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Rapid and Accurate Quantification of RNA in Lipid Nanoparticles by Scatter-Free UV/Visible Spectroscopy

Eric C. Le Ru,* Roland Böttger,* Darren Andrews,* Patrick Baumhof, Jelena V. Rakonjac, Geoffry Laufersky, and Brendan L. Darby

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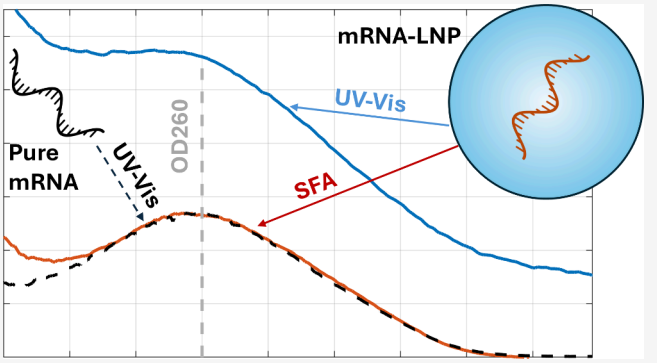
ABSTRACT: UV/visible spectroscopy is the method of choice for RNA quantification, thanks to its simplicity and accuracy. However, it cannot be used to quantify RNA in lipid nanoparticles (LNPs), such as those used for drug delivery in mRNA vaccines, because of light scattering by LNPs. Alternative methods such as the RiboGreen fluorescence assay require much more sample preparation and lack reproducibility and accuracy. Here we propose and demonstrate an alternative approach using an integrating sphere setup to measure scatter-free absorption spectra. RNA spectra of RNA-loaded LNPs can then be directly measured, and the total RNA concentration can be deduced. The method shows very good linearity and precision (∼1.5%), and the accuracy is estimated to be ∼5% when applied to various mRNA-LNP formulations. This work paves the way for the routine characterization of payload concentration in LNP formulation research.



KEYWORDS: *Lipid nanoparticles, mRNA-LNP, RNA quantification, UV/vis spectroscopy, Scatter-free absorption spectroscopy*

Lipid nanoparticles (LNPs) have become the vector of choice for delivery of many therapeutic payloads such as RNA and DNA,[1,2] which are otherwise easily degraded. They have been used extensively for example for mRNA vaccines,[3] notably for Covid-19.[4] The nature of the different lipidic components in LNPs, their relative concentration, the type of RNA, and the manufacturing method all affect crucial RNA-LNP properties—size, concentration, RNA payload, and encapsulation efficiency—which are ultimately linked to their therapeutic capabilities.[5] LNP formulation is therefore a very active area of research. To support this research and the many commercial endeavors related to RNA-LNPs, a battery of analytical methods have been and are still being developed for their characterization. Among them, simple, fast, and accurate techniques are highly desirable. For example, dynamic light scattering (DLS)[6] is commonly used for size characterization.[7,8] Accurately quantifying RNA concentration in LNPs however remains a challenge.

UV/visible (UV/vis) spectroscopy is commonly used for quantification of pure RNA, through its absorption at 260 nm.[9−11] However, LNPs with typical sizes in the range 60−150 nm strongly scatter light, preventing the use of standard UV/vis techniques. Scattering correction techniques based on data processing of the extinction spectrum can be used for small particles such as viruses or proteins where the scattering is weak compared to absorption,[12,13] but these do not remain accurate at larger scattering-to-absorption ratios. To circumvent this problem, extraction techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS),[14] reverse phase high-performance liquid chromatography (RP-HPLC), capillary electrophoresis, or field flow fractionation have been used to extract the RNA from the LNPs and then measure the free RNA with conventional techniques,[15] but these are time-consuming and at risk of losing RNA in the extraction process. The most common approach is the use of fluorescence assays such as RiboGreen,[16−19] after disrupting the lipidic structures with surfactants to expose the RNA. However, the method suffers from poor precision and accuracy.[19] Although widely accessible, it remains time-consuming, with preparation/measurement time in the range 10 min to more than an hour depending on the specific approach taken.

In this work, we propose and demonstrate an alternative approach based on a modified version of UV/vis spectroscopy, where only the absorption component of the sample is measured, independent of scattering. The approach is based on measuring the transmission of the sample located in the middle

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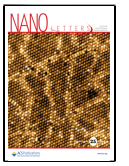

