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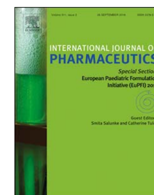

Critical evaluation of quantification methods for oligonucleotides formulated in lipid nanoparticles

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A R T I C L E I N F O

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A B S T R A C T

There is a very large variety in the types of nanoparticulate lipid formulations for oligonucleotides, and remarkably, also a very large heterogeneity in the methods that are used for analyzing oligonucleotide load, encapsulation efficiency and oligonucleotide release. Furthermore, a literature survey showed that the extent to which these procedures are reported in scientific literature varies greatly, with some of them not even reporting any quantification at all. This greatly hampers the reproducibility of nanoparticle preparation between different researchers and between different laboratories, which slows down the clinical translation of such nanomedicines. In this work, a standardized extraction method from liposomes is proposed, in which potential contaminants from the carrier are removed by a simple extraction of the oligonucleotides. These extracts were then analyzed with seven commonly used methods for oligonucleotide quantification, including several absorbance based methods and the most commonly applied dye binding assay. Strikingly, differences in absolute values up to fourfold were found when the same sample was analyzed using different methods which should be taken into consideration when reports using different methods are compared. Furthermore, these results indicate that the most commonly applied method, the dye binding assay, may -without adaptations- not be suitable for short oligonucleotides like siRNAs. The found differences in quantification methods as described here underscore the need for proper documentation of methods to correctly interpret published results, which –with regard to oligonucleotide analysis- is currently lacking in many reports.

1. Introduction

Lipid nanoparticles (LNP) are amongst the most advanced nucleic acid delivery systems currently in clinical trials (Wittrup and Lieberman, 2015; Yin et al., 2014). But also in pre-clinical and academic settings a lot of efforts are put in further improving and developing such systems (Wan et al., 2014). Despite an explosion in the number of papers and groups working on nanomedicine, clinical translation has lagged behind. An increasing number of voices from within the field is now arguing that instead of making even more and more advanced delivery systems, the focus should be on a more rational evaluation and better characterization of the ‘simple’ types (Crommelin and Florence, 2013; Kirsh et al., 2013; Lammers, 2013; Volk et al., 2015; Wang and Grainger, 2014). When taking LNPs as example, a head-to-head comparison of published lipid systems is hampered by the wide variety in encapsulation efficiency, load per particle and nucleic acid to lipid ratio in different systems. When nucleic acids are not encapsulated in the carrier but are merely complexed to them, it has been reported that the association of nucleic acids is incomplete and reversible (Buyens et al., 2008). This implicates that it cannot be assumed that the whole load of nucleic acids that is added is associated (complexed) with the carrier system but many researchers appear to do so and proceed without any quantification steps. Strikingly, in a quick literature scan in Pubmed (keywords “lipid nanoparticle” “liposomes” “oligonucleotides” “siRNA” “RNA” “DNA”, 2015–2016) to search for recent publications about lipid systems for nucleic acids, one third of all papers (13/36) did not quantify the total load of nucleic acids and encapsulation efficiency, nor did they refer to earlier work in which this characterization was indeed performed (See Supplementary Table S1). The most widely used method of characterization is with a gel-retardation assay (10/36), where the optimal ratio between carrier and cargo is determined, but this is rarely applied in a quantitative way. The

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second most-applied method is the Ribogreen® dye-binding assay (6/36) but some limitations to the two most commonly used methods have been reported (Buyens et al., 2008). More worrisome is the finding that many of the surveyed reports proceed to in vivo studies without reporting any quantification at all (7/13 non-quantified preparations are used in vivo). Each method will have its benefits and limitations under certain conditions and there is probably no gold standard, but the high heterogeneity in quantification protocols or the total lack thereof contributes to the low reproducibility of current scientific literature and the lack of clinical translation (Baker, 2016; Volk et al., 2015).

In this work, the degree of loading and the encapsulation efficiency of three nucleic acid lipid formulations is compared by using a selection of commonly used quantification methods for nucleic acids. All three formulations are prepared from the same composition of lipids and with the same feed ratio of oligonucleotides. Three different preparation methods are used that led to different encapsulation rations, to test the quantification methods in the full working concentration range. To get an impression of the robustness of the various analytical assays, their interchangeability and their accuracy, we have made a head-to-head comparison of the most frequently used analytical techniques for nucleic acid quantifications in pharmaceutical formulations. These results should guide the formulation scientists in deciding which assay would work best for their application.

Many of these analytical methods rely on fluorescence or absorbance detection, but particulate systems (or excipients thereof, like lipids or detergents) interfere with such methods. Therefore, we propose a simple extraction method for lipid-based formulations to remove most compounds that may interfere with the tested analytical assays. The extraction method has been validated for two types of small ribonucleic acids (an siRNA and a single stranded antisense oligonucleotide) and the quantification methods have been evaluated for three types of LNP formulation methods, with varying nucleic acid loads. Quantification of the nucleic acid load can be done spectroscopically, measuring the absorbance of the nucleobases, or with an intercalating reagent that produces a fluorescent signal (dye binding, Ribogreen® assay). For some purposes, formulations are loaded with (a fraction of) fluorophore labeled cargo, for example to study the stability in complex fluids (Buyens et al., 2008) or cellular uptake (Vercauteren et al., 2010). The fluorescent fraction of the cargo can also be used to determine the concentrations in the formulations. Finally, two methods of quantitative PCR (originally designed for measuring intact siRNA in the target cell) are compared and employed to quantify the nucleic acid load of the LNPs in a highly sensitive and sequence based manner.

2. Results

2.1. LNP preparation methods and physicochemical properties

The formulations used to compare the different quantification methods were prepared with different techniques. All of them consisted of the same lipid composition, namely DSPC:DODAP:Cholesterol:Ceramide-C16-PEG2000 at a ratio of 25:25:40:10. The first formulation was made using the 'preformed vesicle method', in which preformed empty vesicles are destabilized with ethanol to make them more permeable for the large nucleic acids. Because formulation is done at pH 4.0, the cationic lipids are charged so they can complex the nucleic acids which makes the vesicles coalesce, entrapping the nucleic acids in between the bilayers of the formed multi-lamellar vesicles (Maurer et al., 2001a). The ethanol in the formulation putatively diminishes the charge interaction of the cationic lipids and anionic nucleic acids, thereby avoiding aggregation (Maurer et al., 2001b). Therefore it was also attempted to use the 'conventional' film hydration method in presence of ethanol and at pH 4.0 followed by high pressure extrusion. As a comparison, the third formulation was made using the same method but without ethanol. All three methods were used to make a formulation encapsulating double stranded siRNA and a single stranded

