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Research paper

Influence of polymer hydrolysis on adjuvant effect of Gantrez®AN nanoparticles: Implications for oral vaccination

Katrien Vandamme [a,1], Vesna Melkebeek [b,1], Eric Cox [b], Peter Adriaensens [c], Sandra Van Vlierberghe [d], Peter Dubruel [d], Chris Vervaet [a], Jean Paul Remon [a,*]

[a] Laboratory of Pharmaceutical Technology, Ghent University, Ghent, Belgium
[b] Laboratory of Veterinary Immunology, Ghent University, Merelbeke, Belgium
[c] Applied Chemistry Group, Hasselt University, Diepenbeek, Belgium
[d] Polymer Chemistry and Biomaterials Group, Ghent University, Ghent, Belgium

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ABSTRACT

The adjuvant effect of methylvinylether-co-maleic anhydride (Gantrez®AN) nanoparticles was investigated during oral vaccination of mice with F4 adhesins of F4-positive *Escherichia coli*. To differentiate whether the adjuvant effect originated from a nanoparticle effect or a polymer effect, 20 μg F4 was administered as slightly crosslinked F4-containing nanoparticles ($g(F4)_{0.01}$) or as F4 mixed with slightly crosslinked pure nanoparticles ($F4 + g_{0.01}$).

The F4-specific immune response was reduced using F4-containing nanoparticles due to complete shielding of F4, whereas oral administration of $F4 + g_{0.01}$ increased the level of F4-specific antibody-secreting cells (ASC) in the spleen. When repeating the vaccination study after 6 months using freshly prepared nanoparticles, the adjuvant effect of $F4 + g_{0.01}$ was lost due to an altered polymer reactivity caused by partial hydrolysis of anhydride groups of Gantrez®AN. Combining F4 with nanoparticles stabilised with a higher crosslinker amount during nanoparticle synthesis ($F4 + g_{0.22}$) could overcome the effect of partial polymer hydrolysis, as higher levels of ASC were detected. Hence, an in-depth characterisation of the Gantrez®AN polymer is required as stability issues can alter its biological effect during oral vaccination.

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1. Introduction

Oral vaccination has gained interest due to the ease of administration via the oral route and the advantages of mucosal vaccination as it is more effective compared with non-mucosal vaccination for the induction of mucosal immune responses [1]. However, oral vaccination remains challenging as the antigen often degrades in the gastrointestinal tract and the vaccination efficiency is often low due to limited uptake of soluble antigens [2]. The use of polymeric nanoparticulate delivery systems has been proposed to protect the antigens in the gastrointestinal tract and enhance the uptake of intact antigens. Moreover, conversion of soluble antigens into particulate antigens enhances their processing by antigen-presenting cells such as dendritic cells [3].

In search of new materials for the preparation of nanoparticulate dosage forms, the linear alternating methylvinylether-co-maleic anhydride copolymer (marketed as Gantrez®AN) has been identified as a suitable antigen carrier [4]. To avoid the hydrolysis of the anhydride groups and increase the half-life of Gantrez®AN nanoparticles in aqueous media, 1,3-diaminopropane (DP) was suggested as crosslinker [5], forming amide bounds via covalent binding of the primary amine groups to the anhydride groups in the polymer [6,7]. When Gantrez®AN nanoparticles were applied for oral antigen delivery, nanoparticles were only crosslinked with a low amount of crosslinking agent ($\leqslant$0.01 mg DP/mg polymer) [8–11]. However, in a previous study, we found that nanoparticles required a higher amount of crosslinking agent ($\geqslant$0.22 mg DP/mg polymer) to maintain their nanoparticle characteristics under physiological conditions in the gastrointestinal tract. Using lower amounts of crosslinker resulted in nanoparticle hydrolysis and the formation of carboxylic groups, which caused mild irritation at the mucosal border [12]. Hence, it was questioned whether the chemical reactivity of Gantrez®AN

Abbreviations: F4, F4 fimbriae (solution); NP, nanoparticle; DP, 13-diaminopropane (nanoparticle crosslinking agent); g0.01/g0.22, pure Gantrez® AN nanoparticles, crosslinked with 0.01 or 0.22 mg DP/mg polymer; F4+g, F4 + pure Gantrez® AN nanoparticles; g(F4), F4-containing Gantrez® AN nanoparticles; ASC, antibody secreting cells.

* Corresponding author. Ghent University, Laboratory of Pharmaceutical Technology, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: +32 9 2648054; fax: +32 9 2228236.
E-mail address: jeanpaul.remon@ugent.be (J.P. Remon).

[1] These authors equally contributed to this work.

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polymer contributed to the adjuvant effect of slightly crosslinked Gantrez®AN nanoparticles rather than the nanoparticulate nature of Gantrez®AN nanoparticles.

