JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Colocalized Delivery of Adjuvant and Antigen Using Nanolipoprotein Particles Enhances the Immune Response to Recombinant Antigens

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Supporting Information

ABSTRACT: Subunit antigen-based vaccines can provide a number of important benefits over traditional vaccine candidates, such as overall safety. However, because of the inherently low immunogenicity of these antigens, methods for colocalized delivery of antigen and immunostimulatory molecules (i.e., adjuvants) are needed. Here we report a robust nanolipoprotein particle (NLP)-based vaccine delivery platform that facilitates the codelivery of both subunit antigens and adjuvants. Ni-chelating NLPs (NiNLPs) were assembled to incorporate the amphipathic adjuvants monophosphoryl lipid A and cholesterol-modified CpG oligodeoxynucleotides, which can bind His-tagged protein antigens. Colocalization of antigen and adjuvant delivery using the NiNLP platform resulted in elevated antibody production against His-tagged influenza hemagglutinin 5 and *Yersinia pestis* LcrV antigens. Antibody titers in mice immunized with the adjuvanted NLPs were 5−10 times higher than those observed with coadministration formulations and nonadjuvanted NiNLPs. Colocalized delivery of adjuvant and antigen provides significantly greater immune stimulation in mice than coadministered formulations.

Developing vaccines based on subunit antigens is one approach to improve vaccine safety. Subunit vaccines use immunogenic antigens, either purified from pathogenic microorganisms or prepared recombinantly, in lieu of the whole-pathogen formulations used in inactivated or attenuated vaccines. The use of subunit antigens is an attractive prospect for a number of reasons. First, subunit antigens are a safe alternative to live attenuated or killed vaccines, as they are nonpathogenic, easier to manufacture, and safe for administration to immunosuppressed individuals. Second, since each component can be purified and analyzed independently, quality control of the preparations can be improved. However, subunit antigens alone, especially recombinant proteins, often lack the immunostimulatory properties required to elicit protective immune responses[1] and thus require coadministration with immune-stimulating adjuvants, such as monophosphoryl lipid A (MPLA)[2] and unmethylated CpG oligodeoxynucleotides (CpGs).[3] Although coadministration formulations can be effective, high adjuvant doses are often needed to elicit a significant immune response to the antigen[4] and to ensure that antigen-presenting cells encountering antigen are also stimulated by the adjuvant. Thus, vaccine delivery platforms enabling colocalized delivery of adjuvant and antigen hold great promise for significantly increasing the efficacy of subunit vaccines.[5,6] The realization of this goal requires a delivery platform that can accommodate myriad functionalities for inclusion of adjuvant(s) and antigen, allows for surface exposure of the adjuvant for targeted delivery to the innate immune system, and is composed entirely of biocompatible components.

One platform ideally suited for vaccine applications is based on nanolipoprotein particles (NLPs). NLPs, also known as nanodiscs, are nanoscale (6−25 nm), discoidal, biocompatible particles analogous to naturally occurring high-density lipoproteins found in blood that form through spontaneous self-assembly of a scaffold protein (apo-lipoprotein) and lipids.[7] The versatility of the NLP self-assembly reaction allows the incorporation of lipids bearing functional groups at the solvent interface of the lipid bilayer, enabling multifunctional NLPs to be prepared in a single step. Thus, a large range of functional groups can be incorporated into NLPs, including chemical groups enabling covalent and non-covalent conjugation of biomolecules. Another key attribute of NLPs is their ability to accommodate cholesterol or any fatty acid within the lipid bilayer, providing a facile route for incorporating cholesterol- or fatty acid-modified biomolecules. Importantly, NLPs have been used in a variety of in vivo applications such as hydrophobic drug delivery,[8] MRI imaging,[9] and antigen delivery,[10] underscoring their biocompatible nature.

