Department of Pharmaceutics[1], School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom Campus, Potchefstroom, Lung Function Unit[2], Pretoria Academic Hospital, School of Pharmacy[3], Tshwane University of Technology, Pretoria, South Africa

Low molecular weight quaternised chitosan (II): *In vitro* assessment of absorption enhancing properties

C. Jonker-Venter[1], D. Snyman[1], C. Janse van Rensburg[1], E. Jordaan[1], C. Schultz[2], J. H. Steenekamp[1], J. H. Hamman[3], A. F. Kotzé[1]

Received May 5, 2005, accepted May 16, 2005

J. H. Hamman, PhD, School of Pharmacy, Tshwane University of Technology, Private Bag X680, Pretoria 001, South Africa
hammanjh@tut.ac.za

Pharmazie 61: 301–305 (2006)

N-Trimethyl chitosan chloride (TMC; high molecular weight) and *N*-trimethyl chitosan oligosaccharide (TMO; low molecular weight) with different degrees of quaternisation were synthesised and evaluated for their absorption enhancing properties across mucosal epithelia. These quaternised chitosan derivatives (0.0625% w/v–0.5% w/v) showed a significant decrease in the transepithelial electrical resistance (TEER) of cultured rabbit tracheal epithelial cell monolayers as compared to the control. The degree of quaternisation and concentration of the compounds influenced the extent of the reduction in TEER. Higher degrees of quaternisation and an increase in the concentration of the compound were associated with a more pronounced reduction in the TEER. The TMO derivatives seemed to be more effective in lowering the TEER of tracheal cell monolayers as compared to the TMC polymers. Ciliary beat frequency (CBF) is the main defence mechanism of the respiratory tract and is therefore a useful parameter in evaluating the toxicity of nasally administered drugs and additives. The effect of the synthesised chitosan derivatives on the CBF of human nasal epithelial cells at pH 7.4 was determined by a method based on an analogue contrast enhancement technique. The TMO oligomers exhibited lower inhibition of the CBF of human nasal epithelial cells compared to that of the TMC polymers. It was proposed that this reduced effect on the CBF is due to the lower viscosity and molecular weight of TMO. However, no acute toxicity was found with any of the synthesised chitosan derivatives by means of the CBF tests conducted in this study.

1. Introduction

In recent years, various new therapeutics such as peptide and protein drugs have been produced due to tremendous progress in drug design and biotechnology, which include recombinant DNA and hybridoma technologies (Torchilin and Lukyanov 2003). These therapeutic agents are mostly indicated for treatment of chronic conditions and require the parenteral route of administration due to their poor permeation across mucosal surfaces. However, suitable alternative delivery systems have to be developed to utilise the full benefit of these technologically advanced drugs. Indeed, the emphasis in the development of therapeutic protein and peptide products has shifted towards the demand for improved delivery systems (Cleland et al. 2001) in order to produce optimal therapeutic responses, controlled and site-specific release of the active ingredients, cost-effectiveness and patient compliance (Kotzé et al. 1999a).

Although the oral route is regarded as the most convenient and acceptable route of drug administration (Fasano 1998), most peptide drugs cannot be administered perorally because apart from their physical and chemical instabilities such as their susceptibility to enzymatic degradation, poor absorption at the site of administration is a major limiting factor (Lee 1991). The nasal route is an attractive alternative for the delivery of a wide variety of drugs because of the rich vascularity of the nasal membranes, the ease of intranasal administration and the fact that first-pass hepatic metabolism is eliminated (Chien et al. 1989). Since the discovery of very potent peptide and protein drugs in the 1970's, the nasal route has gained special interest as a potential site of administration for these new drug compounds (Schipper et al. 1991). Furthermore, the absorption efficiency of intranasally administered peptides can be improved by the use of absorption enhancers. However, the acceptability of absorption enhancers is not only dependent on their absorption enhancing effect, but also on their safety and toxicity profile (Merkus et al. 1991).

