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Research Paper

# Influence of reaction medium during synthesis of Gantrez® AN 119 nanoparticles for oral vaccination

Katrien Vandamme [a], Vesna Melkebeek [b], Eric Cox [b], Dieter Deforce [c], Joke Lenoir [a], Els Adriaens [a], Chris Vervaet [a,*], Jean Paul Remon [a]

[a] Laboratory of Pharmaceutical Technology, Ghent University, Belgium
[b] Laboratory of Veterinary Immunology, Ghent University, Belgium
[c] Laboratory for Pharmaceutical Biotechnology, Ghent University, Belgium

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ABSTRACT

Two synthesis methods of poly(methyl vinyl ether-co-maleic anhydride) (Gantrez® AN 119) nanoparticles (NP) (used for oral vaccination) were compared. Wheat germ agglutinin (WGA) was used as ligand to enhance the bioadhesive properties of NP and β-galactosidase as antigen. The first method encapsulated β-galactosidase in NP by co-precipitation in an acetone/water mixture containing 44% acetone. In the second method, antigen addition occurred in 100% acetone. To improve stability, NP were crosslinked with 1,3-diaminopropane. The stability of WGA-conjugated NP with encapsulated antigen diminished at lower pH and when decreasing the amount of crosslinker. The binding type between WGA and polymer depended on the synthesis method: predominantly ionic bonds were formed using the 44% acetone method, whereas synthesis via the 100% acetone method resulted in covalent bonds. The biological activity of the WGA coating, evaluated via a pig gastric mucin binding test, was lower in NP prepared via the 100% acetone method. No release of native antigen was detected after hydrolysis of NP, due to the covalent antigen binding during antigen encapsulation and the high reactivity of the polymer. Moreover, the mucosal irritation capacity was evaluated upon nanoparticle hydrolysis using a slug mucosal irritation assay. Herein, hydrolysed NP of the 44% acetone method were classified as mild irritative.



## 1. Introduction

Oral delivery is the preferred administration route of drugs and vaccines, thanks to its neither invasive nor painful nature and less expensive production processes [1]. In addition, oral administration of vaccines allows mucosal immunisation, which is needed to induce a mucosal immune response [2]. This is advantageous since most pathogens (bacteria, viruses and toxic or allergenic particles) interact with mucous membranes [3]. Nevertheless, the oral route includes several challenges for vaccine delivery. The harsh conditions in the gastrointestinal tract can cause antigen degradation and absorption from the gastrointestinal tract is restricted by digestive fluids, peristalsis and viscous mucus overlaying the epithelium [4]. To improve the mucosal bioavailability of antigens, nanoparticles have been proposed as particulate carrier systems. These submicron-sized colloidal systems are manufactured to protect labile molecules such as antigens against chemical, enzymatic or immunological degradation [5]. However, polymers can affect the antigen and modify antigen presentation by the antigen-presenting cells, resulting in an altered cytokine pattern [6].

The biocompatible copolymer between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) is widely employed for pharmaceutical applications and has been proposed as a new polymer to prepare bioadhesive nanoparticles for mucosal drug delivery [7]. The use of Gantrez nanoparticles as drug carrier is mainly based on three polymer features: (1) the ease to formulate nanoparticles with homogeneous size distribution [8], (2) the ease to incorporate proteins like ovalbumine [9] and hydrophobic bacterial extracts [10] and (3) the ease of ligand binding without the need of time-consuming chemical activation of functional groups [11].

To increase bioadhesion to enterocytes in the gastrointestinal tract, targeting ligands (such as lectins) are of special interest, as they mediate highly specific binding to epithelial cell subpopulations, effected by interaction of carbohydrate-binding sites of lectin with sugar residues in the glycocalyx of epithelial cells. Exploiting lectins for bioadhesive drug delivery purposes, this cell-specific interaction can result in active receptor-mediated endocytosis and/or transcytosis of the drug delivery system [12].

In this paper, the lectin wheat germ agglutinin (WGA) was selected as ligand considering its carbohydrate specificity, stability, low toxicity and low immunogenicity. WGA was found to bind

* Corresponding author. Laboratory of Pharmaceutical Technology, Harelbekestraat 72, Ghent, Belgium. Tel.: +32 9 2648069; fax: +32 9 2228236.
*E-mail address:* chris.vervaet@ugent.be (C. Vervaet).

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specifically with *N*-acetylglucosamine [13], which contributes to the oligosaccharide moieties of glycoproteins and glycolipids forming the glycocalyx of enterocytes [14]. As enterocytes represent the majority of the epithelial cells in the gastrointestinal tract, a large surface for absorption is provided. However, *N*-acetylglucosamine residues are also found in mucus overlaying the epithelial lining and may limit the availability of WGA-conjugated drug delivery systems at the epithelial surface. On the other hand, the initial but reversible mucus binding might enhance absorption by prolonging the contact time with the epithelial cell surfaces [15]. Only low amounts of *N*-acetylglucosamine are found in the common diet of man, hence interference of nutrition is expected to be of minor extent [16]. In addition, WGA showed no susceptibility to gastrointestinal enzymes [17], which makes it appropriate for oral delivery. Targeting of drug delivery systems with WGA is also favorable, since WGA has a low toxicity [12] and elicits only low local and serum-specific antibodies [18]. Nevertheless, toxicity studies are needed before safety in humans can be declared with certainty.

