Development of a novel vaccine delivery system based on Gantrez nanoparticles

Sara Gómez[1], Carlos Gamazo[1], Beatriz San Roman[1], Christine Vauthier[2], Marta Ferrer[3], Juan M. Irache[1*]

[1] *Adjuvant Unit, Department of Pharmaceutical Technology and Microbiology, University of Navarra, 31080 Pamplona, Spain.*

[2] *Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie UMR CNRS 8612, Faculté de Pharmacie, Université Paris-Sud, 5 av. J.-B. Clément, 92296 Châtenay-Malabry, France.*

[3] *Allergy Department, Clínica Universitaria, 31080 Pamplona, Spain.*

*Corresponding autor:

Dr. Juan M. Irache

Centro Galénico. University of Navarra

Ap.177, 31080 Pamplona. Spain

Tel: +34 948 425600; Fax: +34 948 425649

e-mail: jmirache@unav.es

Abstract

The adjuvant capacity of a novel vaccine vector "Gantrez-nanoparticles" (NP) towards coated or encapsulated ovalbumin (OVA) was investigated. OVA nanoparticles were prepared by a solvent displacement method previously described. The protein was incorporated during the manufacturing process (OVA-encapsulated nanoparticles) or after the preparation (OVA-coated nanoparticles). The mean size of the different nanoparticle formulations was lower than 300 nm, and the OVA content ranged approximately from 67 µg/mg nanoparticles (for OVA-coated nanoparticles) to 30 µg/mg nanoparticles (for OVA-encapsulated nanoparticles). All the OVA-NP formulations were capable of amplifying the antibodies titres (IgG1 and IgG2a) in mice after a single subcutaneous inoculation with respect free OVA or OVA adsorbed to Alum. Furthermore, the elicited response was, for some formulations, predominantly Th1 subtype. Thus, the formulation that contained mainly the antigen inside, and with a low concentration of cross-linking agent, displayed the best potential to induce a Th1 response after 35 days post-immunisation. These results are highly suggestive for the use of Gantrez nanoparticles as an efficient antigen delivery system, especially when a long lasting Th1 cytokine response is required.

1. Introduction

The optimization of the efficacy of vaccines relies on the development of effective adjuvants including the antigen delivery systems. Alum (aluminium hydroxide) and MF59 (an oil-in-water emulsion containing metabolizable oil squalene and the surfactants Tween 80 and sorbitan trioleate Span 85) are currently the only adjuvants that have been approved in most countries for vaccine administration in humans [1]. Unfortunately, they produce sometimes adverse reactions, they are poor inducers of cell mediated immunity, and multiple injections are generally necessary to maintain an adequate level and duration of immunity [2]. The single dose administration is an important pilar of the "gold standard" for vaccination, and controlled release of encapsulated antigens from biodegradable polymeric vectors (micro or nanoparticles) or other types of formulations such as liposomes [3], virosomes [4-6], ISCOMs (immunostimulatory complexes) [7] or virus-like particles [8,9] may have here a key role. In this context, formulations based on PLGA copolymers, which include poly(lactide) homopolymer (PLA) and poly(lactide co-glycolide) copolymer (PLGA), have been approved for human use [10]. This kind of particulate vaccine delivery systems has a lot of advantages such as: i) the protection of the encapsulated active product against its enzymatic inactivation, ii) the increase of the stability of the material incorporated during the manufacturing process, transport and storage of such active product, iii) the improvement of the efficacy of the presentation to the antigen presenting cells (APC), and iv) the increase of the half life of the active product in the organism [11-13].

One of the main criteria in the choice of a vaccine depends on the sort of the immune response elicited. The vaccine should be able to induce an immune response resulting in protection, and here, the sort of the immune response is very relevant.

Generally, the type (Th1 or Th2) and duration of the immune response mainly depends on physicochemical properties of the adjuvant vector such as size, polymeric nature, release profile (stability of the vectors) and antigen location on the particle [14]. The summation of these physicochemical properties deeply affects the magnitude and the pathway of antigen presentations. Another important factor that can affect the antigen presentation is the complement activation and coating of the antigen loaded carriers. This property is also extremely related to the surface of the carriers [15].

