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High Resolution Particle Characterization to Expedite Development and Regulatory Acceptance of Nanomedicines

Darby Kozak[1*], Murray Broom[2] and Robert Vogel[2,3]

[1]*Izon Science US Ltd, 85 Bolton Street, Cambridge, MA, USA;* [2]*Izon Science, 8C Homersham Place, Christchurch, New Zealand;* [3]*School of Mathematics and Physics, The University of Queensland, Brisbane, Australia*

Abstract: The pharmaceutical industry as well as European and US governing agencies have indicated the need for more accurate, high resolution, characterization of complex drug materials, nanomedicines, to facilitate their development and eventual approval. In particular, accurately measuring the size, zeta-potential, and concentration of nanomedicines is desired. Herein we demonstrate the comprehensive and high resolution analysis capabilities of tunable resistive pulse sensing (TRPS) on the most widely approved nanomedicines to-date, liposomal particles. The number-based size distribution, concentration and volume fraction of liposomes formed by extrusion through a 100 nm or 200 nm Nucleopore filter membrane are shown as well as how freeze-thaw aggregation changes individual liposomes and the overall size distribution. In addition, the simultaneous size and zeta-potential analysis capabilities of TRPS is used to characterize the homogeneity and difference between liposomes made with and without the addition of PEGylated phospholipids.

Keywords: Coulter counter, particle characterization, pore sensor, TRPS, qNano.

INTRODUCTION

The efficacy of many therapeutics (*e.g*. small molecule drugs and biopharmaceutics) can be improved by compounding them with a secondary carrier material to form a nanomedicine particle [1]. Nanomedicines improve the solubility, release profile, and circulation time of the therapeutic agent as well as help direct their localized delivery within the body [2]. One of the best known examples of this is Doxil, a ~90 nm PEGylated liposomal formulation of the anti-cancer drug doxorubicin hydrochloride. By encapsulating doxorubicin within a liposomal delivery vehicle of specific size and surface properties (*e.g*. degree of PEGylation), the complex drug has a longer circulation time and effective therapeutic window, as well as passively targets tumors *via* the enhanced permeability and retention effect [3].

Key to the development and quality assurance of nanomedicines is controlling the chemical and physical properties that make these complex drugs effective and/or even potentially toxic. In addition to the chemical composition, it is believed that the size, zeta-potential, and concentration of nanomedicine particles are critical to their therapeutic performance [1-3]. In addition, these physical properties also play a fundamental role in the colloidal stability of the complex drug product, which influences the eventual nanomedicine formulation and shelf life [4]. For these reasons, there is an inherent need for instrumentation in the development and in the quality assurance/regulatory process that can accurately measure the size, electrophoretic mobility (zeta-potential), and concentration of nanomedicines.

*Address correspondence to this author at the Izon Science US Ltd, 85 Bolton Street, Cambridge, MA, USA; Tel: +1 617 945 5936;
E-mail: darby@izon.com

A range of instrumental techniques have been used to characterize particle dispersions. These technologies can be broadly categorized as ensemble or single particle analysis techniques [5]. Ensemble techniques such as static and dynamic light scattering (SLS and DLS), acoustic spectroscopy, field flow fractionation (FFF), and analytical ultra centrifugation (UAC) measure the bulk dispersion and calculate the average particle properties. This averaging often results in low measurement resolution and can be biased by or exclude information on small sub-populations within the dispersion. Measurement resolution is improved by pre-separating the dispersion, *e.g*. FFF and UAC, prior to analysis. A key limitation of all ensemble techniques for nanomedicines applications is the inability to measure a true number-based distribution or particle concentration.

In contrast, single particle analysis techniques such as imaging (*i.e*. TEM, SEM, microscopy) nanoparticle tracking analysis (NTA), and tunable resistive pulse sensing (TRPS) measure and collate the properties of individual particles within a dispersion. The resulting number-based histograms often provide a more accurate measure of the true dispersion properties. Furthermore, particle concentration can be calculated if the corresponding measured dispersion volume is known. A general limitation of single particle analysis techniques are that they are often time consuming and only measure a small sample amount, typically only hundreds or a few thousand particles. Sample amount is important to ensuring the statistical significance of a measurement as well as particles at very low concentrations within a dispersion are accurately represented.