In literature, polymeric Gantrez-based nanoparticles have already been prepared using a solvent displacement method [7], in which antigen addition occurred in an organic solvent (i.e. 100% acetone). However, dispersion of antigens in acetone, which is required for antigen incorporation into Gantrez nanoparticles using the solvent displacement method, is not universally applicable, since some antigens do not form a sufficient fine dispersion in acetone for the preparation of nanoparticles. In order to broaden the application of these polymeric NP, a second synthesis method was developed using a water/acetone mixture as solvent during nanoparticle preparation. This allows to dissolve water soluble antigens into a water phase before mixing with the organic Gantrez solution. β-Galactosidase was selected as model antigen, since it could be encapsulated via both methods. In addition, nanoparticles prepared via both synthesis methods were conjugated with WGA to modify their bioadhesive properties. To investigate the influence of the solvents used during NP synthesis, the chemical and biological characteristics of the nanoparticles (stability, type of protein binding, activity of the protein coating, antigen release and mucosal irritation) were determined.

## 2. Materials and methods

### 2.1. Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) (Gantrez® AN 119) was kindly gifted by ISP (Barcelona, Spain). Benzalkoniumchloride (BAC), β-galactosidase (b-gal) from Aspergillus oryzae (~10 units/mg solid), human epidermal growth factor (hEGF), maleic anhydride (MA), wheat germ agglutinin (WGA), phosphate buffered saline (PBS), *N*-acetyl-D-glucosamine (NAG) and pig gastric mucin Type II (PGM) were purchased from Sigma–Aldrich (Steinheim, Germany). 1,3-Diaminopropane (DP) was acquired from Acros Organics (Geel, Belgium). Hydrochloric acid buffer pH 1.2 and phosphate buffer pH 5.8 were prepared as described in USP 27. Phosphate–citrate buffer (PC buffer) was prepared using 20 mM disodium phosphate solution, adjusted to pH 4.5 with 10 mM citric acid solution. o-Nitrophenyl-β-D-galactoside (ONPG) solution contained 10 mM ONPG (Sigma–Aldrich, Steinheim, Germany) in PC buffer. Borate buffer was made using 200 mM boric acid, adjusted to pH 9.8 with 1 M NaOH.

### 2.2. Synthesis of Gantrez nanoparticles

Gantrez NP with encapsulated b-gal were prepared in an optimized water/acetone mixture containing 44% acetone (further referred to as the 44% acetone method): 20 mg Gantrez, dissolved in 0.8 ml acetone was mixed using a magnetic stirrer (speed 5, IKA RO 15, Artisan Scientific Corporation, Champaign, USA) for 1 h with 1 ml of 1 mg b-gal/ml distilled water. After removal of the acetone phase via evaporation under nitrogen flow, NP were stirred for 1 h with 1 ml water (resulting in unconjugated NP, identified as gb NP) or 1 ml 1 mg WGA/ml water (resulting in WGA-conjugated NP, identified as gbw NP). Afterwards, both types of nanoparticles were crosslinked with 5 μl DP (equivalent to 0.22 mg DP/mg polymer) for 5 min while stirring at speed 5. Uncoated antigen-free NP (identified as g NP) were obtained by replacing both protein solutions by distilled water.

When synthesizing Gantrez NP with encapsulated b-gal via the solvent displacement method (further referred to as the 100% acetone method as only acetone is used as solvent during the first synthesis step), 20 mg Gantrez was dissolved in 0.6 ml acetone and magnetically stirred at speed 5 for 1 h with 1 mg of b-gal dispersed in 0.4 ml acetone. Next, 2 ml EtOH and 2 ml water (in case of gb NP) or 2 ml EtOH and 2 ml 0.5 mg WGA/ml water (in case of gbw NP) were added. The organic phase was evaporated under nitrogen flow and after 1 h incubation while stirring at speed 5, the nanoparticles were crosslinked with 0.22 mg DP/mg polymer.

Afterwards, NP were purified by fractional centrifugation (5–20–20–20 min, 30,000*g*). Herein, the supernatant of the first centrifugation step was further centrifuged in the second step (and so on) to recover all nanoparticles. This procedure was used to avoid difficulties when redispersing a large NP pellet. Finally, NP were redispersed in PBS until 2 ml. As a result, 2 ml NP suspension of both the 44% and 100% acetone method contained 20 mg Gantrez.

### 2.3. Nanoparticle characterization

For the assessment of the NP characteristics, purified NP were 1/40 diluted in distilled water, and size and zèta potential measurements were carried out using a Nano Zetasizer system (Malvern Instruments, Worcestershire, UK). Size results were obtained by dynamic light scattering and were expressed by the mean size and mean polydispersity index (PI). The value for PI ranges from 0 and 1; particles with a PI value below 0.2 are regarded as monodisperse. Moreover, NP yield of both methods was evaluated after centrifugation of g NP and air-drying for 1 month at 4 °C.

### 2.4. Statistical analysis of NP characteristics

Statistical analysis on the NP characteristics was performed using a two-way ANOVA, with Bonferroni correction for multiple comparisons.