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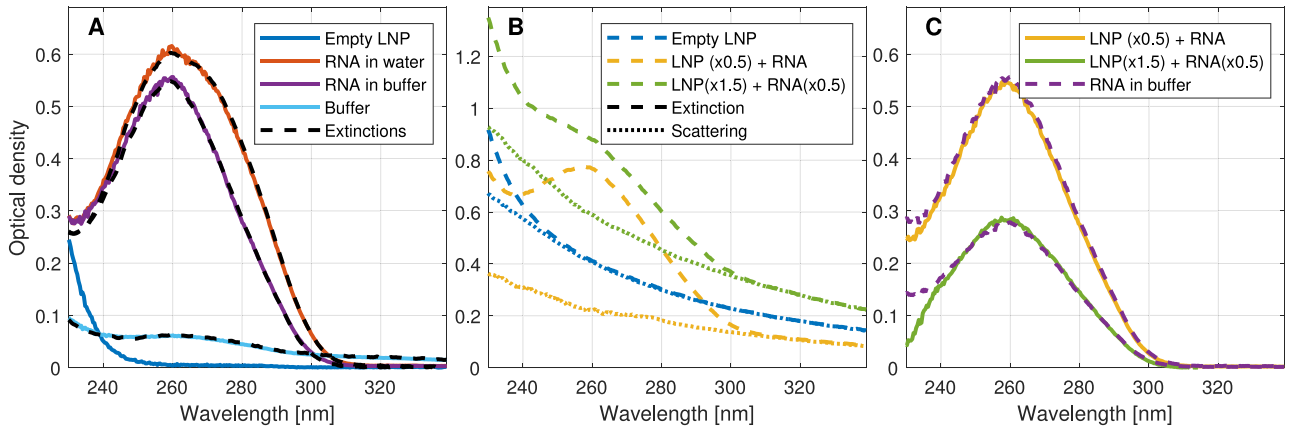

Figure 1. (A) Absorption spectra of empty SM102 LNPs and mRNA1 (20 μg/mL) in water and buffer; note that the buffer absorption (also shown) is subtracted from all spectra for samples in buffer. Dashed lines are extinction spectra, which should be identical for nonscattering samples. (B) Extinction (dashed) and scattering (dotted) spectra for mixtures of empty SM102 LNPs and mRNA1 of different concentrations as indicated in the legend. Full concentration RNA corresponds to 20 μg/mL. (C) Absorption spectra for the same mixtures as in B after subtraction of the empty LNP absorption. In the range 250−280 nm, the resulting spectra are within ±3% (higher concentration) or ±5% (lower concentration) of the spectra for corresponding pure RNA (dashed lines).

of an integrating sphere, which is a spherical cavity with highly diffusely reflecting walls,[20−22] typically made of sintered polytetrafluoroethylene (PTFE). Any scattered light is then trapped within the cavity, and the transmission spectrum is, in a first approximation, independent of scattering.[23] As a trade-off, the path length inside the sample is not well-defined and depends nonlinearly on the sample properties. In practice, careful calibration is therefore needed to transform the measured transmission spectrum into a true absorption spectrum, independent of scattering.[24] This method has been applied successfully to seawater analysis[25−28] and metallic nanoparticles.[29−32] We will refer to this approach as scatter-free absorption spectroscopy (SFAS). With this technique, the absorption spectrum of RNA and other absorbing components such as lipids can be directly measured, from which RNA concentration can be quantified based on absorption at 260 nm. This works regardless of whether the sample is scattering (as is the case for LNPs) or not. As recently stated in ref 14, "The quantification of RNA is a more complex and challenging issue. The total RNA content can be measured by using fluorescence methods. However, these methods show questionable accuracy when applied to measure mRNA loading." Because there is no reliable method to compare our results to, we demonstrate its validity indirectly through a number of tests. We first tested this approach using mixtures of mRNA-loaded and empty (unloaded) LNPs, which allows us to quantify the accuracy and test the scattering independence of the technique. We then tested the method precision and linearity on real mRNA-LNP samples. Different mixtures of loaded and unloaded LNPs are probed to test the linearity in different scattering environments. Finally, we apply it to several types of mRNA-LNPs and compare it to other methods such as RiboGreen, HPLC, and standard UV/vis following RNA extraction from the LNPs (see Methods). Overall, SFAS exhibits much higher precision and accuracy than RiboGreen. We believe this method brings back the convenience and speed of UV/vis spectroscopy for accurate quantification of RNA in LNPs. It can therefore be an invaluable tool for LNP formulation research. Besides, its applicability naturally extends to other similar systems containing various payloads and scattering components, such as DNA-LNPs, liposomes, viruses, capsids, etc.

METHOD VALIDATION WITH CONTROLLED MIXTURES

The validity of the technique can first be checked by considering samples with well-known scattering properties such as (*i*) samples with no scattering (such as pure RNA), for which the extinction and absorption should be identical, (*ii*) samples with no absorption, for which the absorption should be zero, and (*iii*) mixtures of the above, for which the absorption and scattering should be the sum of the (known) components of the mixtures. Such tests are summarized in Figure 1 using samples relevant to our study: pure RNA and empty LNPs. Figure 1A shows the absorption and extinction spectra of pure RNA. The two are effectively identical (within 1.5% at 260 nm), as expected since RNA is nonscattering. These spectra can in principle be used to determine the RNA concentration from Beer−Lambert law, providing that its extinction coefficient ϵ at 260 nm is known:[9]

$$c = \mathrm{OD260}/\epsilon \tag{1}$$

where OD260 is the optical density (OD) at 260 nm for a 1 cm path length. It is worth pointing out that the RNA spectrum appears to depend slightly on the medium into which it is dissolved, i.e., water or buffer, in terms of both its spectral shape and ϵ (even after subtraction of the intrinsic buffer absorption). The value $\epsilon \approx 0.025$ mL/μg (sometimes quoted as $1/\epsilon = 40$ μg/mL) is commonly used for RNA,[9] which would here give $c = 24.5$ μg/mL for RNA in water in the example of Figure 1A. This is higher than the nominal concentration (20 μg/mL), but it is known that ϵ can vary with exact RNA sequence, so we should instead use the nominal concentration and eq 1 to deduce ϵ. We find

$$\epsilon_{\mathrm{water}} = 0.0306\ \mathrm{mL}/\mu\mathrm{g} \tag{2}$$

$$\epsilon_{\mathrm{buffer}} = 0.0277\ \mathrm{mL}/\mu\mathrm{g} \tag{3}$$

This experimental value for $\epsilon_{\mathrm{water}}$ is in fact very close to the one found from the theoretical RNA sequence using the

