

Characterization and immunological evaluation of chitosan nanoparticles as adjuvants for bovine coronavirus N protein

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Abstract. Chitosan nanoparticles (CNP) loaded with nucleocapsid protein of bovine coronavirus (BCV N) were prepared by ionic cross-linking method using sodium tripolyphosphate (TPP) as cross-linking agent. CNP loaded with BCV N protein was intramuscularly administered into Balb/c mice. Serum levels of anti-N protein IgG, IgM, and IgA antibodies were dynamically monitored by indirect ELISA method. Results showed that in the BCV N-loaded chitosan group, both IgA and IgG levels were found to increase significantly after the second immunization comparable to those of the Montanide ISA 206 groups. The IgM content in serum increased after the second immunization in the BCV N protein-loaded CNP group. These findings indicate that CNP can be used as adjuvant in veterinary vaccine field.

1 Introduction

Chitosan, $\alpha(1\rightarrow4)$ -2-amino-2-deoxy- β -D-glucose (N-acetylglucosamine), is a deacetylated form of chitin and is gaining interest because it is biocompatible, biodegradable, non-toxic, and extremely moldable [1-4]. Chitosan also possesses many advantageous features, such as antibacterial, and antiviral activities, as well as the ability to promote tissue repair and stop bleeding [5,6]. Thus, chitosan has wide prospects in the field of biomedicine. For example, Han et al. [7] developed chitosan hydrogel for delivery of doxorubicin on the tumor tissue, which showed good anti-tumor effects.

Chitosan microspheres [8] and chitosan nanoparticle (CNP) [9] loaded with model drugs can attain good sustained release efficacy. In addition, adsorption of model drugs onto nanoparticle surface is also an option. Evidence exists that chitosan, as a novel adjuvant, can enhance antibody levels [10-12]. Therefore, chitosan shows a wide prospect of clinical applications. At present, the drugs enveloped by chitosan carriers include proteins [13,14], hydrophobic drugs [15], anti-cancer drugs [16], vaccines [17-19] and enzymes [20].

Bovine coronavirus (BCV) is one of the pathogens that lead to diarrheic disease of cattle [21]. The key to preventing diarrheic disease caused by BCV is to stimulate the formation of specific antibodies *in vivo*. A newborn calf can be passively immunized *via* colostrums and milk. The level of BCV antibody in the milk is highest during pregnancy and it is rapidly decreased thereafter. In newborn calves, the immunoglobulin adsorbed is secreted into the enteric cavity and adheres to the mucosa of the small intestine, which prolongs the time of passive immunization. Natural infection of BCV in the newborn calves can produce active immunization, but diarrheic disease occurs easily after the decreases of passive immunization and before the formation of active immunization. Therefore, how to prolong the residence time of antibodies *in vivo* is currently a challenge for veterinary medical scholars. Coronavirus nucleocapsid protein is the protein-nucleic acid complex of BCV. The nucleocapsid protein of BCV (BCV N protein) appears to be highly conservative in the carboxy-terminus and also exhibits strong immunogenicity, so it can serve as a diagnostic antigen for BCV.

In our previous study, the NO. 487-1287 bases at 3' side of N protein from bovine coronavirus DB2 strain were synthesized and expressed in *E. Coli* [17]. The present study prepared CNPs by an ionic cross-linking method using sodium tripolyphosphate (TPP) as a cross-linking agent, immunized BALB/c mice after BCV N protein adsorption to the CNP surface, and performed immunological evaluation by indirect ELISA. These results provide experimental foundation for animal vaccine research and clinical application.

2. Materials and methods

2.1. Materials and instruments

Chitosan (deacetylation degree = 94%, $M_v = 400\ 000$, purity $\geq 95\%$) was purchased from Sinopharm Chemical Reagent Co., Ltd., China. TPP, an analytical reagent, was purchased from Tianjin Dongli District Tianda Chemical instruments, Tianjin, China. BCV N protein ($MW=39.48\text{ku}$) was prepared as before with expression yield at 50.5%[17]. The Bradford protein assay kit (P1510) was purchased from Applygen Technologies Inc., China. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgA, and IgM antibodies were purchased from Sigma, Louis, MO, USA. The other reagents used in this study were of analytical grade.

