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Tresyl-based conjugation of protein antigen to lipid nanoparticles increases antigen immunogenicity

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ABSTRACT

The present studies were aimed at investigating the engineering of NPs with protein-conjugated-surfactant at their surface. In order to increase the immunogenicity of a protein antigen, Brij 78 was functionalized by tresyl chloride and then further reacted with the primary amine of the model proteins ovalbumin (OVA) or horseradish peroxide (HRP). The reaction yielded Brij 78–OVA and Brij 78–HRP conjugates which were then used directly to form NP–OVA or NP–HRP using a one-step warm oil-in-water microemulsion precursor method with emulsifying wax as the oil phase, and Brij 78 and the Brij 78–OVA or Brij 78–HRP conjugate as surfactants. Similarly, Brij 700 was conjugated to HIV p24 antigen to yield Brij 700–p24 conjugate. The utility of these NPs for enhancing the immune responses to protein-based vaccines was evaluated in vivo using ovalbumin (OVA) as model protein and p24 as a relevant HIV antigen. In separate in vivo studies, female BALB/c mice were immunized by subcutaneous (s.c.) injection with NP–OVA and NP–p24 formulations along with several control formulations. These results suggested that with multiple antigens, covalent attachment of the antigen to the NP significantly enhanced antigen-specific immune responses. This facile covalent conjugation and incorporation method may be utilized to further incorporate other protein antigens, even multiple antigens, into an enhanced vaccine delivery system.

1. Introduction

Recent vaccine development efforts have moved away from traditional vaccines containing whole organisms and towards safer subunit vaccines containing only the components, such as protein, peptide or plasmid DNA, necessary to elicit useful immune responses. However, these new generation vaccines typically have lower immunogenicity and higher cost. Thus, novel formulations comprising adjuvants or a delivery system are needed to improve immunogenicity, reduce antigen dose, and reduce cost (Gupta and Siber, 1995; Hagmann, 2000; Yan and Huang, 2007). For a successful peptide or protein antigen delivery system to effectively induce humoral- and cell-mediated immune responses, an adjuvant or delivery system is essential since peptides or the protein itself is usually a weak immunogen (Guinn et al., 2007). However, despite an intensive search for newer and better adjuvants, there are relatively few approved adjuvants (e.g., Alum, MF59, ASO_4) for human use (O'Hagan and De Gregorio, 2009; O'Hagan et al., 2006; Gabutti et al., 2005; Pichichero, 2008).

Several groups have reported on the use of particles to deliver protein antigens more effectively to affect the breadth and depth of immune responses (Yoshikawa et al., 2008; Cui and Huang, 2005; Elamanchili et al., 2007; Patel et al., 2006; Cui and Mumper, 2003; van den Berg et al., 2010; Aline et al., 2009; Salman et al., 2009; Akagi et al., 2007; Allison and Gregoriadis, 1974; Alving, 1991). In addition, other groups have shown that the direct covalent or high-affinity conjugation of protein antigen to particles further enhances immune responses (Patel et al., 2007b; Sloat et al., 2010a,b; Dominguez and Lustgarten, 2010).

To this end, our laboratory has reported on the engineering of solid lipid-based nanoparticles (NPs) from oil-in-water microemulsion precursors and the use of these NPs for enhancing immune

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responses to DNA vaccines (Cui and Mumper, 2002b,c), cationized β-galactosidase protein (Cui and Mumper, 2002a), and HIV Tat and Gag p24 proteins (Cui et al., 2004; Patel et al., 2006; Patel et al., 2007a). These NPs are approximately 100 nm in size and have the advantage of being engineered in a single step, one vessel process. The physical and surface properties of the engineered NPs may be modified by utilizing surfactants with various charges (i.e., neutral, anionic, or cationic) or reactive groups (i.e., sulfhydryl, amine, carboxyl). Moreover, the oil phase for the engineering of these solid lipid NPs comprises cetyl alcohol and polysorbate 60; both of which are found in many pharmaceutical products and are biocompatible (Koziara et al., 2005). It was hypothesized that increasing the interaction of the antigen for the particles could lead to increased accumulation of the antigen in the antigen presenting cells (APCs) as compared to antigen coated on the surface of charged NPs and thus, enable greater enhancement in immune responses in vivo. Indeed, his-tagged protein antigen was able to bind NPs decorated with a small amount of accessible nickel on the surface, leading to enhanced immune responses (Patel et al., 2007b). Apparently, the interaction between chelated nickel and the short sequence of histidine residues increased the delivery efficiency of the protein antigen to APCs.

