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## 2. Materials and Methods

### 2.1. Materials

**Drugs:** acetaminophen (APAP, Synopharm GmbH, Barsbüttel, Germany); estradiol (Schering AG, Berlin, Germany); diprophyllin (Knoll AG, Ludwigshafen, Germany); oxymetazoline hydrochloride (Procter&Gamble Pharmaceuticals Germany, Weiterstadt, Germany).

**Commercial drug products:** Aerodiol<sup>®</sup> (Les Laboratoire Servier, Neuilly-sur-Seine, France).

**Vaccine:** monovalent split inactivated influenza vaccine NIB16 (H1N1, strain A/Singapore/6/86, batch #SVA2219) in PBS buffer at a concentration of 250 µg hemagglutinin / ml (Aventis Pasteur, Research & Development, Marcy l'Etoile, France). The vaccine contained also an equivalent amount of nucleoprotein and traces of matrix proteins.

**Polymers:** polyacrylic acid (Carbopol<sup>®</sup> 971P NF, BFGoodrich, Cleveland, Ohio, USA); iota-carrageenan (Genuvisco<sup>®</sup> carrageenan type TPH-1, Copenhagen Pectin A/S, Lille Skensved, Denmark); chitosan glutamate (Protasan<sup>®</sup> G213, Pronova Biomedical A.S., Oslo, Norway); hydroxypropyl methylcellulose (HPMC, Methocel<sup>®</sup> E5 and K15M, Colorcon Ltd, Dartford, UK); polyoxypropylene-polyoxyethylen block copolymers poloxamer 188 (Lutrol<sup>®</sup> F68) and poloxamer 407 (Lutrol<sup>®</sup> F127), polyethylene glycol (PEG 4000, Lutrol<sup>®</sup> E 4000), polyvinyl pyrrolidone vinyl acetate (PVP VA, Kollidon<sup>®</sup> VA 64), polyvinyl pyrrolidone (PVP, Kollidon<sup>®</sup> 25, 30, and 90F) (BASF AG, Ludwigshafen, Germany); polyvinyl alcohol (PVA, Mowiol<sup>®</sup> 6-98), sodium carboxy methylcellulose (NaCMC, Tylopur<sup>®</sup> C 600 G1) (Clariant GmbH, Wiesbaden, Germany); sodium alginate (Na-alginate, Protanal<sup>®</sup> LF 20/200 [used if not further specified], Pronova Biopolymer A.S., Drammen, Norway) and sodium alginate HV, MV, and LV (Na-alginate, Sigma-Aldrich Chemie GmbH, Steinheim, Germany); xanthan gum (Keltrol<sup>®</sup> F, Kelco International, Tadworth, UK).

**Absorption enhancers:** sodium lauryl sulfate (SDS, Texapon<sup>®</sup> K12, Henkel KGaA, Düsseldorf, Germany); poly-L-arginine hydrochloride (molecular weight 70 - 150 kDa), protamin sulfate, sodium glycocholate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany);

3 $\beta$ [N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-cholesterol, Avanti Polar Lipids Inc., Alabaster, AL, USA).

**Other chemicals:** ammonio methacrylate copolymer, type A, USP (Eudragit<sup>®</sup> RL 30D, Röhm GmbH & CO KG, Darmstadt, Germany); randomly methylated  $\beta$ -cyclodextrin (M $\beta$ CD, Wacker-Chemie GmbH, Burghausen, Germany); agar agar, xylitol, and sorbitol (Caelo & Loretz GmbH, Hilden, Germany); mannitol (Roquette Frères, Lestrem, France); mucin (crude porcine stomach mucin), phenolphthalein (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

**Solvents:** Demineralized water; ethanol 96 % (V/V) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

## 2.2. Methods

### 2.2.1. General data presentation in diagrams and tables

The data points in diagrams represent the average (mean) value of n repetitions of the experiment. The error bars stand for the corresponding standard deviations. Data given in tables and in the text are average (mean) values of n repetitions  $\pm$  the standard deviation (SD) thereof. In some diagrams the coefficient of variation ( $CV = 100\% \cdot SD / \text{mean}$ ) is given instead of the SD. If a data presentation does not follow this scheme, it is stated in the table or figure caption.

### 2.2.2. Preparation of samples

**Solutions.** Polymer(s) (1, 2 or 3% w/w) and drug (5, 10, 20, 30, 40, 50, 60 or 100% w/w based on polymer) were dissolved in purified water. If desired, additional excipients were added (M $\beta$ CD 0 - 40% w/w based on total solution; sorbitol, mannitol, and xylitol 0 - 100% w/w based on polymer). Solutions of polymer blends were prepared by dissolving polymers at mass ratios of 1:1, 1:3, 1:19, and 1:39 in purified water (total polymer content 2% w/w).

*Sonication of carrageenan solutions:* 20, 10 or 5 g of carrageenan solution (2% w/w) with / without drug (5% w/w based on polymer) were sonicated with a sonication rod (type UW 200, Bandelin Electronic GmbH, Berlin, Germany) at maximum frequency for given time intervals (0, 1, 3, 5, 10 min).