Chitosan is a mucoadhesive polymer that has attracted a great deal of attention as a potential absorption enhancer across mucosal epithelia. It is a polycationic polymer with numerous applications in food, agricultural, cosmetic and recently, in the pharmaceutical industry (Felt et al. 1998; Illum 1998). Chitosan has a pK_a of 5.5–6.0 and is there-

fore only soluble at acidic pH levels. In neutral and basic pH environments most chitosan molecules loose their charge and precipitate from solution rendering them ineffective as absorption enhancers (Kotzé et al. 1999b). The normal pH of nasal secretions in the adult ranges from 5.5–6.5, whereas in children and young infants it ranges from 5.0–6.7 (Fabricant 1964). During acute rhinitis and acute sinusitis the pH of nasal secretions was found to be on the alkaline side and then shifted back to acidity when the stage of clinical resolution was reached. *N*-Trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan, which is soluble over a wide pH range (pH 1–10) and its potential use as an enhancer of absorption across mucosal surfaces has been reported previously (Kotzé et al. 1999c; Thanou et al. 2000; Hamman et al. 2001). Both chitosan and TMC act by opening tight junctions between adjacent epithelial cells to increase paracellular transport of hydrophilic compounds. The relatively high molecular weight of chitosan and TMC may make them unfavourable for inclusion into nasal formulations as the high viscosity of their solutions may cause a substantial decrease in ciliary movement. For this reason, medium and low molecular weight quaternised chitosans are currently being developed as a substitute for high molecular weight quaternised chitosan polymers. In a previous study, the synthesis and characterisation of several medium and low molecular weight quaternised chitosan derivatives were described (Snyman et al. 2003). It has been suggested that *N*-trimethyl chitosan oligosaccharide (TMO), a low molecular weight quaternised chitosan derivative, possesses gene transfection properties (Thanou et al. 2002) and it is hypothesised that due to its lower molecular weight and viscosity it may have the same or even better characteristics as an absorption enhancer, especially for nasal administration, compared to high molecular weight TMC.

The effect of absorption enhancement via the paracellular route of drug absorption can be determined with *in vitro* methods such as measurement of the transepithelial electrical resistance (TEER) of epithelial monolayers (Borchard et al. 1996). Toxic effects of drugs and compounds administered to the nasal route can be determined by ciliary beat frequency (CBF) measurements (Thanou et al. 1999) and mucus transport time measurements (Florea et al. 1999). Since the mucociliary clearance in the nose is the main defence mechanism of the respiratory tract, it is a useful parameter in evaluating the safety of drugs and additives. Ciliary movement is the most important parameter in mucociliary clearance and plays a major role in preserving the functional integrity of the airways and should therefore not be altered considerably by nasal medication (Duchateau et al. 1985). Ciliostasis may be the result of localised toxicity and prevents the defence barrier from functioning properly (Hermens et al. 1987). To investigate how drugs, additives, diseases and inhaled substances change CBF, it is necessary to measure the beat frequency quantitatively and objectively (Van de Donk et al. 1982). Although the measurement of CBF *in vitro* is mostly reproducible and accurate, the use of excised tissue without the protective effect of the mucus barrier would render it impossible to make predictions regarding the chronic use of a formulation on mucociliary clearance *in vivo* (Ingels et al. 1992; Rusznak et al. 1994). Data obtained from *in vitro* CBF experiments is therefore useful as an indication of acute nasal toxicity, which may be followed up by more extensive *in vivo* chronic toxicity studies.

The aim of this study was to compare the absorption enhancing properties of TMC (high molecular weight chitosan derivative) with TMO (low molecular weight chitosan derivative) in cultured rabbit tracheal epithelial cells as indicated by TEER measurements. The effects of these quaternised chitosan derivatives on the CBF of human nasal epithelial cells were evaluated to determine their safe application in the nasal route of administration.

