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European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 273–281
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Development of an enteric-coated pellet formulation of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections

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Received 4 May 2004; accepted in revised form 16 August 2004
Available online 26 October 2004

Abstract

A multi-particulate formulation of F4 fimbriae was developed for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. A feasibility test showed that incorporation of F4 fimbriae in a disintegrating pellet formulation consisting of 87.5% Pharmatose® 200 M, 2.5% Avicel® CL 611 and 10% Explotab® by extrusion/spheronisation and subsequent fluid bed drying resulted in the maintenance of 69 ± 12% of the biological activity. But subsequent coating resulted in pellets with poor enteric properties, although good in vivo immunising results were obtained after administration to piglets. From the economical point of view, a pellet formulation was optimised to decrease vaccine dose and dosing frequency. After disintegration testing, pellets consisting of lactose (α-lactose monohydrate 90 mesh/β-lactose 75/25 (w/w)) and microcrystalline cellulose in a ratio of 80/20 (w/w) showed a sponge-like structure from which F4 fimbriae could be released. Coating of these pellets resulted in good enteric properties. To improve disintegrating properties of the pellets, the lactose concentration was increased or sodium carboxymethyl starch was added. But this resulted in poor enteric properties after coating. Dissolution test showed that F4 fimbriae were released from the optimised enteric-coated pellets but interaction between F4 fimbriae and the coating polymer was seen. This incompatibility leads to unpredictable in vitro quantification of F4 biological activity.

Keywords: Pellets; Enteric coating; Oral vaccination; F4 fimbriae; Piglet

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) cause diarrhoea and mortality in neonatal [1] and recently weaned [2] piglets and hence leads to economic losses in pig farming. Passive colostral and lactogenic immunity can effectively prevent neonatal infections [3,4] but active intestinal mucosal immunisation is needed for protection of newly weaned piglets since they are deprived of passive lactogenic immunity. This can occur following oral infection but is not obtained by parenteral immunisation, which tends to stimulate the systemic rather than the mucosal immune system [5]. As vaccination of piglets against postweaning infections is still an important challenge, there is clearly a need for competent oral vaccines for induction of mucosal protection.

Some of the ETEC strains bear F4 fimbriae, allowing adherence of the bacteria to F4-specific receptors (F4R) present on brush borders of villous enterocytes and subsequent colonisation of the small intestine. Newly weaned piglets can be orally immunised with isolated F4 fimbriae in solution against F4$^+$*E. coli* infection [6]. However, to protect the piglets against postweaning F4$^+$ETEC infection, the piglets have to be immunised

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doi:10.1016/j.ejpb.2004.08.007

during the suckling period. As oral solutions are unpractical for administration to suckling animals, a multi-particulate formulation of F4 fimbriae would have a considerable advantage as it could be mixed with creep feed. Moreover, from an economical point of view, in order to reduce dose and dosing frequency, the F4 administered should be delivered efficiently to the mucosal surfaces to induce immunisation. Therefore, the formulation has to be enteric-coated to protect the F4 fimbriae against detrimental effects of acids, pepsin, bile and of neutralising antibodies present in the mother's milk, the most important feed of suckling piglets, present along the gastro-intestinal (GI) tract [7,8]. Furthermore, the F4 fimbriae have to be targeted to the major inductive sites of the F4-specific intestinal immune response, namely the jejunal Peyer's patches (Snoeck et al., submitted). In order to select an appropriate coating thickness and coating material for protecting and targeting of the F4 fimbriae, the transit time as well as pH along the GI tract of suckling piglets was studied [8,9]. They showed that the pH at the beginning of the jejunum is 6.3 and that 1.5–3.5 h post oral administration of non-disintegrating radio-opaque pellets, more than 75% was removed from the stomach.

The use of enteric coating polymers for mucosal delivery of vaccines has been extensively reviewed [10]. Klipstein et al. [11] formulated the B subunit of *E. coli* heat-labile enterotoxin by tabletting the freeze-dried toxin and subsequent enteric coating. Subsequent oral administration to rat aroused a strong degree of serum and mucosal antitoxin response. Jain et al., Wong et al. and Flanagan et al. [12–14] described the coating of ovalbumin, *Vibrio anguillarum*, and heat-killed *E. coli*, respectively, on non-pareil seeds and subsequent coating with aqueous Eudragit® L30D-55. Oral administration to mice, salmonid fish and mice, respectively, induced an immune response.

Since binding of F4 to the F4R present on the villous enterocytes in the small intestine is a prerequisite for the induction of a protective intestinal immune response [6], maintenance of the correct F4 confirmation to ensure this binding is of crucial importance. A feasibility study was performed to evaluate the sensitivity of F4 towards different formulation techniques.

The aim of this study was to develop an enteric-coated multi-particulate formulation, which ensures primarily protection of the F4 fimbriae against the detrimental gastro-intestinal influences and subsequently release of the protein at the target site in an immunising conformation.

