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Review

Physicochemical characterization techniques for lipid based delivery systems for siRNA

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ABSTRACT

siRNA based therapeutics is an emerging class of molecules with a high potential for fulfilling the promise of gene medicine. The high selectivity of siRNAs for their targets and subsequent gene ablation has been effectively demonstrated in a wide range of pre-clinical models. siRNA delivery *in vivo* has been most successfully achieved using lipid-based drug delivery systems. These lipid based formulations are designed to entrap siRNA molecules, ensure stability in *in vitro* and *in vivo* milieu, facilitate uptake, enhance cellular targeting, and facilitate delivery in the desired intracellular compartment. As more siRNA-based therapeutics enters the clinic with the associated regulatory scrutiny, there is a clear need to develop well-characterized systems that ensure consistent quality and thus reliable performance. Early clinical trials can be conducted using formulations with limited short-term stability manufactured on a small scale. However, a thorough understanding of the factors that influence the structure and stability of these particulate formulations is required to prevent any issues with optimization of large-scale industrial manufacturing, scale-up, and long-term shelf-life required to support large clinical trials and eventual market use. As newer targets for siRNA are identified and novel lipids are synthesized to optimize their *in vivo* efficiency, concomitant development of bio-physical methodologies that can improve understanding of the assembly and stability of these complex systems is critical. Along with bio-physical characterization, these assays are also required to reliably design, screen, develop and optimize formulations. Physicochemical characterization thus forms the basis of developing an effective analytical control strategy for siRNA delivery systems. In this review, analytical techniques used to characterize lipid-based siRNA delivery systems are discussed in detail. The importance of these physicochemical characterization techniques and analytical assays is explained. Case studies illustrating their use in siRNA formulation development and optimization are presented.

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1. Introduction

Nucleic acid therapeutics offer one of the most potent strategies for the treatment of genetic disorders caused either due to a single gene defect (*e.g.* severe combined immunodeficiency disease, autosomal dominant keratin disorder pachyonychia congenita) (Anderson, 1998; Shearer, 2004), or due to a combination of multiple gene defects (*e.g.* cancer, inflammation) (Ganly et al., 2000; Wahlfors et al., 2005). These agents can achieve a high level of therapeutic success due to selective and targeted up regulation (by DNA) or down regulation (by antisense/antigene oligonucleotide) of the implicated genes *in vivo* with limited non-specific or off-target effects. As a result, these molecules hold tremendous promise and it is anticipated that treatments may be developed for a wide range of chronic diseases associated with pathological up or down regulation of proteins. Diseases often considered incurable and life-long or those that have unmet therapeutic needs due to a lower prevalence in the general population are also attractive targets for this class of therapeutics.

The remarkable progress in the field of nucleic acid therapeutics is apparent from the more than seventeen hundred gene therapy based clinical trials that are currently underway world-wide (www.wiley.com//legacy/wileychi/genmed/clinical; Gene Therapy Clinical Trials Worldwide. J. Gene Med). Gendicine (Ad-p53), the first gene therapy-based product, was approved in China for the treatment of head and neck squamous cell carcinoma in conjunction with radiotherapy. Mutated p53 transcription factor is one of the most common genetic marker for cancer and is also implicated in the resistance of cancer cells to chemotherapy. Gendicine is comprised of a recombinant adenovirus vector designed to express a plasmid transgene for the wild-type p53 transcription factor in cancer cells that leads to the restoration of innate tumor suppressor pathways (Huang et al., 2009; Shi and Zheng, 2009). The efficacy of Gendicine was demonstrated in a Phase III clinical trial wherein patient populations were randomized either in a combination therapy arm (Gendicine and radiotherapy) or radiotherapy alone. Results of the clinical trial showed that the overall response rate with combination therapy (96%) was greater than radiotherapy alone (80%) using standard tumor shrinkage criteria established by the WHO (Peng, 2005). A second gene therapy product (H101 or Ocorine) based on oncolytic virotherapy was approved for human clinical use for advanced head and neck cancers in China. Oncolytic virotherapy exploits the ability of recombinant adenoviral vectors to selectively replicate in and lyse p53-deficient cancer cells. Combination of H101 and chemotherapy have showed significantly higher tumor response rate compared to either agent alone in a range of clinical trials for advanced head and neck and nasopharyngeal cancers (Xia et al., 2004).

siRNA discovery in the late 1990s proved to be a major breakthrough in the field of nucleic acid-based therapeutics. Compared to other nucleic acid therapeutics such as recombinant plasmids and oligonucleotides, siRNA molecules lead to more selective and efficient down regulation of genes due to their unique and specific mechanism and site of action. Unlike plasmid-based nucleic acid therapeutics, siRNA molecules function in the cytoplasm and therefore do not require uptake by the cell nucleus. Additionally, siRNA molecules are theoretically more potent than antisense oligonucleotides (which also have a target in the cytoplasm) since one siRNA molecule can interfere with the translation of several mRNA molecules of one kind (Haley and Zamore, 2004). Consequently, the advent of siRNA has inspired and accelerated the drug development pipeline for several hereditary and acquired genetic diseases such as respiratory synctial virus (Bumcrot et al., 2006), Huntington's disease (DiFiglia et al., 2007) and cancer (Oh and Park, 2009). siRNA molecules have shown promise in treating conditions with implications of over-expression of proteins in monogenetic as well as polygenetic disorders. Furthermore, technological advances in the complete mapping of the human genome, identification and validation of disease-implicated genes for potential drug targets, and faster and efficient tools in pharmacogenomics have opened additional avenues for 'personalized medicine' using nucleic acid therapeutics.

Despite their exceptional success in preclinical models and encouraging promise in human trials, the true therapeutic potential of siRNA remains to be completely exploited. Due to the overall similarity in the nucleic acid back-bone, the field of siRNA formulation development has also experienced similar development and delivery hurdles that have impacted commercial product development of other nucleic acid therapeutics. These include physico-chemical and enzymatic instability, low cellular uptake, limited bioavailibility due to *in vivo* degradation, and inefficient intracellular trafficking. To overcome these limitations, viral and non-viral vectors have been used for efficient *in vivo* siRNA delivery. Of the delivery vectors for siRNA in development, non-viral systems are beginning to emerge in prominence over the viral vectors as these offer significant advantages in terms of control, consistency and superiority over their design characteristics along with better manufacturability, biological activity, immunogenicity and safety (Patil et al., 2005b).

Among the non-viral vectors, liposomes are by far the most advanced due to their efficient interaction with lipidic cell membranes, effective facilitation of endosomal escape which leads to efficient delivery of the entrapped nucleic acid. Furthermore from a drug development perspective, the decades-long existence of numerous safe and well-tolerated commercial liposomal products for human clinical use offers a large knowledge base and

Table 1
Clinical Trials worldwide for siRNA therapeutics (www.clinicaltrial.gov).

Company	Target	Product	Disease	Route	Formulation	Status
Alnylam Pharmaceuticals	N-protein of RSV	ALN-RSV-01	Respiratory synctial virus	Intranasal	Naked siRNA	Phase IIb
Alnylam Pharmaceuticals	Kinesin spindle protein (KSP) and VEGF	ALN-VSP-01	Liver cancer	Intravenous	SNALP (stable nucleic acid-lipid particle)	Phase I
Alnylam Pharmaceuticals	Transthyretin (TTR)	ALN-TTR-01	Transthyretin-mediated amyloidosis	Intravenous	SNALP	Phase I
Alnylam Pharmaceuticals	Huntington's protein	ALN-HTT	Huntington's disease	CNS (implantable infusion)	–	Preclinical
Acuity Pharmaceuticals	VEGF	C and 5 (Bevasiranib sodium)	Wet Age-related macular disease	Intravitreal	–	Phase III (completed)
Sirna Therapeutics	VEGF	Sirna-027 (AGB211745)	Wet Age-related macular disease	Intravitreal	Chemically modified siRNA	Phase II (terminated)
Silence Therapeutics	Proteinkinase N3	Atu-027	Advanced solid tumor	Intravenous	Lipoplex	Phase I
Silence Therapeutics	RTP801	PF-4523655 (RTP801i-14 or REDD14NP)	Diabetes macular edema	Intravitreal	–	Phase II (completed)
Quark Pharmaceuticals	Human p53	QPI-1002 (Akli-5 or I5NP)	Acute kidney injury	Intravenous	–	Phase I/IIa (recruiting)
Quark Pharmaceuticals	Caspase 2	QPI-1007	Non-arteritic-anterios ischemic optic neuropathy (NAION), glaucoma	Intravitreal	–	Phase I
Calando Pharmaceuticals	M2 subunit of ribonuclease reductase (R2)	CALAA-01	Solid tumors	Intravenous	Cyclodextrim containing CAL-101, PEG, PEG-Tf	Phase I (ongoing)
Pachyonychia Congenita Project	Pachyonychia congenital keratin	TD101	Pachyonychia congenita	Callus injection	–	Phase I

technical experience that can ensure confidence in these delivery systems.

Lipid-based and liposomal delivery systems have demonstrated their potential by fast entry and growth in clinical trial programs for siRNA molecules (Table 1). Despite their widespread use, however, no official regulatory guidance exists that can support a consistent product development strategy for liposomal based drug delivery systems for siRNA applications. Although a draft technical guidance for the development of liposomal products exists, the industrial design and control strategy for siRNA liposomal systems remains empirical and mostly dependant on a case-by-case basis (www.fda.gov, Guidance for Industry, Liposome Drug Products, 2002). The delivery of siRNA using liposomes has unique structural requirements and stability implications compared to small molecules or biologics. With the growing emphasis from regulatory agencies to develop formulations using risk-based strategies and Quality by Design principles, there is a clear need to develop well characterized and stable delivery systems for siRNA-based therapeutics. The application of these principles for the exploration of the formulation design space as well as in the improvement of process understanding can result in consistent drug product screening, development, optimization and approval. Furthermore, strategies to develop well-characterized systems can be used to improve manufacturability and reduce batch-to-batch variability during industrial manufacturing, scale up, and thus influence consistent results in the clinic.

This review discusses physico-chemical characterization techniques and assays of lipid based and liposomal siRNA delivery systems (lipoplexes) and their use to support robust product development. Using literature examples, the application of these techniques in improving formulation design space understanding will be demonstrated. Furthermore, the relevance of such assays to yield systems with consistent physicochemical and functional properties is presented. The importance of physicochemical characterization in understanding correlation (if any) between formulation parameters and activity of lipoplexes *in vitro* as well as *in vivo* is discussed in detail. Additionally strategies that can support development of stable liposomal delivery platforms for siRNA-based therapeutics are elaborated.

2. Classes of RNA based therapeutics

RNA-based therapeutics can be classified on the basis of their mechanism of action. There are mainly three types that include shRNA (short hairpin RNA), miRNA (micro RNA) and siRNA (silencing RNA). shRNA are short hairpin expression vectors that after the nuclear entry, transcribe to small RNA molecules that bind to complementary mRNA and inhibit the expression of corresponding protein. miRNA and siRNA are small 21–25 base nucleotides that function directly in the cytoplasm where they cause interference with the target mRNA translation process. miRNA inhibits this translation process by binding non-specifically to the 3′UTR (untranslated region) of the mRNA (Bartel, 2004). Therefore miRNAs are effective against more than one (usually 1000) mRNA with similar sequences (Pillai et al., 2007). On the contrary, siRNA (small interfering RNA), binds specifically to the complementary mRNA thereby knocking down protein expression. Due to their non-specificity, miRNA are more indiscriminate and have off-target activity. For example, Alvarez et al. (2006) demonstrated the efficacy of miRNA against multiple targets but also indicated the non-specificity of miRNA in different species. From the delivery perspective, shRNA delivery is the most challenging of the three, since it requires entering the nucleus which also makes it less efficient in slowly dividing and non-dividing cells. For example, Mantei et al. (2008) and Shen et al. (2004) demonstrated efficient silencing in cell lines such as T lymphocytes and dendritic cells, respectively, which are otherwise difficult to transfect with molecules

that require nuclear entry such as shRNA. However, since siRNA is highly specific, compared to miRNA and does not require nuclear delivery (as in case of shRNA) it is the most preferred in the category of RNA-based therapeutics.

Several siRNA molecules are currently being evaluated in Phase 1 and Phase 2 clinical trials world-wide as summarized in Table 1. Some of the most advanced molecules include Cand5 and PF-655, siRNA molecules targeted against genes implicated in neovascularization such as VEGF and RTF801, respectively. These siRNA molecules have been shown to be well tolerated in wet age-related macular degeneration (AMD) patients in early stage clinical trials. Other siRNA molecules are being evaluated in human clinical trials for a wide range of indications that include renal failure, solid tumors, viral infections, cardiovascular diseases, edema associated with diabetes and prevention of rejection in kidney transplants.

3. Mechanism of siRNA activity

The most typical uptake mechanism of siRNA along with the associated delivery vector (siRNA-liposome complex) involves uptake via the endocytosis process. Other processes such as macro-pinocytosis and receptor mediated pathways have also been implicated in cellular internalization. In this process, the siRNA-complexes upon internalization get entrapped in early endosomes (pH 6.5) (Gruenberg and Maxfield, 1995; Kronenberger et al., 1997; Murphy et al., 1984). Early endosomes then either mature or fuse together, to form more acidic late endosomes (pH 5.5). Thereafter, the siRNA complexes are transported to highly acidic lysosomes (pH 4.5) where they can be degraded. In order to avoid such degradation, it is important for the siRNA molecules to escape from the early/late endosomes. This is usually assisted by inclusion of a delivery vector component in the lipid membrane such as cationic or fusogenic lipids (*e.g.* diC14-amidine (Benatti et al., 2004) and DOPE, respectively).

Upon endosomal release into the cytoplasm, siRNA binds to Argonaute2 (and other) proteins (also known as Ago2 or slicer) and forms RNA induced silencing complexes (RISC) (Fig. 1). siRNA interaction occurs with Ago2 proteins when 5′ phosphate of the least paired strand (known as antisense or guide) of siRNA binds to the PIWI domain of Ago2 proteins via the divalent ion Mg^{2+} (Ma et al., 2005). RISC formation activates the slicer protein which unwinds siRNA thereby deserting the guide strand. The guide strand directs the RISC towards the target mRNA sequence complementary to the guide strand sequence. Following this, the PIWI domain of Ago2 proteins (Ma et al., 2005; Rand et al., 2004) cleaves the phosphodiester bond of mRNA, which is facing the mid region of the 10th and 11th nucleotides of guide strand, thereby rendering mRNA dysfunctional (Elbashir et al., 2001). This process of the mRNA cleavage occurs several times by the same guide strand, the rate and extent of which depends on binding affinity of the 5′end of the siRNA guide strand to the Ago2 proteins and the rate of cleaved mRNA dissociation from the RISC (Haley and Zamore, 2004). It is due to this RISC recycling that siRNA therapeutics has long term effects on their targets when compared to other nucleic acid therapeutics such as antisense oligonucleotides and ribozyme based therapies.