Table 1: The degree of quaternisation (DQ), molecular weight (MW) and relative viscosity (η_{rel}) of the synthesised TMC polymers and TMO oligomers

Polymer	DQ (%)	MW (g/moles)	η_{rel}
Chitosan Seacure 244	/	148000	1.4664
Chitosan oligosaccharide	/	13490	1.0460
TMC 22	22.15	202000	1.3760
TMC 42	42.75	211000	1.0710
TMC 59	59.20	143000	1.1580
TMO 31	31.20	9815	1.0360
TMO 55	54.60	7597	1.0160

2. Investigations, results and discussion

2.1. Chitosan derivatives

The characteristics of the synthesised TMC polymers and TMO oligomers are shown in Table 1. It is important to note the considerable difference between the degree of quaternisation and molecular weights of the different TMC polymers and the different TMO oligomers. The differences in the quaternisation degree are a function of the number and duration of reaction steps used in the synthesis process and is in agreement with results obtained from previous studies (Snyman et al. 2003). The differences in the molecular weights between the TMC and TMO are a result of the different starting compounds (high molecular weight chitosan for TMC and low molecular weight chitosan oligosaccharide for TMO). The molecular weights of TMC and TMO decreased with an increase in the number of reaction steps. This reduction in the starting material's molecular weight is due to the temperature and pH conditions during the synthesis process and is in accordance with results from previous studies (Hamman and Kotzé 2001; Snyman et al. 2003).

2.2. Transepithelial electrical resistance (TEER)

Measurement of TEER is believed to give a good indication of the tightness of the junctions between epithelial cells and has been used to predict the paracellular transport of hydrophilic compounds (Borchard et al. 1996; Kotzé et al. 1999a). The nasal epithelium can be classified into olfactory and respiratory epithelia. The anterior part of the nasal cavity is lined with squamous epithelium, which gradually changes further back in the cavity to pseudostratified columnar epithelium that constitutes the respiratory epithelia (Schumacher and Schumacher 1999).

The effect of TMC and TMO (0.5% w/v and 0.125% w/v) on the TEER of cultured rabbit tracheal epithelial cells is graphically presented in Fig. 1A and 1B. The reduction in TEER of the epithelial monolayers, 2 h after incubation with the different chitosan derivatives (0.0625% w/v, 0.125% w/v, 0.25% w/v and 0.5% w/v), is given in Table 2. Statistical analysis confirmed that the TEER values of the test groups differ significantly from that of the control group ($p < 0.05$).

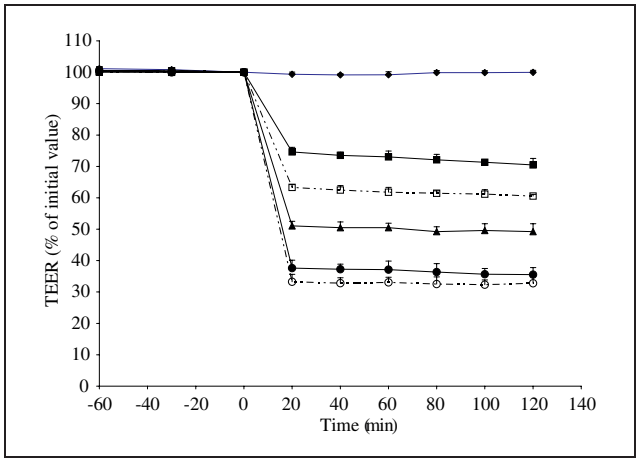

Fig. 1A: The effect of TMC and TMO (0.5% w/v) on the TEER of cultured rabbit tracheal epithelial cells at pH 7.4. Each point represents the mean ± S.D. of 4 experiments. Control (◆), TMC 22 (■), TMC 42 (▲), TMC 59 (●), TMO 31 (□), TMO 55 (○)

