1 **Multi-enveloping of particulated antigens with biopolymers and**
2 **immunostimulant polynucleotides**

3
4 J.F. Correia-Pinto^{a,b}, Mercedes Peleteiro^c, N. Csaba^{a,b}, África González-Fernández^c,
5 M.J. Alonso^{a,b}

6 ^a Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy,
7 University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

8 ^b Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Av.
9 Barcelona s/n, Campus Vida, University of Santiago de Compostela,
10 15707 Santiago de Compostela, Spain

11 ^c Immunology, Institute of Biomedical Research (IBIV), Biomedical Research Center
12 (CINBIO), University of Vigo, Vigo, Spain

13 *Corresponding author at: Department of Pharmacy and Pharmaceutical Technology,
14 School of Pharmacy, University of Santiago de Compostela, 15782 Santiago de
15 Compostela, Spain. Tel.: +34 881815252; fax: +34 981547148.

16 E-mail address: mariaj.alonso@usc.es (M.J. Alonso).

17

18 **Keywords**

19 Hepatitis B; VLP; Multi-enveloped nanoparticle; Polyamino acid; Polysaccharide;

20 Poly (I:C)

21

22

23 **Abstract**

24 The main objective of this work is to study the possibility of enveloping particulated
25 antigens using biomaterials and polynucleotides with immunostimulant properties, and
26 to assess the value of this potential adjuvant strategy. The nanoparticulate structure (22
27 nm) of the recombinant Hepatitis B surface antigen (rHBsAg) has inspired us to use it
28 as a substrate in the design of multi-enveloped nanoparticles. Our approach relies on
29 the adsorption of polymers and immunostimulants by electrostatic interactions.
30 Considering the negative charge of the rHBsAg, as a first layer we adsorbed the
31 cationic polymers protamine (PR) and polyarginine (PARG). Subsequently, these
32 nanostructures were enveloped using the anionic polymers dextran sulfate (DS) and
33 alginate (ALG), and the immunostimulant polynucleotide poly (I:C) (pIC). The
34 resulting multi-enveloped nanoparticles (HB:PR; HB:PR:DS; HB:PARG:ALG;
35 HB:PARG:pIC) presented very small sizes (< 50 nm) and were shown to be
36 internalized by macrophages. With regard to the capacity of these new
37 nanocompositions to elicit immune responses, the results observed following either
38 intranasal or intramuscular administration indicated that despite the modest IgG titers,
39 the IgG1/IgG2a ratio was more balanced than the one observed for the alum-adsorbed
40 antigen. Overall, this work highlights the possibility to protect antigens and modify
41 their presentation using a simple layer-by-layer approach.

42

43 **1. Introduction**

44 Vaccination is the most cost-effective preventive intervention against disease to date
45 [1], and while the majority of the marketed vaccines are based on attenuated and
46 inactivated whole-cell vaccines, the current tendency is to design vaccines based on
47 subunit antigens. These subunit vaccines are based on specific components of
48 pathogens, and are less prone to provoke adverse reactions [2]. Among the subunit
49 antigens, virus-like particles (VLP), formed by self-assembled viral proteins, are
50 particularly interesting due to their superior immunogenicity [3, 4]. Notable examples
51 of VLP-based vaccines are the current hepatitis B (HB) and the human papillomavirus
52 (HPV) vaccines, which are based on the recombinant hepatitis B surface antigen
53 (rHBsAg) [5] and on the HPV L1 protein, respectively [6]. In addition, other VLP-
54 based vaccines, such as RTS,S for malaria and ACAM-FLU-A for Influenza A, are
55 currently in the clinical development stage [7]. Despite their immunogenicity, these
56 particulated antigens still need to be associated to a suitable vaccine adjuvant in order
57 to generate an effective immune response. Most commonly, subunit antigens are
58 adsorbed onto alum salts as adjuvant, due to their long record of safe use and
59 effectiveness [8]. However, these adjuvants present several limitations. The use of
60 alum requires a tight control of the storage conditions, as freezing provokes
61 aggregation and loss of effectiveness [9]. This is a particularly important problem
62 because the maintenance of the global cold chain has significant limitations [10] and
63 can lead to a decreased immunization coverage worldwide [11]. Additionally, the use
64 of alum is limited to parenteral administration, which requires specific training to
65 perform and can cause blood-borne pathogen contamination by the re-use of syringes
66 [12]. Finally, alum salts generate a T_H2 biased immune response and they lack the
67 capacity to stimulate cell-mediated immunity [13].

68
69 The use of nanotechnology-based approaches is known to offer opportunities for
70 improving the formulation of vaccines. Specific advantages of the nanoformulations
71 described in the literature include: i) their improved thermostability, as compared to
72 the alum-adjuvanted vaccines [14, 15]; ii) their capacity to facilitate the transport of
73 antigens across mucosal barriers and generate humoral and mucosal responses [15,
74 16]; iii) their ability to enhance and prolong the immune responses (single-dose
75 vaccination) [17]; iv) their potential to modulate T_H1/T_H2 immune responses [16, 17].

76

77 It has been hypothesized that nanocarriers may facilitate antigen presentation to T cells
78 due to their resemblance to virus in terms of size and surface characteristics [18, 19].
79 These antigen-loaded nanocarriers are captured in the administration area by local
80 antigen-presenting cells (APC), particularly dendritic cells (DCs) [20]. Then, DCs
81 process the antigen while migrating to the lymph nodes through the lymphatic vessels
82 [18]. In the lymph nodes, once the antigen is processed, the DCs present individual
83 peptides associated to the major histocompatibility complex (MHC) class II molecules
84 to specific T lymphocytes, triggering the adaptive immune response, with the
85 generation of memory T and B cells [21]. In addition to this peripheral uptake of
86 antigen-loaded nanocarriers by DCs, it has been recently proposed that small
87 nanoparticles (< 100 nm) could target antigens directly to the DCs in the lymph nodes
88 [18, 22, 23]. As the immune cell population in these locations is much more
89 concentrated than in peripheral tissues [24], such strategy could potentially provide a
90 better and higher antigen presentation, increasing the immune response and decreasing
91 local reactions to the vaccine [23, 25]. An additional advantage of these ultra-small
92 nanocarriers, as compared to larger particles or alum- based vaccines, relies on the fact
93 that they could be sterilized by filtration.