Of these particle analysis techniques few can provide a comprehensive and accurate measure of size, electrophoretic mobility, and concentration. One such technique is TRPS, which provides a direct measure of particle concentration [6]

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as well as high resolution analysis of the sample size [7] and electrophoretic mobility (zeta-potential) [8] distribution. A review of TRPS and the associated fundamental analysis principles of the technique can be found in Kozak *et al*. [9] and the book chapter by Willmott *et al*. [10].

Herein we demonstrate the analysis capabilities of TRPS on a range of liposome formulations. Liposomes are currently one of the most commercially evolved and regulatory accepted nanoparticles for drug delivery. TRPS enables particle-by-particle size and zeta-potential distribution as well as the number concentration and volume fraction of liposomes to be determined. In addition to liposomes, the presented TRPS measurement methodology can be readily applied to any drug delivery particulate system, *e.g*. nanobubbles, emulsions, and metallic or polymeric particle materials, in which the particles are dispersed in aqueous electrolyte solutions.

MATERIALS AND METHODS

All measurements were made using an Izon qNano (NZ). Polystyrene calibration particles with a concentration of 1.2×10^{13} particles / mL and mode size of 115 nm were purchased from Bangs Laboratories (USA). Soy phosphatidylcholine liposomes, SPC liposomes, were prepared by the thin film hydration method and extruded through a 100 or 200 nm Nucleopore Track-Etch membrane using a Lipex Extruder at room temperature (Northern Lipids, Canada) as per the method described in Yang *et al.* [11]. Liposomes and PEGylated liposomes, composed of DPPC/CHOL (55:45 mol/mol) and DPPC/CHOL/mPEG2000-DSPE (50:45:5 mol/mol, respectively, were purchased from FormuMax (USA). Commercially available Caelyx and a generic reproduction were kindly supplied by Azaya Therapeutics (USA). All samples were dispersed in phosphate buffered saline (pH 7.2 and 137 mM NaCl, 2.7 mM KCl, ~12 mM phosphates) for analysis. Particle concentration, size, and zeta-potential were calculated using Izon Control Suite Software V2.4 on a minimum of 500 particle events.

RESULTS AND DISCUSSION

TRPS instruments measure the properties of individual particles as they pass through a small hole or 'pore' produced in a membrane [12]. In addition to characterizing each particle, the collation of hundreds to thousands of particles is used to give an accurate depiction of the overall number-based distribution of these properties within the sample.

TRPS has been routinely used to quantitatively size and determine the concentration of synthetic and biological nanoparticles including liposomes, exosomes, and viruses [6, 7, 11-13]. The size of individual particles traversing the TRPS systems is calculated from the linear relationship between the particle volume and the magnitude of the resistance pulse signal ΔR it generates [7a]. In addition to first principle methods, which require measurement of the pore size and resistivity of the media, [7b] direct calibration of the sample can also be used to quantitate particle size. Using direct calibration, the volume of an unknown particle, V_s, is calculated by,

$$\frac{\Delta R_s}{V_s} = \frac{\Delta R_c}{V_c} \qquad \text{Eq. (1)}$$

where V_c is the volume of the calibration particle and ΔR_s and ΔR_c are the pulse signals generated by the sample and calibration particles, respectively.

This one-to-one calibration negates the need to measure the properties of the pore or the media so long as the analysis conditions (*e.g*. pore size, dispersion media, applied pressure, and voltage) of the calibration and sample are the same.

As TRPS is a volume based measurement, it is very sensitive to small differences in particle size. For example, doubling the radius of a spherical particle gives rise to an eight fold increase in the pulse signal magnitude. This makes TRPS one of the most sensitive, and therefore accurate, measurement techniques, especially for complex samples such as multimodal or aggregated particle dispersions [14].

The high resolution of TRPS has many benefits in characterizing, understanding and optimizing the production, formulation, and stability of complex drug particles. Furthermore, the measurement sensitivity of TRPS can be beneficial in the regulation and quality assurance of nanomedinces. Both the EMA and FDA have been moving toward new tools, standards, and approaches in the characterization and assessment of nanomedicines [15]. This is becoming increasingly important as the number of complex nanomedicines under development go from clinical trials to commercial production to off-patent generics.