We recently demonstrated that Ni-chelating NLPs (NiNLPs) assembled with the 22 kDa N-terminal fragment of apolipoprotein E4 (E422k) and Ni-chelating lipids readily bind His-tagged proteins.[11] The resulting particles present a lipid bilayer surface decorated with chelated Ni^{2+} ions capable of binding recombinant proteins containing a poly-His peptide tag.[11] The kinetics of binding between His-tagged proteins and NiNLPs was previously rigorously analyzed by surface plasmon resonance (SPR) spectroscopy, and the retention time (t_R) of the His-tagged protein on the NiNLP was shown to be dependent on the Ni−lipid content in the NiNLP and the protein density.[12] Furthermore, the utility of the NiNLP platform as an antigen delivery vehicle was assessed using a His-tagged truncated West Nile virus envelope protein (trE) as a His-tagged subunit antigen. After inoculation in mice, trE:NiNLPs showed enhanced protection against a viral challenge relative to trE alone.[10a] However, to our knowledge, the use of NLPs for colocalized delivery of adjuvant and antigen has not been demonstrated.

Received: July 5, 2012
Published: January 18, 2013

dx.doi.org/10.1021/ja3063293 | J. Am. Chem. Soc. 2013, 135, 2044−2047

In this work, the use of multifunctional NiNLPs for colocalized delivery of adjuvant and antigen was examined and tested in vivo. Two adjuvants with disparate physicochemical properties, synthetic MPLA (amphipathic, nonpolar, 1.7 kDa) and cholesterol-modified CpGs (amphipathic, highly charged, 7.1 kDa), were incorporated into the NiNLPs along with the His-tagged recombinant viral and bacterial antigens influenza hemagglutinin 5 (H5) and *Yersinia pestis* LcrV, respectively.

Adjuvanted NiNLPs (MPLA:NiNLPs and CpG:NiNLPs) were prepared as outlined in Figure 1 [see the Supporting Information (SI) for further details]. Initially, MPLA:NiNLPs were assembled with the saturated lipid dimyristoylphosphatidylcholine (DMPC). However, we observed no significant MPLA incorporation into these NiNLPs (data not shown). This is perhaps not surprising, as MPLA contains six saturated hydrocarbon chains that are acylated to the disaccharide and might not readily be accommodated into a bilayer consisting of saturated lipids because of tight lipid packing and steric hindrance. Thus, NiNLPs were assembled with the unsaturated lipid dioleoylphosphatidylcholine (DOPC), which forms more loosely packed bilayers than lipids featuring fully saturated acyl chains. NiNLPs were purified by analytical size-exclusion chromatography (aSEC) (Figure 2A). NiNLP t_R values ranged between 7 and 9 min (see the SI), differing from those for the column void volume (6.2 min) and free scaffold protein (12 min). The collected aSEC fractions were further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and a band corresponding to the E422k scaffold protein (22 kDa) was observed exclusively in the NLP peak. Next, adjuvant incorporation into the DOPC-based NiNLPs was tested. In preliminary experiments, an optimal MPLA/NLP molar ratio of 6 was found; higher MPLA content resulted in both unincorporated lipid and MPLA as determined by aSEC, suggesting that MPLA either preferentially associates with free lipid aggregates or initiates particle disassembly at molar ratios greater than 6 (data not shown). To assess qualitatively the incorporation of MPLA into NiNLPs at a ratio of 6:1, aSEC fractions corresponding to the MPLA:NiNLP peak were subjected to both SDS-PAGE and immunodot blot (IB) analysis. The fractions containing E422k were also positive for MPLA in the IB (Figure 2B). To assess the incorporation of CpGs into NiNLPs, an initial CpG incorporation screen at different CpG/NiNLP molar ratios was performed, and a ratio of 20 was found to provide more efficient CpG incorporation (data not shown). At higher ratios, a significant amount of free CpGs was observed in the aSEC profile (t_R = 13.1 min), indicating that significant amounts of CpG were not incorporated into the NiNLPs at these higher CpG/NiNLP ratios. To assess the incorporation of CpGs into the NiNLPs qualitatively, aSEC fractions corresponding to the CpG:NiNLP peak were subjected to SDS-PAGE and Urea-PAGE analysis (Figure 2C), which showed that fractions

