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Research paper

The influence of gellan gum on the transfer of fluorescein dextran across rat nasal epithelium in vivo

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Abstract

The nasal uptake of a 3000 Da fluorescein dextran (FD3) was investigated in rats, using fluorescence microscopy. The uptake from a formulation containing deacetylated gellan gum, an in situ gelling agent, was compared to that from a mannitol solution. Additionally, the rheological behavior of the gellan gum in water and saline was studied. It was shown that the gellan gum solution was easily administered owing to its low viscosity, and upon contact with the mucosa, a gel was formed. The epithelial uptake and transfer of FD3 appeared to be increased and prolonged using the gellan gum formulation. This increase was not accompanied by qualitative changes of the epithelial FD3 distribution or any visible harmful effects.

Keywords: Intranasal administration; In situ gel; Fluorescein dextran; Deacetylated gellan gum; Nasal mucosa; Olfactory mucosa; Fluorescence microscopy

1. Introduction

Nasal administration is considered to be a convenient route of administration when oral administration of a drug gives an undesirably slow effect, or when a drug is highly metabolized or incompletely absorbed in the gastrointestinal tract. Nasal sprays may be preferred to injections because of higher patient compliance and may have applications in, e.g. pain management [1] and vaccinations [2]. Moreover, several animal studies show that a large number of substances are transferred directly to the central nervous system (CNS) after nasal administration, bypassing the blood–brain barrier (BBB) [3–5]. Some studies indicate that this transfer along the olfactory nerves also occurs in man [6,7]. A field where the olfactory pathway may become important is the delivery of peptides (molecular weights around 1000 Da) intended for neuroprotection, as discussed by Gozes [8]. The olfactory pathway, and other aspects of nasal drug delivery, has been discussed by Illum [9].

In the present study, we investigate the nasal uptake of a 3000 Da fluorescein dextran (FD3) from a gellan gum formulation using a previously reported method [10]. Salmon calcitonin is an example of a substance in the same molecular weight range, currently marketed for intranasal administration.

Gellan gum is a linear, anionic polysaccharide that is secreted by the microbe *Sphingomonas paucimobilis* (formerly known as *Pseudomonas elodea*). Marketed as Gelrite® or Kelcogel®, deacetylated gellan gum is approved in the USA and EU as a gelling, stabilizing and suspending agent in food products [11]. Because of its ability to form strong clear gels at physiological ion concentration, deacetylated gellan gum has been widely investigated for use as an in situ gelling agent in ocular formulations. It has been reported to provide a significantly prolonged corneal contact time in comparison with conventional solutions [12–14] and is currently marketed in the controlled-release timolol formulation Blocadren® Depot (Timoptic-XE®). It has also been suggested that gellan gum is a promising polymer for use in nasal formulations [15]. To our knowledge, however, it has only been included in one

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study [16] on this subject where it was shown to moderately enhance the local and serum antibody response in mice after nasal administration of viral antigens. Other in situ gelling systems, such as temperature and pH responsive gels, have, on the other hand, appeared more frequently in nasal drug delivery studies and have been shown to increase the residence time and improve drug absorption, see, e.g. Zhou and Donovan [17], Aikawa et al. [18] and Park et al. [19].

Enhanced bioavailability has been reported after administration of viscous polymer solutions in some studies (e.g. [20,21]), but it has not been clearly established whether this is caused by a prolonged residence time in the nasal cavity or an effect on the mucosa. Pennington et al. [22] reported clearance half-times of 1 and 2.2 h for 0.6% and 1.25% HPMC, respectively, although the difference was not statistically significant. The dependence of bioavailability and residence time on viscosity may be caused by the larger droplet size arising from the higher viscosity of the formulation rather than the higher viscosity itself [23]. The nasal spray pumps used today give deposition primarily in the anterior nasal cavity, and it was reported that this pattern was further amplified with increasing viscosity [23,24]. The advantage of using an in situ gelling formulation is that, owing to its low viscosity, it can be readily administered. After gelling is induced by some physiological stimulus at the absorption site, the formulation attains semisolid properties.