To further investigate the adjuvant effect of Gantrez®AN nanoparticles for oral vaccination, F4 (K88) fimbriae (adhesins) of enterotoxigenic *Escherichia coli* were selected as antigen. F4 fimbriae are long polymeric appendages mainly composed of several hundreds identical 27.5-kDa Faeg subunits. They enable the bacteria to bind to the intestinal epithelium of pigs resulting in the colonisation of the small intestine [13]. In addition, oral immunisation with Faeg fragments (the major part of F4 which is responsible for the adhesion function) can induce immune responses in mice [14].

In the present study, the adjuvant effect of slightly crosslinked (0.01 mg DP/mg polymer) Gantrez®AN nanoparticles was investigated in mice following the oral administration of F4-containing Gantrez®AN nanoparticles and a mixture of F4 fimbriae and pure Gantrez®AN nanoparticles in order to differentiate whether the adjuvant effect was caused by antigen encapsulation (nanoparticle effect) or by the chemical properties of the polymer (polymer effect). As the reactivity of anhydride polymers can change during storage, additional experiments were carried out to evaluate the influence of polymer stability on the biological effect of the polymeric nanoparticles.

2. Materials and methods

2.1. Materials

E. coli strain IMM 01, serotype O147:F4ac, LT+STb+, was used as a source of F4 fimbriae [15], and F4 fimbriae were purified as described by Van den Broeck et al. [16]. F4 fimbriae were isolated by mixing a bacterial suspension of the IMM 01, serotype O147:F4ac, LT+STb+, with a Heidolph DIAX 900 homogenizer, followed by two centrifugation steps to remove bacterial residues. The isolated fimbriae were subsequently concentrated by ammonium sulphate (40% w/v) precipitation. Thereafter, the pellet was dissolved in PBS and dialysed overnight against a large volume of PBS. The concentration of F4 fimbriae was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as standard (ICN Biomedicals, Belgium). Fimbrial solutions isolated from *E. coli* strain IMM 01, serotype O147:F4ac, LT+STb+, do not contain flagellin [15].

2.2. Optimisation of Gantrez®AN nanoparticle synthesis

During optimisation of the nanoparticle synthesis, the amount of water used for nanoparticle precipitation was varied, while the total water volume used during nanoparticle synthesis remained constant (1 ml). Ten milligrams Gantrez®AN (Gantrez®AN 119, ISP, Barcelona, Spain) was dissolved in acetone and precipitated with a hydroalcoholic mixture containing 1 ml ethanol, supplemented with 0.2, 0.5 or 1 ml water. These nanoparticles are identified by the code g NP. In case of F4-containing Gantrez®AN nanoparticles (identified as g(F4) NP), 0.5 mg F4 was added to the mixture via the water phase. After 1 h, the nanoparticles were mixed with the remaining amount of water (i.e. 0.8, 0.5 or 0 ml) and the organic phase was evaporated under nitrogen flow. After an additional hour of incubation, nanoparticles were used either as such (i.e. no crosslinking) or stabilised with 0.01 mg 1,3-diaminopropane (DP) (Acros Organics, Geel, Belgium) per mg Gantrez®AN).

2.2.1. Nanoparticle characteristics

To assess the nanoparticle characteristics, size and zèta potential were measured using 50 μl nanoparticle suspension (1/40 diluted in distilled water) and a Nano Zetasizer system (Malvern Instruments, Worcestershire, UK). Size results were obtained using dynamic light scattering and were expressed by the mean size and mean polydispersity index (PI). The PI value varied between 0 and 1; particles with a PI value below 0.2 were regarded as monodisperse. Statistical analysis of the nanoparticle characteristics was performed using ANOVA, with Bonferroni correction for multiple comparisons (SPSS 16).

2.2.2. In vitro activity of F4 formulations

The *in vitro* activity of F4 was assessed via an F4-specific ELISA (Section 2.2.3) to evaluate the reactivity of the Gantrez®AN polymer in the different nanoparticle formulations of the optimisation study. Pure Gantrez®AN nanoparticles were mixed with free F4 (in a 10/0.5 w/w ratio), and the activity of F4 was measured after 1 h incubation. In case of F4-containing nanoparticles, F4 activity was tested after incorporation and complete hydrolysis of the Gantrez®AN nanoparticles. NP hydrolysis was obtained by stirring the NP suspension overnight at room temperature. Only uncrosslinked Gantrez®AN nanoparticles were used as they have the fastest hydrolysis rate and highest reactivity.

