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Research Paper

Evaluation of Bioadhesive Capacity and Immunoadjuvant Properties of Vitamin B_{12}-Gantrez Nanoparticles

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Purpose. To design bioadhesive Gantrez AN (poly[methyl vinyl ether-co-maleic anhydride], PVM/MA) nanoparticles (NP) coated with vitamin B_{12} (Vit B_{12}), and investigate their application in oral antigen delivery.

Methods. The association of Vit B_{12} to Gantrez AN nanoparticles was performed by the direct attachment of reactive Vit B_{12} to the surface of the nanoparticles (NPB), or linking to the copolymer chains in dimethylformamide prior to NP formation (NPB-DMF). Nanoparticles were characterized by measuring the size, zeta potential, Vit B_{12} association efficacy, and stability of Vit B_{12} on the surface of the nanoparticles. *In vivo* bioadhesion study was performed by the oral administration of fluorescently-labeled nanoparticle formulations to rats. Both systemic and mucosal immune responses were evaluated after oral and subcutaneous immunization with ovalbumin (OVA) containing Vit B_{12}-coated nanoparticles.

Results. The Vit B_{12} nanoparticles displayed homogenous size distribution with a mean diameter of about 200 nm and a negative surface charge. The association efficiency of Vit B_{12} to NPB-DMF formulation was about two times higher than to the NPB, showing also a higher surface stability of Vit B_{12}. The bioadhesion study demonstrated that NPB-DMF had an important tropism to the distal portions of the gut, which was about two and 3.5 times higher than the tropism observed for NPB and control NP, respectively ($p<0.05$). Oral administration of OVA-NPB-DMF induced also stronger and more balanced serum anti-OVA titers of IgG2a (Th1) and IgG1 (Th2) compared to control OVA-NP. In addition, oral immunization with OVA-NPB-DMF induced a higher mucosal IgA response than subcutaneous administration.

Conclusions. These results indicate the benefits of bioadhesive Vit B_{12}-coated nanoparticles in oral antigen delivery eliciting systemic and mucosal immune response.

KEY WORDS: bioadhesion; nanoparticles; oral antigen delivery; vitamin B_{12}.

INTRODUCTION

Vitamin B_{12} (cobalamin) has its own intestinal uptake pathway by which it can be transported to the systemic circulation. By this mechanism, a molecule of intrinsic factor (IF), secreted by the parietal cells in the stomach, interacts and binds to one molecule of free vitamin B_{12}. The resulting complex (vitamin B_{12}–IF) moves down the gastrointestinal tract until it reaches the ileum. In this region, the complex is recognized by the intrinsic factor specific receptors (IF-CR) which are located on the surface of the ileal epithelial cells. Then, this complex is internalized by receptor-mediated endocytosis. Vitamin B_{12} is subsequently transported to the systemic circulation and complexed with cobalamin-binding protein [Transcobalamin II (TCII)] (1,2). This specific of vitamin B_{12} uptake system has been explored in the last years to enhance the oral delivery of poorly available therapeutic molecules including, granulocyte colony stimulating factor, erythropoietin, and luteinizing hormone-releasing hormone (3–5). However, in spite of the numerous advances for the oral delivery of small peptides and proteins, some disadvantages have limited the efficiency of this strategy. The directly linked macromolecular drugs to vitamin B_{12} are susceptible to the degradation within the gastrointestinal tract. In addition, according to the limited uptake capacity of vitamin B_{12} (2.4 μg/day for adult) (6), the amount of the therapeutic molecule that can be delivered as conjugate is limited. An attractive solution to overcome theses problems would be the incorporation of drug in nanoparticles coated with vitamin B_{12}. This can offer both high loading capacity of the

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therapeutic molecules and the protection against the aggressive gut conditions (pH and enzymatic degradation).

Studies involving vitamin B_{12}-nanoparticles conjugates have been carried out *in vitro* using Caco-2 cells. They have clearly demonstrated the effective cellular uptake of nanoparticles (7) or polymeric micelles containing cyclosporine A coated with vitamin B_{12} (8). Recently, *in vivo* results indicated the beneficial use of vitamin B_{12}-coated dextran nanoparticles for the oral delivery of insulin (9). However, to date, no published studies have been reported on the application of nanoparticles coated with vitamin B_{12} derivatives in the mucosal immunization.

Oral antigen delivery using nanoparticles is being considered as a promising strategy for oral vaccination. Orally delivered vaccines can induce high levels of mucosal immune response and, thus, protect the host against many pathogens that use the mucosal route to invade the body (10,11). Unfortunately, oral immunization using conventional antigen-loaded nanoparticles does not generally elicit a sufficient systemic immune response compared to the parenteral routes, and consequently, high and multiple oral doses are required (12). This fact is related to the low capacity of conventional antigen-loaded nanoparticles to target lymphoid tissues within the gut after an oral immunization. The association between antigen-loaded nanoparticles and specific ligands (i.e., lectins) may enhance this targeting, resulting in a successful antigen delivery to the mucosal surfaces and the induction of the immune response (13,14).

Consequently, our work strategy was to explore the beneficial use of vitamin B_{12} as nanoparticles' targeted ligand to achieve specific bioadhesive profiles within the gut. Thus, the main tasks were to optimize the preparation of vitamin B_{12}-coated Gantrez AN nanoparticles, and to investigate the *in vivo* bioadhesive capacity after their oral administration to laboratory animals. In addition, the potential immunoadjuvant capacity of vitamin B_{12} nanoparticles was investigated using ovalbumin as standard antigen model.

MATERIALS AND METHODS

Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) (PVM-MA) Gantrez® AN 119 was supplied by ISP (Barcelona, Spain). Reactive vitamin B_{12}–5′OCONH$[CH_2]_6$–NH_2 was kindly gifted by Access Pharmaceuticals Australia (Chatswood, Australia). Rhodamine B isothiocyanate (RBITC), 1,3-diaminopropane, ovalbumin (OVA, Grad V), and protease inhibitor cocktail [(4-(2 aminoethyl) benzenesulfonyl fluoride, trans-epoxysuccinyl-leucyl-amido (4 guanidino) butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA] were purchased from Sigma (Madrid, Spain). Peroxidase conjugated antibodies for IgG1, IgG2a and IgA were supplied by Nordic Immunology Labs (Via Cultek S.L, Madrid, Spain). All other reagents were of an analytical grade and supplied by Merck (Darmstadt, Germany).

Preparation of Vit B_{12}–Gantrez AN Nanoparticle Conjugates

The association between Gantrez AN nanoparticles and reactive vitamin B_{12} was performed by two optimized methods (Fig. 1A,B).