4. siRNA Barriers to cellular entry and trafficking

4.1. Nuclease degradation

The *in vivo* efficacy of siRNA molecules is directly dependant on their ability to overcome barriers that prevent absorption, distribution and intracellular trafficking. Nucleic acids are prone to enzymatic degradation by nuclease enzymes in the serum. However, siRNA is more unstable towards this enzymatic degradation when compared to DNA, due to the presence of the 2′OH on the ribose sugar in the structure that is susceptible to alkaline hydrolysis (Lipkin et al., 1954).

Several approaches have been adopted to improve the siRNA serum stability of siRNA which primarily include chemical modification of the molecule. One approach considers backbone modification wherein the nuclease-prone phosphodiester bond can be replaced by other groups such as phosphorothioate (Choung et al., 2006), boranophosphates (Hall et al., 2004), phosphoramidate, and methylphosphonate. While the latter two groups are not very common in siRNA, modification with phosphorothioate and boranophosphate has been successfully utilized to improve serum stability (Behlke, 2008). A second approach that has been utilized to improve siRNA stability is modification of the ribose sugar with groups such as 2′ deoxy, 2′NH_2, 2′F or 2′ OMe (Layzer et al., 2004; Manoharan, 2004). These substitutions are utilized to eliminate the 2′-OH group that is known to participate in alkaline hydrolysis of siRNA. A third approach is base modification but it usually results in poor efficiency due to the effect of the modification on the base pairing ability of the molecule with the target. An example of base modification involved substitution of guanosine with inosine that resulted in lowered efficacy of siRNA duplexes (Parrish et al., 2000).

Although chemical substitution or modification-based strategies are used for improving siRNA stability these can also cause increased toxicity depending on the degree of substitution. For example it has been reported that 100% replacement of phosphodiester with phosphorothioate though improved serum stability but also increased the cytotoxicity (Amarzguioui et al., 2003). However, limited substitution ensured no substantial cytotoxicity while maintaining the activity (Braasch et al., 2003). This is not the case with 2′ OMe substitution in ribose sugar (Amarzguioui et al., 2003).

4.2. Immunogenicity

Another challenge related to siRNA efficacy *in vivo* is its immunogenicity. This is because siRNA molecules are recognized as foreign and suspected to be associated with pathogens such as bacteria and virus (Hornung et al., 2005; Judge et al., 2005). Therefore, siRNA molecules can activate inflammatory receptors such as TLR (toll-like receptors). It has been found that it is mainly TLR7 and/or TLR 8 that cause activation of interferon (IFN-α) (Hornung et al., 2005) and interleukins (IL-6) (Gorden et al., 2005), respectively, once they recognize the RNA molecule. This activation can also trigger other cytokines associated with innate immunity. The key structural component in siRNA responsible for such triggering mechanism has been found to be the ribose sugar and multiple uridine bases, which are predominantly identified by TLR7 (Diebold et al., 2006). Therefore attempts have been made to synthesize chemically modified siRNA with substitution of such immune-triggering moieties and thus prevent the associated immune response. For example, it was found that few substitutions of 2′OMe in place of 2′ hydroxyl (Karikó et al., 2005; Kim et al., 2007), in the ribose sugar significantly reduced the immunogenic potential of siRNA. The precise mechanism was later discovered by Robbins, who found that 2′OMe actually acts as a TLR7 antagonist that is the key culprit for immune stimulation (Robbins et al., 2007). Cekaite also supported these results and reported that 2′ OMe antagonizes TLR7 without signaling (Cekaite et al., 2007). Other substitutions that have been shown to be useful to inhibit the immune response were 2′F (Cekaite et al., 2007) and locked nucleic acid (LNA) (Hornung et al., 2005) configurations.

In addition to TLR, other cellular receptors such as PKR (dsRNA-binding protein kinase) (Saunders and Barber, 2003) and RIG-1 (retinoic acid-inducible gene-I) (Kato et al., 2005) have been implicated in immunostimulation by siRNA. PKR was found to recognize

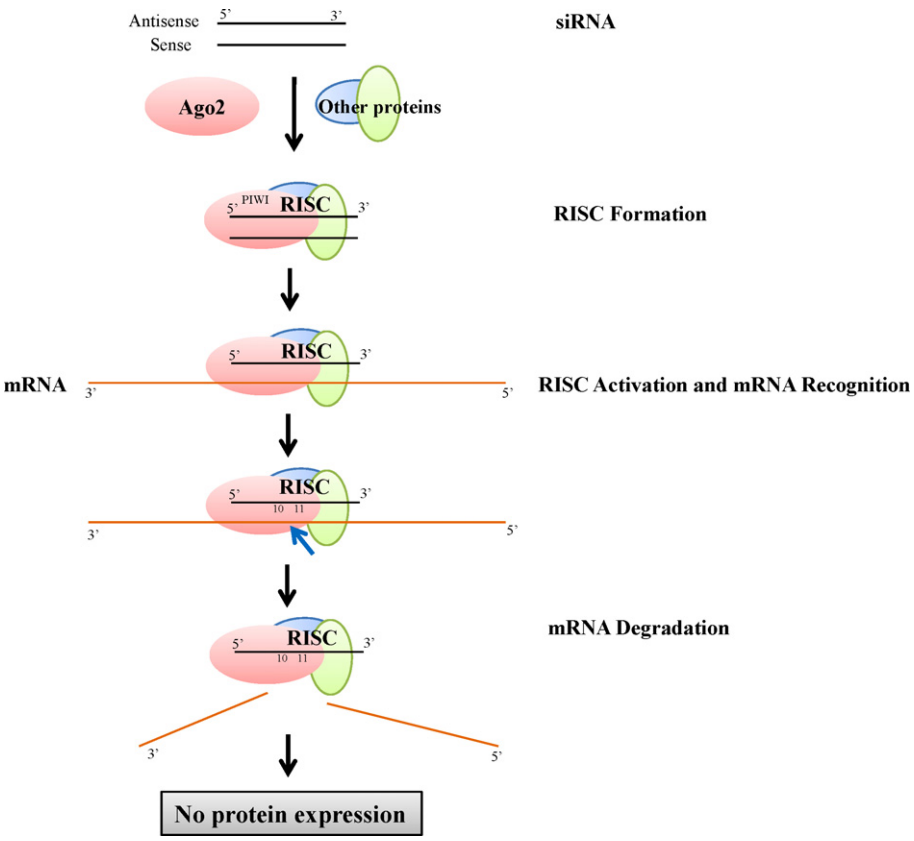

Fig. 1. *siRNA Mechanism*: Mechanism of action of silencing RNA (siRNA) initiated by recognition of the antisense strand by the PIWI domain of the Ago2 proteins, followed by formation of RISC (RNA-induced silencing complex). RISC activation causes mRNA recognition, mRNA complementary binding and mRNA cleavage thereby shutting off expression of the corresponding protein.

long dsRNA molecules (Saunders and Barber, 2003) while RIG-1 recognizes blunt siRNA and ssRNA with 5′-triphosphate siRNA. Kim et al. (2004) synthesized T7 RNA to inhibit 5′-triphosphate mediated response while Rose et al. (2005) incorporated DNA to the blunt ends to avoid triggering the immune response.

4.3. siRNA cellular uptake and endosomal escape

siRNA cellular uptake has also been identified as a barrier for delivery and efficacy. This is attributed to the relatively large molecular weight (approximately 14,000 Da) compared to small molecules and the presence of a highly negatively charged backbone. Due to the lack of innate endosome-disrupting properties, upon internalization, siRNA molecules are incapable of escaping from these vesicles, wherein they get concentrated and thus subjected to degradation. The primary reason is the inability of negatively charged siRNA to interact with the negatively charged endosomal membrane and accordingly siRNA is retained in these highly acidic endosome vesicles resulting in siRNA degradation via acid based hydrolysis of the phosphodiester bonds (Oivanen et al., 1998). Furthermore, low endosomal pH also inhibits base pairing of dsRNA due to unfavorable ionization of the bases in the low pH environment (Huber et al., 2003).

5. Strategies to improve siRNA cellular uptake

The barriers to cellular uptake and intracellular trafficking need to be overcome to ensure target interaction and efficacy of the siRNA molecule. Two key approaches therefore, have been adopted to improve siRNA delivery—(1) direct conjugation and (2) utilization of delivery vectors.

Direct conjugation to siRNA molecules involves covalent modification of the nucleotide sequence by attachment of other chemical entities. There are several reports wherein covalent conjugation of siRNA to moieties such as: lipids (cholesterol (Muratovska and Eccles, 2004), lithocholic acid and lauric acid (Lorenz et al., 2004)); polymers (PEG (Kim et al., 2008b,c; Lee et al., 2007; Oishi et al., 2007)); peptides (TAT (Chiu et al., 2004) cell penetrating-peptides (Moschos et al., 2007; Muratovska and Eccles, 2004)); and aptamers (Chu et al., 2006; McNamara et al., 2006; Zhou et al., 2008), has resulted in significant enhancement of siRNA delivery. This is because; the attached moieties can facilitate intracellular uptake and endosomal escape of siRNA. For example, cholesterol conjugated Apolipoprotein B-siRNA (apo-B siRNA) has been utilized to successfully downregulate apo-B gene expression (57 + 6% compared to control), that is otherwise responsible for hypercholesterolemia (Soutschek et al., 2004). Cell penetrating peptides (CPP) such as penetratin or transportan have been conjugated to siRNA for knockdown of many target proteins such as luciferase and green fluorescent protein in several cell lines (Muratovska and Eccles, 2004). TAT peptide has been used for siRNA internalization (Chiu et al., 2004). Cationic polymers such as polyethylemeimine (PEI) have been conjugated to siRNA via PEG (PEI-PEG-siRNA). Such polyelectrolyte complexes have been utilized to treat systemic and local tumors in animal models (Kim et al., 2008c).However, there are certain limitations to a direct conjugation approach. These include impact on siRNA activity due to the conjugation process. Therefore the conjugation site on siRNA must be judiciously selected in order to avoid potential impact on activity. Conjugation of moieties to the 5′ end of the antisense strand may result in loss of activity (Chiu and Rana, 2003) due to interference in binding of siRNA to the Ago2 proteins which occurs via the 5′ terminus.

Therefore, modifications are preferred on the 3′ or 5′ end of sense strand, or on the 3′ end of the antisense strand. Covalent conjugation of a cationic moiety to anionic siRNA may neutralize the conjugate and reduce the overall efficacy for uptake associated with the modification. For example, conjugation of CPP with 6–8 charges to siRNA (with 40 charges), resulted in neutralization of the peptide thereby restraining the CPP to penetrate into the cells (Meade and Dowdy, 2007). Furthermore, although moieties are able to improve cellular uptake these species may be unable to protect siRNA from nucleases thereby requiring the need for chemical modification of the siRNA to improve its nuclease resistance. Finally, conjugation and purification of high quality starting materials could be a time consuming and challenging process especially when there are electrostatic interactions between conjugating species and siRNA.

In view of avoiding the aforementioned issues, delivery vectors have gained popularity. Delivery vectors help protect siRNA from nuclease and improve their delivery. The details of delivery vectors are discussed in Section 6.

6. siRNA delivery vectors

Naked siRNA molecules retain some degree of silencing ability by overcoming the barriers to uptake and stability if directly administered to the site of action. Formulations based on direct delivery are typically intended for local or regional administration in specific tissues such as the eye or direct tumor administration. Similar to DNA molecules, siRNAs undergo rapid nuclease digestion upon intravenous administration and are rapidly cleared form the system. Hence to improve their delivery, several vectors or delivery systems have been utilized that protect siRNA from enzymatic degradation and facilitate targeted intracellular uptake and promote endosomal escape.

Based on their mechanism of action and origins, delivery vectors can be categorized as viral and non-viral. Viral vectors employ attenuated viruses that can deliver entrapped nucleic acids in their genomes into cells. Examples of attenuated viruses used for gene medicine include lentivirus, adenoviruses, herpes simplex virus, adeno-associated viruses and retroviruses. With millions of years of evolution on their side, viruses have been shown to have extremely high transfection efficiency (more than 99%) in delivering the entrapped gene medicine. However, viral delivery systems can be toxic and immunogenic (Yang et al., 1994, 1995). They can lead to genomic integration and non-specific effects. Furthermore, virus preparations can be difficult to manufacture and standardize on an industrial scale. However, despite these technical hurdles, gene regulation using recombinant adenoviral vectors has achieved the most clinical success and currently remain the only known gene medicine-based treatments on the market (Patil et al., 2005b; www.clinicaltrial.gov). Two drugs using attenuated viruses, Gendicine and H-101 (ocorine) for treatment of advanced head and neck cancer have been approved in China for human clinical use (Huang et al., 2009; Shi and Zheng, 2009; Wilson, 2005). Recombinant adenoviruses are modifications to wild-type adenoviruses wherein virulent segments in the viral gene that are responsible for immunogenicity have been removed. Another product named Advexin which is also a recombinant adenovirus with p53 plasmid DNA, is in Phase III clinical trials for treatment of head and neck cancer (Patil et al., 2005b).

Non-viral vectors can be non-immunogenic and relatively safer when compared to the viral vectors and offer a high level of control with respect to their physicochemical properties and manufacturability. Commonly used non-viral vectors for siRNA delivery are cationic as well as anionic polymeric nano- and micro-particles, lipid based systems (such as liposomes and solid lipid nano particles), micelles, conjugates and complexes with cationic cell penetrating peptides, and complexes with cyclodextrins and proteins. Many strategies include a combination of several types of non-viral delivery vectors and use of systems that incorporate combinations of several agents. Of these systems, polymers easily interact electrostatically with siRNA based on electrostatic charge-based interactions. Multiple cationic charges on the polymer helps in effective condensation of the nucleic acid and improves delivery, however this also increases their cytotoxicity. For this reason, low molecular weight polymers are preferred over high molecular weight polymers (Fischer et al., 1999; Godbey et al., 1999). Additionally, multiple charges on the polymers facilitates multi-contact points with the nucleic acid thereby slowing siRNA dissociation from the polymers, after endosomal escape. Furthermore, polymers usually have high polydispersity and it is difficult to control their molecular weight distribution (Patil et al., 2005b). Micro and nanospheres, usually made of poly(lactic-*co*-glycolic) acid are biocompatible (non-toxic). However, microspheres do require the use of a cationic moiety to interact with siRNA (Andersen et al., 2010; Howard et al., 2006).

