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ORIGINAL RESEARCH

Lipid nanoparticles with accessible nickel as a vaccine delivery system for single and multiple his-tagged HIV antigens

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Abstract: Lipid-based nanoparticles (NPs) with a small amount of surface-chelated nickel (Ni-NPs) were developed to easily formulate the human immunodeficiency virus (HIV) his-tagged Tat (his-Tat) protein, as well as to formulate and co-deliver two HIV antigens (his-p24 and his-Nef) on one particle. Female BALB/c mice were immunized by subcutaneous injection with his-Tat/Ni-NP formulation (1.5 μg his-Tat/mouse) and control formulations on day 0 and 14. The day 28 anti-Tat specific immunoglobulin G titer with his-Tat/Ni-NPs was significantly greater than that with Alum/his-Tat. Furthermore, splenocytes from his-Tat/Ni-NP-immunized mice secreted significantly higher IFN-γ than those from mice immunized with Alum/his-Tat. Although Ni-NPs did not show better adjuvant activity than Tat-coated anionic NPs made with sodium dodecyl sulfate (SDS/NPs), they were less toxic than SDS/NPs. The initial results indicated that co-immunization of mice using his-p24/his-Nef/Ni-NP induced greater antibody response compared to using Alum/his-p24/his-Nef. Co-delivery of two antigens using Ni-NPs also increased the immunogenicity of individual antigens compared to delivery of a single antigen by Ni-NPs. In conclusion, Ni-NPs are an efficient delivery system for HIV vaccines including both single antigen delivery and multiple antigen co-delivery.

Keywords: nanoparticle, nickel, HIV, antigen co-delivery, vaccine

Introduction

A protein-based vaccine can induce both antibody responses and T-cell responses including CTL cytotoxic T lymphocytes (CTL) in the immunized host. Furthermore, a protein contains multiple epitopes including T-helper (Th) epitopes, which play a key role in T-cell immunity, and potentiate CD8+ T-cell responses during priming and/or memory generation.[1,2] The utility of adjuvants to enhance immune responses to protein-based vaccines is widely recognized.[3,4] Specifically related to a potential human immunodeficiency virus (HIV) vaccine, it is critical to design and develop a vaccine that induces both neutralizing antibody and CTL activity, both of which have been shown to be essential for prevention and treatment of this disease.[5,6]

One direction of adjuvant design is to utilize particulate systems including liposomes,[7,8] and other micro- and nanoparticles (NPs)[9] to deliver antigens. Our laboratory has reported on the preparation of lipid-based NPs from oil-in-water microemulsion precursors and on the use of these NPs for enhancing immune responses to protein based vaccines.[10,11] These NPs are approximately 100 nm in size and have the advantage of being prepared in a single step, one-vessel, process. It is also relatively easy to modify the physical characteristics of the particles by using appropriate charged surfactants. Moreover, the oil phase for preparation of these NPs is comprised of cetyl alcohol

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and polysorbate 60, both of which are found in several pharmaceutical products and are biocompatible.[12] In previous studies, the antigens were simply coated on oppositely charged (either cationic or anionic) nanoparticles wherein it is possible that some dissociation of the coated protein from the particle may occur *in vivo*, resulting in a decreased accumulation in antigen-presenting cells (APCs). In addition, the strong ionic surfactants for making the cationic or anionic NPs may be toxic to cells. To solve these problems, lipid NPs decorated with a small amount of accessible nickel on the surface were developed and this system led to enhanced immune responses.[13] In these previous studies, the optimal binding of the his-tagged proteins (GFP or Gag p24) to Ni-NPs was about 1:33 w/w (protein-to-oil phase ratio) and was independent of the size and charge of the his-tagged protein. Apparently, the interaction between chelated nickel and the short sequence of histidine residues increased the delivery efficiency of the protein antigen to APCs.

Multivalent vaccines containing several antigens from different pathogens in one single formulation are commercially available for vaccinating against diphtheria, tetanus, pertussis, polio, hepatitis B and *Haemophilus influenzae* type B.[14,15] Multivalent vaccines not only have significant cost advantages but also have potential to induce synergistic effects between the antigens, since CD4 T helper epitopes in one antigen may enhance B- and T-cell responses induced by other antigens.[2] Thus, ideally, a viable vaccine delivery system should be able to deliver multiple antigens. Recently, Lamalle-Bernard and colleagues developed multivalent vaccines based on anionic polylactic acid (PLA) NPs for co-delivery of HIV p24 and gp120 proteins and they showed that the NPs preserved antigenicity and immunogenicity of p24 and gp120.[16]

The HIV gag gene encodes for four major Gag proteins in the mature virus.[17] Gag proteins are currently under investigation as potential candidates for the HIV vaccines. Of these four proteins, the Gag p24 protein has been shown to be the most conserved among the different HIV subtypes and many groups have also identified Gag p24 as the target of Gag-specific cellular responses.[18,19] More importantly, a recent study of HIV-infected patients highlighted the significance of strong Gag p24-specific cellular responses in controlling viral replication and CD4+ T cell counts.[20] Thus, these features make the HIV-1 Gag p24 protein an attractive and relevant choice for inclusion in a multivalent HIV vaccine.

HIV-1 Nef is an early-expressed regulatory protein which plays an important role in CD4 and major histocompatibility complex (MHC) class I downregulation, the stimulation of virus infectivity and the alteration of the activation state of cells.[21] Moreover, Nef is essential for the maintenance of high levels of viral replication and acquired immunodeficiency syndrome (AIDS) pathogenesis in simian immunodeficiency virus (SIV)-infected macaques and has also been shown to influence progression to AIDS in HIV-infected individuals.[22,23] These *in vitro* and *in vivo* observations suggest that Nef represents another possible attractive target for the development of a multivalent therapeutic HIV vaccine.