2.2. Preparation of CNPs

CNPs were prepared by an ionic cross-linking method using TPP as a cross-linking agent. Chitosan was dissolved in 2% (v/v) acetic acid solution to prepare a 2 mg/mL chitosan/acetic acid solution. Following adjustment of pH to 4.8, the mixture was centrifuged at 4 000 r/min for 5 min to discard non-dissolved chitosan. TPP aqueous solution with TPP concentrations at 0.5, 0.75, 1.0 and 1.25mg/mL was prepared, respectively. Under stirring at 600 r/min, 14 mL TPP water solution was slowly dropped into 35 mL chitosan/acetic acid solution and the reaction was continued for 1 hour at room temperature. The resultant CNP suspension was preserved at 4 °C in an airtight container for later use.

2.3. Preparation of BCV N protein-loaded CNPs

4 mL CNP suspension with optimal TPP concentration was placed in a 6-well plate. After the addition of 2 mL BCV N protein solution (diluted with pH 4.8 PBS to 1.0mg/mL), the 6-well plate was decolorized at 4 °C on a shaking table (100 r/m) for 24 hours, and then BCV N protein-loaded CNPs were acquired.

2.4. Characterization of different CNPs

Particle size analysis: CNP suspension prepared by four concentrations of TPP was placed into the sample dish for particle size analysis using a Zeta PALS-90n granulometer (Brookhaven Instruments Corp., New York, USA.).

Fourier transform infrared (FTIR) spectroscopy analysis: KBr pellets of chitosan, CNP and TPP were prepared. Group changes in surface structure of different materials were analyzed by FTIR spectroscopy (spectrum one, PerkinElmer Inc., Waltham, MA, USA).

2.5. Adsorption efficiency of CNPs to BCV N protein

The standard curve was plotted as follows: 2 mg/mL BSA in PBS at pH7.4 was prepared accurately as stock solution, then the solution was serially diluted to 1.000, 0.750, 0.500, 0.250, 0.125, 0.0625 and 0.03125 mg/mL. Bradford protein assay kit (P1510) was used to determine the OD_{595} values versus BSA concentrations according to the manufacturer's protocol. The linear regression equation was determined as follows:

$$A = 0.6913C + 0.0205; R^2 = 0.9934.$$

Where A and C represent the adsorbance value at OD_{595} and the BSA concentration (mg/mL), respectively.

For BCV N-loaded CNP, 1 mL BCV N-loaded CNP suspension was centrifuged at 20 000 r/min at 4 °C. The BCV N concentration in the supernatant was determined using a Bradford protein assay kit (5 \times). The adsorption efficiency of BCV N protein to CNPs was calculated according to the regression equation mentioned above.

2.6. *In vivo* tests

Animal grouping Twenty-four healthy female Balb/c mouse, aged 6-8 weeks, purchased from the Laboratory Animal Center, Harbin Veterinary Research Institute, were acclimatized at 15–20 °C for 1 week before subsequent tests. The mouse were randomly divided into four groups: BCV N-loaded CNP group (group 1), BCV N protein emulsified in Montanide ISA 206 (Seppic, Paris, France) group (group 2), BCV N naked protein group (group 3, positive control), and CNP group in which the mouse were administered CNP suspension only (group 4, negative control).

Antigen preparation For group 1, the antigen was prepared as described in 2.3. For the Montanide ISA 206 group, the BCV N protein was emulsified in Montanide ISA 206 by peptizing using 2.5 mL syringe repeatedly.

Immunization and serum preparation Each mouse was injected at multiple points in Interfemus. The dose in all groups was calculated based on BCV N amount, i.e., 50 µg BCV N per mouse. The immunizations were boosted once at 2 week interval. The serum was isolated from blood collected from tail bleeds at 0, 10, 20, 28, 36, 43, and 53 days after first immunization, and preserved at –70 °C for use.