Liposomal vesicles are some of the most commonly used non-viral vectors used to facilitate delivery of siRNA molecules. Liposomes are spherical bilayer structures with an aqueous core that help encapsulate siRNA molecules either in combination due to complexation with the lipid bilayers and/or entrapment in the aqueous compartment. Lipids can directly complex with siRNA via electrostatic interaction improving the entrapment efficiency of the system. A range of cationic, neutral and anionic liposomes have been successfully used for siRNA delivery (discussed in Section 7.0). Furthermore, liposomal system properties using these lipids can be designed and modified to promote entrapment, cellular uptake, targeting and endosomal escape of siRNA molecules. It is due to the versatility in design of these formulations and their efficiency in delivery that lipid-based siRNA delivery vehicles are the most clinically advanced among all the vector systems. The design and engineering of liposomal systems with specialized applications for siRNA delivery are described in detail in Section 7. The clinical *in vitro* and *in vivo* applications of liposome based delivery systems have been summarized in Tables 2 and 3.

In addition to liposomes, solid lipid nanoparticles (SLNs) represent an emerging class of non-viral vectors for siRNA delivery applications. In contrast to the bilayer structure observed in liposomes, solid lipid nanoparticles are monolithic lipid matrices made up of triglycerides, waxes, or cholesterol, etc. (Loxley, 2009). These are stable sub-micron sized particles that can encapsulate siRNA molecules in the lipid core or on the particle surface. These systems protect siRNA from *in vivo* degradation, improve circulation half –life and facilitate delivery (Kim et al., 2008a; Montana et al., 2007).Similar to liposomes, these systems also demonstrate tremendous control and versatility of formulation design properties and can be tailormade to support targeted and localized delivery of siRNA molecules. For example, siRNA loaded SLNs composed of cholesteryl ester, triglyceride, cholesterol, DOPE and DC-Chol (weight ratio as 45:3:10:14:28) showed efficient siRNA uptake in prostrate cancer cells (Kim et al., 2008a). Montana et al. (2007) employed SLNs (composed of Compritol ATO 888 as matrix lipid, Pluronic F68 as tenside, and dimethyldioctadecylammonium bromide (DDAB) as the cationic lipid) as a suitable RNA carrier in sea urchin model. Targeting and delivery of siRNA using solid lipid nanoparticles have been successfully demonstrated in a wide range of animal cancer models including those for breast, liver and lung (Oh and Park, 2009; Ozpolat et al., 2010). ALN-RSV and Atu-027, developed by Alnylam and Silence Therapeutics represent examples of SLNs that are currently under Phase 1 clinical trials for respiratory synctial virus (RSV) and advanced solid tumor, respectively (Table 1).

Table 2
In vitro lipid based siRNA delivery systems.

Liposome Composition	Target Gene	Particle Size (nm)	Zeta Potential (mV)	EE (%)	N/P Ratio	Silencing Efficiency (%)	Cell Lines	Reference
DOPE/CDAN (55:45)	β-gal	300–400	NM	NM	13:1	80–90	HeLa, IGROV-1	(Spagnou et al., 2004)
DPPG:DPPC (18:77:5)	EGFP	100	−20	7–9	NA	Poor	HeLa	Foged et al. (2007)
DOTAP:DOPE:Chol: PLR-PEG (70:20:5:5)	GFP	148.6 ± 8.5	+32.3 ± 3.7	100	30:1	60	H4II-E, Hep-G2	Kim et al. (2010)
OH-Chol:Tween 80:fPEG2000-DSPE (10:1.3:0.65)	HER-2	140–150	−9.4 ± 3	60 (∼)	2:1	NM	KB	Yoshizawa et al. (2008)
Anti-E-selectin-SAINT	TNFR2, VE-Cadherin	230	+25	NM	250:1	90	HUVEC, HMEC-1	Asgeirsdottir et al. (2010)
DOTAP:Chol:DSPE-PEG2000+ calcium	Luciferase	150	+40	39.8 ± 2.8	800:1	70	H-460	Li et al. (2010)
DG:DOPE:Chol (3:1:1)	RFP	150	NM	100	1.8:1	70 (∼)	B16F10	Suh et al. (2009)
DODAG:DOPE	HBV, EGFP	200–300	NM	NM	3:1	70–80 (∼∼)	OVCAR-3, IGROV-1, HeLa	Mével et al. (2010)

Abbreviations—NA: Not applicable; NM: Not mentioned; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; CDAN: N1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; DPPG: dipalmitoylphosphatidylglycerol; DPPC: Dipalmitoylphosphatidylcholine; DOTAP: 1,2-dioleoyl-3-trimethylammonium propane; Chol: Cholesterol; PLR-PEG: poly-L-arginine-conjugated polyethylene glycol; OH-Chol:cholesteryl-3β-carboxyamidoethylene-N-hydroxyethylamine; f-PEG2000-DSPE: folate-poly(ethylene glycol)-distearoylphosphatidylethanolamine conjugate; DSPE-PEG2000: 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000) ammonium salt; DG: N,N″-dioleylglutamide; DODAG: N′,N′-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide; (∼) approximately.

7. Liposome based siRNA delivery vectors

7.1. Cationic liposomes

Following the pioneering work of Felgner et al. (1987) on synthesis of the cationic lipid, DOTMA (commercially available as Lipofectin), a number of other lipids such as DOTAP (Crook et al., 1998; Kim et al., 2000) (commercially available as DOTAP:DOPE, DOTAP:Chol) and DOSPA (Lipofectamine) (Felgner, 1993) have been synthesized and utilized for nucleic acid delivery. These lipids have been very effective in improving cellular uptake of nucleic acids. Additionally, incorporation of fusogenic lipids such as DOPE in cationic liposomal sytems enormously helped in improving endosomal escape consequently resulting in significant improvement in transfection efficiency (Cullis and De Kruijff, 1979; Farhood et al., 1995; Felgner et al., 1994; Hafez and Cullis, 2001; Hafez et al., 2001; Koltover et al., 1998). Subsequently, several other liposomal formulations such as DOSPER (Kott et al., 1998) and Oligofectamine (Simeoni et al., 2003) were also utilized for nucleic acid delivery in preclinical models.

However, cationic lipids are cytotoxic due to their charge that is responsible for non-specific interaction with negatively charged cellular components (such as opsonins, serum protein and enzymes) consequently leading to serum inactivation (Liu et al., 1997; Yang and Huang, 1997; Zelphati et al., 1998), interference with the activity of ion channels; and reduction in cellular adhesion (Burger et al., 1992; Litzinger and Huang, 1992). Recent studies by Kedmi et al. (2010) using cationic lipid-siRNA nanoparticles revealed that cationic nanoparticles resulted in hepatotoxicity as well as significant weight loss in mice when compared to neutral and negatively charged nanoparticles. Furthermore, there was increased release of pro-inflammatory cytokines (such as, IL-2, TNF-α) via activation of toll like receptors-4 (TLR4) that may result in progression of cancer and increased risk of angiogenesis (Ikebe et al., 2009). Another study has shown that cationic lipids generate reactive oxygen species (ROS) that indirectly triggers TLR4 (Park et al., 2004; Soenen et al., 2009).

In order to overcome this limitation, the current focus is on developing second generation synthesis of newer cationic lipids that have minimal toxicity but still retain their transfection efficiency. This is primarily being achieved by lipid structure modification in one or more domains, (head group, linker and hydrophobic tail) which is known to regulate transfection (silencing) efficiency as well as cytotoxicity. Consequently, a number of novel lipids have been synthesized and modified to study the structure activity relationship that may help in achieving efficient and safe transfection reagents. For example several reports on hydrophobic tail modification have concluded that transfection efficiency is enhanced by unsaturation (Delepine et al., 2000; Heyes et al., 2005; Loisel et al., 2001), shorter chain length (Balasubramaniam et al., 1996; Felgner et al., 1994, 1981, 1983) and double-tailed lipids.(Cameron et al., 1999; Pinnaduwage et al., 1989) With respect to the lipid head group, the presence of guanidinium (Aissaoui et al., 2002; Mével et al., 2010; Patel et al., 2001), hydroxyalkyl (Felgner et al., 1994), or cyclic groups (Majeti et al., 2004), increases the transfection efficiency. With respect to the Linker (between the hydrophobic tail and hydrophilic head), ester linkages have been reported to be less toxic due to their biodegradability when compared to non-biodegradable linkers such as ether and carbamate (Behr, 1994; Leventis and Silvius, 1990; Obika et al., 1997). However, ether linkages have been reported to be more efficient than ester linkages (Ghosh et al., 2000). Thus, lipid structure modification has been able to effectively synthesize efficient and safer cationic lipids. The newer transfection reagents Lipofectamine 2000 (Invitrogen) and RNAiMax (Invitrogen) are two of the most commonly used for siRNA delivery that are both efficient and relatively safer compared to other primitive reagents such as Lipofectin and Lipofectamine (Dalby et al., 2004; Zhao et al., 2008).

7.2. Anionic and neutral liposomes

Due to the toxicity issues of the early cationic lipids, there has been exploration into the feasibility of anionic or zwiterionic lipids to enact as potentially safe siRNA delivery vectors (Foged et al., 2007; Halder et al., 2006; Pulford et al., 2010; Srinivasan and Burgess, 2009). The siRNA entrapment and delivery efficiency with these lipids on their own, however, is debatable due to absence of complexation-enhancing electrostatic interactions between lipids

Table 3
In vivo lipid based siRNA delivery systems.

Liposome Composition	Route	Targeted gene	Particle Size (nm)	Zeta Potential (mV)	%EE, N/P	Silencing Efficiency	*In vivo* Model	Reference
Chol:DSPC:PEG-cDMA:DLinDMA (48:10:2:40)	i.v.	ApoB	80	+2 to +4	NM	11 days	Dyslipidemia in monkeys	Zimmermann et al. (2006)
DOPC	i.v.	FAK	NM	Neutral	NM	4 days. Tumor wt reduction by 72%	Ovarian tumor in mice	Halder et al. (2006)
Transferrin antibody targeted liposomes with histidine lysine peptide	i.v.	HER-2	100	NM	NM	30 days (with multiple dosing)	Pancreatic tumor in mice	Pirollo et al. (2007)
DSPC:Chol:PEG-cDMA:DLinDMA (20:48:2:30)	i.v.	HBV	141 ± 14	NM	94 ± 4	7 days (with weekly dose upto 6 weeks)	Mouse liver	Morrissey et al. (2005)
DOPC	i.v.	IL-8	NM	Neutral	NM	Tumor weight reduction by 52%	HeyA8 and SKOV3ip1 mouse model	Merritt et al. (2008)
DOPC	i.p.	EphA2	NM	Neutral	NM	Tumor reduction by 86%	HeyA8 and SKOV3ip1 mouse model	Landen et al. (2005)
AtuFECT01 + DPhyPE+DSPE-PEG2000 (50:45:1)	i.v.	PTEN	117 ± 46.4	NM	NM	4 days (daily dosing)	Mice	Santel et al. (2006)
DOTAP:DOPE:DSPE-PEG2000 (4.75:4.75:0.5)	s.c.	V600EB-Raf, Akt3	90 (∼)	NM	100, 10:1	Effect upto 21 days	Melanocytic lesions in nude mice	Tran et al. (2008)
DDAB:Chol (1:1)	i.v.	Caveolin-1	NM	NM	NM, 5:1	90% (in 96 h)	Mouse lung model	Miyawaki-Shimizu et al. (2006)
98N12-5(1):Chol:PEG lipid (42:48:10)	i.v.	FVII	70	+2 to 4	97, 7.5:1	More than 3 weeks (single dose)	Liver tumor in mice	Akinc et al. (2009)
Chol:DSPC:PEG-cDMA:DLinDMA (48:10:2:40)	i.v.	KSP, PLK1	80	NM	NM, 9:1	Significant tumor reduction till day 28	Hepatic and subcutaneous tumor in mice	Judge et al. (2009)
Gd.DOTA.DSA:CDAN:DOPC:DSPE-PEG2000:DOPE-Rhodamine (30:31:31:7.5:0.5)	i.v.	Anti-survivin	80	NM	76	Significant reduction 72 h post-injection	Xenograft tumor model in mice	Kenny et al. (2011)

Abbreviations—NM: Not mentioned; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammonium propane; Chol: Cholesterol; DSPE-PEG2000: 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000) ammonium salt; DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; DLinDMA: 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane; PEG-cDMA: 3-N-[(ώ-methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine; DOPC: 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; AtuFECT01: β-L-arginyl-2,3-L-diaminopropionic acid-N-palmityl-Noleyl-amide trihydrochloride; DPhyPE: 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine; DDAB: dimethyldioctadecylammonium bromide; Gd.DOTA.DSA: Gadolinium (III) 2-[4,7-bis-carboxymethyl-10-[(N,N-distearylamidomethyl-Nα-amido-methyl]-1,4,7,10-tetra azacyclododec-1-yl]-acetic acid; i.v.: intravenous; s.c.: subcutaneous; i.p.: intraperitoneal; (∼) approximately.

and the siRNA. This is because siRNA is negatively charged and the lipids are either anionic or neutral in nature. Therefore, such delivery systems require a third moiety to achieve intense association to form lipoplexes. Examples of such systems include anionic lipoplexes for DNA delivery using calcium as the bridging agent which showed good activity in CHO-K1 cells (Patil et al., 2004, 2005a; Srinivasan and Burgess, 2009). Others such as Huang's group utilized the cationic polymer poly-L-lysine to prepare anionic lipid (CHEMS) associated lipoplexes (Lee and Huang, 1996) with high levels of efficiency. Foged et al. (2007) attempted preparing siRNA associated anionic liposomes without utilizing a bridging agent. Consequently, the prepared formulations showed poor encapsulation efficiency (7–9%) with no activity in HeLa cells. Whereas these groups focused on anionic lipids, Sood's group utilized neutral lipids to achieve efficient siRNA delivery. For example, Halder et al. (2006) (Sood's group) prepared neutral liposome (DOPC) associated lipoplexes that indicated efficient knockdown of the *focal adhesion kinase* gene in an ovarian tumor mice model. The tumor growth inhibition was observed for 4 days with overall reduction in tumor weight by 72%. Although in this case, neutral liposomes were efficient, there may be potential issues with their long-term colloidal stability due to absence of the repulsive forces between the particles.