Therefore, we describe here, for the first time, the adjuvant capacity of Gantrez nanoparticles using OVA as antigen model. The effect of OVA location (coating the surface or encapsulated into the matrix) as well as the effect of cross-linkage of nanoparticles on the antibody immune response was also studied. Gantrez AN is a copolymer of methyl vinyl ether and maleic anhydride which can easily react with amino groups. In this context, previous studies have demonstrated its efficacy to load or link different types of proteins, such as bovine serum albumin (BSA)[16] and flagellin[17].

2. Material and methods

2.1. Chemicals

Gantrez® AN 119 [poly(methyl vinyl ether-co-maleic anhydride); MW 200,000] was kindly gifted by ISP (Barcelona, Spain). Ovalbumin (OVA) (grade V), 1.3-diaminopropane (DP), 2,2´-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), alhidrogel and the anti C3 and C3b goat antiserum, were purchased from Sigma-Aldrich Chemie (Germany). The MicroBCA protein assay was supplied by Pierce (USA). The peroxidase immunoconjugates (GAM/IgG$_1$/PO and

GAM/IgG$_{2a}$/PO) were obtained from Nordic Immunology (The Netherlands). All other chemicals used were of reagent grade and obtained from Merck (Spain).

2.2. Preparation of Gantrez nanoparticles

Gantrez nanoparticles were prepared by a solvent displacement method previously described [16]. The model protein was incorporated during the manufacture process (OVA-loaded nanoparticles) or after the preparation of the adjuvant vectors (OVA-coated nanoparticles).

2.2.1. Preparation of OVA-coated Gantrez nanoparticles (NP I)

Gantrez copolymer 100 mg was dissolved in 5 mL acetone and poured into 20 mL of an ethanol: water phase (1:1 by volume) under magnetic stirring at room temperature. The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland). The freshly prepared nanoparticles (NP) were incubated with 10 mg ovalbumin in 5 mL of water for 1 h at room temperature under magnetic stirring. The resulting carriers were purified by centrifugation at 27,000 x g for 20 min. The supernatants were removed and the pellets resuspended in water. The purification procedure was repeated twice and finally, the formulations were freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5%) as cryoprotector.

2.2.2. Preparation of OVA-encapsulated Gantrez nanoparticles (NP II, NP III and NP IV)

Briefly, 5 mg OVA were dispersed in 1 mL acetone by ultrasonication (Microson™) for 1 min under cooling. The OVA dispersion was then added to 4 mL acetone containing 100 mg Gantrez and the mixture was stirred for 30 min at room temperature. Then, the polymer was desolvated by the addition of 20 mL ethanol: water phase (1:1 by volume). The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland). Some batches were cross-linked by incubation with either 5 µg or 10 µg 1,3-diaminopropane/mg copolymer (NP III and NP IV, respectively) for 5 minutes under magnetic stirring at room temperature. Non cross-linked (NP II) and cross-linked nanoparticles (NP III and NP IV) were purified by centrifugation and lyophilised as described above.

2.3. Characterisation of Gantrez nanoparticles

The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser doppler anemometry, respectively, using a Zetamaster analyser system (Malvern Instruments, UK). The samples were diluted with distilled water and measured at room temperature with a scattering angle of 90º. All measurements were performed in triplicate.

The morphological characteristics of the nanoparticles were obtained by scanning electron microscopy (LEO Electron Microscopy Inc, Thornwood, NY) operating at 3kV with a filament current of about 0.5 mA. Prior to observation, the nanoparticles were coated with a platinum laker of about 2 nm using a Cressington sputter-coated 208HR with a rotatory-planetary-tilt stage, equipped with a MTM-20 thickness controller.

The yield of the nanoparticles preparation process was determined by gravimetry from freeze-dried samples as described previously [16].

The quantification of the amount of associated ovalbumin to nanoparticles was determined using the microbichinchoninic acid (Micro BCA) protein assay. For this purpose, the nanoparticulate pellet, obtained after centrifugation, was digested with NaOH 0.1N for 24 hours at 4ºC. Then, the resulting solutions were analysed in a spectrophotometer at 570 nm. Calibration curves from 1 to 100 µg/mL of OVA (r^2>0.999) were performed using a control ovalbumin solution in NaOH 0.1N. Each sample was assayed in triplicate and results were expressed as the amount of ovalbumin (in µg) per mg nanoparticles. Similarly, the encapsulation efficiency (E.E.) was estimated as follows:

$$E.E. (\%) = (Q_{associated}/Q_{initial}) \times 100 \quad [Eq. 1]$$

where $Q_{initial}$ is the initial amount of ovalbumin added per mg of polymer that form the NP and $Q_{associated}$ is the amount of encapsulated OVA per mg of NP.