However, p24 and Nef proteins have negative and positive charges, respectively, under physiological pH (pH 7.4) precluding the use of either cationic or anionic NPs to bind these two proteins together on the same particle. As an alternative, Ni-NPs have roughly equal binding affinity to his-tag proteins having similar sizes. Since both his-p24 and his-Nef have molecular weights of around 25–27 kDa, they have the potential to be formulated in Ni-NPs and co-delivered to dendritic cells.

HIV-1 Tat protein is an RNA binding transcriptional regulatory protein expressed early in HIV-1 infection, and necessary for high level expression of viral proteins.[10,11] Tat shows very little variability and is highly conserved in the first exon among the different subtypes, with the exception of the O subtype.[24,25] Neutralization of Tat might prevent the transactivation of other infected cells, and also prevent the neurotoxic properties. In previous studies by our group, negatively-charged nanoparticles were utilized to coat the positively-charged Tat (1–72 aa) on the surface of the nanoparticles by charge interaction. Surface coated Tat (1–72 aa) led to significant enhancements in antibody titer, Tat-neutralizing antibodies, and inhibition of Tat-mediated long terminal repeat (LTR) transactivation versus Alum-adjuvanted Tat.[26] In addition, the nanoparticle vaccine delivery system was shown to be dose-sparing.

In order to further investigate the utility of Ni-NPs for vaccine formulation, we have extended previous studies of Ni-NPs to include his-Tat antigen. In addition, we have compared the adjuvant activity and toxicity of Ni-NPs with the previous reported ionic SDS/NPs. Finally, the co-delivery of multiple antigens, p24 and Nef, using this platform vaccine delivery system was also studied.

Materials and methods

Materials

Emulsifying wax (E-wax), comprised of cetyl alcohol and polysorbate 60 in a molar ratio of 20:1, and aluminum hydroxide were purchased from Spectrum (New Brunswick, NJ). PBS/Tween 20 buffer, horseradish peroxidase (HRP), cetyltrimethyl

ammonium bromide (CTAB), SDS and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). Alum was purchased from Spectrum Chemicals and Laboratory Products (Gardena, CA). Brij 78 was purchased from Uniqema (New Castle, DE). 1,2-Dioleoyl-sn-Glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel and ammonium salt, abbreviated DOGS-NTA-Ni and DOGS-NTA, respectively, were purchased from Avanti Polar Lipids (Alabaster, AL). Goat anti-mouse immunoglobulin G (IgG) and peroxidase-linked species specific $F(ab')_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Interferon-γ (IFN-γ) ELISA kit, streptavidin horseradish peroxidase (Sv-HRP), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11c antibody and biotinylated rat anti-mouse IgG1 and IgG2a monoclonal antibodies were from BD Biosciences Pharmingen (San Diego, CA). Tetramethylbenzidine (TMB) Substrate Kit and NHS-fluorescein were purchased from Pierce (Rockford, IL). The antigens his-p24 (a.a. 133–363) and his-Nef (a.a. 3–190) were obtained from Bioclone Inc (San Diego, CA) with purity of >90% by SDS-Page. NHS-fluorescein and Zeba de-salting column were purchased from Pierce Biotechnology (Rockford, IL).

Expression of his-Tat

Tat 1–72 cDNA was polymerase chain reaction (PCR) amplified from HIV-1 strain HXB2 (from NIH AIDS reagent repository), using primers 5′-TAGGATCCGCCACCATGGAGCCAGTAGATC-3′ (forward) and 5′-GCGAATTCTCATTGCTTTGATAGAGAAAC-3′ (backward), and inserted into pHAT10 vector (BD Biosciences, San Jose, CA) at BamH I/EcoR I sites. The sequence containing both his-tag Tat and Tat was then PCR amplified for two rounds from the resulting pHAT-Tat plasmid using primers 5′-CTGTTCCAGGGGCCCTTGAAGGATCATCTC-3′ (forward, for first round PCR), 5′-AGAATTCTGGAAGTTCTGTTCCAGGGGCCC-3′ (forward, for second round PCR), and 5′-ACTCGAGTCATTGCTTTGATAGAGAAAC-3′ (backward, for both), and inserted into pGEM-4T2 vector (GE Healthcare, Piscataway, NJ) at EcoR I/Xho I sites. A new cleavage site for PreScission Protease (GE Healthcare) was also incorporated between GST and his-tag sequence. The resulting pGET-his-Tat plasmid was transformed into BL21-T1 competent cells. Expression of the fusion protein was induced with 3 mM IPTG for four hours. Cells were then harvested and extracted with lysis buffer (1 × phosphate-buffered saline [PBS] with 100 μM phenylmethanesulphonylfluoride [PMSF]). For his-tag Tat protein, the clarified cell extract was applied to a 5 ml GSTrap Sepharose column (GE Healthcare). The column was washed with 10 column volumes of PBS. The fusion protein bound on the column was then digested with 80 units of PreScission Protease overnight at 4 °C. His-tag Tat protein released from the column by digestion was then collected and analyzed by SDS-PAGE and western blot. The eluted fractions were separated by 12.5% SDS-PAGE gel and immunoblotted with anti-Tat antibody. The immunoreactive bands showed the monomer (~10 kDa) as well as multimers of Tat. Coomassie bright blue (CBB) staining showed a similar pattern of protein bands as the western blot.

