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Polymeric nanoparticles as delivery system for influenza virus glycoproteins

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Abstract

The objective of this work was to develop a new delivery system which could enhance the mucosal immune response to influenza virus antigens. Poly(D,L-lactide-co-glycolide) nanoparticles of about 200 nm containing hemagglutinin were chosen as the delivery system. Due to the amphiphilic nature of hemagglutinin (hydrophilic HA1 and hydrophobic HA2), nanoparticles were prepared by both classical oil in water solvent evaporation technique as well as by a [(water-in-oil) in water] solvent evaporation technique. Hemagglutinin was well encapsulated in nanoparticles prepared by both techniques. Molecular weight and antigenicity of entrapped hemagglutinin were not affected by the entrapment procedure. © 1998 Elsevier Science B.V.

Keywords: Influenza virus; Hemagglutinin; Nanoparticles; Poly(D,L-lactide-co-glycolide)

1. Introduction

The influenza virus vaccines currently in use do provide satisfactory immunity in healthy adults, but not in young infants and the elderly [1]. They contain viral proteins including an envelope glycoprotein, hemagglutinin (HA), and residual viral lipids. They are administered by intramuscular or subcutaneous injection and induce high serum IgG response. However they do not induce IgA response in nasal secretions, site of entry of influenza virus [2]. Therefore the introduction of an effective carrier system to enhance the immune response to influenza virus antigens would be a considerable benefit. It has also been shown that HA constitutes the major surface antigen of influenza virus. It by itself stimulates the synthesis of antibodies which neutralise infectivity [3]. Thus among the influenza virus proteins (three P proteins, hemagglutinin, nucleoprotein, neuraminidase, matrix protein) HA is the most important antigen for the induction of neutralising antibody responses.

Mucosal immunity (secretory IgA) can be induced as a result of events initiated at specialised mucosal lymphoid tissues: gut-associated lymphoid tissue (GALT), nasal-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT). It is only rarely induced by systemic injections [4–6]. Accumulated experimental evidences suggest that small polymeric particles are promising antigen delivery systems able to induce mucosal immunity

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after oral or nasal administration. These particles protect the antigen and are taken up by mucosal lymphoid tissues. Therefore they facilitate the interaction of entrapped and/or adsorbed antigen in particles with GALT or NALT enhancing IgA production in mucosal secretions [5–8].

The polymer nature, the technique of particle preparation and the particle size have to be considered. The primary candidates for the development of such polymeric particles have been the poly(D,L-lactide-co-glycolide) copolymers (PLGA) because they are biocompatible, biodegradable and their capture by the Peyer's patches (GALT) has been demonstrated [9–11].

Poly(D,L-lactide-co-glycolide) particles have been prepared by several techniques [12,13]. Oil in water solvent evaporation technique (O/W) has been used successfully by several groups to entrap hydrophobic materials into PLGA nanoparticles and microparticles [14,15]. In contrast, poor levels of encapsulation have been observed with hydrophilic agents [16]. Consequently, a [(water-in-oil) in water] ($W_1/O/W_2$) solvent evaporation technique was developed to encapsulate peptides and proteins in PLGA microparticles [17,18]. This method has not been reported for the preparation of nanoparticles.

Several studies have shown the importance of the particle size on the uptake. Jani et al. [19] showed that optimal particle uptake was observed with particles below 1 μm (nanoparticles): 3 mm and 1 μm diameter microspheres were taken up by the lymphoid tissue less efficiently than smaller particles (100 nm and 500 nm). In contrast, Eldridge et al. [11] showed that when administered orally to mice, microparticles <5 μm in diameter passed through the Peyer's patches within the macrophages to induce a systemic immune response. Only microparticles 5 to 10 μm in diameter remained in the Peyer's patches where they induced mucosal immunity. The observation that microspheres 5 to 10 μm in diameter were retained in the Peyer's patches led to the speculation that these would be more effective than smaller sized microparticles in the induction of mucosal responses.

The aim of this study was to develop a new delivery system which could enhance the mucosal immune response to influenza virus antigens. Poly(D,L-lactide-co-glycolide) nanoparticles of about 200 nm containing HA were chosen as the delivery system. Due to the amphiphilic nature of HA (hydrophilic HA1 and hydrophobic HA2 glycopeptides) [20], nanoparticles were prepared by classical oil in water solvent evaporation technique as well as by a [(water-in-oil) in water] solvent evaporation technique. The efficiency of HA encapsulation as well as the molecular weight and antigenicity of encapsulated HA were assessed.

2. Materials and methods

2.1. Antigen

Hemagglutinin (HA) was prepared from the influenza virus (A/Singapore/6/86 (H1N1)) and provided by Smithkline Beecham Biologicals (Rixensart, Belgium). Briefly, the virus was inactivated with organic solvents, disrupted with detergents and HA was extracted and purified. Protein content was evaluated by the Bradford method. Molecular weight and antigenicity of HA were evaluated by polyacrylamide gel electrophoresis (PAGE) and Western-blot.

2.2. Protein quantification

The Bradford method [21] was used to quantify total proteins. Protein reagent [Coomassie brilliant blue G-250 0.01% (w/v) (Sigma Chemical Co., Bornem, Belgium), ethanol (95%) 4.7% (w/v), phosphoric acid (85%) 8.5% (w/v)] was added to protein solutions (0.1 to 1 mg/ml). The absorbance was measured at 595 nm after 2 min.