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Table 1. Summary of the Composition of mRNA1-Loaded SM102 LNP Samples Measured to Test Linearity and Scattering Independence[a]

	Buffer	Empty LNP	Loaded LNP	RNA conc.	LNP conc.	Abs. OD260	Error [%]	Sca. OD260
SM2	0%	100%	0%	0	1	0.009	-	0.34
SM3	0%	0%	100%	1	1	0.558	0.59	0.52
SM4	33%	33%	33%	1/3	2/3	0.178	1.2	0.3
SM5	50%	50%	0%	0	1/2	0.007	-	0.19
SM6	0%	50%	50%	1/2	1	0.278	0.79	0.45
SM7	50%	0%	50%	1/2	1/2	0.265	0.24	0.30
SM8	25%	25%	50%	1/2	3/4	0.275	-	0.38
SM9	25%	50%	25%	1/4	3/4	0.139	1.2	0.32
SM10	50%	25%	25%	1/4	1/2	0.138	0.85	0.25

[a]Nominal RNA and LNP concentrations are given relative to the concentrations of the starting loaded mRNA-LNPs (SM3), for which c_{RNA} = 20 μg/mL. LNP concentration is only approximate, as it assumes that empty LNPs (SM2) and loaded LNPs (SM3) have the same concentration. Also included in the table are the measured absorption OD at 260 nm obtained from 3 repeats and their relative standard deviation (given as relative error). Note that only one spectrum is measured for SM8.

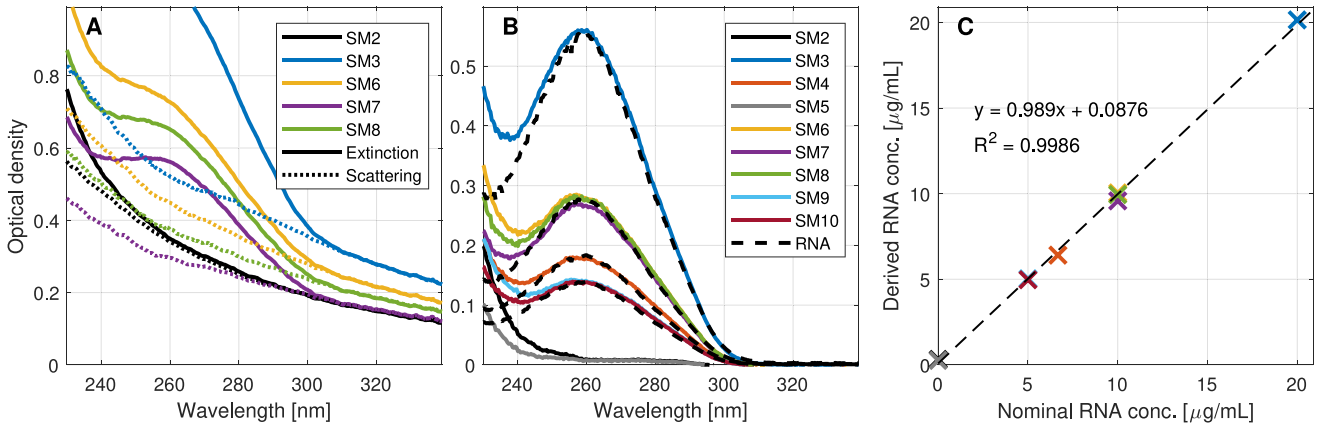

Figure 2. (A) Extinction (solid lines) and scattering (dashed lines) spectra for a selection of mRNA1-SM102-LNP samples from Table 1. Note that SM6/SM7/SM8 have the same RNA content. (B) Absorption spectra of LNP samples (solid lines). Dashed lines show the reference mRNA1-in-buffer spectrum for the respective nominal RNA concentrations. (C) Derived RNA concentration from the absorption OD at 260 nm (assuming ϵ = 0.0277 mL/μg) vs nominal concentration.

Integrated DNA Technologies OligoAnalyzer tool (0.0299 mL/μg) and the RNAcalc[10] tool (0.0312 mL/μg).

Figure 1B shows the extinction and scattering spectra of empty SM102 LNPs in buffer, by themselves or mixed at various concentrations with RNA. The absorption of these empty LNPs is close to zero down to 260 nm but increases slightly below, as shown in Figure 1A, likely due to absorption by the lipids. It is clear that scattering for these particles is non-negligible at this concentration, with a scattering optical density at 260 nm between 0.2 and 0.6. This prevents any accurate RNA quantification from the extinction spectrum only. The absorption spectra, however, match very well the previously measured RNA-in-buffer absorption; they agree within 5% in the region of interest from 250 to 280 nm; see Figure 1C. Note that the (small) intrinsic absorption of the empty LNPs is subtracted from the spectra; otherwise, it affects the spectra below 260 nm, as noted earlier. Overall, the results of Figure 1 validate the use of the SFAS technique for RNA quantification, even in the presence of scattering. The estimated accuracy from these tests is on the order of 5%.