*Drug / M $\beta$ CD complexes:* Complexation of estradiol with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) was achieved by dissolving both in ethanol at a molar ratio of 1:2 (estradiol hemihydrate 10.3 mg/ml and methyl- $\beta$ -cyclodextrin 94.3 mg/ml), followed by drying of the solution under air stream. The estradiol / M $\beta$ CD powder and the hydrophilic polymers (2% w/w) were dissolved in demineralized water (final estradiol concentration: 100  $\mu$ g / 200  $\mu$ l, final M $\beta$ CD concentration: 0.47% w/w). For inserts with increasing estradiol dose (molar ratio 1:2) solutions with final estradiol dilutions of 200, 400 and 1000  $\mu$ g / 200  $\mu$ l were prepared. Alternatively, the estradiol dose (100  $\mu$ g / 200  $\mu$ l) or the M $\beta$ CD content (4.7% w/w) were kept constant while increasing the M $\beta$ CD concentration of the solutions (0.47 - 9.4% w/w) or the estradiol dose (100 - 1000  $\mu$ g / 200  $\mu$ l) thus varying the estradiol:M $\beta$ CD ratios.

*Estradiol / Eudragit<sup>®</sup> RL formulations:* Formulations containing estradiol bound to Eudragit<sup>®</sup> RL 30D were prepared by adding estradiol (5% w/w based on polymer) into the aqueous nanoparticle dispersion (30% w/w) and stirring over 48 h. The absence of estradiol crystals in the dispersion was checked with a polarized light microscope (Axioskop, Carl Zeiss, Oberkochen, Germany), which confirmed the uptake of estradiol by the colloidal polymer particles. The dispersion was then diluted to 40  $\mu$ g estradiol / 20  $\mu$ l and further processed into inserts as described above. HPMC K15M 1% (w/w) was dissolved in the estradiol / Eudragit<sup>®</sup> RL 30D system to increase the viscosity of the polymer dispersion.

*Vaccine solutions:* The vaccine solution was diluted with purified water to a hemagglutinin concentration of 45  $\mu$ g / 200  $\mu$ l = 225  $\mu$ g/ml. Then the absorption enhancers (0.5% w/V) and the polymer (0.5%, 2% w/V) were added and stirred until uniformly distributed.

**Inserts.** Aliquots of the above prepared solutions ( $V = 1.5, 0.2$  or  $0.1$  ml) were placed into blister moulds. For in vivo studies aliquots of  $V = 20$   $\mu$ l were given into silicon tubing (Rotilabo Silicon Tubing TR60 2 mm / 4 mm, Carl Roth GmbH + Co, Karlsruhe). The samples were then frozen at  $-25^{\circ}\text{C}$  for 1 h and freeze-dried (0.25 mbar over 24 h with increasing shelf temperature  $-15$  to  $0^{\circ}\text{C}$  and final drying for 2 h at  $+15^{\circ}\text{C}$  and 0.01 mbar) (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The inserts were stored in a desiccator until use. The polymer content of inserts given in % (w/w) refers always to the polymer content of the solutions from which they were prepared.

**Films and microparticles.** Films were prepared by casting the polymeric solutions with / without drug on Teflon foil (Otto Köhnel & Sohn, Berlin, Germany) and drying over 48 h at 60°C. The film thickness was determined on 6 different positions on each film (Minitest 600, Erichson GmbH & CO KG, Hemer, Germany). All films were stored in a desiccator until use. Microparticles were obtained by grinding films in a Teflon ball mill (MM2000, Retsch GmbH & CO KG, Haan, Germany) at maximum frequency for 10 min under liquid nitrogen cooling. The resulting particles were sieved and the fraction 50 - 100 µm was used for further experiments. All microparticles were stored in a desiccator until use.

### 2.2.3. Polymer solution rheology

All samples were prepared, left unagitated overnight and then equilibrated to the measurement temperature for 1 h in a water bath (Haake G with thermostat Haake D8, Haake Meßtechnik GmbH und Co., Karlsruhe, Germany) before measurement.

**Viscosity.** The viscosity of the polymer solutions was determined with a rotational viscosimeter (Rheostress RS 100, Haake Meßtechnik GmbH und Co., Karlsruhe, Germany) in the controlled rate mode ( $D = 5 \text{ s}^{-1}$ ,  $10 \text{ s}^{-1}$ ,  $30 \text{ s}^{-1}$ ) over a time interval of 150 s at constant temperature (22°C, 37°C) utilizing a plate-cone-geometry (20 mm / 4°, 60 mm / 1°) ( $n = 3$ ). Flow curves of polymer solutions were determined with the geometry 60 mm / 1° by running a shear rate ramp from  $20 \text{ s}^{-1}$  to  $300 \text{ s}^{-1}$  and back to  $20 \text{ s}^{-1}$  at 22°C. Thixotropy was assessed by comparing the area under the flow curve (AUGC) on the upward and downward measurement. When the downward AUGC was more than 1% smaller than the upward AUGC, thixotropy was stated. Yield stress measurements were performed by running a ramp from 5 to 5000 µNm over 180 s at 22°C.

The influence of the MβCD concentration on the polymer solution viscosity was determined in solutions which were prepared by dissolving the polymer (0.5% or 2% w/w) in the appropriate amount of purified water. MβCD (0 - 40% w/w based on the total solution) was then added and dissolved.

**Viscoelasticity.** A viscosimeter (Rheostress RS 100, Haake Meßtechnik GmbH und Co., Karlsruhe, Germany) equipped with the plate-cone-geometry 20 mm / 4° was used in the

oscillatory mode for the measurement of the viscoelastic properties of solutions. Prior to the actual measurement the linear viscoelastic region, i.e. the region, within which the storage modulus  $G'$  is independent of the applied stress, had to be detected by a stress sweep. Here a stress ramp was run from 2.5 Pa to 2500 Pa at 3 different frequencies: 0.1 Hz, 1 Hz, and 10 Hz. The maximum stress in the  $\log G' - \log \text{stress}$  - diagram at which at all three frequencies the storage modulus was still constant was chosen for the following measurements for the sake of measurement accuracy. A frequency sweep (frequency ramp from 0.1 Hz to 10 Hz at constant stress) was then performed for the determination of the storage modulus  $G'$ , the loss modulus  $G''$ , and the complex viscosity. All measurements were performed at constant temperature (22°C or 37°C).