2. Materials and methods

2.1. Preparation of F4 stock solution

The F4ac fimbriae of the bacteria were isolated as previously described [6]. The protein concentration of the isolated solution was determined using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Bornem, Belgium). The purity was assessed by electrophoresis on a SDS-12% polyacrylamide slab gel, followed by analysis of the Coomassie stained gel using the gel analysis software, Image Master 1D® (Amersham Pharmasia biotech, Newcastle upon Tyne, England) and the concentration of the F4 fimbriae in the solution was determined.

2.2. Freeze-drying of the F4 solution

A PBS solution of F4 (3.1 mg/ml) was filled in vials (glass type 1, Gaash Packaging, Mollem, Belgium). The vials were covered with a freeze-drying stopper (V9032 FM 257/2 SAF1, bromobutyl with magnesium silicate as filler, kindly donated by Helvoet Pharma, Alken, Belgium). Prior to freeze-drying, the vials were kept on ice. The vials were loaded on the precooled shelves (−25 °C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to −45 °C over 105 min at 1000 mbar. The primary drying (12 h) was performed at −15 °C and 0.8–1 mbar and the secondary drying (9 h) at 10 °C and 0.1–0.2 mbar. After freeze-drying, the vials were closed under vacuum. Samples were kept on ice until analysis.

2.3. Production of the pellets

2.3.1. Feasibility study

As the isolation of F4 fimbriae from the *E. coli* is a very time-consuming and expensive process, a small-scale production method was used for the feasibility study on the incorporation of F4 in pellets (three batches). Microcrystalline cellulose containing 11.3–18.8% (w/w) sodium carboxymethyl cellulose (Avicel® CL 611, FMC Europe, Brussels, Belgium) and α-lactose monohydrate 200 mesh (Pharmatose® 200 M, kindly donated by De Melkindustrie, Veghel, the Netherlands) were used as excipients. Sodium carboxymethyl starch (Explotab®, received from Penwest Pharmaceuticals, NY, US) was used as a disintegrant. The powders were pre-blended in a ratio of 2.5% (w/w) Avicel® CL 611, 87.5% (w/w) α-lactose monohydrate 200 mesh and 10% (w/w) Explotab® in a mortar and a pestle. Exactly 6.36 ml F4 solution (3.1 mg/ml PBS) was added as the granulation fluid in order to obtain a concentration of 0.2% (w/w) of fimbriae in the pellets. Extrusion was performed using a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. Eight gram extrudate was spheronised on a mini-spheroniser (Caleva model 120, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1680 rpm with a residence time of 2 min. The pellets were dried either by fluid bed or freeze-drying. During fluid bed drying, the wet spheres were dried in a fluid bed dryer (Uniglatt D7852, Glatt, Binzen, Germany) for 8 min at an inlet air temperature of 25 °C. For freeze-drying, the pellets were transferred into glass vials and freeze-dried

Table 1
Composition of the pellet formulations

Excipient (%, w/w)	I	II	III	IV	V	VI	VII	VIII	IX	X
Microcrystalline cellulose	20	20	20	20	20	20	19	18	10	5
α-Lactose monohydrate 200 mesh	80	–	–	–	–	–	–	–	–	–
α-Lactose monohydrate 90 mesh	–	80	–	20	40	60	57	54	67.5	71.5
β-Lactose	–	–	80	60	40	20	19	18	22.5	23.7
Sodium carboxymethyl starch	–	–	–	–	–	–	5	10	–	–

as described above. Samples were taken of the wet and the dry pellets.

2.3.2. Optimisation of a disintegrating pellet formulation, downscaling and incorporation of F4

Different pellet formulations were prepared and evaluated for their disintegration properties (Table 1). The composition of the pellets was changed either by changing the type and ratio of lactose (α-lactose monohydrate 200 mesh (Pharmatose® 200 M) and 90 mesh (Pharmatose® 90 M) and β-lactose (Pharmatose® DCL21) (De Melkindustrie), by adding 5 and 10% (w/w) sodium carboxymethyl starch (Explotab®) or by varying the ratio lactose/microcrystalline cellulose (Avicel® PH 101, FMC, Brussels, Belgium) from 80/20 to 90/10 and 95/5 (w/w).

The pellet excipients were weighted (700 g in total), preblended and granulated with demineralised water in a planetary mixer (Kenwood Major Classic, Hampshire, UK) at 60 rpm for 2 min. Extrusion was performed in a single screw extruder (Dome extruder labo model DG-L1, Fuji Paudal Co., Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. The extrudates (600 g) were spheronised on a spheroniser (Caleva model 15, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1000 rpm with a residence time of 4 min. The wet spheres were dried in a fluid bed dryer (Uniglatt D7852, Glatt, Binzen, Germany) for 20 min at an inlet air temperature of 25 °C. The 700–1250 μm fraction was separated using a vibratory sieve (VE 1000, Retsch, Haan, Germany) for 20 min at amplitude 2.