94
95 Despite these data, most of the antigen delivery carriers described until now present a
96 size larger than 100 nm [26]. Therefore, acknowledging this technological gap, our
97 main goal was to rationally design enveloped virus-like particle antigens with a final
98 size of less than 50 nm. For this purpose, our approach has been to use the antigen
99 itself as a substrate for the adsorption of polymers and polynucleotides with adjuvant
100 properties. As a model antigen we have chosen the recombinant hepatitis B surface
101 antigen (rHBsAg) in its VLP form [5]. This antigen is formed by the self-assembly of
102 rHBsAg proteins with associated lipids, and presents an average particle diameter of
103 22 nm with negative surface charge [27]. Because of this negative surface charge, we
104 have selected for the first enveloping layer two different cationic polymers:
105 polyarginine (PARG) and protamine (PR). The second enveloping layer consisted of
106 anionic polysaccharides, such as dextran sulfate (DS) or alginate (ALG), or a synthetic
107 double-stranded based RNA, poly (I:C) (pIC).

108
109 The rationale for the selection of the polymers was as follows. Polyarginine is a
110 cationic polyamino acid, which has been shown to favor the uptake of antigens by

antigen-presenting cells [28] and, as a consequence, to enhance the T cell responses *in vivo* against associated antigens [29]. These interesting adjuvant properties have led to its inclusion in a hepatitis C vaccine (HCV), which is currently being tested in clinical trials [30]. On the other hand, protamine is a mixture of arginine-rich polypeptides, with membrane translocating properties [31], which has also shown the capacity to increase the uptake of both microparticles [32] and nanoparticles by phagocytic cells [31]. With regard to the selection of the negatively charged polysaccharides, dextran sulfate and alginate are both linear anionic polymers exhibiting immunoadjuvant properties. Alginate has been found to activate macrophages [33] inducing the production of pro-inflammatory cytokines [34], possibly through the interaction with Toll-like receptors (TLR) [35]. There is also evidence of the capacity of dextran sulfate to increase cell-mediated immunity [36] and antibody responses [37] in several animal models. Finally, the polynucleotide poly (I:C) is an agonist of the Toll-like receptor 3 (TLR-3) present in dendritic cells. TLR-3 recognizes double-stranded RNA (dsRNA) associated to viral infection, stimulates dendritic cell activation and maturation and the secretion of interferon alpha/beta, which, in turn, plays an important role in the defense against viruses [38]. Through these mechanisms, poly (I:C) can potentiate both humoral and cellular immune responses [39, 40]. The adsorption of the poly (I:C) onto the enveloped antigen was thought to be particularly important in order to achieve simultaneous delivery of the antigen and the immunostimulant to the immune cells.

In brief, this work describes the development of small nanovaccines (<50 nm) through a multi-enveloping method using HB VLP as model antigen, and their characterization through the study of their physicochemical properties, uptake by immune cells and *in vivo* capacity to generate an effective immune response to the HB antigen after intramuscular (i.m.) and intranasal (i.n.) administration.

2. Material and methods

2.1. Materials

Poly-L-arginine hydrochloride with molecular weight of 5 – 15 kDa (P4663), dextran sulfate sodium salt with molecular weight of 9 – 20 kDa (D6924) and Phosphate Buffer saline (PBS) were obtained from Sigma–Aldrich (Madrid, Spain). Alginate with molecular weight of 4 kDa was obtained from Danisco (Copenhagen, Denmark).

145 Protamine sulfate was kindly donated by LEO Pharma (Denmark). Polyinosinic-
146 polycytidylic acid of low molecular weight (0.2 – 1 kB) (Poly (I:C)-LMW) was
147 purchased from Invivogen (San Diego, USA). The recombinant hepatitis B surface
148 antigen (rHBsAg) was kindly donated by Shantha Biotechnics Ltd. (Hyderabad, India)
149 as an aqueous suspension in PBS with a protein concentration of 1.13 mg/ml.
150 The 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE) *single
151 isomer* (Catalogue # C2211) and the Slide-A-Lyzer Dialysis Cassettes, 2K MWCO
152 were purchased from ThermoFisher Scientific (Massachusetts, USA). Amicon Ultra-4
153 ml Centrifugal Filters with 100 kDa pore and low-binding regenerated cellulose
154 membrane were purchased to Merck Millipore (Massachusetts, USA).

155

156 **2.2. Preparation of multi-enveloped particles using hepatitis B antigen as** 157 **substrate**

158 An aliquot of the antigen suspension diluted with ultrapure water was introduced in an
159 Amicon-4 100 kDa filtration tube and centrifuged at 5,000 rpm for 5 minutes at 15 °C
160 in a Universal 32 centrifuge (Hettich, Tuttlingen, Germany). After repeating this
161 procedure three times, the antigen was recovered from the filter and diluted to the final
162 concentration of 0.1 mg/ml. This final solution was then used as template for the
163 adsorption of the first polymer layer.

164

165 **2.2.1. Preparation of HB:PR and HB:PARG – First layer adsorption**

166 A volume of 0.8 ml of protamine or polyarginine solution (0.1 mg/ml) was mixed in a
167 glass tube with 0.8 ml of HB solution (0.1 mg/ml) under magnetic stirring (200 rpm).
168 The magnetic stirring was maintained for 5 minutes. The resulting suspension was
169 incubated for 20 minutes at room temperature in order to allow the formation of the
170 complex. The formulation was filtered then, using an Amicon-4 100 kDa and diluted
171 to a final volume of 4 ml with ultrapure water. This ultrafiltration procedure was
172 repeated 3 times and subsequently the concentrated dispersion of the HB:PR or
173 HB:PARG complexes was recovered and diluted to a final HB concentration of 0.1
174 mg/ml.

175

176

177

178 **2.2.2. Preparation of HB:PR:DS, HB:PARG:ALG and HB:PARG:pIC – Second**
179 **layer adsorption**

180 A volume of 0.785 ml of dextran sulfate (0.4 mg/ml), alginate (0.4 mg/ml) or poly
181 (I:C) (0.1 mg/ml) solution was mixed in a glass tube with 0.785 ml of HB:PR or
182 HB:PARG formulation (HB at 0.1 mg/ml) under magnetic stirring (200 rpm) to
183 constitute the HB:PR:DS, HB:PARG:ALG or the HB:PARG:pIC formulation,
184 respectively. The formulations were kept under stirring for 5 minutes and then they
185 were incubated at room temperature for 20 minutes. As in the case of the first layer,
186 the formulations from the previous step were filtered with an Amicon-4 100 kDa and
187 diluted to a final volume of 4 ml. This ultrafiltration procedure was repeated three
188 times and subsequently the concentrated dispersion of the complexes HB:PR:DS,
189 HB:PARG:ALG and HB:PARG:pIC was recovered and diluted to a final HB
190 concentration of 0.1 mg/ml. The final theoretical mass ratio for HB:PR:DS,
191 HB:PARG:ALG and HB:PARG:pIC was estimated as 1:1:4, 1:1:4 and 1:1:1,
192 respectively.