#### **2.2.4. Spreading of polymer solutions**

Polymer solutions (400  $\mu\text{l}$ ) were dropped from a height of 20 mm onto a plane agar gel (pH 6.0) to mimic a hydrated surface. The diameter of the solution on the agar gel was measured in a time-dependent fashion ( $n = 3$ ).

#### **2.2.5. Bioadhesion**

Adhesion studies were performed by adapting a method of Nakamura et al. (1996) (Werner and Bodmeier, 2002). 100 g of a hot agar / mucin solution (1% and 2% w/w, respectively in phosphate buffer pH 6.0, USP XXVI), was cast on a glass plate (20 cm x 20 cm) and left to gel at 4 - 8°C for 3 h. The gel was then equilibrated for 1 h to the test conditions of 22°C and 79% relative humidity (storage above saturated ammonium chloride solution) in a tightly closed glass chamber. The inserts ( $V = 0.1 \text{ ml}$ ) were placed on top of the gel and the glass plate was then turned into a vertical position. The bioadhesion potential was determined as the displacement (in cm) of the insert, which hydrated / formed a viscous solution / gel and moved downward on the agar / mucin gel due to gravity as a function of time ( $n = 3$ ). Thus, the adhesion potential was inversely related to the displacement of the insert.

#### **2.2.6. Water uptake and mass loss**

A sponge (5 cm x 6.5 cm x 3 cm, Santex household sponge, Santex GmbH, Wald-Michelbach, Germany) was fully soaked in the hydration medium (phosphate buffer pH 6.0,

USP XXVI if not otherwise stated) and placed in a petri dish filled with the same buffer to a height of 1 cm in order to keep the sponge soaked during the experiment. Round filter paper ( $d = 55$  mm, Schleicher & Schuell GmbH, Dassel, Germany) was also soaked in the medium and positioned on top of the sponge. This experimental set-up was equilibrated for 30 min. Accurately weighed inserts ( $V = 1.5$  ml) were then placed on the filter paper and the water uptake was determined as weight change of the insert (weight of hydrated insert and filter paper minus weight of wet filter paper) over time normalized to the initial dry insert weight. In case of water soluble additive (M $\beta$ CD, sorbitol, xylitol, mannitol) the weight increase of inserts was normalized to the initial dry polymer weight of the inserts due to loss of additive and therefore decreasing total insert weight over time.

The theoretical polymer content of the formed gel was calculated from the water uptake after 1, 2, 5, and 8 h. Aliquots of the gel were weighed and dried to a constant weight at 60°C over 24 h and the actual polymer content was determined. The recovery of the polymer is given as the ratio of actual to theoretical polymer content, expressed in %. All measurements were performed under ambient conditions ( $n = 3$ ).

To evaluate the effect of the buffer on the water uptake behavior of inserts, different buffers were used as medium (Table 2. 1). For the investigation of the effects of medium composition, phosphate buffer pH 6.0 (USP XXVI) was adjusted to the chosen osmolality by addition of sorbitol (Osmomat 030, Gonotec Gesellschaft für Meß- und Regeltechnik mbH, Berlin, Germany). Sodium ion content was adjusted by addition of sodium chloride. Alkaline borate buffer pH 10 (USP XXVI) and hydrochloric acid buffer pH 2 (USP XXVI) were used with the necessary adjustments of osmolality and sodium ion content. In all buffers the potassium ion content was constant at 0.05 mol/l and no other positively charged ions were present.

### **2.2.7. Moisture sorption**

Inserts ( $V = 1.5$  ml) were exactly weighed, placed in aluminum dishes, and stored in a tightly closed glass vessel above a saturated ammonium chloride solution (79% relative humidity) at room temperature. The moisture sorption was determined as weight increase of the inserts over time normalized to the initial dry polymer weight of the inserts, which was kept constant for all inserts for the sake of comparison ( $n = 3$ ).

Table 2. 1 Composition of buffers for the investigation of the effects of the release medium on water uptake and drug release behavior.

Buffer type	pH	Na <sup>+</sup> - conc., mol/l	Osmolality, mOsmol/kg	Buffer composition, per 1000 ml
Hydrochloric acid	2	0.044	196	3.728 g KCl , 1.339 g HCl conc, 2.554 g NaCl
Phosphate	6	0.044	170	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, 2.227 g NaCl
Alkaline borate	10	0.044	185	3.093 g H <sub>3</sub> BO <sub>3</sub> , 3.728 g KCl, 1.748 g NaOH
Phosphate	6	0.006	103	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH
Phosphate	6	0.006	189	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, Sorbitol q.s.
Phosphate	6	0.006	272	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, Sorbitol q.s.
Phosphate	6	0.006	364	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, Sorbitol q.s.
Phosphate	6	0.051	272	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, 2.67 g NaCl, Sorbitol q.s.
Phosphate	6	0.100	274	6.805 g KH <sub>2</sub> PO <sub>4</sub> , 0.224 g NaOH, 5.517 g NaCl

### 2.2.8. Contact angle

Polymer films were cast from solutions of 2% (w/w) polymer in water on microscope slides and dried at 50°C. The contact angle of phosphate buffer pH 6 (USP XXVI) on these films after 1 min contact time was determined under ambient conditions with a goniometer (Type I, Krüss GmbH, Hamburg, Germany).