Recently, the hydroxyl group of poly(ethyleneglycol) (PEG)–sterol was used to conjugate a protein via tresyl chemistry and the protein conjugate was successfully incorporated into a liposome (Steenpass et al., 2006). It was hypothesized that similar chemistry could be used to conjugate a protein antigen to NPs engineered with Brij 78 (polyoxyethylene-20 stearyl ether) and Brij 700 (polyoxyethylene-100 stearyl ether) as the surfactants. The present studies were aimed at investigating the engineering of NPs incorporated with protein conjugated surfactant in NPs. In addition, the utility of these NPs for enhancing the immune responses to protein-based vaccines was evaluated in vivo using OVA as model protein and p24 as the relevant HIV antigen.

2. Materials and methods

2.1. Materials

Emulsifying wax (E-wax), and aluminum hydroxide were purchased from Spectrum Quality Products (New Brunswick, NJ). PBS/Tween 20 buffer, ovalbumin (OVA), horseradish peroxidase (HRP), cetyl trimethylammonium bromide (CTAB), tresyl chloride, and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). HIV p24 was purchased from ARP American Research Products Inc. (MA, USA). Brij 78 and Brij 700 were purchased from Uniqema (New Castle, DE) and Sigma–Aldrich (USA), respectively. Goat anti-mouse IgG and peroxidase-linked species specific $F(ab')_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Tetramethylbenzidine (TMB) Substrate Kit was purchased from Pierce (Rockford, IL). Complete Freund's adjuvant (CFA) and incomplete freunds adjuvant (IFA) were purchased from Fisher Scientific (Hampton, NH).

2.2. Synthesis of tresylated Brij 78 (T-Brij 78) and tresylated Brij 700 (T-Brij 700)

The tresylation of Brij 78 was performed as described previously (Nilsson and Mosbach, 1984). Briefly, Brij 78 (1.15 g, 1 mmol) or Brij 700 (4.67 g, 1 mmol) (Fig. 1a) was dissolved in 10 ml dichloromethane and incubated at 0 °C. Tresyl chloride (120 μl, 2 mmol) (Fig. 1b) and 250 μl pyridine were added to the Brij 78 solution drop-wise. The solution was mixed and the reaction was allowed to continue at room temperature with constant stirring for 18 h under nitrogen atmosphere, after which the solution was rotary evaporated under reduced pressure and the precipitate was re-dissolved in 60 ml ethanol with 250 μl HCl. The solution was kept overnight at −20 °C and then the formed precipitate was centrifuged at 2500 rpm at −5 °C. The supernatant was discarded and the pellet was re-suspended in 50 ml ethanol acidified with 50 μl HCl. The washing steps were repeated until pyridine was removed as verified by a minimum and constant OD at 255 nm. The solid T-Brij 78 or T-Brij 700 (Fig. 1c) was stored at −20 °C under nitrogen. Similarly, Brij 700 (4.67 g, 1 mmol) was also tresylated.

2.3. Preparation of Brij 78–HRP conjugation & preparation and characterization of NP–HRP

A simple standard coupling procedure was established with HRP as a model protein to prepare the Brij 78–HRP conjugate (Steenpass et al., 2006). HRP (100 μl of a 200 μg/ml in 0.1 M HEPES buffer, pH 8.0) was added to T-Brij 78 (8.0 mg) and allowed to react for 3 h at room temperature. The reaction mixture was then heated to 55 °C and added to 4 mg of melted E-wax. The mixture was stirred for 3 min, after which 2 ml de-ionized water was added under stirring to form the warm oil-in-water microemulsion which was allowed to stir for an additional 30 min followed by direct cooling to room temperature while stirring to obtain solid lipid NPs. The engineered (200 μl) NPs were passed down a gravity packed sepharose CL4B gel permeation chromatography column (15 mm × 80 mm) using PBS buffer, pH 7.4 as the mobile phase. Fractions (1 ml) were collected and the fractions containing the NPs were determined by measuring the particle size intensity. The fractions were diluted 200-fold, and 10 μl samples were assayed for HRP enzyme activity

