Quantification and *in vitro* analysis of nanolipoproteins (NLPs) containing adjuvants

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Nanolipoprotein particles (NLPs) self-assemble into nanoscale structures that can be used as vaccines or drug delivery agents. Due to the nature of the NLPs, a variety of immune stimulating compounds or adjuvants can be readily incorporated into NLPs: a characteristic difficult to engineer into most other nanoscale platforms. In light of this, a method for quantifying the amount adjuvant actually incorporated into NLPs is a question of high importance. Through the use of reverse phase High-Performance Liquid Chromatography (HPLC) and an Evaporative Light Scattering Detector (ELSD), standard curves can be constructed by analyzing mixtures of NLP components of known concentration, which can then be used to quantify samples of NLPs and determine the amount of adjuvant incorporated. After constructing NLPs containing different combinations of the adjuvants monophosphoryl lipid A (MPLA) and muramyl dipeptide (MDP), they were tested *in vitro* to determine the intensity of immune response using enzyme-linked immunosorbent assays (ELISAs). ELISA results indicate that when both adjuvants are incorporated into a single NLP, the amount of cytokine production is significantly increased, compared to adjuvants individually incorporated into the NLPs as well as non-NLP formulations of the combined adjuvants. Therefore, the NLPs represent an attractive platform for delivering and enhancing adjuvant combinations for immunological therapeutics.

Nanolipoprotein particles (NLPs) are small particles composed of lipid and protein. The lipid in the NLP is arranged in a bilayer fashion. The protein wraps round the hydrophobic tails which allows them to be soluble in aqueous buffers.

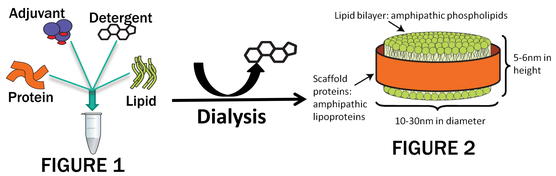


FIGURE 1

FIGURE 2

The different components (lipids, protein and adjuvants) are combined with a detergent in a buffered solution. As the detergent is removed from the solution by dialysis, the particles self-assemble into disk-shaped structures (Figure 2, above). Adjuvants are molecules that initiate an immune response, and can be added to NLPs during the assembly process or to pre-assembled particles. The adjuvants used in this study are shown below (Figures 4 and 5).

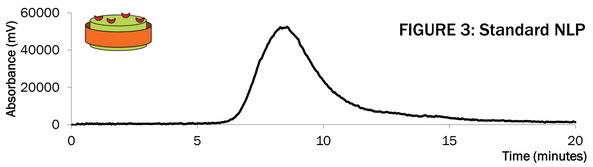


FIGURE 3: Standard NLP

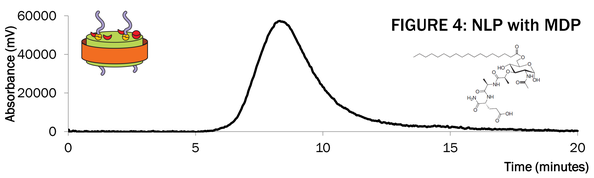


FIGURE 4: NLP with MDP

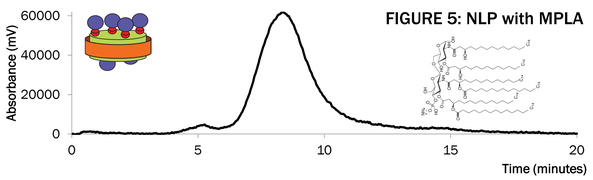


FIGURE 5: NLP with MPLA

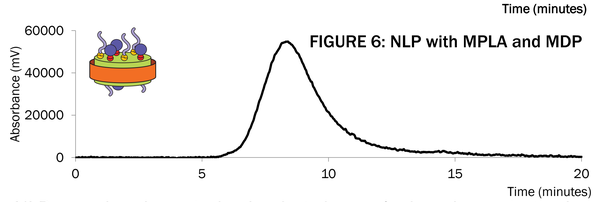


FIGURE 6: NLP with MPLA and MDP

NLPs can be characterized using size exclusion chromatography, a technique used to separate the components of a mixture by their size. The graphs above (Figures 3-6) show that all NLPs elute at approximately 8.5 minutes, regardless of composition. When NLPs are analyzed with a spectrophotometer using size-exclusion chromatography alone, there is little difference between NLPs composed of different molecules.

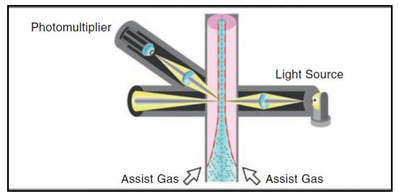


FIGURE 7: The diagram above shows how the amount of solute in the sample is detected.