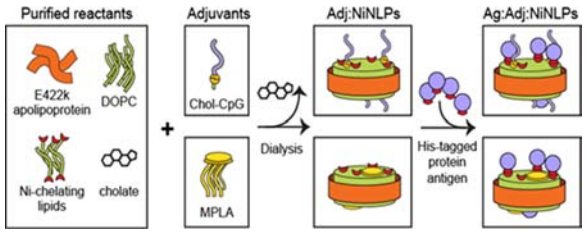

Figure 1. Adjuvant:NiNLP assembly and antigen attachment.

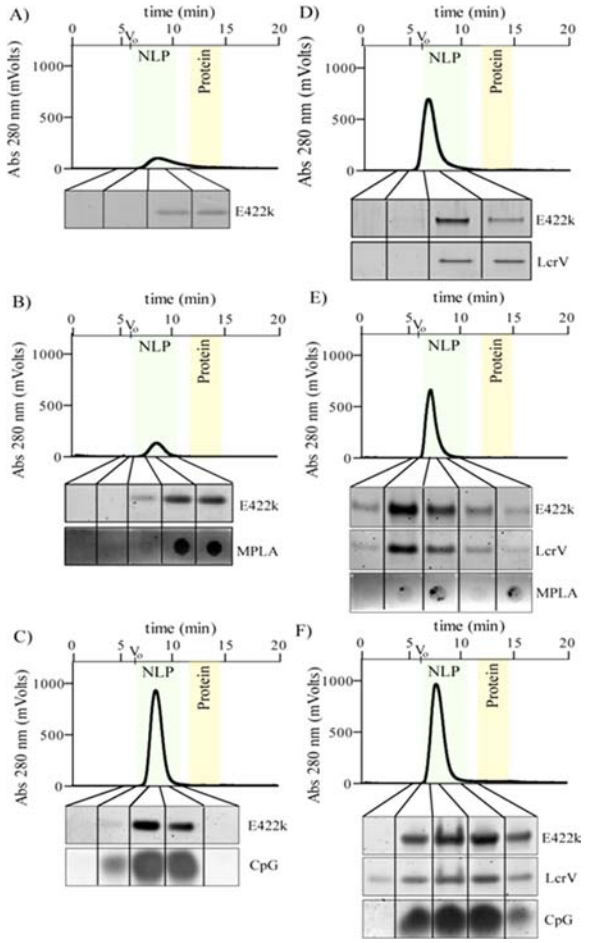

Figure 2. (A, B) aSEC and SDS-PAGE analysis of (A) NiNLP assembly and (B) conjugation of LcrV to NiNLPs. (C, D) aSEC, SDS-PAGE, and IB analysis of (C) MPLA:NiNLP assembly and (D) conjugation of LcrV to MPLA:NiNLPs. (E, F) aSEC, SDS-PAGE, and Urea-PAGE analysis of (E) CpG:NiNLP assembly and (F) conjugation of LcrV to CpG:NiNLPs. Incorporation of MPLA and CpGs into NiNLPs was assessed by analyzing fractions corresponding to the NLP peak upon aSEC separation. Binding of LcrV to NiNLPs and adjuvant:NiNLPs resulted in shorter t_R, indicating increased particle size. Protein components were analyzed by SDS-PAGE. CpGs were detected by Urea-PAGE. MPLA was detected by IB using an anti-Lipid A primary antibody.

containing E422k also contained CpGs. To assess the number of MPLA or CpG molecules per NiNLP quantitatively, the fractions corresponding to the adjuvanted NiNLPs were pooled and concentrated, and the concentrations of E422k, MPLA, and CpGs were determined (see the SI). Adjuvant incorporation was consistent over multiple assemblies, with 3 ± 1 MPLA and 6 ± 1 CpG molecules incorporated within a single NLP (using the established value of six E422k per NiNLP).[7c] Importantly, purification of the adjuvanted NiNLPs by aSEC removed any unincorporated adjuvant from the formulations.