### 2.2.9. Polymer-drug precipitation

Polymer solutions (2% w/w) with increasing oxymetazoline HCl content (% w/w based on polymer) were prepared. The turbidity of the solutions at room temperature was measured as absorption in the visible light range ( $\lambda = 500$  nm, UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). The oxymetazoline HCl concentration, at which precipitation started, was determined by extrapolating the linear correlation of the measured data points to an absorption of zero. Due to the high initial cloudiness of xanthan gum solutions, absorption at 500 nm exceeded the measurement range and precipitation was only observed visually in the case of xanthan gum.

### 2.2.10. In vitro drug release

**Large inserts (V = 1.5 ml).** A self-made diffusion cell (Figure 2. 1) was used for drug release studies mimicking the humidity properties of nasal mucosa. The lower end of a polypropylene tube (inner diameter 3.5 cm) was closed with a tightly stretched, thin sponge (Flinka Allzwecktuch, Kornbusch & Starting GmbH & Co. KG, Borken, Germany). This tube was placed vertically into a release medium container, which contained the release medium buffer, and adjusted exactly to the height of the release medium surface so that the sponge was wetted but not submersed. Inserts (V = 1.5 ml) were placed on the thin sponge and the whole system was closed with Parafilm<sup>®</sup> “M” sealing film (American National Can Company, Chicago, Illinois, USA) to avoid evaporation of release medium and to allow the establishment of a constant relative humidity around the insert (approx. 85 - 95% r.h.). The experiments were performed in a horizontal shaker (GFL 3033, Gesellschaft für Labortechnik mbH, Burgwedel, Germany) with 75 rpm and at 37°C. Samples of 2 ml were taken at predetermined time points and replaced by fresh medium.

The drug content of the samples was analyzed by UV spectrophotometry (oxymetazoline HCl:  $\lambda = 280.0$  nm, diprophyllin:  $\lambda = 273.5$  nm, APAP  $\lambda = 243.6$  nm, UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). Placebo inserts were also subject to the drug release test to quantify the contribution of the polymers to the UV absorption at the specific wavelength of the investigated drug. At each time point the placebo value was subtracted from the value of the drug-loaded inserts.



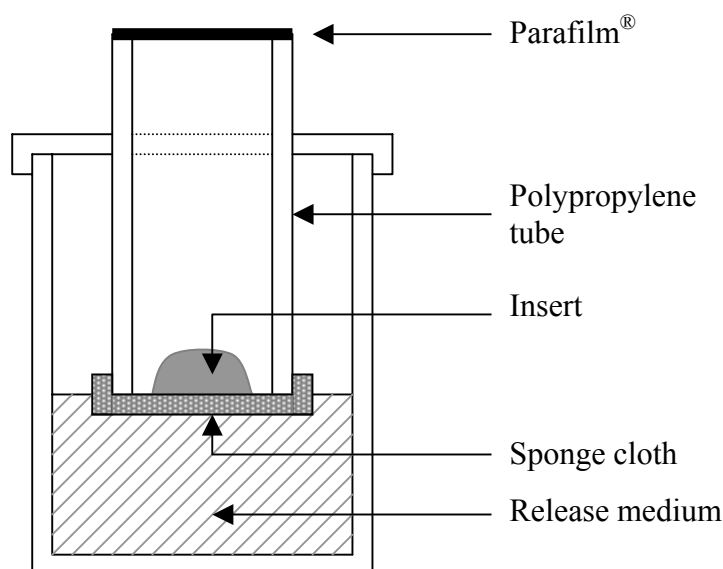


Figure 2.1 In vitro drug release device for large inserts ( $V = 1.5$  ml).

If not otherwise stated, phosphate buffer pH 6.0 (USP XXVI) was used as release medium buffer. Different buffers were used as medium to evaluate the effect of the buffer on the drug release from nasal inserts, as stated previously for the water uptake studies (see section 2.2.6, Table 2. 1).

**Inserts containing estradiol ( $V = 0.2$  ml).** For the low drug dose of the small inserts ( $V = 0.2$  ml) the diffusion cell design was adapted to a smaller vessel (Figure 2. 2). The release device consisted of a release medium container (polypropylene reaction tube 1.5 ml with conic bottom; Plastibrand®, Brand GmbH & Co. KG, Wertheim, Germany) with a small magnetic stirrer (length = 10 mm) inside. The lid of the vial was cut opened from the top and a regenerated cellulose filter membrane (pore size =  $0.45\mu\text{m}$ ,  $d = 13$  mm; RC - fleece supported [Sartorius AG, Göttingen, Germany] or RC-L 55 [Schleicher&Schuell GmbH, Dassel, Germany]) was fixed between the lid and the edge of the vial. A small hole was made in the vial just below the membrane to allow the escape of air during refilling of the vial. Through a second hole in the lower third of the vial a needle (20G; Sterican® 0.90 x 40 mm, B. Braun Melsungen AG, Melsungen, Germany) was inserted and fixed with superglue (UHU® Sekundenkleber, UHU GmbH, Bühl, Germany). The vial was filled with approximately 1.5 ml (accurately weighed) preheated ( $37^\circ\text{C}$ ) phosphate buffer pH 6.0 (USP

XXVI) to wet but not submerge the filter membrane. Solutions ( $V = 0.2$  ml), inserts ( $V = 0.2$  ml), films or microparticles were placed through the opening in the lid on top of the filter membrane and the release system was closed with Parafilm® “M” sealing film (American National Can Company, Chicago, Illinois, USA) to avoid evaporation of release medium. The whole assembly was kept at  $37^{\circ}\text{C}$  in an oven (T 6120, Heraeus GmbH, Hanau, Germany) and the medium was continuously stirred at 750 rpm (Variomag multipoint HP 15, H+P Labortechnik AG, Oberschleißheim, Germany). Samples of 0.5 ml were taken at predetermined time points and replaced by fresh medium.