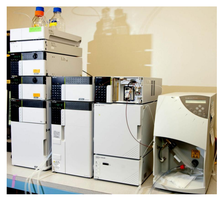


FIGURE 8: The HPLC-ELSD used for these studies.

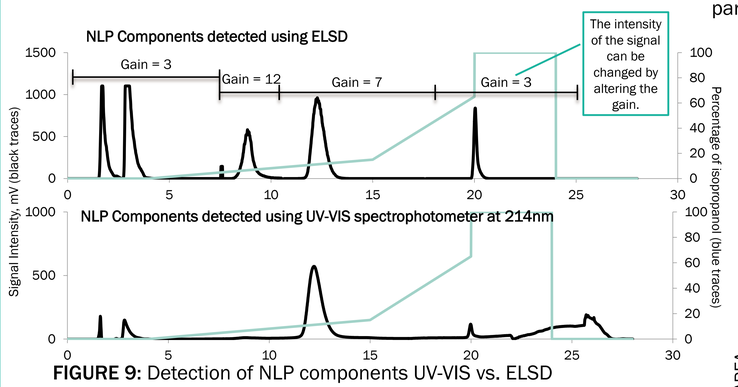


FIGURE 9: Detection of NLP components UV-VIS vs. ELSD

The traces above show NLPs analyzed using two methods: UV-Vis and ELSD. In contrast to UV-Vis absorbance measurements, the ELSD can detect the components of the NLP that have no intrinsic absorbance (e.g. MDP and DOPC lipid) can be visualized. The gain can be changed to detect both small and large amounts of material.

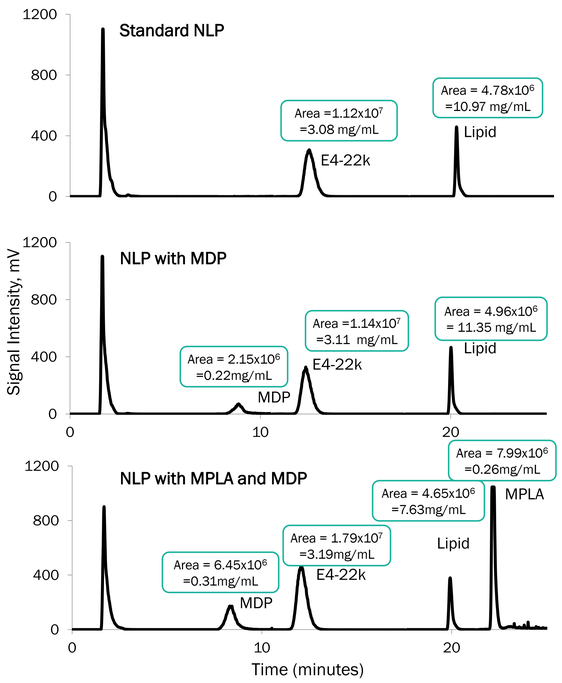


FIGURE 11: ELSD graphs of NLPs of varying composition

The traces above (Figure 11) show data from samples of NLPs containing different components. The elution time identifies the compound. The integrated peak area is then analyzed using the standard curve to determine the concentration of the component within the sample.

Through the use of an evaporative light scattering detector (ELSD), the different components of the NLP can be detected and quantified. After the sample is separated by reverse phase HPLC, the sample is nebulized to form tiny droplets. The solvent within the resulting droplets is then evaporated using a stream of nitrogen gas, leaving behind the solute containing the compound of interest. Prior to detection the solute passes through an oven to ensure that any residual solvent is removed. The solute particles then pass through a beam of light, and the amount of light scattered by the solute particles is detected by a photomultiplier.

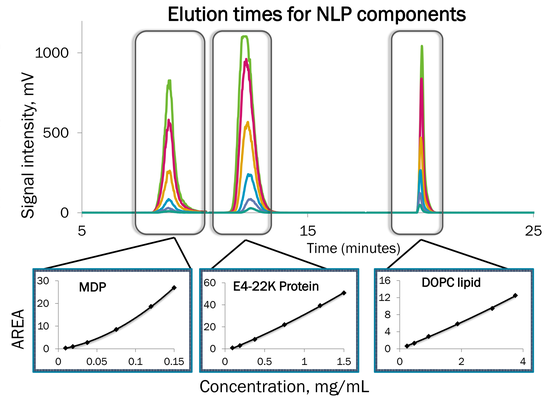


FIGURE 10: ELSD graphs for NLP component standards

Reverse phase HPLC separates mixtures based on polarity, in this case using a gradient of methanol and isopropanol to elute the components off of a column packed with hydrophobic resin. Depending on the its affinity to the hydrophobic resin, each component will elute off of the column at a characteristic solvent ratio, as depicted in the graph above (Figure 10). Each peak corresponds to a particular component of the NLP mixture. The area of each component peak is directly related to its concentration within the sample. Standard curves were prepared using known concentrations of purified compounds and are shown in the boxes below the chromatogram (Figure 10).