2.4. In vitro release study of ovalbumin from nanoparticles

OVA-loaded nanoparticles (8 mg) were dispersed in eppendorf tubes by vortexing in 1 mL phosphate buffer saline (PBS, pH 7.4). Release study was conducted at 37±1ºC under rotating agitation during 7 days. At defined times, the sample tubes were centrifuged at 26,500 x *g* for 20 min and the protein content was determined in the supernatants by micro-BCA assay and performed in a 96-well multiscaner autoreader (Labsystems iEMS Reader MF). Empty nanoparticles were used as control and

subjected to the same procedure. Release profiles were expressed in terms of cumulative release, and plotted versus time.

2.5. Immunisation studies

Animal protocols were performed in compliance with the regulations of the Ethical committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU).

BALB/c mice, females of 8 weeks old (supplied by Harlan Interfauna Ibérica, Spain), were randomized into seven groups of 5 mice. Animals were intradermally immunised with 10 µg OVA incorporated in one of the following formulations: i) OVA-coated nanoparticles (NP I); ii) OVA encapsulated in conventional nanoparticles (NP II); iii) OVA encapsulated in cross-linked nanoparticles with 5 µg DP/mg (NP III); iv) OVA encapsulated in cross-linked nanoparticles with 10 µg DP/mg (NP IV); v) OVA adsorbed in alhydrogel (OVA-Alum); vi) free OVA dissolved in sterile buffered saline solution; and, vii) empty Gantrez nanoparticles (NP).

Blood samples from the the retroorbital plexus were collected on days 0, 7, 14, 28 and 35 post-immunization. The samples were centrifuged (3000 x g, 10 min) and the resulting sera were pooled. Finally, each pool was diluted 1:10 in PBS and stored at -80°C until analysis.

2.6. Quantification of anti-OVA antibodies in serum

Specific antibodies against OVA (IgG_1 and IgG_{2a}) were determined in the pooled sera by indirect ELISA. Briefly, microtiter wells (cliniplatte EB, Labsystems, Finland)

were coated with 1 µg OVA in 100 µL sodium carbonate–bicarbonate buffer (0.05 M; pH 9.6) at 4°C for 15 h. The plates were washed with PBS-Tween 20 (1%) and serum samples were added in two-fold serial dilutions in PBS-Tween 20 (1%) starting with 1:40, and incubated at 37 °C for 4 h. After washing again with PBS-Tween 20 (1%), the plates were incubated, at 37ºC for 2 h, with anti-mouse IgG_1 or IgG_{2a} peroxidase conjugates diluted 1:1000 in PBS-Tween 20 (1%). The plates were washed and, finally, incubated with the substrate chromogen solution (H_2O_2-ABTS). The optical density (OD) was determined at λ_{max} 405 nm (iEMS Reader MF de Labsystems, Finlandia). Measurements were performed by triplicate and data were expressed as the reciprocal of a serum dilution whose optical density was 0.2 above blank samples.

2.7. Evaluation of complement activation by 2-D immunoelectrophoresis of C3

The complement activation in the presence of different nanoparticles was evaluated by quantification of the conversion of C3 into C3b by 2-D immunoelectrophoresis using a polyclonal antibody to human C3. This technique was performed by a modification of a protocol previously described by Labarre et al [18]. Briefly, human serum was obtained after calcifying plasma from healthy donors and stored at -80ºC until use. The electrophoresis buffer contained 63 mmol/L Tris, 27 mmol/L tricine, 1 mmol/L calcium lactate and 3 mmol/L sodium azide (pH 8.6) as described before by Nanjee et al [19]. Veronal buffer saline (VBS) containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} ions (VBS^{2+}) and VBS containing 40 mM ethylenediamine tetra-acetic acid (VBS-EDTA) were prepared as described previously [20]. For the experiment, 100 µL of an aqueous dispersion of nanoparticles (containing a surface area of nanoparticles of 10 cm^2) were incubated under gentle agitation for 1h at 37ºC with 50