2.2.3. F4-specific ELISA

To test the *in vitro* activity of F4, a 96-well microtiter plate (NUNC, Maxisorp Immuno Plates, Life Technologies, Roskilde, Denmark) was coated with the F4-specific monoclonal antibody IMM 01. After 2 h incubation at 37 °C, the remaining binding sites were blocked overnight at 4 °C with PBS supplemented with 0.2% (v/v) Tween 80. Following blocking, 100 μl sample corresponding to 5 μg F4/ml ELISA dilution buffer (PBS + 0.2% (v/v) Tween 20 + 3% (w/v) BSA) was added and serial diluted using twofold dilutions. After 1 h incubation at 37 °C, 100 μl porcine serum containing polyclonal antibodies against F4 (1/500 in ELISA dilution buffer) was added and further incubated for 1 h at 37 °C with 100 μl of crossabsorbed goat anti-pig HRP-conjugated serum (Bethyl Laboratories) 1/5000 diluted in ELISA dilution buffer. Fifty microlitres ABTS solution (Roche Diagnostics, Mannheim, Germany) containing H_2O_2 was added and the optical density was measured spectrophotometrically at 405 nm (OD_{405}) after incubation at 37 °C. Between each incubation step, the plates were washed three times with ELISA washing buffer (PBS + 0.2% (v/v) Tween 20). The detection limit was determined by calculation of the mean OD_{405} plus three times the standard deviation of the optical density of aliquots of dilution liquid.

2.3. Immunisation studies

2.3.1. Nanoparticles for immunisation studies

For the immunisation studies, nanoparticles were prepared using a hydroalcoholic mixture of 1 ml ethanol and 0.5 ml water during nanoparticle precipitation (Section 2.2). The nanoparticles were purified by fractional centrifugation at 30,000*g* (twice for 1 h) and resuspended in sterile PBS (1 ml in total).

2.3.2. Design of immunisation studies

Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium. Ten-week-old balb/c mice were housed in isolation units with water and food ad libitum. Each formulation was given to at least three mice on three successive days and 15 and 29 days postimmunisation (Table 2). The nanoparticles used in the different formulations were daily synthesised and subsequently purified (Section 2.3.1).

In the first immunisation study, negative control mice were orally vaccinated with slightly crosslinked (0.01 mg DP/mg polymer) antigen-free Gantrez®AN nanoparticles ($g_{0.01}$). Other groups were vaccinated with 100 μl PBS containing 20 μg F4, either as

F4 solution, F4-containing slightly crosslinked nanoparticles ($g(F4)_{0.01}$) or as F4 physically mixed with antigen-free slightly crosslinked nanoparticles ($F4 + g_{0.01}$). For the latter group, an F4 solution was added to the pure nanoparticles after resuspension in PBS.

The vaccination study was repeated after 6 months using another group of ten-week-old balb/c mice. The mice were treated with 100 μl PBS containing 20 μg F4, as F4 solution, $g(F4)_{0.01}$ and $F4 + g_{0.01}$. This study also included highly crosslinked (0.22 mg DP/mg polymer) nanoparticles: F4-containing nanoparticles ($g(F4)_{0.22}$) or F4 physically mixed with antigen-free nanoparticles ($F4 + g_{0.22}$). Since two levels of crosslinking were used in this vaccination study, the negative control group was vaccinated with 100 μl PBS instead of 100 μl PBS containing antigen-free Gantrez®AN nanoparticles.

2.3.3. Detection of F4-specific serum antibodies

F4-specific serum antibodies were determined by ELISA from pooled sera as described by Melkebeek et al. [17]. Briefly, the wells of maxisorb plates were coated with 5 μg/ml of IMM 01 F4 fimbriae suspended in PBS at 37 °C for 2 h. Thereafter, the remaining binding sites were blocked overnight at 4 °C with 0.2% (v/v) Tween 80 in PBS. Sera were added for 1 h at 37 °C in series of twofold dilutions in ELISA dilution buffer (PBS + 0.2% (v/v) Tween 20 + 3% (w/v) BSA) starting at a dilution of 1/10. The plates were incubated with rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) at 37 °C 1/1000 diluted in ELISA dilution buffer. Finally, ABTS (Roche Diagnostics, Brussels, Belgium) containing H_2O_2 was added and after 30 min incubation, the optical density was measured at 405 nm. Before each incubation, the plates were washed three times with washing buffer (PBS + 0.2% (v/v) Tween 20 in PBS). Titres were determined as the inverse of the highest dilution that still had an OD405 higher than the cut-off value. The cut-off value was determined by calculation of the mean OD405 plus three times the standard deviation of the optical densities of the 1/10 diluted samples measured at day 0.