Immunoglobulin measurement by ELISA Serum levels of IgG, IgM and IgA were determined by indirect ELISA as described previously [17] to investigate the dynamic changes of immune factors in each group. Briefly, the purified BCV N antigen was diluted to working concentration using pH 9.6 carbonate buffer, and added into 96-well corning plates at 100 µL per well. The plates were coated at 4°C overnight, and then washed using 1:1000 PBST (PBS+Tween20) 4 times. 200 µL 5% skimmed milk was added to each well as blocking fluid and blocked at 37°C for 1h; then washed the plate as above. All the serum was diluted at 1:300 using PBST, and then 100 µL diluted sample was added to each well. The plates were incubated at 37°C for 1h. The HRP-labeled anti-mouse IgG, IgM and IgA were diluted at 1:4000 and added to each well, with 100 µL per well. The incubation and washing process were the same as above. Then 60 µL TMB substrates were added, and incubated at room temperature for 10 min. Finally, 60 µL terminate solution was added to each well and the absorbance at OD₄₅₀ nm were measured on ELISA reader (Versa MAX PLUS 384, Molecular Devices Corporation, Sunnyvale, California, USA).

2.7. *Statistical analysis*

All the tests were repeated at least in triplicates, and the results were expressed as $\bar{X} \pm \text{Std}$.

3. Results and discussion

3.1. *Effects of TPP concentrations on CNPs preparation*

Chitosan is a kind of cationic polymer, which can interact with TPP to form polyelectrolytes through electrostatic interactions. Therefore, in this study, the electrostatic interactions-based ionic cross-linking method was used to prepare the chitosan nanoparticles. Fig. 1 showed the effects of TPP concentrations on the size distribution of chitosan nanoparticles. With the increase of TPP concentrations, the transmittance of the chitosan nano-emulsions decreased (data not shown). In the preparation of chitosan nanoparticles through ionic cross-linking process, the electrostatic interactions among the negative charges from the phosphate groups of TPP and the positive charges from the amino groups of chitosan caused the inter- or inner-molecular cross-linking, thus formed the nanoparticles. Under lower TPP concentration, the less negative charges leads to partial cross-linking of chitosan molecules, therefore, the chitosan solution was clear. Under higher TPP concentration, the aggregation process may cause the production of larger particles. In this study, the TPP concentrations ranged from 0.5mg/mL to 1.25mg/mL. At four TPP concentrations, the sizes of the CNP nanoparticles were 14.128±0.026, 29.132±0.032, 39.333±0.104 and 70.284±0.063nm, respectively. When the TPP concentration was 1.0mg/mL, the polydispersity of size distribution was the least as shown in Fig. 1. Therefore, TPP concentration at 1.0mg/mL was selected for the following study.

