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Chitosan as a nasal delivery system: Evaluation of the effect of chitosan on mucociliary clearance rate in the frog palate model

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Abstract

The effect on mucociliary transport velocity (MTV) of 0.25% solutions of five different types of chitosan, with varying molecular weights and degrees of deacetylation, was evaluated using the frog palate model. The MTV was determined by monitoring the speed of movement of graphite particles placed on the palate before and after 10 min exposure times to chitosan solutions, using a video camera and a novel image analysis system. This system can be used for manual or automatic tracking of all or selected particles on the palate. The five types of chitosan tested were shown to have no toxic effect on the frog palate clearance mechanism. The transient decrease in MTV seen was considered to be the result of an ionic interaction between the positively charged chitosan and the negatively charged mucus. The use of image analysis for the determination of MTV is an improvement in comparison with established manual techniques. A large number of particles can be tracked simultaneously and accurately and changes in transport velocity of the particle speed and direction during the recording period can be identified. Operator fatigue due to laborious manual tracking procedures is also avoided.

Keywords: Chitosan; Mucociliary clearance rate; Image analysis; Frog palate model

1. Introduction

It has been shown that substances such as penetration enhancers administered intranasally can adversely affect the mucociliary clearance rate (Van de Donk et al., 1980; Hermens et al., 1990). Efficient nasal mucociliary clearance depends upon the interaction between cilia, mucus and the periciliary fluid. Alterations in any of these may decrease the transport rate which, whilst not fatal, can cause an accumulation of secretions in airways and retention of toxic materials in the respiratory tract. This may lead to a variety of conditions including chronic rhinitis, Youngs disease and sinusitis (Afzelius and Mossberg, 1980; Wolff, 1986).

Chitosans are biodegradable, high molecular weight cationic polysaccharides. Industrially they are produced from chitin, the world's second most abundant biopolymer, by a deacetylation process involving alkaline hydrolysis. The term chitosan refers to a family of polymers, individually characterised by their ratio of acetylated to

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deacetylated units and molecular weight, both parameters being equally responsible for the properties of the polymer. Chitosan has been used for a range of applications as diverse as for water purification, as a food ingredient and as a pharmaceutical excipient in oral drug formulations for the improvement of the dissolution of poorly soluble drugs or to obtain controlled drug release (Li et al., 1992; Hou et al., 1985). It has previously been shown that chitosan has great potential as a nasal delivery system, facilitating the passage of large hydrophilic molecules such as salmon calcitonin and insulin, through the nasal mucosa and into the systemic circulation (Illum et al., 1994). However, the clinical use of chitosan in nasal preparations necessitates the evaluation of their effect on the nasal epithelium and the mucociliary clearance system.

Numerous methods using different models and markers of MTV have been described for measuring mucociliary clearance rates. In vivo techniques used previously include the application of saccharin, charcoal or ^{99m}Tc-tagged resin particles to the anterior portion of the human nasal cavity and the timing of their appearance in the throat via taste, observation and radiation detection, (Andersen et al., 1974; Passali et al., 1984; Takeuchi et al., 1989). Sackner et al. (1973) used dogs to monitor the movement of particles along the trachea using a small camera inserted between the cartilaginous rings.

The effect of substances on cilia beat frequency in various areas of the respiratory tract has also been used as an indicator of toxicity. High-speed video recordings of cilia motion have been used to monitor cilia beat frequency over long periods of time. Jian and Li Wan Po (1993) used rat tracheal rings to test the effect of the penetration enhancers aprotinin and sodium cholate on cilia movement and Levrier et al. (1989) assessed the effect on cilia, of various preservatives used in nasal preparations on the septal tissue from nasally dosed guinea pigs.

The frog palate epithelium is ciliated with numerous mucus producing and secreting glands, similar to mammalian nasal and tracheo-bronchial mucosa. Several studies have shown the usefulness of the ex vivo frog palate model for evaluating the effects of agents that act on mucus or on ciliary activity (King et al., 1974; Braga, 1981, 1988; Puchelle et al., 1982; Batts et al., 1989, 1990).

Changes in the transport rate of particles placed on the palate after the application of a substance are used to infer similar effects on human mucociliary clearance rates, since the transport rates and mechanisms of the two systems are similar (Puchelle et al., 1980). This system is also used as a rapid toxicity test for agents with the potential for nasal absorption (Braga et al., 1992; Gizurarson et al., 1990).

In the present study the effect of five different types of chitosan on the mucociliary clearance rates of frog palates was investigated employing a video camera and a novel image analysis system based upon a Semper 6.4 software kernel and a Sprynt 40 image processing board.

2. Materials and methods

2.1. Materials

The characteristics of the chitosans used in this study are given in Table 1. All chitosans were hydrogen chloride salts with the exception of Seacure G 210 which was supplied as a glutamate salt. All the chitosans were donated by Pronova Biopolymer, Drammen, Norway, and were used as supplied.

Table 1
Various characteristics of the chitosans under study

Chitosan	Cl 113	Cl 211	G 210	Cl 313	Cl 411
Molecular weight ($\times 10^3$)	< 50	130	165	340	350
% deacetylation	85	50	89	84	50
Intrinsic viscosity (dl/g, 25° C)	153	621	1029	1303	1086
Salt type (% in batch)	hydrochloride (16)	hydrochloride (9.3)	glutamate (45)	hydrochloride (15.6)	hydrochloride (9.3)

All other chemicals used were of analytical grade and supplied by Sigma. The Locke Ringer and phosphate buffer solutions used were made up weekly and stored at 4° C.