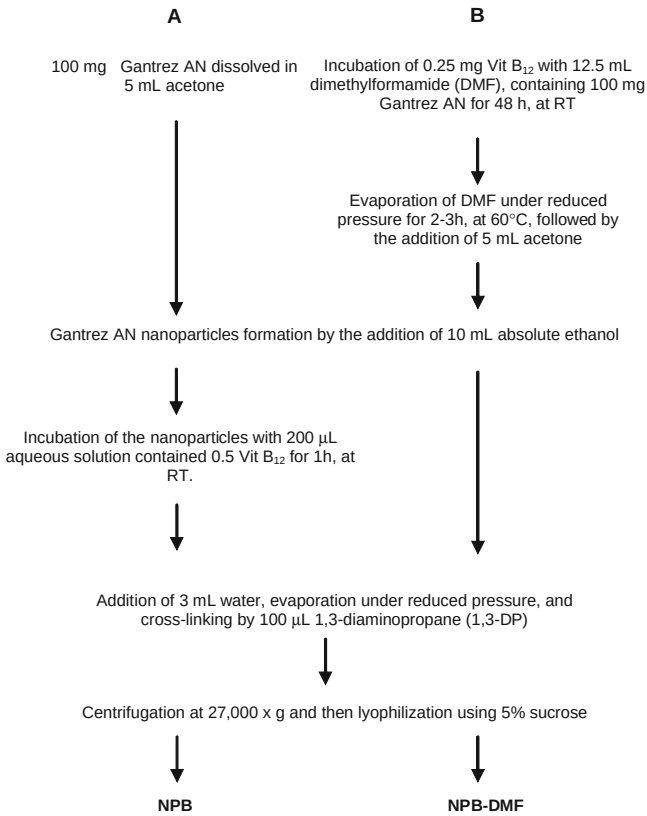

Fig. 1. Schematic representation of the methods A and B used for preparing the Gantrez AN nanoparticles and associating vitamin B_{12} to the nanoparticles.

In method A, Gantrez AN nanoparticles were prepared using a solvent displacement method (15). In brief, 100 mg of PVM/MA copolymer were dissolved in 5 ml acetone, after which the nanoparticles were formed by the addition of 10 ml absolute ethanol under magnetic stirring. The organic polymer solution was mixed with 200 µl deionized water containing 0.5 mg reactive vitamin B_{12} and magnetically stirred for 1 h at RT. Then, 3 ml of deionized water were added, and the organic solvents eliminated under reduced pressure (Büchi Rotavapor R-144, Switzerland). The resulting nanoparticles were cross-linked for 5 min by the addition of 100 µl of 1,3-diaminopropane (1,3-DP, 1% *w/v*) and pelleted by centrifugation at 27,000×*g* for 20 min (Sigma 3K30, Germany). The supernatant was collected for quantifying the unbound vitamin B_{12}.

For *in vivo* bioadhesion studies, the centrifuged nanoparticles were fluorescently labelled by incubation with 1.25 mg RBITC for 5 min at room temperature under magnetic stirring after which, the suspension was pelleted again. Both RBITC- loaded and non-loaded nanoparticles were lyophilized using sucrose 5% as cryoprotector for 48 h in a Virtis Genesis lyophiliser (Virtis, New York, USA).

In method B, Vit B_{12}–Gantrez AN nanoparticle conjugates were prepared by the reaction between 0.25 mg of reactive vitamin B_{12} and 100 mg Gantrez AN copolymer in 12.5 ml dimethylformamide (DMF), under magnetic stirring for 48 h at RT. Then, the organic solvent was removed under reduced pressure at 60°C for 2–3 h. The formed thin film was dissolved in 5 ml acetone, and the nanoparticles were formed and processed as described in method A.

Characterization of Vitamin B_{12}-Gantrez AN Nanoparticle Conjugates

The particle size and zeta potential of nanoparticles were analysed by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a zetamaster analyser system (Malvern Instruments, Malver, UK). The morphology of the nanoparticles was observed by scanning electron microscopy in a Zeiss DSM940 digital scanning electron microscope (Oberkochen, Germany).

The amount of nanoparticles formed from the original Gantrez AN copolymer (% yield) was determined by gravimetry (16). The amount of vitamin B_{12} bound to the surface of the nanoparticles was estimated from the Vit B_{12} concentration difference (colorimetric assay, λ 540 nm) measured in the supernatant before nanoparticle incubation and after the first centrifugation step. The extent of RBITC loading onto the nanoparticles was similarly estimated from the RBITC concentration difference measured before nanoparticle incubation and after the second centrifugation step of the nanoparticles.

In Vitro Release of Vitamin B_{12} from Nanoparticles

The stability of the linked vitamin B_{12} to Gantrez AN nanoparticles was considered to be a critical factor for the planned *in vivo* study and was, therefore, used to select the appropriate nanoparticles formulation; indeed, the presence of unbound vitamin B_{12} can exert an auto-inhibitory effect on the binding capacity of Vit B_{12}–Gantrez AN nanoparticles to the mucosal cells. Therefore, 10 mg Vit B_{12}–Gantrez AN nanoparticles were incubated in 1 ml simulated gastric fluid [SGF (USP XXVIII): pH 1.2, pepsin 0.32% *w/v*] for 1 h at 37±1°C. Then, the nanoparticles were collected by using dialysis tubes (Vivaspin® 100,000 MWCO; VIVASPIN, Hannover, Germany). The nanoparticles were then re-incubated in 1 ml of simulated intestinal fluid [SIF (USP XXVIII): pH 7.5, pancreatin 1% *w/v*] for different time periods until 24 h at 37±1°C. The nanoparticles were collected by dialysis, as described above, after each incubation time point. The concentration of free vitamin B_{12} in all of the collected dialysates was determined by colorimetry at 540 nm.

