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Synthesis and Characterization of Gold Nanoparticle Conjugated Recombinant Aprv2 of *Dichelobactor nodosus* and its Immunogenicity

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Abstract

The present study involves the synthesis, characterization and immunogenicity of Gold Nanoparticle (GNP) conjugated recombinant AprV2 protein of *Dichelobactor nodosus*. To achieve this, GNPs of size 20 nm were prepared and characterized. The gold nanoparticles effectively adsorbed 3.3 µg of recombinant AprV2 protein over the surface. TEM studies revealed corona of AprV2 over GNPs (1-2 nm thick) in GNPs-AprV2 conjugate. Further, GNP-AprV2 conjugate revealed a red shift from 523 nm to 532 nm, indicating increased size of GNPs after conjugation with AprV2. To evaluate the immunogenic response, mice were allotted to 5 different groups. Mice in group I injected with GNP-AprV2 conjugate, group II with GNP-AprV2 with MPLA (Monophosphoryl lipid A), group III with AprV2 on its own, group IV with GNPs and group V with Phosphate Buffered Saline (PBS). The highest antibody responses against the AprV2 were recorded in pooled sera from mice of group II compared to group me and III on day 28. Among all groups, the group II mice vaccinated with GNP-AprV2/MPLA exhibited the highest IgG levels. The third dose of vaccine formulations did not enhance antibody levels. This study is a first report studying the immunogenic effect of conjugation of GNPs with rAprV2 of *D. nodosus*.

Keywords: Foot rot • Nano-vaccine • Antibodies • Gold nanoparticles • AprV2 • *Dichelobactor nodosus*

Introduction

Vaccines have saved millions of humans and animals from deadly diseases [1]. Advances in omic approaches have facilitated therapeutic strategies including vaccine development by clarifying immunological and pathological mechanisms. Nanotechnology is a promising way for synthesizing vaccines using nanoparticles as adjuvants or nanocarriers to protect against deadly pathogens causing various diseases [2]. Nanoparticles improve stability and functionality of antigens, facilitate transport of antigen and adjuvant to specific antigen-presenting cells to control immune tolerance and also help in targeted delivery. Gold Nanoparticles (GNPs) due to their smaller size, high surface to volume ratio, ability to cross cellular components can circulate longer or even achieve a targeted action by surface modification for improving immunogenicity of antigens serve as traditional delivery vehicles [3].

GNPs can be fabricated with biomolecules including proteins forming bio-corona because of high surface area to volume ratio and high versatility. Immunologically, GNPs allow efficient delivery of antigen to target cells and facilitate cross-presentation and cytotoxic T-lymphocyte responses [4]. Keeping in view the importance of nanovaccines, the present study was focused on development and evaluation of a gold nanoparticle conjugated recombinant AprV2 protein so as to further explore its potential as a vaccine against foot rot. Recombinant and purified AprV2 was obtained from *E. coli* Rosetta-gami (de3) plyss harbouring pet-22b. Adsorption/desorption profiling of GNP-AprV2 conjugate and the potential vaccine was used for immunogenicity. Vaccines against foot rot disease have evolved from whole cell vaccines to recombinant vaccines but successful studies are limited as the protection is serogroup-specific which renders animal susceptible for infection by other serogroups [5]. On the other hand, multivalent vaccines suffer from the disadvantage of antigenic competition. The ability of *dichelobacter nodosus* to

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undergo serogroup conversion, the need arises for heterologous protection against all serogroups with a better adjuvant and delivery vehicle for effective vaccination against foot rot [6].

Materials and Methods

Recombinant protein AprV2

The recombinant AprV2 cloned and preserved in *E. coli* Rosetta-gami (DE3) plyss was revived from the glycerol stock previously stored at department of veterinary microbiology, Dr. G.C. Negi college of veterinary and animal sciences, Palampur [7]. The purification of recombinant AprV2 was done based upon previous publication. The expression and purification of recombinant AprV2 was again optimized and extracted from periplasm instead of cytoplasmic fraction. Histrap ff crude Ni-NTA (Nickel-Nitrilotriacetic Acid) column (GE healthcare, USA) was used to purify the recombinant protein [8].