### 2.5. Stability of Gantrez NP

To investigate the stability of WGA-conjugated Gantrez NP with encapsulated b-gal, NP prepared via both the 44% and 100% acetone method were incubated in different media in function of the amount of crosslinker (concentration range: 0.044–0.22 mg DP/mg polymer). Hereto, 70 μl NP crosslinked with varying DP amounts were diluted with 280 μl hydrochloric acid buffer pH 1.2 or phosphate buffer pH 5.8. The remaining turbidity was used as stability marker and was monitored every 30 min (Bioscreen C, Growth Curves AB, Helsinki, Finland) during 3 days at 37 °C.

### 2.6. Identification of WGA binding

To determine the binding efficiency via both synthesis methods, 400 μl gb and gbw NP (crosslinked with 0.22 mg DP/mg polymer)

were centrifuged for 1 h at 30,000*g*. Next, the WGA content in the supernatant was determined via the Lowry method. To identify the type of interaction between WGA and polymeric nanoparticles, a salting out experiment was performed. To 1 ml of different NaCl solutions, 400 μl gb and gbw NP (crosslinked with 0.22 mg DP/mg polymer) was added for 1 h at room temperature (final NaCl concentration: 0–1 M) while shaking. Afterwards, the mixture was centrifuged for 1 h at 30,000*g*, and the amount of WGA in the supernatant was analyzed. The salting out experiment for nanoparticles prepared via the 44% acetone method was also run using different experimental conditions: incubation time up to 16 h and increasing the NaCl concentration to 2 M.

For the Lowry method, 1 ml (diluted) supernatant was mixed for 10 min with 1 ml of an alkaline copper solution containing 0.5 M NaOH, 10% (w/v) $Na_2CO_3$, 0.1% (w/v) $NaKC_4H_4O_6$ and 0.05% (w/v) $CuSO_4{\cdot}5H_2O$. Subsequently, 4 ml of a Folin–Ciocalteu phenol solution (0.066 N) was added and after 30 min, the absorbance was measured at 660 nm. WGA binding and release were calculated based on a WGA standard curve (concentration range: 0-0.1 mg/ml).

### 2.7. Activity and specificity of WGA coating

To explore the activity and specificity of the WGA coating of purified gbw NP, pig gastric mucin (PGM) was used, since PGM contains *N*-acetyl-D-glucosamine (NAG) residues, which can interact with the bound WGA, leading to nanoparticle clustering. In the presence of free NAG, NP clustering is inhibited, provided that the specificity of bound WGA is not damaged. 200 μl gbw and gb NP (crosslinked with 0.22 mg DP/mg polymer) were 1/2 diluted in PBS and mixed via shaking for 30 min with 1 ml PBS or 1 ml 0.4 mM NAG in PBS. Next, 500 μl 0.5 mg PGM/ml PBS was added, and samples were shaken for another 30 min, before they were stored overnight at 4 °C. Afterwards, NP clustering was determined visually, and conclusions were drawn between samples concomitantly treated.

### 2.8. Evaluation of antigen binding by mass spectrometry

To investigate the antigen binding via mass spectrometry (QTOF Micro, Waters, Milford Massachusetts, USA), some modifications to the synthesis procedure were needed. Because of the high molecular weight of b-gal (>100 kDa), human epidermal growth factor (hEGF) was selected to replace b-gal, thanks to its smaller size (6 kDa) and identical isoelectric point (4.6). Besides, to avoid precipitation during the experiment, the Gantrez polymer was replaced by maleic anhydride (MA), the monomer of Gantrez which contains the anhydride groups. MA is water soluble, in contrast to the entire polymer. During measurement in the electrospray positive modus, the mass range varied from 50 to 1900, with a mass flow from 6100 to 6400. 50 μl of a solution containing 1 mg hEGF/ml water and a solution containing 1 mg hEGF and 0.65 mg MA per ml water (this corresponds to the ratio of the amino acids to anhydride groups in nanoparticles containing 1 mg b-gal per 20 mg Gantrez) were analyzed via mass spectrometry after 10-fold dilution in 0.1% formic acid.

### 2.9. B-gal encapsulation and release

To monitor the encapsulation of b-gal in Gantrez nanoparticles during both synthesis methods, the interaction of b-gal with the Gantrez polymer was interrupted after 1, 10, 30, 60, 105 (after evaporation) and 165 min (after ligand binding) by centrifugation of the NP suspension (1 h at 30,000*g*). Next, the unbound b-gal fraction in the supernatant was determined using a b-gal activity test.

To investigate the release of b-gal during hydrolysis of b-gal-entrapped Gantrez NP, uncrosslinked NP were used for practical reasons: they have the highest rate of hydrolysis, which is completed within 24 h. 2 ml freshly prepared gb NP suspensions were stirred for 0, 4 and 24 h at room temperature, after which the NP suspension was centrifuged (1 h at 30,000*g*). In addition, 1 mg b-gal was treated accordingly to both synthesis methods in the absence of Gantrez, to determine the influence of reaction media on b-gal activity during synthesis and during subsequent incubation in aqueous medium. To determine the influence of polymer hydrolysis on the activity of free b-gal, freshly prepared g NP (without crosslinking) were incubated during 24 h with 1 mg b-gal.

The b-gal activity test is based on the enzymatic cleavage of the substrate ONPG into o-nitrophenol and β-D-galactose by b-gal. The linear area of the test corresponds to 0–0.4 units b-gal/ml, and the supernatant was diluted accordingly. Next, 100 μl (diluted) supernatant was mixed with 400 μl PC buffer and 500 μl ONPG solution. After exactly 10 min, the enzymatic reaction was stopped by the addition of 3 ml borate buffer, and the yellow color originating from o-nitrophenol was measured at 410 nm.