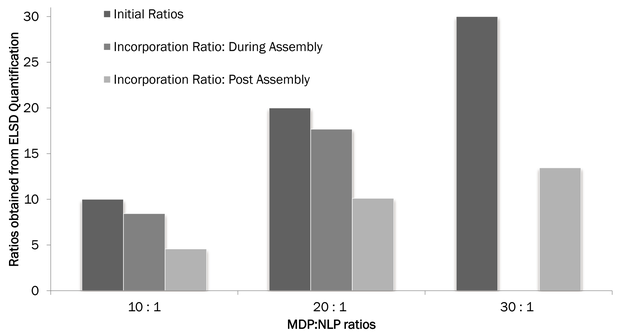
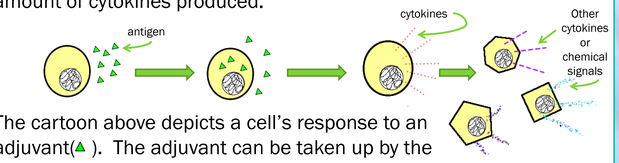


FIGURE 12: Comparison of the addition of adjuvants during the assembly process vs. after assembly

NLPs can be adjuvated during the assembly process or after the NLPs have been formed, depending on the adjuvant. However, it is far more efficient to incorporate adjuvants during the assembly process, as shown in Figure 12 above.

The immunological response of the NLPs were compared to that of the adjuvant alone using an enzyme-linked immunosorbent assay (ELISA). ELISAs were used to measure the response of macrophages exposed to adjuvant formulations by measuring the amount of cytokines produced.



The cartoon above depicts a cell's response to an adjuvant(▲). The adjuvant can be taken up by the cell and cause the cell to produce signals called cytokines. Cytokines then signal other cells, producing an immunological response

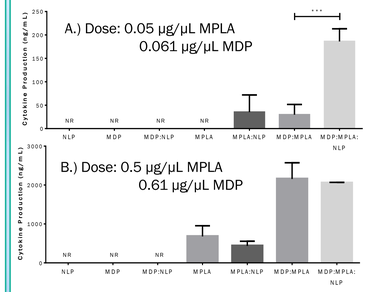


FIGURE 13: IL-6 cytokine response of J774 macrophages to adjuvated NLPs

Two different amounts of adjuvanted NLPs were used to elicit immune responses, measured by macrophage production of IL-6 (Figure 13,left). When NLPs contain a single adjuvant the amount of cytokine production is either modest (MPLA) or below the limit of detection (MDP). However, when particles are made with a combination of MPLA and MDP there is a significant amount of IL-6 production. At the lower dose (0.05 µg/mL MPLA) there is a significant increase in the amount of IL-6 produced between the adjuvants incorporated into the NLP versus the adjuvant alone. At the higher dose (0.5 µg/mL MPLA), the strong stimulation by MPLA appears to mask any contribution of the relatively low levels of MDP. These results are similar to the measured production of a second cytokine, TNF-α (Figure 14).

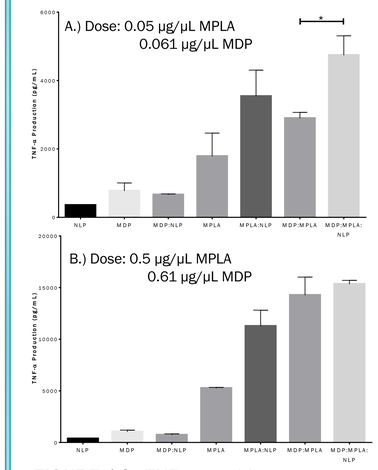


FIGURE 14: TNF-α cytokine response of J774 macrophages to adjuvated NLPs

Conclusion/Future Research

Although there are many different types of adjuvants that can be incorporated into NLPs, there are many factors to consider in relation to immune response that these adjuvant or adjuvant combinations will elicit: the chemical characteristics of the adjuvants, the adjuvant-to-NLP ratios, the dose, and the combination of different adjuvants. The *in vitro* results presented here indicate that while only MPLA incorporated into NLPs individually elicits a significant response, the combination of MDP with MPLA within the NLP further enhances cytokine production. It can be concluded that the two adjuvants act in concert, especially when incorporated into the NLPs, to increase the potency compared to each adjuvant alone.

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