In 2013, the FDA approved one of the first generic complex drugs, a generic form of Doxil. Regulatory approval of a generic is currently dependent upon a manufacturer showing bio- and physical- equivalence to the name brand drug. For generic forms of Doxil, the FDA guidelines include specific reporting statistics (d10, d50, d90 and span) and acceptable tolerances (confidence interval of 95%) of the particle size and zeta-potential distribution [16]. In recommending this information, the FDA advocated that the physical properties of these nanomedicines are also an important component to the drug's therapeutic performance, and as such, must be regulated to ensure their quality.

An example of a physically equivalent generic to Caelyx (European brand equivalent of Doxil), as measured by TRPS, is shown in (Fig. **1**). The high resolution particle-by-particle analysis of TRPS enables an accurate comparison of the size distributions. In turn, this level of measurement detail provides better understanding and confidence in the physical properties of the dispersion. In contrast, ensemble techniques such as DLS can miss subtle yet critical differences in the sample properties, in particular for polydisperse samples. Interestingly, it has been shown that although different particle analysis techniques have varying measurement accuracy and resolution they all have very similar reproducibility [14].

The D10, D50 and D90 values for Caelyx and the generic, measured by TRPS and DLS (% number) are given in Table **1**. As shown, TRPS indicates a more monodisperse sample with spans half of that measured by DLS. The broader span observed by DLS may be due in part to its lower measurement resolution as well as the fact that the

Table 1. Size distribution statistics of Caelyx and generic equivalent by TRPS and DLS.

	D10 (nm)	D50 (nm)	D90 (nm)	Span	Conc (part/mL)
Generic	73	83	108	0.42	1.7×10^{12}
Caelyx	75	84	109	0.40	1.4×10^{12}
Caelyx (DLS)	47	68	109	0.91	-

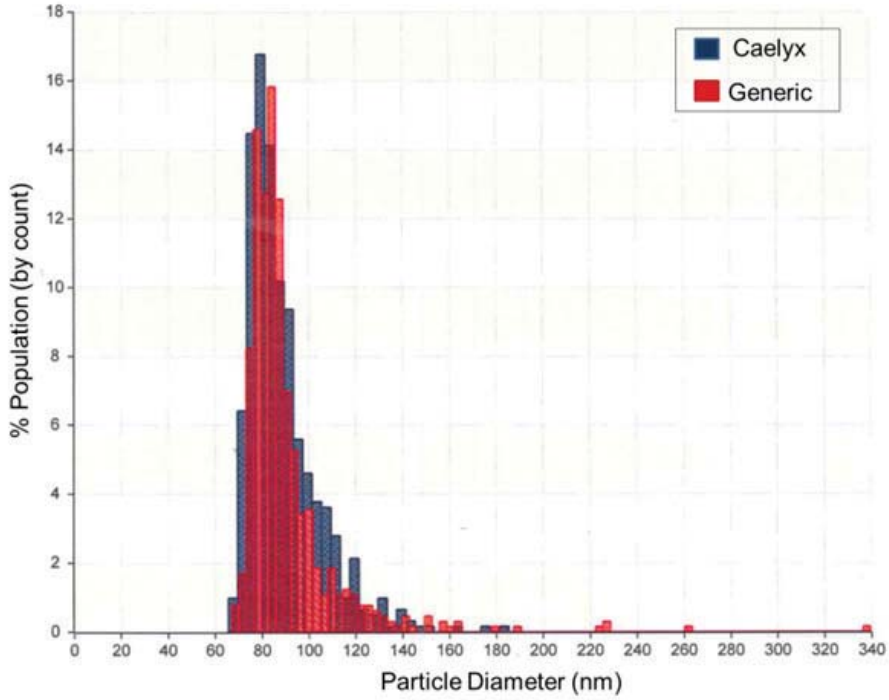

Fig. (1). Size distribution of commercially available Caelyx and a generic equivalent.

DLS number distribution is an approximation algorithmically calculated from the ensemble measurement. Furthermore, DLS assumes a normal or log-normal particle distribution which is often not the case for polydisperse, multimodal or aggregated samples.

In contrast, the particle-by-particle analysis of TRPS is a true number-based measurement, equally weighting each particle event, and does not fit the distribution data to a mathematical model. However, it should be noted that the measureable particle size range of TRPS is highly dependent on the instrument pore size. In general, the measurable particle-to-pore size ratio is from ~1.5-80% [17]. Particles larger than this cannot pass through the pore and those much smaller do not give rise to signal greater than the background noise.

