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# Chitosan-based particles as biocompatible delivery vehicles for peptide and protein-based vaccines

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**Abstract**

It has become increasingly recognized that polymer particle size can have a profound effect on the interactions of particle-based vaccines with antigen presenting cells (APCs) thereby influencing and modulating ensuing immune responses.With the aim of developing chitosan particle-based immunocontraceptive vaccines, we have compared the use of chitosan nano- and microparticles as delivery vehicles for vaccine candidates based on luteinising hormone-releasing hormone (LHRH). Both particle types were taken up effectively by dendritic cells with similar efficacies. Inoculation with nano- and microparticles containing conjugated peptide or protein microparticles also resulted in the induction of high levels of LHRH-specific antibodies. In the case of protein-conjugated particles, the levels of antibodies elicited were similar to those elicited following inoculation with antigen emulsified with complete Freund's adjuvant. The approach to vaccine design that we have described here could represent another useful method for inducing immune responses against microbial, viral and tumorigenic protein antigens.



*Keywords:* antibody; chitosan, dendritic cells, LHRH, nanoparticles, microparticles

## 1. Introduction

Chitosan is a non-toxic linear polysaccharide composed of β-1,4-linked D-glucosamine derived from the deacetylation of chitin, a naturally occurring polymer found in the exoskeletons of crustaceans and insects. Because of its biocompatibility and biodegradability into non-toxic and non-allergenic products, chitosan is commonly used for many applications in pharmaceutical and medical fields. Many studies on various forms of chitosan, including chitosans with varying degrees of acetylation and chemically modified chitosans, have investigated their ability to act as vaccine delivery systems[1]. The uses of both solution and particulate forms of chitosan to increase the efficacy and response to immunization with model protein antigens [2, 3], DNA plasmids [4], viral antigens [5, 6], bacterial derived toxins [7, 8] and antigens [9, 10]are well documented.

With the aim of developing chitosan particle-based vaccines, we have compared the use of chitosan-based nano- and micro-particles as vaccine carriers for peptide and protein antigens based on luteinising hormone-releasing hormone (LHRH). LHRH is a 10 amino acid peptide hormone that is

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secreted by the hypothalamus and initiates a cascade of events that regulates gametogenesis[11]. Antibodies generated against this hormone can lead to the inhibition of reproductive capabilities of vaccinated animals [12, 13]. Because its sequence is conserved in all mammals, the use of an immunocontraceptive vaccine based on LHRH has potential as an alternative to surgical castration in companion animals and livestock [14]and even for the control of hormone dependent malignancies in humans [13, 15].

## 2. Results

### *1.1. Chitosan-based nano- and microparticles*

To produce chitosan-based particles for this study, a high-sheer emulsification process was employed. Varying the hydrophilic/lipophilic balance (HLB) of the surfactant used in this technique allowed for greater control over particle size distribution. Table 1 lists the mixing ratios of the surfactants used to obtain the necessary HLB values and scanning electron microscopy images in Figure 1A show distinct size ranges for the resulting chitosan particles. Figure 1B shows that a high HLB value (8.2) produced particles with a centroid of 2.10 μm such that 92% of the microparticles were in the range 1.62-2.58 μm while the lower HLB value (4.3) produces nanoparticles with a centriod of 163 nm 92% in the range 113-213 nm.

For the covalent attachment of macromolecular cargo to these particles, a chloroacetylation strategy was adopted[16]. Incorporation of chloroacetyl groups provides functional sites to tether protein or peptide cargo containing free thiol groups such as those found in cysteine residues. FTIR spectroscopy was used to confirm the chloroacetylation process. The peak at ~788cm$^{-1}$ (assigned to C-Cl stretching) confirmed the presence of the chloroacetyl functionality, no amine functionality is available in the crosslinked particles this is apparent from the disappearance of the N-H symmetric and asymmetric stretching peaks at 3444 and 3357cm$^{-1}$ peaks (Figure 1C). All particle types examined were found to contain varying amounts of chloroacetyl groups (Table 2) with nanoparticles containing more chloroacetyl groups than microparticles.