The in situ gelling properties of deacetylated gellan gum are attributed to its responsiveness to cations. In an ion-free aqueous medium, the polymer chains form double helices, resulting in a fluid that has a viscosity close to that of water. In the presence of gel-promoting cations (Na^+, K^+, Ca^{2+}), a portion of the helices associates and the cation-mediated aggregates cross-link the gel network [25]. A rapid gelling can be expected upon contact with the mucosa since, even at low polymer concentrations, small quantities of ions suffice for the formation of a strong gel [26].

The purpose of the present study was to investigate whether the use of a gellan gum formulation would change the extent or the time profile of the uptake and transfer of FD3 across rat nasal epithelia in vivo in comparison to a plain isotonic water solution. In addition, the distribution of fluorescence and histological changes in the mucosa were to be evaluated.

2. Materials and methods

2.1. Materials

The deacetylated gellan gum (Kelcogel F®) was kindly given by the Kelco division of the Monsanto Company (USA). Stock solutions of gellan gum (0.5% w/w) were prepared by dispersing the polymer powder in ultra-pure water and then stirring the dispersion in a sealed vial for 20 min at 100 °C by using a water bath. After cooling to room temperature, 4% (w/w) of D-mannitol (Sigma Chemical Co., USA) was added to achieve isotonicity. The resultant stock solution was used within 2 days of preparation. For the rheological analysis a gellan preparation was made with both 4% mannitol and 0.9% NaCl, using the same heating and stirring procedure as for the stock solution. On the same day as the animal experiment, 0.5% (w/v) aldehyde fixable fluorescein dextran (FD3, Molecular Probes Inc., USA) was dissolved in isotonic mannitol or gellan stock solution and protected from light.

The rats were anesthetized using a 1:1:2 mixture of Hypnorm® (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml; Janssen Animal Health, Belgium), midazolam 5 mg/ml and water. Bouin's fluid (saturated picric acid, 40% formaldehyde and concentrated acetic acid; 15:5:1) was used for the perfusion and the immersion fixation. The stock solution of propidium iodide (1 mg/ml in water, Molecular Probes Inc., USA) was diluted to 5 μg/ml in phosphate buffered saline prior to use.

2.2. Rheological measurements

The rheological measurements on the gellan stock solution and the gellan preparation made in 0.9% NaCl were carried out at 37 °C using a Bohlin VOR rheometer (Bohlin Reologi, Lund, Sweden). The measuring systems used were a double gap cylinder (DG 24/27) and a concentric cylinder (C14). Silicone oil was added to the surface of the sample to prevent evaporation during measurements. The gellan stock solution was directly equilibrated at 37 °C, whereas the hot, freshly prepared gellan sample in 0.9% NaCl was poured into the measuring system at 90 °C and subjected to controlled cooling (0.5 °C/min) from 90 to 37 °C [26]. Having equilibrated at 37 °C, a strain sweep measurement was performed at a constant frequency of 1 Hz to determine the maximum strain amplitude. Then a frequency sweep (0.01–5 Hz) was performed at a selected strain amplitude chosen to be within the linear region of the sample. The rheological behavior of the samples was evaluated in terms of the elastic (storage) modulus (G') and the viscous (loss) modulus (G'') obtained in the frequency sweep. Furthermore, the viscosity of the gellan stock solution was determined using the rheometer in the rotational viscometry mode.

2.3. Animal experiments

A more detailed description of the procedures is provided in our previous study [10]. The rats (male Sprague–Dawley, B&K Universal, Sweden) were housed with a normal 12 h light–dark cycle for 1 week prior to the experiment and weighed 241–308 g at the time of the experiment. They were fed a standard pellet diet and tap water ad libitum and were anesthetized with intraperitoneal hypnorm-midazolam (0.27 ml/100 g body weight).

