Accurate Size, Charge & Concentration Analysis of Liposomes using Tunable Resistive Pulse Sensing

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Tunable resistive pulse sensing (TRPS) represents a new and more comprehensive technology for measuring the properties (size, charge, and concentration) of liposomal dispersions where highly sensitive and accurate analysis is required. Based on particle-by-particle analysis TRPS enables the accurate measurement of all these properties to be made from a single analysis run. As demonstrated in this white paper, TRPS provides information on both the particle concentration and the volume fraction of delivered liposome to administered solution volume that is unbiased by the size or polydispersity of the sample. In addition to measuring concentration, TRPS is a highly sensitive technique for analyzing liposome size distribution, which is critical for liposome manufacture, formulation and delivery applications. The size distribution of liposomes formed by extrusion through a 100 nm and 200 nm Nucleopore filter membrane, as well as the aggregation caused by freeze-thawing is shown. Finally we present the newly developed TRPS feature of particle-by-particle size and charge (zeta-potential) analysis for characterizing liposome surface modification (e.g. PEGylation), making TRPS an accurate and comprehensive liposome and nanoparticle analysis tool.

Introduction

Biologically inspired and compatible nano-scale particles, such as liposomes and their derivatives, have generated considerable interest as tunable vehicles for *in vivo* therapeutic delivery.[1, 2] The encapsulation of therapeutics and/or surface modification of liposomes can protect and improve the solubility and circulation time of these therapies as well as direct their localized delivery within the body.[3-8] For these reasons, liposomes are widely studied and are currently one of the most commercially evolved vesicular systems for pharmaceutical delivery.[1]

In general, the efficacy of liposome therapeutic delivery is dependent on the liposome properties, namely their size, charge, and concentration. A number of studies have shown that these properties dictate the circulation time, localized delivery, cellular uptake, drug release profile and even potential systemic toxicity in the body.[9-11] For example, it has been shown that drugs and therapies have an optimal concentration delivery window, below which they are ineffective and above which they can become fatally toxic. Furthermore, the size, polydispersity and charge of nano-scale delivery vehicles can determine their effluent pathway from the body (e.g. via the liver or kidneys),[12-15] preferential localized accumulation (e.g. EPR effect)[16-18] as well as govern if and how they interact with cells (e.g. passive penetration or endocytosis).[19, 20]

In addition to their *in vivo* behavior, particle size, charge and concentration also play a fundamental role in the stability of liposomal solutions. Highly concentrated, polydisperse and uncharged nanoparticles are often unstable.[21] This leads to their rapid aggregation and separation from solution, and in turn reduces their shelf-life and drug stability.[22, 23] Thus, knowledgeable and proper engineering of these systems is not possible without a correct understanding of the particle system properties.[24] *In vivo* trials and formulation development are time consuming and expensive, and it is therefore critical that particle measurements are precise and accurate to give researchers a clear understanding of what is being produced and delivered. This is essential to avoid random outcomes or quality control issues due to out of specification or poorly understood particles. For these reasons, there is an inherent need in both academia and industry for instrumentation that can accurately measure the size, charge and concentration of liposome solutions.

A range of instrumentation techniques have been used to characterize liposome dispersions. These techniques can be roughly classified into three basic characterization categories: ensemble, separation, and particle-by-particle counting.[25, 26] Ensemble techniques, such as multi-angle (static) and dynamic light scattering (MALS and DLS, respectively), calculate the average particle size and charge from the signal generated by multiple particles within the sample. Although these methods often have the advantage of rapid analysis time, they have the disadvantages of low measurement resolution and the inability to quantify particle concentration. Separation techniques, such as disc centrifuge and field-flow fractionation, have the advantage of improving size analysis resolution by using differences in the sample properties, typically sedimentation rates, to pre-separate the sample prior to light based (absorbance or scattering) analysis. However, separation techniques do not measure particle concentration or charge, and they often suffer from separation-based issues.

In contrast, particle-by-particle counting techniques, such as tunable resistive pulse sensors (TRPS), have the advantage of measuring and collating the properties of individual liposomes. This analysis methodology provides a direct measurement of the particle concentration[27] as well as high resolution and more accurate analysis of the particle size[28-31] and charge (zeta-potential)[32, 33] distribution. This ability to simultaneously measure the distribution of both the size and zeta-potential represents a new and effective means of analyzing liposome properties.