QUANTIFYING RNAS IN LNPS

Principle and Demonstration. We now use SFAS to study LNPs loaded with mRNA. We start with SM102 LNPs, as their intrinsic absorption is small at 260 nm, as shown in Figure 1A. We first test the linearity of the technique and its independence of scattering by measuring different dilutions of the mRNA-LNPs, some mixed with empty LNPs to artificially vary the amount of scattering. A total of 9 samples are prepared, as summarized in Table 1 and measured as shown in Figure 2. The buffer spectrum is subtracted from all spectra. All spectra are measured in triplicate, and the precision derived from these repeats is on the order of 1%; see Table 1. Note that the loaded LNPs are larger than the empty LNPs (see sizes from DLS given in the Supporting Information), which accounts for the apparent discrepancy in scattering OD for example between sample SM2 and SM3.

The scattering spectra, Figure 2A, vary from sample to sample (by design), which prevents one from using extinction spectra for RNA quantification. In contrast, the absorption spectra, Figure 2B, match perfectly the reference RNA-in-buffer spectrum measured for the same RNA sequence (mRNA1, see Figure 1A). It is interesting to note that the mRNA1-in-water spectrum (also shown in Figure 1A) does not match as well; therefore, it is likely that the mRNA inside the LNPs is primarily surrounded by buffer. The absorption OD at 260 nm can be used to extract the mRNA-in-LNP concentrations (eq 1), and the results are very linear (see Figure 2C, R^2 = 0.9986) despite the very different scatterings (see Table 2). Samples SM6/SM7/SM8 for example have the

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Table 2. Summary of Measured mRNA Loadings (in μg/mL) in the LNPs Considered in This Study, as Determined by Different Analytical Approaches as Described in the Text and Supporting Information[a]

	Nominal	RiboGreen	UV/RNA-extraction	HPLC	SFAS
SM/RNA1	20 (2)	21 (3.1)	17.7 (0.9)	14.7 (0.4)	20.1 (0.3)
SM/RNA2	20 (2)	17.6 (2.6)	17.4 (0.9)	15.9 (0.5)	20.4 (0.3)
CV/RNA1	20 (2)	20.2 (3)	16.1 (0.8)	14.2 (0.4)	23.1 (0.3)
MC/RNA1	4 (0.4)	3.6 (0.5)	3.5 (0.2)	3.1 (0.1)	3.7 (0.1)

[a]In brackets is the estimated uncertainty based on a precision of 10% (nominal), 15% (RiboGreen), 5% (UV/RNA-extraction), 3% (HPLC), and 1.5% (SFAS).

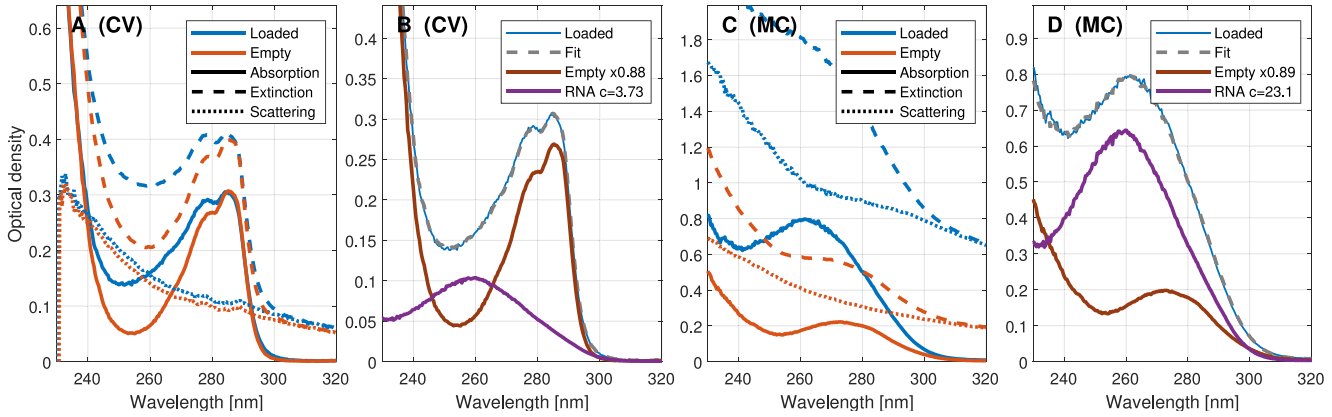

Figure 3. (A) Absorption (solid), extinction (dashed), and scattering (dotted lines) spectra of proprietary CV-LNPs, either loaded with mRNA1 or empty. (B) Linear decomposition of the absorption spectrum of the loaded CV-LNPs, showing the fit and the two components (empty LNPs and pure RNA in buffer). (C, D) Same as (A, B) for MC3 LNPs.