7.3. Long-circulating liposomes

Liposomes with undecorated surfaces interact with blood components (such as antibodies, fibronectins, etc.), attract macrophages and are consequently cleared rapidly from the blood circulation (Gregoriadis, 1988) with clearance rates of anionic and cationic liposomes being more than neutral ones (Kabanov, 1999). However, for *in vivo* purposes, liposomes must remain in blood for longer periods at least until they reach their site of action. This has been accomplished by use of polymers such as poly-ethylene glycol (PEG). PEG is a hydrophilic polymer that when bedecked on liposome surfaces, reduces liposome recognition and uptake by RES (reticulo-endothelial system) thereby enhancing the circulation time of liposomes (Woodle, 1993). Long-circulation also helps in localization of the liposomes to tumor site where fenestrae are 200–780 nm in size (Gaumet et al., 2008) (phenomena known as

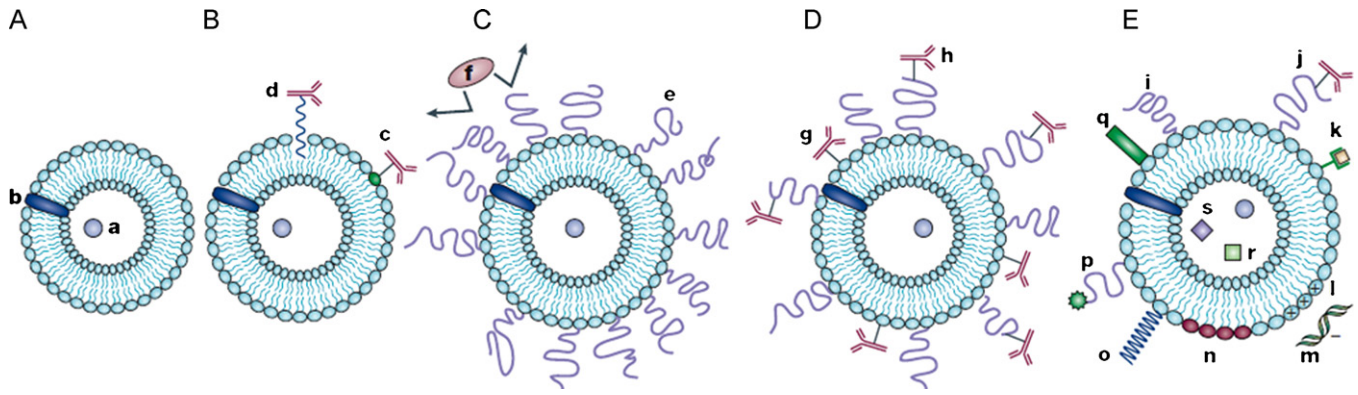

Fig. 2. *Evolution of liposomes as drug delivery systems*: (A) Early traditional phospholipids 'plain' liposomes with water soluble drug (a) entrapped into the aqueous liposome interior, and water-insoluble drug (b) incorporated into the liposomal membrane (these designations are not repeated on other figures). (B) Antibody-targeted immunoliposome with antibody covalently coupled (c) to the reactive phospholipids in the membrane, or hydrophobically anchored (d) into the liposomal membrane after preliminary modification with a hydrophobic moiety. (C) Long-circulating liposome grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f). (D) Long-circulating immunoliposome simultaneously bearing both protective polymer and antibody, which can be attached to the liposome surface (g) or, preferably, to the distal end of the grafted polymeric chain (h). (E) New-generation liposome, the surface of which can be modified (separately or simultaneously) in different ways. Among these modifications are: the attachment of protective polymer (i) or protective polymer and targeting ligand, such as antibody (j); the attachment/incorporation of the diagnostic label (k); the incorporation of positively charged lipids (l) allowing for the complexation with DNA (m); the incorporation of stimuli-sensitive lipids (n); the attachment of stimuli-sensitive polymer (o); the attachment of cell-penetrating peptide (p); the incorporation of viral components (q). In addition to a drug, liposomes can be loaded with magnetic particles (r) for magnetic targeting and/or with colloidal gold or silver particles (s) for electron microscopy. With permission from ref (Torchilin, 2005).

passive targeting). Additionally, PEG chains also provide steric stabilization to liposome particles, the efficiency of which depends on the PEG chain length (Mosqueira et al., 2001). PEG inhibits close association of lipid nanoparticles due to steric repulsion between the PEG chains. Despite this, if an overlap occurs between the polymer chains, a region with high osmotic pressure is created in the overlapped region that forces the solvent to enter in, and pushes the PEGyalted nanoparticles away, thereby helping the stabilizing of the formulations. Several reports have indicated the use of PEG associated siRNA lipoplexes for long-term gene silencing. For example, Morrissey et al. prepared PEGylated cationic lipoplexes (SNALP) for efficient gene knockdown of Hepatitis B virus. Accordingly, single dose injection in mice showed silencing for upto 7 days and weekly dosing indicated efficient knockdown for upto 6 weeks (Morrissey et al., 2005). Others achieved even longer silencing effect with single dose intravenous injection where tumor suppression was achieved for as long as 3 weeks (Akinc et al., 2009). There are several other groups that have utilized PEGylated siRNA lipoplexes to achieve long-circulation and efficient knockdown *in vivo*. For example, Kenny et al. (2011) prepared PEGylated lipoplexes with anti-survivin siRNA and obtained significant tumor reduction 72 h post-injection in mice. Santel reported effective silencing of PTEN (phosphatase and tensin homolog) in mice tumors for upto 4 days with daily dosing (Santel et al., 2006). Numerous others have reportedly used PEG for efficient siRNA delivery (Foged et al., 2007; Judge et al., 2009; Kim et al., 2010; Santel et al., 2006; Tran et al., 2008; Yoshizawa et al., 2008; Zhang et al., 2010).

7.4. Targeted liposomes

Site-specific delivery has gained attention due to its advantages of reduced dose requirement and minimal systemic toxicity. Targeted delivery is critically important for life threatening diseases such as cancer where it is crucial to reduce the systemic adverse effects and achieve maximum dose efficacy at the tumor site. Tumor cells are fast growing and have a unique cellular environment (pH and temperaute) as well as a unique requirement for nutrition compared to normal cells (for nutrients such ass iron, folic acid and sugars). Consequently, these have over-expressed cell surface receptors required for uptake of nutritional agents such transferrin receptor for iron (Pirollo et al., 2007), glycosylated receptor for carbohydrates (Wang et al., 2002), and folate receptor for folic acid (Yoshizawa et al., 2008). Liposomal systems that are designed to incorporate lipids or their modified derivatives that can be selectively internalized by tumor cell types due to the preponderance of such receptors on their surface are therefore more effective and selective in delivering their payload. In addition to small molecules, several other agents such as peptides (Song et al., 2005; Zhang et al., 2006; Adami et al., 2011), and antibodies (Khaw et al., 2001), have also been utilized to achieve targeted delivery to specific sites in the body. Localized delivery at desired sites can therefore be achieved by utilizing targeting ligands that selectively recognize and bind to target antigens or receptors over-expressed or selectively expressed on desired cell type. Targeted systems therefore improve efficiency of non-viral vectors by delivering siRNA to specific sites. For example, Pirollo et al. (2007) used transferrin antibody targeted lipoplexes that resulted in knockdown of HER-2 in mice tumors after intravenous administration for upto 30 days after three dosings. In another report, DOTAP/DOPE liposomes modified with hyaluronic acid were used for site specific delivery of anti-telomerase siRNA to CD44-expressing lung cancer cells (Taetz et al., 2009). Streipe et al. (2010) prepared RGD-peptide (arginine-glycine-aspartic acid tripeptide) and anti-IGF1-receptor antibody modified liposomal siRNA delivery systems for specific recognition by Integrin and IGF1-receptor in alveolar rhabdomyosarcoma cells thereby ensuring improved siRNA delivery to the targeted site. Bedi et al. (2011) prepared siRNA loaded liposomes with phage fused MCF-7 cell specific peptide DMPGTVLP to efficient targeted delivery to breast cancer cells.

7.5. Multifunctional liposomes

The design strategy of multifunctional liposomal systems, utilize surface decoration using a range of ligands with specific properties. These surface moieties include targeting ligands, fusogenic molecules, stealth strategies and thus offer a highly derivatized and well-engineered platform with high efficiency *in vivo*. Evolution of liposomes for drug delivery has been very well defined by Torchilin (2005). Accordingly, conventional or first generation liposomes were utilized for delivery of water soluble and water insoluble drugs (Fig. 2A). Liposomes were further modified with targeting ligands to improve their site specificity (Fig. 2B). Stepping forward,

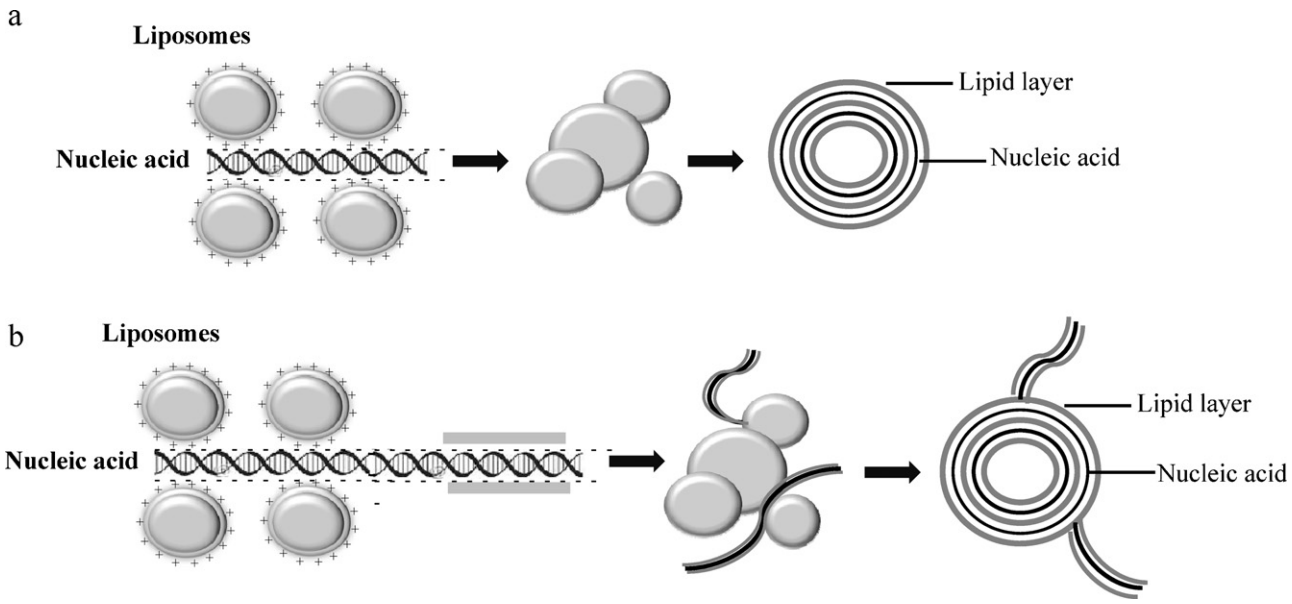

Fig. 3. *Lipid-DNA assemblies*: Arrangement of lipid and nucleic acids in lipoplex assemblies.at different charge ratios. (a) At low charge (DNA to lipid) ratios there are meatballs (liposomes) condensing DNA in between (intermediate stage) eventually forming multilayered structures sandwiching DNA (black) in between the lipid layers (grey). (b) At high charge (DNA to lipid) ratios the meatball spaghetti arrangement involves excess nucleic acid (*e.g.* DNA in black) surrounded by lipid layers (grey) forming spaghetti (fibrillar) structure.

the particle stability and rapid blood clearance were reduced by utilizing hydrophilic polymers such as PEG on the liposome surface (Fig. 2C). The two strategies were then combined where targeted ligands were attached onto PEGylated surfaces to facilitate long circulation along with targeting (Fig. 2D). Recently, several other modifications are possible with liposome systems for example, modification in part of the liposomes with cell penetrating peptides, cationic lipids (for nucleic acid association), stimuli-sensitive lipids and polymers (Fig. 2E). Additionally, liposomes can also be encapsulated with magnetic particles to facilitate microscopic imaging. Such classical explanation on evolution of liposomal surfaces suggests utilization of multifunctional liposomes for nucleic acid delivery. There are several examples in the literature where multiple features have been incorporated into liposomal systems to obtain multifunctional nanocarriers. For example, Li et al. (2010) prepared anisamide conjugated PEGylated liposomes that indicated efficient silencing of luciferase in H-460 cells. Yoshizawa et al. (2008) used PEGylated-folate lipoplexes for efficient knockdown of HER-2 in KB cells. Mendonca prepared transferrin-conjugated PEGylated liposomes loaded with anti-BCR-ABL siRNA for treatment of chronic myeloid leukemia in K562 and LAMA-84 cells (Mendonca et al., 2010). Kim et al. synthesized poly-L-arginine conjugated PEG (PLR-PEG) and prepared liposomes using the cationic lipid DOTAP, fusogenic lipid DOPE, cholesterol and PLR-PEG. Such arginine conjugated targeted liposomes were utilized to knockdown GFP in H4II-E and HepG2 cells. Accordingly, it was found that PLG-PEG liposomes were 30% more efficient than conventional liposomes (non-PEGylated and non-targeted) at N/P ratio of 30:1 (Kim et al., 2010). This was mainly due to site specificity resulting from arginine and particle stability as a result of PEG. Arginine has been used as a targeting ligand by Zhang et al. (2006) where a PEGylated octamer of arginine (R8) was decorated on siRNA-loaded cationic liposomes for efficient silencing of HDM2 gene (human double minute gene2). Santos et al. (2010) prepared antagonist G associated targeted PEGylated liposomes for downregulation of Bcl-2 in SCLC SW2 tumor cells. There are several other examples utilizing multifunctional nanocarriers briefly reviewed by Oliveira et al. and others (Cardoso et al., 2009, 2007; Herringson and Altin, 2009; Musacchio and Torchilin, 2011; Oliveira et al., 2006).