For the coating experiments, 1% (w/w) thymidine was incorporated in the pellets as marker substance by means of preblending with the other excipients. To evaluate the F4 release from and the F4 stability in the optimised pellets, 0.04% (w/w) F4 was incorporated during the granulation step as a F4 solution in PBS.

For the downscaling experiments, the pellet production was performed as described above except that 40, 100 and 300 g of excipients was used instead of 700 g.

2.4. Coating of the pellets with Eudragit® L30D-55

The pellets were coated with Eudragit® L30D-55, an anionic copolymer of methacrylic acid and ethylacrylate (1:1). Previous experiments showed that Eudragit® L30D-55 dissolves from pH 6.0 (Huyghebaert et al., submitted). The preparation of the Eudragit® L30D-55 coating dispersion and the coating process parameters are previously described (Huyghebaert et al., submitted). If only a small amount of pellets was available, coating was performed together with microcrystalline cellulose pellets, containing 0.3% carmin red. After coating, the pellets of interest were selected based on colour. The pellets were coated with 15 or 30% (w/w) Eudragit® L30D-55. As no spray drying occurred during the process, the amount of polymer applied to the pellets was calculated based on the amount of polymer remaining after the process. After coating, the pellets were cured for 24 h or 4 days at 4 °C and 10% RH.

2.5. Storage of the pellets

For the feasibility test, pellets were stored for 1 month at 8 °C and 10% RH (above silica) and at room temperature (RT) (23 ± 2 °C) and 10 or 60% RH.

2.6. Evaluation of the pellets

2.6.1. F4 biological activity

To test the biological activity of F4 fimbriae, 10 ml PBS (pH 7.4) was added to 1 g of pellets for the feasibility test, whereas 1.2 g of the coated pellets of the optimised formulation was first pulverised in a mortar using a pestle after which 10 ml PBS was added. Subsequently, the pellets were homogenised on a rotating wheel for 1 h at RT and centrifugated at 3000 rpm and 18 °C. The supernatant was filtered using a Whatman filter 50 and stored at −20 °C until analysis. The extraction control was performed by redissolving the centrifuged pellet in 10 ml PBS (pH 7.4). It was homogenised on a rotating wheel at 4 °C, overnight and subsequently centrifugated at 3000 rpm and 18 °C. The supernatant was filtered and stored at −20 °C until analysis.

The amount of F4 in the samples was determined by ELISA using an F4-specific Mab recognising the c epitope of the F4ac which is involved in the interaction with the F4R. Moreover, analysis of the F4R binding capacity of the F4 in the samples using an in vitro competitive inhibition villous adhesion assay (bioassay) demonstrated that the ELISA results correlated very well with the F4R binding capacity. Consequently, this ELISA can be used to determine the biological activity of the samples and because of its high sensitivity was preferred above the competitive

inhibition villous adhesion assay. Furthermore, the samples were assessed by 12%-SDS-PAGE with and without previously boiling the samples followed by immunoblotting as described by Snoeck et al. [9] to evaluate the degradation of the multimeric F4 fimbriae and of its major subunit.

The ELISA used has been described by Van der Stede et al. [15]. Briefly, the wells of a 96-well microtiter plate were coated with F4-specific monoclonal antibodies (Mab) (clone CVI F4ac-5, ID-DLO, Lelystad, The Netherlands) [16] followed by blocking of the remaining binding sites. Subsequently, the F4 stock solution and the sample were added in series of twofold dilutions in ELISA dilution buffer (PBS, pH 7.4 with 0.05% (v/v) Tween® 20 and 3% (w/v) BSA). The dilution of the stock solution was started at a concentration of 25 μg/ml. Thereafter, an optimal dilution of an F4-positive serum from a pig repeatedly immunised intramuscularly with purified F4, and an optimal dilution of biotinylated-swine-specific IgG Mab and peroxidase-conjugated streptavidin were added. Finally, an ABTS solution containing H_2O_2 was added after which the optical density was spectrophotometrically measured at 405 nm.

The in vitro competitive inhibition villous adhesion assay was based on the in vitro inhibition villous adhesion assay of Van den Broeck et al. [17]. Briefly, 50 villi with F4-receptors were incubated with different dilutions of the F4 stock solution (concentration of F4: 0, 25, 50, 100, 200, 300, 400 μg/ml) or the samples, and 4×10^8 F4ac$^+$ *E. coli* in 0.5 ml PBS supplemented with 1% D-mannose during 1 h at RT while being gently shaken. Subsequently, the number of bacteria adhering per 250 μm villous brush border length was calculated using phase-contrast microscopy at a magnification of 600. As standard for the concentration determination of the biologically active F4 in the samples, the F4 stock solution was used in both tests. The F4 biological activity was expressed as % of theoretical activity.