Table 1
Hydrodynamic diameter and zeta potential of the siRNA and SCO formulations.

siRNA formulations	Z-average (nm)	PDI	Zeta (mV)
1. Preformed vesicles	109.8 ± 0.7	0.05 ± 0.00	−6.45 ± 0.36
2. Film hydrated (with 30% ethanol)	117.1 ± 1.8	0.07 ± 0.01	−6.42 ± 0.12
3. Film hydrated (without ethanol)	134.1 ± 1.3	0.04 ± 0.03	−6.75 ± 0.30
SCO formulations			
1. Preformed vesicles	104.2 ± 0.8	0.04 ± 0.03	−9.06 ± 0.19
2. Film hydrated (with 30% ethanol)	121.5 ± 0.4	0.06 ± 0.00	−7.13 ± 0.17
3. Film hydrated (without ethanol)	152.8 ± 0.1	0.20 ± 0.01	−8.39 ± 0.47

RNA splice correcting oligonucleotide (SCO) in a lipid:NA weight ratio of approximately 15:1. No strong differences regarding size distribution and zeta potential were observed between formulating siRNA and the SCO. The most obvious difference between formulation methods was that the extrusion of the vesicles without ethanol required significantly more pressure and time than when extruding them with 30% ethanol. This could indicate aggregation but if this occurred it was only partially reflected in the size distribution and polydispersity index (PDI) of the final formulation, see Table 1. Formulations extruded in ethanol had a diameter closer to that of the membrane they are extruded through (100nm) but only the SCO formulation that was hydrated and extruded without ethanol had a markedly increased size and PDI. Formulation methods differed more significantly in encapsulation efficiency as will be discussed next.

2.2. Interference of intact particles on RNA quantification by dye binding assay

The most widely applied quantitative method for determining the siRNA loading in LNPs is the Ribogreen® dye binding assay. When applying this to the formulations described above, it was found that only a fraction of the formulated siRNA was accessible for dye binding in the intact LNPs. Two alternatives were explored, namely incubation of the LNPs with the Ribogreen® dye in the presence of 0.5% Triton X-100 as suggested in one of the found protocols (Sam Chen et al., 2016a, b b) and a complete extraction of the nucleic acids from the formulation according to Bligh and Dyer. Both methods allowed for complete recovery of the nucleic acid load as compared to only 35% that was accessible for dye binding in the intact LNPs (determined from the stock solution of free siRNA that was used to prepare the formulations as a reference), see Fig. 1. The added detergent did disturb the fluorescent signal, which would result in a strong underestimation (~80%) of the nucleic acid amount but in this case, that could be compensated for by using a standard curve that also contained 0.5% Triton X-100. The medium in which a calibration curve is made are a typical example of details that are lacking in the reports, but clearly also of a detail that can significantly alter the outcome of the measurements.

Other quantification methods may not be compatible with either the detergent or the LNP excipients and for these methods the extraction could be used. For example, spectrophotometric detection with Nanodrop is heavily influenced by the presence of detergent and lipids and the injection of intact LNPs in the UPLC column resulted in irreversible damage to the column. Therefore, to directly compare all quantification methods, the LNP samples were extracted first and quantification was done on the extracts.

2.3. Recovery from Bligh and Dyer extraction

Because intact lipid particles can interfere with nucleic acid detection methods and because the nucleic acids are inside the particle and

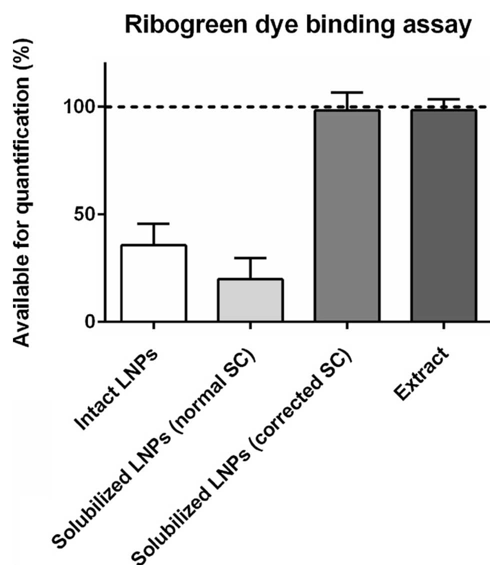

Fig. 1. Accessibility of the nucleic acids formulated in LNPs for the Ribogreen® dye binding assay. Only 35% of the siRNA cargo was quantified when intact LNPs were used, most likely because the nucleic acids inside the particles are not completely accessible for the dye. Solubilization of the LNPs with 0.5% Triton X-100 released all nucleic acids but the detergent strongly influenced the fluorescence signal, resulting in very low values when the normal standard curve (SC) was used. This had to be corrected for by using an additional standard curve that contained the same amount of detergent. Alternatively, extraction of the nucleic acids allowed complete access of the dye to the cargo. The 100% value was determined from the stock solution with which the lipid film was hydrated during formulation.

may thus not be detected by several methods, lipids and nucleic acids were separated before measurement. This was done using a Bligh and Dyer extraction that first solubilizes the particles in a miscible mixture of organic solvent and aqueous buffer. After addition of more chloroform and water, phases separate and lipids are extracted to the bottom phase, while nucleic acids are in the top phase (Bligh and Dyer, 1959). This was visualized by using a formulation labeled ‘red’ (with lipid conjugated Rhodamine) containing a nucleic acid labeled ‘blue’ (with Alexa 647). See Fig. 2.

To more accurately quantify the extraction recovery of lipids, a known concentration of (labeled) siRNA was spiked into liposomes with a positive, negative or neutral (PEGylated) surface. To quantify the amount of phospholipids in the organic phase, the total phosphate concentration was measured and compared to unextracted samples that were used as a 100% value. The extraction recovery of nucleic acids from the liposome samples and a dilution in MilliQ to the aqueous phase was determined using UPLC, measuring both the labeled and the unlabeled fraction. (See Fig. 3.) Recovery of phosphate was almost complete, varying from 97 to 102% and no difference was seen between formulations. This indicates that also the more hydrophilic PEG-lipid is completely extracted to the organic phase. The nucleic acid recovery in the aqueous phase was measured by UPLC and was found to be approximately 100% when extracted from MilliQ water. This shows that none of the nucleic acids participate in the organic phase, as to be expected from their molecular structure. The samples that were spiked with liposomes have a less complete recovery than extraction from MilliQ, indicating that the lipids may pull part of the nucleic acids to the organic phase. This effect is more pronounced when cationic lipids are used, but the recovery is still well above 80%. Typically, ionizable lipids are used that are not or only partially charged at the working pH so the effect will be diminished. In addition, decomplexing agents such as heparin could be added to help dissociate the oligonucleotides from the cationic lipids to further improve the recovery.