8. Formulation optimization of siRNA delivery systems

Efforts to improve the delivery of siRNA molecules have resulted in a wide range of lipid-based systems that vary in terms of their composition and physicochemical properties. The composition and molecular assembly of liposomal systems can be designed so that these particles can be preferentially internalized by cells containing the siRNA target and deliver the desired payload in the intended cellular compartment. Furthermore, both composition as well as physicochemical properties of lipid particles can influence functionality and release of the entrapped siRNA *in vitro* as well as *in vivo*. Due to this intricate engineering, siRNA entrapped lipid-based particles can achieve a high level of complexity on a structural and functional level. The inclusion of lipids with specific functions, targeting moieties, surface decoration ligands and agents that modify the drug release properties can further impact the complexity of particles. It is therefore critical to develop well-characterized systems with consistent properties that are stable over extended periods of time to ensure efficacy and toxicity and eventually to guarantee the intended clinical performance.

With a growing emphasis by regulatory agencies to use Quality by Design principles on formulation development and optimization, it is imperative more than ever to develop well-characterized systems that ensure consistent performance in the clinic. Due to the potential complexity of composition, it is evident that the overall design space in developing lipid-based systems and the resulting permutations and combinations can be challenging. The effect of changing the process and compositions can be difficult to identify and resource-limiting to develop.

The development of physicochemical assays that, at the very least, can be used to correlate to *in vivo* activity or at the very best can be used to predict *in vivo* performance is therefore important in formulation design and optimization. Just as increased complexity of particles can be beneficially exploited to tailor-make release properties and intra-cellular disposition, it can also open up avenues for impacting the stability of the complex and the entrapped siRNA molecule. In addition, physicochemical properties are important stability-indicating indicators that can be used to monitor the long-term stability of these formulations.

Table 4
Formulation parameters for liposome based siRNA delivery systems.

Formulation Attribute	Physicochemical Technique	Preferred Attribute
Lipid Structure	NMR, Mass, FTIR, TLC	Unsaturated double tailed preferably with biodegradable linker Uniform composition in individual liposome
Liposome Composition	HPLC, UV	Cationic/anionic/neutral lipid for membrane and cell-association DOPE for fusogenicity Cholesterol to reduce leakage PEGylated lipids to improve half life Targeting ligands to improve selectivity
N/P Ratio	HPLC, UV	>1:1 molar ratio
Particle Size	DLS, Electron Microscopy	80–100 nm (*in vivo*) Low polydispersity index
Surface Charge	Electrophoresis	−30 mV to +30 mV to prevent flocculation of particles
Encapsulation Efficiency	UV, fluorescence	>80%, high drug to lipid ratio

There are several physicochemical attributes that can be used for formulation optimization for safety and efficacy of liposome based siRNA delivery. These can be classified based on the property of the particles they characterize as summarized in Table 4.

9. Characterization of siRNA liposomal delivery systems

9.1. Morphology of cationic lipoplexes

Morphological characterization of liposomal siRNA vectors helps in understanding the relative structure, orientation of the siRNA molecules and assembly of the complexes. These characteristics can influence the stability of the complex *in vitro* as well as *in vivo* and also can impact the bio-distribution. Morphology of nucleic acid and liposome complexes can be studied using techniques such as cryogenic transmission electron microscopy (cryo-TEM) and freeze fracture electron microscopy.

Cryo-TEM is an electron microscopy technique that uses vitrified sample film obtained by freezing the sample film (−196 °C) on a carbon coated grid. Whereas, in case of freeze fracture microscopy, the sample is frozen (−196 °C), fractured by increasing the temperature to around −100 °C to allow ice sublimation followed by obtaining replica by shadowing on evaporating platinum or gold under high vacuum (Sawyer et al., 2008).

Several electron microscopic studies on DNA lipoplexes have revealed arrangement of lipid bilayers with DNA in one of the two forms as shown in Fig. 3. Based on their charge ratio (Gustafsson et al., 1995; Lasic, 1997b; Radler et al., 1997; Sternberg, 1996; Sternberg et al., 1994). This arrangement wherein negatively charged DNA is condensed between two or more cationic liposomes is commonly known as the 'meatball and spaghetti model' (Fig. 3a). The meatballs with DNA in-between, eventually form concentric multilayered structures as a result of fusion between the lipid layers and consequent encapsulation of DNA between these lipid layers (Lasic, 1997b; Radler et al., 1997). This spaghetti, which are the fibrils formed as a result of DNA being sandwiched between the lipid layers on each side (Fig. 3b), are more prominent at high charge ratios (DNA to lipid ratio) (Sternberg et al., 1994).

Morphological studies of lipoplexes composed of nucleic acids other than DNA, such as antisense oligodeoxynucleotide (ODN) and siRNA have been reported (Ciani et al., 2007; Desigaux et al., 2007; Weisman et al., 2004). Weisman et al. (2004) characterized lipid-ssODN complexes that revealed structures similar to those of DNA lipoplexes. Accordingly, after complexation, the anionic ODN (18-mer anti-bcl2) was observed to be in-between the lipid layers of DOTAP/DOPE liposomes (originally SUV/OLV) forming multilayered structures. From the cryo-TEM images, concentric lamellar structures were observed characterized by 'triplet' parallel lines formed by ODN in between lipid layers on either side irrespective of the charge ratio (Fig. 4). Weisman represents 'triplet' forms at low charge ratios of 0.2 and 0.33 (ODN/lipid). At high charge ratio (ODN/lipid 1:1) other than the triplet structures, some aggregates were also observed. This has been speculated as a result of restricted growth of lamellar particles due to limited lipid concentration and the promotion of aggregate formation due to the association of outer membranes of adjacent particles. Limited lipid concentration also explains lamellar defects that were observed at high charge ratios. Further, cryo-TEM and SAXS studies revealed that these multilayers were more consistently spaced than in the case of MLVs (Weisman et al., 2004). Weisman also highlighted key differences observed between the morphology of DNA and ssODN lipoplexes. Firstly, the aqueous core thickness of DNA (Radler et al., 1997) is much larger (2.3 nm) than ODN (1.18 + 0.23 nm as found by Weisman). Secondly, DNA is more orderly intercalated between the lipid layers compared to ODN. Thirdly, at high charge (DNA/lipid) ratio, spaghetti structures are visible in case of DNA but not ODN. Ciani et al. (2007) obtained similar results with dsODN at different charge ratios.

Lasic (1997a) and later Weisman et al. (2004) proposed a model explaining the mechanism of formation of these multilayered

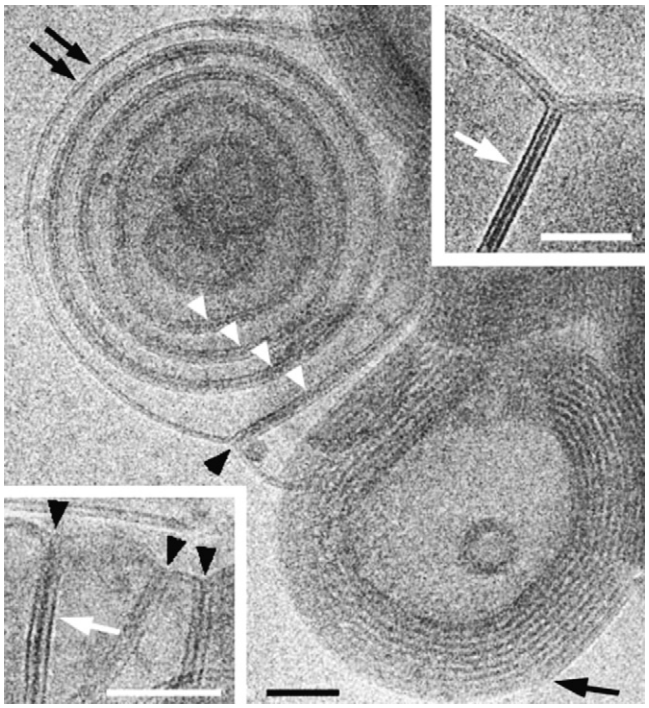

Fig. 4. *Lipid-ODN assemblies*: Cryo-TEM images of systems with excess lipid, showing the coexistence of several nanostructures. A lipoplex of DOTAP/Chol 1:1 with ODN/lipid charge ratio 0.2 contains discrete membranes (double arrow), paired membranes (white arrowheads), and condensed lamellar phase particles (black arrow). Upper inset: DOTAP/Chol 1:1, charge ratio 0.33: a clearer image of paired membrane structures (white arrow). Lower inset: DOTAP/Chol 2:1, charge ratio 0.2: adsorption of liposomes to each other to form paired membrane structures, with black arrowheads indicating membrane junctions and a white arrow pointing to a paired membrane. Scale bars represent 50 nm.
With permission from ref (Weisman et al., 2004).

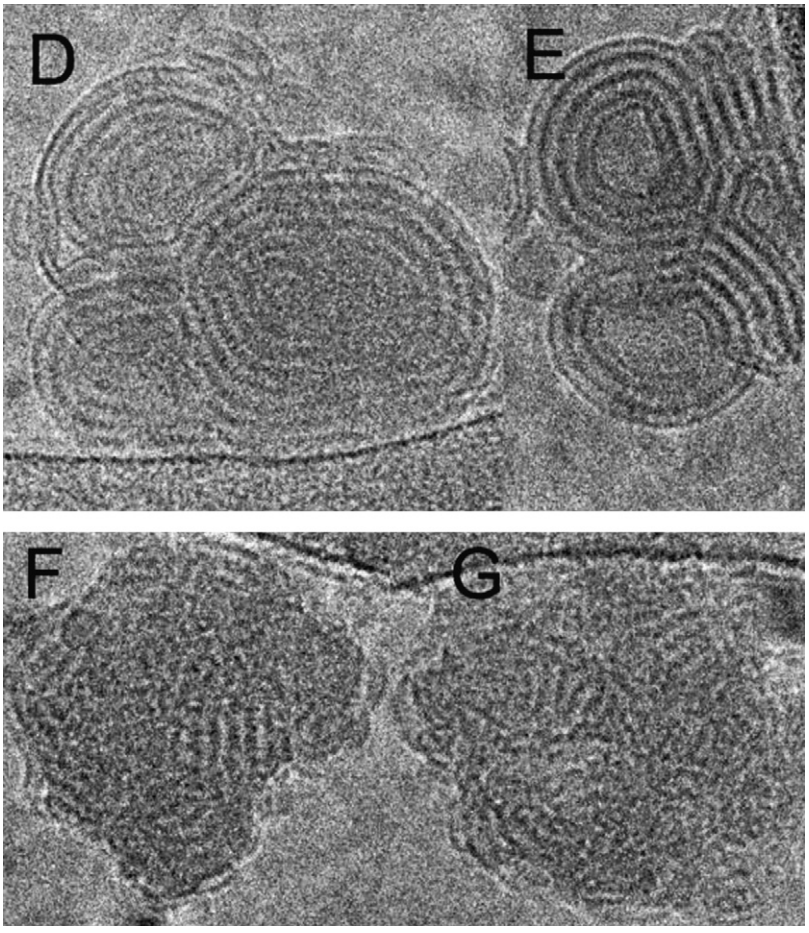

Fig. 5. *Morphology of Lipid-siRNA complexes*: Concentric "onion-like" structures as indicated by Cryo-TEM micrographs of cationic lipid/siRNA complexes. Note the regular arrangement of the RNA molecules at the edge (black arrow). (*D* and *E*). Structure of two DOSK/siRNA complexes at high magnification. (*F* and *G*). Structure of two DOSP/siRNA complexes at high magnification. (Scale bar: 50 nm.)
With permission from ref (Desigaux et al., 2007).

lipoplex structures. Accordingly, ODN first adsorbs onto one liposome. On the other side it adsorbs onto another liposome. This is followed by restructuring of lipid layers (*i.e.* wrapping one layer over the other with condensed nucleic acid in between the layers).

Desigaux et al. (2007) prepared lipoplexes with cationic liposomes and siRNA. Liposomes were prepared using cationic lipids with different aminoglycoside head groups, along with DOPE. All lipoplexes irrespective of their head groups, showed multilayered structures with interlamellar spacing of 7 nm (sum of thickness of lipid bilayer and siRNA). However, the lipids with 4,6 DDS ring (4,6-disubstituted 2-deoxystreptamine) such as dioleyl succinyl kanamycin A (DOSK), dioleyl succinyl tobramycin (DOST), indicated concentric lamellar structures (200–500 nm). Those with a (4,5-DDS) ring (4,5-disubstituted 2-deoxystreptamine) such as dioleyl succinyl paromomycin (DOSP) and dioleyl succinyl ethylthioneomycin B (DOSN), showed irregular structures of size around 60 nm. This was explained on the basis that lipids with 4,6 DDS ring formed larger liposomes than lipids with 4,5 DDS ring. This larger size helped in forming lamellar structures over a long range (onion ring like) (Fig. 5D and E) while smaller liposomes (DOSP, DOSN) helped forming short range ordered structures as shown in Fig. 5F and G (Desigaux et al., 2007).

Formulation optimization based on morphological characterization can be useful as studies have shown correlation of lipoplex morphology to its transfection efficiency. For example, spaghetti structures (at high charge ratio) have been shown to be highly efficacious due to effective interaction of high curvature structure with the cell membrane thereby enabling better penetration (Sternberg et al., 1994). Besides, for siRNA lipoplexes as characterized by Desigaux, the smaller irregular structures (Fig. 5F and G above) are more efficacious in d2GFP, HeLa and HEK293 cells when compared to larger concentric layered structures (Fig. 5D and E). This is due to more efficient endocytic uptake of smaller complexes and better endosomal destabilization due to the cone structure of participating cationic lipids (DOSP and DOSN) (Desigaux et al., 2007).

From the above examples it is clear that morphology affects transfection efficiency. Since morphology is influenced by charge ratio and cationic lipid structure, this explains why these factors affect transfection efficiency. There are several other factors such as incubation time, DOPE content and cationic lipid content that affect the morphology of lipid-nucleic acid complexes and hence efficacy (the details of which are elsewhere, Sternberg, 1998). There are other reports where siRNA lipoplexes have been characterized using cryo-TEM however a detailed study of morphology as a function of formulation paramaters has not been performed (Crawford et al., 2011; Geusens et al., 2009).

9.2. Encapsulation efficiency

Encapsulation efficiency (EE) determines the loading of siRNA in liposomal formulations. EE is critical as it can be used to optimize the formulation composition as well as the manufacturing process. From a formulation design perspective, high EE is advantageous to maximize the drug to lipid ratio and to facilitate development of a

dose-sparing formulation. From a manufacturing process perspective, control of the entrapment process demonstrates a high level of consistency and reduced variability.