The biological activity values obtained after storage for 1 month at different temperature and relative humidity were statistically evaluated with a two-way ANOVA at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov–Smirnov test and the homogeneity of variances by means of the Levene test. A multi-comparison among pairs of means was performed using a Scheffé test with $P<0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

2.6.2. Disintegration test

A disintegration test was performed for 30 min using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 0.5 g pellets per vessel (250 ml) with phosphate buffer (PB) (0.05 M) at pH 6.3. After the test, the pellets were dried and evaluated by optical microscopy (Olympus SZX9 stereomicroscope) or by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared and platinum coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan) before scanning electron microscopy was performed.

2.6.3. Dissolution testing

Dissolution testing ($n=3$) was performed using the reciprocating cylinder method using 1.2 g of coated pellets per vessel with two consecutive media: 0.1 N HCl (250 ml) (2 h) and consequently a phosphate buffer (250 ml) (BP) 0.05 M at pH 6.3. The thymidine concentration was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

2.6.4. F4 release test

A dissolution test was performed as described above ($n=2$). F4 was concentrated from the dissolution samples by filtrating 80 ml of the sample using a Centricon Plus-80 Filter Unit with Ultracel-PL membrane (Millipore, Brussels, Belgium) (MW cut-off 10,000) and a swinging bucket centrifuge for 20 min at 4500 rpm and 4 °C. Retention of recovered F4 was performed using the swinging bucket centrifuge for 2 min at 2000 rpm at 4 °C. The remaining solution was heated to 100 °C for denaturation. SDS electrophoresis and subsequent immunoblotting of the samples (undiluted and diluted 1/2) was performed using an F4ac specific Mab for F4-specific red staining.

3. Results and discussion

3.1. Feasibility study

Preliminary studies have shown that incorporation of F4 fimbriae in a non-disintegrating pellet formulation consisting of pure microcrystalline cellulose (Avicel® PH101) results in a very low release (<5%) of biologically active F4 in phosphate buffer pH 6.3. This could be attributed to the denaturation of F4 during the pellet production process or to a hampered release of the protein from the microcrystalline cellulose pellets especially as F4 fimbriae (consisting of hundreds of identical protein subunits of 27.5 kDa) (0.1–1 μm length and 2.1 nm ∅) have much larger dimensions than conventional drugs (Angstrom-range (0.1 nm)). Besides, these pellets did not disintegrate while Fig. 1 shows that no pores were formed after a 30 min disintegration test in PB pH 6.3 (Fig. 1a). These data clearly indicated the need for a disintegrating pellet formulation or at least a porous pellet, which ensures the release of F4 fimbriae. Preliminary studies have demonstrated that a fast disintegrating pellet formulation consisting of 87.5% α-lactose monohydrate 200 mesh, 2.5% Avicel® CL 611 and 10% Explotab® disintegrated within 10 min in PB pH 6.3. Based on the biological activity of F4 incorporated in this pellet formulation by extrusion/spheronisation, this technique looked very promising for the production of a multi-particulate formulation of F4 (Fig. 2). Immediately

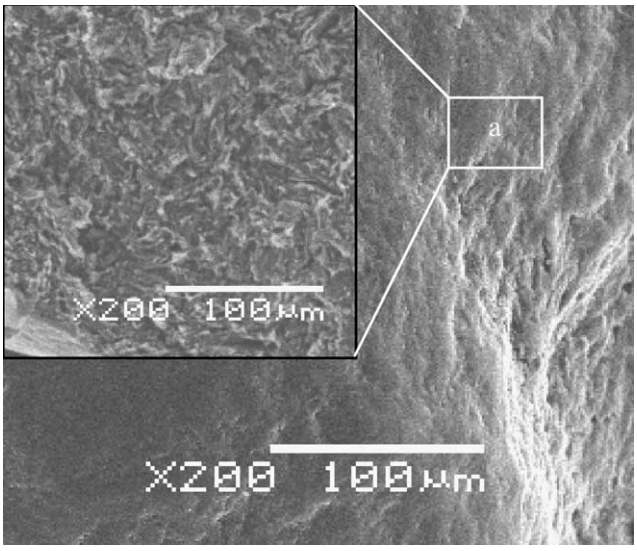

Fig. 1. SEM picture of the surface and cross-section (a) of pellets consisting of microcrystalline cellulose after disintegration test (30 min, PB pH 6.3).

after production, the wet pellets contained 86 ± 7% biologically active F4. No significant decrease in F4 biological activity was seen during drying. Both drying techniques, freeze-drying (77 ± 25%) and fluid bed drying (69 ± 12%) resulted in comparable activity. Freeze-drying of the F4 solution as such resulted in a similar activity (Fig. 2). After freeze-drying in PBS, without any lyoprotectant added, 64% remained active. However, as freeze-drying resulted in a powder, a subsequent compaction was required to obtain a multi-particulate formulation. This could lead to a further decrease in biological activity. Moreover, the compaction of a hygroscopic freeze-dried powder has to be performed at low relative humidity and the production of mini-tablets and the subsequent coating has technical and economical inconveniences.