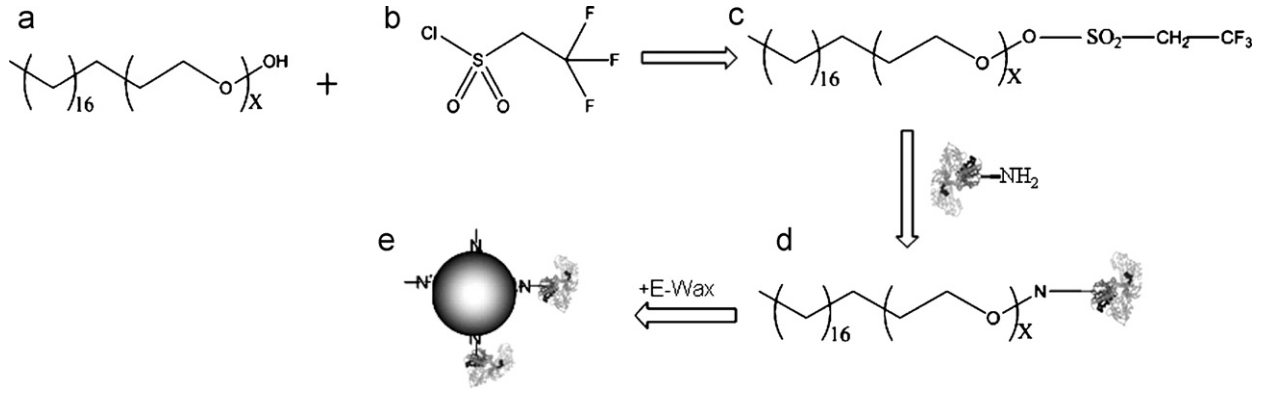

Fig. 1. Synthesis and engineering scheme for NP–protein formulation. Functionalization of Brij 78 ($X = 20$) and Brij 700 ($X = 100$) (a) by tresyl chloride (b). The resulting T-Brij conjugate (c) was reacted with protein to form Brij 78–protein or Brij 700–protein conjugate (d). The NP–protein (e) was engineered via warm oil-in-water microemulsion precursors using E-wax as oil phase and the synthesized protein conjugate (d) as the surfactant.

using 90 μl TMB as a substrate. The color development was stopped by addition of 50 μl of 2 M H_2SO_4 and the OD at 450 nm was read using a Universal Microplate Reader (Bio-Tek Instruments, Inc.).

2.4. Preparation and characterization of Brij 78–OVA conjugate and engineering of NPs

As described in Section 2.3 with slight modification, 100 μg of OVA or p24 was reacted with different weight ratios of Brij 78 and Brij 700, respectively, in 0.1 M HEPES buffer, pH 8.0 at room temperature for 18 h. OVA protein (30 μg total) was subjected to native SDS-PAGE gel. The gel was then stained by coomassie blue and imaged using a digital camera.

Eight (8) mg of T-Brij 78 was reacted with 1.5 mg OVA in 100 μl 0.1 M HEPES buffer, pH 8.0 at room temperature for 18 h to prepare Brij 78–OVA conjugate. The resulting conjugate mixture (Fig. 1d) was heated to 55–60 °C and mixed with 4 mg of melted E-wax. The mixture was stirred for 3 min, after which 2 ml de-ionized water was added under stirring to form the warm oil-in-water microemulsion which was allowed to stir for an additional 30 min followed by direct cooling to room temperature, while stirring to obtain solid NPs. The un-reacted and un-hydrolyzed tresyl group on the surface of the NPs was blocked using 20 mM Tris–HCl buffer, pH 8.0 for 1 h. The 1 ml NP suspension was concentrated to 0.2 ml using 100 kDa cut-off microcon (Millipore), and then subjected to GPC column as described in Section 2.3. Every fraction was assayed for protein concentration using the BCA method (Pierce). Nanoparticles (NP–OVA and NP–p24) were engineered and characterized by measuring their size using the particle sizer at 90° and zeta-potential (surface charge) using a Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