2.4. Quantification of nucleic acids

In order to remove the nucleic acids that were not encapsulated in the LNPs, all formulations were subjected to three rounds of ultracentrifugation to spin down the particles and resuspended in PBS. Samples ‘before’ and ‘after’ separation of unencapsulated nucleic acids were taken from each of the three formulation methods and quantified with seven different quantification/detection methods after extraction.

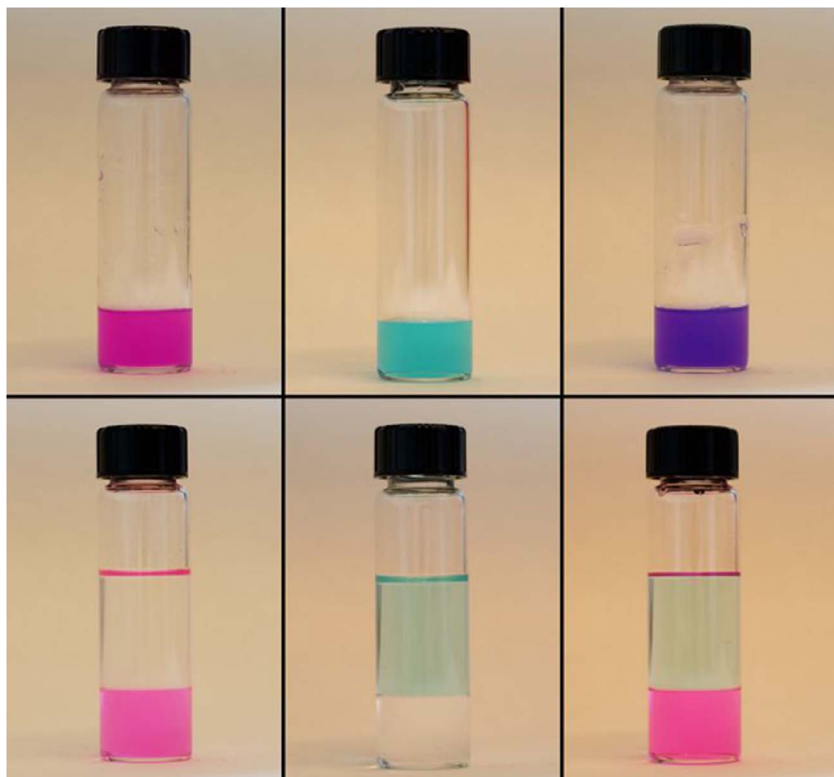

Fig. 2. Top row, from left to right: Rhodamine-PE labeled liposomes, unlabeled liposomes loaded with Alexa 647 labeled siRNA and labeled liposomes loaded with labeled siRNA. Bottom row: same samples as top row, but extracted using the Bligh and Dyer technique. Two clearly separated phases are seen. The labeled phospholipids partition in the bottom organic phase (consisting mostly of chloroform) and the labeled siRNA is extracted to the aqueous phase on the top.

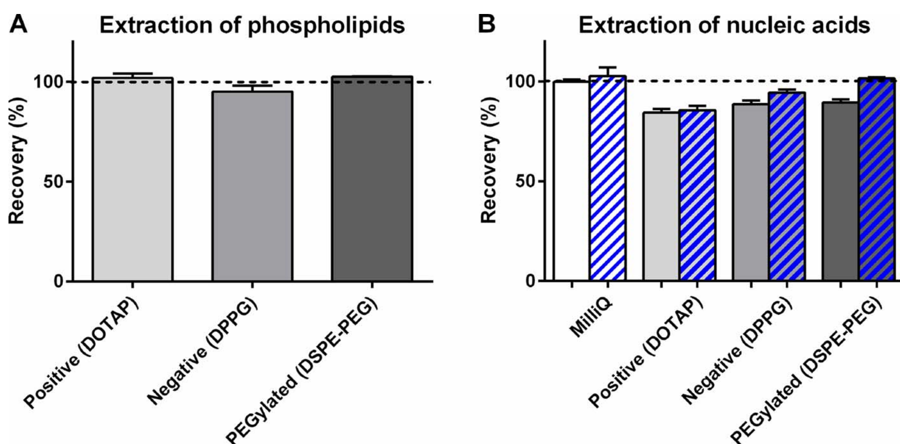

Fig. 3. (A) Recovery of phospholipids in the organic phase and (B) recovery of the nucleic acids in the aqueous phase. Positive, negative and PEGylated liposomes consisted of DPPC:Chol:X in ratio of 6:3:1, with X being DOTAP, DPPG and DSPE-PEG2000 respectively. Unextracted samples were used as 100% values and to calculate recovery percentages. The blue bars represent recovery of the labeled fraction and the grey bars represent the unlabeled GAPDH fraction.

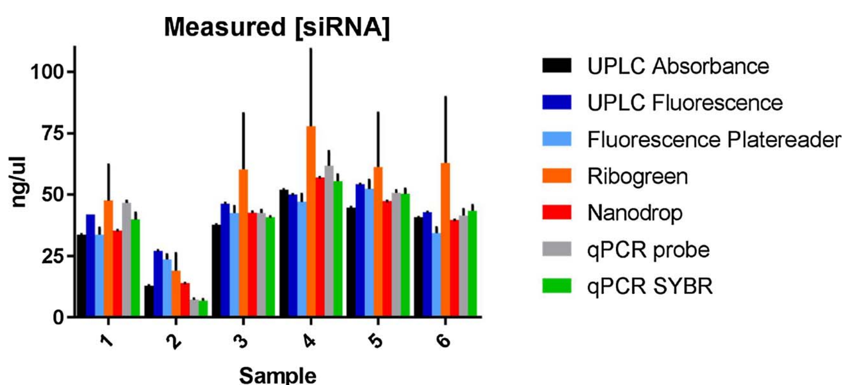

Fig. 4. Absolute values from the six siRNA samples, as obtained by different quantification methods in mean ± SD. Odd numbered samples are 'before' ultracentrifugation and even numbers 'after'. The first pair of samples belongs to formulation method #1, the second pair to method #2 and the third pair to method #3. Samples were diluted or concentrated during processing as is also seen in phospholipid measurements and absolute values have to be corrected accordingly. None of the methods seem to consistently over- or underestimate the value, but Ribogreen® values are the highest in five out of six samples and have a very high standard deviation, suggesting that for this application the dye binding assay may not be the most suitable.