In Vivo Bioadhesion Study

The bioadhesion studies were carried out using a protocol described previously (16), in compliance with the regulations of the European legislation on animal experiments (86/609/EU). Briefly, an aqueous suspension containing 10 mg nanoparticles loaded with RBITC (approximately 45 mg particles/kg body weight) was administered by the oral gavages using 16G X 3″ (76.2 mm) feeding needle (Proper and Sons Inc., N.Y, U.S.A) to male Wistar rats fasted overnight (average weight 225 g; Harlan, Spain). The animals were sacrificed by cervical dislocation at 0.5, 1, 3 and 8 h post administration. The abdominal cavity was opened, and the gastrointestinal tract was removed. Then, the gut was divided into six anatomical regions: stomach (Sto), intestine (I1, I2, I3 and I4) and caecum (Ce). Each mucosa segment was opened lengthwise, rinsed with PBS and digested with 3 M NaOH for 24 h. RBITC was extracted from the digested samples by addition of 2 ml methanol, vortexed for 1 min and centrifuged at 2,000×*g* for 10 min. Aliquots (1 ml) of the obtained supernatants were diluted with water (3 ml) and assayed for RBITC content by spectrofluorimetry at λ_{ex} 540 nm and λ_{em} 580 nm (GENios, TECAN, Austria) to estimate the fraction of adhered nanoparticles on the mucosa. Standard curves were prepared by addition of RBITC solutions in 3 M NaOH (0.5–10 μg/ml) to control tissue segments following the same steps of extraction (r=0.996).

In order to study the kinetic parameters of bioadhesion, the total fraction of adhered nanoparticles in the whole gastrointestinal tract was plotted *versus* time. From these curves, the bioadhesion parameters Q_{max}, AUC_{adh}, T_{max}, MRT_{adh} and K_{adh} were estimated from 0 to 8 h post administration as described previously (15) and calculated using WinNonlin 1.5 software (Pharsight Corporation, USA). Q_{max} represents the maximal adhered quantity. AUC_{adh} is the area under the curve described for the total adhered nanoparticles *versus* time. T_{max} was defined as the time at which maximal adhesion in gastrointestinal tract was obtained. MRT_{adh} indicates the mean residence time of adhering nanoparticles, and K_{adh} the elimination rate of total adhering nanoparticles.

Preparation of OVA-Loaded Vit B_{12}–Gantrez Nanoparticles

To study the adjuvant capacity of Vit B_{12}–Gantrez AN nanoparticles, the model antigen ovalbumin was loaded into the nanoparticle formulations. As the bioadhesion data suggested a benefit of NPB-DMF over NPB, OVA was loaded into the Vit B_{12}–NBP-DMF nanoparticles according to method B (Fig. 1). Briefly, 5 mg OVA were sonicated in 5 ml acetone containing 100 mg Gantrez AN–vitamin B_{12} conjugate. This organic dispersion was magnetically stirred for 1 h at room temperature. The copolymer was desolvated by the addition of 10 ml of absolute ethanol and then 3 ml of deionized water. The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland), and the resulting aqueous nanosuspension was cross-linked with 1,3-DP and pelleted by centrifugation at 27,000×*g* for 20 min. The supernatant was used to quantify the unbounded vitamin B_{12}. Finally, OVA-NPB-DMF nanoparticle dispersion was lyophilized, as described above. Control nanoparticles (OVA-NP) were prepared by the same way, but without the use of vitamin B_{12}.

Characterization of OVA-Loaded Vit B_{12} Nanoparticles

The size and zeta potential of the nanoparticles as well as the amount of vitamin B_{12} associated to OVA-NPB-DMF were determined as described above. The amount of OVA associated to the OVA-NPB-DMF nanoparticles was determined by SDS-PAGE. Typically, 5 mg OVA-NPB-DMF nanoparticles were dissolved in 2 ml DMF/acetone (1:1, *v/v*) and then kept at −20°C for 24 h. Samples were centrifuged at 10,000×*g* for 15 min, and the precipitate was washed with cold acetone and centrifuged again. The amount of OVA was estimated from the average band density of the samples in the SDS-PAGE using Micro Image® software (Version 4.0; Olympus Optical, Hamburg, Germany); an OVA standard curve was established in the range of 2.5–0.25 μg/band.

Immunization Protocol

Animal study protocols were applied in compliance with the regulations of the responsible committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU). Female BALB/c mice of average weight of 20±1 g, supplied by Harlan (Barcelona, Spain), were divided into six groups of ten mice. Mice were fasted overnight, with free access to water. The formulations were administered as single OVA doses by either oral (100 μg OVA) or subcutaneous (20 μg OVA) route. In the oral groups (three groups, $n=10$), animals were fed by oral gavage of 200 μl PBS containing OVA-NPB-DMF nanoparticles or OVA-NP (control nanoparticles), or OVA in solution. In the subcutaneous groups (three groups, $n=10$), the animals were injected with 50 μl PBS containing OVA-NPB-DMF nanoparticles, OVA-NP, or OVA in solution. After 2 h post administration, the animals were allowed unrestricted access to water and food.

Sample Collection

Blood samples were collected from the retroorbital plexus on 0, 1, 2, 4 and 6 weeks post administration. The samples were centrifuged (10,000×*g*, 10 min), the sera pooled for each group (ten mice), diluted 1:10 with PBS and conserved at −80°C untill analysis. Faecal samples were collected and analysed as described previously (16). Pooled fresh pelleted faeces from each group of mice were put into microtubes on 0, 1, 2, 4, 6 weeks post administration and weighed. Non-fatty milk (3%) in phosphate-buffered saline was added at 1 ml/100 mg faecal pellets. The pellets were vortexed for 5 min at room temperature. Then, the tubes were centrifuged at 10,000×*g* for 10 min, and the supernatants transferred into tubes containing 10 μl of protease inhibitor cocktail. Finally, pooled samples were stored at −80°C until analysis.

ELISA Assay

Serum Anti-OVA IgG1 and IgG2a

Anti-OVA serum antibodies (IgG1 and IgG2a) were assayed by ELISA using 96 microtiter plates (Thermo LabSystems, Vantaa, Finland). Wells were coated with OVA (100 μl per well) in a solution of 10 μg/ml of OVA in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6) at 4°C for 24 h. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) using an ELISA plate washer (Thermo Labsystems, Vantaa, Finland). Then, wells were incubated with 200 μl of 1% BSA in PBS-T for 1 h at room temperature. After washing as described above, serum samples (100 μl) were added in twofold serial dilutions in PBS-T starting with 1:40 initial dilution, and incubated at 37°C for 4 h. The washed wells were incubated for 2 h at 37°C with 100 μl of anti-IgG_1 and anti-IgG_{2a} peroxidase-conjugated antibodies (GAM/IgG1/PO and GAM/IgG2a/PO) used in dilution of 1:1,000 in PBS-T. For color development, the substrate-chromogen used was H_2O_2-ABTS (3-ethylbenzthiazoline-6-sulfonic acid). The absorbance (Abs) was determined at 405 nm (iEMS Reader MF de Labsystems, Vantaa, Finland), and the end-point titer determined as the sample dilution giving a mean Abs ≥0.2 above that measured for untreated mice sera.