Besides the enzymatic assay, the release of encapsulated b-gal was also determined using the Lowry assay, which is based on the detection of tyrosine and tryptophan residues of the primary structure of the protein. Uncrosslinked g and gb NP were incubated for 24 h at room temperature to obtain full hydrolysis of the Gantrez polymer. Afterwards, 1 ml of hydrolyzed NP was tested according the Lowry method as described earlier. B-gal concentrations were calculated based on a b-gal standard curve (concentration range: 0–1 mg/ml).

### 2.10. Slug mucosal irritation (SMI) assay

The mucosal irritation and tissue damaging potency of purified Gantrez nanoparticles were evaluated in function of both the synthesis method and the physical form (stabilized using high amounts of crosslinking agent, unstabilised or completely hydrolyzed) with the slug mucosal irritation assay (SMI) [19]. To obtain NP in the hydrolyzed form, purified uncrosslinked g NP were stored overnight at room temperature while stirring prior to assessment.

Five slugs, weighing between 3 and 5 g, were placed on 100 μl 1% (w/v) BAC in PBS as positive control, 100 μl PBS as negative control or 100 μl nanoparticle suspension (corresponding to 1 mg polymer/100 μl PBS) during 30 min a day for five successive days. The irritation potency was predicted based on the total amount of mucus produced (total MP). Total MP is expressed as a percent of the bodyweight of the slugs. For each slug, total MP is calculated by adding up the mucus produced during each 30-min contact period (days 1–5), and a mean value for the slugs in each treatment was calculated. Four categories of irritation potency are defined based on total MP. For liquid formulations, total MP cutoff values are: <2%: non-irritant; between 2% and 8%: mild irritant; between 8% and 15%: moderate irritant and >15%: severe irritant. Moreover, even at low total MP, the incidence of irritation phenomena increases when increased mucus production is observed.

Besides irritation potency, tissue damage was predicted by (1) the number of slugs in each treatment (out of the five per treatment) that show ALP release, (2) the mean LDH release of all the samples and (3) the mean protein release excluding the samples taken on day 1. The SMI is considered valid when the negative control is classified as non-irritant accompanied with minimal tissue damage (mean protein release <25 μg/ml.g, mean LDH release <1 IU/l.g and no ALP release). Moreover, the positive control needs to be classified as a severe irritant accompanied with severe tissue damage (ALP release in 4 of the 5 slugs or mean protein release >100 μg/ml.g). These criteria were met since SMI outcomes for

negative and positive control were, respectively, 0.08 ± 1.5 and 24.8 ± 6.1% of initial body weight for total MP, 9.1 ± 4.3 and 191.2 ± 38.7 μg/ml.g for mean protein release and 0.04 ± 0.09 and 9.0 ± 9.3 IU/l.g for mean LDH release.

## 3. Results and discussion

The biocompatible copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN) is widely employed for pharmaceutical applications and has been proposed as an appropriate copolymer for the preparation of nanoparticulate dosage forms with bioadhesive properties using a simple solvent displacement method [7]. However, some challenges have to be overcome. Although the solvent displacement method resulted in nanoparticles (NP) having a higher bioadhesion than the corresponding Gantrez solution (obtained by NP hydrolysis), unstabilised NP rapidly converted into a Gantrez solution, as the nanoparticles completely dissolved in less than 24 h due to the hydrolysis of anhydride groups. Therefore, the use of 1,3-diaminopropane was advised for crosslinking of the anhydride groups [7]. Moreover, the incorporation of antigens into polymeric Gantrez NP using the solvent displacement method is not generally applicable, due to antigen aggregation in organic solvents such as acetone. Therefore, another synthesis method is proposed, in which proteins are first dissolved in water before encapsulation. As a result, antigen encapsulation occurred by NP precipitation using a mixture of an aqueous antigen solution and an organic polymer solution. A mixture containing 44% acetone as organic phase was selected, since this water/organic solvent combination contained the highest amount of water, allowing efficient NP formation in the absence of NP aggregates. Higher amounts of water resulted in NP aggregates, whereas the turbidity of the obtained NP suspension decreased, probably due to a higher polymer solubility when higher amounts of acetone were used. To be able to investigate the influence of this solvent modification on the chemical and biological characteristics of the obtained nanoparticles, β-galactosidase (b-gal) was selected as model antigen, which could be encapsulated in Gantrez NP using both the solvent displacement method and co-precipitation in water/acetone.

Since crosslinking is known to decrease the bioadhesive properties of nanoparticles [8], one strategy to overcome this lower bioadhesion is modifying the physicochemical properties of the nanoparticles by coating them with macromolecules which provide specific or non-specific bioadhesive interactions at the intestinal surface [11]. However, in this case, the combination of high amounts of crosslinking agent (⩾0.22 mg DP/mg polymer) and subsequent surface modification with wheat germ agglutinin (WGA) was not possible, since insufficient functional groups remained available for WGA binding on the NP surface after crosslinking [7,11]. Therefore, the ligand-binding step was performed prior to crosslinking. As the molecular weight of WGA (36 kDa [13]) is higher compared to DP (74 g/mol), the molar ratio of WGA/polymer was much lower compared to the molar ratio of DP/polymer, hence the number of functional groups necessary for ligand binding did not affect subsequent stabilization. Moreover, due to its smaller size, DP is much more flexible then WGA. As a result, functional groups of the polymer remain available for crosslinking with DP after ligand coating, even when the functional groups are not easily accessible.