The advantage of TRPS is that the pore size can be fine-tuned to the particle size. Detection of the whole particle size distribution is achieved by tuning the pore size, so the signal generated by particles, is above the signal-to-noise threshold, 0.05 nA in this case. In addition, tuning the pore size to the particle sample also improves the measurement resolution [18].

TRPS analysis has many benefits to improving the development and quality control of drug delivery particles. The size and polydispersity of SPC liposomes prepared by extrusion through either a 100 nm or 200 nm Nucleopore track etched filter membrane are shown in (Fig. **2**). The measured mean and size range distribution of the SPC liposomes were 130 nm (110-305 nm) and 186 nm (125-490 nm), respectively. As expected, extruding through the 100 nm Nucleopore filter produced SPC liposomes that were smaller than those prepared by the 200 nm filter. Interestingly, SPC liposomes prepared with the 100 nm Nucleopre filter were larger than the expected 100 nm filter size. Previous findings have shown that this is due to the extrusion process forming large unilamellar vesicles (LUV) with a mean diameter typically between 120-140 nm, [19] which corresponds well with our findings.

The ability to easily discriminate between small differences in particle size using TRPS means that size and polydispersity of the liposome sample can be tailored by further processing, such as additional passes through the filter. Furthermore, the results can be monitored *via* TRPS to create the desired product with a high level of confidence.

To illustrate TRPS sensitivity in measuring the presence of aggregates and/or high polydisperse samples, a 100 nm liposome solution was measured before and after undergoing a freeze-thaw process. Because liposomes are membranous structures, they can be easily damaged when frozen as a result of intra-liposomal ice crystal formation. This results in the formation of larger liposomes, due to liposome aggregation and/or fusion.

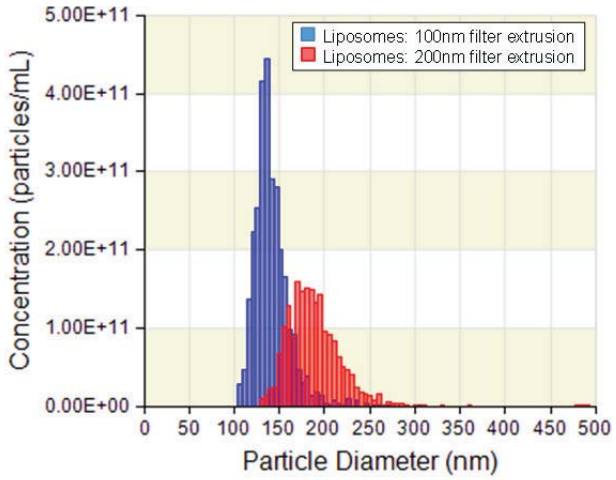

Fig. (2). Size distribution of liposome solutions made by extrusion through a 100 nm or 200 nm Nucleopore track etched pore membrane.

Fig. (**3**) shows the effect that freeze-thaw storage has on liposome structure, *via* the change in size distribution. Although there are still a proportion of liposomes that remain unchanged, that is, there is a population of liposomes approximately 110 nm in size, after freeze thawing, the number of much larger particles is clearly evident. This is seen as a tail in the size distribution and an increased mean size and range of the liposome sample, which went from 108 nm (82-308 nm), before freeze-thaw, to 153 nm (84-600 nm), post freeze thawing. Accurately measuring the size and concentration of aggregate or contaminate particles in an injectable drug sample is a critical safety requirement.

In addition to size distribution, TRPS measurements enable the particle concentration, as well as the liposome volume fraction, to be calculated. Like particle size, particle concentration can be calculated from first principles to determine the number of particle events per volume of fluid analyzed. Alternatively, as the number of particles counted per given time period is linearly proportional to the particle concentration, [6] a one-to-one calibration can also be used. This direct calibration of concentration is similar to size calibration (Eq 1). Thus, the concentration of an unknown sample, C_s, can be calculated by comparing its particle rate, r_s, to a calibration particle sample with known concentration, C_c, by,

$$\frac{C_s}{r_s} = \frac{C_c}{r_c} \qquad \text{Eq. (2)}$$

However, this assumes that the particle dispersion is stable and homogenously dispersed throughout the measurement as well as the forces driving both the sample and calibration particles through the pore are equivalent. To ensure this condition is met, pressure is typically applied to the system, thereby negating any electrokinetic forces. The amount of pressure required depends on the magnitude of the electrokinetic forces in the pore and acting on the particle [20]. In cases where electrokinetic forces become dominant, the concentration of sample particulates is calculated by measuring the blockade rate at multiple applied pressures and comparing the slopes of the linear rate vs P curves of sample and calibration [6].