2.3. Nanoparticle preparation

Poly(D,L-lactide-co-glycolide) (PLGA) (Resomer 502H; Mw=8000; Mn=2700; lactic acid/glycolic acid 50/50) was from Boehringer Ingelheim, Germany. Poly(vinylalcohol) (PVA) (Mw=13 000–23 000; 87–89% hydrolysed) was from Aldrich Chemical Co., Bornem, Belgium. Dichloromethane was purchased from UCB, Braine L'Alleud, Belgium. Span 40 was from ICI, Essen, Germany.

2.3.1. Oil in water solvent evaporation technique (emulsion method) (0/W)

The method of preparation was based on a patent from Vanderhoff et al. [22]. PLGA was dissolved in dichloromethane 1.0% (w/v) (6 ml) (O). This was then emulsified in an aqueous phase (54 ml) (W) containing PVA 0.4% (w/v) and HA (7.4 μg/ml) with a homogeniser Silverson L4R (Silverson Machines, Ltd, Bucks, UK) at 8000 rpm. The organic solvent was then removed and the resulting suspension concentrated by evaporation under reduced pressure (HA 32 μg/ml, PLGA 0.5% (w/v) PVA 1.7% (w/v)).

2.3.2. [(Water-in-oil) in water] solvent evaporation technique (double emulsion method) [(W_1/O)/W_2]

A two-step emulsification procedure was performed. An inner aqueous phase (1 ml) (W_1) containing PVA 2.8% (w/v) and HA (400 μg/ml) was emulsified in dichloromethane (6 ml) containing PLGA 1.0% (w/v) (O) with Ultra-Turrax model T25 (Janke and Kunkel, Staufen, Germany) at 9500 rpm to form the primary emulsion (W_1/O). The first emulsion was then reemulsified in an outer aqueous phase (54 ml) (W_2) containing PVA 0.4% (w/v) with Silverson L4R at 4000 rpm to form the double emulsion [(W_1/O)/W_2]. The organic solvent was then removed and the resulting suspension concentrated by evaporation under reduced pressure (HA 32 μg/ml, PLGA 0.5% (w/v), PVA 1.9% (w/v)).

The physical stability of the first emulsion (W_1/O) was evaluated by visual observation of the phase separation using the cylinder method: the freshly prepared first emulsion was transferred to a cylinder with cover. At intervals, the lengths of the aqueous phase containing indigo carmine, the organic phase and the emulsion were measured [23].

In order to prepare particles of about 200 nm, the influence of some experimental parameters on the nanoparticle size was evaluated by experimental design [24]. These permitted to find the values of independent variables (surfactant nature, surfactant concentration, homogeniser speed, evaporation rate and freeze-drying) that give the most desired value of the dependent variable (size ≈200 nm). For both emulsion and double emulsion methods, a 2^4 factorial design was assessed, where 4 is the number of independent variable, also called factor, and 2 the number of levels for each factor. For the emulsion method, the factors and the levels were PVA concentration (0.1 or 0.4% (w/v)), homogeniser speed (4000 or 8000 rpm), evaporation rate (under reduced pressure or atmospheric pressure overnight) and freeze-drying (no freeze-drying or freeze-drying). For the double emulsion method, the factors and the levels were surfactant type in the internal aqueous phase (Span 40 or PVA), PVA concentration in the external aqueous phase (0.1 or 0.4% (w/v)), evaporation rate (under reduced pressure or atmospheric pressure overnight) and freeze-drying (no freeze-drying or freeze-drying). The results were analysed with a Systat computer programme. Both experimental designs resulted in a mathematical expression like: $y = k + a.x1 + b.x2 + c.x3 + ... + l.x1.x2 + m.x1.x3 + n.x2.x3 + ...$, in which y is the dependent variable (nanoparticle size); k is a constant representing the mean of the dependent variable obtained in each experiment; $x1$, $x2$, $x3$,... are the independent variables; $x1.x2$, $x1.x3$, $x2.x3$, are the interaction terms and a, b, c,...l, m, n,... are the coefficients. This expression gives an insight into the effect of the different independent variables on the nanoparticle size. The optimum condition chosen was a size of about 200 nm with a low polydispersity (measure of the range of nanoparticle size around the mean).

2.4. Particle size and morphology analysis

The mean diameter of nanoparticles was measured using laser light scattering (Coulter N4MD Hialeah, FL, USA). For each experimental conditions, nanoparticle size and polydispersity were measured in triplicate from one batch of nanospheres.

Nanoparticles were coated with gold-palladium and their surface morphologies were observed through a JEOL JSM-840 A scanning electron microscope at 20KV.

2.5. Evaluation of HA encapsulation

2.5.1. Nanoparticle filtration

To separate the unentrapped protein, nanoparticle suspensions were filtered through polyvinylide difluoride membrane of 0.1 μm pore sizes (Millipore, Brussels, Belgium), using Extruder (T001 10 ml Thermobarrel Extruder Lipex Biomembrane,

Canada). The aqueous phase (first filtrate) passed through the membrane, while nanoparticles were retained on the filter. These nanoparticles were then washed in water and filtered again yielding to the second filtrate and the washed nanoparticles.