2.3.4. Detection of F4-specific antibody-secreting cells in spleen

At the moment of slaughter, spleens were aseptically dissected. After the surrounding fat was removed from the specimens, spleen cells were obtained by gentle homogenisation of the tissues in complete RPMI medium (Sigma, Bornem, Belgium). F4-specific serum antibodies were determined by an F4-specific ELISPOT assay. Briefly, the wells of maxisorb plates were coated with 5 μg/ml of IMM 01 F4 fimbriae suspended in PBS at 37 °C for 2 h. Thereafter, the remaining binding sites were blocked overnight at 4 °C with 0.2% (v/v) Tween 80 in PBS. One hundred microlitres of cell suspension (10^6cells/ml) was added and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. After the cells were removed by three subsequent washes with PBS + 0.2% (v/v) Tween 20 in PBS, the plates were incubated for 1 h at 37 °C with rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted in dilution buffer (PBS + 0.2% (v/v) Tween 20 + 3% (w/v) BSA). The unbound conjugates were removed by washing and the substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) working solution and 1 volume of 3% (w/v) agarose, was added. The AEC working solution was prepared by dilution of 0.67 ml AEC stock solution (0.4% AEC in dimethylformamide) and 10 μl 30% H_2O_2 in 10 ml 0.1 M Na acetate (pH 5.2) (BIOzym, Landgraaf, The Netherlands). Dark brown–red spots were counted with an inverted microscope after the plates had been incubated in the dark overnight at room temperature. For each spleen cell suspension, spots in ten wells were counted (10^5 cells/well) in order to obtain the amount of antibody-secreting cells per 10×10^5 (=10^6) cells.

2.4. Polymer stability study

2.4.1. Investigation of the Gantrez®AN polymer during immunisation studies

FTIR spectra of Gantrez®AN powder were obtained when the first immunisation study was carried out and 6 months later (i.e. when the second immunisation study was carried out). Between both immunisation studies, the polymer was stored in the original container, tightly closed, placed in a cool, dry place and out of direct sunlight, according to the recommendations of the supplier. KBr pellets were used to obtain the FTIR spectra on a Bruker Vertex 70 FTIR spectrometer with a DTGS detector at a resolution of 4 cm^{-1}. All spectra were acquired with 32 scans.

2.4.2. Investigation of the Gantrez®AN polymer during stability study

To further understand the nanoparticle reactivity in relation to the (altering) polymer composition, a stability study was carried out using a new batch of Gantrez®AN. Aliquots of this batch were stored for 2 months at different conditions (20 °C at 10%, 30%, 60% and 90% relative humidity; 4 °C and −20 °C). Afterwards, the carboxylic acid groups (via ^{1}H NMR) and the water content (via Karl Fischer titration) were determined in the polymer. In addition, the interaction between F4 and pure nanoparticles was studied using nanoparticles crosslinked with 0.01 and 0.22 mg DP/mg polymer. NP were freshly synthesised using the different polymer aliquots and purified as described in Section 2.3.1. Afterwards, they were mixed with free F4 (in a 10/0.5 w/w ratio) and the F4 activity was measured 1 h after mixing via an F4-specific ELISA (Section 2.2.3).

^{1}H NMR-spectra were recorded at room temperature in d_6-deuterated DMSO using a Bruker WH 500 MHz instrument. The chemical shift was expressed in ppm with tetramethylsilane as internal standard. The number of carboxylic acid hydrogen was calculated via the integration of the peak at 12.4 ppm ($I_{12.4ppm}$) relative to the broad peak around 2 ppm (I_{2ppm}) attributed to the methylene group of methylvinylether.

The water content of 25 mg Gantrez®AN powder was determined by means of a Karl Fisher titration (Mettler DL 35, Beersel, Belgium). Hydroquant-Uniquant 2 and extra dry methanol were the titration reagent and solvent, respectively.

3. Results and discussion

3.1. Optimisation of nanoparticle synthesis

To prepare F4-containing Gantrez®AN nanoparticles, the solvent displacement method for Gantrez®AN nanoparticle synthesis [4,18] was modified since dispersion of F4 in acetone did not result in a sufficiently fine suspension. Synthesis of F4-containing nanoparticles was optimised by varying the hydroalcoholic medium for nanoparticle precipitation and F4 addition. Independent of the synthesis method, negatively charged F4-containing nanoparticles of about 115 nm were obtained after crosslinking with 0.01 mg/mg DP (Table 1). Using a hydroalcoholic solution with a higher water content (ethanol/water ratio: 1/1, v/v), a lower

Table 1
Nanoparticle characteristics of F4-containing nanoparticles, crosslinked with 0.01 mg DP/mg polymer, using varying amounts of water during nanoparticle precipitation. PI, polydispersity index; Zèta, zèta potential ($n = 5$).

EtOH/H_2O (ml/ml)	Size (nm)	PI	Zèta (mV)
1/0.2	114.2 ± 3.8	0.320 ± 0.071	−32.3 ± 2.9
1/0.5	114.5 ± 5.7	0.269 ± 0.053	−34.3 ± 3.3
1/1	118.5 ± 4.1	0.170 ± 0.051	−33.8 ± 5.9

Table 2
Design of immunisation studies.

