Chitosan Nanoparticles for the Effective Vaccine Delivery System

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ABSTRACT

Many percentage of world population has Hepatitis type B disease and is infected by chronic hepatitis type B. All of the infants have to be prescribed by three consecutive injection of hepatitis vaccine just after his born. Adults need inoculation per every five years if they have no antibody. This intramuscular vaccination accompanies patient's pain and is uncomfortable, and more serious to newborn baby. Chitosan, non-toxic and biodegradable polyanion is obtained by deacethylation of chitin which is produced in shell of crustacean. In this study, chitosan nanoparticles were synthesized by ionic cross-linking gelation and then attached hepatitis type B virus surface antigen(Hbs-Ag) for the vaccination. The drug transport efficiency through the intramuscular and nasal cavity was demonstrated *in vivo* test of SD rats. Characteristics of nanoparticles were examined by ELS(electrophorestic light scattering), SEM(Scanning electron microscope), AFM(atomic force microscope),

1 INTRODUCTION

Hepatitis type B(Hb) is one very fatal disease and has trend that more than about eight percentage of all over the world population catches in chronic hepatitis type B and the infection rate of it increases every year. Virus activate to cirrhosis, the inflammation of liver, and liver cancer since it has the latent period of three or four decades. Therefore, all infants shortly after birth have to be prescribed hepatitis vaccine inoculating three times, adults should be vaccinated by intramuscular injection each five years. Hb vaccine can give us antibody for prevention more than 95% when plasma vaccine or gene recombination vaccine inoculated to a person. Antibody shows the highest value after 3 months during inoculation three times, and it is known that the highest value of antibody means prevention is possible for a long time.[1] Recombinant gene vaccine protein(RGVP) in the market is formed irregular cluster with absorbent such as aluminum hydroxide gel.

Table 1. Characteristics of chitosan

Non-toxicity
Vital attraction
Biodegradable material
Cationic polyamine from a natural substance
High surface charge density
Adherences to negatively charged surface
Formation gels with polyanions
Inexpensive material
Amiability to chemical modification
Reactive amino/hydroxyl groups
sterilizing abilities

Chitosan, poly(D-glucosamine) is obtained by deacethylation of chitin which is produced in shells of crab, shrimps, insects, mushroom cell wall, etc. It has very valuable properties in drug delivery, because chitosan is non-toxic and has many useful features; biodegradability, biocompatibility, hydrophilicity, antibacterial activity, protein affinity, and positive polyanion.(Table 1) Chitosan nanoparticles were easily formed by the ionic cross-linking and spontaneously gelation in aqueous solution. Ionic cross-linking is the reaction between amine groups of chitosan and phosphate groups of tripolyphosphate's(TPP), TPP is the most popular because of its non-toxic property and Quick gelling ability.

Nasal cavity's mucosa have a large surface area(150 ㎠), and capillaries of high blood stream are ranging on the mucosa surface. It is useful site in drug delivery because Waldeyer's loop places on nasal cavity inside. Nevertheless there are two barriers in transference of recombinant gene vaccine protein(RGVP) through nasal cavity; short retention time and low membrane permeability. Generally the retention time of particle that is invaded on interior, dust, impurities is no more than about 15 ~ 20 min because of flagellum movement in nasal cavity. The other factor is drug permeability. Drug can cross the epithelial cell membrane either by the trans-cellular route, in compliance with concentration gradients, receptor mediated transport, vesicular transport mechanism, or by the para-celluar route through the tight junction between the cells. Usually, in case of the latter, drug can pass when particles' molecular weights are below 1000Da. Above mentioned problem was investigated that overcome through the high adhesion of chitosan nanoparticles. [2-5]

In this research, we investigated synthesis of chitosan nanoparticles using ionic gelation, attachment it to vaccine protein, and verification of chitosan-HbsAg complex particles transportation into SD rats through intramuscular and intranasal *in vivo.*

2 EXPERIMENTAL

2. 1. Reagents and Apparatus

Chitin from crab shell was purchased from Aldrich. It was deacetylated by reductive reaction until deacetylation degree was 95%, and its molecular weight was 30,000 which was measured by a viscosity meter.

Tripolyphosphate(TPP) was purchased Sigma Aldrich Co., and all other reagents used were of analytical grade unless otherwise specified. Hb vaccine without absorbent and Hepavax-gene were commercial products. Hb vaccine, Antigen indication device was purchased from Green Cross Company. SD Rat from NTacSam, Male SD (MPF SD : murine pathogen free Sprague-Dawley) was purchased from Korea Taconic Company.