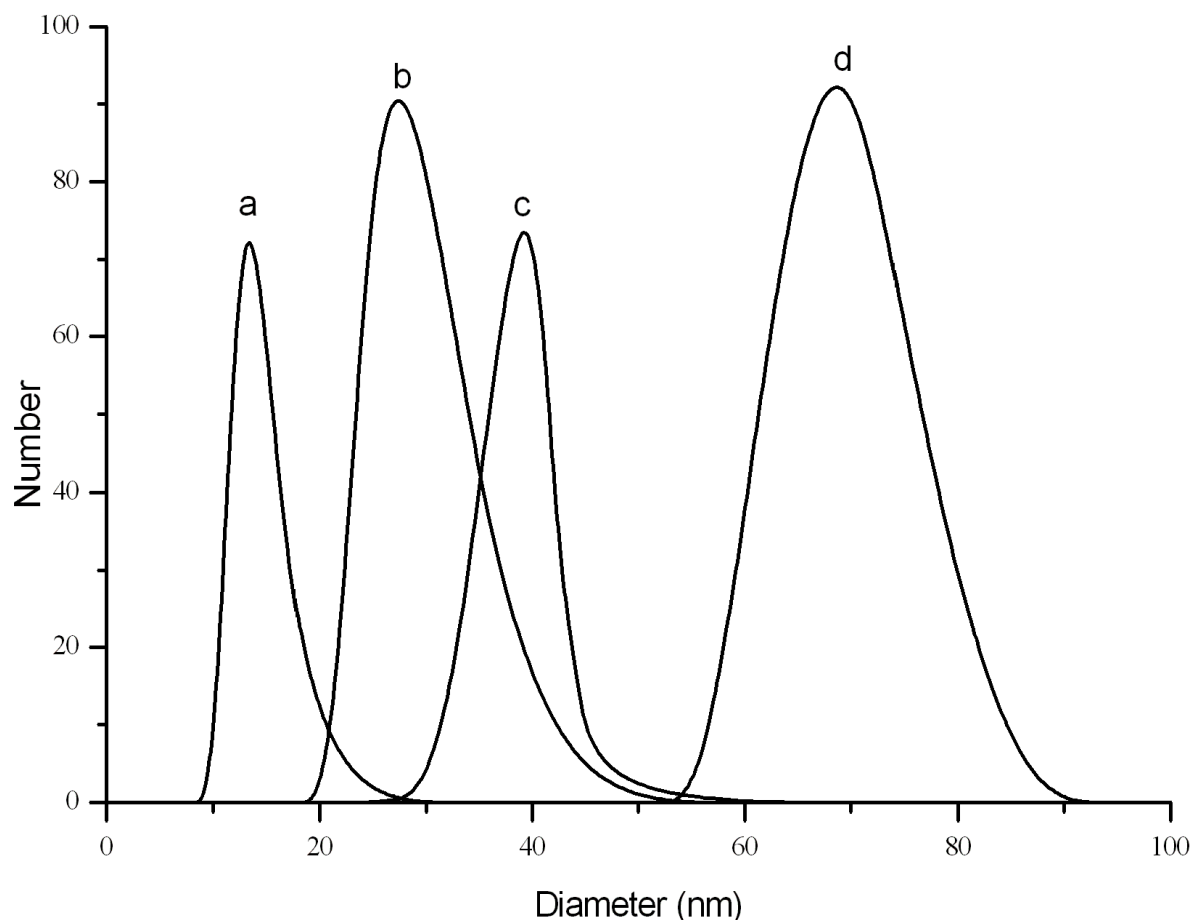


Fig. 1. Particle size distribution of chitosan nanoparticles prepared with TPP concentration at a) 0.5 mg/mL; b) 0.75 mg/mL; c) 1.0 mg/mL and d) 1.25 mg/mL

3.2. FTIR analysis of CNPs

FTIR spectroscopy analysis on chitosan, CNPs and TPP was shown in Fig. 2. All samples showed an adsorption peak in the $3200\text{--}3500\text{ cm}^{-1}$ region, which reflected intermolecular stretching vibration of hydrogen bonds (O-H). The adsorption peak of TPP was weakest, and that of CNP was wider when compared with chitosan. Chitosan molecules showed the bending vibration of N-H in the 1638 cm^{-1} and 1590 cm^{-1} .

3.3. *In vivo* immunological effects

In our previous studies, we have purified and characterized the BCV N protein [17]. In this study, the CNPs loaded with BCV N protein were injected to Balb/c mouse via intramuscular route. According to the standard curve, the concentration of BCV N was 15.31 mg/mL , and CNP had a $(73.26\pm 1.32)\%$ adsorption rate to BCV N.