2.5. Engineering of cationic NPs and coating the OVA

Cationic NPs from oil-in-water microemulsion precursors were engineered as previously described with slight modifications (Patel et al., 2007a). Briefly, 2 mg of E-wax and 3.5 mg of Brij 78 was melted and mixed at ~65 °C. De-ionized water (980 μl) was added to the melted wax and surfactant while stirring to form an opaque suspension. Finally, 20 μl of cetyl trimethylammonium bromide (CTAB) (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 SDS (1 mM). OVA protein was added to NPs (1000 μg/ml) in 5% (v/v) mannitol at a ratio of 10:1 (weight ratio of E-wax:protein). The suspension was gently vortexed and placed on a horizontal shaker at room temperature for a minimum of 30 min to allow for coating. These coated NPs (NP/OVA) were diluted appropriately in de-ionized water for particle size and zeta potential measurements.

2.6. Mouse immunization study

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 14 with NP–OVA, NP/OVA, Brij 78–OVA and OVA adjuvanted with Alum (OVA + Alum). The doses of OVA and Alum in every group were kept as 5 and 100 μg, respectively, per mouse. As a positive control, mice were immunized on day 0 with 5 μg of OVA adjuvanted with complete Freund's adjuvant (CFA) followed by a boost with 5 μg OVA adjuvanted with incomplete Freund's adjuvant (IFA) on day 14. On day 28, mice were bled by cardiac puncture and sera were separated. All sera collected were stored at −20 °C for further characterization.

2.7. Determination of anti-OVA and anti-p24 antibody titers

OVA specific serum IgG, IgG_1 and IgG_{2a} antibody titers were determined using an ELISA as described previously (Patel et al., 2007a). Briefly, 96-well plates (Costar) were coated with 100 μl of OVA (10 μg/ml in 0.01 M carbonate buffer, pH 9.6) overnight at 4 °C. The plates were blocked for 1 h 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 2 h 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 1 h at 37 °C. For IgG_1 and IgG_{2a} determination, the plates were similarly incubated with goat anti-mouse IgG_1-HRP or goat anti-mouse IgG_{2a}-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 incubated for 10 min at RT. The color development was stopped by the addition of 50 μl of 2 M 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 taken from naïve mice. The same procedure was followed for the determination of p24 specific serum IgG antibody titers, but with slight modifications.

2.8. Statistical analysis

Statistical analysis was performed using either one-way analysis of variances (ANOVA) followed by pair-wise comparisons using Tukey's multiple comparison test using GraphPad Prism software or Student's *t*-test. Data were considered statistically significant when p value was less than 0.05.

3. Results and discussion

3.1. Incorporation of HRP into NPs

The surfactant–protein conjugate approach was utilized to attach proteins to NPs. The more conventional method of attaching HRP directly onto pre-made NPs was not successful since it required functionalization of hydroxyl group of Brij 78 on the surface of NPs via tresyl chemistry. When the HRP was reacted with the engineered NPs; HRP enzymatic activity was not detected in the NP fractions, suggesting no HRP conjugation to NPs (data not shown). There were two possible reasons; (1) hydrolysis of tresyl groups on Brij 78 during the process of NP engineering, leading to de-functionalization of the NPs, and (2) steric hindrance of the NPs and protein, both of which prevented the amine group of the protein from reacting with the Brij 78 on the surface of the NPs. To avoid these problems, Brij 78 was first covalently attached to HRP at pH 8.0 resulting in the stable synthesis of the Brij 78–HRP conjugate (Fig. 1d), which was then incorporated into the solid lipid NPs which were successfully engineered via the oil-in-water microemulsion precursor method. These engineered NPs were then fractionated using a GPC column, followed by NP characterization and utilizing an enzymatic assay to quantify retained HRP activity. Particle intensity indicated that NPs were distributed in fractions 4 and 5, which were associated with HRP activity when Brij 78 was reacted with HRP. A control of HRP coated on non-tresyl modified Brij 78 NPs showed no HRP activity in the NP fractions 4 and 5 (Fig. 2), proving that little or no non-specific binding of HRP to the NPs made with non-tresylated Brij 78. Fig. 2 also indicates about 30% of the enzymatic activity was associated with NPs made with Brij 78. These data indicate HRP model protein was able to be conjugated to Brij 78 and subsequently incorporated into NPs as depicted in Fig. 1.