Chitosan powders purification from deacetylation of chitin flakes were dried by a freeze dryer (Freezone plus 6, Labconco, USA). Particles size and density of surface charge were measured by ELS (ELS-6000, Otsuka Electronics, Japan). The morphological characteristics of the nanoparticles were examined using a SEM(S-4700, Hitachi, Japan).

2. 2 Purification of chitosan

Purification of Chitosan was utmost important to ensure that Chitosan intended for biomedical application was free of contamination and had the highest purity. Furthermore, there was a meaning to remove side-effects in the preparation of nanoparticles. Purchased Chitosan flakes were purified according to the preparation method of nanoparticles as a solubility reference at different pH.[6] In 10ml of 1N NaOH solution, 1g of chitosan from the solid chitosan flakes can be purified. This solid-liquid mixture was stirred for 3hours at 70℃, and then filtered off from suction filteration apparatus. Chitosan was insoluable in the alkalis, and the recovered flakes were washed thoroughly and dried purely. This alkali treated chitosan flakes were dissolved in 0.1M acetic acid solution, which was filtered using a filter paper to remove insoluble remained impurity. Filtered chitosan solution was adjusted pH value to pH 8.0 by 1N NaOH, and the purified yellow-white chitosan precipitates were washed thoroughly by deionized water. Finally, uniformed powder was obtained through the sieve.

2. 3 Preparation of chitosan nanoparticles

Purified chitosan powder was dissolved completely in 1% acetic acid solution on the sonicator. Yellowish transparent chitosan solution was filtered using syringe filter, and it was diluted to various concentrations with 1% acetic acid. The solution was mixed with TPP solution as a determined volume ratio and the formation of chitosan-TPP nanoparticles was made spontaneously by the TPP initiated ionic gelation mechanism. Nanoparticles were formed at selected chitosan weight ratios to TPP of 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6. Concentrations of chitosan solution were examined for various percentage(0.1% - 1%). The nanoparticles suspension was stirred for 1hour at room temperature before analysis.

2. 4 vaccine protein complex

Vaccine protein in aqueous phase was added to the purified chitosan powder which was placed for 1hour at room temperature. This chitosan powder - vaccine protein complex was dissolved into 1% acetic acid, the chitosan solution containing suspended vaccine protein was mixed for 1 hour with a TPP solution. Chitosan-vaccine nanoparticles were concentrated by centrifuge for 15min at 12000rpm speed. Concentrated nanoparticles were modulated to optimal quantity and suspended for *in vivo* test.

2. 5 Particles analysis

Particle size and surface charge density of prepared nanoparticles were measured by ELS. Suspended nanoparticles were diluted by deionized water before analysis. Nanoparticles morphology and cross sectional analysis were observed by SEM, AFM, and FIB. These specimen were diluted with distilled water.

Table 2. Particle size and Zeta-potential of synthesized chitosan-TPP nanoparticles (TPP concentration 0.1%)

CS concentration [%]	Additive drug	CS – TPP ratio	Particle size [㎚]	Zeta-potential [㎷]
1	Non	3	1939.3	+60.63
0.8	Non	3	1424.8	+62.26
0.6	Non	3	1265.2	+65.23
0.5	Non	3	818.3	+67.51
0.4	Non	3	1005.9	+62.58
0.3	Non	3	375.2	+61.57
0.2	Non	3	195.5	+62.62
0.1	Non	3	211.0 (aggregation)	+53.35
0.05	Non	3	1896.2 (aggregation)	+23.36
0.2	Non	1	730.4 (aggregation)	+23.29
0.2	Non	2	211.0	+52.91
0.2	Non	4	391.6	+62.62
0.2	Non	5	469.3	+61.94
0.2	Non	6	648.2	+66.69
0.2	10 ㎍ Hepavax	3	368.7	+56.66
0.2	80 ㎍ Hepavax	3	258.5	+53.53
Only Hepavax colloidal solution			87.5	-32.91