Preparation of Ni-NPs and NTA-NPs

Ni-NPs were prepared as described previously with slight modifications.[13] Briefly, in a 7 ml glass vial, 4 mg of emulsifying wax (the oil phase) and 7 mg (3 mM) of Brij 78 (the surfactant) were added and then melted at 65 °C. To this vial, 21.2 μl (0.1 mM) of DOGS-NTA-Ni (10 mg/ml stock in chloroform) was added and the chloroform was evaporated on a hot plate (65 °C) while stirring. De-ionized, 0.2 micron-filtered water (100 μl) was added to the vial at 65 °C and the contents of the vial were mixed on the hot plate for 2 min to form a slurry-like mixture. Then, 1.9 ml water was added to the mixture at 65 °C to form clear oil-in-water microemulsions. NPs were obtained by cooling the vials to room temperature while stirring. NPs of similar composition but without Ni were prepared in the same manner using 0.1 mM of DOGS-NTA lipid instead, referred to as NTA-NPs. Excess DOGS-NTA-Ni or DOGS-NTA was separated from the Ni-NPs by using a gravity packed Sepharose CL4B gel permeation chromatography (GPC) column (15 × 70 mm). Briefly, 200 μl of the Ni-NPs was passed down the GPC column using PBS, pH 7.4 as the mobile phase. Fractions (1 ml) were collected and the fractions containing the NPs (as determined by measuring the particle size intensity with the particle sizer) were stored at 4 °C for further *in vivo* and *in vitro* study. The NPs were characterized by measuring their size, using a Coulter N5 Submicron Particle Sizer (Coulter Corporation, Miami, FL) at 90°, and charge, using a Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

Preparation of fluorescence-labeled his-tag Tat protein (fluo-his-Tat) and binding of fluo-his-Tat to Ni-NPs

Fluo-his-Tat was prepared using NHS-fluorescein, which is an amine-reactive derivative of fluorescein dye. Briefly,

NHS-fluorescein (1 mg) was dissolved in 1000 µl DMSO, and 13 µl of this solution was added to 300 µg his-Tat protein dissolved in 300 µl PBS buffer. The reaction was performed at room temperature for one hour in the dark. Unreacted NHS-fluorescein was removed using a Zeba de-salting column. The degree of modification was calculated using the fluorescein molar extinction coefficient = 68,000 M^{-1} cm^{-1} and the method provided by the vendor. Fluo-his-Tat was stored at −80 °C until use. To determine the optimal binding ratios, fluo-his-Tat was mixed with the GPC column-purified Ni-NPs at a 1:20, 30, 40, 50, 60 w/w ratios in PBS, pH 7.4 at 4 °C overnight. Free protein was separated from NP bound protein by passing the fluo-his-Tat and NP mixtures through the Sepharose CL4B column using PBS, pH 7.4 as the mobile phase. Fractions collected (1 ml) were analyzed by fluorescence to determine the percent of his-Tat bound to Ni-NPs.

Preparation of anionic SDS/NPs and cationic CTAB/NPs

Cationic and anionic NPs from oil-in-water microemulsion precursors were prepared as previously described with slight modification.[10,11] Briefly, 2 mg of emulsifying wax and 3.5 mg of Brij 78 was melted and mixed at ~65 °C. Deionized water (980 µl) was added to the melted wax and surfactant while stirring to form an opaque suspension. Finally, 20 µl of CTAB (50 mM) or SDS (50 mM) was added to form clear microemulsions at 60–65 °C. The microemulsions were cooled to room temperature while stirring to obtain NPs (2 mg/ml). The final concentration of components in the NP suspension was E-wax (2 mg/ml), Brij 78 (3 mM), and either 1 mM SDS (for anionic SDS/NPs) or 1 mM CTAB (for cationic CTAB/NPs).

Mouse immunization study using his-Tat/Ni-NP

Female BALB/c mice (8–10 weeks old) obtained from Charles River Laboratories (Indianapolis, IN) were immunized subcutaneously with 150 µl of the formulations. Mice (n = 8/group) were dosed on day 0 and day 14 with his-Tat/Ni-NP, his-Tat/NTA-NP, his-Tat/SDS/NP, or his-Tat adjuvanted with Alum. The Tat doses in every group were kept as 1.5 µg per mouse and E-wax for preparation of NP or Alum was kept as 100 µg. On day 28, mice were bled by cardiac puncture and sera were separated. All sera collected were stored at −20 °C for further characterization.

Determination of anti-Tat, p24 and Nef antibody titers

Antigen-(Tat, p24 or Nef) specific serum IgG, IgG1 and IgG2a antibody titers were determined by ELISA. Briefly, 96-well plates (Costar) were coated with 100 µl of recombinant Tat, his-p24 or his-Nef (10 µg/ml in 0.01 M carbonate buffer, pH 9.6) overnight at 4 °C. The plates were blocked for one hour at 37 °C with 200 µl of 2% BSA prepared in PBS/Tween 20. The plates were then incubated with 100 µl per well of mouse serum diluted appropriately in 2% BSA/PBS/Tween 20 for two hours at 37 °C. The plates were washed with PBS/Tween 20 and incubated with 100 µl/well sheep-anti-mouse IgG HRP (1:5000 in 2% BSA/PBS/Tween 20) for one hour at 37 °C. For IgG1 and IgG2a determination, the plates were similarly incubated with goat anti-mouse IgG1-HRP or goat anti-mouse IgG2a-HRP diluted 1:5000 (2% BSA/PBS/Tween 20). After washing the plates with PBS/Tween 20, plates were developed by adding 80 µl of TMB substrate and incubating for 10 min at RT. The color development was stopped by the addition of 50 µl of 2M H_2SO_4 and the OD at 450 nm was read using a Bio-Tek Synergy 2 Microplate Reader (Bio-Tek Instruments, Inc.). The endpoint titer was defined as the highest reciprocal dilution of sera yielding an OD_{450} value at least 3 × background obtained using samples obtained from naïve mice.

Cytokine release assay from Tat stimulated splenocytes

Mice were sacrificed on day 28 and spleen cells were isolated. Red blood cells were lysed by adding 1 × ammonium chloride/potassium carbonate (ACK) buffer (156 mM NH_4Cl, 10 mM $KHCO_3$ and 100 µM EDTA) and incubating for 1–2 min at RT. The cells were spun down at 1,500 rpm, 4 °C for 10 min. The cells were resuspended in RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 µM l-glutamine, 10 U/ml penicillin, 100 U/ml streptomycin, 50 µM 2-mercaptoethanol). To measure cytokine release from stimulated splenocytes, parallel 48-well plates were set up using 1×10^6 cells/well (total volume of 400 µl) and incubated with media alone or Tat (5 µg/ml) at 37 °C, 5% CO_2 for 72 hours. The supernatants were collected at 72 hours and stored at −80 °C for IFN-γ analysis by ELISA (BD Biosciences).