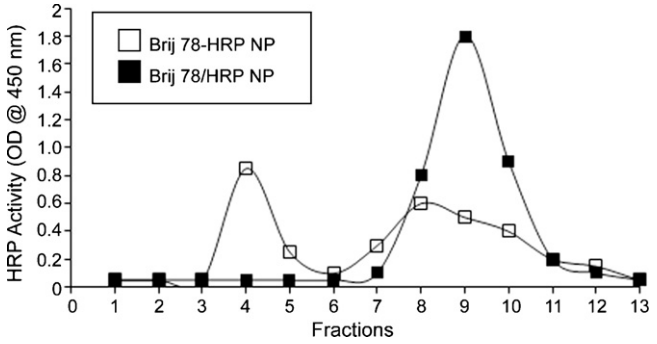

Fig. 2. HRP enzymatic activity associated with NPs made using Brij 78 (■) and T-Brij 78 (□).

3.2. Conjugation of OVA to Brij 78 and incorporation of Brij 78–OVA to NPs

The retention of significant HRP enzymatic activity on the NPs suggested that the tresylation method could be used to formulate protein antigen coupled NPs. However, it did not provide any quantitative information about the amount of protein attached to the NPs. Therefore, the widely used model antigen OVA was utilized to study and optimize the conjugation of OVA to Brij 78, with subsequent incorporation of the conjugate into NPs. Different weight ratios of OVA to Brij 78 were designed to optimize the conjugation conditions. The moderate basic condition of pH 8.0 which significantly reduced the hydrolysis of tresyl groups and at the same time prevented the protonation of primary amines of protein, resulted in high overall conjugation efficiency. The reaction mixtures were then subjected to native SDS-PAGE (Fig. 3a).

The position of a protein on a native gel is influenced not only by the size of the protein, but also by its charge and aggregation status. The Brij 78 portion of the synthesized protein conjugate contains a hydrophobic stearyl moiety which may lead to aggregation of the protein to form oligomers or multimers. Fig. 3a shows that as the amount of T-Brij 78 to OVA was increased, the amount of free OVA gradually decreased and the amount of Brij 78–OVA conjugate was increased. There was no significant difference in staining of SDS-PAGE bands between the weight ratios of 10:1 and 5:1 T-Brij 78:OVA conjugate suggesting the maximal conjugation efficiency was achieved at weight ratio of about 5:1. Therefore, this weight ratio was used in subsequent engineered NPs. According to the molecular weights of OVA and Brij 78, there was about a 160-fold molar excess of Brij 78 in the reaction mixture. Regardless, the unreacted Brij 78 was not removed, but directly utilized together with Brij 78–OVA conjugate and E-wax to form the oil-in-water microemulsion precursor which was then used to form solid NPs since the total Brij 78 (unreacted Brij 78 and the Brij 78–OVA) was precisely the amount of Brij 78 required to engineer the E-wax NPs. Thus, the solid NPs were formed using a combination of Brij 78 and T-Brij 78 that was equivalent to the amount of Brij 78 alone required to form placebo or blank NPs. The formed NPs were fractionalized using a GPC column. The total protein concentrations in the fractions were assayed by the BCA method and are summarized in Fig. 3b. Similar to the results in Fig. 2, the OVA protein was successfully attached to NPs as evidenced by fractions 4 and 5. The NP-associated OVA was around 30% of the total protein input in the initial conjugation reaction as determined by the protein assay. NPs were characterized by both particle size and zeta potential and the data are summarized in Table 1.