193

194 **2.3. Physicochemical characterization of the multi-enveloped particles**

195 The particle size and polydispersity index (PDI) of the multi-enveloped particles were
196 evaluated by Dynamic Light Scattering (DLS) and its surface charge (zeta potential)
197 by Electrophoretic Light Scattering (ELS), using a Zetasizer Nano ZS90 (Malvern
198 Instruments, UK). The measurements were performed at 25°C with a detection angle
199 of 173°, in ultrapure water.

200

201 **2.4. Determination of protamine and polyarginine adsorption and antigen**
202 **enveloping efficiency**

203 The protamine and polyarginine adsorption to the HB particles was evaluated by an
204 indirect measurement based on the free polyamino acid in solution after incubation
205 with the antigen (micro BCA protein assay, Pierce, Thermo Fisher Scientific, MA,
206 USA). The efficiency of adsorption (E.A.%) was calculated subtracting the
207 concentration determined by the micro BCA from the theoretical concentration of the
208 polyamino acid (0.1 mg/ml).

209 The amount of antigen coated by the polymers was determined indirectly using a
210 microtiter plate-based sandwich ELISA kit (Murex HBsAg Version 3, Murex Biotech
211 Ltd.; Dartford, UK). The HB enveloping efficiency (E.E.%) was calculated by the

212 difference between the theoretical amount of antigen and the amount of non-enveloped
213 antigen determined in suspending medium. It was assumed that the enveloped antigen
214 particles with polymers, when incubated in the microwells, would not be recognized
215 by the anti-HB antibodies of the ELISA assay.

216

217 **2.5. Stability of the multi-enveloped particles in aqueous suspension**

218 **2.5.1. Stability during storage**

219 The multi-enveloped nanoparticles, HB:PR 1:1, HB:PR:DS 1:1:4, HB:PARG:ALG
220 1:1:4 and HB:PARG:pIC 1:1:1, at HB concentration of 0.1 mg/ml, were stored at 4 °C
221 for up to 1 month. Then their size and PDI were determined, as described in section
222 2.3.

223

224 **2.5.2. Stability upon dispersion in simulated biological medium**

225 The stability of the multi-enveloped nanoparticles HB:PR 1:1, HB:PR:DS 1:1:4.
226 HB:PARG:ALG 1:1:4 and HB:PARG:pIC 1:1:1, at HB concentration of 0.05 mg/ml,
227 in PBS at 37 °C, was tested for up to 4 hours.

228

229 **2.6. Preparation of fluorescently labeled HB:PR:DS and HB:PARG:ALG multi-** 230 **enveloped particles**

231 In order to perform *in vitro* cell uptake studies, HB:PR:DS and HB:PARG:ALG
232 particles were prepared with fluorescently labeled protamine (PRF) and polyarginine
233 (PARGF), respectively. The dye chosen was the 5-carboxy tetramethylrhodamine
234 succinimidyl ester (TAMRA) and the labeling was performed according to the
235 manufacturer's instructions. Briefly, protamine and polyarginine were dissolved in 0.1
236 M sodium bicarbonate buffer, pH 8.0, at 10 mg/ml. In parallel, TAMRA was dissolved
237 in dimethyl sulfoxide (DMSO) at 10 mg/ml. An aliquot of the TAMRA solution (0.1
238 ml) was added slowly over 1 ml of the polyamino acid solution under magnetic
239 stirring and left for 1 hour at room temperature. After incubation, the labeled
240 polyamino acid solution was dialyzed in Slide-A-Lyzer Dialysis Cassette 2K MWCO
241 for 48 h, to remove free TAMRA. After dialysis, the labeled protamine (PRF) or
242 polyarginine (PARGF) was adsorbed onto the HB antigen and the polysaccharide
243 dextran sulfate or alginate was added over the PRF or PARGF layers to form the
244 fluorescently labeled HB:PRF:DS or HB:PARGF:ALG particles, respectively. The

labeled formulations were then characterized in terms of size, PDI and surface charge, as described in 2.3.

2.7. *In vitro* cell studies

2.7.1. Cells and culture

The adherent RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were cultured in RPMI supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA; Pasching, Austria), 2 mM glutamine and 100 U/ml of penicillin/streptomycin, in a humidified atmosphere at 37 °C in 5% carbon dioxide atmosphere. Cells were split every other day to maintain 70–80% of confluent cultures.

2.7.2. Internalization studies in macrophages

The uptake of HB:PR:DS and HB:PARG:ALG by Raw 264.7 cells was examined using a confocal microscope (Leica SP5, Germany). Briefly, cells (3×10^5) were seeded on a glass cover-slip (Menzel-Gläser; Braunschweig, Germany) in a 24-well plate (Falcon 3047, BD Biosciences, USA) and left overnight at 37 °C to allow cell adhesion. The following day, cells were incubated with TAMRA-labeled HB:PRF:DS or TAMRA-labeled HB:PARGF:ALG formulation (in a mass equivalent to 10 µg of HB), in RPMI with 10% FBS for 30 min at 37 °C, in the dark. After several washes with PBS to remove non-internalized particles, the cells were fixed with 4% formaldehyde for 10 min and then incubated with Alexa Fluor 488-phalloidin (Invitrogen, Thermo Fisher Scientific, USA) for 20 min to stain the cellular cytoskeleton. Finally, the cover-slips containing the attached cells were mounted on slides in the presence of ProLong® Gold Antifade mounting medium (Invitrogen, Thermo Fisher Scientific, USA), containing DAPI as nucleus stain, and analyzed in a confocal microscope (Confocal Laser Scanning Microscopy (Leica SP5)).

2.8. *In vivo* studies

2.8.1. Animals

Female BALB/c mice (4–5 weeks) were housed in filter-top cages in a 12 h light/12 h dark cycle at constant temperature (22 °C) and provided with a standard diet and water *ad libitum*. The animals were kept conscious during the immunizations and bleeding. All protocols were in accordance with the guidelines of the Spanish regulations (Royal

278 Decree 1201/2005) regarding the use of animals in scientific research and under the
279 approval of the Ethical Committee of the University of Vigo.

280

281 **2.8.2. Preparation of the control vaccine using alum as adjuvant.**

282 The HB antigen was used either suspended in PBS for intranasal (i.n.) administration
283 or adsorbed on aluminum hydroxide (HB-Alum) for intramuscular (i.m.)
284 administration, as positive control. Briefly, Alum (Alhydrogel ®, Sigma-Aldrich, St.
285 Louis, MO) was incubated with the HB antigen in a 3:1 (v:v) ratio for 30 minutes at 4
286 °C under gentle agitation. Afterwards, the suspension was centrifuged (10,000 x g, 10
287 minutes, 4°C) and the pellet was resuspended in an isotonic saline solution. The dose
288 of antigen administered (10 µg) was the same in the control vaccines and in the rest of
289 formulations.