Serum levels of IgG, IgM and IgA in different groups at different periods were determined by indirect ELISA (Fig. 3-5). For IgG detection, the OD_{450} value lower than 0.20 was set as negative. Based on the IgG levels detected, CNPs exhibited strong sustained release effects on BCV N protein, indicating that the N protein was maintained at a certain level for a long time in mice. IgG was not detectable in the CNP group, demonstrating that chitosan cannot stimulate the production of IgG in mice. In the BCV N group, IgG was detected after, but not prior to, the second immunization; but IgG levels stayed at a very low level, peaking after 43 days, followed by a significant decline, and tended to be at a level similar to the CNP group after 53 days. In the BCV N-loaded CNP and Montanide ISA 206 groups, IgG was not detectable after the first immunization, but it was significantly increased after the second immunization, reaching a very high level 1 week after the second immunization (i.e., at 28 days). This level was maintained up to 53 days. In addition, IgG levels were similar between the

BCV N-loaded CNP and Montanide ISA 206 groups, following similar profiles with time. These findings indicated that CNP exhibits sustained release potential and immuno-enhancing effects similar to Montanide ISA 206.

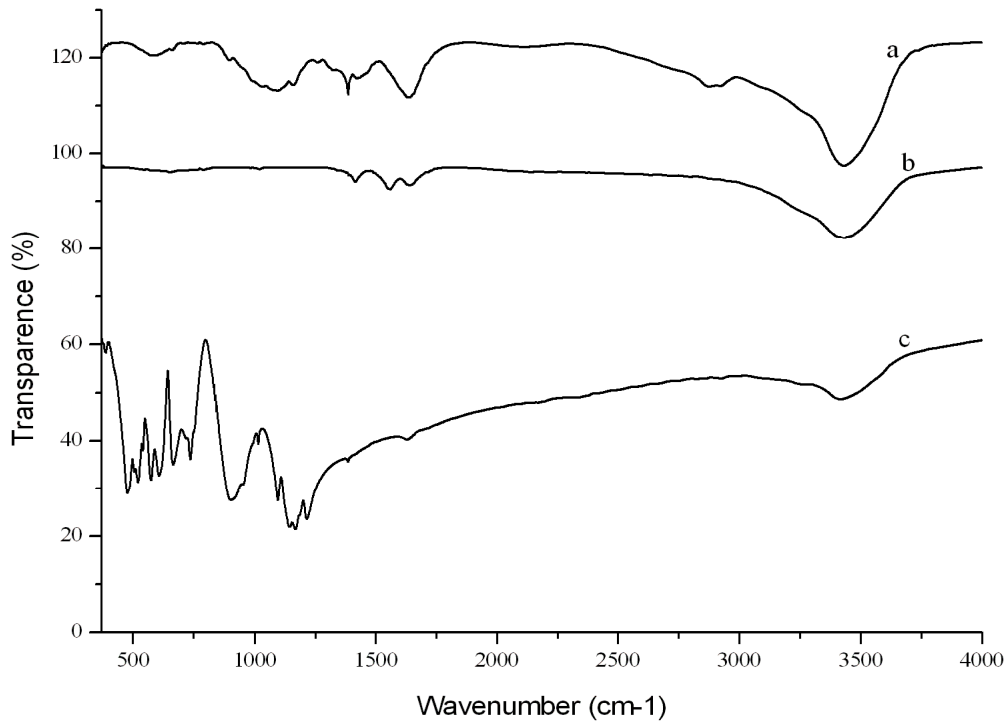


Fig. 2. FTIR spectroscopy analysis. (A) Chitosan; (B) chitosan nanoparticle (CNP); (C) sodium tripolyphosphate