μL human serum and 50 μL VBS^{2+}. The relative surface of nanoparticles was calculated from the average hydrodynamic diameters according to Vittaz et al [21]. After incubation, it was performed the first electrophoresis (600 V, 16 mA, 90 min) on 1% agarose gel and the second-dimension elecrophoresis (500 V, 12 mA, 18 h) on Gelbond® films in agarose gel plates which contained a polyclonal antibody to human C3 (anti C3 and C3b goat antiserum, Sigma, France). As a negative control it was used Human serum diluted in VBS-EDTA (1/4 v/v) and as a positive control Dex-An nanoparticles (Dextran nanoparticles made by anionic polimerization) incubated in serum diluted in VBS^{2+}. In order to analyze the spontaneous complement activation without any activators we used another control with serum diluted in VBS^{2+}. Gels were stained with Coomassie Blue (Sigma, France) and peaks height recorded. The results of the complement activation were expressed as a percentage of C3b detected on the plate regarding the sum of the peaks height of C3 and C3b.

2.8. Statistical analysis

The physico-chemical characteristics were compared using the Student t-Test. *P* values <0.05 were considered significant. For the evaluation of the complement activation, statistical comparisons were performed using the one-way analysis of variance test (ANOVA) and Tukey HSD test. *P*<0.01 was considered as a statistically significant difference. All calculations were performed using SPSS® statistical software program (SPSS® 10, Microsoft, USA).

3. Results

3.1. Characterisation of Gantrez nanoparticles

The main physico-chemical characteristics of Gantrez formulations are summarised in Table 1. The size of OVA-loaded nanoparticles was significantly higher than for empty nanoparticles (NP) (p<0.05). Thus, OVA-coated nanoparticles were found to be 2-times higher than empty nanoparticles. Besides, the size of OVA-encapsulated nanoparticles slightly increased with increasing amounts of cross-linking agent – e.g. from 205 (NP II) to 270 nm when using 10 µg DP/mg (NP IV). Their morphological characterization by SEM showed homogeneous populations of spherical-shaped particles with lower size than that obtained by photon correlation spectroscopy (Fig. 1). The coating of nanoparticles with OVA (NP I) or the use of the cross-linking agent (NP III and NP IV) significantly increased the negative charge of conventional nanoparticles (p<0.05).

Concerning the OVA content, it is interesting to note that, under our experimental conditions, the coating of nanoparticles enabled us to load 2-times more protein that the encapsulation process (i.e 68 vs 36 µg/mg). Furthermore, the cross-linkage process of nanoparticles (NP III and NP IV) also negatively affected the protein content and reductions of about 20% in the OVA entrapment were observed, comparing with NP II. Similarly, with increasing the DP concentration the nanoparticle yield decreased.

Table 1

Figure 1

Figure 2 shows the in vitro release of OVA from nanoparticle formulations in PBS buffer. For OVA-coated nanoparticles, the protein release profile was characterised by a latency period of at least 6 hours, in which less than 5% of the loaded protein was

released, followed by a pulse of release of about 40% of the OVA content. On the contrary, OVA-encapsulated nanoparticles (NP-II, NP-III and NP-IV) displayed a profile characterised by a burst effect of about 10-20% of the loaded protein followed by a sustained period of slow release. Furthermore, cross-linkage with increasing amounts of DP reduce the release rate of OVA.

Figure 2

3.2. Antibody response in BALB/c mice

Figure 3 shows the anti-OVA IgG_1 and IgG_{2a} titres (Th2 and Th1 markers, respectively) in sera after intradermal immunisation with the different formulations.

Concerning Th2 markers, all OVA-loaded nanoparticles (NP I, NP II, NP III and NP IV) displayed higher IgG_1 titres than the OVA-Alum group. In addition, all nanoparticle formulations appear to induce a similar profile of antibodies secretion, which was also different to that observe for the control group. This profile was characterised by a short lag-time, of about 1 week, followed by a rapid an intense period of anti-OVA IgG_1 secretion and, 14 days post-immunisation, a final and longer step of "plateau" of the antibody levels. At the end of the experiment (day 35), the highest IgG_1 titres were found for NP III and NP IV. In fact, cross-linked nanoparticles induced the production of 2-times higher levels of antibodies than NP II or 3-times higher than OVA-coated nanoparticles.

