# Poly-methyl vinyl ether-co-maleic anhydride nanoparticles as innate immune system activators

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## ABSTRACT

Adjuvant research is being oriented to TLR-agonists, but complement activation has been relatively unexplored. In previous studies it was demonstrated that poly-methyl vinyl ether-co-maleic anhydride nanoparticles (PVMA NPs) used as adjuvant differentially activate dendritic cells through toll like receptors (TLR) stimulation, however, a high dose of NPs was used. Now, we demonstrated a dose-response effect, with a concentration as low as 20 μg/mL able to stimulate TLR2 and TLR4 transfected dendritic cells. In addition, we investigated whether these NPs are able to exploit also the immunomodulatory benefits of complement activation. Results indicated that the hydroxilated surface of these NPs highly activated the complement cascade, as measured by adsorption studies and a complement fixation bioassay. Stable binding of C3b to NPs was confirmed as indicated by lability to SDS treatment after washing resistance. Complement consumption was confirmed as the lytic capacity of complement exposed to NPs was abolished against antibody-sensitized sheep erythrocytes, with a minimal inhibitory concentration of 50 μg NPs, equivalent to a surface of 1 $cm^2$. On the contrary, NPs of the copolymers of lactic acid and glycolic acid (PLGA), used as a reference, did not consume complement at a concentration ≥3 mg NPs (≥40 $cm^2$). Complement consumption was inhibited when PVMA NPs were reticulated with diamino groups, indicating the role of hydroxyl groups as responsible of the phenomenon. These results favour a model whereby PVMA NPs adjuvant activate complement on site to attract immature antigen presenting cells that are activated through TLR2 and TLR4.

**Keywords:** nanoparticles, adjuvant, TLR-agonist, complement activation

**Running Title**: Nanoparticles as innate immune system activators

## 1. INTRODUCCION

There is a critical need for safer vaccines. Synthetic or subunit vaccines display important advantages to face the classical live attenuated vaccines handicaps. However, adequate adjuvants must be associated to promote innate immunity and the subsequent right induction of the adaptive immune response. In this context, pattern recognition receptors (PRR), like toll-like receptors (TLRs) and complement proteins are sentinel systems that can be activated rapidly and act synergistically in order to regulate and potentiate the development of the immune response. Thus, strategies for adjuvant technology research have recently focused on TLR activation; nevertheless, complement activation by adjuvants is still relatively unexplored.

Innate immunity can easily be triggered by stimulating TLRs and will lead to strong adaptive immunity. There are many examples of developing vaccines against tumors, allergy or infectious diseases that contain adjuvant combinations based on the use of pathogen-associated molecular patterns (PAMPs, like MPL, flagellin or CpG domains) as PRR-agonists (e.g., MF59 or AS series adjuvants). [1-3]. However, TLR-agonists vaccine adjuvants could induce toxicity [4], and other factors, such as economic costs, may limit their exploitation.

Biodegradable particles represent a strong option as adjuvant systems, showing many advantages since they address the essential issues of the efficient delivery of antigens to antigen presenting cells (APCs) and the subsequent cell activation to trigger adaptive immunity. Other mechanisms underlying their capacity to stimulate the immune system are being elucidated, such as their own ability to stimulate dendritic cells (DC) by TLR activation [5;6].

Among the different types of particulated delivery systems, polymer nanoparticles (NP) are a group of delivery systems with interesting abilities as adjuvants for both conventional and mucosal vaccination [7]. Polyesters and polyanhydrides are the two most widely studied biodegradable materials for controlled release of antigens. Copolymers of lactic acid and glycolic acid (PLGA) have been widely utilized in biomedical applications including as immunoadjuvants [8-10]. However, in order to obtain strong and lasted immune responses, some of these investigations have included the co-encapsulation of a TLR agonist in the particle [11] while others used multiple injection regimens in vivo [12]. Furthermore, as the polyester degrades, an acidic microenvironment is created by the lactic or glycolic acid that may be detrimental to the stability and immunogenicity of the encapsulated antigens [13]. In contrast, polyanhydrides are surface erodible polymers that break down into carboxylic acids, which are less acidic than those of polyesters, non-mutagenic and non-cytotoxic, and have been widely used as carriers for controlled delivery of antigens [13]. Poly-methyl vinyl ether-co-maleic anhydride (PVMA), or poly(anhydride), is a co-polymer of methyl vinyl ether with maleic anhydride. PVMA NPs can develop strong biohesive interactions with components of the gut mucosa [14]. Besides, the incorporation of antigens into these bioadhesive nanoparticles has demonstrated to enhance the immune responses in terms of a potent Th1-adjuvant capacity [15;15-17]. Recently, our attempt to elucidate the mechanisms that underlay the adjuvant effect of PVMA NPs was partially described. Our results revealed that PVMA NPs act as agonists of various TLRs, mainly TLR2 and TLR4 [5]. However, there was a concern since the high concentration used in that study was toxic for some cell lines. Here, we studied the minimal concentration of PVMA NPs able to