NP toxicity to dendritic cells

DC2.4 cells were obtained from bone marrow cells infected with a retrovirus encoding *myc* and *raf* by using supernatant

from NIH J2 Leuk cells, as previously described.[27] DC2.4 cells were provided by Dr. Leaf Huang at the University of North Carolina at Chapel Hill. After incubation of DC2.4 cells with NPs, the cells were stained with CD11c and propidium iodide and analyzed by flow cytometry. The percentage of cell death of DC was determined by the proportion of PI^+ cells within the $CD11c^+$ region using a BD FACSCanto digital flow cytometer (BD Biosciences).

Preparation of his-p24/his-Nef/Ni-NP formulation and *in vivo* immunization study

Ni-NPs were prepared as described above. Since the molecular weights of p24 and Nef are similar (about 24–27 kDa), the previous published optimal P : L weight ratio (1:33.3 w/w) for his-p24 was used for preparation of the formulation his-p24/his-Nef /Ni-NPs.[13] Briefly, his-p24 and his-Nef were bound to Ni-NPs at the weight ratio of 1.5:1.5:100 (his-p24:his-Nef:Ni-NPs) in PBS at 4 °C overnight. The same weight ratio of proteins to Alum was used to prepare the Alum/his-p24/his-Nef formulation. Single antigen formulations his-p24/Ni-NP and his-Nef/Ni-NP were prepared at the weight ratio of 1:33.3 w/w. Blank NPs without DOGS-NTA-Ni were added to the his-p24/Ni-NP and his-Nef/Ni-NP to retain the same amount of NPs among all the formulations. Female BALB/c mice (8–10 weeks old) were immunized subcutaneously with 150 µl of the formulations. Mice ($n = 8$/group) were dosed on day 0 and day14 with 1.5 µg his-p24 and/or his-Nef per mouse. The mice were bled by cardiac puncture on day 28 and the sera were separated and stored at −20 °C for antigen-specific IgG analysis by ELISA.

Statistical analysis

The data were analyzed using Prism 3.0 (GraphPad Software, La Jolla, CA). A one-way ANOVA analysis was performed first followed by the Tukey's test for multiple comparisons. Data were concluded to be statistically different if $p < 0.05$.

Results and discussion

Optimization and characterization of his-Tat/Ni-NP formulations using fluorescent labeled his-Tat

We reported previously on the use of lipid-based NPs with a small amount of Ni-NPs to deliver his-p24 antigen.[13] To test if this system is suitable to deliver other HIV antigens such as cationic Tat, his-Tat was bound to the surface of Ni-NPs. Fluorescent-labeled his-Tat was prepared and used to optimize and characterize the formulation. To avoid the over-modification of his-Tat by fluorescein, a 1:1 molar ratio of protein to NHS-fluorescein was used in the labeling reaction. This reaction yielded fluorescein-his-Tat with a degree of modification of ~10%.

The elution profile of fluorescein-labeled his-Tat mixed with Ni-NPs at five different weight ratios is shown in Figure 1. NPs eluting in fractions 3–6 were monitored by light-scattering intensity (not shown) and free protein was detected in fractions 8–12 and quantified by fluorescence intensity (y-axis). The broadening of the peaks in fractions 3–6 with increasing his-Tat/Ni-NP ratios correspond directly to the amount of Ni-NPs that were loaded onto the column. At a 1:60 w/w ratio of his-Tat to Ni-NPs (P:L), the fluorescence intensity of free his-Tat (fraction 10) was very low while the NP bound his-Tat (fraction 4) was the highest, suggesting the majority of the protein was associated with NP at this ratio. The his-Tat binding to Ni-NPs was found to be stable for four hours in PBS at 37 °C, pH 7.4 with less than 10% of the protein released and particle sizes retained at approximately 120 nm over the time frame (data not shown). Therefore, the ratio of P:L close to 1:60 (w/w) was used for the *in vivo* studies. The optimal P:L ratio for his-Tat is different from a previous report of his-p24.[13]

In vivo immunization using optimized his-Tat/Ni-NP formulations

Based on the optimization studies assessing the fluorescence labeled his-Tat binding to Ni-NPs study, his-Tat was bound to Ni-NPs at a 1:60 w/w ratio for the *in vivo* immunization studies. The binding of his-Tat to the Ni-NPs was also confirmed by SDS-PAGE (data not shown). The use of DOGS-NTA entrapped in NPs was also investigated to control for non-specific adsorption of the protein on the surface of the NPs. The carboxylic groups of the NTA provide an overall slightly net-negative charge to the surface of the NPs and could allow his-Tat, a cationic protein, to be coated on the surface of the particles. The strong negatively-charged NPs made using SDS as a surfactant was used as a positive control.[11] As shown in Figure 2A, the Ni-NPs resulted in significant enhancement in antibody responses compared to both Alum and NTA-NPs ($p < 0.05$). However, the total Tat-specific IgG titer for the Ni-NPs and SDS/NPs was comparable. In this study, the primary binding of protein on NPs is mediated through the interaction of the N-terminal his-tag to Ni on the surface of NPs. Therefore, it is possible that this binding may lead to an orientation of the protein that is different than when the same protein is adsorbed