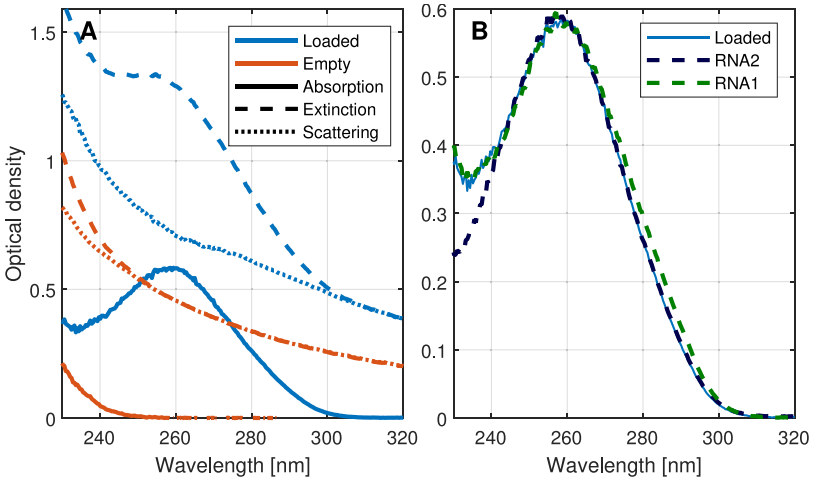

Figure 4. (A) Absorption (solid), extinction (dashed), and scattering (dotted lines) spectra of SM102 LNPs, either loaded with the second type of RNA (RNA2) or empty. (B) Absorption spectrum of RNA2 compared to the spectra of pure RNA1 and RNA2 in buffer. An RNA2 concentration in LNP of 20.4 μg/mL is deduced.

same RNA concentration but scattering ODs ranging from 0.3 to 0.5 at 260 nm, and their absorption or derived concentration has a relative standard deviation of 2.3% (they all agree within ±3%). The slope of the linear dependence is 0.99, so the derived concentrations are, on average, 1% smaller than the nominal concentrations. However, the nominal total RNA concentration for RNA-LNPs was derived from averaging different methods (based on extraction followed by RiboGreen and Nanodrop), but the overall accuracy of this estimate is believed to be only on the order of 5−10%. This accuracy is therefore not enough to conclude on the accuracy of the SFAS, which, from the linearity test, appears to be much better than that.

Absorbing Lipid Formulations. With the SM102 formulation, the analysis is simplified, because the lipids have negligible absorption at 260 nm. However, some formulations may use different lipids, including some absorbing at 260 nm and interfering with RNA quantification. In such cases, a linear decomposition of the measured absorption spectrum in terms of the reference RNA-in-buffer and empty LNP spectra can be performed as illustrated in Figure 3. We consider two types of LNPs: a proprietary formulation (CV) and an MC3-lipid formulation (MC). Both have lipid absorption extending up to

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300 nm, as clearly evidenced in the absorption spectra of the empty LNPs; see Figure 3. From UV/vis measurements of the individual lipid components (not shown here), it appears that the absorption at 260 nm comes primarily from the ionizable lipids in these two cases. The absorption spectra of the loaded LNPs, $A_{\text{Loaded}}(\lambda)$, are now a mixture of the lipid and RNA absorption, which we fit using a linear decomposition:

$$A_{\text{Loaded}}(\lambda) = \alpha A_{\text{empty}}(\lambda) + \beta A_{\text{RNA}}(\lambda) \quad (4)$$

A_{RNA} is the reference spectrum of the same RNA in buffer measured earlier (at c_0 = 20 μg/mL). A_{Empty} is the scatter-free absorption spectrum of the empty LNP, which simply reflects the contribution of lipid to absorption. The linear fit is performed over the range $240 \leq \lambda \leq 310$ nm, and it agrees very well with the measured loaded LNP absorption (Figure 3B,D). The RNA concentration is then deduced as $c = \beta c_0$. These are again within the uncertainty range for the nominal RNA concentrations: 4 μg/mL for CV LNPs and 20 μg/mL for MC LNPs.

Comparing Different mRNA. To further test the methods, we synthesized and measured SM102 particles with a second type of mRNA (denoted RNA2). The corresponding spectra are shown in Figure 4. As for RNA1-SM LNPs, we can deduce the RNA concentration by comparing the absorption spectrum to that of pure RNA2-in-buffer, which differs slightly from that of RNA1. We deduced a concentration of 20.4 μg/mL, within 2% of the nominal concentration. It is interesting to point out that the RNA2-LNP spectrum (solid line in Figure 4B) is almost identical to that of RNA2 (dashed black line) but not to that of RNA1 (dashed green line), as seen in the discrepancy between 260 and 300 nm. This further confirms that the RNA-in-LNP spectrum is unaffected compared to the pure RNA-in-buffer. It also shows that to maximize accuracy, it is necessary to measure a reference pure RNA sample in buffer with the same type of RNA as encapsulated in the LNPs, instead of using the literature value for the RNA extinction coefficient.