Furthermore, Gantrez nanoparticles were able to induce significative Th1 responses, characterized by the IgG_{2a} isotype, however some differences were observed among them. Thus, for NP II and NP III, the lag-time was of at least 7 days, whereas for NP I and NP IV, this perod was of at least 14 days. On the other hand, 35 days post-

immunization, NP I and NP II, appeared to reach a plateau, whereas NP III and NP IV were still increasing their antibody levels. On day 35, NP II displayed a IgG_{2a} titer which was found to be 2-times higher than for NP II and 5-times higher than for NP I.

Figure 3

3.4. Activation of C3 in the presence of the nanoparticles

Figure 4 shows the percentage of C3b (activated C3) as an indicator of the complement activation induced by the nanoparticles after incubation in human serum containing divalent ions.

VBS-EDTA and serum incubated with divalent ions (VBS^{2+}) were used as negative controls. All Gantrez nanoparticles were found to have a higher ability to activate the complement comparing with nanoparticles reported previously to be good activators (Dex-An nanoparticles, described previously as strong activators, were incubated with a surface of 1000 cm^2 [18] whereas in the present study they were incubated at 10 cm^2). Nevertheless, under the experimental conditions described here, no significant differences between the different formulations tested (NP, NP I, NP II and NP IV) were found ($p < 0.001$).

Figure 4

4. Discussion

Vaccination and immunotherapy are basic priorities for human and animal health, and a large number of adjuvants are therefore under investigation. Basically, polymeric adjuvant delivery-systems are able to get a prolonged antigen exposure by

facilitating the antigen uptake by the immune system cells. As a consequence, the delivery of encapsulated antigens results in more efficient immune activation than the equivalent amounts of free antigens [22-24]. Here, we describe the use of a novel vector-adjuvant based on nanoparticles of the polymer Gantrez, which is biocompatible and has been proposed as drug carrier for pharmaceutical applications[16,25]. The use of this copolymer led us to increase dramatically the immunogenicity of a soluble protein like OVA used here as an antigen model. In fact, this is the first description on literature of the use of Gantrez polymer for adjuvant purposes.

The choice of an adjuvant depends on the kind of immune response required to treat pathogenic infections and immunological disorders. On the other hand, the sort of the immune response generated depends on the intracellular fate of the antigen. Thus, exogenous antigens may lead towards a T lymphocyte-MHC II restricted antigen presentation; in contrast, antigens delivered intracellularly tend to elicit a MHC I restricted presentation. Another critical preliminar consideration concerns the main branches of the immune response based on the different Th subsets activation. Th1 cells release predominantly IL-2 and gamma interferon (IFN-γ) in response to the antigenic stimulus, activating the named "cellular" immune response. In contrast, Th2 cells release predominantly IL-4 and IL-5, inducing the "humoral" immune response. In practical means, the generation of a predominantly Th1 response is associated with resistance to intracellular pathogens and allergic processes. The response after Th2 activation is, by contrast, more appropriated for the effective control of extracellular pathogens and autoimmune diseases. Accordingly, in order to induce a different presentation to the immune system cells, and hence, to study the elicited immune response, formulations containing OVA protein either coating the surface or included into nanoparticles of Gantrez were prepared.

First of all, OVA loading percentage data demonstrated the efficiency of the method of preparation used here to get a different physical distribution of that antigen within the particle (OVA coating or encapsulation). All the nanoparticles displayed a similar and homogeneous size (See Table 1 and Figure 1), being slightly higher for NP I (nanoparticles with OVA coating the surface) than for OVA-encapsulated formulations (NP II, NP III and NP IV) (See Table 1). The scanning electron microscopy of freezed-dried formulations showed that the size of the nanoparticles was slightly lower than the one measured by photon correlation spectroscopy, what could be due to the swelling of the particles in aqueous media forming a "sponge" structure. This fact has been previously described for legumin nanoparticles by Mirshahi et al [26]. On the other hand, as it was expected, the outer coating with OVA (NP I) rendered more electronegative particles with respect the ones that were formulated with OVA encapsulated. This fact demonstrated that in NP I, the OVA coated the nanoparticle surface. The zeta potential was also more negative as long as the amount of cross-linking agent increased.