activate TLR2 and TLR4 transfected dendritic cells.

In addition, another important factor that can affect the antigen presentation is the complement activation that, in turn, depends on the surface characteristics of the antigen-loaded carriers. Traditionally, biomaterials have been studied in order to check their un-availability to activate complement, in order to minimize inflammation. However, complement activation could be exploited to generate the appropriate immune response. PVMA NPs offer hydroxylable groups under physiological conditions, offering a potential active surface for complement activation. In order to evaluate it, we determined the complement activation by NPs in vitro. Our findings demonstrated that the PVMA NPs, but not PLGA NPs, were strong activators of the complement system.

## 2. MATERIAL AND METHODS

PVMA NPs were prepared by a modification of the solvent displacement method [18]. Briefly, 100 mg PVM/MA copolymer [poly (methylvinylether-co-maleic anhydride), M.W. 200,000, International Speciality Products, Barcelona, Spain)] were dissolved in 6 mL acetone for 30 min under magnetic stirring at 300 rpm. The acetone/copolymer mixture was poured into a solution containing 0.2 g mannitol in 5.8 mL of dd water. All solvents were eliminated under reduced pressure by Spray drying Büchi B191 (Büchi Labortechnik AG, Switzerland) as described previously [19]

When indicated, nanoparticles were reticulated, that is, cross-linked with 1,3-diaminopropane in order to partially neutralize hydroxyl groups covering NPs. When nanoparticles are incubated with 1,3-diaminopropane, the cross-linking agent easily react with the anhydride groups of the copolymer forming links between these functional groups [18], and the number

of hydroxyl groups in the cross-linked nanoparticles would be lower than for non-reticulated ones. Briefly, the freshly prepared carriers were reticulated by incubation at room temperature for 5 min with 5 mg 1,3-diaminopropane/mg bulk polymer.

Polylactide-co-glycolide (PLGA) nanoparticles were prepared, as a reference control,by a water-oil-water solvent evaporation technique [20]. Briefly, the polymer (Boehringer Ingelheim, Germany).was dissolved in ethyl acetate and mixed by ultrasonication (Branson sonifier 450, Branson Ultrasonics corp., Danbury, USA) under cooling for one minute, to form a $W_1/O$ emulsion. This inner emulsion was added to 2 mL PVA 1% ($W_2$) and homogenized by ultrasonication. The resulting $(W_1/O)W_2$ emulsion was poured to PVA 0.2% and continuously stirred for, at least, 3 h at room temperature to allow solvent evaporation and particle formation. After preparation, nanoparticles were isolated by centrifugation (12,000 x g, 30 min), washed 3 times with dd water and lyophilized.

***Nanoparticle characterization***

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, at 25ºC (Malvern Instruments, Malvern, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1/10) and measured at 25 ºC with a dynamic light scattering angle of 90º. The zeta potential was determined as follows: 200 µL of the samples were diluted in 2 mL of a 0.1 mM KCl solution adjusted to pH 7.4 [5]. All measurements were performed in triplicate, and the average particle size was expressed as the volume mean diameter ($V_{md}$) in nanometers (nm), and the average surface charge in milivolts (mV). The yield of the nanoparticles preparation process was determined by gravimetry from freeze-dried nanoparticles as described previously [21].

The relative surface of nanoparticles was calculated from the average hydrodynamic diameters.