Statistical analysis of the F4 biological activity values after storage for 1 month of the fluid bed dried pellets

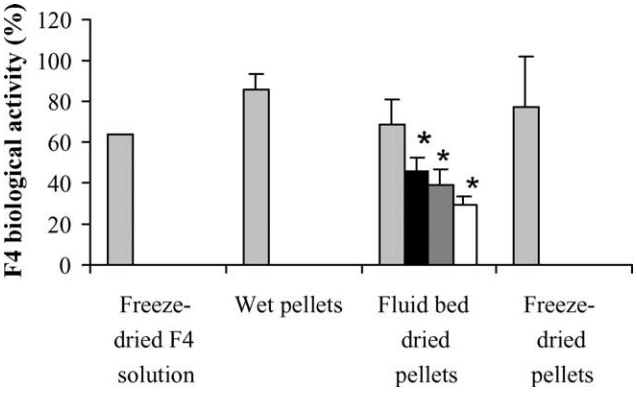

Fig. 2. Biological activity (% of theoretical, mean ± SD) of freeze-dried F4 solution ($n = 1$), of F4 incorporated in wet pellets by extrusion/spheronisation ($n = 1$), in fluid bed dried pellets (mean ± SD, $n = 3$) (after production □, after 1 month storage 8 °C/10% RH ■, RT/10% RH ▒ and RT/60% RH □) and in freeze-dried pellets ($n = 1$) (*: $P < 0.05$).

revealed that its stability decreased significantly at all storage conditions ($P < 0.05$). Although no significant differences were seen between the different storage conditions, the F4 activity tended to be negatively influenced by storage at high temperature and high relative humidity (Fig. 2). From these results, it can be concluded that F4 fimbriae can be incorporated in a pellet formulation by extrusion/spheronisation and subsequent fluid bed drying while maintaining the F4 biological activity.

The F4 pellets were coated with 15% (w/w) Eudragit® L30D-55. Although, a dissolution test showed that these pellets had poor enteric properties (24% release of marker substance in 0.1 N HCl after 2 h), good immunisation results were obtained after administration of the F4 pellets to the piglets [7]. The enteric properties of the pellets could not be improved by increasing the coat thickness to 30% (w/w) Eudragit® L30D-55 (19.35% release of marker substance). SEM revealed that the pellets were not spherical and their surface not smooth at all. However, the coating layer nicely covered the irregular surface (Fig. 3). SEM of a cross-section of the pellet showed that because of the irregular shape of the pellets, the coating thickness was not homogenous (Fig. 3a). The maximum thickness was 58 µm, comparable with values obtained in previous experiments (61.3 ± 8.6 µm) (Huyghebaert et al., submitted). Nevertheless, on some places, the thickness was reduced to 16.3 µm. To reach good enteric properties, a weight gain of 15% (w/w) of Eudragit® is recommended. This corresponds to a layer thickness of

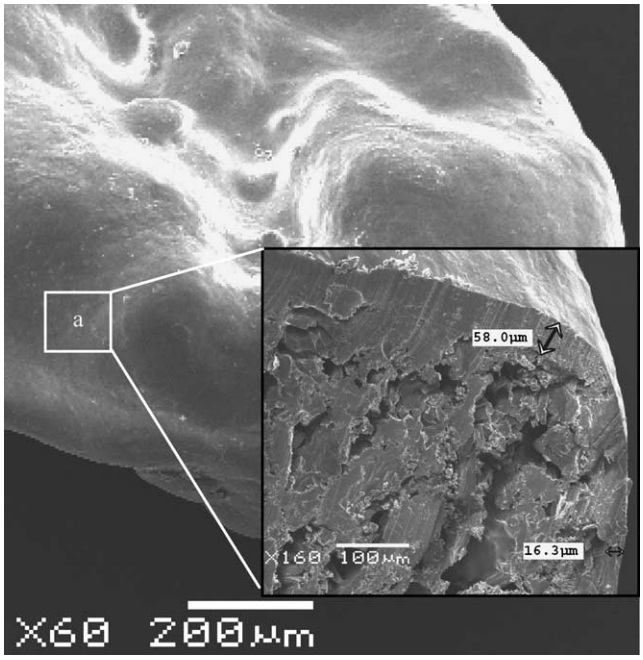

Fig. 3. SEM picture of pellets consisting of 87.5% α-lactose monohydrate 200 mesh, 2.5% Avicel® CL 611 and 10% Explotab®, coated with 30% (w/w) Eudragit® L30D-55 with inserted SEM picture of cross-sections of coating thickness (Fig. 3a).

approximately 30 μm and might explain the high release of marker substance in 0.1 N HCl after 2 h. Another possible explanation is the presence of disintegrants in the pellets. Swelling, as the result of the influx of dissolution medium could cause rupture of the coating and hence drug release. The production of pellets on a small-scale, missing adequate pressure build-up during pelletisation cannot explain the poor enteric properties, as production on a large scale and subsequent coating also resulted in poor enteric properties ($47.79 \pm 0.88\%$ marker substance released in 0.1 N HCl after 2 h).