Fifty microliters of the formulations was administered to the right nostril using a polyethylene tube (PE 90), attached to a micropipette. The tube was inserted approximately 5 mm into the nostril. During and after the administration of FD3, the rats were placed on their backs on a heated plate to maintain body temperature. The rats were perfusion fixed via the heart 15, 60, 120 and 240 min after administration. Two to three rats were used for each time point and formulation. The nasal cavity, including the olfactory bulbs, was isolated and immersed in Bouin's fluid for a maximum of 24 h.

The study complied with approval C6/0, issued by the animal research ethical committee in Uppsala.

2.4. Fluorescence microscopy

After decalcification, the nasal cavity was cut in slices approximately 2–3 mm thick, which were assigned to the levels I–V, as described in Fig. 1. The slices were dehydrated in ethanol, then infiltrated with equal amounts of 99.5% ethanol and glycol methacrylate (GMA) infiltration solution (Technovit 7100, Heraeus Kulzer GmbH and Co., Germany) for 12–24 h, followed by 24 h with pure infiltration solution. The samples were then embedded in GMA according to the manufacturer's instructions.

After sectioning, transfer of the sections to microscope slides, drying and counterstaining with propidium iodide, the sections were investigated in a fluorescence microscope. A number of regions of the nasal cavity were defined: *upper septum* and *lower septum*, *nasoturbinate* and *maxilloturbinate* (only present in the anterior nasal cavity) and *endoturbinates* and *ectoturbinates* (only present in the posterior nasal cavity). The epithelium and lamina propria in every region were evaluated in terms of the occurrence of FD3 fluorescent cells and were given scores in the range 0–5, where 1 indicates fluorescence in sporadic cells and 5 in all cells. Each region was given two scores, one maximum and one minimum. Individual scores were given to columnar cells and goblet cells in the respiratory epithelium, and to olfactory cells and supporting cells in the olfactory epithelium. Mean values were calculated from the fluorescence scores, including all regions and cell types mentioned above. Thus, a *maximum score average* and a *minimum score average* represents each level of the nasal cavity.

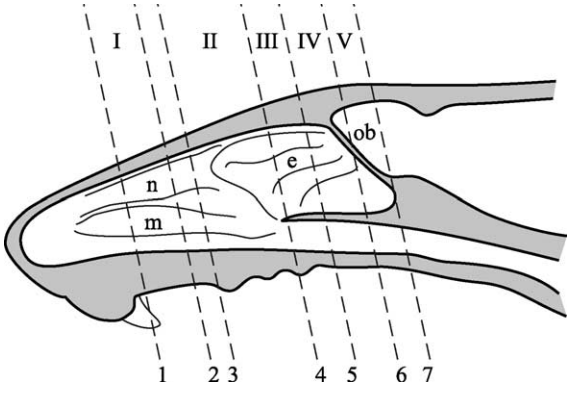

Fig. 1. Cuts were made perpendicular to the hard palate and to the plane of the nasal septum as follows: (1) immediately posterior to the upper incisor teeth. (2) Approximately 4 mm posterior to cut 1. (3) Through the incisive papilla. (4) Through the second palatal ridge. (5) Through the middle of the first upper molar teeth. (6) Through the middle of the third upper molar teeth. (7) At the end of the hard palate. The symbols I–V denote the various levels. Levels I, II and IV were further divided as follows: (1b) approximately 2 mm posterior to cut 1. (3b) At the first palatal ridge. (5b) In the middle of the second upper molar teeth. *e* endo- and ectoturbinates, *m* maxilloturbinate, *n* nasoturbinate, *ob* olfactory bulb.

A confocal laser scanning microscope (Leica TCS 4D, Leica Microsystems, Germany) was used for the imaging. All images were produced with identical settings of the confocal microscope, imaging software and printer.

3. Results

3.1. Rheological properties of the gellan gum formulation

The results from the viscoelastic measurements are shown in Fig. 2. The gellan stock solution exhibited the properties of a polymer solution, i.e. the G' and G'' were relatively low and frequency dependent. However, the sample that was prepared in 0.9% NaCl to simulate the gelling in physiological conditions, was found to have a frequency-independent G' that was considerably higher than G'' over a large frequency range, i.e. it had the rheological behavior of a strong cross-linked gel [27,28].

