Optimization of preparation of NDV F Gene encapsulated in N-2-HACC-CMC nanoparticles

S S Li¹, Y Zhang¹, K Zhao¹,³ and X H Wang²,³,⁴

¹Key Laboratory of Microbiology, School of Life Science, Heilongjiang University, Harbin 150080, China
²Heilongjiang University Library, Harbin 150080, China
³School of Biological Science and Technology, University of Jinan, Jinan 250022, China
⁴E-mail: xiaohua020623@foxmail.com

Abstract. In this study, the biodegradable materials N-2-hydroxypropyl trimethyl ammonium chloride chitosan (N-2-HACC) and N, O-carboxymethyl chitosan (CMC) are used as delivery carrier for the pVAX I -F(o)-C3d6. The optimal preparation condition is as follows: concentration of N-2-HACC is 1.0 mg/ml, concentration of CMC is 0.85 mg/ml, concentration of pVAX I -F(o)-C3d6 is 100 μg ml. The results show that the prepared N-2-HACC-CMC/pFDNA NPs have regular round shape, smooth surface and good dispersion, the particle size is 310 nm, Zeta potential is 50 mV, the entrapment efficiency is 92 %, the loading capacity is 51 % (n=3).

1. Introduction
Newcastle disease is an important disease of poultry and can cause severe economic losses to the poultry industry around the globe [1]. However, the prevention of this disease requires an effective way, chitosan nanoparticles as an excellent delivery carrier appeared in front of people [2].

Nano vaccines have more long-term significance than the traditional inactivated vaccine and live vaccine, inactivated vaccines may cause disease transmission because of inadequate inactivation, and live vaccines are destroyed by enzymes during immunization. The preparation of chitosan nanoparticles has laid a theoretical and practical basis for the study of nano-delivery system [3], which is of great significance.

Chitosan is a promising carrier material for vaccine mucosal delivery, but its narrow soluble range limits the further application of chitosan. In this study, N-2-HACC-CMC / pFDNA NPs were prepared using N-2-HACC and CMC as carriers. N-2-HACC was a quaternary ammonium salt derivative of chitosan. This derivative, under the premise of maintaining the other biological properties of chitosan, greatly improves the solubility in neutral or alkaline environments [4]. And the modified chitosan nanoparticles have the following advantages: non-toxic, biodegradable [5], bacteriostatic [6], biocompatibility [7], and so on. In conclusion, chitosan based modified nanoparticles have strong advantages, so we have synthesized N-2-HACC-CMC/pFDNA NPs nanoparticles containing Newcastle disease F gene in this laboratory.
2. Materials and methods

2.1. Materials
The eukaryotic expression plasmid pVAX-optiF with C3d6 molecular adjuvant (pVAX I -F(o)-C3d6), N-2-hydroxypropyl trimethyl ammonium chloride chitosan (N-2-HACC) and N, O-carboxymethyl chitosan (CMC) were provided by our Laboratory in School of Life Science of Heilongjiang University, China.

2.2. Single factor test
There are five single factors influencing the preparation of N-2-HACC-CMC/pFDNA NPs: the concentration of N-2-HACC (0.5, 1.0, 1.5 and 2.0 mg/ml); the concentration of pVAX I -F(o)-C3d6 (50, 100, 150, 200 and 250 μg/ml); the concentration of CMC (0.75, 1.0, 1.25, 1.5 and 1.75 mg/ml); stirring speed (900, 1000, 1100, 1200, 1300 r/min) and stirring time (20, 30, 40 and 50 min).

2.3. Orthogonal test
According to the single factor results, three factors were selected for the orthogonal test (table 1), the morphology, particle size, encapsulation efficiency (EE) and loading capacity (LC) of N-2-HACC-CMC/pFDNA NPs were taken as evaluation indexes.

2.4. Preparation of NDV F gene encapsulated in N-2-HACC-CMC nanoparticles
N-2-HACC-CMC/pFDNA NPs were prepared by polyelectrolyte complex method, but the specific concentration of each component was determined according to the single factor test and orthogonal test results. The specific flow is as follows: Add 5 ml of a certain concentration of N-2-HACC solution to 50 ml erlenmeyer flask, slowly add 2 ml of a certain concentration of pVAX I -F(o)-C3d6 solution with 1 ml syringe, 300 r/min for 3 min, then raise the stirring speed to the target speed for 1 min, then 2 ml of a certain concentration of CMC solution was added dropwise slowly with 1 ml syringe, and kept in 5 min drops finished, continue magnetic stirring, and maintain a certain mixing time. The solution will be 12000 r/min 4 ℃ for 20 min, discard the supernatant and wash the precipitate with 4 ℃ precooled sterile deionized water, repeat 3 times, and finally resuspend the precipitate with 2 ml of pre-cooled sterile deionized water and lyophilize in vacuo to obtain N-2-HACC-CMC/pFDNA NPs.

3. Results

3.1. Single factor test
(1) Effects of N-2-HACC concentration on the preparation of N-2-HACC-CMC/pFDNA NPs. As shown in figure 1a, with the increasing of N-2-HACC concentration, the EE firstly increased and then decreased. When the concentration of N-2-HACC was 1.0 mg/ml, EE and LC were desirable.