The absolute values are plotted in Fig. 4 to show the differences between the different methods (different symbols) and between triplicates of the same method (error bars, showing standard deviation). Several conclusions can be drawn from the graph. First, absolute values in some cases vary almost 100% between methods. For example, in sample 1.2 the UPLC measurement on the fluorescent fraction gives a value almost twice as high as the measurements on the non-fluorescent fraction by UPLC and Nanodrop. This obviously has a huge consequence when either of the methods is used to dose the amount of oligonucleotides in follow-up experiments. Secondly, apart from the Ribogreen® assay, triplicate measurements using the same method are very accurate, as seen from the small standard deviation. This indicates that the assays themselves may find a reproducible value, but that the obtained values heavily depend on which method is used. The Ribogreen® assay found values that are deviating more from the values of the other methods and also has a very high standard deviation between repeated measurements. These two findings may be related, but in our hands, the Ribogreen® assay failed to produce reproducible results and is clearly the outlier in this study. Although the other values are closer to each other, there is not one method that always produces the highest or lowest values. This suggests that there is some variation between the methods, but there are no interfering factors that consistently over- or underestimate the siRNA content in either of the methods.

2.5. Encapsulation efficiency

The variation in the absolute values is caused by differences in the formulation method and varying dilution factors during the processing and purification. To determine the encapsulation efficiency of the three employed formulation methods, the absolute values were corrected for the amount of phospholipids and then values before and after separation of unencapsulated oligonucleotides were compared. The encapsulation values obtained with seven different methods are shown in Table 2.

The ‘preformed vesicle method’, or formulation #1 clearly has a much lower encapsulation efficiency than the other two formulations that were made with film hydration methods. However, the efficiencies obtained with the two methods that detect the fluorescent Alexa 647 cargo are much higher than the other efficiencies, while in formulation #2 and #3 the fluorescence based methods follow the trend of the other methods. All oligonucleotides are covalently labeled with a single fluorophore, so a wide distribution in the number of labels per molecule cannot be of influence on these measurements. A clear explanation for this discrepancy cannot be given, but it does show that it is not recommended to extrapolate the total encapsulated fraction from measuring the fluorescently-labeled fraction. From these results, it appears that the fluorescence based methods are less reliable, at least when only

Table 2
Encapsulation efficiencies of the three different formulation methods as measured with the seven different quantification methods.

	Nanodrop	Ribogreen®	UPLC (UV)	UPLC (FLR)	Platereader	qPCR (probe)	qPCR (SYBR green)
Formulation #1	29%	26%	28%	47%	50%	11%	13%
Formulation #2	95%	95%	97%	76%	80%	103%	97%
Formulation #3	93%	98%	101%	88%	73%	91%	96%

a small fraction of the total cargo is labeled. Apart from some small differences in absolute values, all other absorbance based methods produce very similar values of encapsulation efficiency. This again shows that the variations within methods are not that high, but that incorrect conclusions are more likely to be drawn when different methods are compared. The qPCR quantifications produced values much in line with the other methods, except for sample two, where the results are much lower. This could be caused by a contamination in the sample that inhibited amplification of the target strand. Because the values of the 'after' sample from the first formulation method (1.2) were underestimated by the qPCR methods, the calculated encapsulation efficiency is much lower. Although the goal of this work was not primarily to compare the three formulation methods, the preformed vesicle method is clearly less favorable than the other two methods, in terms of encapsulation efficiency.

The process of encapsulation, purification, extraction and quantification was repeated using the same preparation methods and lipid composition, but with a short, single stranded RNA splice correcting oligonucleotide (SCO). Except for the qPCR measurements, all methods could be adapted to the different cargo and no significant differences were observed on the outcome. For the Ribogreen® assay, the working range had to be adjusted to 10 times higher concentrations of nucleic acids. This was still in the low nanomolar range (which is more sensitive than the other assays, except qPCR) but it also required 10 times higher concentrations of the Ribogreen® dye, making this assay 10 times more expensive for the SCO than for siRNA.

2.6. UPLC analysis to determine nucleic acid integrity

The quantification methods described above cannot say anything about the sequence and structural integrity of the NA. We have therefore also included a UPLC method with a commercially available column (Waters Acquity UPLC Oligonucleotide BEH C18, 130A, 1.7uM, 2.1 × 100mm) which employs a combination of reversed phase and ion-pairing and is able to separate oligonucleotides based on length and sequence. The high operating temperature and pressure separate the sense and anti-sense strands of siRNAs that can then be detected at different retention times. However, separation is more challenging when oligonucleotides have backbone modifications such as phosphorothioate bonds or 2′O-methyl modifications and the nucleic acids used here have both. It was attempted to optimize the analysis method towards complete baseline separation of the sense and the anti-sense strands of the siRNAs but that was not completely successful for all the used sequences.

Nevertheless, enough structural information could be gathered from the chromatograms to distinguish between different sequences. Moreover, it was possible to identify degradation products and to assess whether the measured samples were still intact. For forced degradation, a small fraction of LV2 control siRNA was enzymatically degraded using RNAse A and then injected. A multitude of smaller peaks was detected after digestion and the original peak had completely disappeared (See Fig. 5). All peaks had lower retention times, as all degradation products are obviously shorter than the original product. The peak at 1.15 min is the injection peak, which may contain individual nucleotides that have no retention on the column. An increasing number of peaks was seen when higher concentrations were injected, showing that less abundant degradation products require higher initial concentrations to be detected. Admittedly, not all degradation products could be detected at feed concentrations that are typical in liposomal formulations, but UPLC analysis remains a powerful method to determine structural integrity of the measured samples. A 'complete' peak of the full length product (that is, identical in triplicate samples) together with the absence of degradation products, can ensure that the sample is completely intact. No degradation products were detected in any of the samples used here.