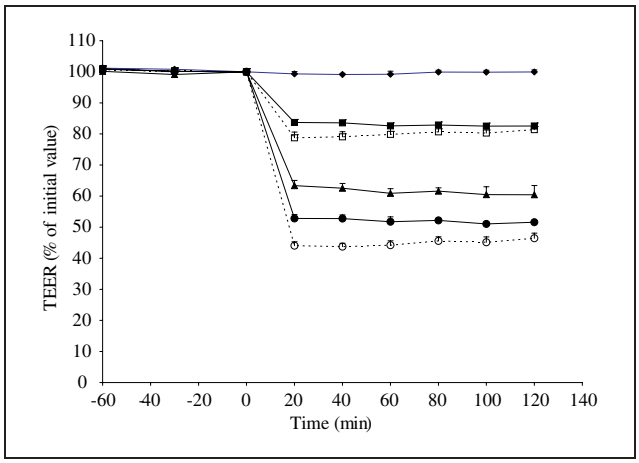

Fig. 1B: The effect of TMC and TMO (0.125% w/v) on the TEER of cultured rabbit tracheal epithelial cells at pH 7.4. Each point represents the mean ± S.D. of 4 experiments. Control (◆), TMC 22 (■), TMC 42 (▲), TMC 59 (●), TMO 31 (□), TMO 55 (○)

In general, the decrease in TEER was dependent on both the degree of quaternisation and the concentration of the applied compound. Higher concentrations of the chitosan derivatives led to a more pronounced decrease in the TEER of the cultured monolayers, while higher degrees of quaternisation of the respective chitosan derivatives also had a more pronounced effect on the TEER of the epithelial monolayers.

Table 2: Reduction in TEER of rabbit tracheal epithelial cells obtained with the TMC polymers and TMO oligomers, 2 h after incubation at pH 7.4

Reduction in TEER (%)				
Polymer	Concentration			
	(0.0625% w/v)	(0.125% w/v)	(0.25% w/v)	(0.5% w/v)
TMC 22	13.71 ± 0.6	17.46 ± 0.3	22.59 ± 1.5	29.54 ± 2.1
TMC 42	30.76 ± 4.5	39.58 ± 3.0	46.62 ± 1.4	50.76 ± 2.5
TMC 59	43.35 ± 1.7	48.39 ± 0.3	56.47 ± 1.1	64.44 ± 2.2
TMO 31	16.44 ± 1.5	18.62 ± 2.0	32.17 ± 1.3	39.45 ± 0.5
TMO 55	46.22 ± 1.9	53.56 ± 1.6	63.02 ± 1.1	66.72 ± 2.1

From Table 2 and Fig. 1A and 1B it can be observed that the TMO oligomers produced more pronounced reductions in the TEER values as compared to the TMC polymers with corresponding degrees of quaternisation (i.e. TMC 22 vs TMO 31 and TMC 59 vs TMO 55) at all the concentrations tested. The larger reduction in TEER achieved with TMO 31 compared to TMC 22 may be explained in two ways. Firstly, the TMO oligomer has a 9% higher degree of quaternisation that could lead to more interactions with the negatively charged sites on the cell membranes resulting in improved opening of tight junctions. Secondly, TMO 31 has a lower molecular weight than TMC 22 (i.e. 0.085×10^5 g/mol vs 2.020×10^5 g/mol). This implicates that at the same percentage weight per volume concentration, TMO has more quaternised groups than TMC, which can result in improved opening of tight junctions. The larger reduction in TEER found with TMO 55 compared to TMC 59 at all the concentrations tested might also be explained in terms of the molecular weight of the two polymers. Although TMO 55 had a slightly lower degree of quaternisation as compared to TMC 59 (i.e. 4%), the effect of the molecular weight and therefore the quantity of quaternised groups in the same weight per volume concentration seemed to outweigh this relatively small difference in the degree of quaternisation.