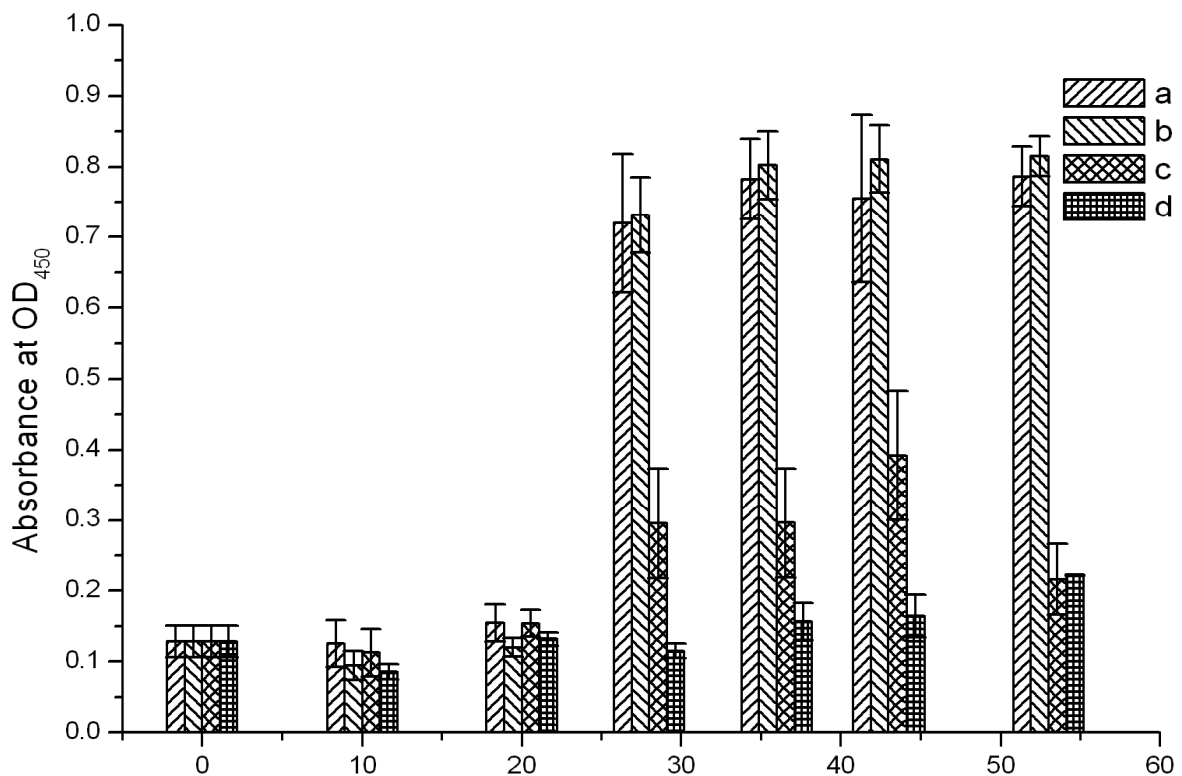


Fig. 3. Serum IgG level in mouse intramuscular injection with different sample: a, b, c, d represent BCV N-loaded CNP, Montanide ISA 206, BCV N, and CNP groups, respectively.

In the IgM detection, the OD value lower than 0.4 was considered as negative. IgM was not detectable in the CNP group. In the BCV N group, IgM was detected after the first immunization, but it returned to the initial level after 20 days, and was only slightly increased after the second immunization, peaking at 43 days. In both the Montanide ISA 206 group and BCV N-loaded CNP group, IgM was not detected after the first immunization, but it was detected after the second immunization and kept higher levels thereafter, compared with that of the other two groups. IgM has been used in diagnosis as it is the earlier emerged antibody after pathogen invasion. In this study, the naked BCV N can cause IgM production at 10d after the first immunization and disappeared quickly, while the IgM tendency in the Montanide ISA 206 group and BCV N-loaded CNP group showed sustained-release profiles of these two kinds of adjuvant to the antigen.