***Scanning electron microscopy and transmission electron microscopy***

The shape and morphological characteristics of both micro and nanoparticleswere obtained by scanning electron microscopy (LEOElectron Microscopy Inc., Thornwood, NY) operating at3kV with a filament current of about 0.5 mA. Prior toobservation, the nanoparticles were coated with a platinum laker of about 2 nm using a Cressington sputter-coated208HR with a rotatory-planetary-tilt stage, equipped witha MTM-20 thickness controller.For this purpose freeze-dried vaccine formulations were suspended in ultrapure water and centrifuged at 27,000 x g for 20 min at 4 ºC. Then, supernatants were rejected and the obtained pellets were mounted on a glass plates adhered with a double-sided adhesive tape onto metal stubs, coated with gold to a thickness of 2 nm (Emitech K550 equipment, United Kingdom).

***Toll like receptors stimulation by Nanoparticles***

PVMA NPs were tested by their capacity to stimulate TLR signaling. Briefly, samples and controls were tested on recombinant HEK293 cell lines that functionally overexpress TLR2 or TLR4 protein as well as a reporter gene which is a secreted alkaline phosphatase. The TLR2 and TLR4 clones were generated by stably transfecting HEK293 cells with the pNifty plasmid, a NF-κB inducible plasmid expressing the secreted alkaline phosphatase reporter gene. Thus, the production of the secreted alkaline phosphatase gene is driven by the NFκβ inducible promoter. Clones were grown in complete DMEM medium with 10% FBS supplemented with blasticidin (10 μg/ml) or blastidicin (10 μg/ml) plus higromicin (50 μg/ml), for TLR2 or TLR4 clones, respectively. Nanoparticles where incubated at different

concentrations from 20 to 500 μg/mL, the highest non-toxic concentration. Known TLR agonists were used as positive controls, including the synthetic lipoprotein Pam3CSK4 (Invivogen, San Diego, CA) (100ng/ml) for TLR2 and *Escherichia coli* K12 LPS (1μg/ml) for TLR4. All results were given as optical density.

***Evaluation of Complement adsorption by Nanoparticles***

The complement adsorption by NPs was evaluated by SDS-PAGE and further immunoblotting using apolyclonal antibody to C3 (Sigma, Madrid, Spain). Briefly, 1 mL of an aqueous dispersion of 1 mg nanoparticles was incubated under gentle agitation for different times at 37 ºC with 1 mg guinea pig complement (Biomerieux, France). After incubation, samples were centrifuged at 10.000 x g, 15 min. The supernatant was recovered for analysis, and the sediment containing NPs was washed three times with saline solution. Then, the sediment was resuspended in 1 mL of SDS-mercaptoethanol and boiled for 10 min. Supernatant and treated sediment were analyzed by SDS-PAGE and immunblotting.

SDS-PAGE was performed in 12% acrylamide slabs (Criterion XT, Bio Rad Laboratories, CA) with the discontinuous buffer system of Laemmli and gels stained with Coomassie blue. Immunoblotting was carried out with a serum from goat anti-C3 (Sigma-Aldrich).

***Evaluation of Complement depletion by a lysis mediated bioassay***

Complement consumption was confirmed by measuring the residual hemolytic capacity of the complement system after incubation with NPs. Double dilutions of NPs resuspended in veronal buffer (5 mM sodium barbital, 142 mM NaCl, 3.7 mM HCl, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) were added to the same volume of complement (previously titered for hemolysis) and incubated at 37 ºC for 60 min under gentle agitation. Sheep erythrocytes were

sensitized with specific antibodies and resuspended to a final concentration of $10^8$ cells/ml in veronal buffer. This suspension was used as bioindicator of the complement not-consumed by nanoparticles. Thus, sensitized erythrocytes were incubated at 37 ºC for 60 min with NPs previously exposed to complement. Lysis of the cells was directly correlated with the presence of residual active complement after contact with the nanoparticles. The bioassay was conducted in duplicate in three independent experiments. Results were expressed as the minimal concentration of nanoparticles able to avoid the lysis of the opsonised erythrocytes.

**Measurement of Endotoxin Activity**

In order to discard the possibility that PVMA polymer present LPS as contaminants that could be partially responsible for the TLR activating properties, the endotoxin activity of the polymer (0.5 mg/mL) was determined using the LAL assay kit (BioWhittaker, Walkersville, MD) according to the manufacturer's recommendation.The lower detection limit of the test used was 0.1 EU/ml.

**Statistical Analysis**

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

### *Nanoparticles characterization*

Table 1 summarizes the main physicochemical properties of nanoparticles. NP formulations displayed homogeneous volume mean diameter of 149 ± 2 nm with low polydispersity index. PVMA NPs displayed an electronegative surface charge with Zeta potential values of –49 ± 2 mV, that diminished after reticulation with diaminopropane (-37 ± 1mV).