2.7. Quantitative PCR

qPCR based detections were performed essentially as described for small RNAs (Chen et al., 2005; Tang et al., 2006; Varkonyi-Gasic et al., 2007). Since the qPCR amplification is sequence dependent, only the GAPDH strands, but not the labelled controls were detected. For the enzymatic reactions, the siRNA solutions needed to be diluted strongly in a stepwise manner. A GAPDH siRNA solution with an initial concentration of 1 µM was used as a standard and diluted and analyzed in parallel to the samples. Two different reverse transcription methods, using either stem-loop primers (Chen et al., 2005) or the S-Poly(T) method (Kang et al., 2012), were tested. The S-Poly(T) gave poor results in the subsequent qPCR, which was attributed to the chemical modification of the siRNA. In particular, the phosphorothioate bond in the 3′-dT overhang seems to impede efficient polyadenylation, a major step for one of two routinely used methods for generating cDNA. Thus, we resorted to use a stem-loop primer method which efficiently amplified the siRNA (Supplementary Information). The samples were subsequently analyzed with both a probe and a dye based qPCR assay. Both analysis methods showed only negligible differences between the two, apart from generally higher values for the dye based detection, which is a known characteristic. (Fig. 4) This was taken into account through the use of a standard solution for calibration which served as the basis for determining the copy number per and the concentrations of the analyte solutions (Kroh et al., 2010).

3. Discussion and conclusion

In the work presented here, seven different methods for quantification of short oligonucleotides that are commonly used in the field of drug delivery were systematically evaluated. Three different formulation methods were compared, by producing lipid nanoparticles with identical lipid composition, containing siRNA or a splice correcting oligonucleotide. The formulation methods used were the 'preformed vesicles method' (Maurer et al., 2001a) and two variations of the lipid film hydration method. All formulations were purified by dialysis and unencapsulated oligonucleotides were removed by ultracentrifugation and washing. Particulate systems can cause scattering in spectroscopic methods and the encapsulation of the oligonucleotides inside the particles can hamper detection (because of fluorescence quenching, or the unavailability to reagents such as the Ribogreen® reagent, see references (Buyens et al., 2008; Lucas et al., 2005; Vader et al., 2010) and our results in Fig. 1. Therefore, the oligonucleotides were separated from the particles first, by a validated extraction method, to liberate the cargo from the carrier and remove interfering substances. All samples were treated exactly the same and the extracts were assayed for oligonucleotide concentration using seven different methods. These methods were spectroscopic detection using a Nanodrop spectrophotometer, fluorescent detection of the Alexa 647 labeled fraction of the cargo (10% of total), the commercial Ribogreen® Assay, UPLC analysis using UV detection (260nm) and fluorescence detection (Alexa 647) and two methods of qPCR. Most of these methods are readily available to most labs and routinely used in publications to quantify siRNA and characterize formulations (See Supplementary Table S1).

Remarkably, when measuring the same sample using different quantification methods, in the most extreme cases differences of up to four times higher than other methods were found. Replicates using the same quantification method were found to be consistent in most cases, which means that differences are predominantly caused by differences between analytical methods. This makes the comparison between for example, formulation methods, encapsulation efficiency and transfection efficiency (based on measured doses) impossible if different measurement methods are used, even more so when inter-lab variation also plays a role. This is worrisome as different labs all use their own methods and protocols while there appears to be a clear need for standardization. Findings like these can partially explain the reported

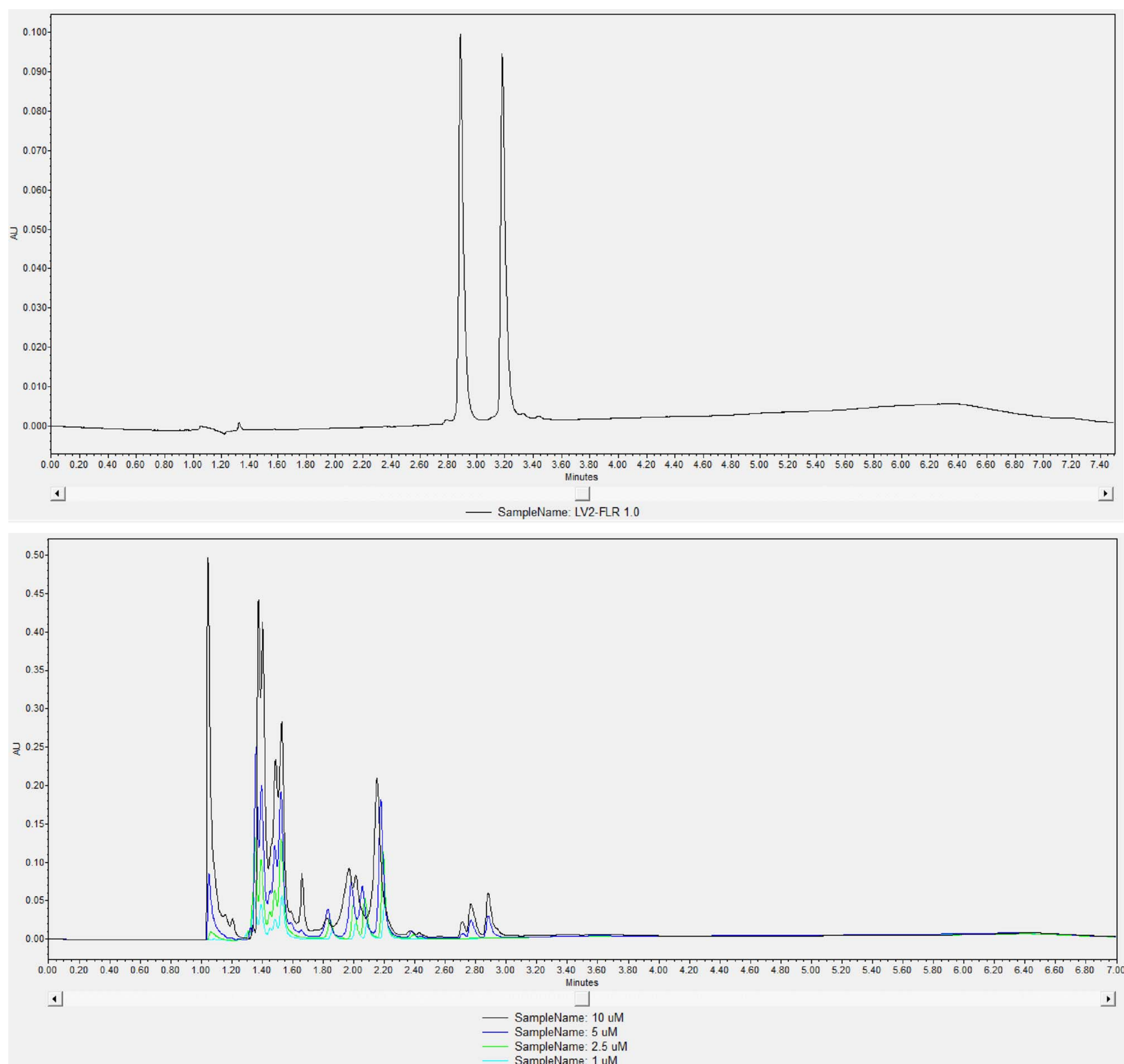

Fig. 5. Strand separation of siRNAs for identification and analysis of degradation products Top: Complete strand separation of the sense and anti-sense strands gives a unique pattern for each sequence and aids in identifying sequences and whether they are intact. Bottom: Peak distribution of RNAse A degraded LV2 siRNA. Shorter fragments have shorter retention times and are more abundant than the longer fragments. Less abundant degradation products are only detected when higher initial concentrations of siRNA were used.

low reproducibility of scientific literature and more within-lab validation and external-lab validation could definitely improve this reproducibility issue (Baker, 2016).