Encapsulation of siRNA into the liposomes can be achieved during liposome preparation or after liposome preparation. The dried lipid film can be hydrated with an aqueous solution of siRNA to form siRNA encapsulated MLVs which are then freeze-thawed followed by extrusion. This passive method of siRNA loading results in poor EE of around 10–20% if the lipids are negative or neutral (Auguste et al., 2008; Foged et al., 2007) and hence poor knockdown. Strategies to improve entrapment efficiency of passive systems include inclusion of highly charged cationic molecules such as as poly-L-lysine (PLL) R8-MEND (octaarginine modified multifunctional envelope-type nanodevice) and protamine (Nakamura et al., 2007). For example, when siRNA precondensed with peptide (R8-MEND) was encapsulated into DOPE/CHEMS (9:2 mol/mol) lipids during the hydration process, the EE was approximately 87% (Nakamura et al., 2007).

For siRNA entrapment after liposome preparation, the entrapment occurs as a result of lipid–siRNA electrostatic interaction, followed by restructuring of the lipid layers. This method results in increase in EE as the N/P ratio increases, thereby facilitating efficient knockdown (Akinc et al., 2009; Kim et al., 2010; Morrissey et al., 2005; Suh et al., 2009).

EE is calculated using either formula 1 or 2:

Formula 1:

$$\%EE = \frac{\text{Encapsulated siRNA concentration}}{\text{Initial siRNA concentration}} \times 100$$

Encapsulated siRNA concentration is obtained after solubilising the liposomes using surfactants such as TritonX-100 (most common).

Formula 2:

$$\%EE = 100 - \left(\frac{(\text{Free siRNA concentration})}{(\text{Initial siRNA concentration})} \times 100\right)$$

Initial siRNA concentration is measured theoretically or determined in the solution during the unit operation processes.

There are several techniques to determine EE namely absorbance/fluorescence assay, ultrafiltration centrifugation and gel retardation assay. In the first technique, initial concentration and encapsulated siRNA (formula 1) or free siRNA (formula 2) is determined using absorbance (λ 260 nm) or fluorescence (ribogreen, (490 nm/520 nm). Percentage EE is then calculating using formula 1 (encapsulated) or formula 2 (free), respectively. Auguste et al. (2008) determined EE of PEGylated liposomes using fluorescence (ribogreen) assay wherein EE was ca. 19%. Another technique called ultrafiltration centrifugation is similar to the first technique. In this method, lipoplexes are filtered through a membrane of approximate size 30–100 kDa, so that free siRNA can pass through while entrapped siRNA is retained. The concentration of free siRNA is then determined using an absorbance or fluorescence assay to determine EE (using formula 2). Zhang et al. used this method to determine the effect of various formulation parameters (liposome composition, N/P ratio) on EE. They passed the lipoplexes through 100 kDa ultra centrifugal filters at 3000 × *g* for 10 min. The complexes were then washed 5 times and the siRNA concentration in the filtrate was determined using UV absorbance at 260 nm (Zhang et al., 2010). The third technique to determine EE is gel retardation assay, whereby the free siRNA band migrates towards the cathode (+) under electrophoresis. The concentration of the initial and free siRNA is determined using gel band quantifying software such as Image J. Often this method is used for qualitative rather than quantitative purposes due to lack of a universal method for quantification.

EE depends on a number of factors such as the N/P ratio (Kim et al., 2010; Suh et al., 2009), liposome size, cationic lipid content (Zhang et al., 2010) and the nature of the nucleic acid (Spagnou et al., 2004). Usually higher lipid concentration with fixed siRNA concentration (high N/P ratio) and larger liposomes have greater EE since there is more of lipid or lipid area available to encapsulate the siRNA. After a certain lipid concentration, any further increase brings no change in EE indicating that saturation has been reached. For example, Tran et al. performed gel retardation assay to determine N/P ratio that gives 100% EE. There was nearly 20% encapsulation at a N/P of 5:1 which did not improve even when the complex incubation time was increased from 0.5 to 6 h (Tran et al., 2008). However, when the N/P ratio was increased to 10:1, 100% encapsulation was achieved. Yoshizawa also studied migration of siRNA in folate lipoplexes on gel as a function of charge ratio and accordingly achieved 100% encapsulation at a charge ratio of 4:1 (Yoshizawa et al., 2008). However, since at this ratio, complexes were cytotoxic, they used a 2:1 ratio for silencing studies where there was approximately 50% encapsulation. Even at this low encapsulation, significant silencing of HER-2 protein was achieved (compared to non-folate lipoplexes) indicating that EE may not be the key determinant of *in vitro* activity. Zhang et al. performed a gel retardation assay on DC-Chol/DOPE–siRNA lipoplexes at N/P of 5:1 (w/w). They observed approximately 100% encapsulation when an ultrafilteration method was adopted (Zhang et al., 2010), but approximately 80% EE using the gel retardation assay at DC-Chol/DOPE ratio of 2:1 (Fig. 6A). This indicates that the lipoplexes may have weak ionic interactions and so they may be unable to retain some siRNA during electrophoresis. Consequently, gel retardation assay is not always a true representation of EE. This is in agreement with the results of Han et al. (2008). Besides studying the effect of *N/P* ratio, Zhang et al. (2010) also studied the effect of liposome composition on EE using a gel retardation assay. It was determined that as the DC-Chol/DOPE ratio was reduced (Fig. 6A–F), there was improvement in encapsulation indicating that a limited amount of DC-Chol is required to appropriately interact with the siRNA. Several other reports have been published on the effect of formulation parameters on the EE (Kim et al., 2010; Suh et al., 2009; Yang et al., 2011).

9.3. Particle size

Size and size distribution measurements are formulation parameters that indicate homogeneity of the particles in liposomal formulations and can be used for formulation and process optimization. The poly-dispersity index of a formulation reflects the range of particle species present around the target average particle size. Uniformly sized particles with a lower poly-dispersity index (<0.2) are preferred. Changes in the average particle size and the poly-dispersity index can be used as indicators of long-term stability. From an *in vivo* perspective, particle size and size distribution determine the suitability of the route of administration and the clearance of the lipoplexes upon administration.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) are the most common techniques to determine particle size of nano-range lipoplexes. DLS is based on the measurement of intensity fluctuations (as a result of Brownian motion) and obtaining correlation to the particle size using Stokes-Einstein equation. DLS can determine the particle size of the entire sample. However, it provides a hydrodynamic size which is larger than the actual size. TEM determines the actual size however due to time involved only a small population can be measured.

The size of the lipoplexes is usually larger than the liposomes due to lipid structural rearrangement involved in siRNA encapsulation (Santel et al., 2006; Suh et al., 2009). There are several reports that have shown that lipoplex particle size of approximately 200–400 nm results in high *in vitro* silencing efficiency *in vitro*. However others have reported that even lipoplexes of size 1 or

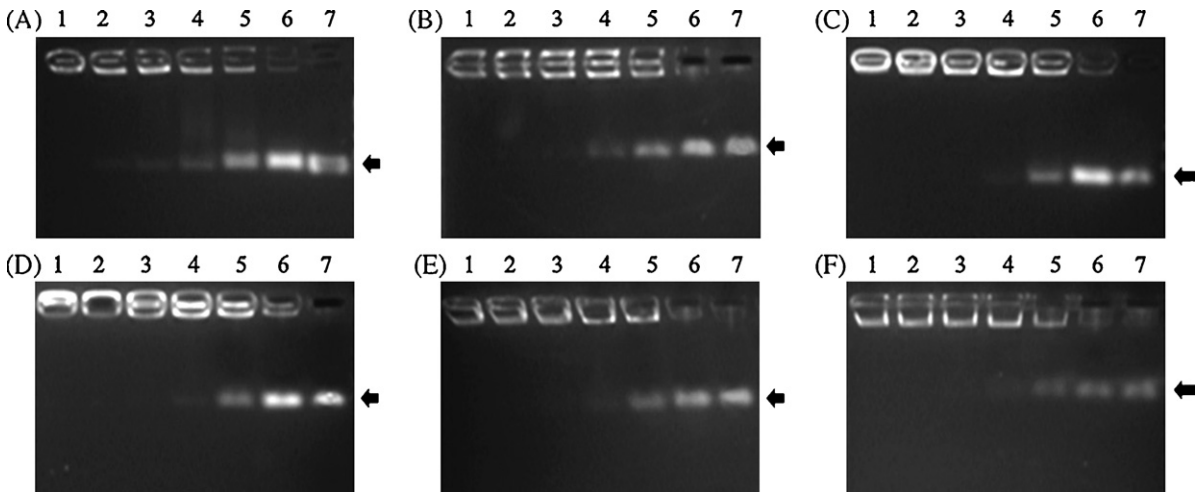

Fig. 6. *siRNA gel retardation assays*: DC-Chol/DOPE liposomes were complexed with siRNA at various weight ratios, and then run through a 2% agarose gel. The mobility of siRNA was visualized by ethidium bromide staining. The weight ratio of DC-Chol/siRNA was 15, 10, 7.5, 5, 3 and 1 (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane 7, 0.3 μg siRNA. (A) DC-Chol/DOPE = 2/1; (B) DC-Chol/DOPE = 3/2; (C) DC-Chol/DOPE = 1; (D) DC-Chol/DOPE = 1/2; (E) DC-Chol/DOPE = 1/3; (F) DC-Chol/DOPE = 1/4. The bars were used to indicate the siRNA bands in the gels.
With permission from ref (Zhang et al., 2010).

2 μm result in high transfection efficiency *in vitro* (Esposito et al., 2006; Masotti et al., 2009; Zuhorn et al., 2005). Conversely, several studies have reported that smaller particle size of (100 nm or less) is optimal for *in vivo* silencing efficiency (see Table 3).

Several factors determine particle size such as charge (N/P) ratio, liposome composition and media ionic strength. High positive or negative charge (N/P) ratio forms smaller complexes relative to when the charge is near neutral (Almofti et al., 2003; Eastman et al., 1997). Zhang et al. prepared cationic lipoplexes with different N/P ratios and observed a decrease in particle size with increase in the N/P ratio. At a charge ratio of 5:1, the average particle size was 3000 nm and this reduced to 400 nm at charge ratios of 20:1 onwards (Zhang et al., 2010). This was reported to be due to charge neutralization at low N/P ratio. Contrary results were obtained by Yoshizawa et al. (2008) who reported an increase in particle size with increase in N/P ratio from 2:1 to 3:1.

Besides N/P ratio, liposome composition has also been shown to affect the particle size. When particle size analysis on liposomes with different compositions (DC-chol/DOPE) was performed, it was reported that the particle size reduced 10-fold when the composition was changed from 2:1 (w/w) DC-Chol/DOPE ratio to 1:2 (w/w). It is interesting to note that this difference persisted only at low N/P ratio of 5:1–10:1 (Zhang et al., 2010).

The ionic strength of the media plays a crucial role in determining colloidal sizes, specifically with charged surfaces as in the case of cationic or anionic lipoplexes. Suh et al. studied the effect of NaCl concentration on DG-siRNA lipoplex size (DG: N,N″-dioleylglutamide) (N/P of 3.6). As indicated in Fig. 7, the size remained consistent up to 300 mM NaCl after which it consistently increased from around 200 nm (at 300 mM NaCl) to as high as 800 nm at 500 nM NaCl (Suh et al., 2009). This might be due to aggregation of particles in the presence of excess electrolytes as a result of charge shielding. For other examples please refer to the cited references (Ferrari et al., 2001; Hays et al., 2007; Yoshizawa et al., 2008).

9.4. Surface charge

Surface charge determination is critical for understanding the cellular uptake of particles. siRNA entrapped lipoplexes are usually considered to be internalized into cells via endocytosis (Molnar et al., 1977; Straubinger et al., 1983) although some cells also take up macromolecules by the fusion process (Papahadjopoulos et al., 1973). For lipoplexes to interact with the negatively charged cell membranes (due to heparin sulfate proteoglycans and integrins), it is believed that a slight positive charge is usually helpful. This is why cationic lipoplexes have been enormously successful in siRNA delivery. For example, Zhang et al. prepared DC-chol/siRNA lipoplexes at different N/P ratios. It was determined that with increase in surface charge of ca. (−30 mV) to (+50 mV) from 1:1 to 5:1 (N/P ratio) (Fig. 8a), transfection efficiency increased from 50 to almost 90% (Fig. 8b). Beyond 5:1 N/P ratio, the positive charge was constant and so was the transfection efficiency indicating that sufficient positive charge has already been obtained at the 5:1 ratio

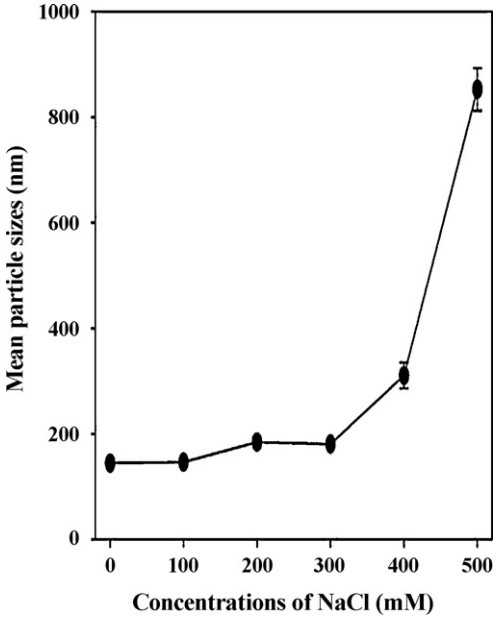

Fig. 7. *Effect of ionic strength on particle size*: DG-based cationic liposomes were complexed with siRNA at the N/P ratio of 3.6. The size of the lipoplexes at different NaCl concentrations was measured by light-dynamic scattering.
With permission from ref (Suh et al., 2009).

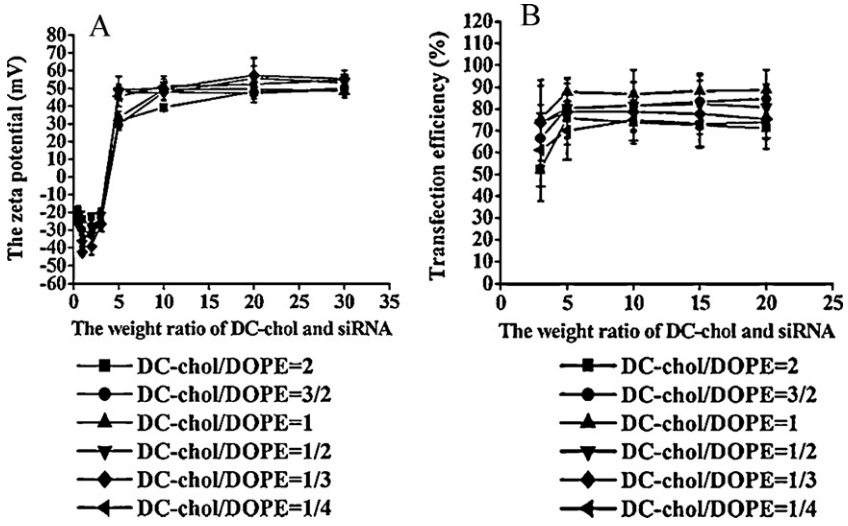

Fig. 8. (A) The zeta potential and (B) transfection efficiency (serum-free media), of DC-Chol/DOPE liposomes/siRNA complexes at different liposome compositions (in serum free media).
With permission from ref (Zhang et al., 2010).