### *Nanoparticles-stimulated Toll-like receptors*

In order to evaluate the capacity of NPs to trigger a TLR response, recombinant dendritic HEK293 cell lines overexpressing TLR2 or TLR4 were used. As shown in figure 1, when these cells were incubated with the LPS-TLR4 agonist or PAM2-TLR2 agonist, repectively, the increase in phosphatase alkaline activity was signficantly higher compared with non-stimulated cells. Similarly, when incubated with PVMA NPs, TLR2 and TLR4 expressing cells resulted significantly activated in a dose dependent manner, being the minimal concentration used (20 μg/mL) TLR-stimulant with respect basal level.

### *Evaluation of complement consumption*

Poly(anhydride) NPs were found to have a high ability to adsorb complement. Figure 2 shows a representative result obtained after incubation of complement with PVMA NPs (reticulated and non reticulated with diaminopropane) or PLGA NPs. Figure 2A shows the supernatants, i.e, proteins do not attached to the NPs, and panel B shows the proteins covalently attached to the NPs (see material and methods). By comparisons, Fig. 2 lane shows the protein profile of the complement sample used at 10 and 30 μg (lanes 1 and 2, respectively). The 75-kDa band

corresponds to the ß-chain of C3 (C3ß), not cleaved during activation. C3α presents an AMW of 120 kDa under reducing conditions. The protein profile depicted for PLGA NPs (lanes 6 and 8) was identical to the one shown by free complement (lanes 1 and 2). In contrast, the intensity of the proteins from samples containing PVMA NPs was lower. Furthermore, reticulation of PVMA NPs affected the attachment of proteins (lanes 3-4 vs. 6- 7). A time-effect was also observed, being the effect of adsorption after 10 min of incubation higher than that observed after 5 min incubation (lane 7 vs. 6).

The stable binding of C3b to PVMA NPs was demonstrated by releasing of C3b after treatment of NPs with SDS under reducing conditions and 100 ºC, where covalent ester bonds could not be maintained. Upon activation, a small fragment of the C3α-chain is cleaved and released, resulting in the reactive C3b of 110-kDa. This activation results in the exposure of a reactive thiolester domain, which can then bind to the target via a covalent linkage. Our study was performed under reductive conditions with mercaptoethanol, so, the major surface-bound C3 fragment is iC3b that will run with AMW of 68 Kda and 49 kDa, corresponding to the fragments iC3b α´1 and iC3b α´2, respectively. In fact, after 10 min of incubation, the most prominent protein attached to PVMA NPs was iC3b (Fig. 2B, lane 7). However, reticulated PVMA NPs did not show this major band (Fig. 2B, lane 6), confirming the results obtained with the supernatants (Fig. 2A).

Complement consumption by PVMA NPs was confirmed with a bioassay, using antibody-sensitized sheep erythrocytes, susceptible to be lysed by free complement. Thus, the lytic capacity of the serum exposed to nanoparticles was abolished with a minimal concentration of 50 µg NPs, equivalent to a surface of 1 $cm^2$. When the serum was not previously exposed to the nanoparticles, no complement consumption occurred, and the lysis of erythrocytes was complete. In contrast, PLGA NPs exhibited a worthless level of complement proteins adsorption, nor complement fixation activity (≥3 mg NPs, equivalent to ≥40 $cm^2$).

***Discussion***

Considering the current knowledge on adjuvants mechanisms of action, a possible scheme for a rational vaccine may well consist in the use of multiple adjuvants acting in synergy. One of the most interesting approaches might be based on polymers acting as TLR-agonists and complement activators as an alternative to the microbial derived adjuvants, such as microbial derived components, as PAMPs. It has been described that TLRs on DCs have evolved to recognize and react to hydrophobic portions of PAMPs [22]. For that reason, the use of hydrophobic polymers as both adjuvant and delivery system are being explored. Polyanhydrides based on hydrophobic moieties such oligomeric ethylene glycol-containing anhydrides or methylvinylether-co-maleic anhydrides have been shown to modulate the immune response [13-17;23-26]. We have previously shown that antigenic complexes loaded PVMA NPs shift the immune response from Th2 to Th1 [14-16]. Usually, induced by internalization of pathogens through TLRs, DCs induces the naive T cells to differentiate into IFNγ-secretingTh1 T cells [27]. Therefore, to gain knowledge on the biological properties of PVMA NPs in the context of activation of innate immunity, we studied its capacity to interact and activate DCs by TLR-stimulation. When the potential of NP to act as TLR ligands was screened, we observed a high ability to stimulate TLR2, and in a lesser extent TLR4 signaling. The absence of TLR-minus cell line activation suggested that specificity should drive the activation [5]. However, a high dose of NPs were used (500 μg/mL), therefore, in order to preclude some unspecific effect, lower concentrations of NPs were tested. Results confirmed a positive TLR2 and TLR4 stimulation, with a minimal activating concentration of 20 μg/mL.