DISCUSSION AND CONCLUSION

The RNA concentrations deduced from SFAS for each type of loaded LNPs are summarized in Table 2 and compared to other methods, namely, (*i*) RiboGreen and (*ii*) UV/vis (Nanodrop) measurement of RNA following extraction from LNPs, (*iii*) HPLC, all as described in the Method section. RNA quantification in LNPs by RiboGreen is known to be subject to a large uncertainty,[19] which we estimate to be about 15% in our case. The HPLC method is not yet validated, but the estimated precision is about 3% (the accuracy is unknown). A major challenge in RNA-based HPLC is the low elution recoveries caused by adsorption of the instrument surfaces and the column material. This issue is often addressed by applying harsh conditions such as high elution temperatures, elevated pH levels, or high concentrations of salts in buffers. However, these conditions, particularly alkaline pH and elevated temperatures, can compromise the integrity of RNA. As a result, quantification of RNA in LNPs by HPLC can be subject to large systematic errors.[14] We have not studied in detail the UV/RNA-extraction method, but we believe the precision is on the order of 5% (but accuracy is unknown). The nominal concentration in Table 2 is determined on concentrated samples using an average of Ribogreen and Nanodrop, within an estimated accuracy of 10%. Note that the UV/RNA-extraction and HPLC ultimately use UV/vis of RNA after separation but assume an extinction coefficient of 0.025 mL/μg, so we have applied a factor of 0.025/0.0277 to the raw analytical data to correct for that. The values in the table are the corrected values, together with estimated precision in brackets.

From these, it is difficult to assess the accuracy of SFAS because the precision of the other methods is less than that of SFAS. SFAS agrees with RiboGreen and nominal concentration within experimental errors, but the uncertainty is very large for these methods. Compared to RiboGreen, SFAS, and nominal concentrations, it would appear that UV/RNA-extraction and HPLC suffer from systematic errors that underestimate the RNA concentration. This could be understandable, as these techniques are fundamentally based on extraction/separation of the RNA inside the LNPs, and the process may result in losses of RNA material. In the absence of a reference technique with an accuracy comparable to the SFAS precision (about 1.5%), we believe that the SFAS results are the most reliable. This technique is also by far the simplest and fastest: a measurement typically takes a few minutes for sample pipetting/handling and reference/sample measurements (each 10 s). The mixture tests suggest that the accuracy of SFAS is on the order of 5%. This may, however, be affected by any change in the RNA optical properties once encapsulated. We cannot exclude this, but note that this effect is not observed in capsids,[12] and we also see no evidence of spectral change in our results. Other important characteristics of these methods are estimated and compared in Table 3.

Table 3. Comparison of the Main Characteristics of the Two Methods of RNA Quantification in LNPs: Ribogreen (Fluorescence) and the Scattering-Free Absorption Spectroscopy (SFAS)

	RiboGreen	SFAS
Total time needed	∼1 hour	1−2 min per sample
Raw material cost	∼500 USD for a 200-sample kit	None
Limit of detection	∼1 ng of RNA	∼1 μg of RNA
Precision	∼15%	∼1.5%
Accuracy	∼20%	∼5%

SFAS is better than Ribogreen in most aspects except the limit of detection. A minimum total RNA mass of ∼1 μg is needed for SFAS, which is much larger than Ribogreen. It should however be noted that the sample can be recovered and used for example for a DLS measurement, which typically requires the same concentration and volume of LNPs.

In conclusion, we proposed and validated an alternative approach to RNA quantification in RNA-LNP systems. The method is based on the use of an integrating sphere to measure the scatter-free absorption spectrum, from which the RNA concentration is derived from the OD at 260 nm, as in conventional pure RNA quantification. The precision (around 1.5%) and accuracy (under 5%) are better than existing, much more time-consuming, methods. The absence of a more accurate technique to compare against prevents us from proving this accuracy more rigorously. For LNPs with lipids absorbing in the same region as RNA, especially around 260 nm, a linear decomposition of the spectra is necessary, which could slightly affect the accuracy, especially at low RNA-to-lipid ratios. However, we have shown that the method still works very well for an RNA concentration of 4 μg/mL, and we

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can expect from the measured signal-to-noise ratio that this could be extended down to at least 1 μg/mL. Such a method could therefore prove a game-changer for routine characterization of RNA-LNPs, from formulation development to quality control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.5c01491.

Methods and experimental details: reagents, mRNA molecules, mRNA-LNP quantification by NanoDrop, HPLC, and Ribogreen assay, LNP synthesis and composition, dynamic light scattering, scatter-free absorption spectroscopy (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Eric C. Le Ru − *Marama Labs Limited, Wellington 6012, New Zealand; The MacDiarmid Institute for Advanced Materials and Nanotechnology, School of Chemical and Physical Sciences, Victoria University of Wellington, Wellington 6140, New Zealand;* orcid.org/0000-0002-3052-9947; Email: eric.leru@vuw.ac.nz

Roland Böttger − *CureVac SE, 72076 Tübingen, Germany;* orcid.org/0000-0002-5764-6844; Email: roland.boettger@curevac.com

Darren Andrews − *Marama Labs Limited, Dublin D11KXN4, Ireland*; Email: darren.andrews@maramalabs.com

Authors

Patrick Baumhof − *CureVac SE, 72076 Tübingen, Germany*

Jelena V. Rakonjac − *Marama Labs Limited, Wellington 6012, New Zealand*

Geoffry Laufersky − *Marama Labs Limited, Wellington 6012, New Zealand*

Brendan L. Darby − *Marama Labs Limited, Dublin D11KXN4, Ireland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.nanolett.5c01491

Notes

The authors declare the following competing financial interest(s): E.C.L.R., D.A., J.V.R., G.L., B.L.D. are employees and shareholders of Marama Labs, which commercializes the CloudSpec spectrophotometer that was used in this study. R.B. and P.B. are employees of CureVac.