Table 3. *In vivo* test for SD rat

Formulation / dosed quantities (μg)	Age of SD rat (weeks)	Administration route (interval / frequency)	Anti-body verification After dose (days)
Commercial hepavax / 10	4	Intramuscular injection	7
Commercial hepavax / 10	6	Intramuscular injection	8
Commercial hepavax / 10	8	Intramuscular injection	8
Commercial hepavax / 10	7	Intramuscular injection	6
Commercial hepavax / 6	7	Intramuscular injection	6
Commercial hepavax / 2	7	Intramuscular injection	6
Hepavax without absorbents / 10	7	Intramuscular injection	None
Chitosan – hepavax / 40	7	Intramuscular injection	5
Chitosan – hepavax / 10	7	Intramuscular injection	8
Chitosan – hepavax / 6	7	Intramuscular injection	8
Chitosan – hepavax / 2	7	Intramuscular injection	None
Commercial hepavax / 10	7	Intranasal absorption	None
Chitosan – hepavax / 10	7	Intranasal absorption (1day/ 4)	None
Chitosan – hepavax / 40	7	Intranasal absorption (1day/ 4)	None
Chitosan – hepavax /120	7	Intranasal absorption (2h / 4)	None
Chitosan – hepavax / 80	7	Intranasal absorption (6h /16)	None

2. 6 *in vivo* test

In vivo test was schemed to three directions. First, SD Rat's susceptibility test about hepatitis vaccine protein. Vaccination was injected into a thigh muscle of 4 – 8 weeks old SD rat and injection volume was 0.1~0.5ml. Second, chitosan nanoparticles' ability as an absorbent was definited between the plasma in blood and vaccine proteins. Chitosan-vaccine nanoparticles instead of aluminum hydroxide-vaccine particles which was usually used for vaccine drug were identified as an adsorbent. Finally, prepared chitosan – vaccine protein was absorbed into the nasal cavity of SD rat. All experiments repeated 3 times.(Table 3)

2. 7 Blood sampling and analytical method

For all SD rats, 0.5 ㎖ sample of blood was drawn from the tail vein at 24 hours intervals during one month after vaccine delivery. Collected blood was placed about 40min, and identified antibody by anti-body indicate device after separating serum from coagulated blood using centrifuge(3000g × 15min) at normal temperature.

3 RESULTS AND DISCUSSION

3. 1 Chitosan nanoparticles

The results at variously modulated concentrations and mixing ratios were showed on Table 2. Figure 1 and Figure 2 show particles size was the smallest when chitosan concentration and chitosan–TPP ratio were 0.2% and 3:1 respectively. The highest zeta-potential was observed at 0.5% and 6:1. The SEM image of chitosan nanoparticles was shown in Figure 3. It has a small aggregated but confirmed a each division size 150nm ~ 300nm.

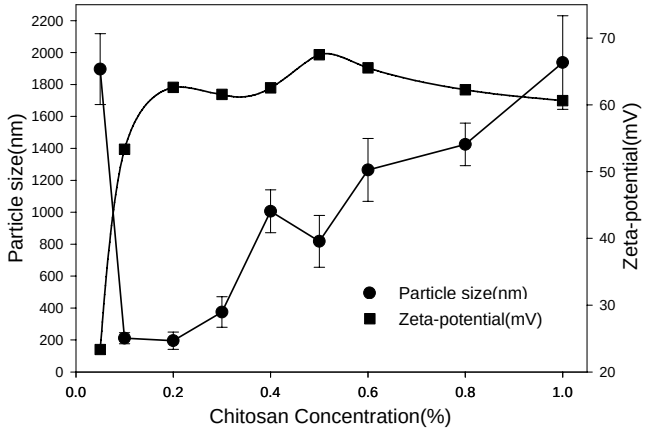

Figure 1. Effect of chitosan concentration from 1% to 0.1% on particle size and zeta-potential.

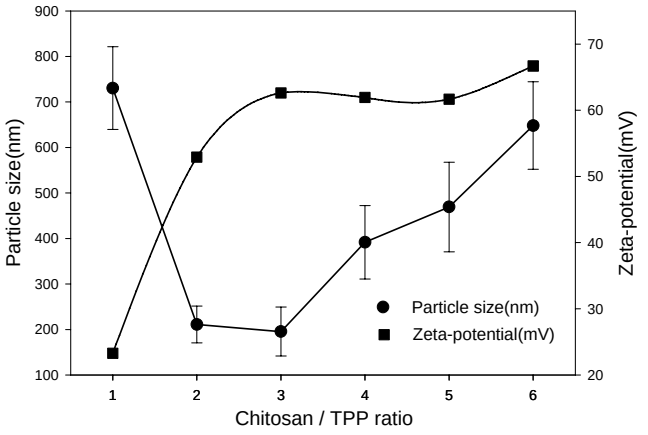

Figure 2. Effect of chitosan – TPP ratio from 1:1 to 6:1 on particle size and zeta-potential.