The more effective reduction in the TEER of the epithelial monolayers by the TMO oligomers as compared to the TMC polymers may therefore be explained by the concentration of fixed positive charges applied to the cells and possibly better interactions with the cell membranes by the smaller molecules. However, permeability studies with suitable marker molecules are needed to confirm if TMO indeed causes more effective absorption enhancing effects than TMC. An important factor to consider is the low molecular weight of the TMO polymers as indicated in Table 1. Although the TMO oligomers are able to open epithelial tight junctions more effectively as compared to the TMC polymers, which was indicated by the reduction in TEER, the oligomers may be able to move into the intercellular spaces due to their relatively small size and this could hamper the diffusion of substances via the paracellular route.

Previous toxicity studies with TMC polymers, involving nucleic acid staining in Caco-2 cell monolayers and CBF measurements, have already indicated that these chitosan derivatives are non toxic (Thanou et al. 1999; Kotzé et al. 2002). However, the safety of TMO oligomers needs to be investigated to confirm the non toxic status of these low molecular weight chitosan derivatives before they can be recommended as absorption enhancers across nasal and/or other epithelia.

2.3. Ciliary beat frequency (CBF) measurements

Ciliary beat frequency (CBF) measurements were performed with the different quaternised chitosan derivatives to determine their acute toxicity on nasal epithelial cells. A statistically significant inhibition in the CBF for the TMC polymers and TMO 31 oligomer (0.125% w/v and 0.5% w/v) as compared to the control was observed and these effects are shown in Fig. 2A and 2B. However, the inhibition in CBF by TMO 55 at both concentrations did not differ significantly from the control group ($p > 0.5$). The results obtained for TMC in this study are in good agreement with previously published results (Kotzé et al. 2002), which were explained by the high viscosity of the TMC solutions. It is clear that the low molecular weight

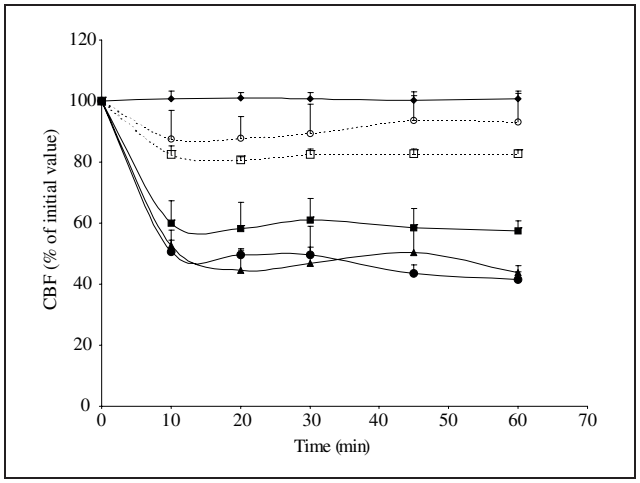

Fig. 2A: The effect of TMC and TMO (0.5% w/v) on the CBF of human nasal epithelia at pH 7.4. Each point represents the mean ± S.D. of 3 experiments. Control (◆), TMC 22 (■), TMC 42 (▲), TMC 59 (●), TMO 31 (□), TMO 55 (○)

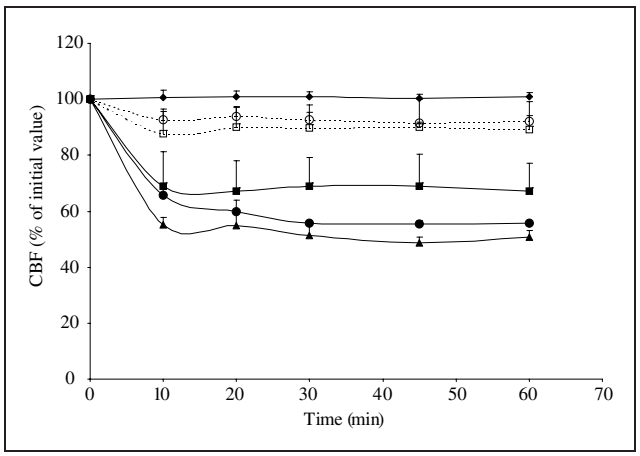

Fig. 2B: The effect of TMC and TMO (0.125% w/v) on the CBF of human nasal epithelial cells at pH 7.4. Each point represents the mean ± S.D. of 3 experiments. Control (◆), TMC 22 (■), TMC 42 (▲), TMC 59 (●), TMO 31 (□), TMO 55 (○)