290

291 **2.8.3. Immunization and bleeding schedule**

292 In total, three *in vivo* trials were performed in mice, in order to evaluate the capacity of
293 1) the HB:PR, 2) the HB:PR:DS and HB:PARG:ALG and 3) the HB:PARG:pIC
294 systems, to stimulate an immune response against the HB antigen by i.n. and i.m.
295 routes.

296 In all cases, the initial pool of mice was randomly distributed in groups of ten animals
297 for each formulation and administration route (i.m. or i.n.). The antigen dose was 10
298 µg, for both test formulations and controls. In the i.m. mice test groups, the test
299 formulations and control were administered at days 0 and 28 (0 and 4 weeks) in the
300 hind leg of the mice, in a volume of 0.06 ml (HB at 0.167 mg/ml). In the i.n. mice test
301 groups, the formulations and control were administered at days 0, 28 and 112 (0, 4 and
302 16 weeks), in a volume of 0.02 ml (HB at 0.5 mg/ml) by instillation of 0.01 ml in each
303 nostril. Blood samples were collected in all cases from the mouse maxillary vein at
304 days 42, 126 and 183 (corresponding to weeks 6, 18 and 26).

305

306 **2.8.4. Determination of anti-HB IgG in the serum of immunized mice by ELISA**

307 Specific antibody levels against HB in mice sera were evaluated by ELISA at different
308 time points. For this purpose, MaxiSorp ® 96-well plates (Nunc, Denmark) were
309 coated with a HB solution at 5 µg/ml, in carbonate buffer (pH 9.6), and incubated
310 overnight at 4 °C. Plates were then blocked with BSA 1% in PBS for 1 hour at 37°C.
311 In order to quantify specific anti HB-IgG, both serum samples and a mouse

monoclonal anti-HB IgG antibody (Acris Antibodies GmbH; Hiddenhausen, Germany), were serially diluted and incubated for 2 hours at 37 °C. Afterwards, secondary antibodies (goat anti-mouse IgG from Southern Biotech, AL, USA) were added to the wells and incubated for 1 hour at 37 °C. Bound antibodies were finally revealed with 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and the absorbance read at 405 nm. To transform antibody levels in µg/ml to international units (mIU/ml), a similar protocol was carried out in parallel using a control rabbit anti-HB IgG (Biokit, Barcelona, Spain) of known concentration (mIU/ml). The IgG1/IgG2a ratio was also evaluated in a pool of sera from mice immunized with HB:PARG:pIC and HB-Alum by i.m. route. In this case, the same protocol was followed but specific anti-mouse IgG1 and IgG2a secondary antibodies were used. The ratio IgG1/IgG2a was then calculated based on the absorbance levels detected. All serum samples were tested at least twice and in duplicate.

325

3. Results and discussion

Virus-like particles (VLP) antigens are having an increasing presence in the immunization protocols. Despite their good safety profile and immunogenicity, these new vaccines need to be administered in association with adjuvants in order to generate robust immune responses. Inspired by the nanoparticulate nature of the VLP, we have designed a strategy intended to increase the immune response against these antigens. This strategy relies on the use of a particulated antigen as a substrate for the adsorption of polymers with immunoadjuvant properties. The antigen chosen was the hepatitis B VLP antigen (HB), due to its nanometric size (22 nm) and negative surface charge. The biomaterials selected for the enveloping process were: protamine (PR), polyarginine (PARG), dextran sulfate (DS) and alginate (ALG). Finally, the immunomodulator poly (I:C) (pIC), was also associated to the nanostructures in order to evaluate its potential for enhancing the immune response against the enveloped antigen. With this strategy, four multi-enveloped systems (HB:PR; HB:PR:DS; HB:PARG:ALG; HB:PARG:pIC) (**Figure 1**) were developed, characterized and their behavior upon contact with dendritic cells and upon *in vivo* administration was also assessed.

343

344

345

3.1. Preparation and characterization of multi-enveloped nanoparticles

The HB antigen in a PBS stock solution was washed with ultrapure water (by ultrafiltration) in order to decrease the salt content before its enveloping with the polymers. As the adsorption of the polymers was based on electrostatic interactions, it was important to reduce the presence of salts that could shield the electrostatic attraction between molecules of opposite charge [41]. This operation led to a decrease of the antigen sample number-distribution: before ultrafiltration it presented a size of 53 nm while after this process the size was of 22 nm (data not shown). After incubation of the antigen with the polymers, the formulation was also ultrafiltered to separate the multi-enveloped nanosystem from the free polymer and to allow the concentration of the formulation. This was considered an important step, particularly after the first coating (protamine and polyarginine), to eliminate the free polymer molecules that could, otherwise, bond with the negatively charged polymers (dextran sulfate, alginate and poly (I:C)) and interfere with the antigen enveloping process. The DLS and ELS techniques were considered the most practical techniques to characterize the antigen and to monitor its progressive coating by the polymers. In order to have a reliable signal with these techniques, the minimal HB concentration needed was 0.05 mg/ml and this concentration was taken as reference for the characterization of the multi-enveloped systems. The initial DLS intensity-distribution analysis of the antigen alone indicates a size greater than 100 nm (**Annex 1A**), which contradicts previously published work [27, 42]. However, when the DLS signal was evaluated through a volume-distribution (**Annex 1B**) and number-distribution analysis (**Annex 1C**), we observed that the majority of the HB particles in solution had in fact a size close to 22 nm (22 ± 1) and that particles of this size represented the majority of the antigen volume. Such difference between intensity and number evaluation could arise from the presence of a small number of big particles resultant from HB particles agglomeration. In DLS, the intensity signal is directly proportional to the size of the particles, so even if the quantity of small particles is greater than that of large ones, the intensity-distribution profile will predominantly reflect the presence of large particles [43]. As the number-distribution size matched perfectly with the referenced size of the HB particles, this parameter was considered to be the most adequate for the antigen characterization and to follow its progressive coating through the multi-enveloping process. The presence of the referred HB particles aggregates could explain the high polydispersity (PdI 0.3). As this nanostructure was used as a template for the polymer

adsorption, all the multi-enveloped structures developed presented also high PdI (**Figure 2**). The ELS analysis of the antigen alone confirmed its negative surface charge. After the addition of the protamine or polyarginine, this surface charge became positive, which suggest that the antigen surface was coated by the cationic polyamino acids. Finally, when the dextran sulfate, alginate or poly (I:C) were added to the formulations, the zeta potential detected was again negative, which indicates that the antigen particles had been coated by the anionic polymers. The adsorption of the different polymer layers led to a sequential negative-positive-negative surface charge inversion and also to a subtle increase of the size from the original 22 nm to about 40 nm, upon formation of the multilayer structure.