Although OVA loading on NP I formulation was higher than OVA encapsulation in the other formulations (NP II, NP III and NP IV), the encapsulation efficiency was almost the same for all formulations. The yield of the process was also similar for the different nanoparticles, being approximately 80% as described previously by Arbos [16].

The in vitro release studies, demonstrated that, as expected, the OVA release from NP I formulation (OVA coating the surface) was faster than from NP II, NP III and NP IV (OVA encapsulated) [27,28].

This fact can be easily related to the immune response. In this context, we observed that the elicited immune response after NP I administration was of lower

intensity than for the other formulations (See Figure 3). Thus, we can estimate that most of the elicited antibody response by NP I was due to the OVA released just after the inoculation. These results suggest that both physical distribution of OVA and the consequent controlled release of the antigen exert an effect in the immunostimulating capacity of the nanoparticles.

On the other hand, cross-linkage with increasing amounts of DP slightly diminished the OVA in vitro release from the nanoparticles. Concerning in vivo results, some differences were found. For IgG1 titres, cross-linked nanoparticles slightly increased the antibody levels. On the contrary, IgG2a titres increased more rapidly for NP II (non cross-linked nanoparticles) and NP III (cross-linked with 5 µg DP/mg) than for NP IV (cross-linked with 10 µg DP/mg). In fact, for this last formulation, no presence of this antibody isotype was observed before 2 weeks post-administration. In addition, 5 weeks post-administration, the levels of antibodies of the animals immunized with NP III continued to increase whereas for NP II, the levels of antibodies reached a plateau. All of these results may suggest than an optimal cross-linking concentration is required.

Sustained presentation of antigen by these systems could be the decisive factor in inducing long lasting immune responses. Maximizing a desired response by controlling delivery pathways is crucial in vaccine development and immunotherapy. It has long been suggested that the sustained release characteristics of the antigen delivery system, related to the "depot effect" contribute to the immunogenicity-enhancing effect of adjuvant by producing a prolonged immunological response [23,29]. However, in this case, Th2 would be expected to predominate in response to a free-released OVA. On the contrary, our data on a selective Th1 response suggest that the associated antigen-Gantrez-nanoparticles, particularly those cross-linked with DP, is more important than a

depot effect. The potent, long-lasting immune responses induced after a single injection of antigen-loaded NP III may be due to its efficient uptake by dendritic cells (DCs), efficient antigen processing and sustained presentation to T-helper cells. However, the use of Gantrez copolymer, diaminopropane as cross-linking agent and physical location of the antigen may contribute to its presentation to APCs (antigen presenting cells).

The complement activation data demonstrated that Gantrez nanoparticles can strongly activate the cleavage of C3 into C3b. This fact was corroborated comparing Gantrez particles with Dex-An nanoparticles, used as positive control. These nanoparticles were described previously as strong activators when incubated with a surface of 1000 cm^2 but their capacity to activate complement was considerably reduced for the such low surface area used in the present work (10 cm^2)[18]. As well known, complement activation induces phagocytosis by macrophages [30], thus, this strong activation of Gantrez nanoparticles could enhance the particle uptake by APCs. But the direct or indirect roles in the activation of APCs and induction of Th1/Th2 mediated immunity remain to be determined.

Finally, as a conclusion of this work, it can be remarked that Gantrez AN nanoparticles seemed to be a strong adjuvant. In this context, the titres elicited by intradermal administration of 10 µg of OVA in combination with the new nanoparticle-adjuvant were found to exceed titers induced by immunization with the same amount of antigen. Furthermore, the induced titers were elicited with a relatively smaller amount of antigen used by other autors[31-33], supporting the potential feasibility of our system. Further benefits of this new antigen delivery system are currently being studied in vaccination and immunotherapy in animal models.

Acknowledgements

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Development of a novel vaccine delivery system based on Gantrez nanoparticles

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**Table 1**. Physico-chemical characteristics of Gantrez nanoparticles. Data were represented by mean ± SD (n=10).