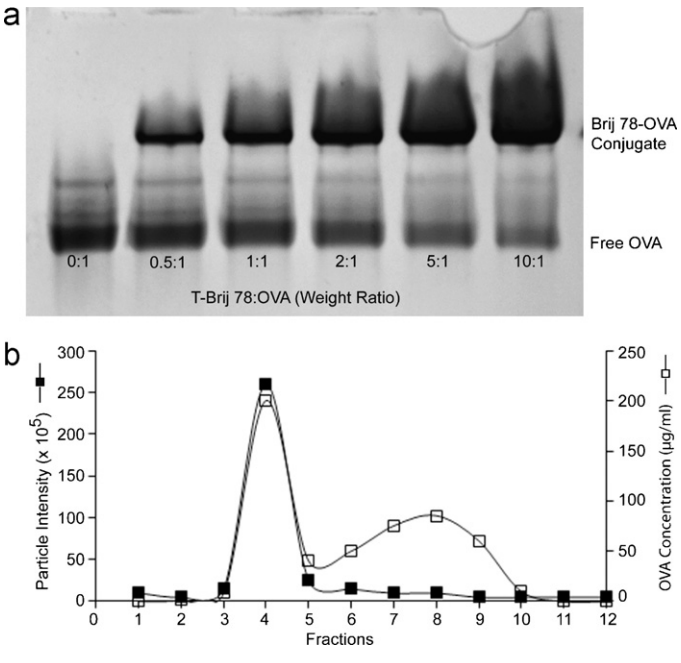

Fig. 3. (A) Conjugation of OVA protein to T-Brij 78: different weight ratios of T-Brij 78:OVA were used to conjugate OVA to T-Brij 78, followed by native PAGE gel separation. Thirty micrograms of OVA was loaded onto the gel and stained by coomassie blue and imaged using a digital camera. (B) The GPC purification profile for particle intensity and protein concentration of OVA associated with NPs.

Table 1
Physical properties of NP–OVA, NP/OVA and blank NPs.

Nanoparticles (NPs)	Mean diameter (nm) ($n=5$)	Mean PI ($n=5$)	Mean charge (mV) ($n=5$)
NP/OVA[a]	111.2 ± 13.2	0.199 ± 0.069	+25.6 ± 4.4
NP–OVA[b]	81.8 ± 1.0	0.270 ± 0.065	−19.3 ± 0.7
NPs[c]	82.8 ± 3.5	0.120 ± 0.099	−3.2 ± 0.3

[a] Cationic NPs engineered with E-wax, Brij 78 and 1 mM CTAB were coated with OVA at a final weight ratio of 10:1 (w/w) (E-wax:OVA).
[b] Purified NPs conjugated with OVA.
[a,b]OVA concentration was 0.1 mg/ml formulation of NPs.
[c] Blank NPs made with E-wax and Brij 78.

Fig. 3b. Similar to the results in Fig. 2, the OVA protein was successfully attached to NPs as evidenced by fractions 4 and 5. The NP-associated OVA was around 30% of the total protein input in the initial conjugation reaction as determined by the protein assay. NPs were characterized by both particle size and zeta potential and the data are summarized in Table 1.

The NP–OVA had similar particle size and PI values compared to blank NPs formed by E-wax and Brij 78 alone, but had a more negative zeta potential due to negative charged OVA protein on the surface of NPs. However, both NP–OVA and blank NPs had smaller sizes than NP/OVA complex. The NP–OVA formulation, filtered through a 0.22 μm filter, was stable for at least one week at 4 °C. It should be noted that no formal stability study of the formulations was performed.