Tunable Resistive Pulse Sensing

Tunable resistive pulse sensors (TRPS) are instruments that measure the properties of individual particles as they pass through a small hole or 'pore' produced in a membrane.[34-36] In addition to characterizing each particle, the collation of hundreds to thousands of particles is used to give an accurate depiction of the overall distribution of these properties within the sample. Particle-by-particle analysis using resistive pulse sensing has been shown to provide greater analysis resolution and sensitivity compared to other particle characterization techniques,[29, 35, 37] e.g. ensemble and light scattering based analysis, which can be biased by small subpopulations of particles present in the suspension.[25, 26]

Historically, pulse sensors have been limited to characterizing particles larger than a micron. Commercially known as 'Coulter counters', these first large particle pulse sensor instruments are still routinely used in research and histology labs for the high throughput automated counting and sizing of cells. Recent advancements in nanofabrication techniques have led to the miniaturization of this technique. This miniaturization enables the same high throughput particle-by-particle characterization, originally pioneered by W.H. Coulter,[38] to be applied to nano-scale systems such as liposomes.

The Izon qNano and qViro-X (Figure 1) are commercially available TRPS systems that have been used for a diverse range of applications, from molecular diagnostic detection[39, 40] to analysis of particulates in wine.[41] Unlike current commercial Coulter Counters and research-based resistive pulse sensors that have pores of fixed size, the Izon instruments use an elastic, size-tunable pore.[42] The size of the pore can be easily and rapidly changed in real-time by axial stretching of the elastic membrane. In this way, the pore diameter is tuned to suit the size of the particle dispersion being interrogated.[30] Furthermore, the Izon instruments enable the user to control and optimize the key features (pressure and voltage) that drive particle transport.[43, 44] By tuning the system, a user has the ability to further improve the measurement sensitivity as well as measure the particle concentration or zeta-potential. This unique tunability of the Izon instruments has been shown to increase the dynamic measurement range and improve the measurement sensitivity and resolution, often beyond that of other measurement techniques. In addition to improving the measurement quality, tuning the system also enables users to rapidly and easily clear a sample blockage in the pore, which is often not possible with fixed pore devices. A review on TRPS and the associated fundamental analysis principles of the technique can be found in the recent article by Kozak *et al.*[35] and the book chapter by Willmott *et al.*[45

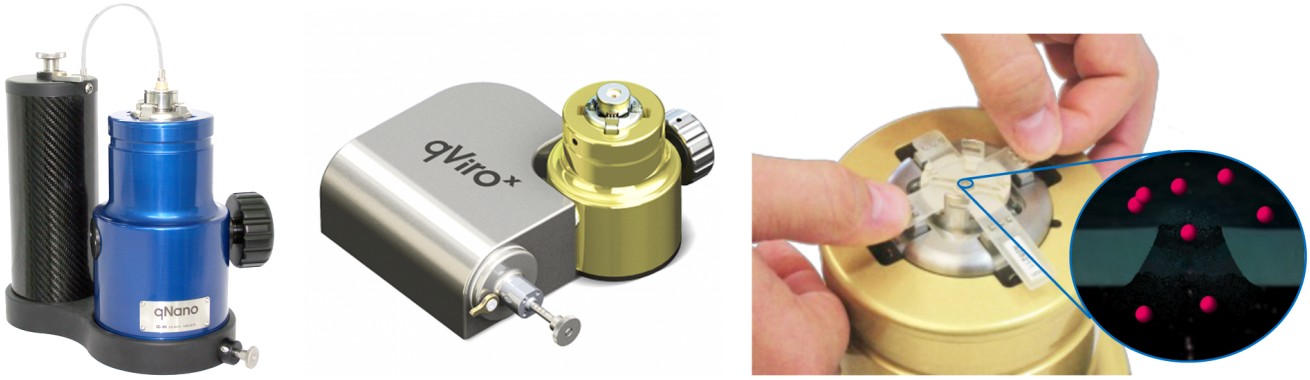

Figure 1. Commercially available tunable resistive pulse sensors (TRPS). Izon qNano and qViro-X measure the size, charge and concentration of nano- and micro- size particles via a size-tunable pore. The pore, which is made in a four arm elastic membrane, is mounted on the adjustable jaws of the instruments. The pore size is tuned to the particle sample in real-time by adjusting the axial strain applied to the membrane. The properties of individual liposome particles (size, charge and concentration) are measured from the resistive pulse signal they generate as they pass through the pore.