	Size (nm)	Zeta Potential (mV)	OVA content (μg/mg)	Encapsulation efficiency (%)	Yield (%)
NP	158 ± 3	-45.1 ± 0.5	_____	_____	85.3 ± 0.8
NP I	300 ± 4	-61.3 ± 3.8	67.8 ± 20.2	47.5 ± 2.1	87.2 ± 0.6
NP II	205 ± 1	-41.4 ± 2.5	36.1 ± 3.8	50.5 ± 5.3	83.9 ± 0.8
NP III	239 ± 4	-50.8 ± 2.9	30.1 ± 4.5	42.1 ± 6.3	78.2 ± 1.1
NP IV	270 ± 2	-57.5 ± 3.1	31.4 ± 4.2	44.1 ± 5.9	74.9 ± 0.2

NP: empty nanoparticles; **NP I**: OVA-coated nanoparticles; **NP II**: OVA encapsulated in nanoparticles; **NP III**: OVA encapsulated in cross-linked nanoparticles with 5 µg DP/mg; **NP IV**: OVA encapsulated in cross-linked nanoparticles with 10 µg DP/mg.

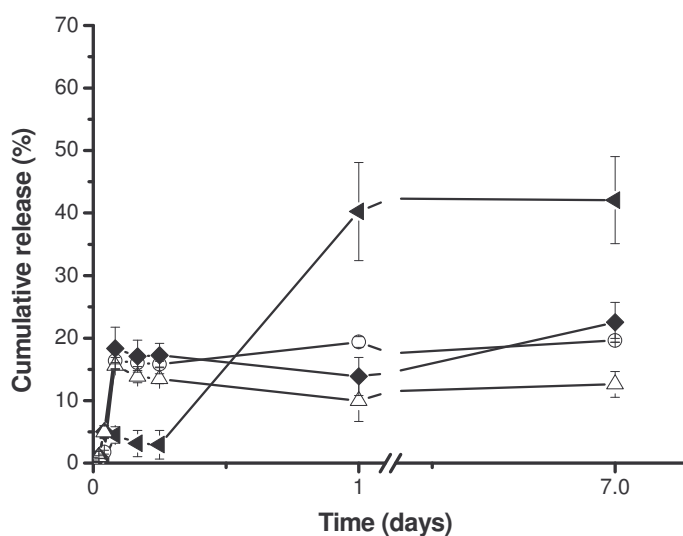# Development of a novel vaccine delivery system based on Gantrez nanoparticles

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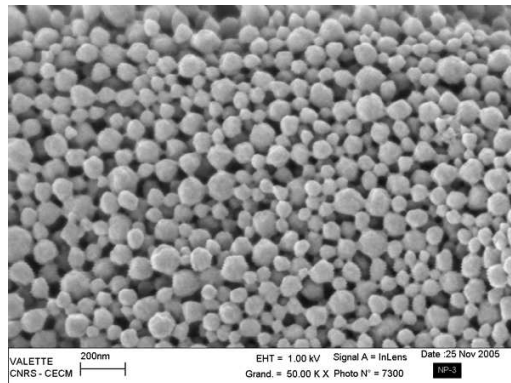
Figure 1. Scanning electron microscopy photograph of NP III (OVA encapsulated in cross-linked nanoparticles with 5 µg DP/mg).

# Development of a novel vaccine delivery system based on Gantrez nanoparticles

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Figure 2. In vitro release of OVA from Gantrez formulations in PBS. **NP I** (◄): OVA-coated nanoparticles; **NP II** (○): OVA encapsulated in nanoparticles; **NP III** (♦): OVA encapsulated in cross-linked nanoparticles with 5 µg DP/mg; **NP IV** (△): OVA encapsulated in cross-linked nanoparticles with 10 µg DP/mg. Data express the mean of the cumulative amount of OVA released vs. time (mean ± SD, n=3).