390

391 **3.2. Determination of protamine and polyarginine adsorption and antigen** 392 **enveloping efficiency**

393 In order to evaluate the percentage of protamine and polyarginine adsorbed onto the
394 antigen particles, the concentration of the non-associated polymers was determined by
395 mBCA. Under the selected experimental conditions, the percentage of polyarginine
396 and protamine adsorbed was 23% and 13%, respectively (**Table 1**). This indicates that
397 the amount of polymers used, exceeded the amount required for the enveloping
398 process. These cationic polymers are hydrophilic and the mechanism driving their
399 adsorption is based on their ionic interaction with the negatively charged surface of the
400 antigen, as illustrated in **Figure 1**.

401

402 The amount of HB antigen coated by the polymers was determined by ELISA.
403 Interestingly, although the amounts of protamine and polyarginine adsorbed to HB
404 were quite different, the HB enveloping efficiency was relatively high (> 60%) for
405 both polyamino acids (**Table 1**). A possible explanation for the higher amount of
406 polyarginine adsorbed could be the greater density of positive charges in this polymer.
407 In addition, the higher molecular weight of polyarginine (5 – 15 kDa) vs. protamine (5
408 kDa) could have also influenced the amount of polymer adsorbed. The slightly larger
409 size and higher positive surface charge identified for HB:PARG compared with the
410 HB:PR also support this hypothesis (**Figure 2**).

411

412

413

414 **3.3. Stability of multi-enveloped particles in aqueous suspension**

415 A critical requisite of colloidal carriers is the preservation of their stability upon
416 storage and also upon incubation in simulated biological fluids. Indeed, very small
417 nanoparticles, especially those formed by ionic complexation, often have a tendency to
418 destabilize under such conditions. Interestingly, irrespective of the nature of the
419 coating polymer, the nano-enveloped particles remained stable in terms of particle size
420 distribution, upon storage for at least one month at 4 °C (**Annex 2**). On the other hand,
421 the behavior of the different nanostructures upon incubation in PBS at 37 °C, was
422 slightly different. The HB:PR formulation showed a slight and rapid size increase
423 (from 40 to 60 nm size) (**Figure 3A**) upon incubation in PBS. However, following this
424 initial increase, the size and also the PDI remained stable for the rest of the assay. On
425 the other hand, the HB:PR:DS prototype exhibited an adequate stability in PBS
426 (**Figure 3B**) while the HB:PARG:ALG system, aggregated rapidly when dispersed in
427 this media. Finally, the HB:PARG:pIC preserved its size and PDI for up to 4 hours in
428 PBS (**Figure 3C**). The different behavior of HB:PARG:ALG and HB:PARG:pIC
429 systems (which share the same HB:PARG core) in PBS, could be related to the
430 different ionic forces between the first and second enveloping layers. The great
431 negative charge density of poly (I:C) might be responsible for the strong association
432 between these two layers.

433 Overall, the conclusion is that the stability of the multi-enveloped antigen
434 nanoparticles is dependent on the balance between the attractive forces of the polymer-
435 polymer and polymer-antigen layers, and between the polymer layer and the
436 components of the incubation medium.

437

438 **3.4. *In vitro* internalization studies of fluorescently labeled multi-enveloped** 439 **particles**

440 As indicated in the introduction, in order to facilitate the antigen presentation to the
441 immune system, nanoparticles should have the capacity to enter the APCs. To test the
442 conditions for this internalization process, the HB:PR:DS and HB:PARG:ALG
443 nanosystems were prepared using TAMRA-labeled polymers and incubated with
444 macrophages (RAW 264.7). The dye was covalently linked to the polyamino acids
445 prior to the enveloping process. The DLS and ELS analysis of the labeled formulations
446 confirmed that all the physicochemical parameters of the multilayer systems remained

unaltered, with only a small decrease in the zeta potential values of the HB:PARG:ALG formulation (**Annex 3**).

The internalization process of the nanosystems in macrophages was visualized by confocal microscopy. The images obtained show that both are internalized, with a more pronounced uptake of the HB:PR:DS nanoparticles as compared to the HB:PARG:ALG ones (**Figure 4**).

3.5. *In vivo* studies

The ability of the HB:PR, HB:PR:DS and HB:PARG:ALG or HB:PARG:pIC formulations to promote a systemic humoral immune response by i.n. or i.m. routes against the hepatitis B antigen was assessed in three different *in vivo* trials with BALB/c mice. The antigen dose was 10 µg both for the formulations and for the alum-adsorbed antigen used as positive control (same mass ratio as in licensed vaccines) [44]. The specific anti-HB IgG concentration (µg/ml) in mouse sera was determined by ELISA and converted to international units (mIU/ml) to permit its comparison with the protective anti-HB IgG levels observed in humans (10 mIU/ml) [45].

Following intramuscular immunization, it was found that the prototypes investigated, led to a steady anti-HB IgG profile close to 100 mIU/ml (values between 179 and 62 mIU/ml) during the whole experiment (**Figure 5**). Although these levels were lower than those observed for the alum-adjuvanted vaccine, it is important to mention that they were always superior to 10 mIU/ml, a concentration known to be protective in humans. On the other hand, the effect of poly (I:C) in the formulation was found only at early times.

When testing the formulations by intranasal route and in order to better understand the adjuvant effect of enveloping the antigen, the antigen alone was also administered by the i.n. route. Despite being a highly immunogenic antigen, the HB alone generated almost undetectable IgG titers (2 mIU/ml) by this route. Overall, the responses were similar for the different prototypes. The IgG concentration levels were low (below 10 mIU/ml) when measured at short time periods, however, they increased over time, reaching IgG levels close to 60 mIU/ml (**Figure 6**). These values were also significantly higher than those attained when using the free antigen.

481 The modest humoral response observed for all the multi-enveloped systems, suggests
482 that there might be an intrinsic limitation in the multi-enveloping strategy for the
483 hepatitis B antigen. This limitation might be related to the recent theory that underlines
484 the importance of the exposure of a repetitive epitope array in the generation of an
485 effective immune response [3]. Such theory has been used to justify the superiority of
486 VLP antigens over soluble antigens [6, 7]. However, despite this, it is clear that the
487 rHBsAg antigen, as many others, needs to be associated with an adjuvant like Alum,
488 in order to activate the immune cells and generate an effective immune response. On
489 the other hand, the improved behavior of the multi-enveloped formulations as
490 compared to the VLP antigen alone, following intranasal administration, suggests that
491 the enveloping polymers exert a certain role. This role could be simply related to the
492 capacity of polyarginine to facilitate the transport of proteins across the nasal mucosa
493 [46] or it could also be related to the adjuvant effect of the biomaterials [29, 31, 33,
494 37, 39].