(2) Effects of CMC concentration on the preparation of N-2-HACC-CMC/pFDNA NPs. As shown in figure 1b, with the increasing of CMC concentration, the EE firstly increased and then decreased. When the concentration of CMC was 1.0 mg/ml, the EE and LC were desirable.

(3) Effects of DNA concentration on the preparation of N-2-HACC-CMC/pFDNA NPs. As shown in figure 1c, with the increasing of pVAX I -F(o)-C3d6 concentration, the EE firstly increased and then decreased. When the DNA concentration is 100 μg/ml, the EE and LC were desirable.

(4) Effects of stirring rate on the preparation of N-2-HACC-CMC/pFDNA NPs. As shown in figure 1d, with the increasing of stirring rate, the EE firstly increased and then decreased. When the speed was 1200 r/min, the EE and LC were desirable.

(5) Effects of stirring time on the preparation of N-2-HACC-CMC/pFDNA NPs. As shown in figure 1e, with the stirring time prolonging, the EE and LC increased to 40 min, and reached the highest.
3.2. Orthogonal experiment
Three factors that influence the preparation of N-2-HACC-CMC/pFDNA NPs are N-2-HACC concentration, CMC concentration and DNA concentration. The optimal preparation condition of N-2-HACC-CMC/pFDNA NPs were as follows: N-2-HACC concentration was 1.0 mg/ml, CMC concentration was 0.85 mg/ml, DNA concentration was 100 μg/mL (table 1), the entrapment efficiency was 92 %, the loading capacity was 51 % (n=3).

3.3. Preparation of NDV F Gene Encapsulated in N-2-HACC-CMC Nanoparticles
The optimal preparation process is as follows: Add 5 ml 1.0 mg/ml N-2-HACC solution to 50 ml erlenmeyer flask, slowly add 2 ml 100 μg/mL pVAX 1 -F(o)-C3d6 solution with 1 ml syringe, 300 r/min for 3 min, then raise the stirring speed to 1200 r/min for 1min, then 2 ml 0.85 mg/ml CMC solution was added dropwise slowly with 1 ml syringe, and kept in 5min drops finished, continue magnetic stirring, and maintain for 40 min. The solution will be 12000 r/min 4 ℃ for 20 min, discard the supernatant and wash the precipitate with 4 ℃ pre-cooled sterile deionized water, repeat 3 times, and finally resuspend the precipitate with 2 ml of pre-cooled sterile deionized water and lyophilize in vacuo to obtain N-2-HACC-CMC/pFDNA NPs.

It can be seen from figure 2, the N-2-HACC-CMC/pFDNA NPs prepared by the optimal condition had regular round shape, smooth surface and good dispersion. The average particle size was 310 nm (figure 3a) and the Zeta potential was 50 mV (figure 3b).
Table 1. Results of orthogonal experimental design.

| Experiment No. | N-2-HACC concentration (mg/ml) | CMC concentration (mg/ml) | pVAX I -F(o)-C3d6 concentration (µg/ml) | Encapsulation efficiency (%) | Loading capacity (%) |
|----------------|--------------------------------|---------------------------|----------------------------------------|-------------------------------|---------------------|
| 1              | 0.8                            | 0.85                      | 80                                     | 79                           | 31                  |
| 2              | 0.8                            | 1.0                       | 80                                     | 79                           | 31                  |
| 3              | 0.8                            | 1.15                      | 80                                     | 79                           | 31                  |
| 4              | 1.0                            | 0.85                      | 100                                    | 92                           | 51                  |
| 5              | 1.0                            | 1.0                       | 80                                     | 88                           | 41                  |
| 6              | 1.2                            | 0.85                      | 80                                     | 88                           | 41                  |
| 7              | 1.2                            | 1.0                       | 80                                     | 88                           | 41                  |
| 8              | 1.2                            | 1.15                      | 80                                     | 88                           | 41                  |
| 9              | 1.2                            | 1.15                      | 80                                     | 88                           | 41                  |
| K_{EE} 1       | 76%                            | 86%                       | 80%                                    |                              |                     |
| K_{EE} 2       | 89%                            | 82%                       | 86%                                    |                              |                     |
| K_{EE} 3       | 82%                            | 78%                       | 81%                                    |                              |                     |
| R_{EE}         | 13%                            | 8%                        | 6%                                     |                              |                     |
| K_{LC} 1       | 34%                            | 41%                       | 40%                                    |                              |                     |
| K_{LC} 2       | 46%                            | 40%                       | 43%                                    |                              |                     |
| K_{LC} 3       | 41%                            | 39%                       | 38%                                    |                              |                     |
| R_{LC}         | 13%                            | 1%                        | 5%                                     |                              |                     |

Figure 2. TEM morphology of N-2-HACC-CMC/pFDNA NPs.
4. Conclusion
The optimal preparation condition of N-2-HACC-CMC/pFDNA NPs was achieved, the N-2-HACC-CMC/pFDNA NPs have regular round shape, smooth surface and good dispersion, the particle size is 310 nm, Zeta potential is 50 mV, entrapment efficiency is 92 %, loading capacity is 51 %.

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