Group	Oral vaccine (days 0, 1, 2, 15 and 29)	Study 1 # mice	Study 2 # mice
F4	F4 solution	5	3
F4 + $g_{0.01}$	F4 + slightly crosslinked pure Gantrez®AN NP	5	3
$g(F4)_{0.01}$	F4-containing slightly crosslinked Gantrez®AN NP	5	4
$g_{0.01}$	Slightly crosslinked pure Gantrez®AN NP	5	
F4 + $g_{0.22}$	F4 + highly crosslinked pure Gantrez®AN NP		3
$g(F4)_{0.22}$	F4-containing highly crosslinked Gantrez®AN NP		4
PBS	PBS solution		3

polydispersity index was achieved compared with nanoparticles prepared using a hydroalcoholic solution with a lower water content (ethanol/water ratio: 1/0.2, v/v) ($p = 0.028$).

As the hydroalcoholic F4 solution was added to an organic medium in which the anhydride groups of the polymer remain intact, formation of stable amide bonds between the polymer anhydride groups and the nucleophilic amine groups of the F4 antigen was favoured [6,7]. The latter will inhibit the diffusion of the F4 antigen from the polymeric nanoparticles. As a result, antigen accessibility will only be promoted by nanoparticle hydrolysis. After the complete hydrolysis of uncrosslinked F4-containing nanoparticles, no *in vitro* F4 activity was detected, independently of the nanoparticle synthesis method (Fig. 1A). The lack of *in vitro* activity can be attributed to the fact that the fimbriae remained covalently bound to hydrolysed Gantrez®AN nanoparticles, inhibiting the Faeg fragments to interact with the monoclonal IMM 01 antibody of the F4-specific ELISA.

When uncrosslinked pure nanoparticles precipitated with the different hydroalcoholic mixtures were incubated with free F4, the *in vitro* activity of F4 was reduced after 1-h incubation time. This effect was most pronounced for nanoparticles precipitated using a hydroalcoholic mixture with a high water fraction (ethanol/water ratio: 1/1, v/v) (Fig. 1B). As the water amount used during the nanoparticle synthesis was the same in all cases, the time of water addition during synthesis (i.e. at nanoparticle precipitation or later in the synthesis) was very important for the reactivity of the resulting nanoparticles. A longer reaction time between water molecules and the anhydride groups of the polymer resulted in more anhydride hydrolysis with a higher number of carboxylic acid groups available on the surface of the nanoparticles. As these carboxylic acid groups induce ionic or H-bonds with F4 [10], the activity of F4 reduces when incubated with these nanoparticles.

For the immunisation studies, nanoparticle precipitation using an ethanol/water ratio of 1:0.5 (v/v) was selected as a compromise between acceptable nanoparticle characteristics of F4-containing nanoparticles and remaining F4 activity after addition to pure partly hydrolysed nanoparticles. In addition, a low amount of crosslinking agent (0.01 mg DP/mg polymer) was selected in analogy with Salman et al. [10], further decreasing the interaction of the pure Gantrez®AN nanoparticles with F4.

3.2. Immunisation results

Vaccination of the positive control group with an F4 solution resulted in F4-specific serum antibodies and antibody-secreting cells (ASC) in spleen (Figs. 2 and 3). The immunogenicity of the major subunit Faeg of soluble F4 fimbriae after oral delivery in mice is already described by others [14] but is in contrast to the observations in pigs, the natural hosts for F4 fimbriae, because mice do not have a receptor for F4 fimbriae (F4 receptor (F4R)) on their enterocytes. In the pig model, there exist pigs with and pigs without an F4R on their small intestinal enterocytes and presence of an F4R is required to obtain immunity after oral immunisation. In F4R-negative pigs, F4 behaves like a common food antigen upon oral delivery [20]. As a result, the immunogenicity of F4 in mice suggests that in these animals, the uptake of F4 and the induction of immunity occur in another, F4R independent, manner. Moreover, the lack of LPS purification in the F4 extract used can also partly explain the immunogenicity of this F4 extract in mice.

Encapsulation of F4 fimbriae in slightly crosslinked nanoparticles ($g(F4)_{0.01}$) affected their immunogenicity as the F4-specific serum antibodies and ASC in spleen were reduced (Figs. 2 and 3). This was probably due to the covalent interaction of the antigen with the Gantrez®AN polymer.