TLR4 binds to the LPS of the outer membrane of Gram-negative bacteria, and TLR2 recognizes and signals bacterial lipoproteins, peptidoglycans, and lipoteichoic acid from Gram-positive bacterial cell walls. Agonists for TLR4 may be related to TLR2 agonists since both may require acylated saturated fatty acids in their molecules. Both TLR appears to contain several regions within the molecule that might contribute to the recognition of specific agonists. Thus, some kind of promiscuity has been related for both ligands [28], including the recognition of some other polymers used in nanotechnology [29;30]. Hence, computational molecular modeling studies will enable rational insight with respect to the ability of PVMA NPs to stimulate DC through TLR recognition [31].

Complement research is throwing new lights into the integration of innate and adaptive immune responses [32]. Classically, opsonization by complement proteins was considered to play a critical role in the uptake of foreign particles, and so, to initiate innate and adaptive immunity. However, nowadays, complement is being described as a key player for optimal T cell function [33]. Therefore, we were interesting to study if complement was related with the high adjuvant capacity of the PVMA NPs. In order to determine NP-complement interactions, several assays were performed in vitro, including SDS-PAGE analysis of C3b deposition on NPs, and measuring the hemolytic activity on sensitized sheep erythrocytes. With independence of these in vitro experimental systems, results demonstrate that PVMA NPs shown to be strong complement fixing surfaces.

Complement may be initiated by three different pathways after activation by different systems. Thus, the classical pathway is initiated by antigen-antibody interaction; the lectin pathway is initiated by lectin-polysaccharide, and the alternative pathway is initiated spontaneously but activated by contact with a variety of surfaces, such as carbohydrate structures on microorganisms and other surfaces. Particles, like liposomes, depending on

composition and other physicochemical properties, can activate complement via alternative pathway, thereby leading to enhanced uptake by APCs [34]. The alternative pathway is spontaneously activated and C3b is continually generated, but at a low rate and with a short live, unless it is covalently attach to an activating surface, recruiting then the serum factor B which is a substrate for the protease factor D, generating the active C3 convertase (C3bBb). In our hands, PVMA NPs were active in adsorbing complement, and in producing C3b. In any case, we used complement fixation bioassay since deposition of complement proteins on particles does not mean activation. For instance, in some cases, following complement activation, C3 may deposit on the surface in a non-covalent manner, a process that may not lead to complement activation. However, the right active surfaces, strongly activate C3, leading the opsonic products C3b and iC3b, that are expected to be linked covalently to the particle surface via their highly reactive thioester moiety. This was the case of PVMA NPs, where C3b was covalentely attached to the NPs since it was necessary boiling in SDS-mercaptoethanol to release it. It is then plausible to suppose that alternative pathway can be initiated after contact with NPs.

The strength of protein adsorption to the NPs surface depends not only on the protein type but also on the chemical composition of the particle or the conformational characteristics of the surface. Hydrophobic surfaces are more potent activators than hydrophilic ones, and incorporation of chemical groups such as $NH_3$, OH or COOH influences the activation of complement. Biomaterial surfaces with free OH and $NH_3$ groups are generally regarded as more dynamic to activate complement than others, since these groups are essential for the covalent binding of C3b [35]. PVMA polymer reaction with water under physiological conditions yields two carboxylic groups, highly reactive hydroxyl nucleophiles able to form hydrogen bonds with C3b [36]. This mechanism has been described with other hydroxylated materials [35;37]. To explore whether complement was activated exclusively by surface

hydroxyls on the PVMA NPs, we blocked hydroxyl groups with diaminopropane. These reticulated NPs reduced complement adsorption, indicating that the strong capacity of PVMA NPs to adsorb and activate complement was related with that high hydroxylated surface.