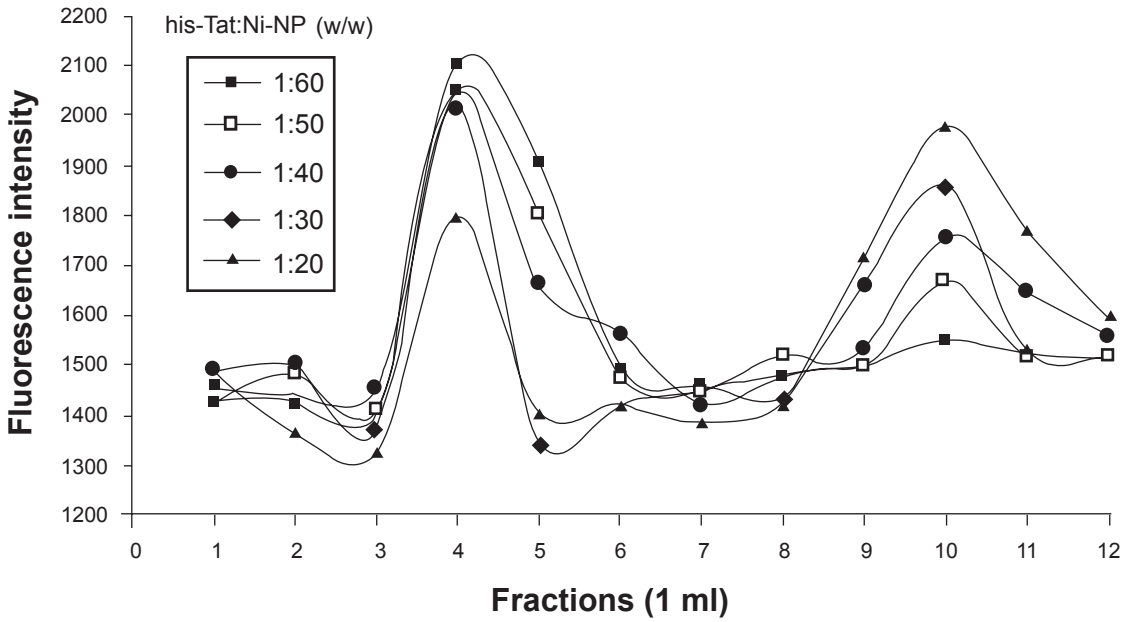

Figure 1 Binding of fluo-his-Tat to Ni-NPs. The GPC purification profile for fluo-his-Tat bound to Ni-NPs at 1:20, 30, 40, 50, 60 w/w ratio of protein to Ni-NPs. Ni-NPs eluted in fraction 3–6 as determined by particle size intensity (not shown). The extent of his-Tat bound to Ni-NPs was determined by separating Ni-NP bound protein (fractions 3–6) from free protein, which elutes in faction 8–12 as determined by fluorescence intensity measurements.
Abbreviations: GPC, gel permeation chromatography; NPs, nanoparticles.

onto SDS-NPs or adjuvanted with Alum thereby resulting in qualitative differences in the induced immune responses. In fact, in previous studies we have shown by epitope mapping that Tat adsorbed on SDS-NPs led to differences in immune responses to several different peptide regions on Tat as compared to Tat adjuvanted with Alum.[10]

It is known that during an immune response, the release of Th1 or Th2 cytokines will affect the production of antibody isotypes. Th1 cytokines like IFN-γ promote class switching to the IgG2a isotype. BALB/c mice bias the immune response towards a Th2 profile, resulting in a relative lack of IFN-γ and more antibodies of IgG1 isotype. The serum isotype levels, IgG1 versus IgG2a, were measured to assess the type of immune response generated (ie, Th1 or Th2). The isotype analysis in these studies revealed that the Ni-NPs resulted in higher levels of IgG2a compared to the Alum group ($p < 0.01$), while the IgG1 levels were comparable to the other three immunized groups (Figure 2B). The data indicated that all the NP groups (Ni-NP, NTA-NP and SDS/NP) showed a balanced IgG2a/IgG1 ratio of approximately 0.5. In contrast, the IgG2a/IgG1 ratio in the Alum group was 0.153 indicating a more Th2-biased response.

The cellular immune responses in these studies were evaluated by IFN-γ release assays. Splenocytes from immunized mice that were stimulated *in vitro* with recombinant Tat protein demonstrated significantly higher IFN-γ release than those from naive mice. Moreover, the IFN-γ release from stimulated splenocytes of Ni-NP immunized mice was significantly higher than that from the Alum and NTA-NPs groups (Figure 3). Although Ni-NPs showed superior adjuvant activity than Alum and NTA-NPs, it had comparable activity with the previously developed SDS/NPs. Thus, although the different NP types induced similar Th1/Th2 profiles inferred by antibody isotype differences, Ni-NP and SDS/NP induced a higher Th1 response inferred by IFN-γ release from spleen cells. This may reflect a greater stimulation of CD8 T cells by the Ni-NPs and SDS/NP which might not contribute to antibody isotype switching *in vivo*.

Dendritic cell toxicity induced by NPs

The immunization results above showed that the Ni-NP vaccine delivery system was suitable for his-Tat delivery and generated enhanced immune responses versus Alum adjuvant, but comparable to that of the previously developed SDS/NPs. However, we have previously noted that the highly anionic SDS NPs showed a dose-dependent toxic effect on bone marrow-derived macrophages (Woodward and Mumper, unpublished results). To ascertain potential differences in the toxicity of the various NPs used in these studies, the toxicity of Ni-NPs, NTA-NPs, SDS/NPs (anionic) and CTAB/NPs (cationic) were investigated