Synthesis and characterization of nanoparticles

Gold nano particles were synthesized by citrate reduction method. The reaction involved 1.25 ml of 20 mm hydrogen tetrachloroaurate (III) hydrate $HAuCl_4.3H_2O$ mixed with 48.75 ml deionised water. The mixture was placed over magnetic stirrer with constant heating at 150°C and vigorous shaking. Further, 1.75 ml of 1% trisodium citrate dihydrate was added quickly as soon as solution started to boil. Color of the solution gradually developed into black followed by ruby red indicating reduction of gold (III) into colloidal gold (0) by citrate. Once ruby red color was visible, magnetic stirrer was turned off and colloidal Gold Nanoparticles (GNPs), thus formed were left at room temperature for overnight. Buffer system was optimized in which GNPs and AprV2 both would remain stable. One ml of colloidal GNPs was centrifuged for 10 minutes at 10,000 rpm [9]. Supernatant was removed and pellet was resuspended in test buffers. Development of violet color indicated irreversible loss of colloidal GNPs while pink color indicated stable GNPs in test buffer.

The nanoparticles were synthesized by DLS corresponding Surface Plasmon Resonance (SPR) band was measured by UV-Vis spectroscopy (Shimadzu, Japan) and transmission electron microscopy was performed for size confirmation [10].

Preparation of AprV2-GNPs conjugates

AprV2-GNPs conjugate was prepared by centrifuging 2 ml colloidal GNPs for 10 minutes at 10,000 rpm. Colorless supernatant was exchanged with 1 ml borate buffer (ph 7.4) and pellet was dissolved in it. One ml of purified AprV2 (10 µg/ml in 0.1 m borate buffer, ph 7.4) was added to colloidal GNPs dropwise by continuous mixing and incubated at room temperature for 1 h followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant thus obtained was kept at 4°C and the pellet was resuspended in 1 ml 0.1 m borate buffer (ph 7.4). Concentration of rAprV2 was quantified and optimized until the actual concentration of rAprV2 adsorbed over the surface of GNPs became ~10 µg. AprV2-GNP conjugate was characterized by transmission electron microscopy for the possible increase in size and AprV2 corona over the surface of GNPs. The shift in corresponding Surface Plasmon Resonance (SPR) band was measured by UV-Vis spectroscopy. Fluorescence quenching of tryptophan moiety of AprV2 by GNPs was also observed (RF-6000 spectrophotometer, Shimadzu, Japan) [11].

Quantification of AprV2 adsorbed over the surface of GNPs/ protein release assay

Concentration of AprV2 adsorbed over the surface of GNPs was measured with quick start bradford protein assay, kit. To get actual concentration of AprV2 adsorbed over GNPs, the concentration of AprV2 in supernatant was subtracted from the initial concentration of AprV2 (5 µg/ml).

Animals

For experimental studies, 6 weeks-8 weeks old 50 female balb/c mice procured from CSIR-Institute of Himalayan Bioresource Technology (IHBT) were maintained in experimental animal house of department of veterinary microbiology, Dr G.C. Negi college of veterinary sciences, Palampur, H.P. The experimental animal room was thoroughly cleaned, disinfected and then fumigated three days prior to experiment initiation. Cages and water containers were also washed thoroughly with water followed by potassium permanganate solution. Before experimentation, mice were examined for any abnormality and signs of ill health. They were housed in cage system and were provided water and feed ad libitum. Mice were maintained for four days for the adaptation before the start of experiment [12].

Vaccine studies

Healthy mice were divided into groups which includes four treatments and one group with no treatment (control) (Table 1). The groups are as follows:

Group I (10 mice)	Group II (10 mice)	Group III (10 mice)	Group IV (10 mice)	Group V (10 mice)
GNP-AprV2 nano-vaccine	GNP-AprV2 with MPLA	AprV2 on its own	GNP	PBS (Sterilised)

Table 1. Experimental groups used for the evaluation of the immune response.

Animals in group I were immunized with nanovaccine *i.e.* rAprV2 conjugated with gold nanoparticles, group II with GNP-AprV2 with MPLA (5 µl of lyophilised MPLA (1 mg/ml) added to the GNP-rAprV2 before immunization), group III with AprV2 on its own (10 µg/100 µl 0.1 M borate buffer (ph 7.4) prepared from purified rAprV2 stock, group IV with GNPs only (2 ml colloidal gold centrifuged and suspended in 100 µl borate buffer) and group V administrated with 100 µl of autoclaved PBS per dose which serve as control.