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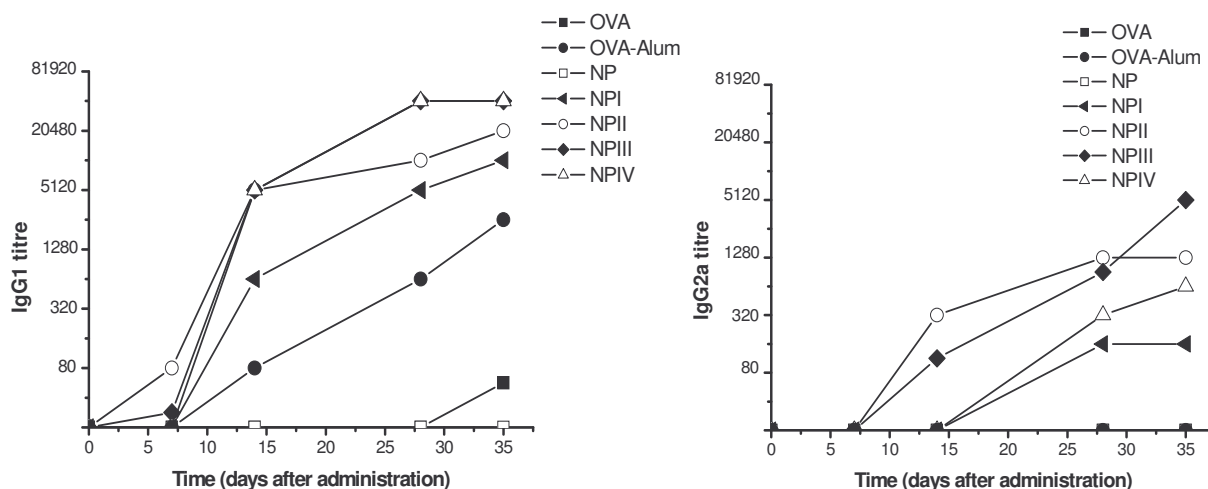
Figure 3. Anti-OVA IgG$_1$ and IgG$_{2a}$ titres in sera after intradermal immunisation with: ovalbumin solution (OVA) (■), ovalbumin adsorbed in alhydrogel (OVA-Alum) (●), blank nanoparticles (NP) (□), ovalbumin coated nanoparticles (NP I) (◄), ovalbumin encapsulated nanoparticles (NP II) (○) and cross-linked ovalbumin encapsulated nanoparticles (NP III (♦) and NP IV (△)). The antibody titre is defined as the reciprocal dilution giving an optical density. Female BALB/c mice were immunised intradermally with 10 µg ovalbumin as follows: i) OVA in solution; ii) OVA adsorbed in alhydrogel (OVA-Alum); iii) OVA coated nanoparticles (NP I); iv-vi)
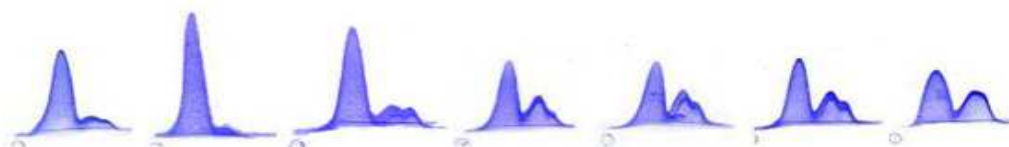
OVA encapsulated in nanoparticles (NP II), and nanoparticles cross-

linked with DP (NP III and NP IV); and vii) empty nanoparticles (NP).

 (OD) reading at 405nm of ≥0.2.

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	Serum VBS-EDTA	Serum VBS^{2+}	Dex-An	NP	NP I	NP II	NP III
% C3b	6.2 ± 2	12.7 ± 0.4	14.5 ± 4.7	31.1 ± 1.4	34.1 ± 1.1	32.5 ± 0.1	35.8 ± 2

Figure 4. Percentage of C3 activated (% C3b) measured by 2-D immunoelectrophoresis after 60 min incubation of 10 cm^2 of samples with 50 µL of human serum at 37ºC. The relative amounts of C3 and C3b are indicated by the first and second peak respectively. The samples were as the following: Serum VBS-EDTA (negative control), Serum VBS^{2+} (experimental conditions), Dex-An (nanoparticles previously reported as strong complement activators by Labarre et al [18]), and Gantrez AN nanoparticles (NP, NP I, NP II and NP III). (mean ± SD, n=3). *** p<0.001 for Gantrez AN nanoparticles vs Dex-An.