Measuring Liposome Concentration

Knowledge and an accurate measure of liposome concentration is an important factor in all stages of liposome development and application. For example, liposome particle concentration affects its colloidal stability and remote drug loading efficacy. When used in combination with the overall drug loading, it also allows the drug dose to particle ratio to be calculated.

This is especially critical as drugs have an optimal therapeutic concentration range, so minute differences in the amount of drug delivered can affect therapeutic effectiveness. With further studies looking at single liposome internalization, a correlation between liposome number (e.g. delivered dose amount) and desired cellular therapeutic effect (i.e. cell death, transfection, and protein regulation) can be determined.

Currently the most common methodologies to determine liposome concentration rely on indirect measurements.[46, 47] These methodologies are often based on colorimetric light absorption techniques (e.g. Stewart assay) or scattering measurements of the solution turbidity or liposome particle size. A key limitation of these methods is that lipid type, liposome size, and polydispersity all affect the calculated concentration. For example, Stewart assays cannot detect glycol modified phospholipids, whereas light scattering techniques are strongly biased by larger particles in the suspension which scatter more light.[48] Thus, liposome concentrations calculated from these measurements do not accurately represent the true liposome particle concentration.

In contrast, TRPS enables a direct, particle-by-particle measurement of particle concentration. This is because TRPS counts each liposome that passes through the pore, and the resulting measure of concentration is, in general, independent of the liposome composition, size, and polydispersity. The particle concentration (number of particles per mL) is determined in TRPS from the number of particles counted over a given time period (particle count rate).[27] This count rate is linearly proportional to the total particle concentration; doubling the liposome concentration corresponds to a two fold increase in the particle count rate.

Figure 2 shows the linear relationship between the count rate and the concentration of a 1:10, 1:25, 1:75, and 1:100 diluted liposome solution. As expected, a tenfold dilution of the liposome sample (1:10 to 1:100) proportionally reduced the measured count rate from 1545±88 to 142±23 particles / min. This corresponded to a tenfold decrease in the measured particle concentration, which was $5.75 \pm 0.33 \times 10^{10}$ and $5.28 \pm 0.88 \times 10^{9}$ particles / mL, respectively. Measured values and errors are the average and standard deviation of three analysis runs. The linear relationship between count rate and concentration means that TRPS provides a sensitive measurement of particle concentration.

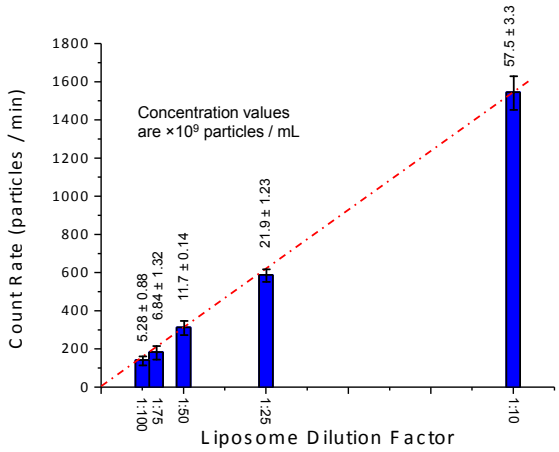

Figure 2. Direct measurement of liposome concentration. The liposome concentration (particles / mL) is directly proportional to the particle count rate (particles / min) which is independent of the liposome size or polydispersity. Diluting the sample 1:10, 1:25, 1:50, 1:75, and 1:100 gives rise to a corresponding linear decrease in the particle count rate (red line). The measured count rates and corresponding concentration values are the average and standard deviation of three analysis runs.