For semi-quantitative comparison between immune responses induced by the different nanoparticle formulations, the areas under the curves of the antibody response profiles [$AUC_{th1(IgG2a)}$ and $AUC_{th2(IgG1)}$] were calculated for the period of 0–6 weeks post immunization using the WinNonline 1.5 software (Pharsight Corp, USA), as described previously (16).

Faecal Anti-OVA IgA

After the washing step of OVA-coated plates, as described above, the plates were blocked with 200 μl 3% non-fatty milk in PBS-T for 1 h at room temperature. Faecal extract samples of 100 μl were added starting with undiluted samples and followed by twofold serial dilutions up to 1:64 in PBS-T; the plates were incubated at 37°C for 4 h. After washing the wells, 1:1,000 dilutions of anti-IgA peroxidase conjugated antibody (GAM/IgA/PO) were added. The detection step and the end titers were determined as described above.

Statistical Methods

The bioadhesion data and the physico-chemical characteristics were compared using the nonparametric Mann–Whitney *U* test and Student's *t* test, respectively. *P* values of <0.05 were considered significant. All calculations were performed using SPSS® statistical software program (SPSS® 10, Microsoft, USA).

RESULTS

Characteristics of Nanoparticle Formulations

Vit B_{12} nanoparticle conjugates were prepared by two different methods. In method A, vitamin B_{12} was bound to the surface of previously prepared nanoparticles. In method B, a Vit B_{12}–Gantrez AN conjugate was first prepared, which was subsequently used to prepare the nanoparticles. All nanoparticle formulations were prepared with similar yields (about 80%) and showed homogenous size distributions with a mean diameter of about 200 nm (Table I, Fig. 2). Both types of Vit B_{12} nanoparticles (NPB and NPB-DMF; see Fig. 1) displayed a similar negative zeta potential, which differed significantly from that measured with control nanoparticles ($p<0.05$; Table I). The content of vitamin B_{12} was higher in the NPB-DMF nanoparticles than in the NPB nanoparticles ($p<0.05$). In addition, the association efficiency of vitamin B_{12} to the nanoparticles prepared by method B was two times higher than that obtained with the nanoparticles prepared by method A (Table I). Finally, the amount of loaded RBITC was similar, i.e., approximately 13 μg/mg nanoparticles, for all the nanoparticle formulations.

In vitro Release of Vitamin B_{12} from the Nanoparticles

Figure 3 illustrates the release of vitamin B_{12} from nanoparticles after their subsequent incubation in simulated gastric fluid (SGF) and intestinal fluid (SIF). After 1 h incubation in SGF (pH 1.2), NPB produced a significant release of vitamin B_{12}, which was about 30% of the total

Table I. Physicochemical Characteristics of the Vit B_{12} Nanoparticles

	Size (nm)[a]	PDI[b]	Zeta potential (mV)[c]	Yield (%)	Vit B_{12} content (μg/mg)[e]	Association efficiency of vitamin B_{12} to NP (%)[f]
NP	203±1	0.15±0.04	−47±1	78±1	–	–
NPB	200±3	0.16±0.03	−24±3*	83±1	2.32±0.07	38±1
NPB-DMF	198±1	0.09±0.08	−26±2*	79±2	2.45±0.05	77±1*

Data expressed as mean±SD (n=6). NPB and NPB-DMF: Vit B_{12} nanoparticles prepared by methods A and B (see Fig. 1)
*$p<0.05$ for MNPB and NPB-DMF *versus* control nanoparticles (NP; Student's t test)
[a] Measurement of the nanoparticle size by photon correlation spectroscopy
[b] Polydispersity index
[c] Measurement of the zeta potential by electrophoretic laser Doppler anemometry
[d] Percentage of nanoparticles formed relative to the initial amount of polymer used
[e] Amount of vitamin B_{12} associated to the nanoparticles expressed in μg /mg NP
[f] Association efficiency of vitamin B_{12} to the nanoparticles formulation expressed in percentage relative to the initial amounts of materials used

amount of associated vitamin B_{12}. When this formulation was subsequently incubated in SIF (pH 7.5), the release of vitamin B_{12} continued in a more sustained way and reached approximately 50% of the total dose within 24 h. On the contrary, less than 3% of vitamin B_{12} were released from NPB-DMF after their incubation in SGF (1 h) and SIF (23 h).

In Vivo Bioadhesion Study

Figure 4 shows the distribution of the adhered nanoparticles throughout the different sections of the GI tract after a single oral administration of 10 mg RBITC-loaded nanoparticles. Thirty minutes after administration, the Vit B_{12} nanoparticles (NPB, NPB-DMF) and control nanoparticles (NP) displayed a similar capacity to adhere to the gut, with a preferential distribution in the stomach and upper regions of the small intestine (Fig. 4a–c). Interestingly, at 3 h post administration, both Vit B_{12}-nanoparticle formulations displayed a higher tropism for the distal part of the gut (I3 and I4 portions) than the control nanoparticles (NP). Furthermore, this tropism was two and 3.5 times higher with the NPB-DMF nanoparticles than with the NPB and control nanoparticles (NP), respectively.

Analysis of the bioadhesion kinetics (Fig. 5) revealed that control nanoparticles achieved their maximal gut adhesion within the first 30 min, whereas the Vit B_{12}-nanoparticle formulations required 3 h to reach T_{max} (Table II). On the other hand, both the maximum amount (Q_{max}) and total amount (AUC_{adh}) of adhered nanoparticles were significantly higher for the NPB-DMF nanoparticles than for the NPB or control nanoparticles ($p<0.01$). The mean residence time of the adhered nanoparticle fraction (MTR_{adh}) was similar, i.e., approximately 3.5 h, for all nanoparticle formulations. Finally, the elimination constant (K_{adh}) for the adhered fraction of nanoparticles was significantly higher for the NPB-DMF nanoparticles than for the other formulations (Table II).

