Rapid High Resolution Multi-Parameter Characterization of Liposome-Protein Complexes by Nanoparticle Tracking Analysis

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ABSTRACT

Aggregation of both biological and synthetic materials is a source of tremendous concern, posing problems at various stages of the discovery and formulation processes. Liposomes have been used in drug discovery and drug delivery for some time, and the biophysical characterization of these systems and their payloads is critical to understanding their function. One such payload, are the membrane-associated receptor tyrosine kinase (RTK) targets which continue to be the focus of many discovery campaigns. RTKs are typically single-pass transmembrane signaling proteins which are difficult to purify intact, and are important targets in many disease pathways. Our aim is to correlate previously published activity data[1, 2] with the changes in size and aggregation state of the liposome-RTK complex as measured by Nanoparticle Tracking Analysis (NTA) from the NanoSight product range within Malvern Instruments.

Keywords: nanoparticle tracking analysis, liposome, insulin receptors, size, and concentration

1 INTRODUCTION

The ability to identify and characterize aggregates is of broad utility, and the experiments described here demonstrate NanoSight' unique ability to understand dynamics of aggregation in a biological system through high resolution particle-by-particle analysis. For this work, we'll utilize a model system that has been used in drug discovery and presents some unique challenges in reagent characterization. This system was generated to allow functional assembly of cloned/recombinant membrane receptors on liposomes, without the necessity of reconstitution of full length receptors. Our aim is to correlate previously published activity data[1, 2] with the changes in size and aggregation state of the liposome-RTK complex as measured by Nanoparticle Tracking Analysis (NTA).

It is expected that the experiments described here represent characterization methods which will be of broad utility to the nanoparticle field. Determination of size, assessment of polydispersity, and identification of components which promote aggregation, are all of interest, and can all be assessed in these simple experiments. The model characterization system was chosen for this work because of the abundance of activity data that exists.

For this work, we first investigate the aggregation state of a representative RTK target (kinase domain of insulin receptor). Then we analyze the RTK on an engineered cell-like scaffold, specifically a liposome functionalized with nickel-chelated head groups (described in references 1 and 2). Briefly, the nickel head groups facilitate template-directed self-assembly of his-tagged RTKs, such that the kinase domains (cytoplasmic fragments) are oriented as though within the cell. This analysis is performed using magnesium (Mg) or manganese (Mn) as the available counter-ion in the assay buffer, in order to understand why manganese might be increasing the activity level of kinases despite the understanding that manganese is not a physiologically relevant counter-ion.

The NanoSight range from Malvern Instruments presents a unique capability to rapidly characterize this well-studied drug discovery system by directly visualizing nanoscale particles in suspension (between 10 to 2000 nm), with high-resolution, in real-time and with minimal sample preparation. Nanoparticle Tracking Analysis (NTA) software delivers an unprecedented insight into size distributions through a particle-by-particle approach to particle sizing. NanoSight provided visual validation and high resolution analysis of particles within the nanoscale range with both size and concentration measurements. NTA offers a robust analysis of this liposome-protein system and shed light on this method of kinase activation.

2 MATERIALS AND METHODS

Extruded liposomes and purified protein were obtained from Blue Sky BioServices. Samples were diluted in HEPES buffer prior to analysis by a NanoSight NS300 equipped with a high sensitivity Hamamatsu sCMOS camera, 20x objective lens, and a 50 mW green 532 nm laser. Samples were analyzed using NTA 3.1 Build 46 software. NanoSight technology observes the Brownian motion of particles in suspension and calculates their size through the Stokes-Einstein equation. It derives concentration by observing particles on a frame-by-frame basis through the use of the sCMOS camera (25 frames per second). The software notes the number of particles per frame in a two-dimensional field of view and then extrapolates to a known third dimension and calculates an absolute number average of concentration.

3 RESULTS AND DISCUSSION

Nanoparticle Tracking Analysis afforded an appreciation of the rapid kinetics with high resolution analysis of aggregation on a [real-time, particle-by-particle] basis. We could see a simultaneous decrease in concentration and increase in size with time in the presence of manganese buffer. The liposomes clearly aggregate in the presence of Mn (Figure 2 and 4). Analysis of the insulin receptor RTK domain in HEPES (or Tris) buffer suggest that they further aggregate in presence of manganese (Figure 3 and 4) This analysis was challenging because the protein itself appears aggregated to a degree in absence of either counter ion by noting the initial time curve in both Figures 3 and 4, though there is clearly a tremendous increase in aggregate size in presence of manganese that is not observed with addition of magnesium.