An interesting review has been recently published on the crosstalk between TLR and the complement system [38]. Thus, it has been documented that full maturation of DCs and further CD4 and CD8 T cell responses upon antigen uptake does not occur in C3-deficient mice. Besides, the C3a/C5a intracellular signalling pathways on APCs intersect with those of the Toll-like receptor (TLR) family. Hence, the optimization of the development of new effective adjuvants based on the use of pleiotropic polymers should be continued for antigen delivery to improve current vaccine strategies. Chemical surface differences may affect TLR recognition and complement activation, and so, conducting different biological behavior. Therefore, we are currently studying this phenomenon by using NPs obtained by different methods and polymers to validate this hypothesis.

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Reference List


[1] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124(4):783-801.

[2] Garcon N, Chomez P, Van MM. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 2007;6(5):723-39.

[3] Chu DW, Hwang SJ, Lim FS, et al. Immunogenicity and tolerability of an AS03(A)-adjuvanted prepandemic influenza vaccine: a phase III study in a large population of Asian adults. Vaccine 2009;27(52):7428-35.

[4] Krieg AM. Toll-free vaccines? Nat Biotechnol 2007;25(3):303-5.

[5] Tamayo I, Irache JM, Mansilla C, Ochoa-Reparaz J, Lasarte JJ, Gamazo C. Poly(anhydride) nanoparticles act as active Th1 adjuvants through Toll-like receptor exploitation. Clin Vaccine Immunol 2010;17(9):1356-62.

[6] Sharp FA, Ruane D, Claass B, et al. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A 2009;106(3):870-5.

[7] Mann JF, Acevedo R, Campo JD, Perez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev Vaccines 2009;8(1):103-12.

[8] Chong CS, Cao M, Wong WW, et al. Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery. J Control Release 2005;102(1):85-99.

[9] Hamdy S, Elamanchili P, Alshamsan A, Molavi O, Satou T, Samuel J. Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles. J Biomed Mater Res A 2007;81(3):652-62.

[10] Raghuvanshi RS, Katare YK, Lalwani K, Ali MM, Singh O, Panda AK. Improved immune response from biodegradable polymer particles entrapping tetanus toxoid by use of different immunization protocol and adjuvants. Int J Pharm 2002;245(1-2):109-21.

[11] Schlosser E, Mueller M, Fischer S, et al. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine 2008;26(13):1626-37.

[12] Najar HM, Dutz JP. Topical TLR9 agonists induce more efficient cross-presentation of injected protein antigen than parenteral TLR9 agonists do. Eur J Immunol 2007;37(8):2242-56.

[13] Mallapragada SK, Narasimhan B. Immunomodulatory biomaterials. Int J Pharm 2008;364(2):265-71.

[14] Irache JM, Salman HH, Gomez S, Espuelas S, Gamazo C. Poly(anhydride) nanoparticles as adjuvants for mucosal vaccination. Frontiers in Bioscience 2009.

[15] Gomez S, Gamazo C, Roman BS, Ferrer M, Sanz ML, Irache JM. Gantrez AN nanoparticles as an adjuvant for oral immunotherapy with allergens. Vaccine 2007;25(29):5263-71.

[16] Ochoa J, Irache JM, Tamayo I, Walz A, DelVecchio VG, Gamazo C. Protective immunity of biodegradable nanoparticle-based vaccine against an experimental challenge with Salmonella Enteritidis in mice. Vaccine 2007;25(22):4410-9.

[17] Gomez S, Gamazo C, San RB, Vauthier C, Ferrer M, Irachel JM. Development of a novel vaccine delivery system based on Gantrez nanoparticles. J Nanosci Nanotechnol 2006;6(9-10):3283-9.

[18] Arbos P, Campanero MA, Arangoa MA, Renedo MJ, Irache JM. Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties. J Control Release 2003;89(1):19-30.

[19] Ojer P, Da Costa MR, Calvo J, et al. Spray-drying of poly(anhydride) nanoparticles for drug/antigen delivery. 2010.
Ref Type: Unpublished Work

[20] Blanco-Prieto M, Lecaroz C, Renedo M, Kunkova J, Gamazo C. In vitro evaluation of gentamicin released from microparticles. Int J Pharm 2002;242(1-2):203-6.

[21] Arbos P, Wirth M, Arangoa MA, Gabor F, Irache JM. Gantrez AN as a new polymer for the preparation of ligand-nanoparticle conjugates. J Control Release 2002;83(3):321-30.