In the viscosity measurements, the gellan stock solution exhibited Newtonian behavior with a viscosity of 7.5 mPa s. The solution could be readily administered to the nasal cavity of the rats through the polyethylene tube.

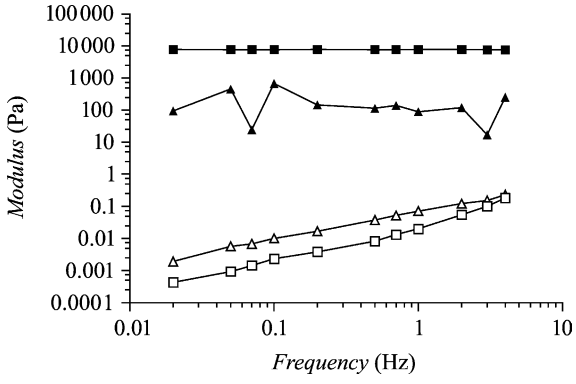

Fig. 2. The frequency dependence of the elastic modulus, G' (squares), and the viscous modulus, G'' (triangles), for the 0.5% gellan gum stock solution (open symbols) and for the gellan gum preparation with 0.9% NaCl (filled symbols), simulating the in vivo gelation. The behavior of the stock solution is typical of that for a polymer solution, while that of the saline preparation corresponds to the rheological definition of a gel.

3.2. Fluorescence microscopy

No general adverse effects, related to the intranasal administration of the mannitol solution or the gellan gum formulation (such as bleeding from the nose or respiratory dysfunctions) were observed in the rats. Furthermore, there were no obvious histological changes such as swelling, cilia loss or loss of epithelial cells.

The FD3 gellan formulation was mainly located in the anterior nasal cavity at all times studied. When investigating the different levels of the nasal cavity, gel was observed over large areas of levels I and II in all animals. More posterior, at level III, the gel was frequently observed in discrete regions whilst at level IV the gel was only occasionally observed.

Figs. 3 and 4 illustrate the FD3 uptake at different times after administration of the two formulations. The diagrams show the minimum and maximum score averages in the epithelium and the lamina propria at level I and level IV (2–3 rats/condition, four regions/rat, all cell types). The sections were collected approximately half-way through the levels. At level I (Fig. 3), both the minimum and maximum score averages were higher after administration of the FD3 gellan formulation than after administration of the FD3 mannitol solution. There was a clear tendency for the extent of fluorescence to decrease with time after the administration of the FD3 mannitol solution, a trend that was less pronounced after administration of the gellan gum formulation.

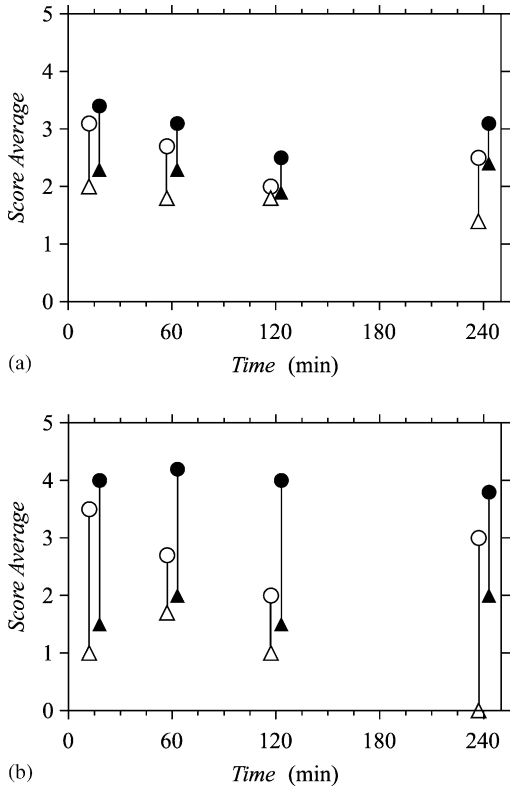

Fig. 3. Minimum (triangles) and maximum (circles) score averages at level I, representing the uptake of FD3. Filled symbols indicate estimated fluorescence after administration of the FD3 gellan formulation and open symbols fluorescence after administration of the FD3 mannitol solution. (a) Respiratory epithelium. (b) Lamina propria.