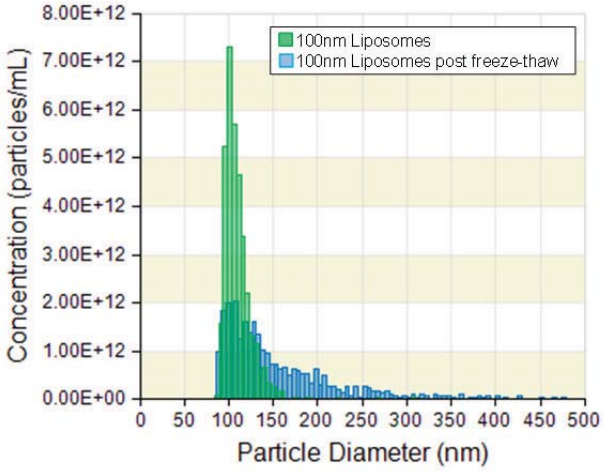

Fig. (3). Using high resolution TRPS analysis to detect and monitor the stability of liposome solutions due to freeze-thaw processing. The mean size and distribution range increase post freeze-thaw.

The 200 nm filtered SPC liposomes had a total concentration of 1.97×10^{12} particles / mL and ranged in size from 125 to 490 nm. Summing the volume of each particle size times its relative concentration provides the liposome volume fraction, that is, the particle to media volume ratio. The volume fraction for the 200 nm liposomes was 0.4%, which corresponds to 0.004 mL of liposomes delivered per mL of administered solution. This is important since the volume of liposomes administered is expected to be directly proportional to amount of drug delivered.

SIMULTANEOUS SIZE AND ZETA-POTENTIAL ANALYSIS

Recently it was shown that TPRS is able to measure the zeta-potential of individual particles based on the duration of the resistive pulse signal [8a] As the pulse signal duration is independent of the particle size, TRPS can simultaneously measure both the size and electrophoretic mobility of each particle passing through the pore. Particle zeta-potential is calculated from the measured electrophoretic mobility of each particle using the Smoluchowski equation, as detailed in Kozak *et al*. [8a] This unique capability of TRPS to simultaneously measure particle size and zeta-potential represents

a new approach for investigating and understanding the properties of particle dispersions.

For example, a simple means of tracking the successful modification of liposomes is *via* a change in their electrophoretic mobility (zeta-potential), which arises from the change in the number of charged surface groups. Phosphocholine lipids, such as the DPPC lipids, used to prepare the liposomes in this study, are zwitterionic, that is, each molecule possesses an equal number of positive and negatively charged groups. Therefore, when assembled into a liposome, the particles have no net surface charge. Reacting to, replacing or adding moieties that change the number of choline (*i.e.* positively charged) groups will often result in a change in the net charge of the lipid. When used to form a liposome, the number or ratio of modified to unmodified lipids can be detected as a difference in the liposome zeta-potential. For example, the number or ratio of glycol chain modified phospholipids incorporated into the liposome, that is, the degree of liposome PEGylation, can be monitored from the corresponding decrease (becoming more negative) in the zeta-potential of the particles [3c].

TRPS was used to measure the size and zeta-potential distribution of a liposomes and PEGylated liposome solutions. As shown in Fig. (**3**), both particle sets had very similar, *i.e.* monodisperse, size distributions and modes of 90 and 95 nm for the normal and PEGylated liposome, respectively. This negligible size change is most likely due to a combination of a low degree of PEG lipid incorporation within the liposome (5% mol/mol) and the PEG being a short chain (2,000 Mw). In contrast to the negligible size change, the addition of the PEG lipid does give rise to a change in the liposome zeta-potential. As expected, the liposomes had an approximately neutral zeta-potential as demonstrated by their narrow distribution and mode of -2.5 mV. In contrast, the PEGylated liposomes had a broader but more negative zeta-potential distribution, with a mode of -10 mV. This seems to indicate that all of the liposomes incorporate some of the glycol modified lipid, but the degree of PEGylation is not homogenous through the system. Although measured by alternative techniques to TRPS, similar magnitudes and shifts in the zeta-potential of liposomes and PEGyliposomes are widely reported in the literature [19, 21].