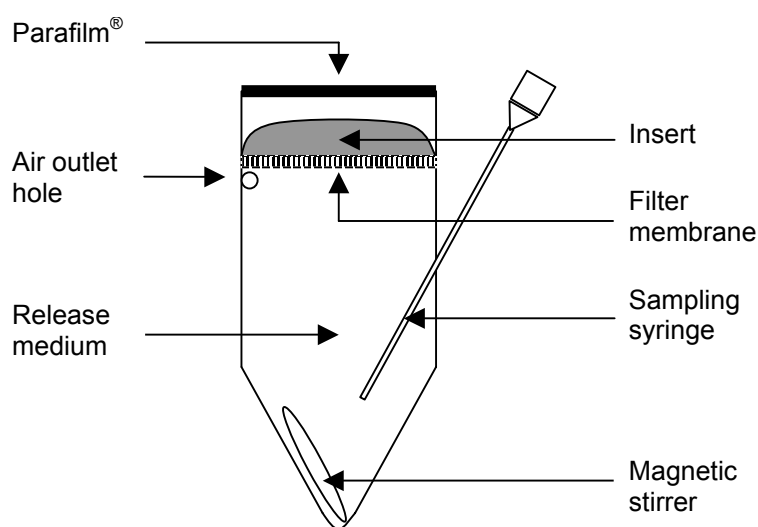


Figure 2. 2 In vitro drug release device for small inserts ( $V = 0.2$  ml).

The drug content of the samples was analyzed by UV spectrophotometry ( $\lambda = 281$  nm; UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). The UV spectrum of the estradiol / M $\beta$ CD complex in water was identical to the spectrum of pure estradiol dissolved in ethanol. Placebo inserts were also subject to the drug release test to quantify the contribution of the polymers to the UV absorption. At each time point, the placebo value was subtracted from the value of the drug loaded inserts.

The actual drug loading of the inserts was determined by complete dissolution of inserts in phosphate buffer pH 6.0 (USP XXVI) followed by UV analysis. All measurements were performed in triplicate (mean  $\pm$  SD).

The absorption of estradiol to the release system was determined by incubating a solution of the estradiol / M $\beta$ CD complex in release buffer (10 and 30  $\mu\text{g}$  estradiol / ml) in the complete

assembly under drug release test conditions and by measuring the estradiol concentration after 6 h by UV spectrophotometry ( $n = 3$ ). The estradiol recovery was  $86 \pm 3 \%$  for the Sartorius filter membrane and  $96 \pm 3 \%$  for the Schleicher&Schuell filter membrane independent of the original concentration. These values were in good agreement with the plateaus of the drug release tests.

**Inserts containing influenza vaccine ( $V = 0.2$  ml).** The release test device was identical to the one described in the previous section (Figure 2. 2) using the filter membrane RC - fleece supported (Sartorius AG, Göttingen, Germany). Phosphate buffer pH 6.0 (USP XXVI) was used as release medium. Protein release was performed from solutions ( $V = 200 \mu\text{l}$ ) and inserts ( $V = 200 \mu\text{l}$ ), corresponding to a hemagglutinin dose of  $45 \mu\text{g}$ .

The protein content of the samples was analyzed using a commercial colorimetric protein assay (Micro BCA Protein Assay, Pierce, Rockford, Illinois, USA; sample volume 0.5 ml, incubation time 120 min) with UV detection ( $\lambda = 562$  nm; UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). The actual drug loading of the inserts was determined by complete dissolution of inserts in phosphate buffer pH 6.0 (USP XXVI) followed by the Micro BCA Protein Assay. With each assay, a standard curve was run which was prepared by dilution of the original vaccine solution ( $250 \mu\text{g}$  hemagglutinin / ml) and was based on the hemagglutinin and not the total protein content. All measurements were performed in triplicate ( $n = 3$ ). Placebo inserts were also subject to the drug release test to quantify the contribution of the polymers to the UV absorption. At each time point the placebo value was subtracted from the value of the drug loaded inserts.

The absorption of protein to the release device was determined by incubating a solution of the vaccine in release buffer (concentration  $45 \mu\text{g}$  hemagglutinin / vial) in the complete assembly under drug release test conditions and measuring the protein concentration after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h by the Micro BCA Protein Assay ( $n = 3$ ). The protein recovery decreased with time and reached a constant value of 69.2% after 2 h.

At the end of each experiment a mass balance study was performed. The residual amount of protein above the filter membrane was determined by dissolving the remaining gel in 5 ml of release medium and analysis using the Micro BCA Protein Assay. The sum of the adsorbed, released and remaining protein was calculated and expressed as percent of the original protein loading of the test sample ( $45 \mu\text{g}$  hemagglutinin).

### 2.2.11. Release of M $\beta$ CD

The release of M $\beta$ CD from nasal inserts was performed in the release device for small inserts using the membrane RC-L 55 (Schleicher&Schuell GmbH, Dassel, Germany) (see section 2.2.10 and Figure 2. 2) and under the same conditions as for the drug release (n = 3). A colorimetric assay was developed for the quantification of M $\beta$ CD, which was based on the color depression of a phenolphthalein solution by inclusion of the dye into M $\beta$ CD at alkaline pH (Zarzycki and Lamparczyk, 1998). The sample (0.5 ml) was diluted 1:1 with a phenolphthalein solution (0.1 mg/ml) in borate buffer pH 10 (USP XXVI, 5 x more concentrated) giving a final pH of  $9.64 \pm 0.02$  (n = 10). The color intensity of this solution was analyzed by UV spectrophotometry (phenolphthalein:  $\lambda = 553.5$  nm; UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany).