3.2. Optimisation of the pellet formulation

The pellet formulation was optimised to improve the enteric properties of the coated pellets as this could lead to a dose reduction of enteric-coated F4 pellets needed for oral vaccination. Therefore, a disintegrating pellet formulation had to be developed with spherical appearance and without incorporation of disintegrants, resulting in good enteric properties after coating and the subsequent release of F4 in buffer. α-Lactose monohydrate was chosen as main excipient of the disintegrating pellets as α-lactose monohydrate is freely water-soluble (1 g/5 l). As pelletisation of lactose alone is impossible [18], microcrystalline cellulose was chosen as an additional excipient because of its excellent pellet-forming capacity [19]. It was used in a concentration of 20% (w/w) (Formulation I, Table 1) [20]. In order to evaluate the influence of lactose solubility and particle size on disintegration properties, pellets were prepared with either α-lactose monohydrate 200 mesh (I), 90 mesh (II) or β-lactose (III). Although the lactose ratio in the pellets is large, none of the pellets disintegrated after testing for 30 min in PB pH 6.3. Probably, compaction forces applied on the pellets during extrusion and subsequent spheronisation lead to dense pellets and hence poor disintegration properties. Optical microscopy revealed that after the disintegration testing, the size of the pellets consisting of α-lactose monohydrate 200 mesh or β-lactose decreased significantly, in contrast to pellets consisting of α-lactose monohydrate 90 mesh. This could be explained by the higher solubility of β-lactose (1 g/2.2 ml). SEM revealed that although some ruptures appeared after the disintegration test, the remaining pellet consisting of β-lactose was still dense (data not shown). Comparison of Figs. 4 and 5 clearly shows that the remaining α-lactose monohydrate 90 mesh pellets were more porous than

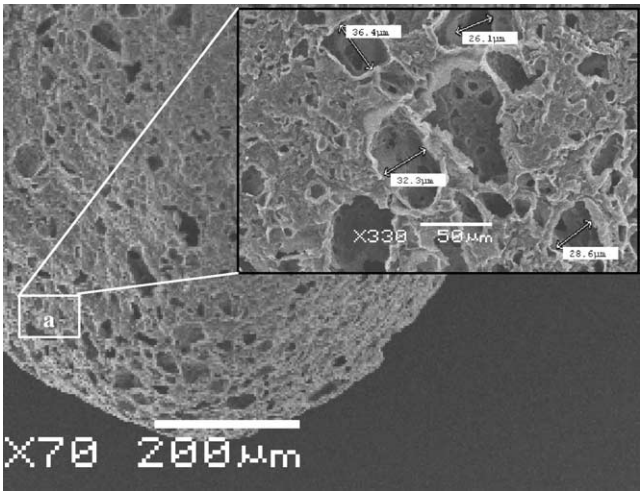

Fig. 4. SEM picture of the surface and cross-section (a) of pellets consisting of 80% (w/w) α-lactose monohydrate 200 mesh and 20% (w/w) microcrystalline cellulose (Formulation I) after disintegration test (30 min, PB pH 6.3).

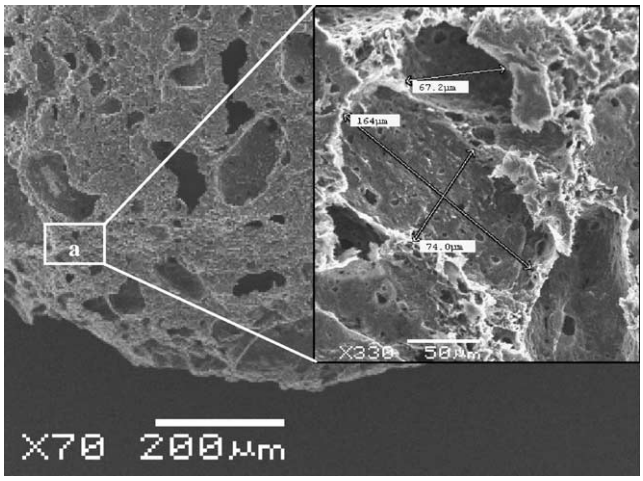

Fig. 5. SEM picture of the surface and cross-section (a) of pellets consisting of 80% (w/w) α-lactose monohydrate 90 mesh and 20% (w/w) microcrystalline cellulose (Formulation II) after disintegration test (30 min, PB pH 6.3).