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Supporting Information for Rapid and accurate quantification of RNA in lipid nanoparticles by scatter-free UV/Visible spectroscopy

Eric C. Le Ru,[*,†,‡] Roland Böttger,[*,¶] Darren Andrews,[*,§] Patrick Baumhof,[‖] Jelena V. Rakonjac,[†] Geoffry Laufersky,[†] and Brendan L. Darby[§]

†*Marama Labs Limited, 32 Salamanca Road, Wellington, 6012, New Zealand*

‡*The MacDiarmid Institute for Advanced Materials and Nanotechnology, School of Chemical and Physical Sciences, Victoria University of Wellington, P.O. Box 600, Wellington 6140, New Zealand*

¶*CureVac SE, 72076 Tübingen, Germany*

§*Marama Labs Limited, DCU Alpha, Old Glasnevin Road, Dublin, D11KXN4, Ireland*

‖*CureVac SE, Friedrich-Miescher-Str. 15 ,72076 Tübingen, Germany*

E-mail: eric.leru@vuw.ac.nz; roland.boettger@curevac.com; darren.andrews@maramalabs.com

Methods and experimental details

Reagents. All chemicals were purchased from commercial suppliers with purities of $\geq 98\%$ and used as received without further purification. Ethanol (absolute), sucrose, Pluronic F-127, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 1 M, pH 7.4), cholesterol, and Delbucco's phosphate buffered saline (PBS) were purchased from Merck KGaA

(Darmstadt, Germany). Triethylammonium acetate (TEAA, 1 M) was purchased from AppliChem GmbH (Darmstadt, Deutschland). Heparin-Natrium-5000 was purchased from Ratiopharm GmbH (Ulm, Germany). Triton X-100 (10%, w/v) was purchased from G-Biosciences (St. Louis, MO). Quant-iT^{TM} RiboGreen^{TM} RNA reagent and 20x Tris-EDTA (TE) buffer were purchased from Life technologies Corporation (Eugene, OR). NaCl (5 M) was purchased from Lonza Ltd (Basel, Switzerland). Sodium citrate (100 mM, pH 4) was purchased from Alpha Teknova Inc. (Hollister, CA). Isopropanol was purchased from VWR International SAS (Briare, France). (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(Dimethylamino)butanoate (DLin-MC3-DMA) and 9-Heptadecanyl 8-(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]aminooctanoate (SM102 ionizable lipid) were purchased from MedChemExpress LLE (Monmouth, Nu). 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG) was purchased from NOF America Corp. (San Mateo, CA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

mRNA-LNP quantification by NanoDrop. mRNA-LNPs (100 μL) were mixed with Pluronic F-127 (5 μL, 10% v/v in water) and Heparin (50 μL, 500 I.E. in 10 mM Tris-HCl buffer) and incubated in a ThermoMixer (15 min, 45°C, 1000 rpm, Eppendorf SE, Hamburg, Germany) to disrupt vesicles. NaCl (100 μL, 5 M), sodium citrate buffer (100 μL, 50 mM), isopropanol (100 μL) and chilled ethanol (1200 μL, -20°C) were added and mixed thoroughly to precipitate RNA. Samples were centrifuged (20 min, 4°C, 13200 rpm, Centrifuge 5430, Eppendorf SE). The supernatant was carefully removed and the RNA containing pellet was dried in a Thermomixer (60 min, 37°C, 500 rpm). The pellet was resuspended in Tris-HCl buffer (250 μL, 10 mM) and the RNA concentration was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Madison, WI).

RiboGreen Assay. mRNA-LNPs diluted in TE buffer (95 μL, $< 2\,\mu$g/mL RNA) were added to 96-well black bottom plate (Thermo Fisher Scientific) and either mixed with 5 μL TE buffer to keep LNPs intact, or with 5 μL Triton X-100 (10% w/v, G-Biosciences, St.

Louis, MO) to release encapsulated RNA. Quant-iT RiboGreen RNA reagent (100 μL, 200-fold diluted in TE buffer) was added to each well and the plate was shaken in a plate reader (TriStar LB949, Berthold Technologies GmbH, Bad Wildbad, Germany) for 2 min. The fluorescence intensity (excitation/emission maxima: 500/525 nm) was recorded to detect RNA. To estimate the concentration, standard dilutions of equivalent RNA ($0.063-2\,\mu$g/mL) were used instead of samples to achieve a linear calibration curve.

mRNA-LNP quantification by HPLC. mRNA-LNPs (5 μL) were injected into a Vanquish Flex chromatography system (Thermo Fisher Scientific GmbH, Dreieich, Germany) coupled on-line to a diode-array detector recording the absorbance at 260 nm. Separation relied on a BEH-C18 column (inner diameter: 2.1 mm, length: 50 mm, particle size: 1.7 μm, pore size: 130 Å, column temperature: 60°C, Waters Corp., Milfiord, MA) at a flow rate of 0.5 mL/min using a binary gradient of two eluents A and B consisting of 0.1 M aqueous TEAA and 0.1% ammonium hydroxide in 50:50 (v/v) methanol/acetonitrile, respectively. The following gradient was applied: 1 min: A/B (90/10), 9 min: A/B (0/100), 26 min: A/B (0/100), 28.5 min: A/B (90/10), 31 min: A/B (90/10). The area under the curve of the RNA signal was used to estimate the RNA concentration using the Lambert-Beer equation and extinction coefficient of 0.025 mL mg^{-1} cm^{-1}.

mRNA molecules. The mRNAs used in this study code either for Rabies virus glycoprotein (RNA1) or for photinus pyralis luciferase (RNA2). Both mRNA contain a 5′ untranslated region (UTR) from the human hydroxysteroid 17-beta dehydrogenase 4 gene (HSD17B4) and a 3′ UTR from the human proteasome 20S subunit beta 3 gene (PSMB3), followed by a histone stem loop and a polyA stretch.[1] RNA1 contained 100% N1-methylpseudouridine (m1ψ)-modification and RNA2 was unmodified.