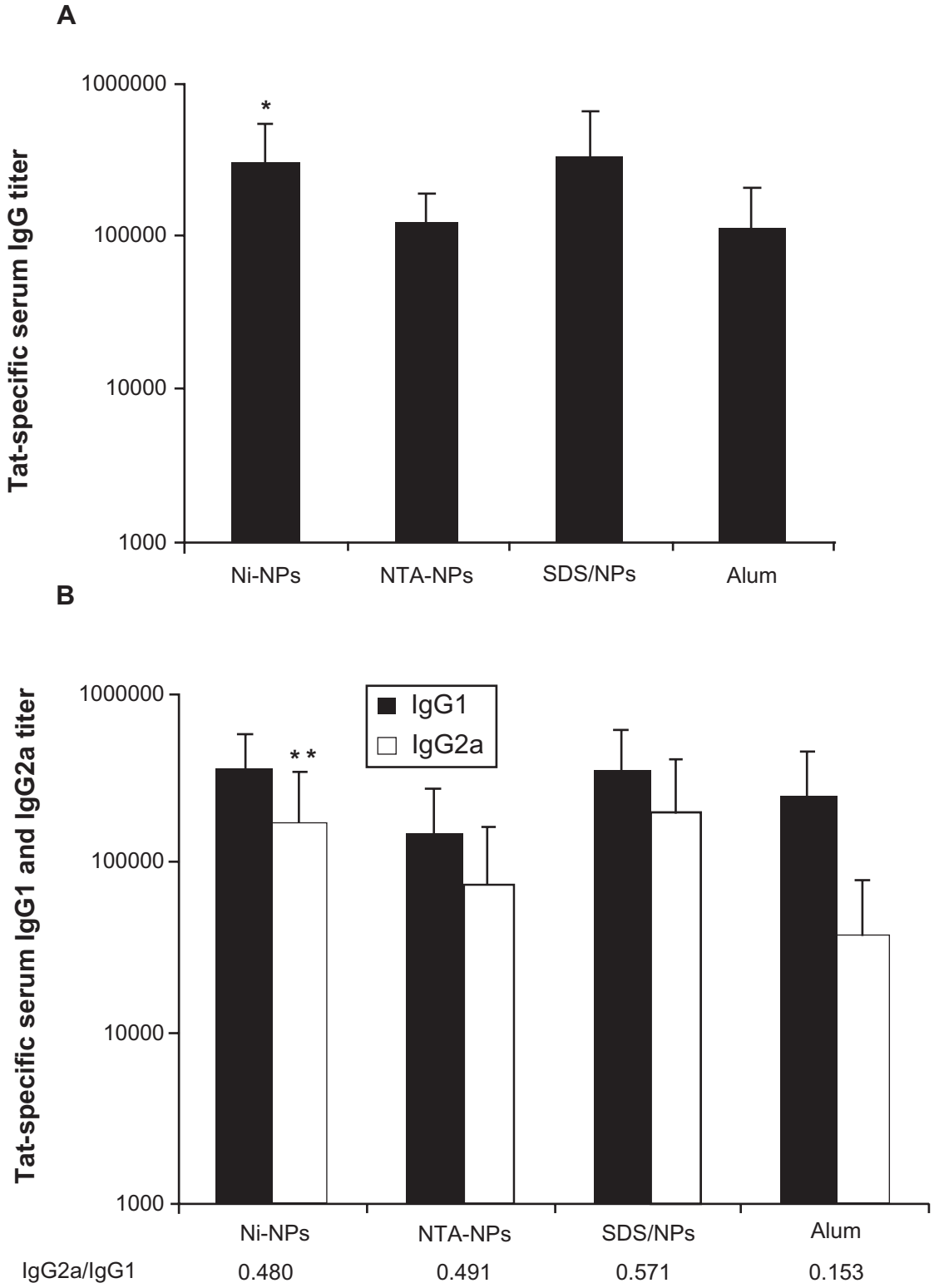

Figure 2 Tat-specific serum IgG titer. BALB/c mice were immunized on day 0 and day 14 with 150 μl of each formulation containing 1.5 μg his-Tat and 100 μg NPs or Alum. **(A)** Tat-specific total serum IgG titers were evaluated on Day 28 by ELISA. Data represent the mean ± S.D. $^{*}p < 0.05$ compared to NTA and Alum groups. **(B)** Tat-specific serum IgG2a and IgG1 titer were evaluated on day 28 by ELISA. The mean IgG2a/IgG1 ratio is indicated on the bottom of the graphed titer for each group. Data represent the mean ± S.D. $^{**}p < 0.01$ compared to Alum group.

Abbreviations: IgG, immunoglobulin G; NTA, nitrilotriacetic acid; NPs, nanoparticles.