aSEC was also used to assess the conjugation of antigens to the NLP constructs. LcrV (37 kDa) was incubated for 30 min with purified NiNLPs and adjuvant:NiNLPs at an LcrV/NLP ratio of 5. The NiNLPs were fractionated by aSEC and analyzed as described above (Figure 2D–F). LcrV was found to be colocalized in the same fractions as E422k and the adjuvant (Figure 2D–F), indicating that the LcrV antigen was successfully conjugated to the NiNLPs and adjuvant:NiNLPs. Similar results

dx.doi.org/10.1021/ja3063293 | *J. Am. Chem. Soc.* 2013, 135, 2044–2047

were observed for H5 (60 kDa) (data not shown). All of the parameters associated with the aSEC analysis [t_R, Stokes radius (R_S), and peak width] are shown in Table S1 in the SI. It is worth noting that the adjuvant:NiNLPs were slightly larger than the unloaded NiNLPs. The size increase was more pronounced for the CpG:NiNLPs, which we attribute to protrusion of the CpG molecules from the face of the NLP bilayer, leading to an increase in R_S.

Since the experiments described above were conducted at an antigen/adjuvant:NiNLP ratio of 5, we next assessed the ability of the adjuvant:NiNLPs to accommodate multiple antigens. Adjuvant:NiNLPs were incubated with LcrV at LcrV/adjuvant:-NiNLP ratios of 0−40, as described previously.[12] For MPLA:NiNLPs, as the LcrV/MPLA:NiNLP ratio increased, the intensity of the MPLA:NiNLP:LcrV aSEC peak gradually increased to a ratio of ∼20:1 (Figure 3A), concomitant with a

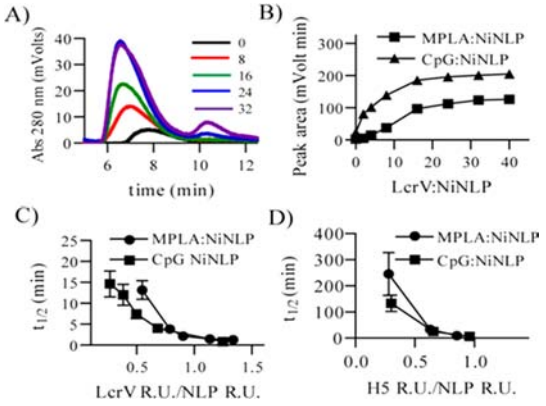

Figure 3. (A) Representative aSEC traces for MPLA:NiNLPs incubated with increasing amounts of LcrV. Increasing the LcrV/MPLA:NiNLP molar ratio resulted in a decrease in t_R and a concomitant increase in peak intensity. The intensity eventually saturated, concomitant with the appearance of a peak corresponding to free, unbound protein (∼10 min). (B) Integrated areas of the adjuvant:NLP:LcrV peaks as functions of the LcrV/NLP molar ratio. (C, D) SPR analysis of the binding of adjuvant:NiNLPs with (C) LcrV and (D) H5.

decrease in t_R. At higher ratios, no further increase in the intensity of the peak was observed, whereas a second peak corresponding to LcrV alone (t_R = 10.2 min) appeared. These results indicate that at LcrV/MPLA:NiNLP ratios lower than 20:1, all of the LcrV was bound to the NiNLPs, while at ratios higher than 20:1, a fraction of the LcrV did not bind to the NiNLPs. Similar results were also observed for CpG:NiNLPs at different antigen ratios (data not shown). To evaluate these aSEC traces quantitatively, the integrated adjuvant:NiNLP peak areas were analyzed as functions of LcrV/adjuvant:NiNLP ratio (Figure 3B). The integrated peak areas increased at higher ratios and eventually saturated. It is also worth noting that the overall higher absorbance of the CpG:NiNLPs is due to the intrinsic absorbance of the CpG moiety at 280 nm. The point of saturation was determined by doubling the ratio where the integrated area of the peak was half the maximal value, which was determined as a fitting parameter to a sigmoidal function.[12] With this approach, the saturation molar ratio for LcrV was determined to be ∼21 for both types of adjuvant:NiNLPs (i.e., the maximal number of LcrV proteins that could bind to a single adjuvant:NiNLP is ∼21). A similar trend was observed for H5 (data not shown).