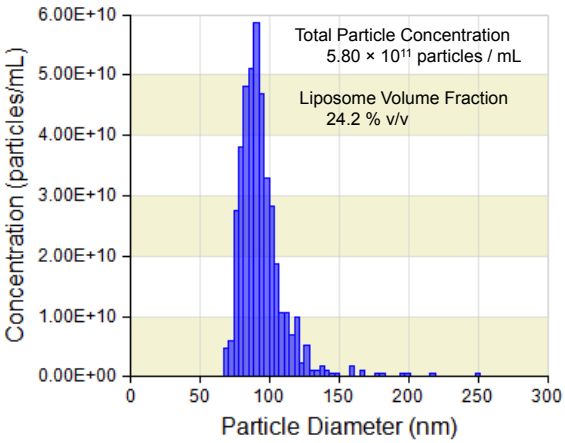

Figure 3. Size-specific concentration. In addition to the total particle concentration, TRPS enables the overall liposome volume fraction and the size-specific concentration, that is number of liposomes of a specific measured size, to be calculated. For the undiluted liposome solution this was 5.80 $\times 10^{11}$ particles / mL and 0.24 mL liposome per mL of solution.

In addition to the total particle concentration, TRPS has the added advantage of accurately measuring and collecting the size of each particle as it passes through the pore. This means that the size-specific concentration for a sample is also recorded. That is, TRPS records the number of particles of each measured size, as shown in Figure 3 for the undiluted

liposome sample. It was found that the majority of particles (7.51×10^{10} particles / mL) in the sample measured 90±2.25 nm in diameter and the sample had an overall liposome size range of 70 nm to 200 nm. TRPS measurements enable the total particle concentration (sum of all the particles measured) and the total liposome delivery volume, that is the volume fraction of liposome to administered solution volume, to be calculated. This is important as the volume fraction of liposome administered is directly proportional to amount of drug delivered. In this study, the undiluted liposome sample had a total particle concentration of 5.80×10^{11} particles per mL, and a corresponding deliverable volume fraction of 24%, that is 0.24 mL of liposome payload is delivered per mL of administered solution.

Measuring Liposome Size, Polydispersity, and Aggregation

Controlling the size and polydispersity of synthesized liposomes is critical to their intended and effective *in vivo* use. Currently there are a range of methods available to make liposomes.[48] One of the most common is lipid hydration and extrusion. This involves passing a lipid solution through a filter with a defined pore size. Varying lipid type, concentration, filter pore size, flow rate and temperature can be used to control the liposome size and polydispersity.[48] Understanding and optimizing these conditions, which is often done via measurement of the size of liposomes produced, is an important factor in liposome manufacture and quality control. TRPS has been routinely used to quantitatively size synthetic and biological nanoparticles including liposomes, cells and polymeric nanoparticles.[27, 31, 49, 50]

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. For example, a particle with twice the volume will give rise to a resistive pulse signal that is two times larger. As the volume of a particle is proportional to the diameter cubed, very small differences in particle size give rise to very large changes in the pulse signal generated. TRPS calculates the particle diameter d from its relationship to the magnitude of the resistance signal ΔR,[31, 51]

$$\Delta R = \frac{4\rho d^3}{\pi D^4} \qquad (1)$$

where D is the pore diameter and ρ is the resistivity of the aqueous electrolyte media the particles are suspended in.

The signal sensitivity and particle size resolution is illustrated in Figure 4, which shows the raw pulse signal generated by a particle suspension with three different sized particles, a 220 nm, 330 nm, and a 410 nm diameter particle set, and an overlay of the signal generated by each particle size. By measuring and collating the size of each particle, a high resolution histogram of size distribution that is representative of the sample is generated.