[22] Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 2004;4(6):469-78.

[23] Kipper MJ, Wilson JH, Wannemuehler MJ, Narasimhan B. Single dose vaccine based on biodegradable polyanhydride microspheres can modulate immune response mechanism. J Biomed Mater Res A 2006;76(4):798-810.

[24] Agueros M, Areses P, Campanero MA, et al. Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles. Eur J Pharm Sci 2009;37(3-4):231-40.

[25] Salman HH, Irache JM, Gamazo C. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccine 2009;27(35):4784-90.

[26] Salman HH, Gamazo C, Campanero MA, Irache JM. Salmonella-like bioadhesive nanoparticles. J Control Release 2005;106(1-2):1-13.

[27] Feili-Hariri M, Falkner DH, Morel PA. Polarization of naive T cells into Th1 or Th2 by distinct cytokine-driven murine dendritic cell populations: implications for immunotherapy. J Leukoc Biol 2005;78(3):656-64.

[28] Jenkins SJ, Hewitson JP, Ferret-Bernard S, Mountford AP. Schistosome larvae stimulate macrophage cytokine production through TLR4-dependent and -independent pathways. Int Immunol 2005;17(11):1409-18.

[29] Flo TH, Ryan L, Latz E, et al. Involvement of toll-like receptor (TLR) 2 and TLR4 in cell activation by mannuronic acid polymers. J Biol Chem 2002;277(38):35489-95.

[30] Xu R, Jiang ZH. Synthesis of beta-(1-->4)-oligo-D-mannuronic acid neoglycolipids. Carbohydr Res 2008;343(1):7-17.

[31] Govindaraj RG, Manavalan B, Lee G, Choi S. Molecular modeling-based evaluation of hTLR10 and identification of potential ligands in Toll-like receptor signaling. PLoS One 2010;5(9):e12713.

[32] Kemper C, Atkinson JP. T-cell regulation: with complements from innate immunity. Nat Rev Immunol 2007;7(1):9-18.

[33] Kemper C, Atkinson JP, Hourcade DE. Properdin: emerging roles of a pattern-recognition molecule. Annu Rev Immunol 2010;28:131-55.

[34] Huong TM, Ishida T, Harashima H, Kiwada H. The complement system enhances the clearance of phosphatidylserine (PS)-liposomes in rat and guinea pig. Int J Pharm 2001;215(1-2):197-205.

[35] Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. The role of complement in biomaterial-induced inflammation. Mol Immunol 2007;44(1-3):82-94.

[36] Sim RB, Twose TM, Paterson DS, Sim E. The covalent-binding reaction of complement component C3. Biochem J 1981;193(1):115-27.

[37] Reddy ST, van der Vlies AJ, Simeoni E, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol 2007;25(10):1159-64.

[38] Hajishengallis G, Lambris JD. Crosstalk pathways between Toll-like receptors and the complement system. Trends Immunol 2010;31(4):154-63.

**Tables**

**Table 1**. Physicochemical characteristics of nanoparticles.

| | [a]Size (nm) | Zeta potential (mV) |
|---|---|---|
| PVMA NP | 149 ± 2 | - 49 ± 2 |
| reticulated - PVMA NP [c] | 161 ± 2 | - 37 ± 1 |
| PLGA NP | 300 ± 2 | -19 ± 1.1 |

[a] Determination of the nanoparticles volume mean diameter (nm) by photon correlation spectroscopy.

[b] The percentage yield of the polymer transformed into nanoparticles.

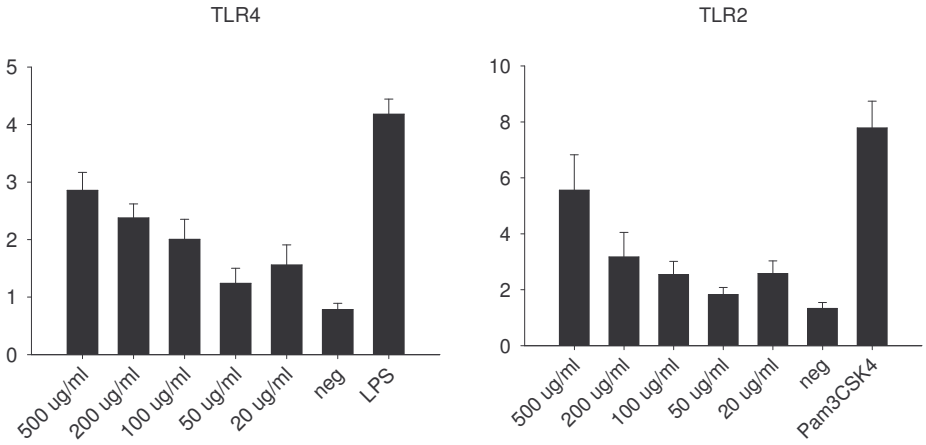
[c] Nanoparticles treated with 1,3-diaminopropane in order to partially neutralize hydroxyl groups covering PVMA NPs.