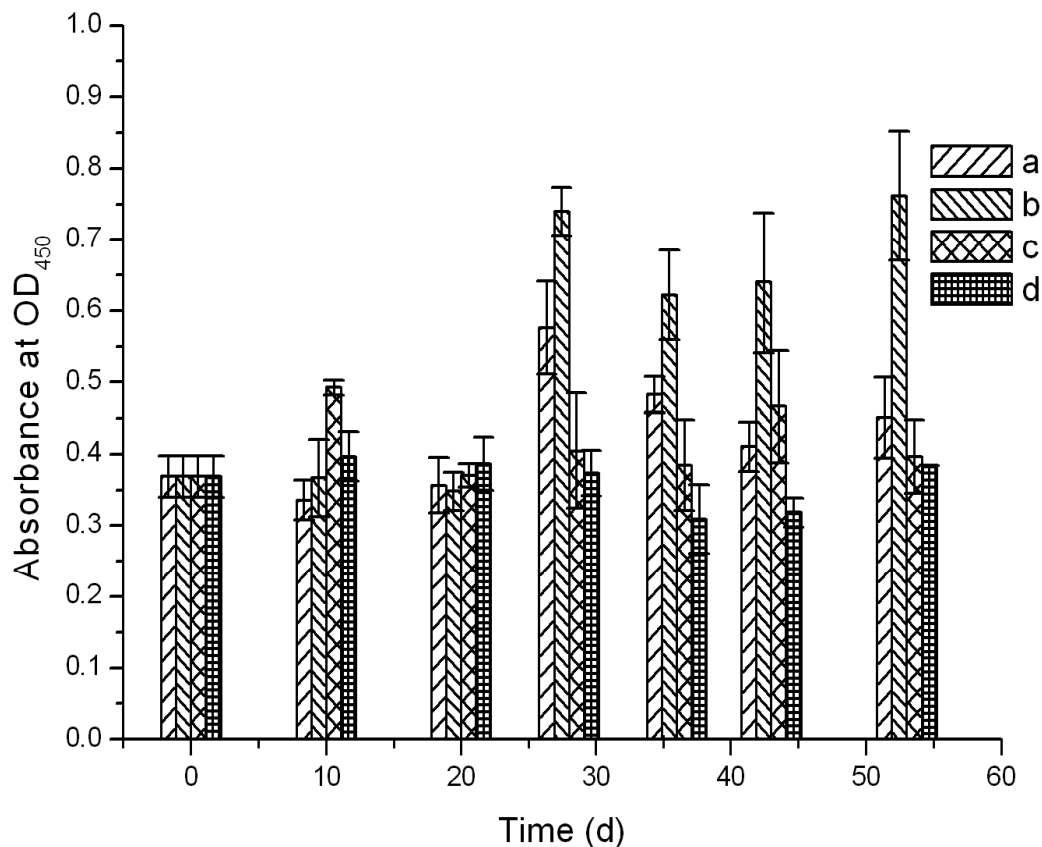


Fig. 4. Serum IgM level in mouse intramuscular injection with different sample: a, b, c, d represent BCV N-loaded CNP, Montanide ISA 206, BCV N, and CNP groups, respectively.

The OD₄₅₀ value lower than 0.25 was set as negative for IgA level. IgA was not detectable in the CNP group. In the BCV N group, the IgA level was detected after the first immunization; this was maintained after the second immunization, peaking at 43 days. In the Montanide ISA 206 group, IgA was not detected after the first immunization, but it was detected after the second immunization and stayed at a very high level thereafter. The BCV N-loaded CNP group showed a similar tendency to the Montanide ISA 206 group, with the exception that the highest IgA level occurred at 36 days.

IgA participates in the immunity of mucous membranes and its level reflects the immune state of the mucosal membrane. In this study, in the CNP and BCV N groups, IgA levels stayed very low level during the whole experimental period. In contrast, in the Montanide ISA 206 and BCV N-loaded CNP groups, serum levels of IgA increased after the second immunization. Evidence exists that suggests chitosan has the potential to break the tight junction of epithelial cells [22, 23]. For this

reason, antigen molecules can be induced to enter into the epithelial inner region to stimulate the immunization of mucous membranes. These results demonstrated that mucous membrane immune pharmaceuticals using CNP as an adjuvant are of good clinical feasibility.