Addition of magnesium to RTK does not cause aggregation and is consistent with previously generated data which shows no appreciable activity with Mg [1.2.5] (Figure 5 and 6). The idea that magnesium is a biologically relevant counterion but does not lead to activity has been puzzling. Adding Mg to RTK and liposome did not promote aggregation but leads to high cell like activity[1.2.5]. The RTK potentially assembles on the outside of the liposome in the same manner as inside the cell (Figure 1). Mn causes aggregation and fortuitously increases activity in a cost-effective and easy manner with reasonable reproducibility and robustness. When RTK is properly self-assembled on a liposome in the presence of magnesium, a biologically relevant counterion, the following benefits are reported[1.2.5]:

a. increase in autophosphorylation and substrate phosphorylation
b. highest catalytic rates (except where autophosphorylation is self-inhibitory, Tie2)
c. improved substrate selectivity

It is expected that template-assembled enzymes are a better screening tool because the target appears to be in cell-like conformation, and allows unique opportunity for screening protein-protein interactions. This work points to the need for better understanding of the difference between forced colocalization (or molecular crowding) vs. functional assembly of RTK complexes (for interesting colocalization discussion see reference 4). The work presented here is consistent with the idea that manganese is increasing catalytic activity of some kinases by facilitating formation of aggregated complexes.

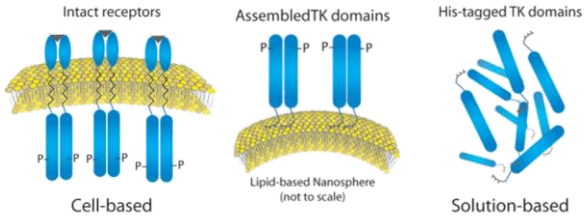

Figure 1. Liposome - Insulin Receptor Complexes

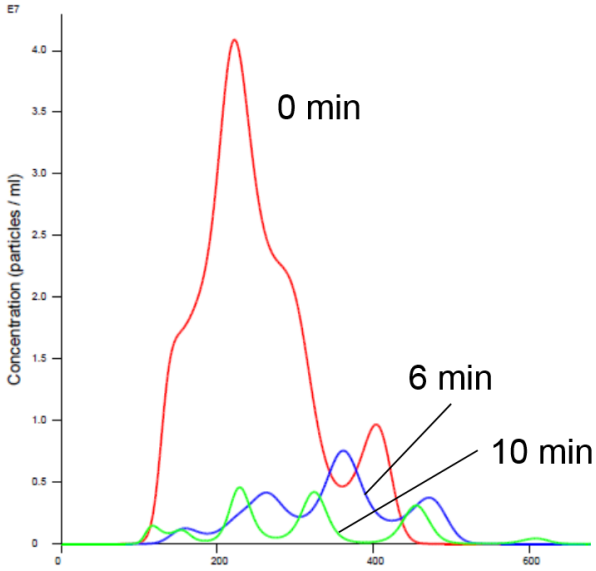

Figure 2. Liposomes in Mn Buffer show aggregation (increased size and decreased concentration)

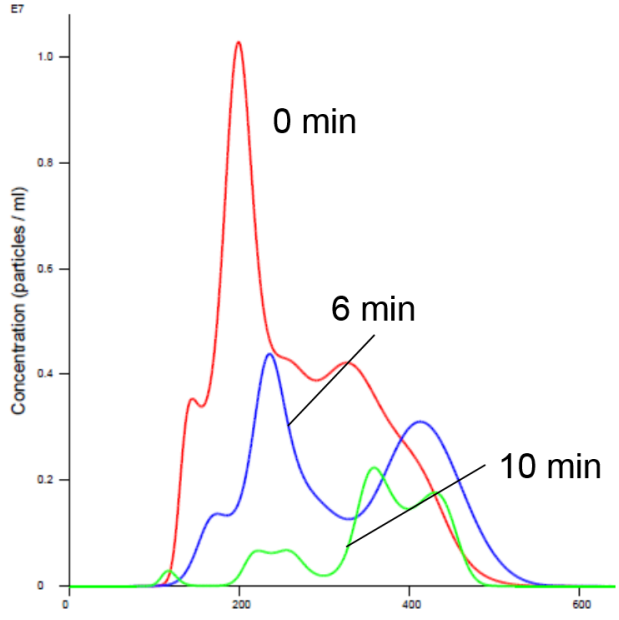

Figure 3. Insulin Receptor alone in Mn buffer show aggregation (increased size and decreased concentration)