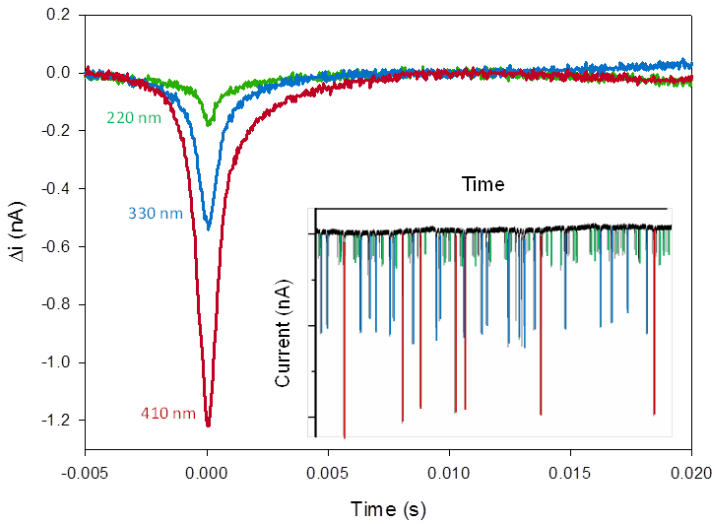

Figure 4. TRPS signal intensity relative to particle size. The magnitude of the pulse signal generated by individual particles is directly dependent on the particle volume. As the volume is proportional to the diameter cubed, the TRPS signal is very sensitive to small changes in particle size as shown by the difference in signal magnitude for 220 nm, 330 nm, and 410 nm particle sets. Insert shows the raw signal generated for a mixed dispersion of these three particle sets. Individual particle pulses have been color coded to illustrate which size particle they were generated from.

An example of the high-resolution particle size distribution analysis provided by TRPS is shown in Figure 5A&B. The size and concentration for each particle set within a mixed sample of three different sized particles are easily resolved by TRPS.[37] In contrast, ensemble based light scattering techniques have a lower size resolution and can be adversely affected by the presence of a relatively low number of larger particles, which can skew the measured size. This difference in techniques is critically important when measuring complex or polydisperse samples. For example, the difference in TRPS and DLS measured size distribution of a complex solution of two different sized polystyrene samples, with mean diameters of 220 nm and 410 nm, is shown in Figure 5B. TRPS easily resolves both particle populations, whilst measurements by dynamic light scattering only show a single population with a broad size range. However, both sizing methods are in good agreement when the particle types are separated and measured individually.

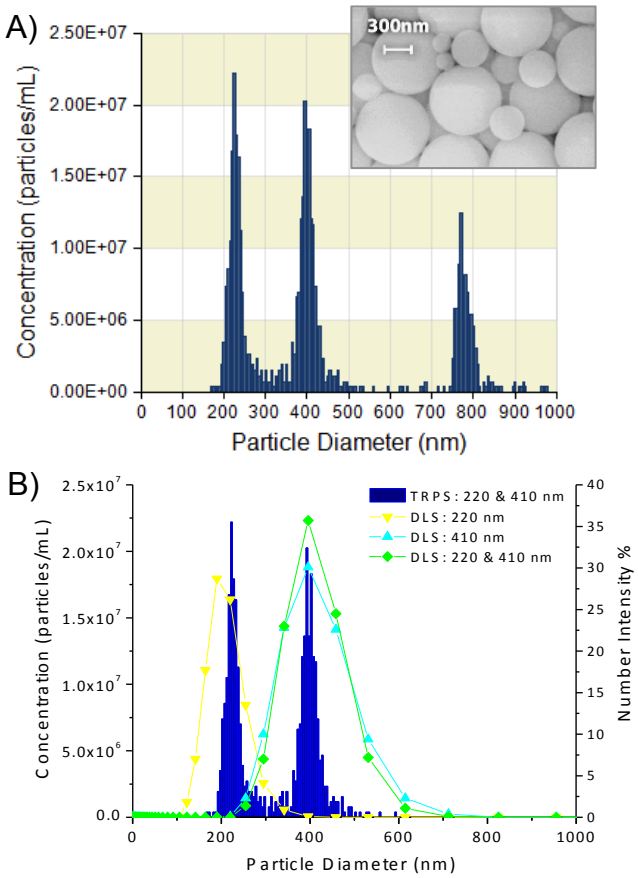

Figure 5. Sensitivity and resolution of TRPS for size distribution measurements. A) The concentration dependent size distribution for a mixed suspension of 220 nm, 410 nm, and 780 nm polystyrene particles is easily resolved by TRPS. Insert shows an SEM of the mixed particle suspension. B) In contrast, other measurement techniques such as dynamic light scattering do not measure concentration and cannot offer the same size measurement resolution as is shown by the single DLS peak for the mixed suspension of 220 nm and 410 nm particles.