495
496 The theory on the limited exposure of the antigen epitopes in the enveloped systems
497 would agree with the positive results previously obtained by our group for a prototype
498 based on chitosan nanocapsules, which have VLP rHBsAg antigen exposed on their
499 surface [16, 17]. However, this theory would be in disagreement with the results of
500 previous works that have shown that the coating of rHBsAg antigen [47] or its
501 entrapment within polymers such as chitosan [14], may also lead to significant
502 responses following either i.m. or i.n. administration. Therefore, given the different
503 nature of the biomaterials and the different sizes of the vaccine nanostructures it is
504 difficult to draw a conclusion at this point. Another explanation for the limited
505 response obtained for the enveloped nanosystems could be related to the small size of
506 the nanostructures. Even though we have postulated that small size could facilitate the
507 direct access of the antigen to the lymph nodes, the possibility that these ultra-small
508 particles would be less recognizable by the antigen-presenting cells, cannot be
509 discarded.

510
511 Finally, based on previous works [39, 40], the association of poly (I:C) to the
512 nanostructure was expected to lead to an enhanced response. Keeping in mind that this
513 immunostimulant would mainly have an impact on the cellular responses (associated
514 to Th1 responses) [48], the IgG1/IgG2a ratios of the mice immunized with the

HB:PARG:pIC formulation were compared with those of the alum-absorbed antigen (Figure 7). As the IgG1 and IgG2a are characteristic of a Th2 and Th1 biased-response, respectively [49], we assumed that a IgG1a/IgG2 ratio superior to 1 was indication of a Th2 polarized response while a IgG1/IgG2a ratio inferior to 1 was the indication of a Th1 polarized response [50]. The activation of lymphocytes Th2 has been associated to a more preponderant humoral response, and the activation of Th1, to an enhanced cellular based immunity [51]. The IgG1/IgG2a ratio found with the alum absorbed antigen, indicates that the alum markedly shifted the lymphocytes towards a Th2 profile, which is a well known characteristic of this adjuvant [13]. On the contrary, in the case of the HB:PARG:pIC formulation, it was found that the initially balanced Th1/Th2 response ($\text{IgG1a/IgG2} = 0.97$) shifted towards a clear polarization of a Th1 response ($\text{IgG1a/IgG2} = 0.54$) at the end of the assay. The increased activation of Th1 cells by the HB:PARG:pIC formulation can be an interesting advantage of this system, as these cells stimulate the development of cellular immune responses capable of eliminating virus infected cells [52].

530

531 4. Conclusion

532 Here we present the design and evaluation of a new strategy for the
533 immunomodulation of immune responses associated to particulated antigens. The
534 strategy involves using the HB particulated antigen as a model template, enveloped
535 with a number of biomaterials and immunostimulators. The HB multi-enveloped
536 systems were capable of generating protective IgG titers (>10 mIU/ml), but the IgG
537 titers achieved were lower than those obtained when using alum-adsorbed antigen.
538 There were no main differences between the activity of the HB:PR, HB:PR:DS and
539 HB:PARG:ALG systems, despite their diverse composition and structure, which
540 suggests an intrinsic limitation of this multi-enveloping strategy. Nevertheless, the
541 association of the immunostimulant poly (I:C) with the enveloped antigen led to a
542 more balanced Th1/Th2 response as compared to the one observed for the alum-
543 absorbed antigen. This could be taken as an indication of enhanced cellular response,
544 which would be of importance for the complete elimination of viral infections.
545 Overall, this work provides new insight on the properties that determine the behavior
546 of the antigen delivery nanocarriers and suggests that a very small size might not be a
547 good option for enhancing the immune response against particulated antigens.

548

549 **Acknowledgments**

550 This work was supported by grants from the Bill & Melinda Gates Foundation (Grand
551 Challenges in Global Health initiative), the Xunta de Galicia (Competitive Reference
552 Groups), the Spanish Ministry of Economy and Competitiveness (SAF2011-30337-
553 C02-02) and the FP7/REGPOT-2012-2013.1-BIOCAPS-316265. We would like to
554 thank Shantha Biotechnics Ltd. (Hyderabad, India) for providing us the HB. Jorge
555 Pinto and Mercedes Peleteiro acknowledge fellowships from the Spanish Ministry of
556 Education (FPU predoctoral grants). The technical assistance of Rafael Romero is
557 highly appreciated.

558 **References**

- 559 1. Zepp, F., *Principles of vaccine design—Lessons from nature*. Vaccine, 2010.
560 **28, Supplement 3(0)**: p. C14-C24.
- 561 2. Huang, D.B., J.J. Wu, and S.K. Tying, *A review of licensed viral vaccines,*
562 *some of their safety concerns, and the advances in the development of*
563 *investigational viral vaccines*. Journal of Infection, 2004. **49(3)**: p. 179-209.
- 564 3. Jegerlehner, A., et al., *Regulation of IgG antibody responses by epitope density*
565 *and CD21-mediated costimulation*. Eur J Immunol, 2002. **32(11)**: p. 3305-14.
- 566 4. Grgacic, E.V.L. and D.A. Anderson, *Virus-like particles: Passport to immune*
567 *recognition*. Methods, 2006. **40(1)**: p. 60-65.
- 568 5. Keating, G.M. and S. Noble, *Recombinant hepatitis B vaccine (Engerix-B): a*
569 *review of its immunogenicity and protective efficacy against hepatitis B*. Drugs,
570 2003. **63(10)**: p. 1021-51.
- 571 6. Schiller, J.T. and D.R. Lowy, *Raising Expectations For Subunit Vaccine*. J
572 Infect Dis, 2014. **24**.
- 573 7. Kushnir, N., S.J. Streatfield, and V. Yusibov, *Virus-like particles as a highly*
574 *efficient vaccine platform: Diversity of targets and production systems and*
575 *advances in clinical development*. Vaccine, 2012. **31(1)**: p. 58-83.
- 576 8. Clements, C.J. and E. Griffiths, *The global impact of vaccines containing*
577 *aluminium adjuvants*. Vaccine, 2002. **20, Supplement 3(0)**: p. S24-S33.
- 578 9. Chen, D., et al., *Characterization of the freeze sensitivity of a hepatitis B*
579 *vaccine*. Hum Vaccin, 2009. **5(1)**: p. 26-32.
- 580 10. Matthias, D.M., et al., *Freezing temperatures in the vaccine cold chain: A*
581 *systematic literature review*. Vaccine, 2007. **25(20)**: p. 3980-3986.
- 582 11. Davaalkham, D., et al., *Administration of hepatitis B vaccine in winter as a*
583 *significant predictor of the poor effectiveness of vaccination in rural*
584 *Mongolia: evidence from a nationwide survey*. J Epidemiol Community
585 Health, 2007. **61(7)**: p. 578-84.
- 586 12. Drucker, E., P.G. Alcabes, and P.A. Marx, *The injection century: massive*
587 *unsterile injections and the emergence of human pathogens*. The Lancet.
588 **358(9297)**: p. 1989-1992.
- 589 13. Gupta, R.K., *Aluminum compounds as vaccine adjuvants*. Adv Drug Deliv
590 Rev, 1998. **32(3)**: p. 155-172.
- 591 14. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization*
592 *against hepatitis B infection*. Vaccine, 2010. **28(14)**: p. 2607-14.
- 593 15. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers*
594 *for nasal vaccine delivery in mice*. Eur J Pharm Biopharm, 2004. **57(1)**: p. 123-
595 31.