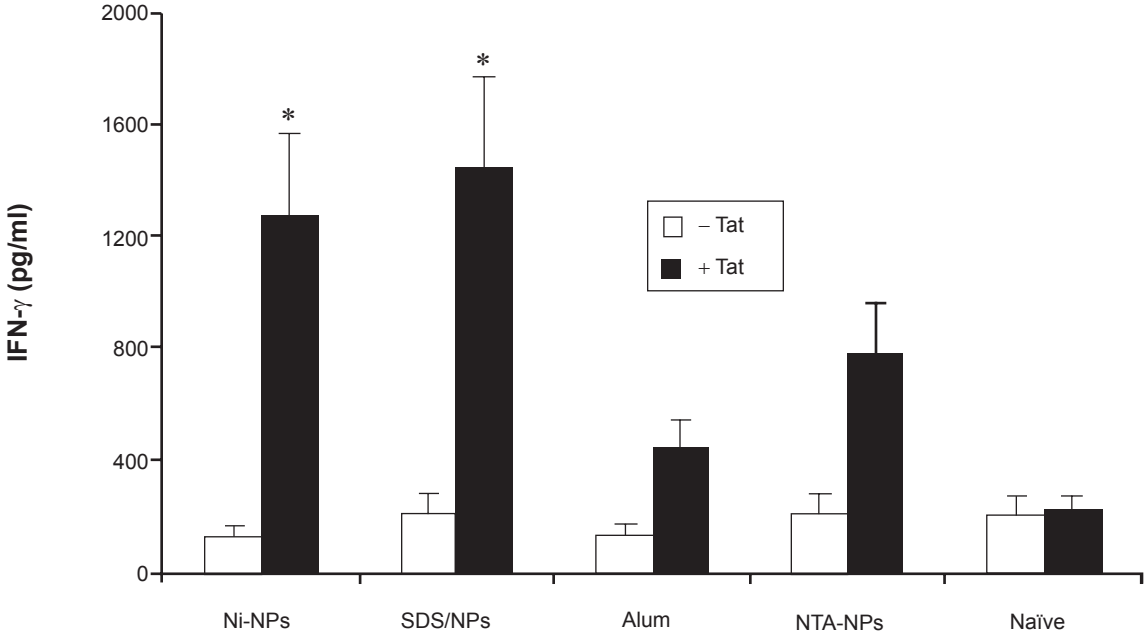

Figure 3 Cellular responses to his-Tat. Mice were immunized with 1.5 μg of his-Tat on day 0 and day 14. Spleens were harvested and pooled for each group on day 28. Cells (1×10^6) were stimulated with recombinant Tat (5 μg/ml) and the supernatants were evaluated for IFN-γ release at 72 h by ELISA. The data represent the mean ± SD (n = 3). *$p < 0.05$ compared to the Alum and NTA groups.
Abbreviations: NTA, nitrilotriacetic acid; NPs, nanoparticle; IFN-γ, interferon-γ; SD, standard deviation; SDS, sodium dodecyl sulfate.

in the DC2.4 dendritic cell line. All the formulations showed low toxicity for 24-hour treatment (around 5% PI-positive cells). However, after 48-hour treatment, the charged NP (SDS/NPs and CTAB/NPs)-treated DC2.4 cells showed more than 20% PI-positive (dead) cells. Both NTA-NPs and Ni-NPs showed less toxicity than either of the charged NPs ($p < 0.05$ compared to SDS/NPs, $p < 0.01$ compared to CTAB/NPs) (Figure 4). The data also showed that positive charged CTAB/NPs were more toxic than negative-charged SDS/NPs ($p < 0.05$) (Figure 4). Thus, Ni-NPs may be superior to charged NP in terms of safety/toxicity.

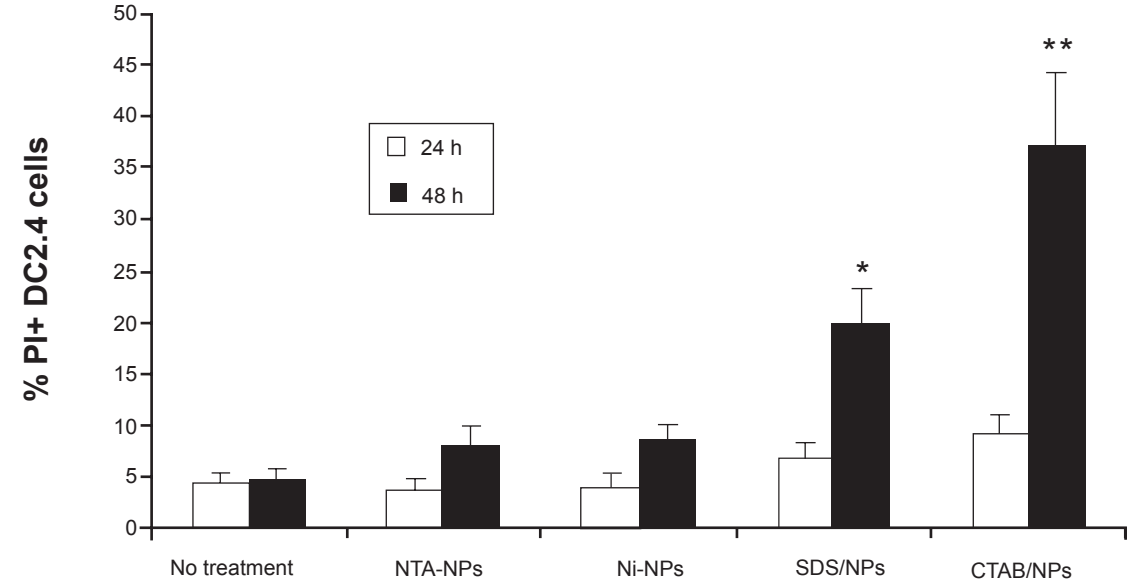

Figure 4 Cytotoxicity induced by NPs in DC2.4 cells. DC2.4 cells were incubated with NPs (11 μg/ml) for the indicated time. The cells were washed and incubated with FITC labeled CD11c and PI. The percentage of cell death was determined by the proportion of PI^+ cells within the $CD11c^+$ region. The data represent the mean ± SD (n = 5).
Notes: *$p < 0.05$ compared to NTA-NPs, Ni-NPs and CTAB-NPs; **$p < 0.01$ compared to NTA-NPs and Ni-NPs.
Abbreviations: PI, propidium iodine; CTAB, cetyltrimethyl ammonium bromide; NPs, nanoparticles; FITC, fluorescein isothiocyanate.

Ni-NPs for the delivery of multiple antigens

Although negatively-charged SDS/NPs are suitable for the delivery of Tat and other cationic antigens, they are obviously not suitable for multiple antigens especially if the antigens are negatively-charged. The toxicity study in DC2.4 cells also showed that the charged NP may not be warranted for larger antigen doses. An alternative strategy would be to utilize Ni-NPs to bind various his-tagged proteins irrespective of their native charge. To test this hypothesis, the his-tagged antigens, gag p24 and Nef, were bound to Ni-NPs. Indeed, mice co-immunized with his-p24 and his-Nef antigens co-bound to Ni-NPs, had higher p24-specific antibodies as compared to mice immunized with p24-alone bound to NPs ($p < 0.05$). In addition, his-p24/his-Nef/Ni-NP resulted in significant enhancement in p24-specific antibody response compared to his-p24/his-Nef adjuvanted with Alum ($p < 0.05$) (Figure 5A). In a similar manner, mice immunized with his-p24/his-Nef/Ni-NP had greater Nef-specific antibodies than mice immunized with Nef alone bound to NPs (Figure 5B). Thus, Ni-NPs provide a distinct advantage of allowing the binding of varied his-tagged proteins, regardless of charge.