The high sensitivity and resolution of TRPS has many benefits for optimizing and understanding the interactions and longevity of liposome solutions. The size and polydispersity of phosphatidylcholine unilamellar liposomes prepared by extrusion through either a 100 nm or 200 nm Nucleopore track etched filter membrane are shown in Figure 6A. Interestingly, the 100 nm Nucleopore filter gave rise to liposomes smaller than the 200 nm filter but the average liposome size was not smaller than 100 nm. The measured mean and size range distribution of liposomes extruded through the 100 nm and 200 nm filters were 130 nm (110 – 305 nm) and 186 nm (125 – 490 nm), respectively.

In addition to characterizing liposome preparation conditions, the size distribution can also be used to test and monitor the stability of liposome solutions. Liposomes are often vulnerable to degradation and aggregation, which can arise from the presence of detergents, or changes in temperature or osmotic pressure.[23] Additionally, they can be mechanically damaged by excessive physical force. Liposome degradation leads to fewer liposome particles (lower concentrations), whereas aggregation and coalescence give rise to a population of larger sized particles in solution. TRPS particle-by-particle analysis enables the detection of these sub-populations of larger particles, as well as an indication of their concentration (ratio) in solution. For example, Figure 6B shows the effect, via the change in size distribution, that freeze-thawing has on liposome structure. In this study, freeze-thawing resulted in the formation of a more polydisperse sample with much larger liposomes. This is due to the rupturing and reforming of liposomes that occurs during freeze-thawing. Freeze-thaw cycling is often used in liposome production and is an important factor to consider for long-term storage and transport, especially considering the often temperature-dependent activity of loaded therapeutics.

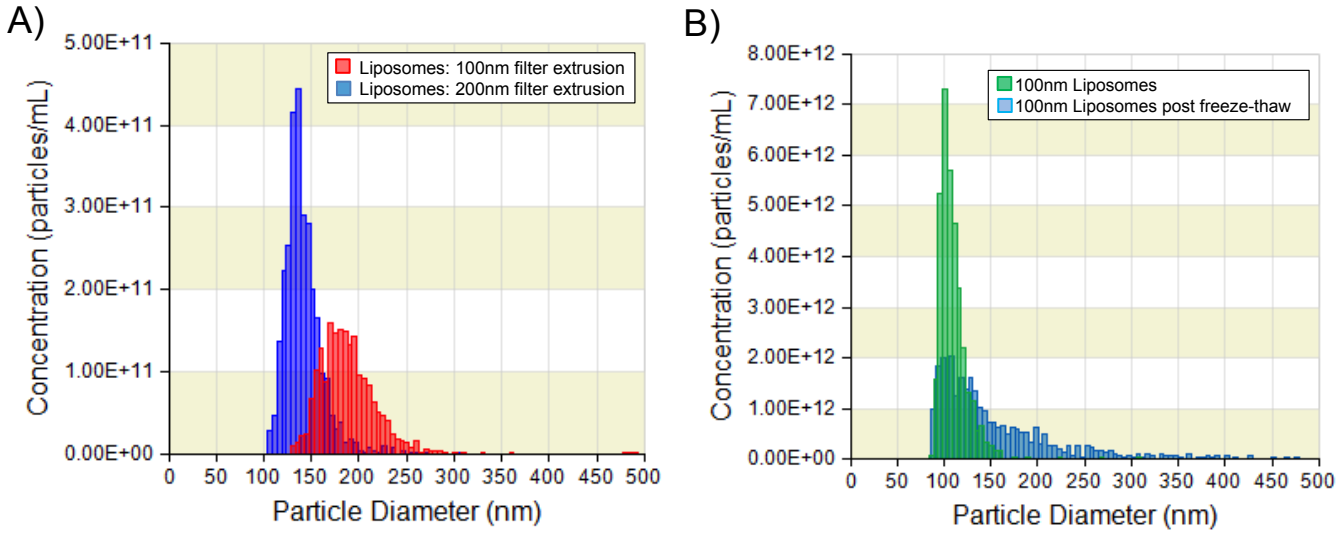

Figure 6. Measuring the size distribution of liposomes. A) Size distribution of liposome solutions made by extrusion through a 100 nm or 200 nm Nucleopore track etched pore membrane. B) The observed increased liposome aggregation due to freeze-thawing.