Characteristics of Ova-Loaded Vit B_{12} Nanoparticles

For the immunization studies, the model antigen OVA was encapsulated in the Vit B_{12}-nanoparticle formulation NPB-DMF and control nanoparticles free of vitamin B_{12} (NP). The OVA-NPB-DMF nanoparticles displayed a slightly

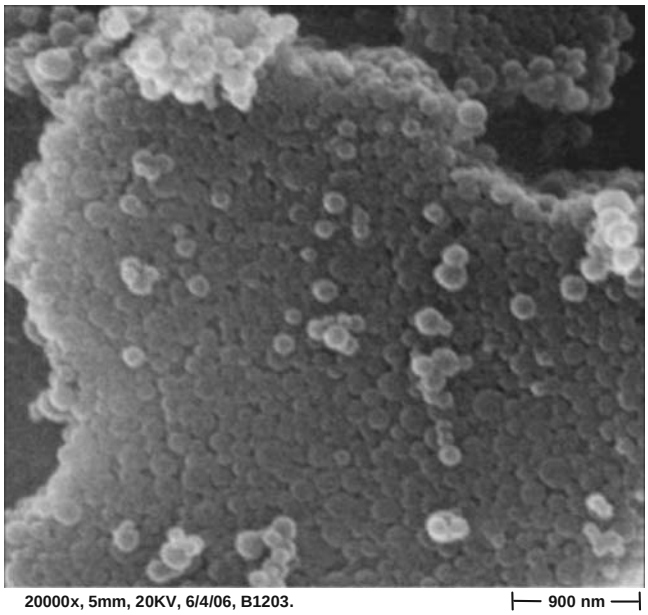

Fig. 2. Scanning electron micrographs (*SEM*) of lyophilized Vit B_{12} nanoparticles (NPB-DMF).

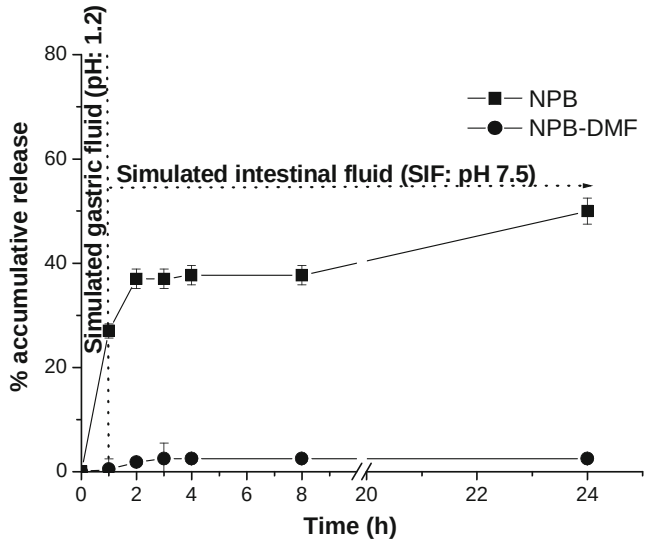

Fig. 3. Vitamin B_{12} release profiles from Vit B_{12}–Gantrez nanoparticles upon their subsequent incubation in simulated gastric and intestinal fluids. The nanoparticle formulations were: (*filled square*) NPB and (*filled circle*) NPB-DMF. Data represented as mean±SD (n=3).

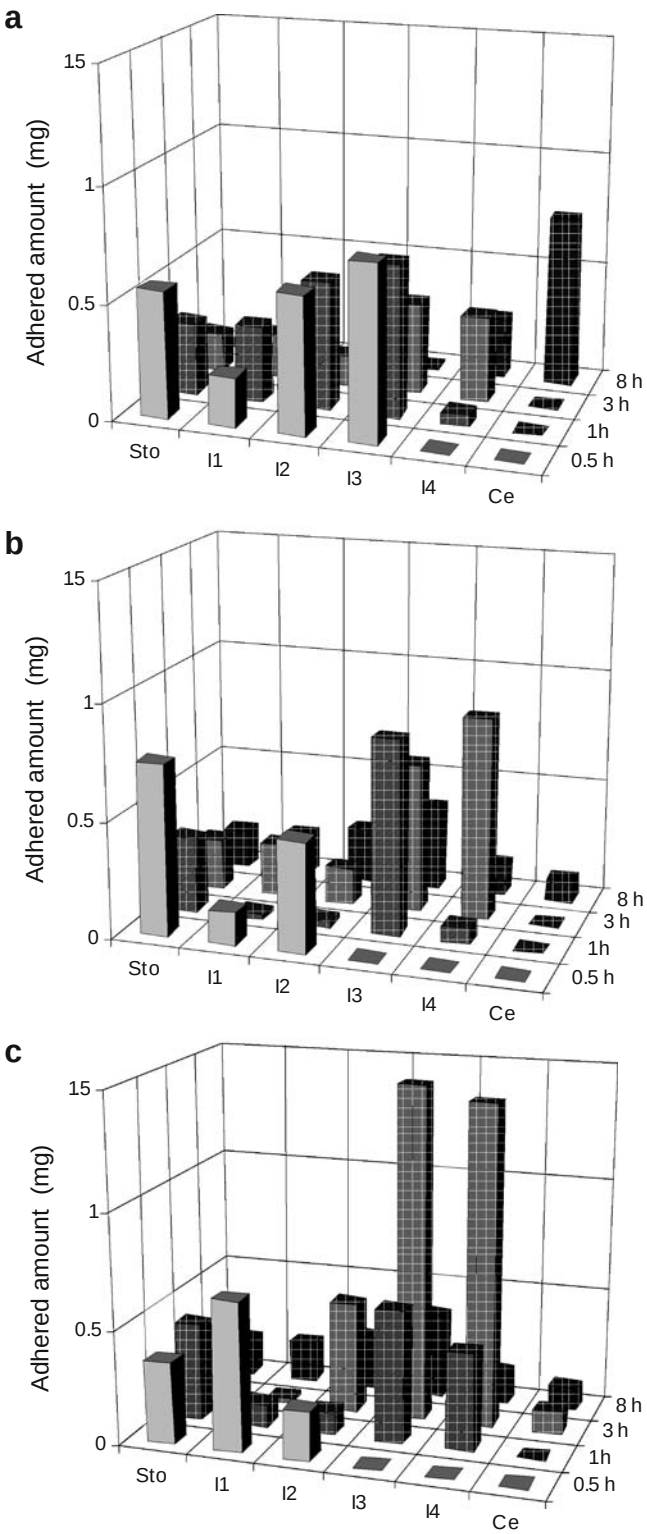

Fig. 4. Distribution of nanoparticles in the gastrointestinal tract of rats after oral administration of 10 mg RBITC-loaded nanoparticles. **a** Control nanoparticles (*NP*); **b** Vit B_{12}-NP prepared by method A (NPB); **c** Vit B_{12}-NP prepared by method B (NPB-DMF). The *x*-axis represents the different gut segments of stomach (*Sto*), intestinal sections (I1,I2,I3,I4), and caecum (*Ce*). The *y*-axis shows the adhered amounts of nanoparticles on the mucosa. The *z*-axis represents the post administration time. Each value is represented by the mean (n=3; SD was less than 20% of the mean).

lower mean diameter (230 nm) than the control OVA-loaded nanoparticles (OVA-NP, 270 nm). The amount of vitamin B_{12} associated to OVA-NPB-DMF nanoparticles was approx. 2.4 μg/mg NP. The amount of OVA loaded in the nanoparticles was approximately 12 μg OVA/mg NP and similar for both the OVA-NPB-DMF nanoparticles and OVA-NP.