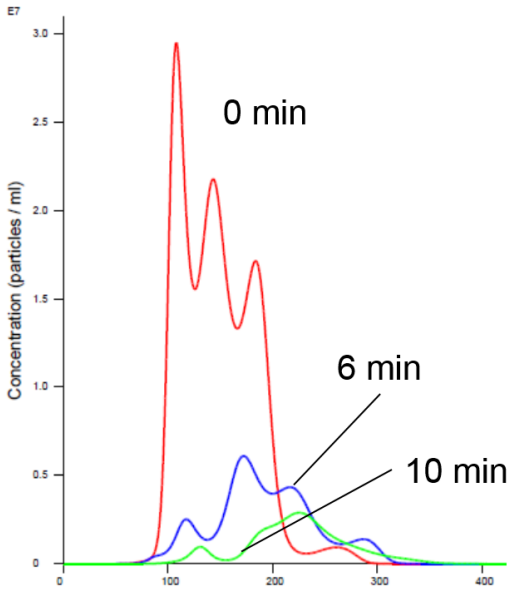

Figure 4. Insulin Receptor and Liposome in Mn buffer showing aggregation (increased size and decreased concentration)

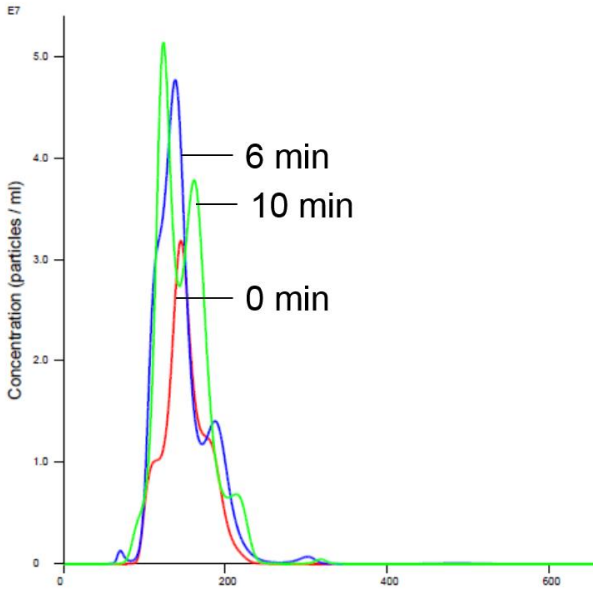

Figure 5. Liposomes in Mg buffer show stabilized size and concentration

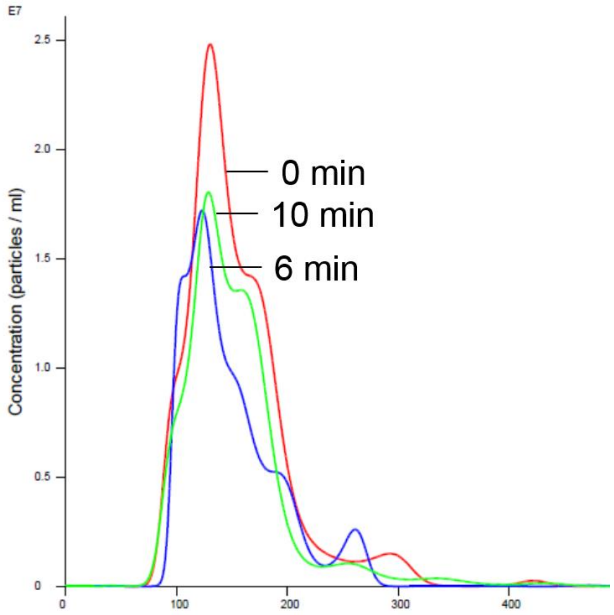

Figure 6. Insulin Receptor and Liposome in Mg buffer show stabilized size and concentration

4 CONCLUSIONS

NTA is a useful technique for accurately characterizing the aggregation behavior of liposome preparations under a range of conditions. The high resolution data provides a unique insight into the aggregation kinetics of these systems. The results here call into question the relevance of compounds selected in high-throughput screening (HTS) assays using Mn as a counter-ion.

REFERENCES

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TDA technology used to prepare the liposomes is protected by patent and available via Blue Sky Bioservices for license: US: 8,541,190.