The recovery of the assay for M $\beta$ CD was  $103.9 \pm 0.8$  % and  $104.5 \pm 6.2$  % for 0.8 and 1.6 mg M $\beta$ CD / ml, respectively (n = 6). M $\beta$ CD was not absorbed to the components of the release system (recovery of M $\beta$ CD after 6 h incubation under release test conditions was  $103.1 \pm 6.9$  % and  $101.1 \pm 5.5$  % for 0.25 and 1.9 mg M $\beta$ CD / ml, respectively, n=3). Carrageenan inserts placed in the release system did not affect the recovery of M $\beta$ CD from the release medium during sampling over 8h. The recovery with inserts was  $98.3 \pm 4.1$  % of the recovery without inserts (n = 3).

### 2.2.12. Flame absorption and flame emission spectroscopy

M $\beta$ CD solutions (0.1, 0.5, 1, 2, 5, and 10%, w/V) in distilled water were prepared. The Mg<sup>2+</sup> and Ca<sup>2+</sup> ion content of the solutions was determined by flame absorption spectroscopy in an acetylene / air flame (Mg<sup>2+</sup>:  $\lambda = 285.2$  nm, Ca<sup>2+</sup>:  $\lambda = 422.7$  nm; PO9100X, Philips Industrial & Electro-Acoustic Systems Division, Almelo, The Netherlands). The detection limits, determined as the mean of 10 measurements of the solvent (distilled water) plus three times the standard deviation, were 0.1 ppm and 0.5 ppm for Mg<sup>2+</sup> and Ca<sup>2+</sup>, respectively. The Na<sup>+</sup> and K<sup>+</sup> ion content of the solutions was determined by flame emission spectroscopy in an acetylene / air flame (Na<sup>+</sup>:  $\lambda = 589.0$  nm, K<sup>+</sup>:  $\lambda = 769.9$  nm; Pye Unicam SP9, Philips Industrial & Electro-acoustic Systems Division, Almelo, The Netherlands). For the K<sup>+</sup>-determination the M $\beta$ CD solutions had to be submitted to nitric decomposition (63% HNO<sub>3</sub>, 5 min boiling). The flame emission spectroscopy studies were performed together with B.H. Behrens (Institute for Chemistry: Inorganic and Analytical Chemistry, Free University Berlin, Germany).

### **2.2.13. Scanning electron microscopy (SEM)**

Inserts were cut with a razor blade to expose the inner structure, fixed on a sample holder with double-sided tape and coated under an argon atmosphere with gold to a thickness of 6.5 nm (SCD 040, Bal-Tec GmbH, Witten, Germany). The samples were then observed with a scanning electron microscope (S-4000, Hitachi High-Technologies Europe GmbH, Krefeld, Germany) using secondary electron imaging at 10 kV in order to examine the surface morphology and structure of the inserts. SEM studies were performed together with U. Gernert at the Zentraleinrichtung Elektronenmikroskopie (Technical University Berlin, Germany).

### **2.2.14. Differential scanning calorimetry (DSC)**

A computer-interfaced differential scanning calorimeter (DSC 821, Mettler Toledo AG, Gießen, Germany) was used to determine the crystalline drug content in films. The film samples were accurately weighed and heated from 20°C to 190°C at a rate of 10 K/min. The presence of an endothermic peak around the melting point of the pure drug was used as a marker of crystalline drug and the enthalpy of melting / sample mass was determined. A linear correlation between the melting enthalpy / sample mass and the drug content of the film was established and extrapolation to zero enthalpy / sample mass revealed the “solubility” of the drug (absence of crystalline drug) in the film matrix.

The crystalline oxymetazoline HCl content of polymer films could not be determined by this method because the drug does not melt but decomposes at 300 - 303°C.

### **2.2.15. Powder X-ray diffraction**

Powder X-ray diffraction was used to study the crystallinity of untreated polymer powders, inserts / powders prepared thereof by freeze-drying from aqueous solutions (2% w/w), as well as freeze-dried inserts and cast films containing various excipients and drugs. Diffraction patterns were measured using a Philips X-ray generator PW 1830 equipped with a copper cathode ( $\lambda = 1.5418 \text{ \AA}$ , 40 kV, 20 mA) coupled to a computer-interfaced Philips PW 1710 diffractometer control unit. The scattered radiation was measured with a vertical goniometer (Philips PW 1820) (Philips Industrial & Electro-Acoustic Systems Division, Almelo, The Netherlands).

### 2.2.16. $^{13}\text{C}$ Nuclear magnetic resonance spectroscopy ( $^{13}\text{C}$ CP/MAS NMR)

Solid state  $^{13}\text{C}$  CP/MAS NMR measurements were performed at the Institute of Chemistry: Physical and Theoretical Chemistry (Free University Berlin, Germany) together with Dr. G. Buntkowsky and T. Emmler. The spectra of powders were measured on a Varian Infinity Plus 600 equipped with Varian Chemagnetics DR T3 (HX) probes (Varian Deutschland GmbH, Darmstadt, Germany) with sample spinning rates of 20 kHz for carrageenan and M $\beta$ CD, and 10 kHz for the ground film of carrageenan and M $\beta$ CD at room temperature. Chemical shifts were referenced to the external standard glycine.