### 3.1. Nanoparticle characterization

The use of different solvents during synthesis of WGA-conjugated Gantrez NP with encapsulated antigen did influence some of the NP characteristics (Table 1). Functionalisation of pure Gantrez NP (g NP) into WGA-coated b-gal-entrapped NP (gbw NP) increased NP size ($p = 0.006$), especially for NP prepared via the 100% acetone method. In addition, as polydispersity index of gbw NP 100% increased up to 0.25, NP aggregates were observed using the 100% acetone method. Since WGA coating of NP 44% occurred by simple incubation of WGA to an aqueous medium (in contrast to WGA coating of NP 100% when the WGA solution was added to an organic medium), the differences in NP aggregation can be explained by conformational changes of the coated ligand. However, the use of different solvents during NP synthesis as well as antigen encapsulation and ligand binding did not change the zèta potential ($p > 0.164$), suggesting that the influence of these modifications was overruled by subsequent crosslinking with high amounts of DP [8]. Moreover, a high NP yield (>98% of initial dry weight) was obtained for gbw NP, prepared via the 44% and 100% acetone methods (data not shown).

**Table 1**
Nanoparticle characteristics of Gantrez NP synthesized via the 44% and 100% acetone method. PI: polydispersity index, zèta: zeta potential. g NP: uncoated antigen-free NP, gb NP: uncoated b-gal-entrapped NP; gbw NP: WGA-coated b-gal-entrapped NP ($n = 5$).

| | | Size (nm) | PI | Zèta (mV) |
|---|---|---|---|---|
| NP 44% | g NP | 175.4 ± 11.9 | 0.070 ± 0.033 | −32.3 ± 0.7 |
| | gb NP | 185.8 ± 18.0 | 0.077 ± 0.013 | −31.6 ± 1.4 |
| | gbw NP | 197.5 ± 21.9 | 0.106 ± 0.021 | −33.8 ± 1.6 |
| NP 100% | g NP | 156.7 ± 7.9 | 0.164 ± 0.041 | −31.6 ± 1.2 |
| | gb NP | 184.7 ± 27.8 | 0.102 ± 0.025 | −31.4 ± 0.9 |
| | gbw NP | 198.7 ± 19.4 | 0.259 ± 0.071 | −32.3 ± 2.6 |

### 3.2. Stability of Gantrez NP

The stability of WGA-conjugated Gantrez nanoparticles was investigated in function of the amount of crosslinker (0.044–0.22 mg DP/mg polymer) using remaining turbidity as stability marker (Fig. 1). The choice for this crosslinker concentration range was based on preliminary results, showing that the mean size of gb NP increased using a higher amount of crosslinking agent (data not shown). However, only gbw NP crosslinked with 0.22 mg DP/mg Gantrez remained stable in conditions simulating the gastrointestinal tract (2 h in pH 1.2 and 8 h in pH 5.8). This indicated that crosslinking with at least 0.22 mg DP/mg polymer was required to provide sufficient protection for the antigens against the prevalent harsh conditions.

Further investigation into the stability of WGA-conjugated NP revealed some differences in the turbidity profiles depending on the synthesis method. This trend was most pronounced with NP crosslinked with the lowest amount of crosslinker used (0.044 mg DP/mg polymer) during incubation in pH 1.2 (Fig. 2). Using the turbidity profiles, two phenomenons can be monitored: first, degradation of polymeric nanoparticles, and secondly, the formation and breakdown of ionic bounds [20]. In case of 'pure (uncoated antigen-free) nanoparticles (g NP), the decrease in turbidity represented the degradation of the polymer. As similar degradation profiles were found for pure nanoparticles and b-gal-entrapped nanoparticles (gb NP), the decrease in turbidity was only due to polymer degradation. In contrast, a fast drop in turbidity was observed during the initial hours of stability testing for WGA-conjugated nanoparticles, synthesised following the 44% acetone method (gbw NP 44%), compared to the corresponding g and gb NP. Since the breakdown of ionic bounds decreased turbidity, this fast drop in turbidity can be explained by the release of ionically bound WGA. During the remainder of the incubation period, a slower decrease in turbidity was observed due to polymer degradation. In case of nanoparticles produced by the 100% acetone method, no major differences were observed between