OVA-Specific Serum Antibody Response

Figure 6 shows OVA-specific IgG2a and IgG1 serum titers measured after oral gavage or subcutaneous (s.c.) administration of a single dose of OVA-loaded Vit B_{12}-nanoparticles (OVA-NPB-DMF), OVA-loaded control nanoparticles free of vitamin B_{12} (OVA-NP) and free OVA. After both the oral and s.c. administrations, both nanoparticle formulations enhanced the serum antibody responses (IgG2a, IgG1) compared to free antigen. Upon s.c. immunization, the IgG2a titers (mirroring a Th1 response) elicited by the OVA-NPB-DMF nanoparticles were at least two times higher than those elicited by the OVA-NP (see AUC-values, Fig. 6a,b), although both OVA-loaded nanoparticle formulations (OVA-NPB-DMF and OVA-NP) induced similar IgG1 titers (mirroring a Th2 response).

Oral immunization with the control OVA-NP showed a predominant IgG1 response (AUC_{IgG1} was seven times higher than AUC_{IgG2a}, Fig. 6c,d). In contrast, oral immunization with OVA-NPB-DMF elicited stronger and more balanced Th1/Th2 responses as compared to the control OVA-NP. The sum of both the AUC_{IgG1} and AUC_{IgG2a} for OVA-NPB-DMF was about three times higher than that calculated for OVA-NP (Fig. 6c,d).

Anti-OVA Specific Intestinal IgA

Figure 7 illustrates the faecal anti-OVA IgA response after subcutaneous and oral immunization of the different formulations tested. Both nanoparticle formulations elicited higher levels of intestinal IgA compared to free OVA when

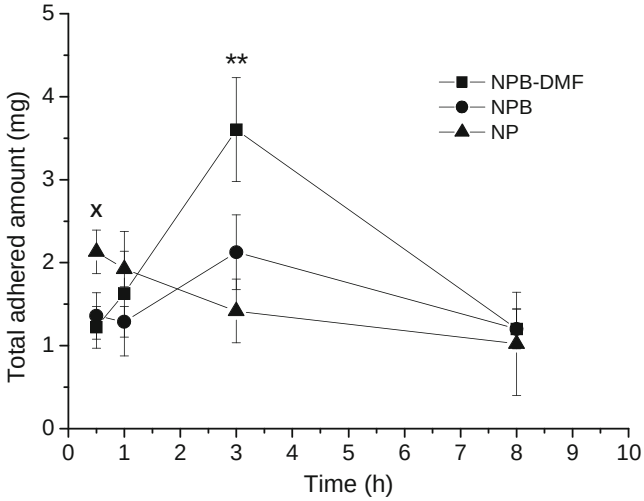

Fig. 5. Kinetics of bioadhesion of Vit B_{12}-nanoparticles through the whole gastrointestinal tract *versus* time: (*filled circle*) NPB, (*filled square*) NPB-DMF, (*filled triangle*)control NP. Each value is represented by the mean±SD (n=3). **p<0.01 NPB-DMF *vs.* NPB and control nanoparticles (NP). ^{x}p<0.05 NP *vs.* NPB-DMF and NPB.

Table II. Bioadhesion Kinetic Parameters for the Tested Formulations

	Q_{max} (mg)	T_{max} (h)	AUC_{adh} (mg h)	K_{adh} (h^{-1})	MRT_{adh} (h)
NP	2.13±0.18	0.5	10.95±0.1	0.13±0.08	3.45±0.32
NPB	2.17±0.46	3	12.87±2.1	0.11±0.01	3.77±0.14
NPB-DMF	3.61±0.73**	3	24.41±2.2**	0.22±0.08*	3.53±0.24

Data expressed as mean±SD (n=3). NPB and NPB-DMF: Vit B_{12} nanoparticles prepared by methods A and B (see Fig. 1)
Q_{max} (mg**)**: Maximal amount of nanoparticles adhering to the intestinal mucosa; AUC_{adh} (mg.h): area under the curve of nanoparticles adhering to the intestinal mucosa; K_{adh} (h^{-1}**)**: terminal elimination rate of the adhered fraction of nanoparticles; MRT_{adh} (**h)**: mean residence time of the adhered fraction of nanoparticles; T_{max}: the time needed to reach the maximal adhesion
*p<0.05, **p<0.01 for NPB-DMF *versus* control nanoparticles NP (Man–Whitney U test)

administered by either the s.c. or oral route. Interestingly, the oral administration of OVA-NPB-DMF elicited higher levels of intestinal secretory IgA compared to the s.c. administration. This mucosal immune response (IgA) was about four log_2-titers (approximately 100-fold) higher than that elicited by OVA-NP.

DISCUSSION

The first objective of this work was to optimize the preparation of Vit B_{12}–Gantrez AN nanoparticle conjugates. For this purpose, the association of vitamin B_{12} to the nanoparticles was done by applying two different methods, where vitamin B_{12} was either directly attached to the surface of the nanoparticles (NPB, method A), or linked to the copolymer chains prior to nanoparticle formation (NPB-DMF, method B). Both Vit B_{12}-nanoparticle types displayed a homogenous size distribution with mean diameter of about 200 nm, and a less negative surface charge compared to control nanoparticles (NP). The high density of negative surface charge of the control NP is due to the exposure of hydrolyzed carboxylic groups of Gantrez AN copolymer. The