In order to evaluate if nanoparticle aggregation occurs during this process, the mean size of the particles were measured before and after filtration.

To verify that HA was not adsorbed on the polyvinylide membrane, a HA solution was filtered through a membrane producing a first filtrate. The membrane was then washed in water and the resulting aqueous solution was filtered again producing a second filtrate. A PAGE of the HA solution, the first filtrate and the second filtrate was performed and stained with silver nitrate. The dried gel was subsequently scanned enabling a semi-quantitative analysis (Dalton, Waalwijk, Netherlands). This method assumes that the degree of staining can be directly correlated with the amount of protein contained in each lane. Coomassie staining or protein quantification by the Bradford method could not be used because of their low sensibility and their lack of specificity.

2.5.2. Nanoparticle dissolution

The classical method for dissolving PLGA particles uses NaOH 0.1N containing sodium dodecyl sulfate 5.0% (w/v) [18]. However since this high concentration in anionic surfactant decreases the HA adsorption on nitrocellulose membranes used for the Dot-blot and Western-blot techniques, an alternative method had to be developed. Nanoparticles (washed nanoparticles) were dissolved with NaOH 0.1N for two min, at 100°C (washed and dissolved nanoparticles). To detect a possible alteration of HA by this technique, a HA solution underwent the same treatment (dissolution control).

2.5.3. Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out under non-reducing conditions. First filtrate, second filtrate and washed and dissolved nanoparticles were loaded onto 12% acrylamide gel (Ready Gel 12%, Bio-Rad) and run using Bio-Rad Mini Protean II Electrophoresis System [25]. Proteins were visualised by staining with silver nitrate (Daïchi Pure Chemicals Co. Ltd., Tokyo, Japan) [26].

2.5.4. Single-radial immunodiffusion (SRID)

A detergent [Zwittergent 3–12 (Calbiochem Corporation, La Jolla, CA)] was added to the first filtrate, the second filtrate, the washed nanoparticles and a diffusion control (HA 28.8 μg+unloaded nanoparticles) prior to assay in SRID to dissociate the components of the influenza vaccine into aggregates which are small enough to diffuse through the agarose gel. The diffusion control allowed to evaluate if free HA was able to diffuse in presence of nanoparticles in the agarose gel. Samples were introduced in wells (4 mm diameter) made in 1.5% (w/v) agarose gel (Sepracor/IBF, Villeneuve-la-Garenne, France) which contains antiserum raised against sheep anti A/Singapore/1/86 (H1N1) glycoproteins (HA1 glycopeptide) [27]. After diffusion through the agarose gel, free HA formed a precipitation ring with the antiserum visible after staining with Coomassie brilliant blue. The diameter of the precipitation rings were compared to those of HA standards (7.5, 15.0, 22.5, 30.0 μg/ml).

2.6. Molecular weight and antigenicity of encapsulated HA

2.6.1. Polyacrylamide gel electrophoresis (PAGE)

An identical procedure to that described above Section 2.5.3 was followed.

2.6.2. Dot-blot

First filtrate, second filtrate and washed and dissolved nanoparticles were twofold serially diluted and filtered under reduced pressure through a nitrocellulose membrane (Bio-Rad). The membrane was incubated with a sheep antiserum raised against sheep anti A/Singapore/1/86 (H1N1) glycoproteins (HA1 glycopeptide). After washing the membrane was incubated with rabbit anti-sheep IgG conjugated to phosphatase alkaline. The ability of the antiserum to recognise HA was demonstrated colorimetrically using nitro blue tetrazolinum (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) (Promega, Leiden, Netherlands) in Tris buffer, pH 9.5 [28].

2.6.3. Western-blot

First filtrate, second filtrate and washed and dissolved nanoparticles were loaded onto 12% acrylamide gel (Ready Gel 12%, Bio-Rad), run and

transferred from the gel onto the nitrocellulose membrane (Bio-Rad) using Bio-Rad Mini Protean II Electrophoresis System and Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The blot was incubated with a sheep antiserum raised against sheep anti A/Singapore/1/86 (H1N1) glycoproteins (HA1 glycopeptide). After washing the blot was incubated with rabbit anti-sheep IgG conjugated to phosphatase alkaline. The ability of the antiserum to recognise HA was demonstrated colorimetrically using nitro NBT and BCIP in Tris buffer, pH 9.5 [29].

2.6.4. Hemagglutination-inhibition test (HI)

Female Balb/c mice, specific pathogen-free, aged 8 weeks (Iffa Credo, Brussels, Belgium) were immunised at day 0 either subcutaneously using split virus (conventional vaccine) (1.5 μg/ml) or intranasally using HA (1.5 μg/ml) free or encapsulated in nanospheres prepared by the double emulsion method (6 mice per group). The mice received a second booster immunisation at day 56, using the same formulation and dose as for the first immunisation. At day 70, each mouse was bled by retro-orbital puncture. Serum HI tests were performed according to standard procedures [30]. Briefly, before HI tests, nonspecific inhibitors were removed by treatment of the sera at 56°C, with kaolin suspension (Behring, Brussels, Belgium) and finally with chicken erythrocyte suspension. Sera were then two-fold serially diluted. Three units of antigen (inactivated A/Singapore/6/86 (H1N1)) and 25 μl of 0.6% chicken erythrocyte suspension were added. The titers were expressed as the reciprocal of the final dilution of the sera after the addition of all reagents.