In addition to monitoring the produced size and environmental effects on liposome stability, sensitive size measurements are valuable for characterizing and understanding the interactions that liposomes undergo *in vivo*. One such interaction of particular interest is the non-specific adsorption of small molecules (e.g. proteins) present in blood onto the liposome surface. Protein adsorption not only obstructs targeting molecules engineered onto the liposome surface, it is also often seen as the first step in the immune response and clearance by the reticuloendothelial system. A recent study by Yang *et al.*[49] using TRPS demonstrated that the adsorption of plasma proteins on liposomes was detected as a 10 nm increase in liposome size. Furthermore, they also observed an indicator that the adsorbed protein layer altered the liposome surface charge.

Measuring Liposome Zeta-potential

It is often desirable to modify the surface of liposome vehicles to improve their *in vivo* drug delivery performance. These modifications can include, but are not limited to, the addition of polyethylene glycol (PEG) chains to reduce aggregation and increase circulation time,[52-56] molecular targeting probes, such as antibodies, and cellular receptor recognition molecules (e.g. the RGD peptide).[57-62] A simple means of tracking the successful modification of liposomes is via measuring the change in their electrophoretic mobility (zeta-potential) which arises from the change in the number of charged surface groups. In general, the phosphocoline lipids that make up a large majority of liposomes are zwitterionic, that is each molecule possesses an equal number of positive and negatively charged groups, and therefore they carry no net surface charge.

Reacting, replacing, or adding moieties which change the number of charged groups, such as replacing the positively charged choline group with a neutral glycol chain, give rise to lipids that carry a charge. When used to form a liposome, the number or ratio of these 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 negative shift in particle zeta-potential.[48]