- 596 16. Vicente, S., et al., *Co-delivery of viral proteins and a TLR7 agonist from*
597 *polysaccharide nanocapsules: A needle-free vaccination strategy*. Journal of
598 Controlled Release, 2013. **172**(3): p. 773-781.
- 599 17. Vicente, S., et al., *A Polymer/Oil Based Nanovaccine as a Single-Dose*
600 *Immunization Approach*. PLoS ONE, 2013. **8**(4): p. e62500.
- 601 18. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size,*
602 *geometry, kinetics and molecular patterns*. Nat Rev Immunol, 2010. **10**(11): p.
603 787-796.
- 604 19. Scheerlinck, J.P. and D.L. Greenwood, *Virus-sized vaccine delivery systems*.
605 Drug Discov Today, 2008. **13**(19-20): p. 882-7.
- 606 20. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of*
607 *immunity*. Nature, 1998. **392**(6673): p. 245-52.
- 608 21. Pozzi, L.A., J.W. Maciaszek, and K.L. Rock, *Both dendritic cells and*
609 *macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop*
610 *effector function, and differentiate into memory cells*. J Immunol, 2005. **175**(4):
611 p. 2071-81.
- 612 22. Manolova, V., et al., *Nanoparticles target distinct dendritic cell populations*
613 *according to their size*. Eur J Immunol, 2008. **38**(5): p. 1404-13.
- 614 23. Reddy, S.T., et al., *In vivo targeting of dendritic cells in lymph nodes with*
615 *poly(propylene sulfide) nanoparticles*. Journal of Controlled Release, 2006.
616 **112**(1): p. 26-34.
- 617 24. Reddy, S.T., et al., *Exploiting lymphatic transport and complement activation*
618 *in nanoparticle vaccines*. Nat Biotech, 2007. **25**(10): p. 1159-1164.
- 619 25. Junt, T., et al., *Subcapsular sinus macrophages in lymph nodes clear lymph-*
620 *borne viruses and present them to antiviral B cells*. Nature, 2007. **450**(7166):
621 p. 110-114.
- 622 26. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, *Vaccine delivery carriers:*
623 *insights and future perspectives*. Int J Pharm, 2013. **440**(1): p. 27-38.
- 624 27. Zhao, Q., et al., *Maturation of Recombinant Hepatitis B Virus Surface Antigen*
625 *Particles*. Human Vaccines, 2006. **2**(4): p. 174-180.
- 626 28. Buschle, M., et al., *Transloading of tumor antigen-derived peptides into*
627 *antigen-presenting cells*. Proceedings of the National Academy of Sciences,
628 1997. **94**(7): p. 3256-3261.
- 629 29. Mattner, F., et al., *Vaccination with Poly-l-Arginine As Immunostimulant for*
630 *Peptide Vaccines: Induction of Potent and Long-Lasting T-Cell Responses*
631 *against Cancer Antigens*. Cancer Research, 2002. **62**(5): p. 1477-1480.
- 632 30. Valneva Austria GmbH, *Phase II Study of Immunization With a Hepatitis C*
633 *Virus (HCV) Antigen Peptide Vaccine*, 2008: In: ClinicalTrials.gov [Internet].
634 Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 March

- 635 17] Available from: <http://clinicaltrials.gov/show/NCT00602784> NLM
636 Identifier: NCT00602784.
- 637 31. Reynolds, F., R. Weissleder, and L. Josephson, *Protamine as an efficient*
638 *membrane-translocating peptide*. Bioconjugate Chemistry, 2005. **16**(5): p.
639 1240-1245.
- 640 32. Martínez Gómez, J.M., et al., *Surface coating of PLGA microparticles with*
641 *protamine enhances their immunological performance through facilitated*
642 *phagocytosis*. Journal of Controlled Release, 2008. **130**(2): p. 161-167.
- 643 33. Yang, D. and K.S. Jones, *Effect of alginate on innate immune activation of*
644 *macrophages*. J Biomed Mater Res A, 2009. **90**(2): p. 411-8.
- 645 34. Kurachi, M., et al., *Comparison of the activities of various alginates to induce*
646 *TNF-alpha secretion in RAW264.7 cells*. Journal of Infection and
647 Chemotherapy, 2005. **11**(4): p. 199-203.
- 648 35. Flo, T.H., et al., *Involvement of toll-like receptor (TLR) 2 and TLR4 in cell*
649 *activation by mannuronic acid polymers*. J Biol Chem, 2002. **277**(38): p.
650 35489-95.
- 651 36. McCarthy, R.E., L.W. Arnold, and G.F. Babcock, *Dextran sulphate: an*
652 *adjuvant for cell-mediated immune responses*. Immunology, 1977. **32**(6): p.
653 963-974.
- 654 37. Kerlin, R.L. and D.L. Watson, *Effect of dextran sulphate on IgG subclass of*
655 *antibody in efferent popliteal lymph of sheep*. Immunol Cell Biol, 1987. **5**: p.
656 411-7.
- 657 38. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and*
658 *cytokine induction by human primary cells: a phenomenon absent from murine*
659 *cell systems*. Vol. 110. 2007. 3245-3252.
- 660 39. Durand, V., et al., *Shaping of adaptive immune responses to soluble proteins*
661 *by TLR agonists: a role for IFN-alpha/beta*. Immunol Cell Biol, 2004. **82**(6): p.
662 596-602.
- 663 40. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen*
664 *Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced*
665 *Strong Mucosal and Systemic Immunities*. Pharmaceutical Research, 2006.
666 **23**(6): p. 1217-1226.
- 667 41. Van de Steeg, H.G.M., et al., *Polyelectrolyte adsorption: a subtle balance of*
668 *forces*. Langmuir, 1992. **8**(10): p. 2538-2546.
- 669 42. Diminsky, D., et al., *Comparison between hepatitis B surface antigen (HBsAg)*
670 *particles derived from mammalian cells (CHO) and yeast cells (Hansenula*
671 *polymorpha): composition, structure and immunogenicity*. Vaccine, 1997.
672 **15**(6-7): p. 637-47.
- 673 43. Instruments, M., *Dynamic light scattering: an introduction in 30 minutes*.
674 Technical Note Malvern, MRK656-01, 2012: p. 1-8.