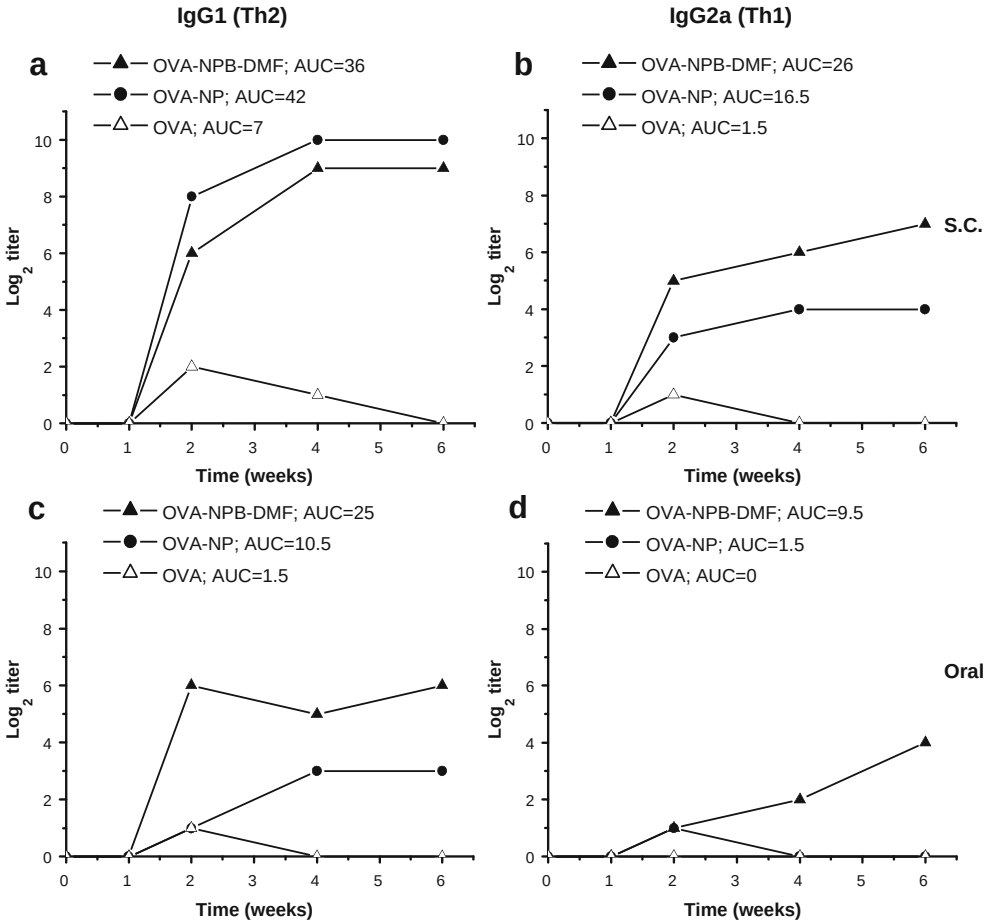

Fig. 6. OVA-specific serum IgG2a and IgG1 in BALB/c mice (n=10). The animals were immunized on day 0 with a single dose of the formulations containing 20 μg OVA for the subcutaneous route (**a**, **b)** or 100 μg OVA for the oral route (**c**, **d**). The formulations were: OVA-NPB-DMF (*filled triangle*), OVA-NP (*filled circle*), free OVA (*open triangle*). AUC is the area under the curve of the immune response profiles calculated over the time of the experiment.

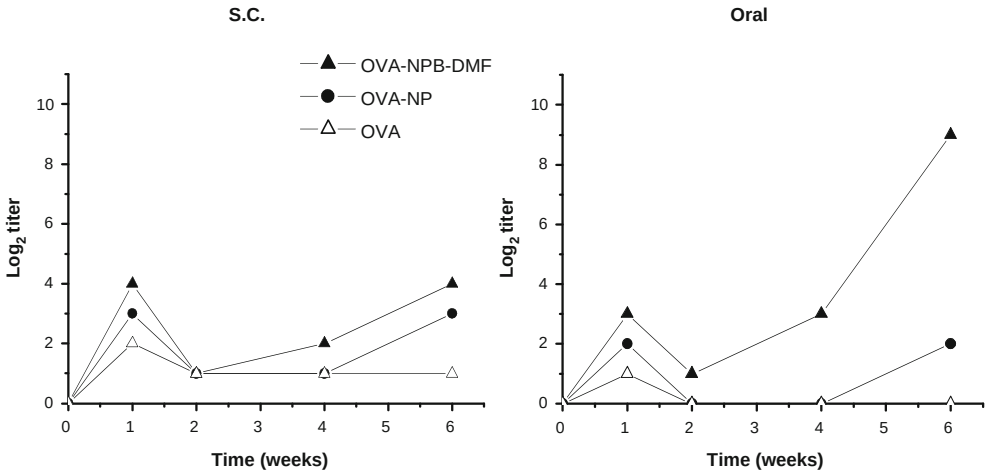

Fig. 7. Faecal secretory OVA-specific IgA in BALB/c mice ($n=10$). The animals immunized on day 0 by a single dose of the formulations containing 20 μg OVA for the subcutaneous route or 100 μg OVA for the oral route. The formulations were: OVA-NPB-DMF (*filled triangle*), OVA-NP (*filled circle*), free OVA (*open triangle*).

attachment of primary amine-containing molecules such as 1,3-diaminopropane, lectins or proteins to the surface of Gantrez nanoparticles decreases their negative surface charge (15,18). This may explain the significant decrease in the negative surface charge of both types of vitamin B_{12} nanoparticles when reactive vitamin B_{12}-NH_2 was directly attached to Gantrez nanoparticles or linked to the copolymer.

During the optimization of the nanoparticle preparation methods (A and B) we noticed that method B (NPB-DMF; 250 μg vitamin B_{12} per 100 mg Gantrez AN in DMF) required only half of the amount of reactive vitamin B_{12} to yield a loading of approximately 2.4 μg/mg NP (Table I) than method A (NPB; 500 μg vitamin B_{12} per 100 mg Gantrez AN nanoparticles, in an aqueous dispersion). This result correlated with the two fold higher association efficiency of vitamin B_{12} to the NPB-DMF as compared to the NPB nanoparticles.

The physical stability of association of vitamin B_{12} and Gantrez AN nanoparticles was considered as a critical factor for the *in vivo* experiments, since free vitamin B_{12} was expected to exert an auto-inhibitory effect on the mucosal affinity of Vit B_{12}–Gantrez AN nanoparticles after their oral administration. Therefore, the release of vitamin B_{12} was studied upon subsequent incubation of the nanoparticles in simulated gut fluids. The release of vitamin B_{12} form both types of nanoparticles showed that the vitamin B_{12} remained more strongly associated to NPB-DMF than to the NPB nanoparticles (Fig. 3).