derivatives (i.e. the TMO oligomers) with corresponding degrees of quaternisation inhibited the CBF to a lower extent when the results obtained for TMC 22 and TMC 59 are compared to the results obtained for TMO 31 and TMO 55, respectively.

TMC inhibited the CBF to a greater extent as compared to TMO possibly due to the higher viscosities of the TMC solutions. It appears that the degree of quaternisation of the chitosan derivatives did not play a major role in the inhibition of the CBF of nasal epithelia. It was further observed that no apparent disruption and damaging effects occurred in the nasal epithelial cells, which were in contact with the TMC and TMO solutions. This lack of damaging effects is an indication that none of these two chitosan derivatives induced local toxicity on the cells. Therefore, both TMC and TMO can be regarded as potentially suitable absorption enhancers for inclusion in nasal preparations as they did not cause ciliostasis and/or cellular damage. Their inhibitory effects on CBF are probably only a function of the viscosity of the solutions. It is recommended that future studies should focus on more extensive toxicity investigations to confirm the safety of TMO oligosaccharides.

3. Experimental

3.1. Materials

Primex chitosan oligosaccharide (79.1% deacytelated chitosan oligosaccharide) was a gift from Primex Ingredients ASA (Avaldsnes, Norway). Chitosan Seacure 244 (93.0% deacytelated chitosan) was purchased from Pronova Biopolymers (Drammen, Norway). The chitosan polymer and oligosaccharide were used as received. Iodomethane, sodium hydroxide pellets, sodium chloride, acetic acid, ammonium acetate, absolute ethanol, diethylether and *N*-methyl-2-pyrrolidone (Riedel-de Haën, South Africa), sodium iodide and iodomethane (Fluka, Switzerland) and potassium bromide (Merck, Germany) were used as received. All chemicals used in the synthesis process of the chitosan derivatives were of analytical grade.

Rabbit tracheal epithelial cells were obtained from New Zealand white rabbits, weighing approximately 3.5 kg each. Jokliks modified Ca^{2+}– free Minimal Essential Medium (MEM), Pen/Strep/Fungizone solution (10000 U/ml penicillin, 10000 μg/ml streptomycin sulfate and 25 μg/ml fungizone), fetal bovine serum (FBS), Dulbeccos Modified Eagle's Medium (DMEM), Hams F-12 Nutrient Mixture, bovine pituitary extract (BPE), insulin, transferrin, hydrocortisone, epidermal growth factor (EGF), retinoic acid, triiodothyronine (T_3), phosphate buffered saline (PBS) and epinephrine were obtained from Sterilab (South Africa). Chlorhexidine was obtained from Zeneca (South Africa), protease (type XIV) from Sigma (USA) and Costar Transwell filter inserts from Costar Corning Incorporated (USA).

3.2. Synthesis of chitosan derivatives

A range of *N*-trimethyl chitosan chloride (TMC) polymers and *N*-trimethyl chitosan oligosaccharide (TMO) oligomers were synthesised by reductive methylation of different chitosan starting materials. Chitosan Seacure 244 was used for the synthesis of TMC polymers and Primex Chitosan Oligosaccharide was used for the synthesis of TMO oligomers. The products obtained from the synthesis were characterised by ^{1}H NMR spectra to determine the degree of quaternisation and SEC/MALLS was used to determine the average molecular weight. The relative viscosities of the products were determined according to the British Pharmacopoiea (1988).