To assess the binding of antigens to the adjuvant:NiNLPs quantitatively, SPR analysis was used. Adjuvant:NiNLPs were immobilized on a lipophilic SPR chip [2500 response units (RU)], and increasing amounts of antigen were injected onto the immobilized adjuvant:NiNLPs. As previously described,[12] dissociation half-lives ($t_{1/2}$) and antigen RU/NiNLP RU ratios were used to examine the effect of antigen density on antigen t_R (Figure 3C,D). The t_R of the antigen on the NiNLP decreased with antigen loading for both antigens. This result corresponds to our previous observations of a decrease in binding of His-tagged protein to the NiNLPs at higher protein/NiNLP ratios due to a decrease in the number of Ni ligands available for protein rebinding.[12] At low antigen loading, t_R was higher for MPLA:NiNLPs than for CpG:NiNLPs, which may be due to electrostatic repulsion from the highly charged CpG molecules on the CpG:NiNLPs. In addition, $t_{1/2}$ was much longer for H5 than for LcrV (Figure 3C vs 3D). We attribute this difference to the reported tendency of H5 to form trimers.[13] Thus, for H5, a single binding event may have involved three poly-His tags, which would have enhanced the overall H5−adjuvant:NiNLP binding affinity.

To assess whether colocalized delivery of adjuvant and antigen improved antibody production in vivo relative to coadministered formulations, groups of 10 mice were immunized intraperitoneally with either H5, H5:NiNLPs, H5+MPLA (coadministration), H5:MPLA:NiNLPs (codelivery), H5+CpG (coadministration), or H5:CpG:NiNLPs (codelivery). Each animal received a total of 2.5 μg of antigen at an antigen/adjuvant:NiNLP molar ratio of 5, corresponding to MPLA and CpG doses of 0.2 and 2.2 μg, respectively. This antigen/adjuvant:NiNLP ratio resulted in significantly lower adjuvant doses than commonly used in coadministration formulations in mice (e.g., MPLA, 20 μg; CpG, 10 μg), so any potential effects of codelivery were not masked by a large adjuvant dose.[14] H5-specific IgG antibody titers were measured at 2−20 weeks postinoculation. H5:MPLA:NiNLP constructs elicited IgG titers 5−10 times higher than those from mice immunized with H5 coadministered with free MPLA (Figure 4A). The antibody titers in mice immunized with H5:CpG:NiNLPs were also approximately 5−10 times higher than those observed with coadministration of H5 and CpG (Figure 4A). Importantly, the significantly higher antibody titers induced by NiNLP codelivery of adjuvant and antigen were sustained over a 20 week

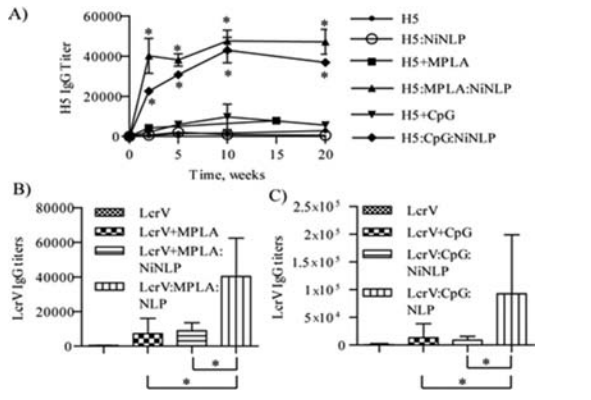

Figure 4. (A) H5-specific IgG titers over a 20 week period after immunization (* indicates $p < 0.001$ relative to coadministration). (B, C) LcrV-specific titers 3 weeks after immunization with (B) MPLA and (C) CpG formulations (* indicates $p < 0.001$).

dx.doi.org/10.1021/ja3063293 | J. Am. Chem. Soc. 2013, 135, 2044−2047

time period with no boost. Notably, no antibodies against E422k were detected, indicating that the scaffold protein is non-immunogenic.