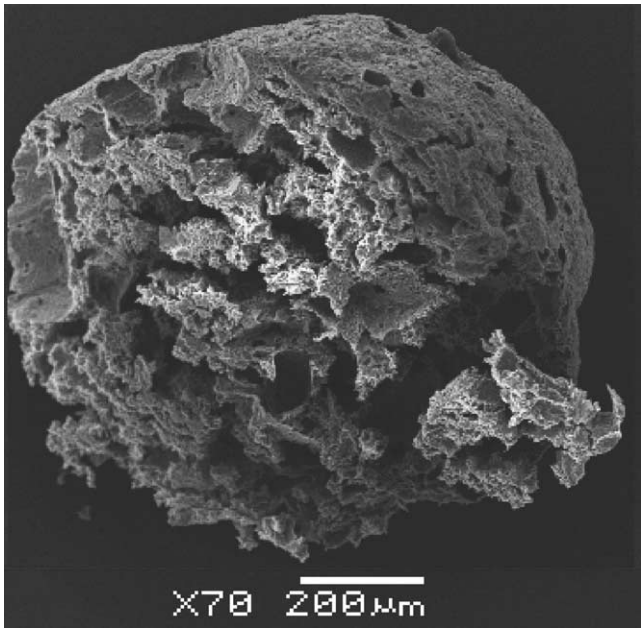

Fig. 6. SEM picture of pellets consisting of 20% (w/w) microcrystalline cellulose and 80% lactose (α-lactose monohydrate 90 mesh/β-lactose ratio 75/25 (w/w)) (Formulation VI) after disintegration test (30 min, PB pH 6.3).

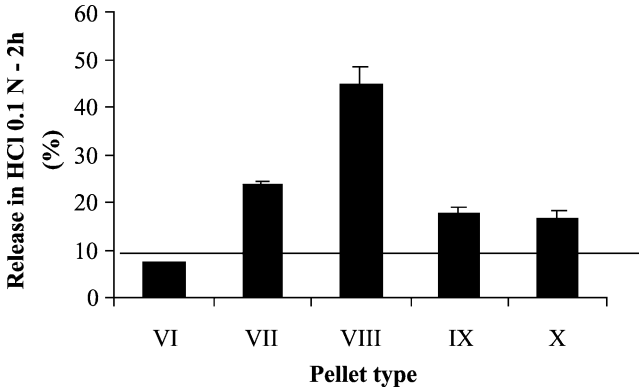

Fig. 7. Release of marker substance (mean ± SD, $n = 3$) after 2 h dissolution in HCl 0.1 N of pellets of different composition (cf. Table 1).

the remaining α-lactose monohydrate 200 mesh pellets (Figs. 4 and 5). This can be explained by the larger particle size of α-lactose monohydrate 90 mesh, leading to larger pores in the remaining pellet after lactose dissolution. The advantageous properties of β-lactose and α-lactose monohydrate 90 mesh were combined in one pellet formulation with three different α-lactose monohydrate 90 mesh/β-lactose ratio's: 25/75 (IV), 50/50 (V) and 75/25 (VI). After the dissolution test, none of the pellets completely disintegrated but the pellets with α-lactose monohydrate 90 mesh/β-lactose ratio of 75/25 (VI) showed a very porous surface, a sponge-like inner structure and some ruptures (Fig. 6). During this test, the pellets density clearly decreased and after the test, the pellets appeared totally wet and soft, and disintegrated by simple touching. This might indicate that during in vivo passage, the pellets will easily disintegrate under the influence of gastro-intestinal motility. Following production, the pellets appeared spherical and had a smooth surface. Coating and subsequent curing for 4 days at 8 °C and above silica (Fig. 7), as previous results showed that F4 stability was best maintained after storage at low temperature and low relative humidity, resulted in good enteric properties (7.29 ± 0.16% marker substance released in 0.1 N HCl after 2 h). Four days of curing was necessary as curing for 2 days at these conditions resulted in poor enteric properties of the coated pellets (17.88 ± 1.50% marker substance released in 0.1 N HCl after 2 h).

Further attempts were made to improve the disintegration of the pellets as Debunne et al. [21] showed that addition of 11% of Explotab® doubled the amount of a poorly water-soluble drug within 45 min in PB pH 6.8. Nevertheless, Fig. 7 shows that addition of 5 and 10% (w/w) sodium carboxymethyl starch (formulation VII and VIII, respectively) resulted in coated pellets with poor enteric properties (23.86 ± 0.54 and 44.65 ± 4.04% marker substance released in 0.1 N HCl after 2 h, respectively). This confirmed the previous hypothesis that incorporation of Explotab® could result in swelling of the pellets during coating and/or dissolution testing in HCl with subsequent rupture of the coating. Increasing the ratio lactose/microcrystalline cellulose from 80/20 (I) to 90/10 (IX) and 95/5 (X) could improve the disintegration but resulted in poor enteric properties (17.68 ± 1.42 and 16.52 ± 1.74% marker substance released in 0.1 N HCl after 2 h, respectively).