3.3. Rabbit tracheal epithelial cell cultures

Primary rabbit tracheal epithelial cell cultures were grown after approval was obtained from the appropriate Ethics Committee for experimentation with animals. After euthanasia the hair of the rabbit's ventral neck and thorax was shaven and the skin was disinfected with chlorhexidine (1% v/v) in 70% v/v ethanol. A mid-ventral skin incision was cut through the skin of the neck and chest. The skin edges were clamped with x-action towel clips to the next layer of drapes so that the surgical procedure could be performed aseptically. The cervical trachea was exposed by blunt dissection and then the chest cavity was opened by median sternotomy. The trachea caudal to the larynx and cranial by the bronchial bifurcation was cut and removed from the body. A 4–5 cm segment of the trachea was excised at a position just posterior to the larynx and then further down just anterior to the bronchus. The excised tracheal segment was rinsed with ice cold Jokliks modified Ca^{2+}-free Minimal Essential Medium (MEM) containing 10000 U/ml penicillin, 10000 μg/ml streptomycin sulfate and 25 μg/ml fungizone (Pen.Strep.Fungizone solution). After rinsing, one end of the tracheal segment was tied off using silk sutures. A 0.1% w/v solution of protease (type XIV) in Jokliks MEM was added to the lumen. The remaining end was tied off and the entire tracheal segment was immersed in Jokliks MEM and incubated overnight (16 h) at 4 °C. The following day the close ends were cut open and the lumen was rinsed with 50 ml cold Jokliks MEM containing 10% fetal bovine serum (FBS) to remove the dissociated cells. The cell suspension was centrifuged for 5 min at 700 rpm and the cell pellet was re-suspended in a mixture of cold Jokliks MEM and 10% FBS and centrifuged again to remove all traces of protease.

This mild protease treatment is sufficient to remove the epithelial cells lining the trachea while limiting contamination by cells such as fibroblasts and endothelial cells (Freshney 1986). Routinely, $3–5 \times 10^6$ cells were obtained from each trachea with a viability of 80–90% as judged by trypan blue dye exclusion. The dissociated rabbit tracheal epithelial cells were seeded on 6.5 mm (0.33 cm^2) Costar Transwell filter inserts (0.4 μm pore size) at a seeding density of 10^5 cells/cm^2.

The cell suspension (0.2 ml) was placed on the apical side of each filter insert, prior to the addition of culture media (1 ml) to the basolateral side of the insert. The culture media consisted of a 50 : 50 mixture of Dulbeccos Modified Eagle's Medium (DMEM) and Hams F-12 Nutrient Mixture. This medium was supplemented with 2 ml bovine pituitary extract (BPE), 5 mg/ml insulin, 10 mg/ml transferrin, 0.5 mg/ml hydrocortisone, 10 mg/ml epidermal growth factor (EGF), 0.1 μg/ml retinoic acid, 6.5 μg/ml triiodothyronine (T_3), 0.5 mg/ml epinephrine, 10000 U/ml penicillin, 10000 μg/ml streptomycin sulfate and 25 μg/ml fungizone. The cells were maintained at a temperature of 37 °C under an atmosphere of 95% air and 5% carbon dioxide in an incubator. During the initial 48 h the cultures usually contained a large population of floating red blood cells which were subsequently removed by washing the inserts twice with phosphate buffered

saline (PBS) prior to re-feeding on day 2. After the initial media change the cultures were re-fed on a daily basis. Under these conditions the cells grew to confluency within 5–6 d. EGF usually induces cell proliferation, which results in multi-layering of the cells and promotes differentiation of the cell monolayer. Therefore, transepithelial electrical resistance (TEER) measurements were done 5–6 d post-seeding as soon as confluency was reached as judged with microscopic inspection.