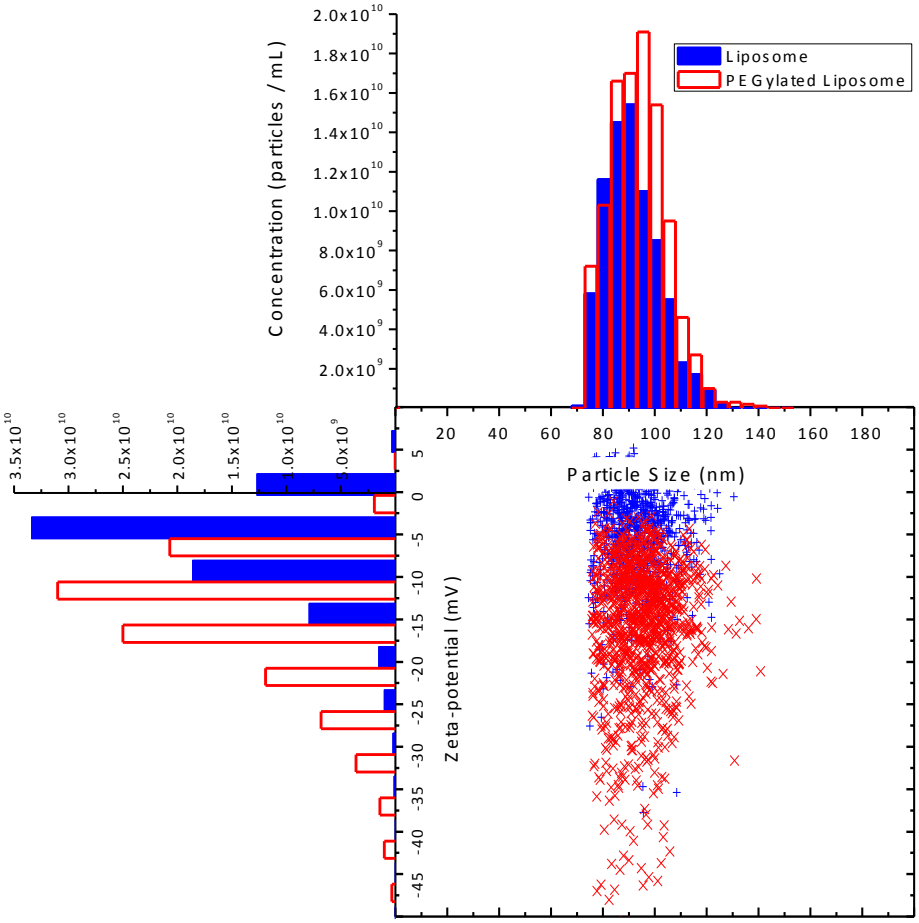

Figure 7. Izon particle-by-particle size and charge analysis. The size and zeta-potential of individual liposome (blue) and PEGylated liposome (red) particles are shown in the 2D dot plot. The associated size (top) and zeta-potential (left) concentration histograms show the distribution of these properties 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.

Currently the most common method for measuring the zeta-potential of liposome particles is via ensemble light scattering techniques, which use a similar principle as dynamic light scattering. These instruments measure differences in the scattered light properties to calculate the average particle electrophoretic mobility, that is an average velocity of all the particles when in the presence of an applied electric field.[26] Thus, like size measurements by DLS, the calculated zeta-potential values cannot measure or can be biased by small subpopulations of particles.

In contrast, TRPS measures the zeta-potential on a particle-by-particle basis from the shape of the resistive pulse signal generated by objects traversing the pore sensor.[33] This means TRPS provides higher sensitivity and resolution when compared to other techniques for size and charge analysis. Furthermore, as the shape of the pulse signal is independent of the particle size, TRPS can simultaneously measure both the size and zeta-potential of each particle passing through the pore.

TRPS was used to measure the size and charge distribution of a 'normal' and PEGylated liposome solution. As shown in Figure 7, both particle sets had very similar, i.e. monodisperse, size distributions and modes of 90 nm and 95 nm for the normal and PEGylated liposomes, respectively. This size information indicates that the glycol chain has only a marginal effect on increasing the overall particle size, which is most likely due to a combination of it being a short chain and it being a low degree of PEGylation. In addition to the size increase, the presence of PEG substituted phospholipids within the PEGylated liposomes was further demonstrated by the more negative shift in their zeta-potential values. This shift is a result of the PEG substituted phospholipids having an overall negative charge arising from the replacement of the positively charged choline group with the neutral PEG chain.

As expected, normal liposomes had an approximately neutral zeta-potential as demonstrated by their narrow distribution and mode of -5mV. In contrast, the PEGylated liposomes had a broader but more negative zeta-potential distribution, with a mode of -10mV. This seems to indicate that all of the liposomes incorporate some of the glycol modified lipid but the degree of PEGylation is not homogenous throughout the system.

Conclusions

Tunable resistive pulse sensors provide researchers and commercial manufactures with an accurate and comprehensive analysis tool to measure and study the size, charge and concentration of liposomes in biologically relevant media. A key advantage of TRPS over other particle characterization instruments is its fundamental particle-by-particle analysis capability, which provides more detailed and often more sensitive measurement of the distribution of sample properties. This was demonstrated using a series of liposome samples where the ability of TRPS to directly measure the total liposome particle concentration as well as the administered liposome volume fraction was shown. Furthermore, the high-resolution size analysis of TRPS was shown to discriminate between populations of particles with similar size, which the other techniques could not. The ability to obtain a detailed distribution of the size and degree of liposome aggregation was shown for two extrusion preparations and following exposure to freeze-thawing. Finally, we demonstrated the use of TRPS to measure the size and charge distribution difference of a normal and PEGylated liposome solution on a particle-by-particle basis. This ability to characterize the properties of liposomes on a particle-by-particle basis and generate a more accurate picture of their distribution, represents a new approach to investigating and understanding liposome function and fundamental behavior. In addition to liposomes, the presented TRPS measurement methodology can be readily applied to any particulate system, e.g. nanobubbles, emulsions, and metallic or polymeric particle materials, in which the particles are dispersed in aqueous electrolyte solutions

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