3.3. Downscaling of the production process

As the F4 solution is only available in low quantities, down scaling experiments were performed in an attempt to produce pellets on a smaller scale. Decreasing the excipient load for production of the pellets from 700, 300, 100 to 40 g resulted in poor enteric properties, i.e. from 7.29, 16.54, 17.18 to 50.32% marker substance released in 0.1 N HCl after 2 h. This shows that a minimum of excipient was

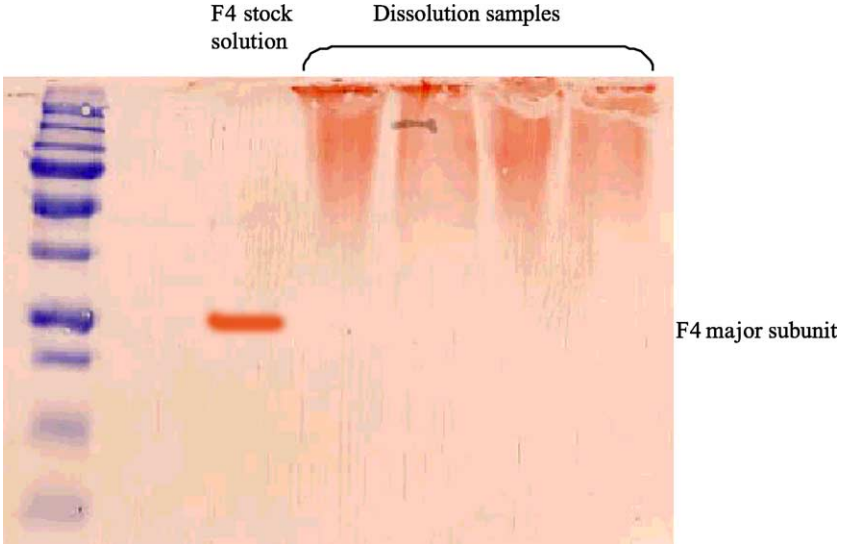

Fig. 8. 12%-SDS-PAGE (with previously boiling of the samples) followed by immunoblotting of native F4 in the F4 stock solution and of F4 in the dissolution samples ($n = 2$, undiluted (lanes 1 and 3) and diluted 1/2 (lanes 2 and 4)).

required to produce pellets by extrusion/spheronisation to result in good enteric properties after coating. Probably, a certain compaction force is necessary to produce compact pellets.

3.4. F4 stability in and release from the optimised pellet formulation

Determination of the biologically active F4, incorporated in the optimised coated pellet formulation, by ELISA showed the presence of interfering compounds as a biological activity of more than 100% was found (values around 150%). F4 biological activity from pure F4 solution, incubated with blanco coated pellets even reached values around 200%. However, blanco coated pellets showed no background signal, demonstrating that the pellets excipients and coating polymer alone were not responsible for the interference. This suggests that an interaction between F4 and the pellet excipients and/or coating polymer might take place causing the interference in ELISA.

The molecular weight of the F4 in the dissolution sample was compared to that of the native F4 in the F4 stock solution by 12%-SDS-PAGE with previously boiling the samples followed by silver staining or immunoblotting (Fig. 8). The 27.5 kDa band of the major subunit of the F4 was absent in the dissolution sample and instead, a smear of high molecular weight proteins reacting with the F4-specific Mab was seen. This demonstrated that the F4 had probably interacted with other compounds, such as the coating polymer (polymer: 135 kDa). The deprotonated carboxylic functions of the coating polymer probably interact with the cationic regions in the F4 protein.

From the present studies, it can be concluded that F4 is released from the optimised and coated pellet formulation but there seems to be an incompatibility between the protein and the coating polymer.

4. Conclusion

The pellet formulation consisting of lactose (α-lactose monohydrate 90 mesh/β-lactose 75/25 (w/w)) and microcrystalline cellulose in a ratio of 80/20 (w/w) has good pore forming (sponge-like structure) as well as enteric properties following coating. Increasing the concentration of lactose or adding sodium carboxymethyl starch results in poor enteric properties following coating.

Incompatibility between the protein and the enteric coating polymer resulted in an unpredictable quantification of in vitro biological activity, resulting in lack of quality control of this dosage form and accurate dosing. Consequently, careful pre-formulation studies are required before an oral enteric-coated protein formulation can be developed in order to exclude incompatibility between the protein and Eudragit® L30D-55. In vivo experiments have to reveal if this interaction will lead to altered immunisation capacity of the F4-fimbriae.

Acknowledgements

This work was supported by the Research Fund of the Ghent University. The authors gratefully acknowledge the technical assistance of Els De Vogelaere and Pharm. Thomas De Beer. Appreciation is also expressed to Prof. Dr. Simoens (Faculty of Veterinary Medicine, Ghent University) for the use of the scanning electron microscope and to Mr. Bart De Pauw for his technical assistance.

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