From Viruses to Cells: Tuneable Resistive Pulse Sensors for High Resolution Characterization of Nano to Micro-Scale Particle Solutions

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ABSTRACT

Tunable resistive pulse sensing (TRPS) represents a new and more comprehensive technology for measuring the properties of biological and synthetic particles. Based on particle-by-particle analysis, TRPS enables the accurate measurement of particle size, charge, and concentration to be made from a single analysis run. Herin we demonstrate the ananlysis capabilites of TRPS on liposomal particles. In addition to measuring concentration and volume fraction, TRPS is a highly sensitive size analysis technique, which is critical for liposome manufacture, formulation and delivery applications. The size distribution of liposomes formed by extrusion through two Nucleopore filter membranes, 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 a very accurate and comprehensive liposome and nanoparticle analysis tool.

Keywords: particle characterization, pore sensor, SIOS, qNano, coulter counter

1 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]. 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 [2-3]. 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 [4-5]. In addition to their *in vivo* behavior, particle size, charge and concentration also plays a fundamental role in the stability of liposomal solutions. For these critical reasons, there is an inherent need for instrumentation that can accurately measure the size, charge and concentration of liposome solutions.

A range of instrumental techniques have been used to characterize nanoparticle and liposome dispersions. In general, few techniques can provide a comprehensive measure of particle concentration, size and charge. Of these particle-by-particle counting techniques, such as tunable resistive pulse sensors (TRPS), have been generating considerealbe intereset as they measure the properties of individual particles as they pass through a pore sensor. This analysis methodology has been shown to provide a direct measure of particle concentration [6] as well as high resolution analysis of the sample size [7-11] and charge (zeta-potential)[12, 13] distribution. A review on TRPS and the associated fundamental analysis principles of the technique can be found in the recent review by Kozak *et al*.[14] and the book chapter by Willmott *et al*.[15].

Herin we demonstrate the accurate, high resolution, and comprehensive analysis capabilites of TRPS on a range of liposome furmulations. 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 particulate system, e.g. nanobubbles, emulsions, and metallic or polymeric particle materials, in which the particles are dispersed in aqueous electrolyte solutions.

2 MATERIALS AND METHODS

Measurements were made using an Izon qNano (NZ). Polystyrene calibration particles were purchased from Bangs Laboratories (USA), unmodified and modified liposomes from FormuMax (USA) and were dispersed in phosphate buffered saline for analysis. Particle concentration, size and charge (zeta-potential) were calculated using Izon Control Suite Software V2.4 on a minimum of 500 particle events.

3 RESULTS AND DISCUSSION

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 [16]. 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.

TRPS has been routinely used to quantitatively size synthetic and biological nanoparticles including liposomes, exosomes, and viruses [6, 9-11, 17-21]. 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. Thus, very small differences in particle size give rise to very large differences in the pulse signal generated as the particle volume is proportional to the diameter cubed. For example, doubling the particle diameter gives rise to an eight fold increase in the pulse signal magnitude. The relationship of particle diameter *d* to the magnitude of the resistance signal ΔR is given by [7, 10],

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

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

The high sensitivity and resolution of TRPS has many benefits in charaterising, understanding and optimizing the production, formulation and stability 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 1. 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.

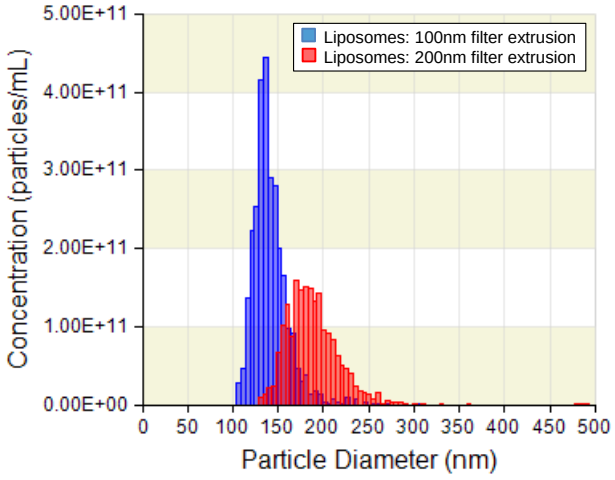

Figure 1 Size distribution of liposome solutions made by extrusion through a 100 nm or 200 nm Nucleopore track etched pore membrane.