3.4. Measurement of TEER

A Millipore Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of chopstick electrodes was used to measure the TEER of the cell monolayers in this study. Solutions of the different TMC and TMO polymers (0.0625–0.5% w/v) were prepared in serum-free culture medium. The pH of each solution was adjusted to 7.4 with 0.1 N NaOH or 0.1 N HCl. TEER measurements started one hour before application of the different test solutions, after which the medium in the apical compartment was removed and replaced by 200 µl of the different chitosan derivative (i.e. TMC and TMO) solutions. The TEER of the cell monolayers was then measured every 20 min for a period of 2 h.

Control experiments were done under the same conditions without application of chitosan derivative solutions. Experiments were done in quadruplet at a temperature of 37 °C under an atmosphere of 95% air and 5% CO_2. The average TEER value for untreated cell monolayers was in the range of 100–170 $\Omega \cdot cm^2$.

3.5. CBF measurements

The system used for CBF measurements is based on the analogue contrast enhancement technique, which has been shown to be highly reproducible and a comparatively safe and simple technique. The technique involves transfer of the microscopic image of the specimen of human nasal epithelia onto a video monitor, via a video camera, followed by electronic stretching of the video signal such that the contrast is dramatically increased to allow visualisation of the specimen far beneath the resolution limits of the optical microscope. Television signals relating to differences in light intensity resulting from ciliary motion at any specific point on the monitor screen, as predetermined by positioning an electronic mouse-operated light-sensing probe directly over ciliated cells, are analysed by an on-line computer specifically programmed for this application. The differences in light intensities are directly converted into units of Hertz (Hz) and are therefore not subject to any further manual or mathematical transformations (Rusznak et al. 1994).

Nasal epithelial cells were collected using a non-invasive brush technique. A nostril of a healthy human volunteer was inspected with a Welch Allyn Diagnostic set. A nylon nasal cytology brush with a 1.73 mm diameter (Hobbs Medical, Inc., South Africa) was inserted through the diagnostic set (window removed) into the left or right nostril. The brush was rapidly moved anteroposteriorly along the lateral wall of the nasal cavity, in the region of the inferior turbinate, to yield a sample. The epithelial cells were immediately suspended in 6 ml preheated (37 °C) Dulbecco's Modified Eagle's Medium (DMEM) and the suspension was kept at 37 °C.

Stock solutions of the TMC and TMO derivatives were prepared in DMEM (pH 7.4) in concentrations of 0.25% w/v and 1.0% w/v, respectively. A volume of 5 ml cell suspension was added to 5 ml of each TMC and TMO solution to obtain final polymer concentrations of 0.5% w/v and 0.125% w/v, respectively. A sample (1 ml) from the cell suspension, before the polymer solutions were added, was used for the control experiments. Samples from the control cell suspension and from the test solutions were transferred to a prepared area surrounded by high vacuum grease on glass microscope slides. A cover slide was gently placed over the high vacuum grease thereby sealing the preparation. The high vacuum grease also ensured that the cover slide did not press directly onto the sample of human nasal epithelium and thereby interfered with ciliary beat activity. The sealed preparation was placed onto the hot stage (37 °C) of a microscope (Olympus BH2, Japan) equipped with a video camera (Panasonic, Japan) connected to a video monitor (Panasonic, Japan). The television signals relating to differences in light intensity resulting from ciliary motion was determined by a PCX video digitiser card (programmed by the CSIR, South-Africa) and converted directly into units of Hertz (Hz). The CBF (Hz) of the nasal cells in the sample was measured over a period of one hour at time intervals of 0, 10, 20, 30, 45 and 60 min (5 readings per time interval). Each experiment was performed in triplicate. Average values were calculated and expressed as a percentage of the respective initial value at $t = 0$ min. The mean CBF (% of initial value) was plotted as a function of time.

Statistical analysis was done with ANOVA/MANOVA Statistica (Statsoft Inc. 2000). Analysis of variance and the post hoc Tukey test were used to determined differences between treatments and groups. Results were considered statistically significantly different with p values ≤ 0.5.

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