(Zhang et al., 2010). However, negatively charged lipoplexes also have been reported to have good transfection efficiencies. Bajoria et al. (1997) have reported better uptake of carboxyfluorescein encapsulated anionic liposomes compared to neutral and cationic ones by human trophoblast cells. Although anionic liposomes show poor association with negatively charged cell membranes due to repulsion of the negative charges, the *in vivo* efficacy of these systems is speculated to be due to adsorption of phagocytic promoting factors such as α_2-macroglobulin, IgG and C-reactive proteins on the surface of liposomes (Bonté et al., 1987; Molnar et al., 1977). Alternately, anionic charge could have altered the conformation of proteins which intern improved cell recognition (Dini et al., 1991; Senior, 1987). The negative charges of anionic liposomes can be shielded using divalent cations (calcium, barium) so that the cellular interaction of such systems can be facilitated by the cation (Patil et al., 2004, 2005a; Srinivasan and Burgess, 2009). In this case, calcium caused lipid scrambling and redistribution which helped with endosome destabilization and release of the cargo (DNA).

Neutral liposomes have been shown to be effective *in vivo*, although the mechanism of their delivery is poorly understood. Landel et al. used neutral liposomes (DOPC) to effectively suppress tumor growth (by 86%) in mice after intraperitoneal injection (Landen et al., 2005). The efficiency of such systems suggests that enhanced cellular interaction due to liposome surface charge is only one of several characteristics that determine transfection efficiency.

Particle charge is critical in determining long-term colloidal stability of lipoplexes (Wiese and Healy, 1970). It is generally believed that particles with a zeta potential of (+30) mV or more provide a sufficient barrier to prevent agglomeration and flocculation of particles. Generally, the overall surface charge of a lipoplex is primarily determined by the N/P ratio. Zhang studied the effect of N/P ratio on surface charge. As expected the zeta potential increased gradually from (−40) mV to (+50) mV when the N/P ratio was increased from 1:1 to 5:1 (w/w). Beyond 5:1 there was a further increase in surface charge indicating that saturation has been reached (Zhang et al., 2010). Similar results were obtained by Li et al. (2010) where there was a gradual increase in surface charge with increase in N/P ratio. Another factor affecting surface charge is the ionic strength of the media. As mentioned in the previous section (particle size), very high ionic strength can cause charge neutralization thereby resulting in particle aggregation and hence large particle size.

9.5. siRNA release

siRNA release from lipoplexes is critical to ensure the nucleic acid can interact with and inhibit the target of interest. From a drug delivery perspective, siRNA release studies can be used as a surrogate to simulate the physiological environment that the siRNA lipoplexes experience prior to and following internalization. The release of siRNA should not occur in circulation prior to internalization by the target cells. Upon cellular uptake, in the low pH (4–5) environment of the endosome compartment, siRNA molecules entrapped in pH sensitive lipid systems are designed to destabilize the endosome and release siRNA in the cytoplasm (pH 7). Accordingly, siRNA lipoplex release studies should be performed at pH 7 to show no release of the siRNA, followed by immediate change to pH 4–5 to show siRNA release. Li et al. prepared lipid coated calcium phosphate nanoparticle and studied the degradation of nanoparticles in the pH range of 4–7 using absorbance spectrometry. They observed no degradation of the nanoparticles at pH 6 and 7 but there was a fast degradation at pH 4 and 5 (Fig. 9) (Li et al., 2010).

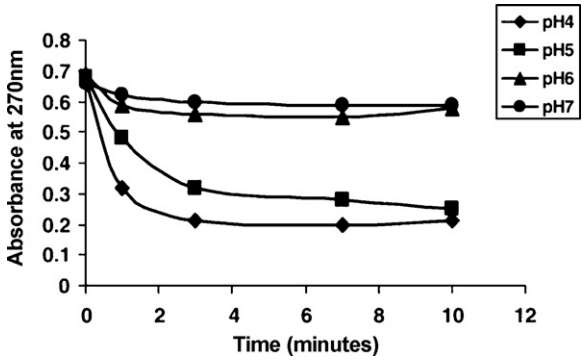

Fig. 9. *pH-release profile*: The release profile of lipid coated calcium nanoparticles, using light scattering method at different pH.
With permission from ref (Li et al., 2010).

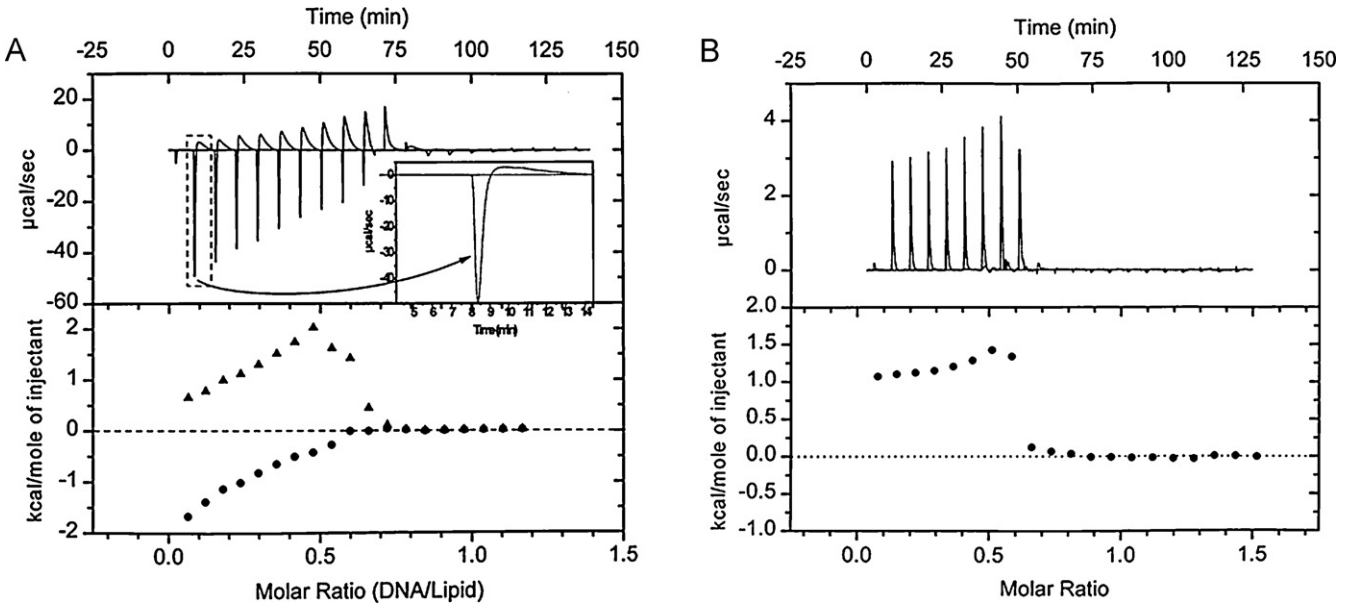

Fig. 10. *ITC of lipid-DNA complexes*: Isothermal titration calorimetry (ITC) of unilamellar diC14-amidine liposomes with plasmid DNA (pcDNA 3.1) in a 10 mM HEPES buffer (pH 7.3) at 28 °C. (A) High lipid concentration. The upper part shows the calorimetric trace as a function of time. Each peak corresponds to the injection of 10 μl of a DNA solution (76.2 mm in nucleotides) in a 1.33-ml cell filled with the liposomal suspension (10.1 mm diC14-amidine total). The inset represents an enlargement of the first peak of titration. The lower part of A shows the binding isotherm resulting from integration with respect to time: reaction enthalpy (kcal/mol of injectant) is plotted as a function of the DNA:lipid molar ratio; ●, exothermal component of the titration curve; ▲, endothermal component of the titration curve. (B) Low lipid concentration. The upper part shows the heat capacity tracings as a function of time. Each peak corresponds to the injection of 10 μl of the DNA solution (8.8 mm in nucleotides) in a liposomal suspension (0.93 mm diC14-amidine total). The lower part of B shows the binding isotherm resulting from integration with respect to time: reaction enthalpy (kcal/mol of injectant) is plotted as a function of the DNA:lipid molar ratio.
With permission from ref (Pector et al., 2000).

9.6. Lipid-nucleic acid interaction studies

For a molecular level understanding of lipid-nucleic acid interaction isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) have been used. Pector et al. (2000) performed ITC studies on cationic lipid (di C14-amidine)–DNA complexes at different charge (DNA/lipid) ratios. It was reported that at low charge ratio (high lipid concentration) a fast exothermic reaction followed by a slow endothermic process occurs. However, at high charge ratio only the endothermic process occurs. Here the exothermic process is due to electrostatic binding of cationic lipid and DNA whereas the endothermic process is attributed to structural rearrangement of the lipid bilayers to encapsulate DNA. These charge ratio processes were explained on the basis of a kinetic model as represented below.

$$\underset{(\text{Step 1})}{D + L \underset{k_2}{\overset{k_1}{\rightleftarrows}} C} \xrightarrow[(\text{Step 2})]{k_3} F$$

Accordingly, lipid (L)-DNA (D) complexation is a two step process. In step 1, lipid and DNA electrostatically interact with each other and form a soluble lipid-DNA complex (C). This process is quick and exothermic as indicated by ITC at high lipid concentration (Fig. 10A). Following this, in step 2, the complex (C) undergoes lipid structural rearrangement (Step 2) resulting from lipid bilayer collapse thereby forming fused complexes with encapsulated DNA (F). This is a slow and entropy driven endothermic process.

At low DNA/lipid ratios (same lipid (L) concentration), the rate for consumption of DNA (D) is equal to rate of formation of the complex (C). Since the transformation of complex (C) to fused (F) form is slow, the complex form accumulates (exothermic peak). This indicates that k_3 (C to F) is the rate limiting step. However, at high DNA/lipid ratios, D and L interact to form C which transforms immediately into F indicating that k_1 (D + L to C) is the rate limiting step. This justifies the absence of exothermic peak at high DNA/lipid ratios (Fig. 10B). Such models can be used to analyze lipid–nucleic acid interactions. Pector also used DSC where heat capacity was shown to increase with decreasing charge (DNA/lipid) ratio. This is due to the lower energy requirement for lipid restructurating at high lipid concentration. DSC also indicated no change in heat at charge ratios greater than 0.6. There was maximum transfection efficiency at this charge ratio in CHO cells.

In a previous report Jaaskelainen et al. (1994) developed a kinetic model for lipid-ODN using resonance energy transfer (RET) experiments. Accordingly, lipid–ODN interaction is a two step process composed of aggregation followed by fusion, both induced by addition of ODN to the cationic liposomes. Charge ratio played a key role in defining the rate constant for the two processes. RET was also used to determine lipid–DNA interactions by Pozharski (Pozharski and MacDonald, 2003). Accordingly, lipid-DNA interaction is entropy driven process where the increase in entropy is due to release of lipid and DNA counterions, upon complex formation. The binding free energy is approximately 1RT per mole of lipoplex which is equivalent to 25% separation of charged pairs at one time. Similar conclusions were drawn by Lobo et al. (2003) thereby confirming that entropy gain during lipoplex formation is due to the release of lipid and DNA counterions.

10. Intracellular trafficking mechanism of lipoplexes

Intracellular trafficking studies are designed to identify barriers to siRNA delivery and improve understanding of the pharmacokinetics of these molecules. These studies have high utility in screening formulations, lipid type, lipid ratios, and identifying lead development candidates. Intracellular trafficking has been studied using flow cytometry and confocal microscopy. Flow cytometry studies help determine the average fluorescence intensity inside the cells. Accordingly, a fluorescent tag attached to siRNA can be

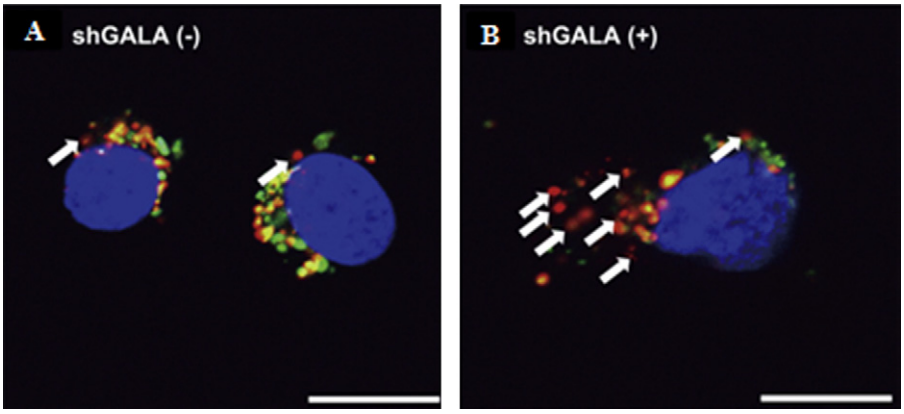

Fig. 11. *Confocal images of HT1080 cells incubated with MEND*: Enhancement in endosomal escape of siRNA encapsulated with MENDs by shGALA-modification. (A) The PEG-MEND or (B) the shGALA-MEND containing cy5-siRNA (480 nM) were added to HT1080 cells and then incubated at 37 °C for 1 h. Endosome/lysosome fractions and nuclei were stained with Lysotracker green and Hoechst33342, and then observed at 2 h post-transfection. Arrows indicated siRNA escaping from lysosome/endosome compartments. Scale bars indicate 20 nm.
With permission from ref (Sakurai et al., 2011).