In addition to size distribution TRPS measurements enable the total particle concentration (sum of all the particles measured) as well as the liposome volume fraction, to be calculated. It was found that the 200 nm filtered liposomes had a liposome concentration of 1.97×10^{12} particles / mL. The highest concentration of partilces (mode) were 168 nm in size and as mentioned before the size range was from 125 to 490 nm. Suming the volume of each partilce size times its relative concentration provides the liposome volume fraction, that is the particle to media volume ratio. The volume fraction for the 200nm liposomes was 0.4% which corresponds to 0.004mL of liposomes are delivered per mL of administered solution. This is important as the volume of liposome administered is expected to be directly proportional to amount of drug delivered.

High resolution, particle-by-particle size analysis is also a powerful tool to test and monitor the colloidal stability of nanoparticles and liposome solutions. This is because TRPS particle-by-particle analysis is sensitive and unbiased to these sub-populations of larger particles. Additioanlly as each particle is counted this provides a measure of the aggreagte ocncentration in solution.

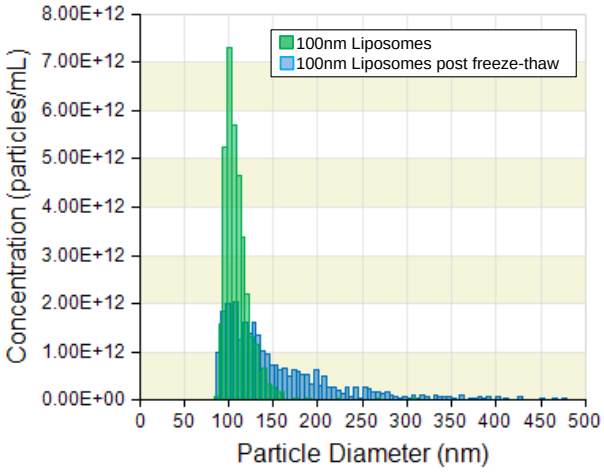

Figure 2 Using high resolution TRPS analysis to detect and monitior the stability of liposome solutions due to freeze-thaw processing. The mean size and distribution range increase post freeze-thaw.

To illustrate TRPS sensitivity in measuring the presence of aggreagates 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 intraliposomal ice crystal formation. This results in the formation of larger liposomes, due to aggregation and/or fusion of liposomes. Figure 2 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 approximatly 110 nm in

size, after freeze thawing a 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.

3.1 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 shape of the resistive pulse signal [13]. 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. 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. In general, the phosphocoline lipids used to make 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 to, replacing, or adding moieties which 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 in the zeta-potential of the particles [22].

TRPS was used to measure the size and charge distribution of a 'normal' and PEGylated liposome solution. As shown in Figure 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 size information indicates that the glycol chain has only a marginal effect on 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. The presence of glycol substituted phospholipids in the PEGylated liposomes was further demonstrated by the PEGylated liposomes more negative zeta-potential shift. 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 through the system.