3. Results

3.1. Nanoparticle preparation

In order to produce particles of about 200 nm by both emulsion (O/W) and double emulsion [(W_1/O)/W_2] methods, influence of processing parameters [surfactant type, surfactant concentration, emulsification speed, evaporation rate and freeze-drying (independent variable)] on nanoparticle size (dependent variables) was studied in factorial designs. For the emulsion method, experimental design (Table 1) showed that increasing the PVA concentration highly decreased the nanoparticle size. The homogeniser speed, the evaporation rate or the freeze-drying slightly influenced the nanoparticle size. For the double emulsion method, the experimental design (Table 2) showed that PVA in the internal aqueous phase (W_1) decreased the nanoparticle size compared to Span 40. The PVA concentration in the external aqueous phase, the homogeniser speed or the freeze-drying slightly influenced the nanoparticle size.

For both methods, experimental conditions producing nanoparticles of about 200 nm with a low polydispersity were chosen. To avoid a long contact between HA and dichloromethane, which could decrease HA antigenicity, dichloromethane was always removed under reduced pressure. The nanoparticles were prepared with PVA 0.4% and at 8000 rpm for the emulsion method (Table 1: experiment 4). The nanoparticles were prepared with PVA in the internal aqueous phase for the double emulsion method (Table 2: experiment 4). Table 3 shows the size of the nanoparticles prepared by the emulsion method and the double emulsion method. The results show that the nanoparticle size is about 200 nm for both methods and does not change when HA is encapsulated or after freeze-drying.

The physical stability of the first emulsion method (double emulsion method) was also evaluated. When PVA 2.8% (w/v) was added in the internal aqueous phase, the emulsion was stable over more than 4 h. In the absence of PVA, the emulsion broke after 10 min. Since nanoparticle preparation lasted 1 h, the stability of the first emulsion containing PVA was satisfactory.

Scanning electron microscopy of nanoparticles prepared by emulsion and double emulsion methods are presented in Figs. 1 and 2, respectively. The figures show spherical nanoparticles with smooth surfaces for both methods of preparation, and confirm the size of nanoparticles measured by the Coulter N4MD.

3.2. Evaluation of HA encapsulation

The first step to evaluate HA nanoencapsulation was to separate free from encapsulated HA. Ultracentrifugation is widely used to separate free from

Table 1
Experimental design of the emulsion method

Experience number	PVA concentration (w/v) − + 0.1% 0.4%	Homogenizer speed (rpm) − + 4000 8000	Evaporation rate (mm Hg) − + ≈0 760	Freeze-drying − + no yes	Nanoparticle size[a] mean±SD (nm) (n=3)
1	−	−	−	−	329±3
2	+	−	−	−	233±3
3	−	+	−	−	321±3
4	+	+	−	−	216±2
5	−	−	+	−	328±5
6	+	−	+	−	239±2
7	−	+	+	−	331±4
8	+	+	+	−	222±2
9	−	−	−	+	333±4
10	+	−	−	+	233±7
11	−	+	−	+	336±1
12	+	+	−	+	218±2
13	−	−	+	+	341±6
14	+	−	+	+	237±4
15	−	+	+	+	334±5
16	+	+	+	+	226±5

Mathematical expression: $y=279.9-51.9x_1^*-4.3x_2^*+2.4x_3^*+2.5x_4^*-3.3x_1.x_2^*+0.6x_1.x_3-2.0x_1.x_4+0.3x_2.x_3+0.6x_2.x_4-0.3x_3.x_4$.
(y=dependent variable (nanoparticle size); 279.9=constant representing the mean of all the nanoparticle size; x_1-x_4=independent variables; x_1=PVA concentration; x_2=homogenizer speed; x_3=evaporation rate; x_4=freeze-drying; $x_1.x_2$=PVA concentration.homogenizer speed; $x_1.x_3$=PVA concentration.evaporation rate; $x_1.x_4$=PVA concentration.freeze-drying; $x_2.x_3$=homogenizer speed.evaporation rate; $x_2.x_4$=homogenizer speed.freeze-drying; $x_3.x_4$=evaporation rate.freeze-drying; *=significant difference; a=mean and standard deviation of nanoparticle size measured in triplicate from one batch of nanoparticles.

encapsulated drug [12]. However, the high speed required to centrifuge nanoparticles can precipitate high molecular weight proteins: 45 700 *g* for 15 min was necessary to precipitate the nanoparticles producing also a precipitation of 26.5% HA as measured by the Bradford method. To overcome this problem, nanoparticle suspensions were filtered through a polyvinylide difluoride membrane of 0.1 μm pore sizes. No modification in the mean size of the particles was noticed during this procedure: the size of the particles were 213±12 nm and 227±5 nm before filtration and 217±5 nm and 229±5 nm after filtration for the emulsion and double emulsion methods, respectively (Table 3) suggesting that no particle aggregation occurred during this process. Moreover HA was not adsorbed on polyvinylide difluoride membranes: HA was almost quantitatively recovered in the first filtrate (Table 4).