### 2.2.17. FT-Raman spectroscopy

Carrageenan and oxymetazoline HCl powders were analyzed in a capillary while the inserts (carrageenan 2% w/w loaded with oxymetazoline HCl 20% w/w based on polymer) were compressed for measurements. An FT-Raman-spectrometer with an integrated, software-controlled Nd:YAG-Laser ( $\lambda = 1064$  nm, RFS 100, Bruker Optik GmbH, Ettlingen, Germany) was employed for the recording of the Raman spectra. The theoretical spectrum, calculated by fractional addition of the spectra of the single substances, was compared to the measured insert spectrum. All measurements were performed together with Priv.-Doz. Dr. W.-D. Hunnius (Institute for Chemistry: Inorganic and Analytical Chemistry, Free University Berlin, Germany).

### 2.2.18. Mechanical properties

The hardness of inserts was measured by compressing inserts, which were obtained by freeze-drying the liquid polymer formulations in glass vials (25 mm diameter, 1 cm filling height), with a flat punch (5 mm diameter) at a speed of 3 mm/min to a fixed distance of 3 mm from the inserts surface and monitoring the compression force (Universal testing machine, Instron Model 4466, Instron Wolpert GmbH, Ludwigshafen, Germany) ( $n = 6$ ). The elasticity was determined as the energy ratio of two consecutive compressions separated by a 5 min time interval (elasticity, % =  $100 * W_2 / W_1$ , whereby  $W_1$  and  $W_2$  are the energies required for the first and the second compression, determined as areas under the force-distance curves obtained by the trapezoidal method).

### 2.2.19. Hemagglutination test

Inserts were reconstituted in the original volume (200  $\mu$ l) of water. The solutions or reconstituted inserts were then diluted 1:10 with phosphate buffered saline (PBS) and the protein content was determined prior to the hemagglutination test using a commercial colorimetric protein assay (Micro BCA Protein Assay, Pierce, Rockford, Illinois, USA; sample volume 0.5 ml, incubation time 120 min) with UV detection ( $\lambda = 562$  nm; UV-2101 PC, Shimadzu Deutschland GmbH, Duisburg, Germany). By further dilution the protein content was adjusted to 0.5  $\mu$ g / 25  $\mu$ l.

25  $\mu$ l of these solutions were given into the first well of a 96-well-plate (V-bottom, Greiner Bio-One BioScience Division, Frickenhausen, Germany) and a series of 1+1 dilutions of each sample was prepared in PBS. Then 25  $\mu$ l of an erythrocyte suspension (1% V/V in PBS, from specific pathogen free (SPF) chicken supplied by the Institute for Poultry Diseases, Department of Veterinary Medicine, Free University Berlin, Germany) and another 25  $\mu$ l of PBS were added to each well. After 40 min of incubation at ambient conditions the agglutination of the erythrocytes was evaluated visually. The hemagglutination (HA) titer was defined as the reciprocal value of the highest dilution (given as the  $\log_2$ ) of the sample which still resulted in agglutination of the erythrocytes, visually detected by the reduced flow of the blood cells (n = 3). On each plate a negative (buffer) and a positive control (vaccine) was run with the sample.

### 2.2.20. In vivo studies

**Estradiol study in rats.** In vivo studies with estradiol were performed at the Centre Européen d'Etudes du Diabète, Université Louis Pasteur, Strasbourg, France) under the supervision of Dr. C. Damgé.

*Animals and experimental procedure.* Male Wistar rats (bodyweight  $330 \pm 21$  g) were anesthetized intraperitoneally with a mixture of xylazin (Rompun; Bayer Animal Health, Monheim, Germany) and ketamine (Imalgene 1000; Merial, Lyon, France). The left jugular vein was catheterized with a polyethylene cannula (Biotrol Diagnostic, Chennevieres les Louvre, France). The cannula was flushed with 0.2 ml Heparin 1000 IU/ml (Heparin Choay 25,000, Sanofi Winthrop, Gentilly, France; diluted in normal saline) to avoid clotting. Formulations containing 40  $\mu$ g estradiol were administered into the right nostril of anesthetized rats being in the supine position (n = 6). Inserts (V = 20  $\mu$ l, d = 1mm) were

inserted into the nostril with thin tweezers until they were out of sight. Solutions and dispersions ( $V = 20 \mu\text{l}$ ) were given with an Eppendorf pipette. Microparticles (approximately 1 mg) were administered by inserting a soft, microparticle filled tube attached to an air filled 5-ml-syringe. The microparticles were expelled from the device into the nose by rapid compression of the syringe. During the microparticle application, the airways of the rats were kept free to avoid pressure damage to the lung. The tubes were weighed after the application to determine the applied microparticle mass.

The animals were kept in the supine position and under a heating lamp to maintain the body temperature. Anesthesia was maintained throughout the experiment by repeated injections of ketamine. Blood samples of 0.2 ml were taken after 0.5, 1, 2, 3, 4, and 6 h and serum was prepared by centrifugation (2400 g,  $4^{\circ}\text{C}$ , 15 min; Heraeus Biofuge 22R, Kendro Laboratory Products GmbH, Hanau, Germany). Samples were frozen at  $-70^{\circ}\text{C}$  until analysis. After the experiment the rats were sacrificed and the nasal cavity was opened to observe any remainder of the dosage form.

*Estradiol-specific radioimmunoassay.* The estradiol concentration in the serum samples was determined using a commercial radioimmunoassay (ultra-sensitive estradiol RIA DSL-4800, Diagnostic Systems Laboratories Deutschland GmbH, Sinsheim, Germany). The highest coefficient of variance of the control values (20 and 250 pg/ml) between 5 assays was 9.4%, while the highest coefficient of variance within one assay was 4.3%. The control values were within the ranges recommended by the supplier. Radioimmunoassays were performed under supervision of Dr. V. Bähr (Department of Endocrinology, Diabetes and Nutritional Medicine, Free University Berlin, Germany) at the Clinic and Policlinic for Radiology and Nuclear Medicine of the University Hospital Benjamin Franklin.