LNP composition. The mRNAs were encapsulated with three types of LNPs, each consisting of 4 lipid components. MC-LNPs consisted of DLin-MC3-DMA[2] (50 mol%), DSPC (10 mol%), Cholesterol (38.5 mol%) and DMG-PEG (1.5 mol%). SM-LNPs consisted of SM102 ionizable lipid[3] (50 mol%), DSPC (10 mol%), Cholesterol (38.5 mol%) and DMG-

PEG (1.5 mol%). CV-LNP relied on proprietary LNP technology from CureVac consisting of ionizable aminolipid, structural lipid, Cholesterol and hydrophilic lipid.

LNP synthesis. mRNA-LNP were prepared by toroidal microfluidics using an Ignite© system (Precision Nanosystems Inc., Vancouver, BC, Canada). Lipids were dissolved in ethanol and mRNAs were dissolved either in 50 mM sodium acetate buffer, pH4 (CV-LNP) or in 50 mM sodium citrate buffer, pH4 (MC3 and SM102) at a concentration of 0.16 g/L. Lipid solution and mRNA solution were mixed using the microfluidic cartridge of the Ignite© at a flow rate ratio of 1:3 (water:ethanol) and a total flow rate of 20 mL/min. A total volume of 12 mL per sample was collected and subsequently dialyzed against 5 mM Tris-HCl buffer, pH 7.5 containing 20 w% sucrose for 16 h using Slide-A-Lyzer© dialysis cassettes (molecular weight cut-off 3.5 kDa, Thermo Scientific, Rockford, IL). The buffer was replaced after 2 h and 4 h. Note that equivalent empty LNPs were also prepared following the same method but without mRNA.

After dialysis, the samples were concentrated 4-fold by centrifugation (1300 g, Eppendorf Centrifuge 5424R, Eppendorf AG, Hamburg, Germany) using Vivaspin® Turbo 15 centrigation filters (molecular weight cut-off 30 kDa, Sartorius Stedim Lab Ltd, Stonehouse, UK) and filtered using Rotilabo© PES syringe filters (pore size 0.2 μm, diameter 15 mm, Carl Roth GmbH+Co. KG, Karlsruhe, Germany). After quantification by RiboGreen assay and Nanodrop (taking the average), the samples were adjusted with formulation buffer to a final mRNA concentration of 400 μg/mL and frozen at -80°C until further use. All samples in this study are obtained by suitable dilution after thawing and the quoted nominal RNA concentrations are deduced from the dilution factor assuming a 400 μg/mL starting concentration.

Dynamic Light Scattering. The hydrodynamic size and polydispersity index (PDI) were measured by dynamic light scattering (DLS) using a Dynapro III plate reader (Wyatt Technologies Europe, Dernbach, Germany). The samples were diluted (1:100, v/v) in phosphate buffered saline (PBS), 35 μL were transferred into a 384-well clear-bottomed plate (Aurora Microplates, Scottsdale, AZ), sealed by a drop of silicon oil and measured at 25°C. The aver-

age of three consecutive measurements of each well was reported. The particle sizes are (PDI in bracket), SM-LNP loaded with RNA 1: 85.2 nm (0.145), SM-LNP loaded with RNA 2: 80.5 nm (0.073), SM-LNP empty: 63.9 nm (0.088), CV loaded: 73.8 nm (0.128), CV empty 55.1 nm (0.098), MC-LNP loaded: 97.1 nm (0.356), MC-LNP empty: 57.0 nm (0.229).

Scatter-free absorption spectroscopy. To measure SFAS spectra, we use the CloudSpec spectrophotometer from Marama Labs (Ireland). For a typical measurement, 1 mL of sample is pipetted into a 1 cm quartz cuvette attached to a special holder, which is then reproducibly suspended inside an integrating sphere. The instrument features two optical pathways. The first corresponds to standard transmission configuration, which measures the extinction spectrum (equal to absorption plus scattering) as in standard UV/Vis. The second uses the integrating sphere and proprietary algorithms to obtain the absorption spectrum. The scattering spectrum is obtained indirectly as extinction minus absorption. All spectra are given as optical density (OD) for an equivalent 1 cm pathlength. As in conventional UV/Vis, a reference must first be measured, ultrapure water in our case. New water references were measured regularly, typically every 30 minutes. For all RNA or mRNA-LNPs samples in buffer (Tris-HCl+20% sucrose), the buffer spectrum is subtracted to account for its intrinsic absorption. Between measurements, the cuvette was cleaned as follows: rinse with water (3x), rinse with ethanol (3x), dry under pressured air stream.

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