The second step to evaluate HA encapsulation was to quantify HA in the nanoparticles. Numerous problems were encountered (Table 5). The Bicinchoninic acid (BCA) method [31] and the Lowry method [32], commonly used to quantify proteins in particles, presented interferences mainly with thiomersal (preservative agent present in influenza virus vaccines) and PVA. The Bradford method [21] was insufficiently sensitive. SDS/NaOH used at the beginning of our study to dissolve nanoparticles interfered with the hemagglutination assay [33]. Classical radio-iodination of proteins [34] failed for HA. Therefore PAGE was used to evaluate the amount of HA in the first filtrate, second filtrate and washed and dissolved nanoparticles (Fig. 3). Five bands were observed for untreated HA solution (lane 1) and correspond to >200 kDa,174 kDa, 130 kDa, 84 kDa and 63 kDa proteins. The >200 kDa and 174 kDa proteins could be hemagglutinin trimer and dimer, respectively [20]. The 130 kDa proteins could be nucleoprotein dimer [35]. The 84 kDa and 63 kDa proteins are hemagglutinin and nucleoprotein monomers, respectively. After Western-blot, only the >200 kDa, 174 kDa and the 84 kDa appeared after incubation with the antiserum raised against sheep anti A/Singapore/1/86 (H1N1) glycoproteins (HA1

Table 2
Experimental design of the double emulsion method

Experience number	Surfactant nature (w1) − + Span PVA	PVA concentration (W2) (% (w/v)) − + 0.1 0.4	Evaporation rate (mm Hg) − + ≈0 760	Freeze-drying − + no yes	Nanoparticle size[a] mean±SD (nm) (n=3)
1	−	−	−	−	269±2
2	+	−	−	−	239±1
3	−	+	−	−	293±3
4	+	+	−	−	229±6
5	−	−	+	−	286±3
6	+	−	+	−	249±1
7	−	+	+	−	317±3
8	+	+	+	−	239±2
9	−	−	−	+	242±4
10	+	−	−	+	241±1
11	−	+	−	+	278±3
12	+	+	−	+	229±5
13	−	−	+	+	250±3
14	+	−	+	+	245±3
15	−	+	+	+	317±3
16	+	+	+	+	259±3

Mathematical expression: $y=261.4-20.1x_1^*-8.8x_2^*+8.9x_3^*-3.8x_4^*-11.0x_1.x_2^*-2.1x_1.x_3+6.0x_1.x_4^*+4.0x_2.x_3+4.4x_2.x_4+1.3x_3.x_4$. (y=dependent variable (nanoparticle size); 261.4=constant representing the mean of all the nanoparticle size; x_1-x_4=independent variables; x_1=surfactant nature; x_2=PVA concentration; x_3=evaporation rate; x_4=freeze-drying; $x_1.x_2$=surfactant nature.PVA concentration; $x_1.x_3$=surfactant nature.evaporation rate; $x_1.x_4$=surfactant nature.freeze-drying; $x_2.x_3$=PVA concentration.evaporation rate; $x_2.x_4$=PVA concentration.freeze-drying; $x_3.x_4$=evaporation rate.freeze-drying; *=significant difference; a=mean and standard deviation of nanoparticle size measured in triplicate from one batch of nanoparticles.

glycopeptide) (data not shown). The comparison of the lanes allowed a good evaluation of HA entrapment. Since the band of HA was clearly more intense in the nanoparticles (lanes 5, 8) than in the filtrates (lanes 3, 4, 6, 7), most HA was within the nanoparticles and not in the filtrates, for both emulsion method (lanes 3, 4, 5) and double emulsion method (lanes 6, 7, 8). However due to high background in the lanes 5 and 8 (residual oligomers and surfactants) semi-quantitative analysed of the dried gel could not be realised.

In order to confirm these results, single radial immunodiffusion was carried out (Fig. 4). We can notice precipitation rings for the diffusion controls (B4, C4) and the absence of precipitation rings for the filtrates (B1, B2, C1, C2) and the washed

Table 3
Mean diameter of nanoparticles before filtration/freeze-drying, after filtration and after freeze-drying

		Before filtration and freeze-drying mean±SD (nm) (n=5)[a]	After filtration mean±SD (nm) (n=5)[a]	After freeze-drying mean±SD (nm) (n=5)[a]
Emulsion method (O/W)	uncharged	213±12	217±5	213±11
	hemagglutinin	223±8	218±10	211±3
Double emulsion [$(W_1/O)/W_2$]	uncharged	227±5	229±5	223±7
	hemagglutinin	223±14	219±14	219±14

[a]The values represent the mean size±SD of at least 5 nanoparticle batches prepared independently.

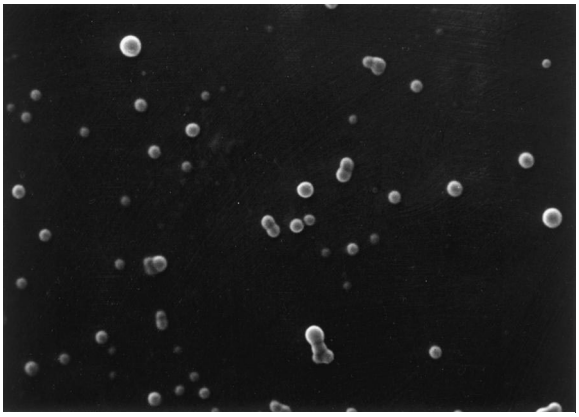

Fig. 1. Scanning electron micrograph of PLGA nanoparticles prepared by the emulsion method (1 μm=17 mm).