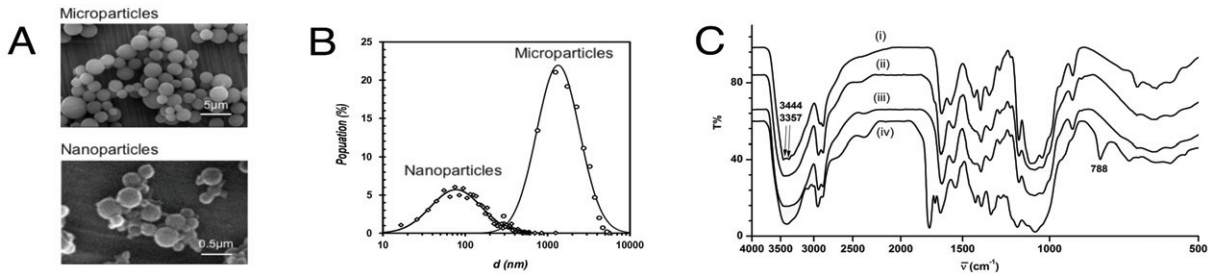


Figure 1. A) Scanning Electron Micrographs of different sized chitosan-based particles. B) The size distribution of chitosan micro and nano-particles showing the diameter (d) in nanometers (nm), C) FTIR spectra of i) chitosan, ii) glutaldehyde cross-linked chitosan particles, iii) reduced cross-linked chitosan particles and iv) chloroacetylated chitosan particles.

Table 1. Weight fractions of Tween 80 ($X_{T80}$) and Span 80 ($X_{S80}$) surfactants used to produce particles

| Particle Type | $X_{T80}$ | $X_{S80}$ | HLB | $V_{surf}$(ml)* | Particle size distribution |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| Microparticles | 0.37 | 0.63 | 8.2 | 1.4 | 1-6μm |
| Nanoparticles | - | 1.00 | 4.3 | 2.2 | 30-500nm |

* $V_{surf}$ is the surfactant volume used in 250:25 o/w emulsion.

Table 2. Total amount of antigen taken up by particles

| Particle type | Amount of peptide (μg)* | Loading Efficiency (%)# | Amount of protein (μg)* | Loading Efficiency (%)# |
|---|---|---|---|---|
| Microparticles | 617μg ± 112.6 | 24.7% ±4.5 | 263.1μg ± 29.1 | 10.4% ±1.1 |
| Nanoparticles | 734.1μg ± 38.4 | 29.4% ±1.5 | 105.3μg ± 31.7 | 4.2% ±1.2 |

*expressed as amount per mg of particles

#expressed as a percentage of the starting amount of peptide or protein used

The ability of synthesised particles to take up peptide or protein cargo was determined by incubation with a 3kD immunocontraceptive peptide-based vaccine candidate [17] or a 68kD proprietary protein-conjugate vaccine. Free thiol groups were confirmed to be present in both vaccines used (data not shown).Each particle type was found to absorb approximately 60-70% of their weight in peptide antigen and 10-20% of their weight in protein antigen over an incubation period of 24 hours (Table 2).

In order to accurately quantitate the amount of antigen that was covalently attached to particles, loaded particles were washed extensively and amino acid analysis performed to quantitate the amount of associatedprotein. It was found that the amount of antigen detected was inversely proportional to particle size with significantly larger amounts of peptide or protein associated on nanoparticles than on microparticles (Table 3) correlating with the amount of chloroacetyl groups present on these particles. Therefore although there were very little differences in the amount of cargo that can be absorbed by these particles, it appears that particle size may be an influential factor for the amount of antigen that can be covalently conjugated.

Table 3. Amount of chloroacetyl groups associated with particles

| Particle type | Amount of $ClCH_2CO$ groups (nmoles)* | Amount of conjugated peptide (μg)* | Amount of conjugated protein (μg)* |
|---|---|---|---|
| Microparticles | 241.6 nmoles | 17.2μg ± 5.9 | 14.3μg ± 3.46 |
| Nanoparticles | 485.0 nmoles | 25.3μg ± 2.9 | 58.6μg ± 6.1 |

*expressed as amount per mg of particles

### *1.2. Dendritic cell uptake of particles*

In order to determine if particles can be internalised by dendritic cells (DCs), non-antigen loaded particles were fluorescenated and uptake by DCs assessed *in vitro*. The results of the experiment (Figure 2A) demonstrate that uptake of fluorescenated particles by DCs occurred in a concentration-dependent manner. At all concentrations examined, both nanoparticles and microparticles show similar abilities to be taken up by dendritic cells. The intracellular localisation of particles was also confirmed by confocal microscopy which demonstrated the presence of fluorescent spherical bodies distributed within the cell interior (Figure 2B).