Although comparing formulation methods was not the primary goal of this work, by using the standardized method described here, we were able to unambiguously demonstrate differences in encapsulation efficiencies. Method #2 was found to be the most ideal in these conditions, as it has a high encapsulation efficiency and a proper size distribution and polydispersity. The mean size and polydispersity of method #3 was higher than that of the other two, possibly because aggregation of the anionic oligonucleotides and cationic lipids occurred to a higher extent in the absence of ethanol. The encapsulation efficiency of method #1 was clearly lower than that of the other two methods and also much lower than was originally reported (Maurer et al., 2001a). The original publication also reports the influence of lipid composition and ethanol concentrations, so if the preformed vesicle method is preferred, it can most likely be optimized by changing those parameters. Here, method #2 was the most efficient and convenient formulation method.

Despite the remarkable differences in absolute values between methods, it is not possible to say which one of them is the most accurate, although it could be said that in this case the Ribogreen® assay gives the biggest variations and is far away from the other values. Possibly, the Ribogreen® reagent is sensitive to ‘environmental influences’, such as temperature, light, time of incubation. This makes it suitable for determining encapsulation efficiencies (when samples are measured in the same assay) but less suitable for comparing samples from different experiments or on different dates. The high variations may also be caused by the short length of the oligonucleotides used here, while the assay was developed to quantify isolated mRNA (and the dye is known to intercalate between the bases, which could be obstructed by backbone modifications). The finding that higher concentrations of dye are necessary to quantify the SCO than for the siRNA indicates that the assay is less sensitive for single-stranded oligonucleotides and may be influenced by the sequence and chemical modifications (the SCO has a full phosphorothioate backbone), and that therefore the Ribogreen® dye may be better suited for long transcripts

or for determining total mRNA in cell extracts.The two quantification methods based on the fluorescence detection of the conjugated Alexa 647 label, produced similar results, but those were quite far away from the values obtained with other methods. This is possibly caused by the fact that only 10% of the cargo used here was labeled and that extrapolation of the recovery of the labelled sequence with altered biophysical properties does not produce reliable results for encapsulation efficiency. These methods may be more suitable when bigger fractions of labeled cargo are used. The reported problem that larger quantities of fluorophores are quenched inside liposomal carrier systems (Buyens et al., 2008; Lucas et al., 2005; Vader et al., 2010) can be avoided by solubilizing the LNPs with Triton X-100 or by the proposed extraction method.

Quantitative PCR is the most complicated method used herein. Its main virtue is the possibility to detect nucleic acids in a sequence-specific manner, even within mixtures of many different sequences, and its low detection limit. In addition, it is suitable for detection after extraction from cells and tissues. For quantification of the samples and the calculation of encapsulation efficiency, the samples needed to be strongly diluted, making several individual diluting steps necessary. In the context of determining encapsulation efficiency, this method may be too complicated, but it can be useful in settings in which low detection limits, high sequence specificity are needed, or within biological settings.

Other factors that could play a role in determining the ‘preferred’ method, are summarized in the table below (Table 3). The ease of use, price and accessibility of a method could play a role if high throughput analysis of the experiments is desired. To make the table less subjective, some considerations for assay validation are also taken into account, such as specificity, accuracy, precision and detection limit. If sample concentrations are low, some options may be unsuitable and obviously, when only unlabeled oligonucleotides are used, the fluorescence based methods are not an option. On the other hand, a fluorescent plate reader is probably available to every lab so this may be the most accessible and cheapest option of all.

*Nanodrop calculates concentrations based on spectroscopy and an average extinction coefficient for RNA or DNA and does not make use of a standard curve of the actual sequence that is analyzed.

In our experimental setup, the UPLC method is the most preferred method. It does require a UPLC system which may not be available to every lab, but HPLC alternatives also exist (although this increases runtime and decreases throughput). Analysis may take some time as each sample and standard has to be run consecutively rather than in parallel but the system is automated and therefore has a high throughput. Of all the methods tested, UPLC analysis has the highest precision and specificity together with qPCR, but is quicker and easier to use. It can detect the backbone and fluorescent label in parallel and separation of strands and potential degradation products gives structural information that cannot be obtained by any other method.

In summary, we give an example of how lipid nanocarrier systems could be compared in a more systematic way. Using a standardized method for separating the oligonucleotides from the lipids, we were able to indicate a clear preference for one of the three tested formulation methods to prepare nucleic acid lipid nanoparticles. More importantly, we demonstrate that there are substantial differences between routinely used detection methods for oligonucleotides, which makes comparison very difficult. Of course, the research laboratories should make their own decision as to which method to use, and these decisions may even be based on practical issues such as price and availability. However, this work illustrates the importance of method validation and brings to light one of the possible causes of the low reproducibility of, and the discrepancies between reports from different labs. It is probably unrealistic to expect that the field could find one method that would be agreed upon to be used as the standard method, given the huge number of laboratories working on nanoformulations for oligonucleotides. Nevertheless, the field would benefit if labs would approach this problem in a more standardized way or at least report their quantification methods in more detail. We would like to give the US. National Cancer Institute’s ‘Nanotechnology Characterization Laboratory’ (http://nanolab.cancer.gov/) as an example of how standardization of characterization techniques might contribute to better therapies and a protocol for quantification of oligonucleotides could be added to their list of standard procedures. We advise to pay more attention to procedures such as the separation of the unencapsulated material, dissociation from the carrier and quantification methods, both when interpreting, as well as reporting results. Neglecting to describe or even perform such procedures could be the cause of the reproducibility issues that hold back the progress of the field.

4. Materials and methods

4.1. Materials

4.1.1. Nucleic acids

All nucleic acids used here are provided by GlaxoSmithKline (Stevenage, UK) for use in the EU IMI COMPACT consortium.

Table 3
Advantages and disadvantages of the employed analysis methods.