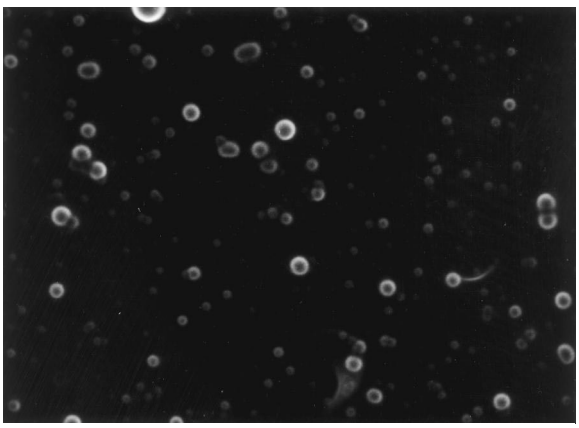

Fig. 2. Scanning electron micrograph of PLGA nanoparticles prepared by the double emulsion method (1 μm=17 mm).

nanoparticles (B3, C3). This indicates that (i) For the diffusion controls, free HA was able to diffuse in the presence of nanoparticles, (ii) For the filtrates, either HA was in the filtrates but conformationally altered by the nanoparticle preparation and thus unable to recognise the antibody in the agarose gel, or HA was not in the filtrates and (iii) For the nanoparticles, either HA was encapsulated in the nanoparticles, or HA was not in the nanoparticles. Since HA recognised the antibody following the entrapment procedure (see below: Molecular weight and antigenicity of encapsulated HA), HA was not conformationally altered. Therefore as also shown by PAGE (Fig. 3), HA was not in the filtrate but within the nanoparticles. From both PAGE and SRID techniques, it was difficult to quantify the exact amount of HA in the nanoparticles and the filtrates. Therefore, we can only deduce that most HA was within the nanoparticles.

3.3. Molecular weight and antigenicity of encapsulated HA

During the nanoparticle preparation, HA was exposed to potentially harsh conditions, such as contact with dichloromethane and shear force. These conditions may result in alteration of the molecular weight and loss of the antigenicity of the protein. Therefore the molecular weight was evaluated by PAGE and the antigenicity by Dot-blot, Western-blot and HI. PAGE (Fig. 3) shows the untreated HA (lane 1), a dissolution control (lane 2) and the encapsulated HA in nanoparticles (lane 5 and 8). The

Table 4
Validation of the filtration method to separate free from encapsulated HA: HA adsorption on polyvinylide difluoride membrane

	HA before filtration (ng)	HA after filtration first filtrate (ng)	HA after filtration second filtrate (ng)
Spot 1 (hemagglutinin trimer)	66	66	0
Spot 2 (hemagglutinin dimer)	254	254	56
Spot 3 (nucleoprotein dimer)	37	37	0
Spot 4 (hemagglutinin monomer)	746	742	7
Spot 5 (nucleoprotein monomer)	223	206	0

Table 5
Problems encountered in the quantification of hemagglutinin in nanoparticles.

	Thiomersal	PVA	SDS	Other problems
BCA [31]	+	+	/	/
Lowry [32]	+	+	/	/
Bradford [21]	−	−	+	insufficiently sensitive
Hemagglutination [33]	−	−	+	/
Radio−iodination [34]	+	−	/	low radiochemical yield low radiochemical purity
Dot−blotting [28]	−	−	+	
Western−blotting [29]	−	−	+	

+ interference; − no interference; / undetermined.

dissolution control (lane 2) indicates that the technique of nanoparticle dissolution, using NaOH, altered HA (84 kDa) (lane 1). It dissociated into 2 proteins of 62 kDa and 22 kDa (lane 2). These bands could correspond to HA1 and HA2 since the theoretic molecular weight of HA1 and HA2 are 60 kDa and 20 kDa, respectively [20]. Moreover, after Western-blot, only the 60 kDa band appeared after incubation with the antiserum raised against sheep anti A/Singapore/1/86 (H1N1) glycoproteins (HA1 glycopeptide) (data not shown). Identical bands were observed for the encapsulated HA (lanes 5, 8) and the dissolution control (lanes 2). Moreover, PAGE of undissolved nanoparticles revealed that small amount of unaltered HA (84 kDa) was able to migrate in the gel (data not shown). Hence these data suggest that