All themice were immunized with a primary dose (day 0) followed by two booster doses on 15th and day 29th day post primary immunization. About 200 µL of blood sample was collected from each mouse on day 14, 28 and 45 by retro-orbital route under mild anaesthesia induced by isoflurane. The serum samples were used to determine the systemic immunoglobulin IgG levels using indirect elisa [13].

In vitro analysis of immunogenicity

Flat bottom ELISA plates were coated with AprV2 (5 µg/100 µl) by incubating in carbonate/bicarbonate buffer, ph 9.6 at 4°C. The plates were washed thrice with PBST (Phosphate Buffered Saline containing 0.05% Tween-20, ph 7.2). Blocking of the wells was done with 3% BSA in PBS (ph 7.2) for 2 h at 37°C, followed by three washes with PBST. Pooled serum samples obtained from each group on 14th, 28th and 45th day were added to each well in dilution of 1:8 with 1% BSA in PBST and then incubated for 1 h and 30 min at 37ºC. Further, plates were washed with 200 µl of PBST thrice and incubated with 100 µl of 1:10,000 diluted anti-mouse Igg hrpo conjugate antibody at 37ºC for 1 h and 30 min. Again, the plates were washed thrice with 200 µl of PBST and incubated in dark with 100 µl of O-Phenylenediamine Dihydrochloride (OPD) substrate for 30 min for color development. Reaction was stopped with 100 µl of 1 m H_2SO_4. Absorbance of color developed was measured using ELISA plate reader at 450 nm [14].

Statistical analysis

All statistical analyses were performed using online OPSTAT tool (IBM Corporation, Armonk, NY). Tukey's HSD post Hoc test was used to compare antibody titers between the non-immune and an indicated immunization group or between two indicated immunization groups. Where applicable, a one-way ANOVA was applied. P values of <0.05 were considered significant [15].

Permission for animal studies

The permission to carryout animal experiment studies was obtained from IAEC through communication number IAEC-259. All the experiments were performed in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC) of CSK HPKV, Palampur [16,17].

Results and Discussion

Confirmation of recombinant AprV2

The presence of AprV2 in the recombinant *E. coli* rosetta-gami (DE3) plyss harboring recombinant plasmid pet22b-AprV2 was confirmed by the release of 1.4 kb insert after restriction digestion. The expression of 37 kda in size was obtained in sds-page (Figure 1).

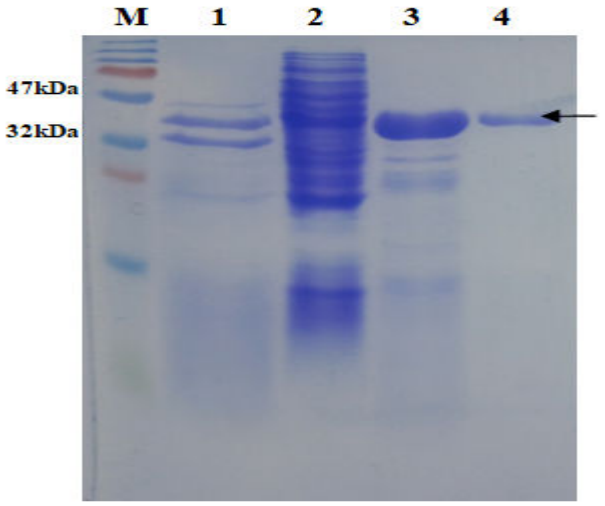

Figure 1. Affinity purification of the AprV2 using Ni NTA column: Lane M: Prestained marker, Lane 1: Induced, Lane 2: Wash, Lane 3: Elute 250 mm imidazole (fraction I), and Lane 4: Elute 250 mm imidazole (fraction II).