To determine the universal applicability of NiNLP-mediated colocalized delivery of antigen and adjuvant, these experiments were repeated using recombinant LcrV, a protein antigen that has previously been shown to protect against a lethal *Y. pestis* challenge in mice.[15] To understand the role of antigen and adjuvant colocalization, additional groups corresponding to antigen not conjugated to the adjuvanted NLPs were included in these experiments. In these formulations, LcrV was coadministered with adjuvant-loaded NLPs lacking the Ni-chelating lipids, abrogating the antigen−NLP interactions. A single immunization dose of 10 μg of LcrV per animal was used[15] at an antigen/adjuvant:NiNLP molar ratio of 5, corresponding to MPLA and CpG doses of 0.13 and 3.1 μg per animal, respectively. This antigen/adjuvant molar ratio was again selected to give lower adjuvant doses than in traditional coadministration formulations.[14] Antibody titers were subsequently measured 3 weeks postimmunization. Consistent with our H5 results, the NiNLP-colocalized formulations elicited significantly higher antibody titers than the coadministered formulations (7- and 5-fold higher for MPLA and CpG, respectively) (Figure 4B,C). Importantly, the specific antibody titers for the coadministered formulations (LcrV + adjuvant and LcrV + adjuvant:NLPs) were nearly identical, and neither was comparable to the antibody titers for the colocalized formulations. Taken together, these results clearly show that colocalized delivery on a single platform results in a significant boost in the overall adaptive immune response.

At present, there exist few technologies for codelivery of antigens and adjuvants that allow for both clustered antigen presentation and targeted delivery to the adaptive immune system through surface-exposed adjuvant molecules. Furthermore, the incorporation of biomolecules with disparate physicochemical properties continues to be technically challenging. In this context, the NiNLP platform is ideally suited for use as a vaccine delivery platform because it is amenable to the incorporation of both antigen and adjuvant. Our data show that the NiNLP platform can be successfully engineered for colocalized delivery of antigen and adjuvant. Importantly, we have shown that incorporating both adjuvant (MPLA or CpG) and antigen (H5 or LcrV) into a single NiNLP significantly enhances antibody production in vivo relative to coadministration formulations and nonadjuvanted NLPs. These findings correlate with our previous studies using nonadjuvanted formulations (trE:NiNLPs), where the overall antibody production when an antigen was conjugated to the non-adjuvanted NiNLP was relatively small.[10a] Taken together, these results clearly demonstrate the potential of using this platform in vaccine formulations for the colocalized delivery of antigen and adjuvant. An evaluation of the efficacy of this platform in protection against a live *Y. pestis* challenge is currently underway. It is noteworthy that the adjuvant doses used in these experiments were significantly lower (~10-fold) than those needed to elicit robust immune responses in coadministration formulations. Thus, the adjuvant dose can be reduced while consistently eliciting high antibody titers through colocalization on the NiNLP platform.

■ ASSOCIATED CONTENT

Supporting Information

Materials and Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Vidadi Yusibov (Fraunhofer Institute) for providing His_6-tagged H5 and Dr. Matthew Coleman and Dr. Brent Segelke for providing the LcrV gene construct and the technical expertise needed to prepare it, respectively. This work was supported by LLNL under Contract DE-AC52-07NA27344, with additional support from LLNL (LDRD 09-LW-077 to C.D.B. and LDRD 11-ERD-016 to A.R.). LLNL-JRNL-523576.

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