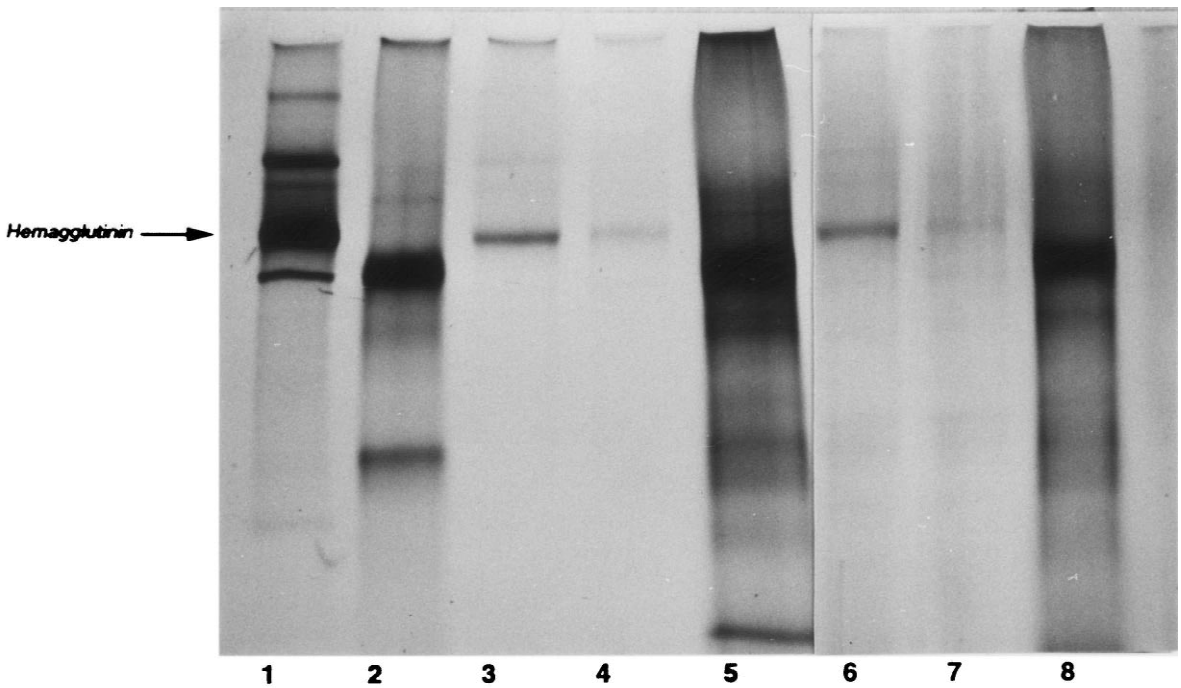

Fig. 3. Polyacrylamide gel electrophoresis of hemagglutinin in nanoparticles prepared by the emulsion and the double emulsion methods: lane 1, hemagglutinin; lane 2, dissolution control; lanes, 3–5 emulsion method (E) (lane 3 first filtrate, lane 4, second filtrate, lane 5, washed and dissolved nanoparticles); lanes 6–8 double emulsion method (EE) (lane 6, first filtrate, lane 7, second filtrate, lane 8, washed and dissolved nanoparticles) (arrow indicates monomeric hemagglutinin) (for details see Section 2).

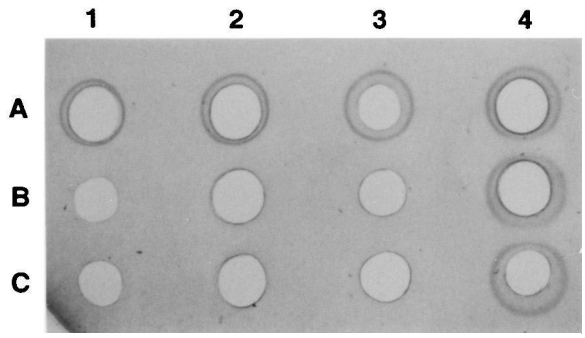

Fig. 4. Single radial immunodiffusion of hemagglutinin in nanoparticles prepared by the emulsion and the double emulsion methods: A1–A4 hemagglutinin standards; B1–B4 emulsion method (B1 first filtrate, B2 second filtrate, B3 washed nanoparticles, B4 diffusion control; C1–C4 double emulsion method (C1 first filtrate, C2 second filtrate, C3 washed nanoparticles, C4 diffusion control (for details see Section 2).

the molecular weight of HA was affected by the nanoparticle dissolution procedure but not by the entrapment procedure.

Dot-blot (Fig. 5) reveals that the colorations were visible until the same dilution for the entrapped HA in the emulsion method (lane 5), the entrapped HA in the double emulsion method (lane 8) and the dissolution control (lane 2). This indicated that an anti-serum raised against non-entrapped HA still recognised encapsulated HA. The preservation of

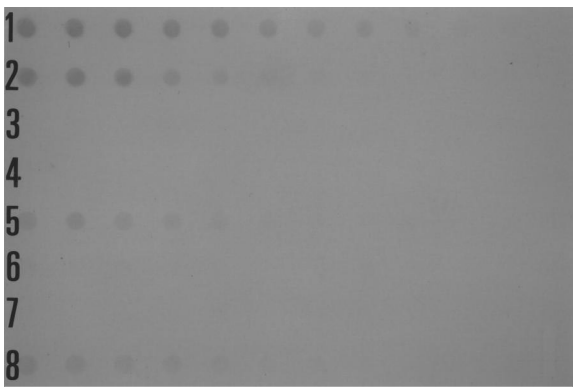

Fig. 5. Dot-blot of hemagglutinin in nanoparticles prepared by the emulsion and the double emulsion methods: lane 1, hemagglutinin; lane 2 dissolution control; lanes 3–5 emulsion method (E) (lane 3, first filtrate, lane 4, second filtrate, lane 5, washed and dissolved nanoparticles); lanes 6–8 double emulsion method (EE) (lane 6, first filtrate, lane 7, second filtrate, lane 8, washed and dissolved nanoparticles) (for details see Section 2).