Method	Ease of use/ accessibility	Working range	Advantages	Disadvantages
Nanodrop	+ + +	10 − 100 ng/ul*	- Low sample volume (1 ul) - Easiest and fastest method described	- Sensitive to impurities, low detection limit - Detection based on average extinction coefficient, not standard curve*
Ribogreen®	+ +	0–5 nM (siRNA) 0–50 nM (ASO)	- Cheap and commercially available kit - Only require fluorescence platereader - Very low detection limit	- Very sensitive to 'environmental influences' (high variability) - ‘High’ working range (ASO) consumes a lot of reagent. - (limits use to 20 plates per assay kit) - Low accuracy compared to other methods
UPLC	+	0–4 uM	- Structural information (double strands and degradation products) - High specificity - Detection of backbone (UV260) and fluorescent fraction	- Requires expensive system and dedicated OST column
Platereader	+ + +	0–1 uM	- Cheapest method described, only requires fluorescence platereader	- Measures fluorescently labeled fraction only
qPCR	+	0–10 pM	- Detects only intact sample (high specificity) - Lowest detection limit of all methods (several copies/µl)	- Most laborious method

Sequences are as follows:

Luciferase siRNA	Sense 5'-CUUACGCUGAGUACUUCGAdTdT-3' Antisense 5'-UCGAAGUACUCAGCGUAAGdTdT-3'
Negative Control siRNA (LV2)	Sense 5'-AUCGUACGUACCGUCGUAUdTdT-3' Antisense 5'-AUACGACGGUACGUACGAUdTdT-3'
GAPDH siRNA	Sense 5'-GGUCAUCCAUGACAACUUUdTdT-3' Antisense 5'-AAAGUUGUCAUGGAUGACCdTdT-3'
Luciferase ASO	5'-CCUCUUACCUCAGUUACA-3' (All PS)
Negative Control ASO	5'-CCTGUUAUACCACUUACA-3' (All PS)

Underlined bases indicate a 2′O-methyl modification. dT indicates a deoxyribonucleic acid base, all of which have phosphorothioate (PS) bonds. Antisense oligonucleotides consist completely out of PS bonds.

Labeled siRNA and ASO have the respective negative control sequences and are labeled with Alexa 647 on a C6 linker. All labeling was done on the 3′ end and in case of siRNA, on the sense strand.

4.1.2. Lipids

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), N-palmitoyl-sphingosine-1-succinyl[methoxy(polyethylene glycol)2000] (C16 Ceramide-PEG2000), L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was from Sigma-Aldrich (St. Louis, MO, USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DPPG), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) were a gift of Lipoid GmbH (Ludwigshafen, Germany).

4.1.3. Liposome preparation

For all formulations, a lipid film was made first by dissolving the lipids in chloroform and evaporating the organic solvent using a rotary evaporator and a 25 ml round bottom flask. The formed dry lipid film was flushed with nitrogen until all residual chloroform was removed. For nucleic acid formulations, all lipid films consisted of DSPC:DODAP:Cholesterol:CeramideC16-PEG2000 at a ratio of 25:25:40:10. For the 'preformed vesicle method', the lipid film was hydrated in 70% 50 mM citrate buffer pH 4.0 and 30% ethanol absolute to a final concentration of 8 mM total lipid. The formed vesicles were then extruded at least 10 times using a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada) through Track etch polycarbonate membranes with a final pore size of 100 nm (Nuclepore, Pleasanton, CA, USA). A solution of 90% GAPDH siRNA and 10% Alexa 647 labeled LV2 Control siRNA or 90% Luciferase ASO and 10% Alexa 647 labeled Negative Control ASO in 70% 50 mM citrate buffer pH 4.0 and 30% ethanol absolute was then added dropwise to the empty vesicles under heavy stirring after both the vesicles and the nucleic acid solution were preheated to 45 °C. The vesicles were then incubated at 45 °C for 90 min to allow reorganization of the vesicles. Ethanol was removed and buffer was replaced by overnight dialysis in a 2K MWCO Slide-A-Lyzer G2 Dialysis Cassette (Life Technologies) at 4 °C against PBS. The other lipid films are hydrated with the same amount of nucleic acids, either in 70% 50 mM citrate buffer pH 4.0 and 30% ethanol absolute or in 100% 50 mM citrate buffer 4.0 and then extruded as above. The final NA/lipid ratio of all formulations was 0.06 (wt/wt). These formulations were also dialysed as above and after dialysis, unencapsulated nucleic acids were removed by three rounds of ultracentrifugation in a Type 70.1 Ti rotor for 50 min at 55.000RPM at 4 °C. The pictures in Fig. 2. were made with formulations containing 0.5 mol% Rhodamine-PE and 50:50% of the labeled:unlabeled siRNA. For the extraction validation, liposomes consisting of 10 mol% DOTAP, DPPG or DSPE-PEG2000 and 60:30 mol% of DPPC:Cholesterol were hydrated in Hepes Buffered Saline (10 mM HEPES, 145 mM NaCl) pH 7.4 to a final concentration of 10 mM total lipid. The hydrodynamic diameter and the polydispersity index of the liposomes were measured by dynamic light scattering, using a Malvern CGS-3 multiangle goniometer with He-Ne laser source (λ = 632.8 nm, 22 mW output power) under an angle of 90° (Malvern Instruments, Malvern, UK). The zeta-potential was measured using laser Doppler electrophoresis in a Zetasizer Nano-Z (Malvern Instruments) with samples dispersed in 10 mM Hepes buffer pH 7.4 (no additional salts). The encapsulation efficiency was calculated using the following formula

$$\textit{Encapsulation efficiency}\ (\%) = \frac{\frac{[\textit{nucleic acids}]}{[\textit{phospholipids}]}\textit{after ultracentrifugation}}{\frac{[\textit{nucleic acids}]}{[\textit{phospholipids}]}\textit{after dialysis}} * 100$$

4.1.4. Extraction of lipids and nucleic acids

Lipids and nucleic acids were separated by an extraction according to Bligh and Dyer (Bligh and Dyer, 1959). For every 100 µl of liposome sample in aqueous buffer, 375 µl of chloroform:methanol in 1:2 ratio was added. This forms one single phase in which both lipids and nucleic acids are dissolved. 125 µl of chloroform was then added and vortexed. 125 µl of MilliQ water was then added and vortexed. Tubes were then centrifuged at 1500xg for 90 s. The top phase contains the water (including the salts in the initial sample) and methanol. This phase was transferred to a separate tube and the bottom phase consisting of chloroform could be assayed for total phosphate, according to the method of Rouser et al. (Rouser et al., 1970). With this method, phospholipids are destructed by addition of perchloric acid and heating at 180 °C to yield organic phosphate. This was then measured colorimetrically with sodium biphosphate as a standard. For more details it is referred to the original work (Rouser et al., 1970). The top phase was evaporated in a vacuum concentrator and reconstituted in MilliQ water to measure the concentration of nucleic acids.