When pure slightly crosslinked nanoparticles were simultaneously administered with F4 fimbriae (F4 + $g_{0.01}$), the level of ASC in spleen increased (Fig. 3A), indicating a systemic adjuvant effect. This is caused by a combination of two factors: (a) in contrast to F4-containing nanoparticles, the antigenicity of the added F4 antigen was preserved as only a limited interaction of F4 and Gantrez®AN nanoparticles was observed when free F4 and uncrosslinked pure nanoparticles were mixed during nanoparticle optimisation (Section 3.1); this interaction was even further inhibited using a low amount of crosslinking agent during the vaccination studies; (b) using Gantrez®AN nanoparticles stabilised with only a low crosslinker amount, nanoparticle hydrolysis occurred during passage in the gastrointestinal tract, resulting in carboxylic acid groups available to interact with the mucosal border. This further highlights the importance of the amount of carboxylic acid groups available on the nanoparticle surface to interact with the mucosal border, thus inducing local inflammation and contributing

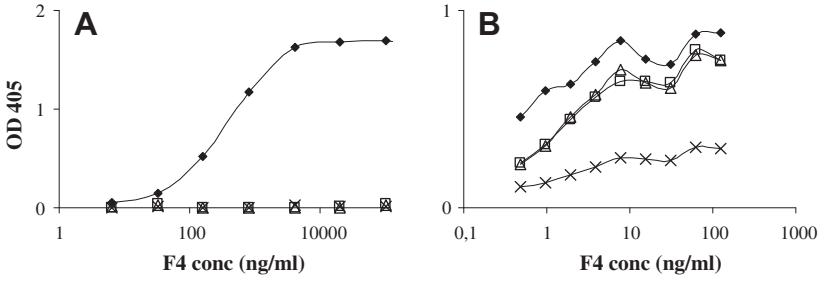

Fig. 1. F4 activity of (A) F4-containing Gantrez®AN nanoparticles after complete hydrolysis and (B) free F4 added to pure Gantrez®AN nanoparticles after 1 h incubation. During synthesis, the polymer was precipitated with a hydroalcoholic mixture (EtOH/water v/v) □ 1/0.2, △ 1/0.5, × 1/1, ♦ F4 reference.

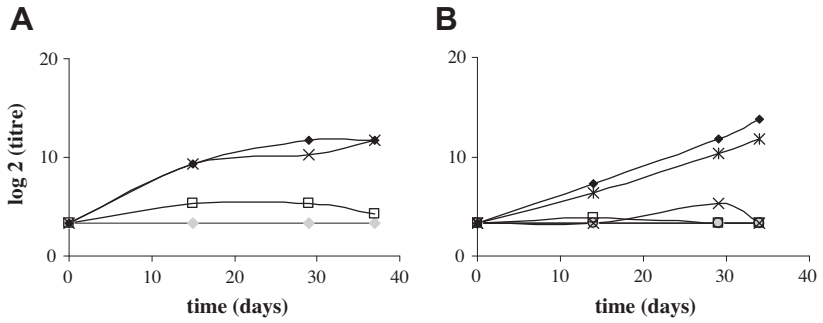

Fig. 2. F4-specific serum antibodies during (A) first and (B) second vaccination study (6-month time interval between both studies). ♦ F4 solution; × F4 + $g_{0.01}$ (F4 + slightly crosslinked pure Gantrez®AN NP); □ $g(F4)_{0.01}$ (F4-containing slightly crosslinked Gantrez®AN NP); ◆ negative control: (A) $g_{0.01}$ (slightly crosslinked pure Gantrez®AN NP), (B) PBS solution; (B) ∗ F4 + $g_{0.22}$ (F4 + highly crosslinked pure Gantrez®AN NP); ○ $g(F4)_{0.22}$ (F4-containing highly crosslinked Gantrez®AN NP).

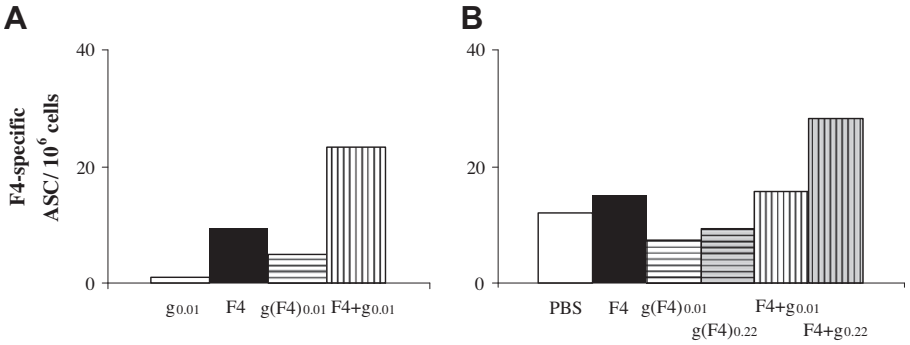

Fig. 3. F4-specific antibody-secreting cells (ASC) in spleen after (A) first and (B) second vaccination study (6-month time interval between both studies). □ Negative control: (A) $g_{0.01}$ (slightly crosslinked pure Gantrez®AN NP), (B) PBS solution; ■ F4 solution; F4 + $g_{0.01}$ (F4 + slightly crosslinked pure Gantrez®AN NP); $g(F4)_{0.01}$ (F4-containing slightly crosslinked Gantrez®AN NP); F4 + $g_{0.22}$ (F4 + highly crosslinked pure Gantrez®AN NP); $g(F4)_{0.22}$ (F4-containing highly crosslinked Gantrez®AN NP).

to the adjuvant effect of Gantrez®AN nanoparticles [12]. Moreover, the higher immune response against the F4 + $g_{0.01}$ formulation suggests that the polymer as such is more important for the adjuvant effect of Gantrez®AN nanoparticles than the presentation of antigen in nanoparticles.