Table 6
Serum HI antibody titers after s.c. or i.n. administration of split virus, hemagglutinin or hemagglutinin encapsulated in nanoparticles

Group	HI titers
Split virus s.c.	448
HA i.n.	224
NP/HA i.n.	224

(Split virus=conventional vaccine; HA=hemagglutinin; NP/HA=hemagglutinin encapsulated in nanoparticles; s.c.=subcutaneously; i.n.=intranasally).

this epitope was confirmed by Western-blot (data not shown). Fig. 5 also confirmed the HA encapsulation for both methods since the colorations were visible at much higher dilutions for the nanoparticles (lanes 5, 8) than for the filtrates (lanes 3, 4, 6, 7). The HI test (Table 6) showed that the sera HI titers were identical for free HA (HI titers=224) and HA encapsulated in nanoparticles (HI titers=224). This indicates that HA within the nanoparticles induced antibodies which were capable of inhibiting influenza mediated hemagglutination.

4. Discussion

The objective of this work was to prepare PLGA nanoparticles of about 200 nm and containing HA. Due to the amphilic nature of HA (hydrophilic HA1 and hydrophobic HA2 glycopeptides), nanoparticles were synthesised by classical oil in water solvent evaporation technique as well as by a [(water-in-oil) in water] solvent evaporation technique. The efficiency of HA encapsulation as well as the physical integrity and antigenicity of the encapsulated HA were evaluated.

Experimental designs showed that changing processing parameters such as the surfactant nature and the surfactant concentration permitted the preparation of spherical nanoparticles of about 200 nm by the classical emulsion method as well as by the double emulsion method. An increase in surfactant concentration resulted in a decrease in particle size (emulsion method). Increasing PVA could provide smaller emulsion droplets resulting in the formation of smaller nanoparticles. Scholes et al. [36] also showed that a significant reduction in PLGA

nanoparticle size was achieved as the concentration of PVA increased. The addition of PVA in the internal aqueous phase allowed the formation of smaller nanoparticles than the addition of Span 40 (double emulsion method). The decrease of the interfacial tension is probably higher for PVA than for Span 40. Jeffery et al. [37] also showed that the nature of the surfactant influences the particle size. For example PVA allowed the preparation of smaller microparticles than sodium dodecyl sulfate. In addition, to avoid hydrolysis of the polymer, drug leakage or drug degradation, nanoparticle suspensions have to be freeze-dried. Our results show that PLGA nanoparticles prepared by the emulsion or the double emulsion methods were easily resuspended in water after freeze-drying without size variation. The addition of surfactant used by Alonso et al. [38] for poly(alkylcyanoacrylate) nanoparticles and the ultrasonication employed by Krause et al. [39] for polylactic acid nanoparticles were not required.

Numerous problems were encountered to assess HA encapsulation in the nanoparticles. We finally used the PAGE and SRID techniques. From both techniques, it was difficult to quantify the exact amount of HA encapsulated within the nanoparticles. However they showed that most HA used during the nanoparticle formulation was encapsulated within the nanoparticles for the emulsion method as well as for the double emulsion method. These results are encouraging. First they show that the double emulsion method seems promising to encapsulate hydrophilic agents in nanoparticles. Second, whatever the preparation method, important entrapment efficiencies of HA are obtained. This is probably due to the amphiphilic nature of HA [20]. Hydrophobic HA2 glycopeptide could be responsible for HA adsorption at the surface of the PLGA nanoparticles prepared by the emulsion method. In contrast the first emulsion could be responsible of the HA encapsulation in the nanoparticles prepared by the double emulsion method. Kreuter et al. [40] evaluated indirectly the influenza virus antigens encapsulation in poly (methylmethacrylate) nanospheres using the hemagglutination test. The particles were centrifuged to separate free from bound antigen and the antigen was measured in the supernatant. They also presented high levels of encapsulation. Since purified HA precipitated at high centrifugation speed, we did not use the centrifugation technique. In addition our technique allows the evaluation of HA in nanoparticles. In other studies with influenza virus antigens encapsulation [41,42] there was no data on the antigens encapsulation levels.

A critical task in developing carrier systems for antigens is preserving their molecular weight and particularly their antigenicity. Our process of encapsulation exposed HA to potentially harsh conditions including exposure to dichloromethane and shear force, even though the contact of HA with the organic solvent was minimised by eliminating dichloromethane under reduced pressure. Our results showed that the encapsulated HA was not affected by the entrapment procedure as shown by PAGE, Dot-blot and Western-blot techniques. Moreover the encapsulated HA was able to induce antibodies capable of inhibiting influenza mediated hemagglutination as shown by HI technique. Inactivated influenza virus vaccines are themselves produced with organic solvents (inactivation) and detergents (disruption), yet keep their molecular weight and antigenicity. Jeffery et al. [19] showed that the molecular weight and the antigenicity of the encapsulated albumin in PLGA microspheres were not altered. In contrast Alonso et al. [43] observed a reduction of the antigenicity of tetanus toxoid encapsulated in PLGA microspheres. In others' reports [40–42] the molecular weight and antigenicity of encapsulated inactivated influenza virus antigen were not presented.

In conclusion, the results described in the present study show that PLGA nanoparticles of about 200 nm can be prepared by both classical oil in water solvent evaporation technique as well as by [(water-in-oil) in water] solvent evaporation technique. HA was well encapsulated in nanoparticles prepared by both techniques. Molecular weight and antigenicity of entrapped HA were not affected by the entrapment procedure. The in vivo adjuvant activity of these polymeric particles is presently under investigation.

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