The differences in vitamin B_{12} association efficiency stability between the two nanoparticle formulations may depend on the reaction conditions under which vitamin B_{12} was coupled to Gantrez AN copolymer (NPB-DMF) or linked to the surface of the nanoparticles (NPB). The expected formation of stable amide bonds between the primary amine group of vitamin B_{12}-5′$OCONH[CH_2]_6$-NH_2 and the anhydride group of Gantrez AN nanoparticles (19) may have been hampered in the presence of water used for this reaction with the NPB. Indeed, the high hydrolysis rate of the anhydride groups of the poly(methyl vinyl ether-co-maleic anhydride) may have produced carboxylic groups that formed less stable charged ionic or hydrogen bonds with the reactive vitamin B_{12} derivative. Conversely, the coupling of vitamin B_{12} to the copolymer (NPB-DMF) in the absence of water must have yielded a high fraction of stable covalent bonds between vitamin B_{12} and the copolymer. These observations are in agreement with previous results obtained for the pegylation of Gantrez AN nanoparticles (20), where a threefold higher pegylation of Gantrez nanoparticles was achieved when polyethylene glycol was reacted with Gantrez in an organic phase prior to nanoparticle formation as compared to the coating of freshly prepared nanoparticles with polyethylene glycol.

The *in vivo* bioadhesive capacity of Vit B_{12} nanoparticles was investigated after oral administration of fluorescently labelled NPB and NPB-DMF to fasted rats. The adhered fractions of nanoparticles were mainly found in the stomach and upper regions of the small intestine at 30 min post administration (Fig. 4a–c). At 3 h post administration, both Vit B_{12}-nanoparticle formulations showed an important tropism to the distal portions of the gut (I3 and I4 regions). The tropism of the NPB-DMF nanoparticles represented about 28% of the given dose and was about two and 3.5 times more than the tropism observed for the NPB and control nanoparticles, respectively ($p<0.05$). From a physiological point of view, the ileum is the region of the gastrointestinal tract where the Vit B_{12}–intrinsic factor (IF) complex is transported (1). Therefore, these results appear to demonstrate the biological property of vitamin B_{12} on the surface of the nanoparticles (NPB-DMF) to bind effectively to the IF, with subsequent binding of the IF-Vit B_{12} nanoparticles to the IF receptors of the mucosal cells.

Interestingly, we noted a good agreement between the vitamin B_{12} release (Fig. 3) and the bioadhesive capacity (ileal tropism) of the two types of vitamin B_{12} nanoparticles (Fig. 4). The twofold enhanced ileal tropism of NPB-DMF nanoparticles over that of the NPB may be related to the strong association of vitamin B_{12} to the NPB-DMF nanoparticles (Fig. 3), whereas the important release of vitamin B_{12} from the NDP (Fig. 3) probably exerted an auto-inhibitory effect on the ileal tropism of the nanoparticles. Such competing effect between orally administered free

vitamin B_{12} and vitamin B_{12}–peptide conjugates, which negatively affected the process of uptake and transport to the circulation of the conjugate, has been described in the literature (21).

NPB-DMF and NPB nanoparticles reached the maximal gut adhesion at 3 h post administration (Figs. 4 and 5, Table II), whereas the control nanoparticles achieved their maximal bioadhesion already at 0.5 h post administration. These results indicate that control nanoparticles without vitamin B_{12} on their surface distribute quickly in the stomach and upper ileal segments, which is mediated by non-specific bioadhesive interactions between the Gantrez AN copolymer and the mucosal surfaces. However, the presence of vitamin B_{12} on the surface of the nanoparticles prolongs substantially the time needed to reach the maximal gut adhesion, which we ascribe to specific bioadhesive interactions between the vitamin B_{12} and the IF-receptor of the mucosal cells.

Arbós *et al.* (16) demonstrated that surface modifications of conventional Gantrez AN nanoparticles with lectins can delay the time of maximal bioadhesion of control nanoparticles from 0.5 to 1 h post oral administration. Similarly, oral administration of vitamin B_{12}-conjugates with peptides to rats yielded the highest tissue concentration in the small intestine after 4 h post oral administration (21). The time of 4 h agrees well with our data suggesting that similar bioadhesive kinetics determined the fate of both the vitamin B_{12}-peptide and vitamin B_{12}-nanoparticle conjugates in the same animal model.

Finally, to investigate the adjuvant capacity of Vit B_{12}-nanoparticles as antigen delivery system, the antibody immune response was studied in BALB/c mice. Both s.c. and oral administration of the OVA-loaded nanoparticle formulations elicited a stronger serum antibody response than free OVA. In the s.c. immunization, the OVA-NP induced a considerable IgG1 (Th2) response, whereas the OVA-loaded Vit B_{12}-nanoparticles (OVA-NPB-DMF) induced higher IgG2a levels (Th1 response) than OVA-NP. The IgG2a response may be related to effective uptake of Vit B_{12} nanoparticles by antigen presenting cells, although this hypothesis requires further investigation. The intragastric inoculation of the OVA-NPB-DMF formulation induced also stronger and more balanced serum titers of IgG2a (Th1) and IgG1 (Th2) compared to the OVA-NP, which induced a typical Th2 response (Fig. 6c,d). For semi-quantitative analysis, the cumulated areas under the curve of the antibody response profiles (AUC_{IgG2a} + AUC_{IgG1}) were found to be approx. three times larger for OVA-NP-DMF than for OVA-NP. This emphasized again the strong adjuvant capacity of Vit B_{12} nanoparticles when loaded with ovalbumin.

These results clearly indicated the capacity of Vit B_{12} nanoparticles to enhance a Th1 response after both s.c. and oral immunization of BALB/c mice, which have a genetic tendency towards a Th2 response (22). This Th1 response enhancement may be related to the high ileal tropism of Vit B_{12} nanoparticles, where Peyer's patches with a high density of antigen presenting cells are found to be concentrated. In contrast, control nanoparticles did not show ileal tropism and elicited only a week Th2 type immune response. These results agree with those reported on the delivery of OVA-loaded microbeads to lower intestine region, where they enhanced a Th1 response (23). Finally, the oral immunization with OVA-NPB-DMF induced a higher mucosal IgA response than the s.c. administration. In summary, these results support further studies in order to apply Vit B_{12}–Gantrez AN nanoparticles in protective mucosal vaccination or immunotherapy purposes.

CONCLUSIONS

The present results demonstrate the adjuvant benefits of vitamin B_{12} association to Gantrez AN nanoparticles for oral antigen delivery. The bioadhesive capacity and ileal tropism of the orally administered OVA-loaded and vitamin B_{12}-decorated nanoparticles appeared to be instrumental for the effective elicitation of both systemic and mucosal immune responses. Further efforts should be focused on exploring the role of vitamin B_{12} in the interaction with immune cells and their potential activation.

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