4.1.5. Extraction validation

To calculate the recovery of lipids in the organic phase and nucleic acids in the aqueous phase, liposomes consisting of DPPC:Cholesterol:X in 6:3:1, with X being (cationic) DOTAP, (anionic) DPPG, (neutral) DSPE-PEG2000 were spiked with nucleic acids. The nucleic acid solution contained 90% GAPDH siRNA and 10% Alexa 647 labeled Negative Control siRNA and was mixed in a 1:1 ratio with the three liposomes samples, with a final phospholipid concentration of 1 mM and a nucleic acid concentration of 1 uM. This mixture was extracted as described above and the organic phase was assayed for phosphate. As a 100% value an unextracted sample and unspiked sample of the same concentration was used. The top phase was evaporated as described and after reconstitution measured by UPLC as described below. As controls, siRNA samples were also mixed 1:1 with MilliQ. These were extracted and of half of the samples the top phase was collected. The other half were evaporated (including the organic phase) until completely dry and then reconstituted in MilliQ (there was no possible loss of sample in this tube and it was therefore used as the 100% value).

4.1.6. uplc

Nucleic acid quantification by UPLC was done using a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) with PDA and FLR detectors on an Acquity UPLC Oligonucleotide Separation Technology (OST) BEH C18, 130A, 1.7uM, 2.1 × 100mm column (Waters Corporation). Waters OST employs a combination of reverse phase and ion pairing to separate (detritylated) oligonucleotides based on length and sequence. Gradient mobile phase at a flow rate of 0.2 ml/

min was changed from 95% solvent A (10/90% Methanol/HFIP 400mM and 15 mM TEA in water) to 40% solvent B (Methanol) in 5 min. Column operating temperature was 60 °C as recommended by the manufacturer.

UV detection with the PDA detector was done on 260nm and the FLR detector was used at 650/665 for detection of the Alexa 647 conjugated nucleic acids. Peaks were integrated and AUCs were compared to standard curves of the corresponding sequence to calculate concentrations. Whenever possible, quantification in the FLR channel was preferred over the PDA channel. For forced degradation, a 10 μM solution of LV2 Control siRNA was incubated with 100 μg/ml RNAse A (Thermo Fischer Scientific, Waltham, MA, USA) for 48 h at room temperature and then injected in concentrations 1, 2.5, 5 and 10 μM.

4.1.7. Nanodrop

Nucleic acid concentrations were determined spectrophotometrically using a NanoDrop ND-1000 (Thermo Fischer Scientific). Absorbance at 260nm and a nucleic acid conversion factor of 60 was used to calculate concentrations.

4.1.8. Fluorescence detection

The concentration of Alexa 647 labeled nucleic acids was measured using a Jasco FP8300 Spectrofluorometer with micro-well plate reader. (JASCO Benelux BV., De Meern, Netherlands) Wavelengths used were 650/665 and a calibration curve was used to calculate concentrations.

4.1.9. Ribogreen® assay

The commercially available Quant-iT™ RiboGreen® RNA Assay Kit (Thermo Fischer Scientific) was used according to the manufacturer's instructions. It was found that the 'low range assay' (1 ng/mL to 50 ng/mL) was not sensitive enough to detect the single stranded SCO so for these formulation the 'high range assay' (20 ng/mL to 1 μg/mL) was used. The corresponding oligonucleotides (in the same ratio of labeled:unlabeled of 10:90) were used as a calibration curve. Measurement was done at 485/520 nm. The solubilized LNP sample was diluted in TE buffer containing 0.5% Triton X-100 and for this quantification an additional standard curve was added that also contained 0.5% Triton X-100.

4.1.10. qPCR

4.1.10.1. Sample processing for cDNA synthesis and qPCR. The reference GAPDH siRNA duplex was diluted from a 1 μM solution to 1 nM as described in E. M. Kroh et al. (Kroh et al., 2010) This stock solution was further diluted stepwise 295,520 fold to receive a 3.38 pM solution. For ensuring quantitative reverse transcription, a dilution array was prepared by diluting the 3.38 pM solution four times eight fold. The 3.38 pM solution contains 32,768 copies/μl. Four μl of this solution were used for cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Thermo FisherScientific) and diluted six-fold before use as template in qPCR reactions. The samples were diluted and analyzed in the same way as the standard.

4.1.10.2. cDNA synthesis. cDNA synthesis was performed with the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit in 40 μl reaction volumes. A GAPDH siRNA specific stem-loop RT primer was designed as described (Chen et al., 2005; Varkonyi-Gasic et al., 2007). Three μl of a 1 μM GAPDH specific stem-loop RT primer were added to a PCR tube, followed by 3 μl of standard or sample solution, and filled up with 19 μl nuclease free water. Content was mixed by flipping and reunited by a short spin at ~200g for 5 s. The RNA-RT primer solution was heated to 65 °C for 5 min, 22 °C for 30 s and 20 °C for 30 s and directly on ice for > 2 min to enable specific binding of RNA to the added stem-loop RT primer. The cDNA reaction was completed by adding 15 μl mastermix, mixed by flipping followed by a short spin at ~200g. To improve sensitivity, reverse transcription was performed with a pulsed program (30 min at 16 °C, 60 cycles at 30 °C for 30 s, 42 C for 30 s and 50 °C for 1 s). The reaction was stopped by heating to 85 °C for 5 min. A reverse transcriptase minus (RT-) sample was included as a control.

4.1.10.3. qPCR. qPCR analysis was performed with a Roche LightCycler® 96 System by using either Roche Probe or SYBR Green I master mix in a 20 μl reaction volume. GAPDH specific forward primer and universal stem-loop reverse primer (both Microsynth, Balgach, Switzerland) were added to a final concentration of 250 nM. The probe based assay was prepared by diluting the ZEN probe (Integrated DNA Technologies, Coralville, IA, USA) to a final concentration of 300 nM. Fluorescence of SYBR Green I was measured in FAM channel and Yakima Yellow of the ZEN probe in the VIC channel. Each cDNA sample was analyzed in triplicate and the qPCR efficiency was determined by performing in run cDNA dilution arrays for both probe and dye based assay (Supplementary information).

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