3.3. Immune response to NP–OVA formulation

In vivo studies in BALB/c mice were carried out to investigate the use of NP–OVA as a potential delivery system to enhance the immune responses to OVA. Mice were immunized with NP–OVA, cationic NP/OVA complex (NP/OVA), OVA adjuvanted with Alum (Alum + OVA), Brij 78–OVA conjugate on day 0 and 14. Our previous results showed that at a neutral pH, negatively charged OVA efficiently bound to cationic NPs made by the addition of CTAB as a co-surfactant and formed a stable NP/OVA complex (Patel et al., 2007a). As a positive control, mice were immunized with OVA adjuvanted with CFA (OVA + CFA) using the same immunization protocol. The OVA antigen dose among all the groups was 5 μg per mouse. Sera collected from mice were analyzed for total OVA-specific IgG at 14 days post second immunization. As expected, animals immunized with OVA adjuvanted with CFA + IFA resulted in the highest total IgG titers among all the groups. More importantly, 5–10-fold greater titers were observed using NP–OVA

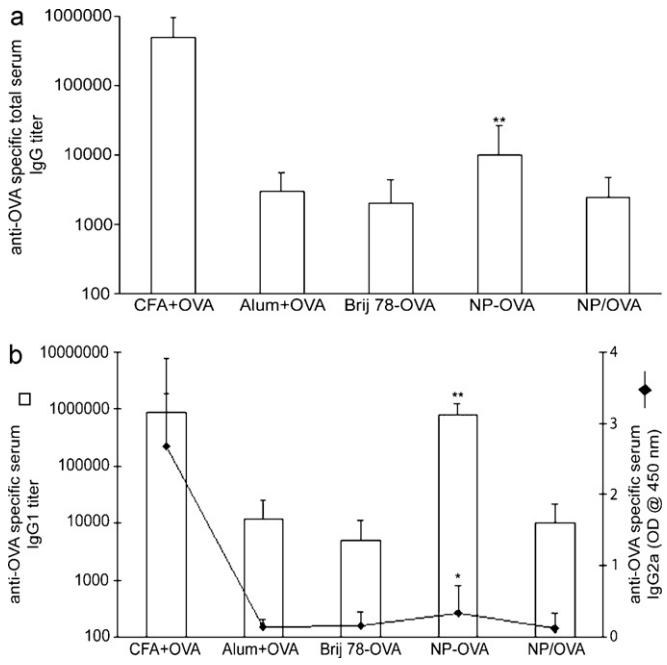

Fig. 4. OVA specific humoral responses after immunization with NP–OVA formulations. Mice were immunized with 5 μg of OVA covalently bound to NPs (50 μg) (NP–OVA), coated on cationic NPs (50 μg) (NP/OVA), adjuvanted with Alum (50 μg) (Alum + OVA), Brij 78–OVA conjugate or adjuvanted with CFA on day 0 and day 14. Total anti-OVA serum IgG levels (A) and IgG_1 (B) were measured on day 28. The data for each group represent the mean ± SD ($n = 8$). $^{**}p < 0.01$ compared to NP/OVA, Alum + OVA and Brij 78–OVA. Serum IgG_{2a} levels were measured on day 28 by ELISA at a 1:100 serum dilution. The data for each group represent the mean ± SD ($n = 6$–8). $^{*}p < 0.05$ compared to NP/OVA, Alum + OVA and Brij 78–OVA.

compared to Alum + OVA, or Brij 78–OVA, or NP/OVA (Fig. 4a). The Brij 78–OVA conjugate control had similar antibody response with Alum + OVA suggesting the protein conjugation itself increased the immunogenicity of the OVA as presented in Fig. 4a. Our previous studies reported anionic NP/Tat complex enhanced humoral immune responses compared with Tat adjuvanted with Alum (Cui et al., 2004; Patel et al., 2006); however, current studies indicated no difference between the cationic NP/OVA complex and Alum + OVA. It has been shown that cationic surfactants are more toxic than anionic and neutral surfactants at the same concentration (Lewis, 1990). Recently, it was reported that high doses of cationic liposome induced apoptosis of dendritic cells and lead to compromised immune responses (Yan et al., 2008). Therefore, the cationic NP-induced dendritic cell apoptosis may explain the weaker adjuvant activity of cationic NPs compared to the anionic NPs. However, the toxicity profiles of the cationic NPs and anionic NPs require further systematic investigation. Overall, these data support that protein covalently bound to NPs was superior to Alum and NP/OVA complexes in enhancing humoral immune responses to model antigen OVA.