**Vaccine studies in mice and rats.** All in vivo vaccine studies were performed in collaboration with and by Dr. J. Haensler, M.-C. Bernard and T.-M. Jourdier (Aventis Pasteur, Marcy l'Etoile, France). All tested formulations were prepared at the Freie Universität Berlin and shipped in cooled containers to Aventis Pasteur for the studies. Additionally to the influenza vaccine (hemagglutinin  $4.5 \mu\text{g} / 20 \mu\text{l}$ ), the liquid formulations contained polymers 0.5% (w/V) with / without absorption enhancers 0.5% (w/V). These concentrations corresponded to  $100 \mu\text{g}$  of each in the application volume of  $20 \mu\text{l}$ . From these solutions nasal inserts were prepared by freeze-drying  $20 \mu\text{l}$  - aliquots. The controls (vaccine solution, vaccine solution with cationic lipid) were provided by Aventis.



*Animals.* Outbred OF1 female mice (Iffa-Credo, St Germain-sur-l'Arbresle, France) and female Sprague-Dawley rats (Iffa-Credo, St Germain-sur-l'Arbresle, France) were housed in an accredited facility and cared for under the guidelines of Aventis Pasteur-France local Ethics Committee.

*Immunization schedule and samplings.* Groups of six OF1 mice were immunized twice at a three weeks interval by the intranasal route without anesthesia with 20  $\mu$ l of the liquid formulation containing a defined amount of the monovalent influenza vaccine NIB16 (A/H1N1) corresponding to 4.5  $\mu$ g of hemagglutinin. Animals were sacrificed two weeks after the last immunization and sampled for blood and nasal secretions for antibody evaluation.

Groups of five rats were immunized twice at a three weeks interval by introduction of a nasal insert ( $V = 20 \mu$ l) into one nostril under anesthesia. The insert were pushed into the nostril until they were out of sight, using thin slightly bent forceps. Each insert containing a defined amount of the monovalent influenza vaccine NIB16 (A/H1N1) corresponding to 4.5  $\mu$ g of hemagglutinin. Two control groups received intranasally 50  $\mu$ l per nostril (total 100  $\mu$ l) of liquid influenza vaccine alone (4.5  $\mu$ g hemagglutinin) or vaccine mixed with DC-cholesterol (4.5  $\mu$ g hemagglutinin + 200  $\mu$ g DC-cholesterol). Animals were sacrificed two weeks after the last immunization and blood sampled for antibody evaluation.

*Antigen-specific ELISA assays.* Sera from immunized animals were analyzed by ELISA for the presence of NIB16-specific IgG. In addition and only in the mouse study, NIB16-specific IgA and total IgA ELISA antibody responses were measured in nasal wash samples. Briefly, 96-well flat-bottom plastic plates (Dynex Technologies Inc., Chantilly, VA, USA) were coated overnight at 4°C with the influenza monovalent NIB16 antigen (reassortant of the two A/H1N1 strains: A/Tawain/1/86 and X31; produced by propagation on chicken eggs, purified, split then inactivated by formaldehyde at Aventis Pasteur, Marcy l'Etoile, France) at 100 ng / 100  $\mu$ l (total protein) per well in 0.05 M carbonate buffer pH 9.6. Plates were then blocked for 1 h with 150  $\mu$ l of phosphate buffered saline (PBS) pH 7.1 containing 0.05 % Tween 20 and 1% (mouse ELISA) or 2% (rat ELISA) (w/V) powdered skim milk (DIFCO™, Becton Dickinson Diagnostic Systems, Sparks, MD, USA).

All further incubations were carried out in the final volume of 100  $\mu$ l, followed each time by four washings with PBS pH 7.1 containing 0.05 % Tween 20. Serial dilutions of the sera in blocking buffer were added to the plates and incubated for 90 min at 37°C. The plates were then washed with PBS pH 7.1 containing 0.05% Tween 20. Peroxidase conjugates diluted in

blocking buffer were added and incubated for 90 min at 37°C. The conjugates used were specific for either mouse IgG (diluted at 1/5,000, Ref: A3673) or mouse IgA (diluted at 1/500, Ref: A4789) (both Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or rat IgG (diluted at 1/20,000, Ref: 112-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The plates were further washed and incubated in the dark for 20 min at room temperature with 3,3',5,5'-tetramethyl benzidine (TMB). The reactions were stopped with 100 µl of HCl 1N.

The optical density (OD) was measured at 490 - 650 nm with an automatic plate reader (VERSAmax, Molecular Devices France, St. Grégoir, France). The mean value of the blanks (the signals obtained in wells incubated with all reagents except serum or secretion) was subtracted from the data. The NIB16-specific IgG antibody titers were calculated from the regression curve of a reference specific sera originating from the same animal species as the samples. A purified mouse IgA (Ref: 02-6500, ZYMED Laboratories Inc., San Francisco, CA, USA), present on each ELISA plate, was used as standard for NIB16-specific IgA. The quantity of total mouse IgA in mouse nasal wash secretions was quantified with the known protein concentration of the purified mouse IgA defined by the supplier.

IgG seric response results are given in arbitrary units (UA\*) (log10). IgA mucosal responses were expressed as normalized specific activity (NSA) with  $NSA = [\text{anti-NIB 16 IgA titers (UA*)} / \text{total IgA titers (ng/ml)}] \times 1000$ .