico-chemical and in vitro characterization have been completed, the polyplex must undergo preclinical in vivo evaluation, including safety/toxicity testing (changes in mice behavior, liver transaminases — ALT/AST levels at 24 h, no changes in tissue gross appearance, cytokine induction at 2, 6 and 24 h post-treatment (Zuckerman et al., 2014), defined DLT and maximum tolerated dose (MTD)) and efficacy experiments (single versus multiple dose, dose response, mRNA reductions of at least 50% relative to controls including non-targeting siRNA control). Albeit immune stimulation and complement activation have always raised concerns in case of naked RNAi and lipid-based delivery systems (Judge et al., 2005; Kanasty et al., 2012; Landesman-Milo et al., 2013), the latest results in Phase I/II clinical trials in patient treated with CALAA-01 revealed that also polymer-based delivery systems suffer from hypersensitivity reactions and immune response adverse effects (Zuckerman et al., 2014). Thus preclinical investigations of these features are fundamental to achieve successful in vivo delivery of RNAi.
Flowchart. Plan of operation and decision-making process in the development of RNAi polyplexes.
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