**Figure legends:**

**Figure 1.** Effects of nanoparticles on the activation of TLR signaling. Bars represent engagement to TLR2 or TLR4 after incubation with different concentrations of poly(anhydride) NPs. Specific agonists for each TLR were used as positive controls: PAM2 (100 ng/ml) for TLR2; LPS K12 (1 μg/ml) for TLR4. TLR non expressing recombinant cell line also included (NEG). Results are given in optical density values (O.D.).

**Figure 2**. Incubation of complement with PVMA NPs [reticulated with diaminopropane (lanes 3, 6) and non reticulated (lanes 4, 7)] or PLGA NPs (lanes 5, 8). The incubation times were 5 or 10 min. Nanoparticles were washed, the first supernatant recovered (panel A) and then, the sediment boiled with SDS-mercaptoethanol to release proteins covalently bound (panel B). By comparisons, lanes 1 and 2 show the protein profile of the complement sample used at 10 and 30 μg, respectively). The molecular mass markers and the positions of iC3b are shown with arrows.

**Figure 1**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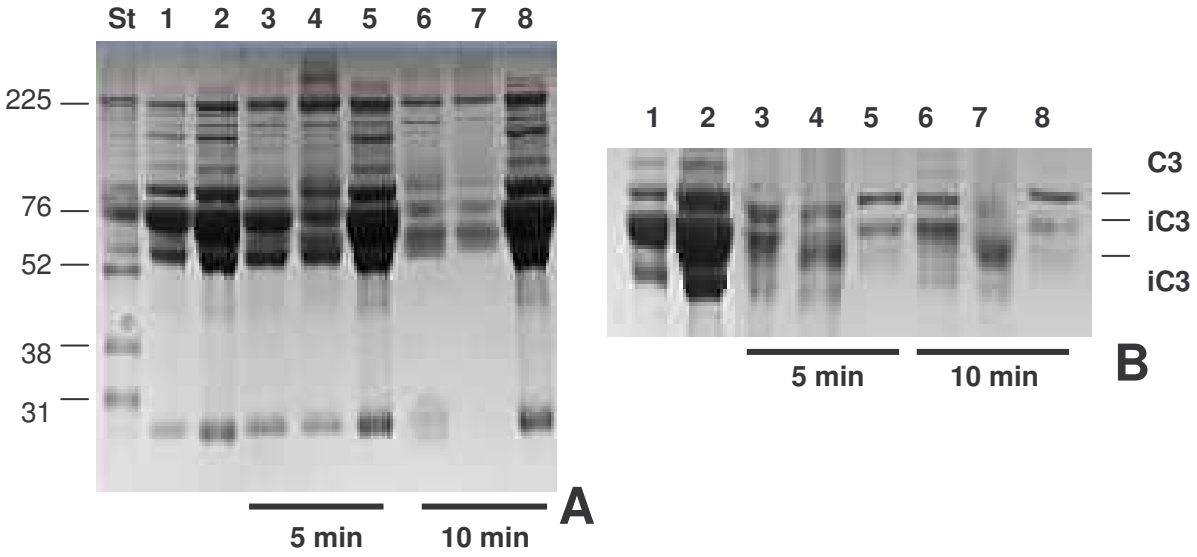
4
3
2
1
0
500 ug/ml
200 ug/ml
100 ug/ml
50 ug/ml
20 ug/ml
neg
LPS
TLR2
10
8
6
4
2
0
500 ug/ml
200 ug/ml
100 ug/ml
50 ug/ml
20 ug/ml
neg
Pam3CSK4

**Figure 2**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76
52
38
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5 min
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A
1 2 3 4 5 6 7 8
C3
iC3
iC3
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