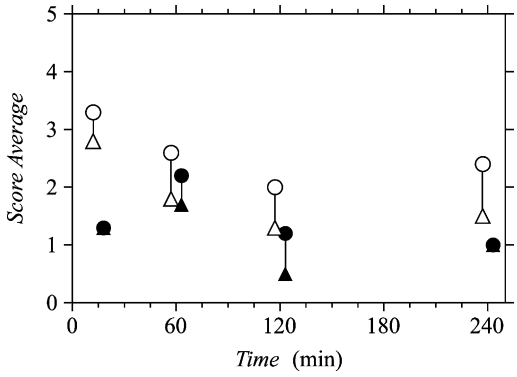

Fig. 4. Minimum (triangles) and maximum (circles) score averages at level IV, representing the uptake of FD3. Filled symbols indicate estimated fluorescence after administration of the FD3 gellan formulation and open symbols fluorescence after administration of the FD3 mannitol solution. The graph shows the values for both the epithelium and lamina propria, since they were closely correlated.

For level IV (Fig. 4), the scoring of the lamina propria was closely correlated to the scoring of the epithelium; therefore this diagram can be considered to be representative of both the epithelium and the lamina propria. Here, the minimum and maximum score averages were higher for the mannitol solution. For levels II and III, the difference between the score averages for the two formulations was less pronounced (data not shown).

Since the localization of the gel seemed to influence the uptake, two regions in which there was a high probability of deposition of both formulations were identified, one comprising respiratory and the other olfactory epithelium. The defined respiratory region was the middle part of the septum at level I (Fig. 5a), and the olfactory region was the superior, medial aspect of the ectoturbinate $1'$ at level III (Fig. 6a).

In the defined respiratory region, the extent of FD3 fluorescence was approximately equal for the two formulations 15 min after the administration. The degree of FD3 fluorescence 60, 120 and 240 min after administration of the gellan gum formulation exceeded that observed after administration of the mannitol solution. Fig. 5 shows representative micrographs from this region in animals euthanized 120 min after administration of the two formulations being investigated.

In the defined olfactory region at level III, gel was visible in the lumen of the nasal cavity in 5 out of the 8 images studied from rats having obtained the FD3 gellan formulation. In all of these cases, the degree of FD3 fluorescence was higher than in the corresponding images from rats having obtained the FD3 mannitol solution. This is exemplified by micrographs (Fig. 6) from rats euthanized 120 min after administration of the formulations.