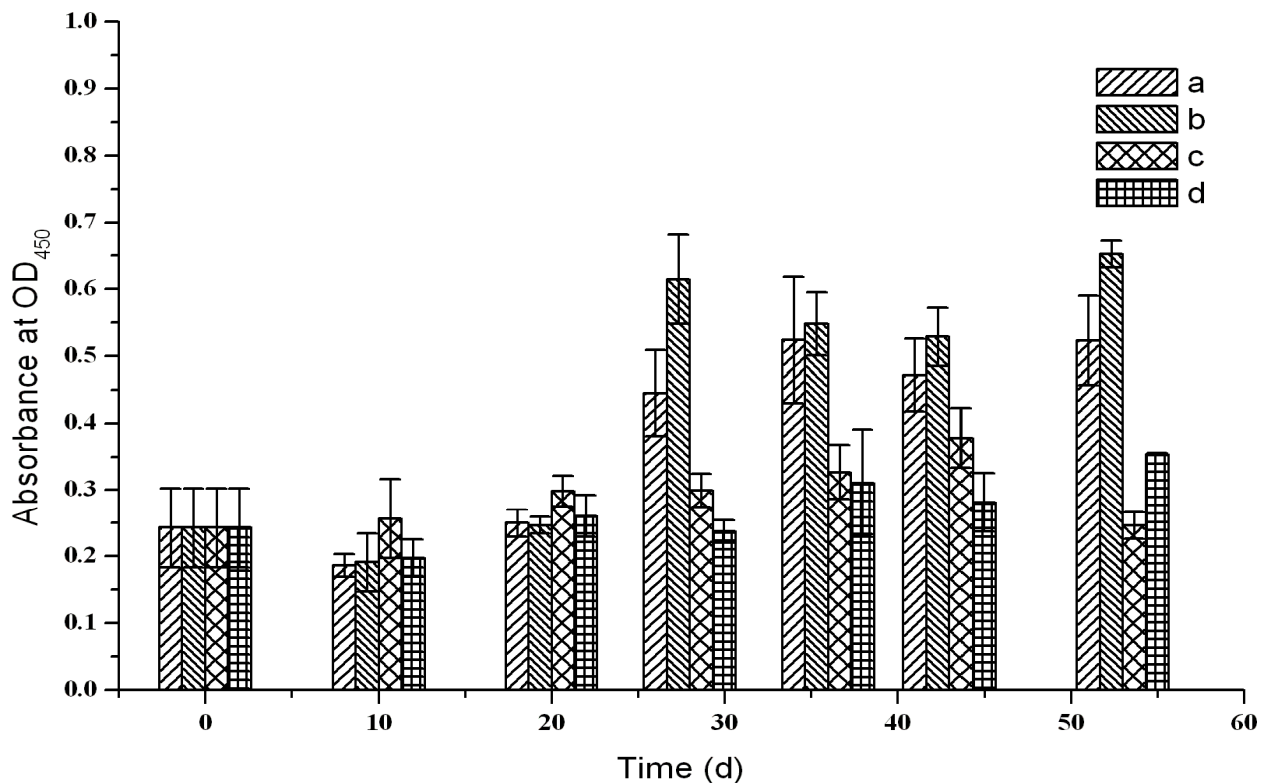


Fig. 5. Serum IgA level in mouse intramuscular injection with different sample: a, b, c, d represent BCV N-loaded CNP, Montanide ISA 206, BCV N, and CNP groups, respectively.: a, b, c, d represent BCV N-loaded CNP, Montanide ISA 206, BCV N, and CNP groups, respectively.

4. Conclusion

Granular CNPs of good disperse, with a particle size of 39.333 ± 0.104 nm, were prepared by an ionic cross-linking method. The adsorption rate of BCV N-loaded CNP was $(73.26 \pm 1.32)\%$. *In vivo* immunological evaluation revealed that CNP can greatly increase serum levels of IgG, IgM and IgA levels in mice after immunization and that CNP has no immunogenicity. Therefore, CNP can be used as a novel adjuvant for clinical study.

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