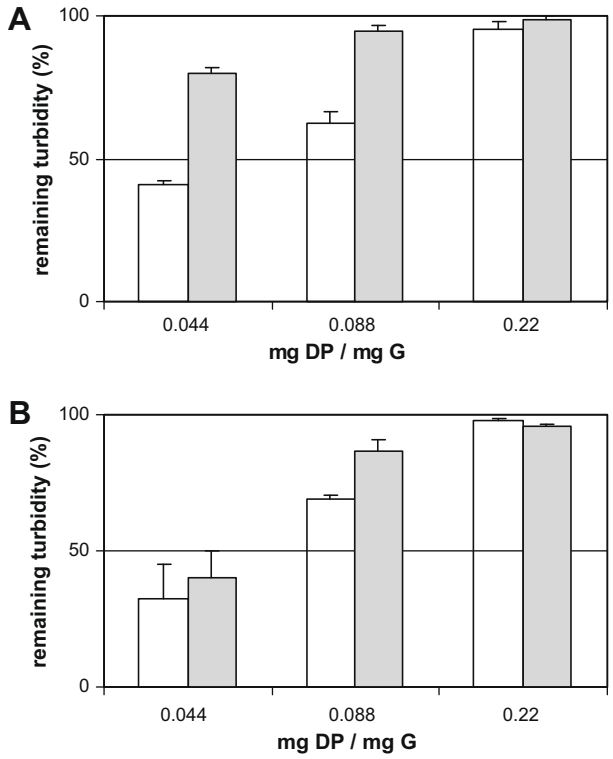


**Fig. 1.** Stability of gbw NP in function of the amount of crosslinking agent/mg polymer during □ 2 h in pH 1.2 and ■ 8 h in pH 5.8. A: gbw NP 100%, B: gbw NP 44% ($n$ = 3).

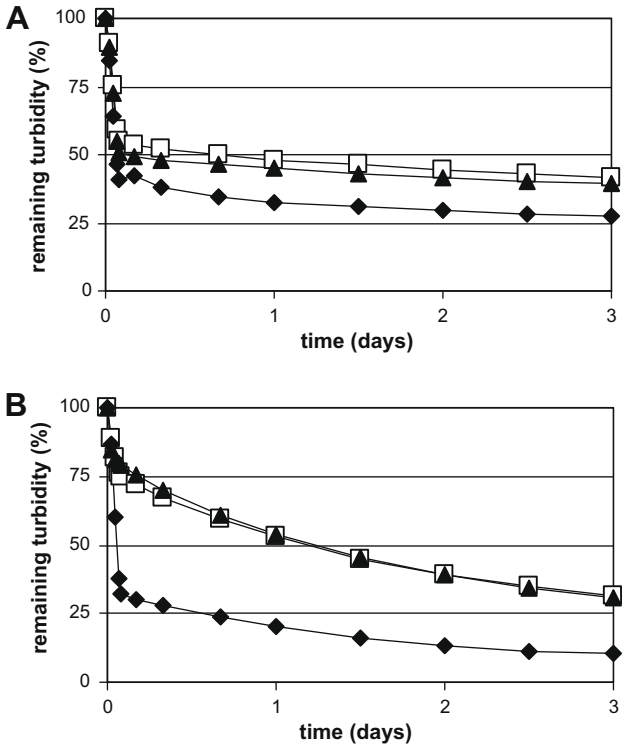


**Fig. 2.** Turbidity profiles of ▲ g NP, □ gb NP and ♦ gbw NP crosslinked with 0.044 mg DP/mg polymer in pH 1.2. A: NP 100%, B: NP 44%.

the degradation profiles of WGA-conjugated NP (gbw NP 100%) and gb NP, showing the absence of ionic bonds between WGA and Gantrez NP after synthesis in an organic solvent.

### 3.3. Identification of WGA binding

In both 44% and 100% acetone method, WGA-binding efficiency after NP synthesis was above 97% (data not shown), resulting in approximately 1 mg WGA bound onto 20 mg polymer. Nevertheless, incubation of those gbw NP in NaCl media confirmed that the interaction between WGA and polymer depended on the synthesis method: after 1 h in 1 M NaCl up to 58.4% WGA was released from nanoparticles prepared via the 44% acetone method, indicating an ionic binding, whereas under the same conditions, only 7.7% WGA released from nanoparticles prepared via the solvent displacement method, suggesting a covalent binding. Increasing the incubation time to 16 h or the molarity of the medium to 2 M did not influence WGA release from gbw NP 44%, showing that during synthesis via the 44% acetone method, WGA was bound to Gantrez via a combination of covalent and ionic interactions.

Although WGA coating of NP occurred by simple incubation in both synthesis methods, the differences in WGA binding can be explained by the different reaction conditions during ligand coating: in the 100% acetone method, the WGA solution is added to an organic medium, in which the anhydride groups of the polymer remained intact, favoring the formation of stable amide bonds between the anhydride groups of the polymer and the nucleophilic amine groups of the coating protein [21]. In contrast, in the 44% acetone method, the aqueous WGA solution is only added after evaporation of the organic phase. As a result, the anhydride groups of the polymer partially hydrolyzed in the aqueous medium, yielding carboxylic groups [8] that can also interact with amine groups of the coating protein, resulting in a combination of ionic and covalent bonds.

### 3.4. Activity and specificity of WGA coating

In order to explore the impact of the different synthesis methods onto the activity and specificity of the WGA coating, pig gastric mucin (PGM) was used. Thanks to the presence of *N*-acetyl-D-glucosamine (NAG) residues on PGM, the activity of WGA can be evaluated. When active WGA is present on the nanoparticle surface, nanoparticle precipitation will occur after incubation with PGM. Moreover, the specificity of the interaction between the conjugated WGA and PGM can be evaluated by pre-incubation with the single sugar residue NAG, blocking the specific binding sites of WGA.

The WGA coating of gbw NP of the 44% acetone method showed a high activity and specificity (Fig. 3). In contrast, the activity of gbw NP 100% was less pronounced and less specific, since pre-incubation with NAG only partially inhibited nanoparticles precipitation. However, this non-specific reaction of NP prepared via the 100% acetone method was not due to non-specific interactions between mucin and gb NP 100%.