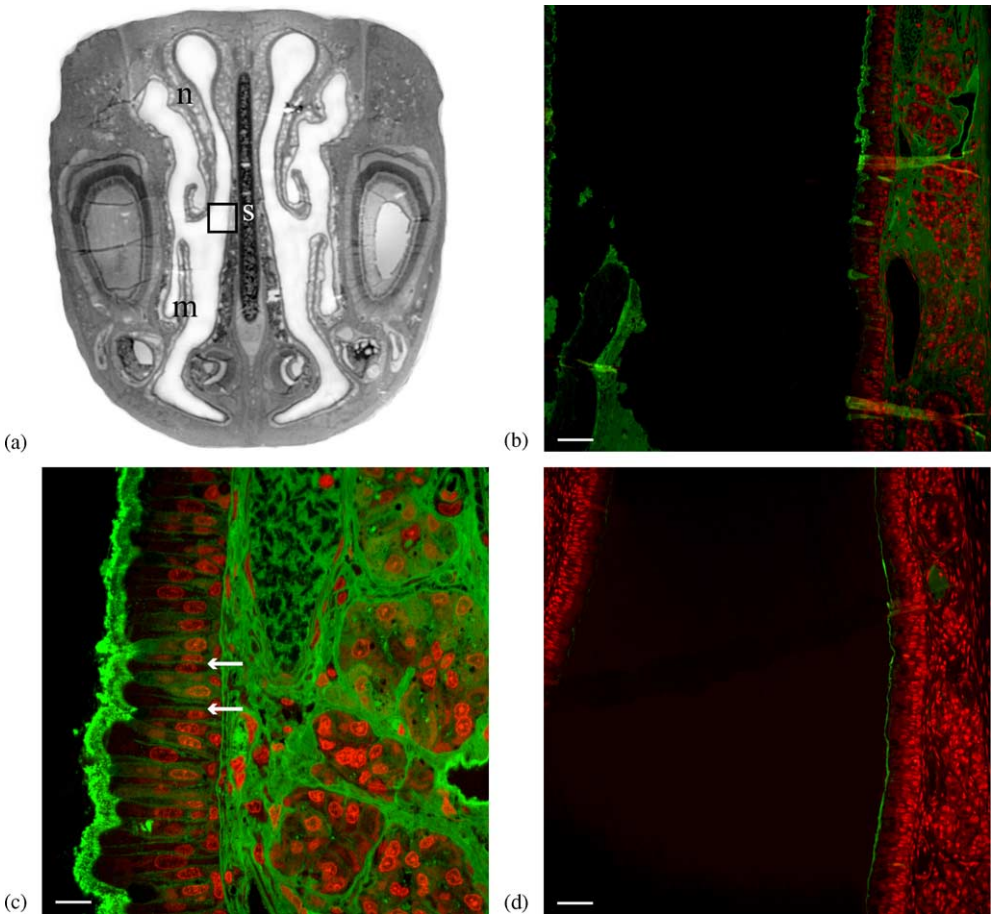

Fig. 5. Representative fluorescence micrographs for the respiratory region defined in (a) below, from rats euthanized 120 min after nasal administration of the FD3 formulations. The cell nuclei are red (propidium iodide). (a) At level I, the septum at the level of the inferior part of the nasoturbinate (square) was defined as a region with a high probability of deposition of both the mannitol solution and the gellan gum formulation. *s* septum, *n* nasoturbinate, *m* maxilloturbinate. (b) FD3 gellan formulation. The gel can be observed to the lower left. Scale bar 50 μm. (c) Higher magnification of (b). Scale bar 10 μm. (d) FD3 mannitol solution. Scale bar 50 μm. There was a higher localization of FD3 fluorescence in the epithelium and lamina propria after administration of the FD3 gellan formulation than after administration of the FD3 mannitol solution. There was intracellular as well as some paracellular (arrows) FD3 fluorescence.

In the respiratory epithelium, both intracellular and paracellular FD3 fluorescence was observed, whereas in the olfactory epithelium, only intracellular FD3 fluorescence could be discerned. At the longer exposure times (120 and 240 min), some of the intracellular FD3 fluorescence in the olfactory epithelium had a point-shaped distribution. No other differences, such as variations in FD3 fluorescent cell types, were found.

FD3 fluorescence in the olfactory bulb was also present, where the degree of fluorescence was closely correlated to that observed in the adjacent olfactory mucosa. There did not seem to be any other, more specific, effect of the gellan gum on the uptake to the olfactory bulb.

4. Discussion

To our knowledge, deacetylated gellan gum has not been used in nasal formulations in clinical studies, and only once in animal studies [16]. The utilized concentration of the deacetylated gellan gum, 0.5%, was selected on the basis of earlier studies [26], showing that the preparation behaves like a fluid but forms a rigid gel when exposed to cations. The low viscosity enabled administration to be made through the thin tube utilized in the present study, and should be suitable for use in nasal spray pumps as well.