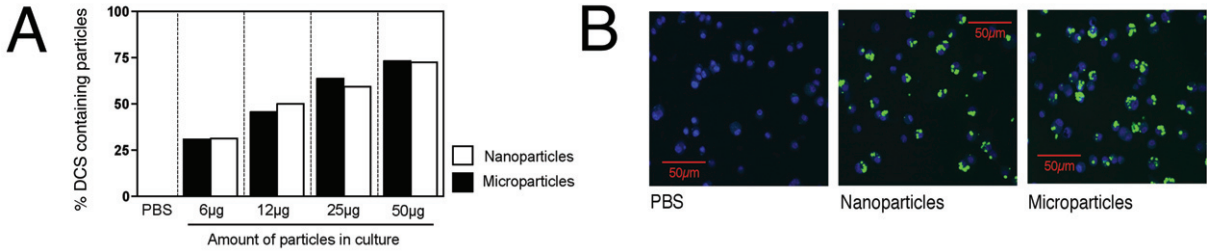


Figure 2. Uptake of fluorescenated nanoparticles and microparticles by dendritic cells. Bone marrow-derived DCs ($5\times10^5$ cells/ml) were incubated with varying amounts of fluorescenated microparticles or nanoparticles for 24 hours at 37°C. (A) Particles that were not associated with cells were removed by Ficoll Paque gradient separation and extracellular fluorescence was quenched using the dye trypan blue before analysis by flow cytometry. (B) Confocal micrographs of cells (nucleus stained in blue DAPI) which have taken up fluorescenated particles.

### *1.3. Immunogenicity of antigen-conjugated particles*

To evaluate the immunogenicity of vaccine-loaded particles, mice were inoculated with two doses of nanoparticles or microparticles that had been loaded with similar amounts of protein or peptide antigen. High titres of LHRH-specific antibodies were induced by vaccination using protein-loaded nanoparticles and microparticles but two doses were necessary to reach the levels that were achieved with CFA. The protein-based vaccine when administered in saline elicited very little if any antibody after a single dose of vaccine and levels of antibody that were only comparable to those achieved by single dose administration with particles.

In the case of the peptide-based vaccine (Figure 3B) a similar pattern of responses was observed although the reproducibility of the antibody response between animals receiving vaccine administered with particles was more variable than that observed when the protein-based vaccine was used. The variation in responses observed when peptide-based vaccine was used does not appear to be related to particle size because the mean difference in the range of antibody levels induced by each particle type was similar ($\log_{2.208}$ vs $\log_{2.362}$).

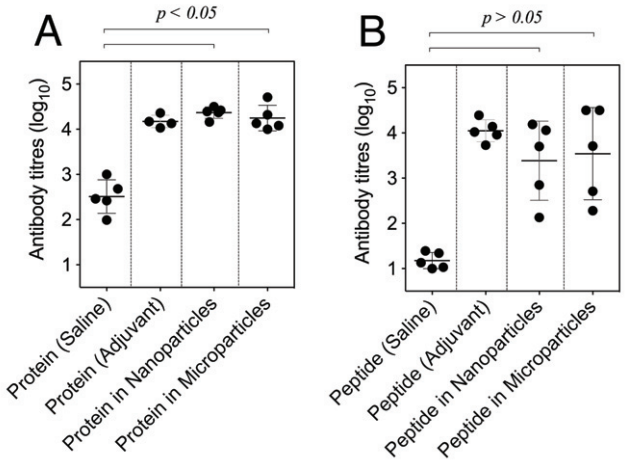


**Figure 6.** Immunogenicity of antigen-loaded nanoparticles and microparticles. Groups of BALB/c mice ($n$ = 5 per group) were inoculated sub-cutaneously at the base of the tail with microparticles or nanoparticles containing 25µg of protein (A) or 20 nmoles of peptide antigen (B) in a volume of 100µl. Mice were also inoculated with each antigen in saline only or antigen emulsified in CFA. Sera was obtained from blood taken 27 days after the primary (○) and 13 days following the secondary (●) inoculation. Antibody levels were then determined by ELISA.

## 3. Conclusion

Immunological approaches to controlling reproduction by modulating hormones such as LHRH represent a non-invasive and often effective form of endocrine therapy. While currently available LHRH-based vaccines require the co-administration of an adjuvant to induce effective responses, we have shown in this study that vaccination with chitosan-based nano- and microparticles that are loaded with LHRH-based peptide or protein antigens in the absence of adjuvant can elicit high titres of LHRH-specific antibodies at levels which have been previously associated with the inhibition of reproductive function [12, 18, 19]. More recently, the use of LHRH-based vaccines for reducing testosterone levels for the control of hormone-dependent malignancies in humans has also resulted in promising clinical outcomes [13, 15]. Given the biocompatibility of chitosan and its apparent adjuvanting effects as well as its amenity to chemical and physical modifications the approach to vaccine design that we have described here could represent another useful method for inducing immune responses against microbial, viral and tumorigenic protein antigens.

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