Synthesis and characteristic properties of gold nanoparticle conjugated AprV2 vaccine

Synthesis of GNPs was confirmed by strong absorption peak (λ_{max}) at 523 nm in UV-Vis absorption spectroscopy indicating average size ~20 nm similar to the reported studies based on absorption bands in UV-Vis and fluorescence measurements. For synthesizing gold nanoparticle mediated platform, size and shape imposes crucial impact in terms of antigen presentation, blood clearance, cellular uptake, biodistribution and immunological response. These nanoparticles were stable in borate buffer (ph 6.5 and 7.5) indicated by the wine-red color of the suspension. GNPs were monodispersed of average diameter 18 nm-20 nm with spherical shape. TEM studies revealed corona of 1 nm-2 nm thick AprV2 over GNPs as conjugate. Further characteristics of GNP-AprV2 conjugate revealed a red shift of 9 nm from 523 nm-532 nm indicating ~30 nm increased size of GNPs after conjugation with AprV2. The results confirmed the successful adsorption of AprV2 over GNPs. FTIR spectrum revealed a well-defined emission band of AprV2 (λ_{em}) at 350 nm while coating of GNPs to AprV2 caused a decrement in the fluorescence intensity. The quantification revealed 3.3 µg of AprV2 adsorbed over GNPs and confirming 81%conjugation efficacy of AprV2 over GNPs. Nanoparticle conjugated vaccines provide better stability in blood flow, avoiding need for booster doses, and facilitate active targeting. It is now an established fact that GNPs indeed have high proficiency for antigen delivery as they enhance the immunogenicity of that antigen (Figures 2-6) [18].

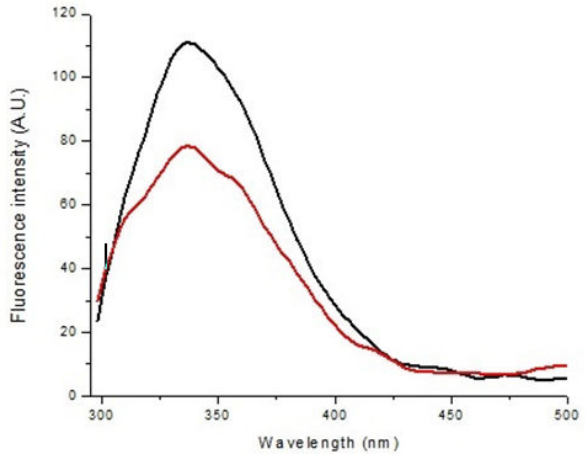

Figure 2. Quenching of AprV2 fluorescence in presence of GNPs, where F_0 and F are fluorescence intensities ofAprV2 in absence and in presence of GNPs.

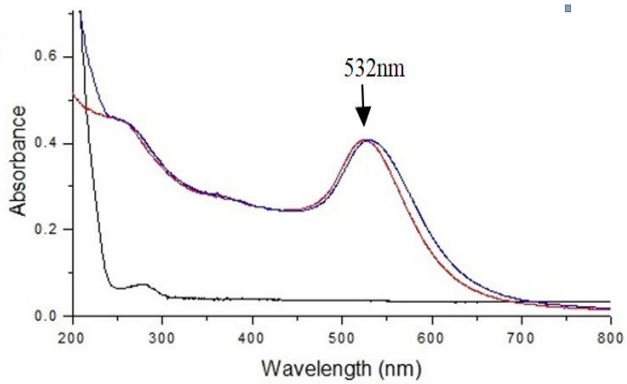

Figure 3. Characterization of AprV2-GNP conjugated nanovaccine absorption spectra of GNPs and AprV2-GNPs conjugate.

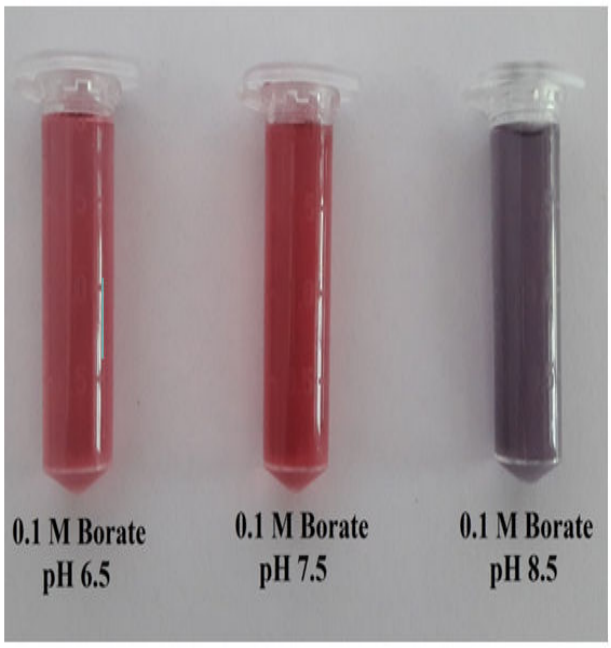

Figure 4. Preparation of stable GNPs in borate buffer (pH 6.5, 7.5).