The formulation seemed to gel rapidly upon contact with the mucosa, as seen by the fact that there was less of the gellan gum formulation localized in the posterior parts of the nasal cavity. Because of the small quantities of ions that are required for gel formation [26], rapid gelation can be expected. In humans, the surface area of the nasal cavity is more than 10 times larger than in rats, while the volume of the formulation would only be 2–3 times larger. Therefore, the gelling is likely to occur rapidly in humans as well. Less spreading of the dose in the nasal cavity would be expected than after administration of a plain solution because of the rapid gelling. The demands made on the deposition from the spray pump would increase as a consequence. When targeting drugs to the olfactory epithelium in humans, the design of the spray pump will be very important. The spray pumps used today mostly deposit the spray in the anterior

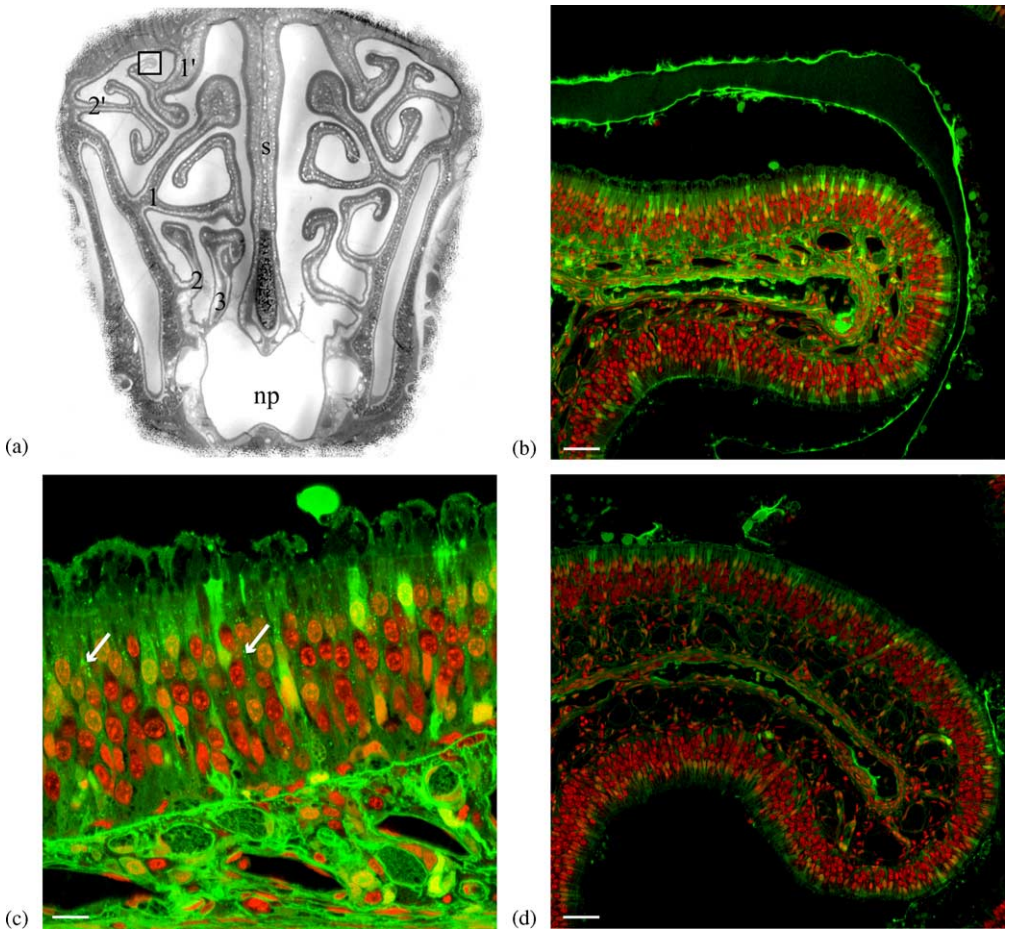

Fig. 6. Representative fluorescence micrographs for the olfactory region defined in (a) below, from rats euthanized 120 min after nasal administration of the FD3 formulations. The cell nuclei are red (propidium iodide). (a) At level III, the defined region was the superior, medial part of ectoturbinate 1′ (square). *s* septum 1′, 2′ ectoturbinates, 1, 2, 3 endoturbinates, *np* nasopharynx. (b) FD3 gellan formulation. The gel can be observed overlying the mucosa throughout the image. Scale bar 50 μm. (c) Higher magnification of (b). Scale bar 10 μm. (d) FD3 mannitol solution. Scale bar 50 μm. There was a higher localization of FD3 fluorescence in the epithelium and lamina propria after administration of the FD3 gellan formulation than after administration than the FD3 mannitol solution. Some point-shaped intracellular FD3 fluorescence (arrows) could be seen at 120 min. No paracellular FD3 fluorescence was evident in the olfactory epithelium.

portion of the nasal cavity, and do not reach the olfactory epithelium at all.