In these current studies, Ni-NPs were successfully used for the delivery of cationic his-Tat antigen to induce enhanced immune responses compared to Alum. Although this system did not show superior adjuvant activity compared

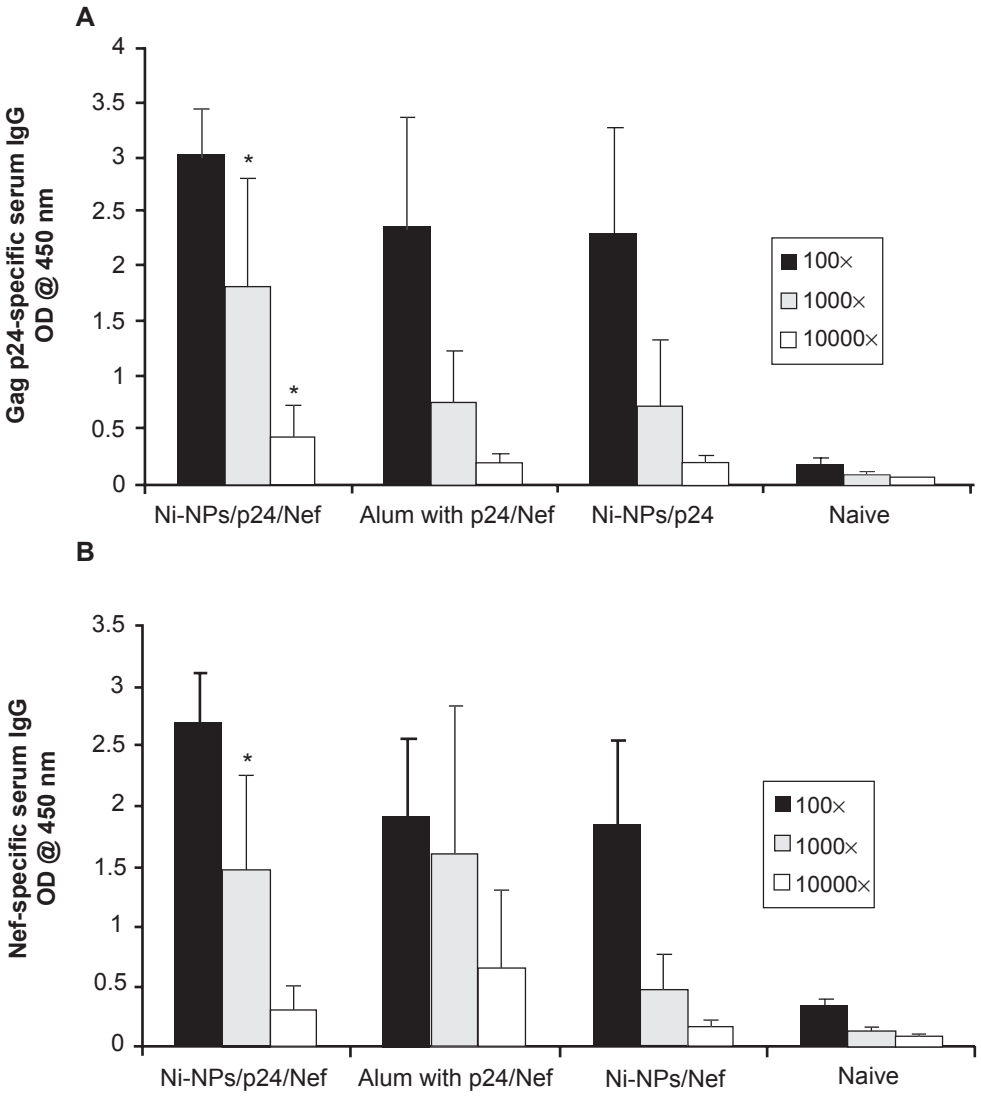

Figure 5 Ni-NPs for co-delivery of his-tagged p24 and Nef antigens. BALB/c mice were immunized on day 0 and day 14 with 150 μl of each formulation containing 1.5 μg his-p24 and/or 1.5 μg his-Nef adjuvanted by 100 μg NPs or Alum. Total serum IgG levels at day 28 were diluted 100, 1,000 and 10,000-fold and measured by ELISA. **A**) Gag p24-specific serum IgG titer. *$p < 0.05$ compared to Alum/his-p24/his-Nef and his-p24/Ni-NP groups. **B**) Nef-specific serum IgG titer, *$p < 0.05$ compared to his-Nef/Ni-NP group. Data represent the mean ± SD.

Abbreviations: IgG, immunoglobulin G; NPs, nanoparticles; SD, standard deviation.

to SDS/NPs, it showed less toxicity to dendritic cells than SDS/NPs. Interestingly, cationic CTAB/NPs were more toxic to dendritic cells than anionic SDS/NPs. Dendritic cells play a central role to link the innate immune responses to adaptive immune responses, so their life-span affects the potency of vaccines. For example, it was reported that a high dose of cationic liposome induced apoptosis of dendritic cells leading to compromised immune responses.[28] In addition, two other reports showed that the potency of a dendritic cell-based vaccine was enhanced by incorporating anti-apoptotic strategies for prolonging dendritic cell life.[29,30] Therefore, utilizing less toxic but equally effective vaccine carriers such as Ni-NPs to bind his-tagged protein antigens have potential advantages.

Conclusions

We have expanded our studies of Ni-NPs as an effective vaccine delivery vehicle to include the delivery of cationic his-Tat antigen, and the co-delivery of gag p24 and Nef. Ni-NPs induced enhanced Tat-specific immune responses compared to Alum. Although this system did not show superior adjuvant activity compared to SDS/NPs, it showed less toxicity to dendritic cells than SDS/NPs. Ni-NPs were found to be suitable for the co-delivery of p24 and Nef protein antigens, and the binding of various his-tagged antigens on the same particle is regardless of the charge. The safety and adjuvant activity of Ni-NPs make this system suitable for HIV vaccine development, especially for multivalent vaccines.

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