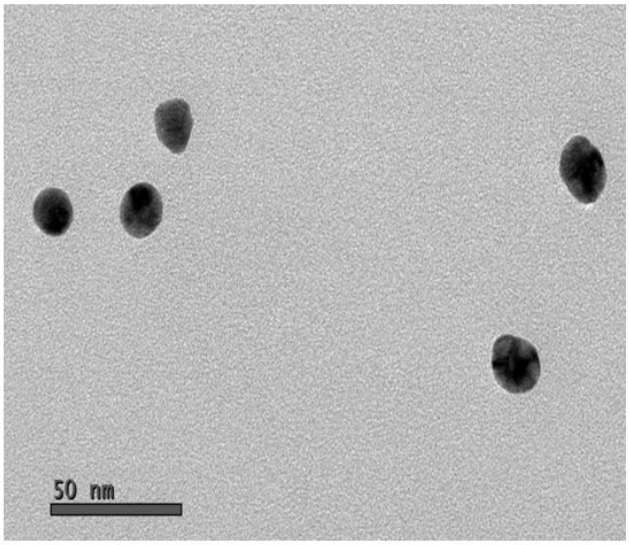

Figure 5. Representative transmission electron microscopy analysis of GNPs.

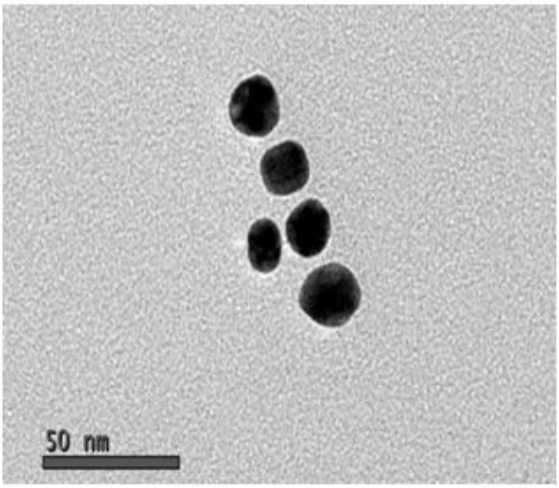

Figure 6. GNP-AprV2 conjugates at 50 nm.

Immune response against recombinant AprV2

In response to the first dose of vaccine delivered on day 0, group I vaccinated with GNP-AprV2, group II with GNP-AprV2 with MPLA and group III with AprV2 showed highest IgG level in comparison to group IV vaccinated with GNP alone and group V as control. After administration, group I and II exhibited significantly higher antibody response compared to group III on 14th day. Similarly, after the second dose administration, a steep rise in IgG levels was observed among groups I, II and III on 28th day. Amongst all, group I and II exhibited significantly higher antibody response compared to group III on 14th and 28th day. GNP-AprV2 nanovaccine could itself generate good immune response as nanoparticle conjugated with protein biomolecules could trigger enhanced cellular and humoral immune responses by expressing various pro-inflammatory cytokines. The uptake of GNPs by dendritic cells followed by the processing of peptides on their surface might resulted in elicitation of cell mediated immunity by stimulation of cytotoxic T-lymphocytes. Studies have also reported protective humoral response in respective animal model besides cell mediated immunity [19].

GNP-AprV2 with MPLA also showed significant immune response on 14th day and reached at peak on 28th day in comparison to group I and group III. GNP-AprV2 nanovaccine with MPLA (a potent TLR4 agonist and proven B-cell and T-cell activator) might have provided an additional advantage over GNPs and thus, best response was observed after its administration. In similar studies, GNP with advax and dio-1 (a TLR4 agonist) adjuvants, and GNPs conjugate with recombinant Pfs 25 surface antigen and Burkholderia thailandensis LPS conjugated to FliC has been reported for developing an effective T-cell vaccine malaria transmission-blocking antibodies and as vaccine for Glanders on rhesus macaques, respectively. Other studies include GNPs conjugated recombinant flagellin protein with Freund's adjuvant produced high levels of anti-flagellin antibodies by recognizing native flagellin of *Pseudomonas aeruginosa*. In another study, GNPs conjugated with proteins Lomw and escc provides protection by expressing high levels of Igg and Iga against diarrhea and hemorrhagic colitis caused by Enterohemorrhagic *E. coli* (EHEC) O_{157}:H_7. Hence, gold nanoparticle-based formulations as nanovaccines can be made for enhanced immunogenicity (Figures 7 and 8) [20].