The lower activity of WGA in NP 100% can be explained by the exposure to acetone during synthesis and by the covalent binding of WGA. A reduced activity of the ligand coating was already observed [7] when too many highly reactive anhydride groups were available for covalent ligand binding, resulting in multiple binding places. As a consequence, the ligand conformation changed, altering the activity of the protein coating. However, when ionic WGA binding is used for targeting purposes, WGA can be released from the nanoparticles during gastrointestinal passage. This creates an autoinhibitory effect [22] as the released WGA can react with the WGA receptors, blocking the accessibility of the receptors for WGA-conjugated nanoparticles. As a result, both the 100% acetone method and the alternative 44% acetone method do not achieve optimal targeting properties via ligand coating.

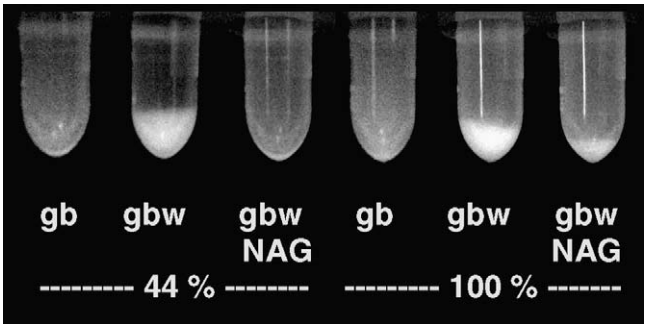


**Fig. 3.** Incubation of pig gastric mucin with purified gb and gbw NP, prepared via both 44% and 100% acetone method. NAG: in the presence of NAG as inhibitor.

### 3.5. B-gal encapsulation

In order to explore b-gal encapsulation in both synthesis methods, the encapsulation efficiency of b-gal in Gantrez NP was measured at different time points during NP synthesis. For the 44% acetone method, the addition of an aqueous b-gal solution to the organic Gantrez solution resulted in a fast encapsulation. After 10 min incubation, less than 1% b-gal was found in the supernatant. After evaporation of the organic phase and ligand binding, no b-gal could be detected in the supernatant. In contrast, in the 100% acetone method, no b-gal encapsulation occurred until water addition during the WGA-binding step. Nevertheless, after evaporation of the organic phase and subsequent incubation until NP synthesis was completed, b-gal encapsulation efficiency was found to be 99.4 ± 0.6% and 99.7 ± 0.4%, respectively (data not shown). As a result, b-gal encapsulation in both synthesis methods occurred rapidly when a water phase was added to an organic phase containing unhydrolysed anhydride groups. As explained before, these reaction conditions favored the formation of covalent bonds. This non-ionic interaction between b-gal and the polymer was also seen in the turbidity measurements during degradation, since the degradation profiles of Gantrez NP with encapsulated antigen were similar to the corresponding NP without encapsulated antigen (Fig. 2).

### 3.6. Evaluation of antigen binding by mass spectrometry

To investigate antigen binding with mass spectrometry, b-gal was replaced by human epidermal growth factor (hEGF), which has a smaller size and identical isoelectric point. Moreover, to avoid precipitation, the Gantrez polymer was replaced by maleic anhydride (MA), representing the anhydride groups of the polymer.

Addition of 0.65 mg solid maleic anhydride to an aqueous solution of hEGF formed a reaction product with a mass of 6314 Da, corresponding to the molecular weight of native hEGF increased with the molecular weight of MA (98 Da). Since no preceding hydrolysis of the anhydride groups of MA could occur when solid MA is used, the fast covalent binding of proteins to unhydrolysed anhydride groups of maleic anhydride was confirmed. The reactivity of unhydrolysed maleic anhydride remained valid when maleic anhydride was polymerized as the formation of covalent bounds was also described between primary amine-containing molecules (i.e. glucosamine, phenylethylamine and lysine residues of BSA) with polymerized maleic anhydride [21,23].

### 3.7. B-gal release

Since the encapsulation data suggested that b-gal encapsulation into Gantrez nanoparticles occurred via covalent binding in both synthesis methods, the availability of the antigen for vaccination purposes will be promoted by nanoparticle degradation. Therefore, uncrosslinked Gantrez nanoparticles were used to test b-gal release as their degradation is completed within 24 h. During hydrolysis of uncrosslinked gb NP, prepared via both 44% and 100% acetone method, no b-gal activity could be detected in the supernatant after 0, 4 and 24 h. However, no significant influence of the reaction media was found on the enzymatic b-gal activity, when b-gal was treated in the absence of Gantrez, according to both synthesis methods and subsequent incubation in aqueous medium. Moreover, after hydrolysis of gb NP, the theoretical b-gal amount was completely recovered via the Lowry method, which is based on the primary structure of the protein. Hence, the absence of enzymatic b-gal activity in the supernatant after complete hydrolysis of gb nanoparticles can be explained by the covalent binding between b-gal and the Gantrez polymer during synthesis. As the covalent bond between antigen and polymer remained intact after NP hydrolysis, the formation of a b-gal tetramer that is required for b-gal activity will be inhibited even after complete hydrolysis of the polymer into water soluble polymer fragments.