used to quantify cellular uptake of siRNA. For example, uptake of Cy3-siRNA encapsulated in transferrin-liposomes was examined in LAMA-84 cells 4 h post-incubation at 37 °C. Flow cytometry studies revealed that the uptake of targeted liposomes was significantly higher than the non-targeted ones (8-fold) and was also capable of competitive inhibition by the free ligand, transferrin (Mendonca et al., 2010). In another report, uptake studies with FAM-siRNA entrapped into folate liposomes, were performed in KB cells 24 h post incubation. Uptake of folate lipoplexes was 275 fluorescence units (FU) compared to 200 FU by non-folate lipoplexes when the complexes were prepared in 5 mM NaCl (Yoshizawa et al., 2008). Alexa 488-siRNA associated with a liposome-peptide delivery system, was taken up by 90% of neuronal cells with mean fluorescence intensity (MFI) of 296 ± 44 (Pulford et al., 2010). Efficient uptake usually corresponds to effective silencing unless endosomal escape is the rate limiting step. This has been the case with all the previous examples (Mendonca et al., 2010; Pulford et al., 2010; Yoshizawa et al., 2008).Endosomal escape can be visualized microscopically using confocal microscopy. Confocal microscopy allows visualization of cells in a single focal plane thereby omitting interference from background fluorescence. Quantification studies using this technique, is also performed using fluorescent labeled siRNA. Additionally, other components of the delivery system for example, lipids and targeting peptides in combination with cellular organelles can be labeled for examination of rate limiting steps to siRNA delivery. These steps include cellular uptake, endosomal degradation and endosomal escape. Therefore, this technique facilitates visual proof for intracellular trafficking studies of the siRNA associated systems. For example, Pulford et al. prepared liposome-siRNA-peptide complexes (LSPC) where the targeting peptide RVG-9r (arginine modified peptide) was labeled with DyLight 649 (red), siRNA was tagged with Alexa 488 (green) while the nucleus was labeled with DAPI (DAPI) in N2a cells. Upon microscopic visualization, the role of the targeting peptide was clearly indicated when higher concentrations of green siRNA was observed in the presence of the peptide (compared to non-targeted), indicating its contribution to efficient siRNA internalization (Pulford et al., 2010). In another example, the endosomal escaping capability of shGALA fusogenic peptide was explored and visualized using confocal microscope. Sakuria et al. labeled the nucleus (blue, hoechst33342), the endosomes/lysosomes (lysotracker green) in HT1080 cells and delivered a PEG modified multi-functional nano device (PEG-MEND) associated cy5-siRNA (red) with and without shGALA peptide (Sakurai et al., 2011). The role of fusogenic peptide was clearly observed when cy5-siRNA (red) was visualized after escaping the endosome/lysosome compartments (Fig. 11B). On the contrary, in the absence of the peptide, (Fig. 11A), the siRNA was observed to be colocalized with the endosome/lysosome compartments indicating poor escaping capability of the delivery system due to the lack of fusogenic peptides. Therefore, overall, confocal microscopy a very useful technique to study siRNA distribution inside the cells and also understand the role of formulation components in overcoming rate limiting steps in siRNA delivery. In addition this technique can also be used to validate siRNA targeting moieties.

Besides fluorescent dyes, quantum dots have also been utilized as tagging molecules to track biomolecules. Quantum dots have the advantages over the organic dyes such as photostability, extreme sensitivity, broad absorption spectra, and tunable emission spectra which makes them a preferable labeling moiety when compared to organic dyes (Resch-Genger et al., 2008). Accordingly, quantum dots allow long-term same cell imaging (Srinivasan et al., 2006). There have been a number of studies utilizing quantum dots for tracking siRNA associated with liposomes (Chen et al., 2005; Derfus et al., 2004, 2007), however, the major concern is on the possibility for alteration of the pharmacokinetics of siRNA which has a relatively low molecular weight when tagged with a large molecular weight entity such as quantum dots.

11. Stability of siRNA lipoplexes

siRNA lipoplexes can be destabilized by physical or chemical means. Physical stability in indicated by significant increase in lipoplex size and the formation of aggregates, while chemical stability is indicated by leakage of encapsulated siRNA or complete loss of the lipoplex assembly. In addition, degradation of the lipids and or nucleic acids may occur.

11.1. Physical

A common issue with colloidal particles such as siRNA liposomal delivery systems is that if they do not have sufficient interparticle repulsion the suspension may shows signs of physical instability characterized by particle growth. Attractive forces such as van der Waals interactions among lipoplexes that are near-neutral may dominate in such systems ultimately resulting in aggregation. It is belived usually a charge of +30 mV is sufficient to prevent physical instability. Therefore, lipoplex physical stability can be monitored using particle size analysis via DLS or electron microscopy (Section 9.3).

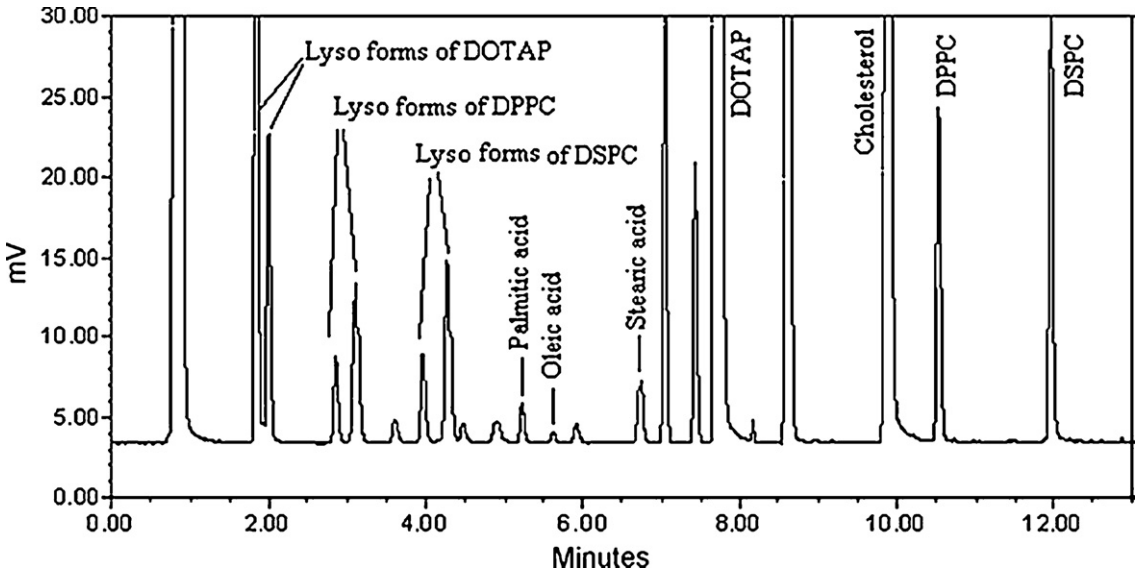

Fig. 12. *Lipid degradation profile using HPLC*: Example chromatogram of forced degradation of a standard mixture using 0.1 N HCl at room temperature for 4 days. With permission from ref (Zhong et al., 2009).

Physical stability of lipoplexes can be improved using a PEG coating where the polymer (PEG) chains help disperse the particles. For example, Kenny et al. (2011) prepared PEGylated cationic lipoplexes with siRNA and examined their stability for 10 days at 4 °C via particle size analysis using light scattering. Geusens et al. (2009) examined stability of cationic lipoplexes (composed of DOTAP/sodium cholate with siRNA) for 28 days at 4 °C and 25 °C. As seen from Fig. 13, they observed the particle size to be consistent at 4 °C but increased from 100 nm to 160 nm in 28 days indicating instability at room temperature.

11.2. Chemical

Chemical stability of lipoplexes can be affected by either lipid degradation or nucleic acid degradation.

11.3. Lipid degradation

This can occur commonly by either or both of the following mechanisms.

(i) Hydrolysis: Lipid chains specifically containing ester or amide groups undergo acid or base catalyzed hydrolysis thereby leading to lipid structure destruction and hence liposome destabilization which causes leakage of the encapsulated contents. Hydrolysis in one of the chains forms lysolipids that are highly toxic since they incorporate into the cell membrane and cause membrane destabilization (Goonesinghe et al., 2005; Henriksen et al., 2010). Besides the lipid tail, the linker (ester or amide) can also hydrolyze and cause lipoplex disassembly. Use of appropriate buffers to maintain the pH helps in inhibiting lipid hydrolysis. Alternately, hydrolysis can be prevented by lyophilizing the siRNA lipoplexes. Yadava et al. (2008) showed siRNA lipoplexes using lyophilization, could have enhanced stability when compared to solution formulations.

(ii) Oxidation: Unsaturated lipids are prone to oxidation (form reactive oxygen species) that is usually catalyzed by metal ion contamination (Fe, Cu), free radicals, high temperature or exposure of the formulation to oxygen (Evans et al., 2000). Even trace amounts (ppm levels) of dissolved oxygen in aqueous vehicle, can cause lipid oxidation. Lipid oxidation affects liposome fluidity (by affecting the double bonds and hence lipoplex efficiency) and occasionally forms toxic products (Kubow, 1992). Maintaining pH (buffers), use of EDTA (chelate metal ions), anti-oxidants and highly pure nucleic acid are common strategies used to avoid lipid oxidation and enhance the shelf-life of lipoplex formulations.

The oxidation and hydrolysis products of the lipids can be obtained by forced degradation of the lipid in extreme conditions of pH (with 0.1N HCl or 0.1N NaOH) in normal phase (Felgner, 1997) or reverse-phase HPLC (Chang and Harris, 1998; Heyes et al., 2006; Meyer et al., 2000; Zhong et al., 2009) with UV or light scattering detector. Zhong et al. studied the lipid degradation profile of DOTAP:Chol:DPPC:DSPC liposomes using reverse phase HPLC. Forced degradation of the liposomes resulted in the HPLC chromatogram as shown in Fig. 12.

11.4. Nucleic acid degradation

As in the case of lipids, the nucleic acids can also undergo hydrolysis of phosphodiester bonds via acid or base catalyzed reactions. These reactions are accelerated by nucleases (phosphodiesterase). Although the presence of liposomes ensures protection of siRNA from degradation, liposome destabilization by physical or chemical means can expose siRNA to unfavorable environment (low pH, enzymes). When compared to the DNA, siRNA is more to sensitive to nucleolytic degradation due to the 2′ OH group of ribose that is prone to alkaline hydrolysis (Lipkin et al., 1954).

Destabilization of lipoplexes due to a combination of physical and chemical degradation can result in loss of silencing efficiency of entrapped siRNA. Therefore, it is critical to monitor the stability of these complexes with time. Geusens et al. prepared cationic lipoplexes and monitored their stability for 28 days at 4 °C and at room temperature using particle size analysis and silencing efficiency. Although the particle size was intact at 4 °C, there was some loss in activity on day 2 (Fig. 13). This could be due to chemical degradation of nucleic acids or lipids in the aqueous environment (Geusens et al., 2009). Similar observations were attained from the stability studies conducted by Kenny and coworkers. They performed stability studies on siRNA lipoplexes for 10 days. Though there was no change in particle size, there was

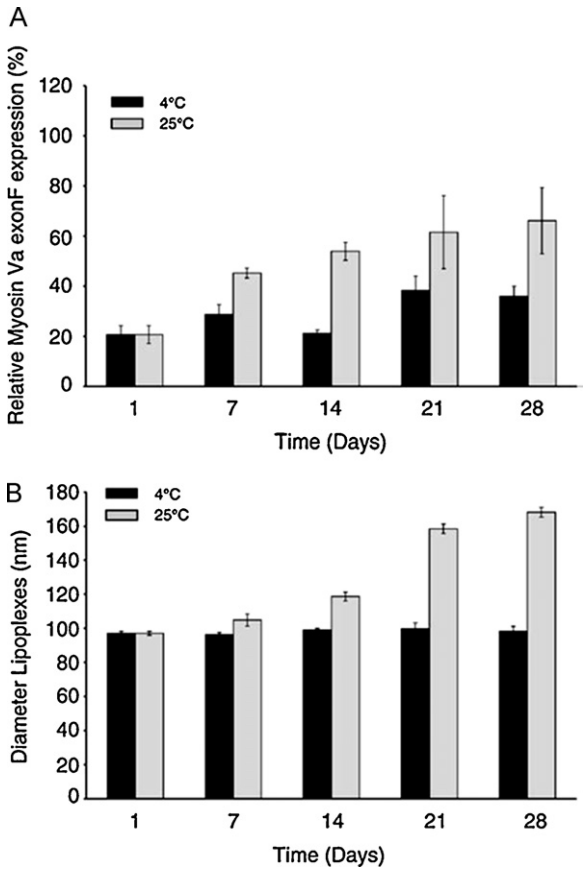

Fig. 13. *Biological and physicochemical stability*: (A) gene silencing capacity and (B) diameter of the ultradeformable cationic liposomes (UCL) and siRNA complexes as a function of their storage time at 4 °C and RT. Each point represents the mean ± SEM of three ($n = 3$).

With permission from ref (Geusens et al., 2009).

leakage from the lipoplexes after day 4 as indicated by encapsulation efficiency studies (Kenny et al., 2011). Besides the techniques mentioned in these examples, other techniques such as gel retardation assay can be used to determine stability of lipoplexes over time.

12. Conclusions

The growing influence of siRNA, subsequent to their discovery in the early nineties, is evident due to the rapid progress in the use of these innovative molecules in early clinical trials. The design and molecular mechanism of gene silencing using siRNA has been demonstrated to be as elegant and innovative as the delivery systems used to deliver them. The extensive use and prominence of non-viral lipid-based nanoparticulate systems for siRNA delivery, is due to their desirable formulation properties that include: safety, non-immunogenicity, high degree of control on material properties, tunability of function, and the ability to impact pharmacokinetics and bio-distribution *in vivo*.

Further advancement of siRNA into reliable and preferred therapeutic strategies requires consistent and robust clinical performance. Early clinical performance demonstrating the safety and efficacy of these molecules has been encouraging in conditions with primarily unmet clinical needs in trials with small numbers of patients. As more siRNA-based nucleic acid therapeutics and their delivery systems are evaluated in placebo-controlled randomized clinical trials in larger patient populations for complicated disease indications, the development of well-characterized formulations is of paramount importance. A thorough understanding and assessment of the formulation design space on the stability and function of these systems and the implication of these factors on clinical performance is necessary. A systematic quality-by-design approach in mapping the design space of the formulation and the critical attributes that affect performance using a range of orthogonal characterization techniques and analytical methods will be essential. A combination of these approaches will ensure that formulation-driven strategies that can retain the long-term physical, chemical, biological and functional stability of siRNA will play an important role in realizing the complete therapeutic potential of these molecules. Additionally, the elucidation of the complex relationships between formulation and clinical performance will help further this important milestone.

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