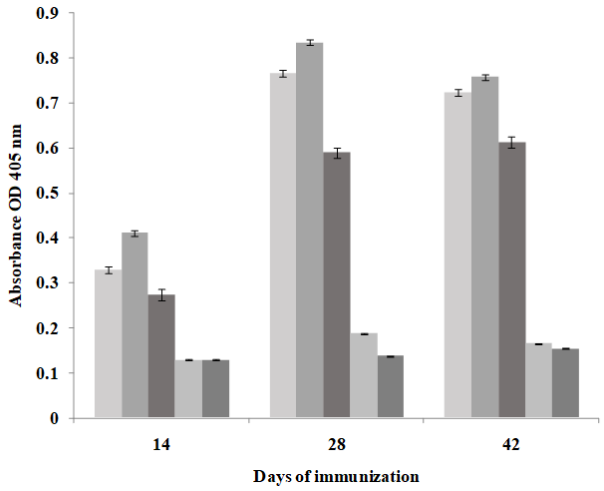

Figure 7. Enzyme Linked Immunosorbent Assay (ELISA): ELISA reactivity of antisera from individual animals immunized with GNP-AprV2 nanovaccine (Group I), GNP-AprV2 with MPLA (Group-II), AprV2 on its own (Group-III), GNPs on their own (Group-IV) and Control (Group-V) at 14, 28 and 45 days. The values are mean of duplicate values and level of significance calculated at CD 0.05%.

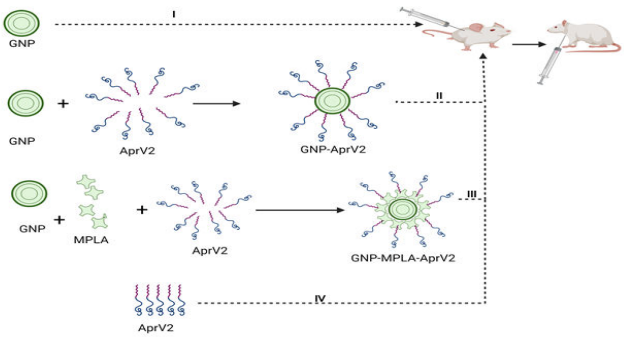

Figure 8. Schematic representation showing design of AprV2 conjugated nanovaccines, immunization in BALB/c mice *via* subcutaneous route, collection of blood samples *via* orbital sinus and generation of immune response after immunization [21].

Following administration of a booster/third dose, the vaccines failed to enhance or maintain the peak Igg levels. This might be due to the reason that second dose would already have resulted in the plateau immune response and any further booster would have no effect on antibody levels. This can be explained on the basis of either antigen saturation or formation of antigen-antibody complexes which may reduce antigen availability for B-cell binding and/or trigger negative feedback mechanisms for generation of antibody. The duration of antibody responses is proportional to the number of plasma cells generated during immunization. Vaccination schedule also controls persistence and magnitude of antibody. Primary doses are administered for rapid and persistent responses while doses administered at longer intervals reflects the generation of limited number of B-cells and thus, requires later boosting. But the selection of best vaccination schedule for a vaccine itself requires a complete study of immune response generated at various intervals between primary and booster doses.

Conclusion

GNPs with properties including high surface area and functionalization serve as excellent candidates for vaccine development. GNPs can pass through cellular compartments *via* endocytosis and affects immunological responses. Such nano-formulations could be practiced besides maintaining consistency, large-scale production, and reproducibility. To understand the interaction of nanoparticle with immune system, formation of bio-corona around GNP surfaces is important. In the current study, the response generated by GNP-AprV2 with MPLA showed significant immune response over other vaccine formulations with respect to immunogenicity in mice. Further investigations to elucidate generation of protective immune response using a target host would further strengthen this study and pave way for designing nano-conjugated vaccine.

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Conflicts of Interest

The authors declared no conflict of interests.

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