- 675 44. Rustgi, V.K., C.J. Schleupner, and D.S. Krause, *Comparative study of the*
676 *immunogenicity and safety of Engerix-B administered at 0, 1, 2 and 12 months*
677 *and Recombivax HB administered at 0, 1, and 6 months in healthy adults.*
678 *Vaccine*, 1995. **13**(17): p. 1665-1668.
- 679 45. West, D.J. and G.B. Calandra, *Vaccine induced immunologic memory for*
680 *hepatitis B surface antigen: implications for policy on booster vaccination.*
681 *Vaccine*, 1996. **14**(11): p. 1019-1027.
- 682 46. Miyamoto, M., et al., *Effect of poly-l-arginine on the nasal absorption of*
683 *FITC-dextran of different molecular weights and recombinant human*
684 *granulocyte colony-stimulating factor (rhG-CSF) in rats.* *International Journal*
685 *of Pharmaceutics*, 2001. **226**(1–2): p. 127-138.
- 686 47. Tafaghodi, M., et al., *Hepatitis B surface antigen nanoparticles coated with*
687 *chitosan and trimethyl chitosan: impact of formulation on physicochemical*
688 *and immunological characteristics.* *Vaccine*, 2012. **30**(36): p. 5341-5348.
- 689 48. Hafner, A.M., B. Corthesy, and H.P. Merkle, *Particulate formulations for the*
690 *delivery of poly(I:C) as vaccine adjuvant.* *Adv Drug Deliv Rev*, 2013. **65**(10):
691 p. 1386-99.
- 692 49. Finkelman, F.D., et al., *Lymphokine control of in vivo immunoglobulin isotype*
693 *selection.* *Annual review of immunology*, 1990. **8**(1): p. 303-333.
- 694 50. Cribbs, D.H., et al., *Adjuvant-dependent modulation of Th1 and Th2 responses*
695 *to immunization with β -amyloid.* *International Immunology*, 2003. **15**(4): p.
696 505-514.
- 697 51. Paul, W.E. and R.A. Seder, *Lymphocyte responses and cytokines.* *Cell*, 1994.
698 **76**(2): p. 241-251.
- 699 52. Graham, M.B., V.L. Braciale, and T.J. Braciale, *Influenza virus-specific CD4+*
700 *T helper type 2 T lymphocytes do not promote recovery from experimental*
701 *virus infection.* *J Exp Med*, 1994. **180**(4): p. 1273-82.
- 702
- 703

Figure captions:

Figure 1: Illustration of the adsorption process of the **(A)** protamine (PR) and **(B)** polyarginine (PARG) layers onto the HB antigen nanoparticles, and the subsequent adsorption of the dextran sulfate (DS) (HB:PR:DS), alginate (ALG) (HB:PARG:ALG) or poly (I:C) (HB:PARG:pIC) layers, respectively.

Figure 2: Evolution on the particle size (Size), polydispersity index (PdI) and surface charge (zeta potential) of the HB nanoparticles coated with multiple layers, namely **(A)** HB:PR and HB:PR:DS coated with protamine (PR), or PR plus dextran sulfate, respectively **(B)** HB:PARG and HB:PARG:ALG, coated with polyarginine (PARG) or PARG plus ALG, respectively and, **(C)** HB: PARG and HB:PARG:pIC, coated with polyarginine (PARG) or PARG plus poly (I:C) (pIC), respectively. Results are presented as the mean $\pm$ standard deviation (n = 4).

Figure 3: Evaluation of size number distribution (Size) and polydispersity index (PdI) after 4 hours at 37 °C in PBS of **(A)** HB nanoparticles coated with protamine (HB:PR), **(B)** HB nanoparticles coated with protamine and dextran sulfate (HB:PR:DS) and **(C)** HB nanoparticles coated with polyarginine and poly (I:C) (HB:PARG:pIC). Results are presented as the mean $\pm$ standard deviation (n = 3).

Figure 4: Confocal microscopy analysis of the uptake of fluorescent nanoparticles: **(A)** HB:PR:DS and **(B)** HB:PARG:ALG, by RAW 264.7 macrophage cells. Red channel: TAMRA-labeled particles). Green channel: Alexa Fluor 488-phalloidin (cytoskeleton). Blue channel: DAPI (nucleus).

Figure 5: Systemic humoral (IgG) immune response observed in mice upon intramuscular (0 and 4 weeks) immunization with HB antigen nanoparticles coated with protamine (HB:PR), protamine and dextran sulfate (HB:PR:DS), polyarginine and alginate (HB:PARG:ALG) or polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen adsorbed to Alum (HB:Alum) was used as control. The plot represents the anti-HB IgG average quantified at different time points after primary immunization. Results are shown as mean $\pm$ standard deviation (n = 10).

Figure 6: Systemic humoral (IgG) immune response observed in mice upon intranasal (0, 4 and 16 weeks) immunization with HB antigen nanoparticles coated with protamine (HB:PR), protamine and dextran sulfate (HB:PR:DS), polyarginine and alginate (HB:PARG:ALG) or polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen dispersed in PBS (HB in PBS) was used as control. The plot represents the anti-HB IgG average quantified at different time points after primary immunization. Results are shown as the mean $\pm$ standard deviation (n = 10).

Figure 7: IgG1 and IgG2a subtype ratios in the sera of mice immunized with HB antigen nanoparticles coated with polyarginine and poly (I:C) (HB:PARG:pIC) (grey squares), and with HB adsorbed onto alum (HB:Alum) (black circle). Results are shown as the mean (n = 10).

Annex 1. Comparison of DLS signal analysis of the HB antigen nanoparticles alone through three different size distributions: **(A)** Intensity, **(B)** Volume and **(C)** Size

Annex 2. Evolution of size number distribution (Size) and polydispersity index (PdI) after one month storage at 4 °C of the HB nanoparticles coated with protamine (HB:PR), with protamine and dextran sulfate (HB:PR:DS), with polyarginine and alginate (HB:PARG:ALG) or with polyarginine and poly (I:C) (HB:PARG:pIC). Results are presented as the mean $\pm$ standard deviation (n = 3).

Annex 3. Comparison of the size number distribution (Size), polydispersity index (PdI) and surface charge (Zeta) of non-labeled and TAMRA-labeled HB:PR nanoparticles **(A)**, and of non-labeled and TAMRA-labeled HB:PARG and HB:PARG:ALG formulations **(B)**. Results are presented as mean $\pm$ standard deviation (n = 3).

Figure 1

A

B

**Polyarginine (PARG)
Protamine (PR)***

Alginate (ALG)

Dextran Sulfate (DS)

Poly (I:C) (pIC)

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Annex 1

Size Distribution by Intensity

Size Distribution by Volume

Size Distribution by Number

Annex 2

Annex 3

Graphical Abstract

Internalization of fluorescent multi-enveloping nanoparticles (Red)

Increased IgG response
Balanced T_h1/T_h2 response