Although the strongest gellan gum gels are obtained with divalent cations such as Ca^{2+} [29,30], the most prevalent cation, Na^{+}, has been shown to be more important in in vivo conditions [26]. In the present study, in vitro experiments demonstrated that a strong gel is formed in 0.9% NaCl and it is evident from the micrographs that the gel formed in vivo was strong enough to remain in the nasal cavity for the whole time interval studied.

Both the semiquantitative score averages and the micrographs clearly illustrate that there was a more extensive localization of FD3 fluorescence in the anterior nasal cavity after administration of the gellan gum formulation than after administration of the mannitol solution. This was true for both the respiratory epithelium and the underlying lamina propria. The reason for the higher degree of fluorescence after administration of the gellan gum formulation was not investigated, but it is most likely related to high local concentrations caused by slower clearance of the gel from the nasal cavity. This is confirmed by the observation that only the degree of fluorescence was different, whereas the distribution of the fluorescence in the epithelial cells was the same, irrespective of whether the gellan gum formulation or the mannitol solution had been administered.

The lower score averages for the gellan gum formulation in the posterior nasal cavity was attributable to lower deposition in these areas. The micrographs clearly show that where the gel indeed was localized, the degree of FD3 fluorescence in the olfactory epithelium and lamina propria was high.

The distribution of FD3 in the rat olfactory epithelium was previously [10] shown to be mainly intracellular, both in olfactory and supporting cells. Since the FD3 fluorescence was distributed in the same manner in the present study, these mechanisms will not be discussed here to any great extent. However, as can be seen in Fig. 6, there was

some point-shaped FD3 fluorescence present in the olfactory epithelium at the longer times studied. Since this was not observed at the shorter times, it is unlikely that it is related to the uptake, but rather to the elimination mechanisms.

In the respiratory epithelium, we observed some paracellular FD3 fluorescence along with the intracellular fluorescence. The relative contribution of these pathways could, however, not be determined. The transport routes in the respiratory nasal epithelium of rats have previously been studied using confocal laser scanning microscopy [31]. It was concluded that the FD3 follows a paracellular transport route, along with endocytotic uptake. In the present study, the intracellular FD3 fluorescence had a quite diffuse distribution, in contrast to the more point-shaped distribution reported by Marttin et al. [31].

There also seems to be a higher degree of FD3 fluorescence in the respiratory lamina propria in the present study than in our previous study [10], where saline was used as a vehicle. Different uptake characteristics arising from the use of different osmolarity agents have been reported before, but, in contrast to the results presented here, the trend is towards higher uptake when saline is used rather than mannitol or sorbitol [32,33].

The present study is, to our knowledge, the first report of modified absorption across the olfactory epithelium associated with the use of a gel or other consistency modifiers. There has been one report [34] of the use of a permeation enhancer and a hypotonic formulation to increase the brain uptake after nasal administration of a peptide to rats. This investigation was performed using a nasal perfusion at 2 mL/min during a period of 30 min. Since no morphological examinations of the nasal mucosa were performed, damage to the olfactory epithelium cannot be excluded. No tissue damage was observed in the present study.

In conclusion, this study shows that the gellan gum formulation had a residence time of at least 4 h in the rat nasal cavity without any visible harmful effects. The epithelial uptake of the model substance, a 3000 Da fluorescein dextran, and its transfer across the epithelium appeared to be increased using the gellan gum formulation in comparison to a mannitol solution. This increase was not accompanied by any qualitative changes of the epithelial FD3 distribution.

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