Surprisingly, when repeating the vaccination study after 6 months with freshly prepared nanoparticles, the adjuvant effect of slightly crosslinked Gantrez®AN was lost in the F4 + $g_{0.01}$ formulation as the level of F4-specific ASC in spleen was not higher compared with that of the F4 and negative control (Fig. 3B). In addition, the level of F4-specific antibodies was reduced compared with that of F4 alone (Fig. 2B). In order to understand this observation, FTIR spectra of aliquots of Gantrez®AN polymer used for both immunisation studies were compared. During the 6-month time interval between both immunisation studies, FTIR data of the polymers showed polymer hydrolysis (Fig. 4), based on the increasing number of carboxylic acid groups upon storing the polymer. In addition to the carbonyl groups of the cyclic anhydride groups (at 1853 and 1779 cm^{-1}) [19], an extra peak (1725 cm^{-1}) appeared, originating from the carbonyl stretch of carboxylic acids.

3.2.1. Effect of polymer hydrolysis on adjuvant effect

As a result of this polymer hydrolysis, the reduced immune response after administration of the F4 + $g_{0.01}$ formulation in the second vaccination study was probably related to differences in $g_{0.01}$ nanoparticle reactivity, influencing F4 antigenicity. To the best of our knowledge, this is the first report in which the effect of the hydrolysis of the Gantrez®AN polymer on vaccination results is described.

During nanoparticle optimisation, it was observed that F4 activity decreased, when F4 was added to pure nanoparticles precipitated with a high water fraction (Fig. 1B). These nanoparticles contained a higher number of carboxylic acid groups due to a longer incubation time with water during nanoparticle synthesis, which increased the interaction of the nanoparticles with F4. Similarly, the $g_{0.01}$ nanoparticles used during the second vaccination study contained a higher number of carboxylic acid groups compared with those of the first vaccination study, due to anhydride hydrolysis upon storage of the polymer. Hence, the loss in adjuvant effect using the F4 + $g_{0.01}$ formulation in the second vaccination study can be explained by a lower F4 activity due to an increased interaction of the nanoparticles with F4. As a result, the amount of carboxylic acid groups on the nanoparticle surface was crucial: on the one hand, carboxylic acid groups are required to interact with the mucosal border in order to obtain an adjuvant effect, whereas too many carboxylic acid groups reduce antigenicity due to the interaction with the antigen.

In the second vaccination study, a Gantrez®AN aliquot with more anhydride hydrolysis was also used to prepare highly crosslinked Gantrez®AN nanoparticles ($g_{0.22}$), thus stabilising the remaining polymeric anhydride groups during nanoparticle synthesis and avoiding the formation of additional carboxylic acid groups upon the passage of these nanoparticles through the gastrointestinal tract. When F4 fimbriae were combined with these nanoparticles (F4 + $g_{0.22}$), the immunisation profile was comparable with the profile of F4 + $g_{0.01}$ in the primary study (Figs. 2B and 3B), establishing the beneficial effect of the addition of Gantrez®AN nanoparticles to enhance the immune response against co-administered antigens.

3.3. Polymer stability study

To further investigate the effect of polymer hydrolysis on the nanoparticle reactivity, an additional stability study was carried

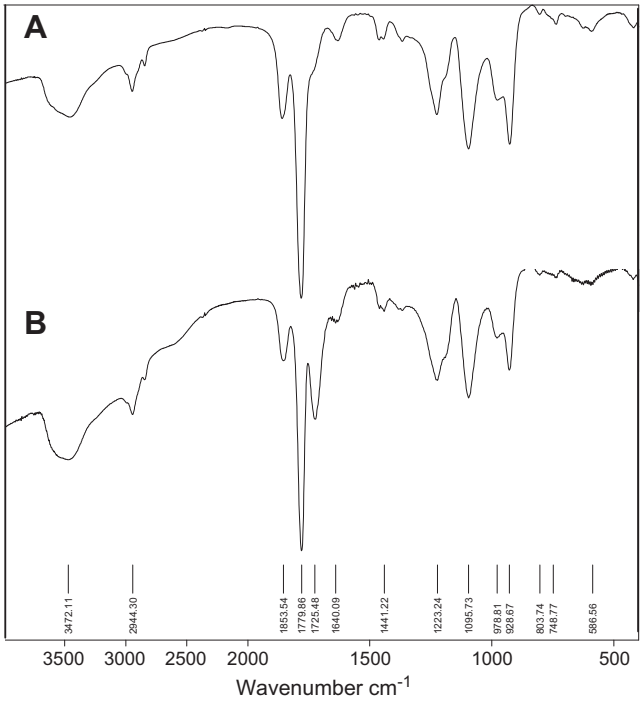

Fig. 4. FTIR spectra of Gantrez®AN powder used for (A) the first immunisation study, (B) the second immunisation study (i.e. after a 6-month time interval).