Moreover, when free b-gal was incubated with uncrosslinked antigen-free Gantrez nanoparticles (g NP), a fast decrease in b-gal activity was observed (Fig. 4), suggesting a rapid interaction between free b-gal and Gantrez upon hydrolysis of g NP. Both the remaining and hydrolyzed anhydride groups can contribute to this interaction, offering, respectively, covalent and ionic bounds with free b-gal. As a result, the fraction of b-gal that is not covalently bound to the Gantrez polymer during synthesis will – upon release from the hydrolized NP matrix as free b-gal molecules – immediately interact with these reactive groups and hence contribute to the absence of enzymatic b-gal activity after hydrolysis of gb NP.

### 3.8. Slug mucosal irritation assay

As cleavage of anhydride groups into carboxylic groups was already associated with increased interaction with the mucosa [8], the administration of unstable Gantrez nanoparticles (uncrosslinked or with a low degree of crosslinking) will result in highly reactive polymer fragments in the gastrointestinal tract, able to interact with the proteins of the intestinal barrier. Indeed, incubation of slugs with uncrosslinked nanoparticles stimulated the production of protective mucus, in contrast to incubation with highly crosslinked nanoparticles (Fig. 5), indicating the influence of available carboxylic groups onto the mucosal irritation potency of the nanoparticles. Moreover, the highest mucus production was observed after repeated exposure to hydrolyzed g NP of the 44% acetone method. When the prediction model was used, this level of total mucus production was classified at the border of mild irritation. However, hydrolyzed g NP 100% were less reactive compared to hydrolyzed g NP 44% and classified as non-irritative. This

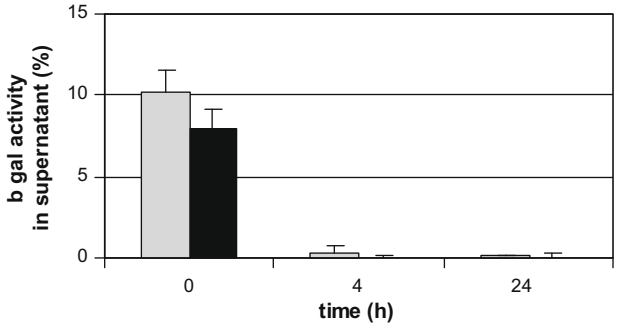


**Fig. 4.** B-gal activity in supernatant during hydrolysis of uncrosslinked ■ g NP 44% and ■ g NP 100% stirred with free b-gal ($n = 3$).

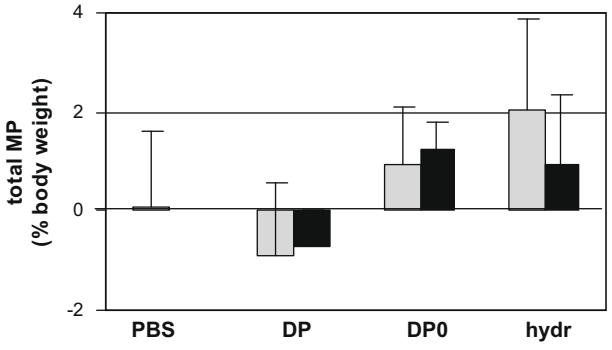


**Fig. 5.** Total mucus production of slugs ($n = 5$) incubated with PBS or pure Gantrez nanoparticles, prepared via the ■ 44% acetone method and ■ 100% acetone method. DP: nanoparticles crosslinked with 0.22 mg DP/mg polymer; DP0: uncrosslinked NP; hydr: hydrolyzed NP.

difference in irritation potency can be explained by the higher organic solvent content of the mixture and the corresponding lower degree of anhydride hydrolysis during nanoparticle synthesis via the 100% acetone method, influencing the final conformation of the obtained carboxylic groups after complete hydrolysis of the nanoparticles. Nevertheless, as protein release was minimal in all NP conditions, mucosal irritation was not accompanied by tissue damage (data not shown).

All in all, these results can elucidate the good vaccination results obtained with slightly crosslinked (0–0.01 mg DP/mg polymer) Gantrez nanoparticles, prepared via the solvent displacement method (100% acetone method) [9,24–28]. When antigen-entrapped Gantrez nanoparticles (uncrosslinked or with a low degree of crosslinking) are administered, nanoparticle hydrolysis occur during passage through the gastrointestinal tractus, resulting in reactive polymer fragments (mainly covalently) bound to the antigen. At the mucosal surface, these reactive polymer fragments cause minor mucosal irritation, which may activate dendritic cells (DC), facilitating immunisation [29]. As a result, antigen presentation and DC activation are concomitantly triggered [30], which can explain the adjuvant activity of these Gantrez NP. However, as mucosal irritation was function of crosslinking, activation of DC depends on amount of functional groups available for hydrolysis, forming carboxylic groups. Hence, in combination with covalently or ionically bound ligands, less carboxylic groups will be able to react with the mucosal border and adjuvant effect will be reduced. For example, in an oral immunotherapy challenge in mice, lipopolysaccharide coating of Gantrez NP with encapsulated ovalbumine did not result in a similar protective effect compared to uncoated ovalbumine-encapsulated NP [26].

As a conclusion of this work, the alternative synthesis method (44% acetone method) enlarged the applicability of Gantrez nanoparticles for oral vaccination as it facilitates the encapsulation of the antigen and offers a mild irritation at mucosal epithelia when unstabilised nanoparticles are used.

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