CONCLUSIONS

The design, performance, and quality assurance of complex nanomedinces can be improved *via* sensitive and accurate analytical characterization. Tunable resistive pulse sensing is a comprehensive particle-by-particle analysis technique that measures the size, electrophoretic mobility, and concentration of particles suspended in electrolyte media. A

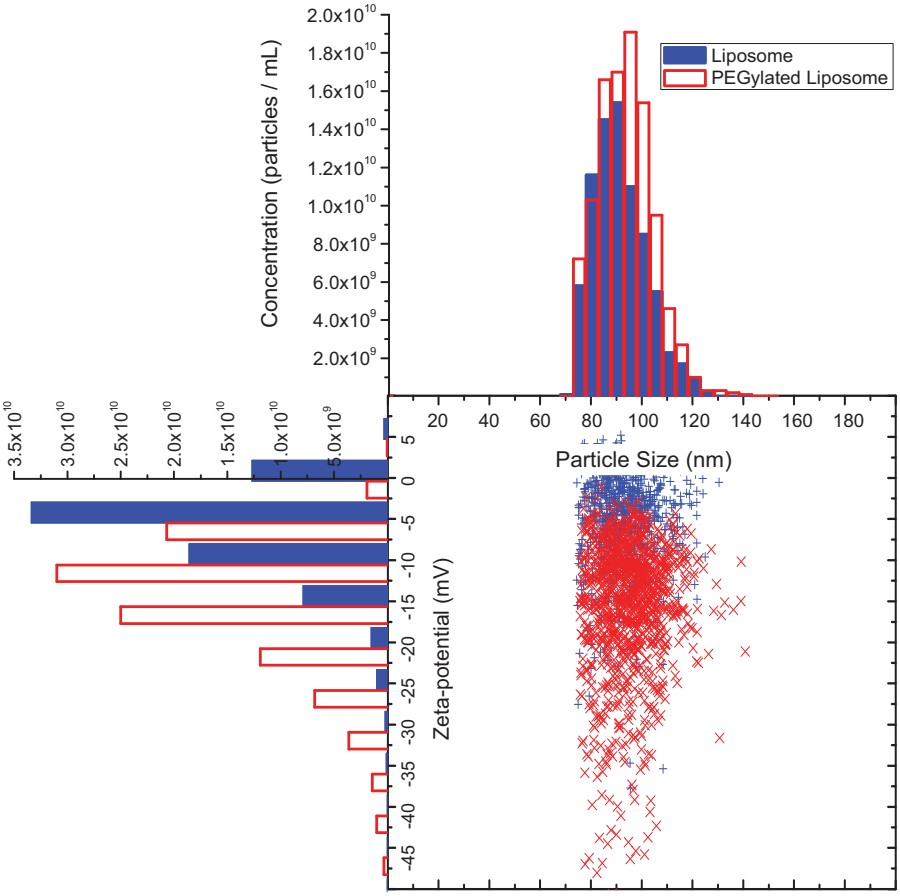

Fig. (4). The size and zeta-potential of individual liposome (+) and PEGylated liposome (x) particles are shown in the 2D dot plot. The associated size (top) and zeta-potential (left) concentration histograms show the distribution of these properties over the whole liposome suspension. PEGylated liposomes are slightly larger and more negatively charged than the unmodified liposomes. The homogeneity of the PEGylation can be related back to the width of the size and zeta-potential distribution.

key advantage of TRPS over other characterization techniques is its fundamental particle-by-particle analysis capability, which provides a more detailed and often more sensitive measurement of the sample property distribution. This was demonstrated using a series of liposome samples in which it was shown that TRPS has the resolution capability to directly measure the total liposome particle concentration as well as the administered liposome volume fraction. The ability to obtain a detailed distribution of the size and degree of liposome aggregation was shown for two extrusion preparations before and after exposure to freeze-thawing. Finally, we demonstrated the use of TRPS to measure the size and zeta-potential distribution difference of a normal and PEGylated liposome solution on a particle-by-particle basis. This ability to characterize the properties of liposomes on an individual basis, generates a more accurate picture of their distribution, and represents a new approach for investigating and understanding liposome function and fundamental behavior. This level of detail is key to expediting the transfer of complex drugs from the lab to the clinic.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Declared none.

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