The serum isotype levels, IgG_1 versus IgG_{2a}, were measured to assess the type of immune response generated (i.e., Th2 or Th1, respectively). During an immune response, the release of Th1 or Th2 cytokines will affect the production of these antibodies. BALB/c mice bias the immune response towards a Th2 profile (Guler et al., 1997; Bix et al., 1998) generating relatively higher levels of antibodies of IgG_1 isotype; however, in the presence of Th1 type immune responses, the cells produce Th1 cytokines which cause a switch in the isotype produced to IgG_{2a}. The isotype analysis in these studies revealed that the NP–OVA resulted in superior IgG_1 (Fig. 4b) and IgG_{2a} (Fig. 4b) levels compared to NP/OVA, Alum + OVA and Brij 78–OVA, suggesting enhanced and balanced Th1 and Th2 immune responses can be generated by NP–OVA formulation.

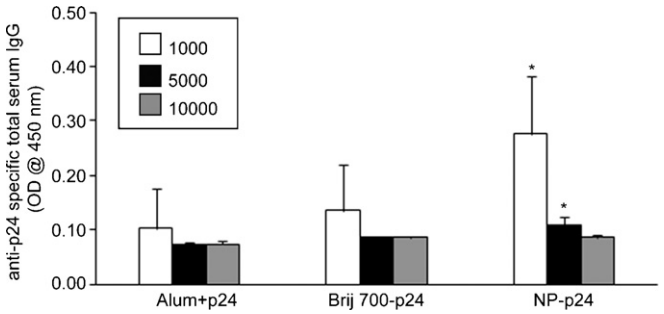

Fig. 5. p24 specific humoral responses after immunization with NP–p24 formulations. Mice were immunized with 5 μg of p24 coated to NPs (50 μg) (NP–p24), adjuvanted with Alum (100 μg) (Alum + p24), Brij 700–p24 conjugate on day 0 and day 7. Total anti-p24 serum IgG levels were measured on day 25 by ELISA at 1:1000, 1:5000, and 1:10000 serum dilutions. $^{*}p < 0.05$ compared to Alum + p24 and Brij 700–p24. The data for each group represent the mean ± SD ($n = 3$).

Human immunodeficiency virus type-1 (HIV-1) capsid protein p24 is the main component of tubular core in the mature HIV particle (Marechal et al., 1998). Studies have found p24 to be relatively conserved among HIV subtypes. Decrease in the level of antibodies to p24 and p24 antigen has been considered to be one of the most useful prognostic markers in HIV infected individuals (Bollinger et al., 1992). Therefore, most assays have been developed to detect HIV antibodies which use p24 as an integral component in addition to envelope glycoproteins. Inhibition of capsid formation can be an effective prophylactic or therapeutic strategy (Tang et al., 2003). Thus, p24 is a rationale target antigen.

Therefore, to assess this proof of concept, Brij 700–p24 antigen conjugate was prepared, as described above, and coated onto the NPs and total IgG was assessed (Fig. 5). The total IgG for NP–p24 was found to be increased as compared to Brij 700–p24 conjugate and p24 adjuvanted with Alum. Brij 700 was utilized in later studies using p24 since other studies have shown that the longer chain polyoxyethylene chain improves both the conjugation efficiency and loading onto nanoparticles due to reduced steric hindrance near the surface of the nanoparticles (unpublished results). These data demonstrate the potential applications of these NPs for vaccine delivery and warrant further investigation of these systems for enhancing immune responses with HIV protein antigen based vaccines.

4. Conclusions

Covalent conjugation of protein antigen to NPs through tresyl chemistry is a novel approach to increase the immunogenicity of the poorly immunogenic proteins. This facile covalent conjugation and incorporation method may be utilized to incorporate other peptide and protein antigens, even multiple antigens, into an enhanced vaccine delivery system.

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