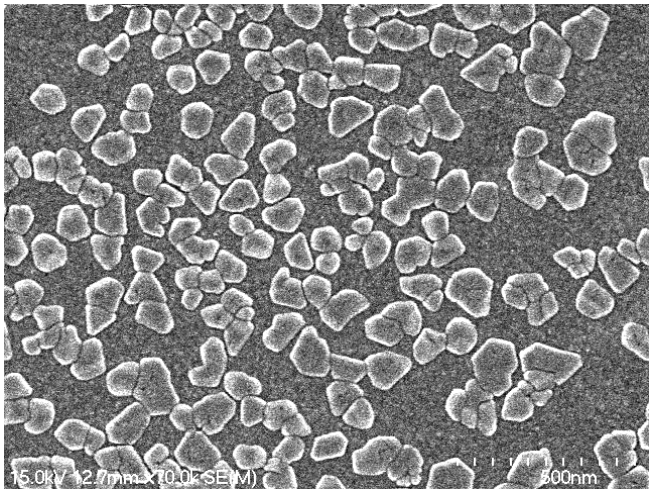

Figure 3. SEM spectroscopy of the synthesized chitosan nanoparticles.

3. 2 Vaccine attached to Chitosan nanoparticles

Chitosan–vaccine nanoparticles size was larger than previously synthesized chitosan nanoparticles and its range was from 258nm to 369nm. Figure 4 shows SEM spectroscopy of the chitosan-vaccine complex. From the figure it was known chitosan nanoparticles was pile of vaccine protein, a distortional spherical shape is similar to each chitosan nanoparticles morphology in Figure 3.

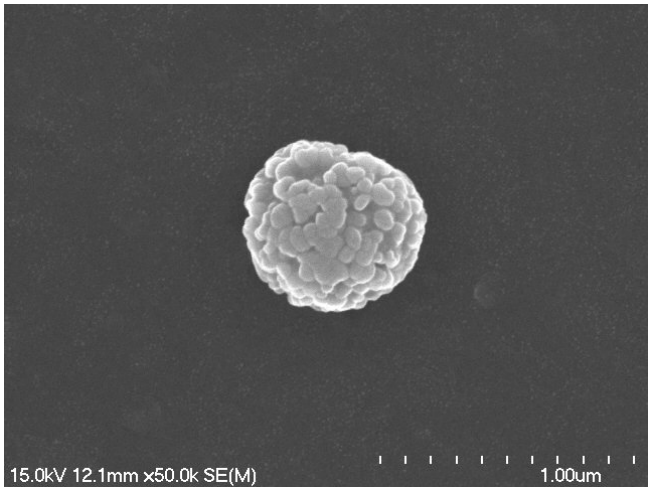

Figure 4. SEM spectroscopy of the prepared chitosan-vaccine complex.

3. 3 *in vivo* Test

Hbv Anti-gene's susceptibility for various dosage and period was identified as *in vivo* experiments. SD rats surface anti-body concentration was risen more than the effective levels. From the results, it was known antibody was induced independently of its age and the dosage in several days after injection. Subsequently, chitosan nanoparticles attached by vaccine protein were dosed through intramuscular and intranasal administrations. Results of intramuscular administrations confirmed its ability of chitosan nanoparticles as Drug adsorbent such as aluminum hydroxide gel, and this connoted that injection of vaccine using chitosan drug carrier was also sustainable and protective of vaccine protein.

Concurrently, intranasal administration was examined at various vaccine concentrations and absorption intervals. It was known antibody induction showed several case and partially effective. It was a notice that continuous contact with vaccine protein was major factor in immunizations than dosage. *In vivo* results were arranged on Table 3.

4 CONCLUSIONS

This experiment has investigated the novel drug carrier and administration route for vaccination of hepatitis type B. Drug carrier was synthesized by ionic-crosslinking between Chitosan and TPP, this chitosan nanoparticles have a 200~300nm particle size and 60mV surface charge density. Synthesized biodegradable nanoparticles had the form of distortional spherical gel, and showed the preparation of Chitosan-vaccine complex as an absorbent that attached to Chitosan nanoparticles. Intramuscular and intranasal administrations by SD rats passed susceptibility test confirmed chitosan ability as an absorbent in drug. But immunization efficiency of intranasal delivery was lower than the former. The future study will aim at the increase of efficiency of transmucosal vaccination.

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