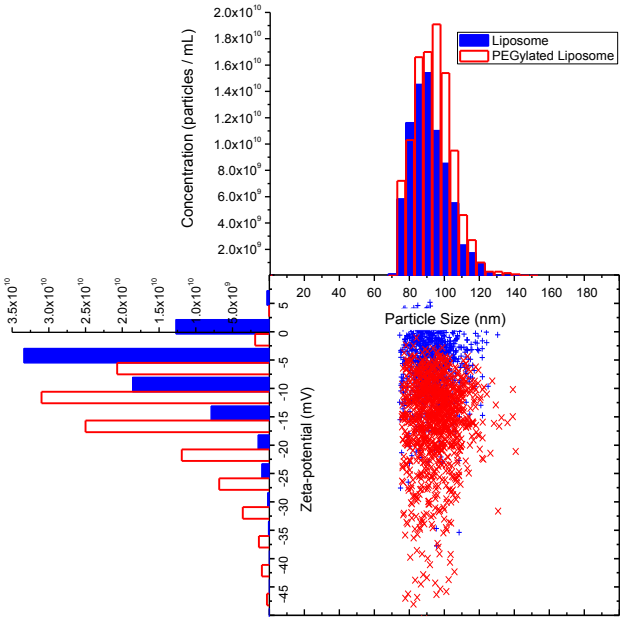

Figure 3. The size and zeta-potential of individual liposome (blue) and PEGylated liposome (red) particles are shown in the 2D dot plot. The associated size (top) and zeta-potential (left) concentration histograms show the distribution of these properties over the whole liposome suspension. PEGylated 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.

4 CONCLUSIONS

Tunable resistive pulse sensors provide researchers and commercial manufactures an accurate and comprehensive analysis tool to measure and study the size, charge and concentration of liposomes under biologically relevant media conditions. A key advantage of TRPS over other particle characterization instruments is its fundamental particle-by-particle analysis, which provides a more detailed and often more sensitive measurement of the sample property distribution. 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 calculated. 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, to generate a more accurate picture of their distribution, represents a new

approach for investigating and understanding liposome function and fundamental behavior.

REFERENCES

[1] Torchilin, V.P., Nat Rev Drug Discov, **4**(2), 145- 160, 2005.
[2] Dabbas, S., et al., Endothelium-Journal of Endothelial Cell Research, **15**(4), 189-201, 2008.
[3] Song, G., et al., Journal of Liposome Research, **22**(3), 177-192, 2012.
[4] Bertrand, N. and J.-C. Leroux, Journal of Controlled Release,. **161**(2), 152-163, 2012.
[5] Torchilin, V.P., Pharmaceutical Research, **24**(1), 1-16, 2007.
[6] Roberts, G.S., et al., Biosensors & Bioelectronics, **31**(1), 17-25, 2012.
[7] Deblois, R.W., et al., Analytical Biochemistry, **90**(1), 273-288, 1978.
[8] Fraikin, J.L., et al., Nature Nanotechnology, **6**(5), 308-313, 2011.
[9] Roberts, G.S., et al., Small, **6**(23), 2653-2658, 2010.
[10] Vogel, R., et al., Analytical Chemistry, **83**(9), 3499-3506, 2011.
[11] Anderson, W, et al., J. Colloid Interface Science, 2013, doi: 10.1016/j.bbr.2011.03.031.
[12] Ito, T., et al., Langmuir, **20**(16), 6940-6945, 2004.
[13] Kozak, D., et al., ACS Nano, **6**(8), 6990-6997, 2012.
[14] Kozak, D., et al., Nano Today, **6**(5), 531-545, 2011.
[15] Willmott, G., et al., *Tunable Elastomeric Nanopores*, in *Molecular- and Nano-Tubes*, O. Hayden and K. Nielsch, Editors. 2011, Springer. p. 209-261.
[16] Sowerby, S.J., et al., Sensors and Actuators B: Chemical, **123**(1), 325-330, 2007.
[17] Ang, Y.S. and L.-Y.L. Yung, ACS Nano, **6**(10), 8815-8823, 2012.
[18] Gazzola, D., et al., Journal of Agricultural and Food Chemistry, **60**(42), 10666-10673, 2012.
[19] Willmott, G.R., M. Platt, and G.U. Lee, Biomicrofluidics, **6**(1), 014103, 2012.
[20] Yang, L., M.F. Broom, and I.G. Tucker, Pharmaceutical Research, **29**(9), 2578-2586, 2012.
[21] de Vrij, J., et al., Nanomedicine, 2013, doi:10.2217/nnm.12.173
[22] Torchilin, V. and V. Weissig, *Liposomes: A Practical Approach*. 2003: OUP Oxford.