out in which aliquots of a new polymer batch were stored under different conditions. At higher temperature and relative humidity, the water content and anhydride hydrolysis of the polymer increased; only at low temperature (⩽4 °C) or relative humidity (10%), the anhydride groups remained intact (Fig. 5).

In addition, nanoparticles were prepared using polymer samples stored under the different conditions to test the nanoparticle reactivity in relation to the polymer storage conditions. Therefore, the *in vitro* F4 activity was measured after 1 h incubation with pure nanoparticles. In case of slightly crosslinked nanoparticles ($g_{0.01}$), no differences were detected as the *in vitro* activity of F4 was already below the detection limit at the start of the stability study. After incubation of F4 with highly crosslinked nanoparticles

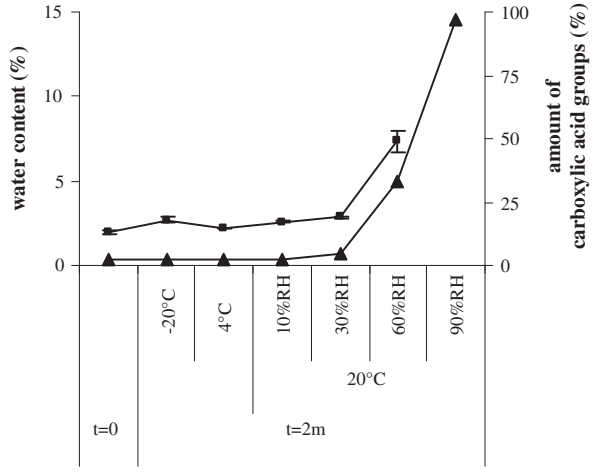

Fig. 5. Amount of anhydride groups hydrolysed into carboxylic acid groups (▲, right axis) and water content (■, ±standard deviation, left axis) of Gantrez®AN polymer in function of storage time and storage conditions. The water content in the polymer batch stored at 90% relative humidity (RH) could not be measured.

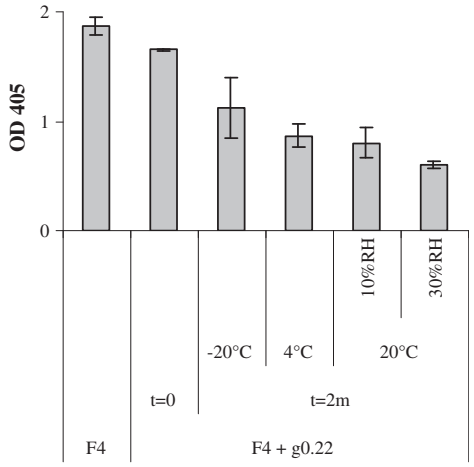

Fig. 6. *In vitro* activity of F4 after 1 h incubation with nanoparticles prepared using Gantrez®AN polymer stored under different conditions and crosslinked with 0.22 mg DP/mg polymer. F4: activity of F4 as reference. Polymer batches stored at 60% and 90% relative humidity (RH) could not be used to prepare nanoparticles.

($F4 + g_{0.22}$), F4 activity was lower using nanoparticles prepared with polymer aliquots having a higher amount of carboxylic acid groups due to water absorption and subsequent anhydride hydrolysis (Fig. 6). These data confirm that stability issues of the polymer during storage alter the adjuvant activity of the nanoparticles for oral mucosal vaccination. Although the chemical reactivity of the anhydride groups of methylvinylether-co-maleic anhydride (Gantrez®AN) offers many attractive features for oral vaccination full control over the stability of the raw polymer is needed. As alternative, biodegradable poly(lactic acid-co-glycolic acid) (PLGA) nanoparticles could be more appropriate for oral vaccination as promising results with antigen-entrapped nanoparticles were already published [21].

4. Conclusions

As the adjuvant effect of methylvinylether-co-maleic anhydride (Gantrez®AN) nanoparticles depends on the number of carboxylic acid functions on their surface, the optimisation of the crosslinking degree is essential to obtain an optimal nanoparticle reactivity and adjuvant effect, which is related to the degree of hydrolysis of the polymer.

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