2.2. Experimental method for determining the mucociliary transport rate

The frog palate model used in this study was a modification of that described by Batts et al. (1989). *Rana temporaria* frogs were double-pithed, their palates excised and placed in a double-walled transparent glass chamber. This was maintained at a relative humidity (RH) of 90–100%, using a layer of water in the bottom of the chamber and sealing the top of the chamber with a piece of cling-film. A temperature of 20 ± 0.5° C was maintained by running thermostatically controlled water through the double walls of the chamber (Fig. 1).

In this experimental set-up it has been shown that cilia continue to beat for at least 72 h, however, the release of mucus declines after 4–5 h (Sade et al., 1970). As mucus is essential for mucociliary transport all the experiments were completed within this time.

To detect the mucociliary clearance of the test graphite particles, a camera (COHU 4912, San Diego, USA) attached to the Sprynt 40 image processing board was situated directly above the chamber. During experiments the cling-film was removed for a maximum of 1 min to apply particles and to rinse the surface of the palate.

The frog palate, with the native mucus intact, was allowed to stabilize in the chamber for 30 min before the start of the experiments. In each experiment graphite particles, 50–100 μm in diameter, produced by scraping a scalpel blade across a pencil lead were used as markers to monitor the changes in MTV. The graphite particles, scattered as uniformly as possible over the surface of the frog palate, were carried along in the mucus streams of the palate. Recordings were made of the graphite particle movements over the central area of the palate surface for a period of 25 s immediately after application. Records of particle movement were stored as a sequence of images taken at 1 s intervals.

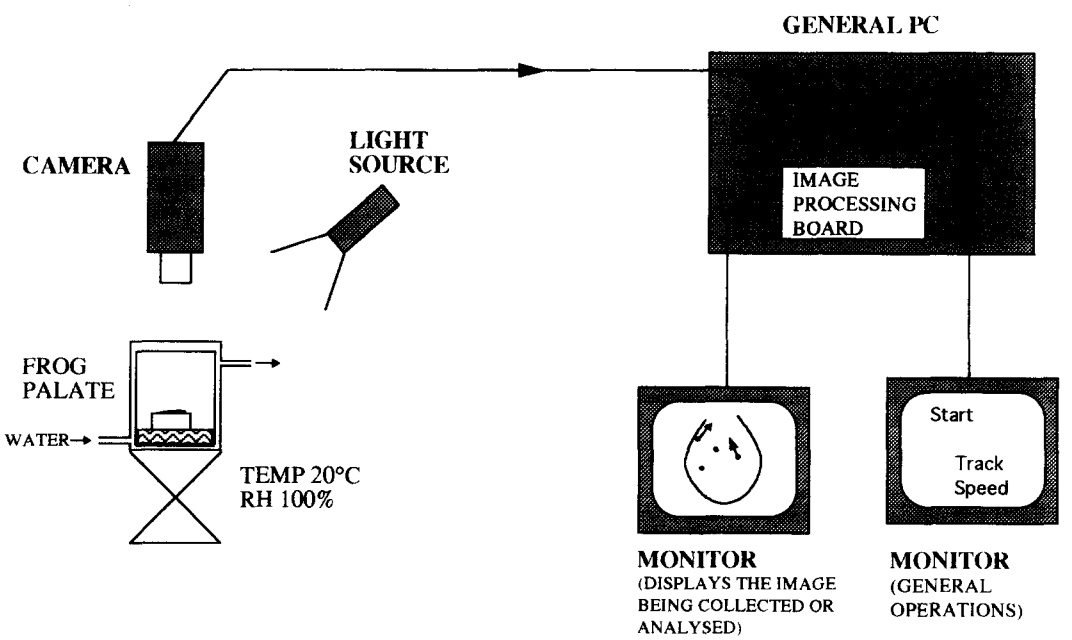


Fig. 1. Schematic representation of the apparatus used in the studies.

Prior to applying chitosan to a palate, between three and four recordings of particle velocity following application of 0.2–0.4 ml of Locke Ringer were made. This determined a basal mucociliary transport velocity (BMTV) for each individual palate. Any particles remaining were washed from the palate using 5 ml of Locke Ringer solution, the palate being allowed to drain by gravity.

The chitosans used were dissolved in phosphate buffer at a concentration of 0.25% w/v and the pH adjusted to 4 with hydrochloric acid. Under this condition no precipitation of the chitosans occurred. The minimum volume sufficient to cover the whole surface of the palate (0.2–0.4 ml) was used. Each palate was exposed to chitosan three times for a 10 min period during each study and each chitosan solution was tested on six different frog palates.

The method was validated by initially determining the effect on MTV of applying Locke Ringer solution at pH 7.4 to three frog palates every 10 min for 90 min and recording the MTV after each application. This procedure was repeated with phosphate buffer at pH 4 in three other palates.

A 1% Laureth 9 solution and 1–10% cocaine solutions were used as positive controls. Laureth 9 has been reported to inhibit irreversibly the MTV (Gizurarson et al., 1990; Hermens et al., 1990), and to cause substantial nasal epithelial disruption (Daugherty et al., 1988; Lee et al., 1988). Cocaine has been found to have a reversible inhibitory effect on mucociliary transport (Van de Donk et al., 1982).

It was previously shown by Braga et al. (1992) that the volume of solutions applied which might physically dilute the mucus lying over the ciliated epithelium did not slow mucociliary transport and hence would not affect the interpretation of the data.

Stewart (1948) investigated the weight carrying capacity and excitability of excised ciliated epithelium and concluded that up to a weight of 20 mg/mm^2 there was no difference in the excitability of the epithelium or acceleration of particles applied. This confirms that the size of the graphite particles themselves have no effect on the MTV recorded.

2.3. Analysis of the results

MTVs were obtained by analysing the stored images of graphite particles moving over the frog palate, using a programme based upon a Semper 6.4 software kernel and running on a Sprynt 40 image processing board (Synoptics, Cambridge,

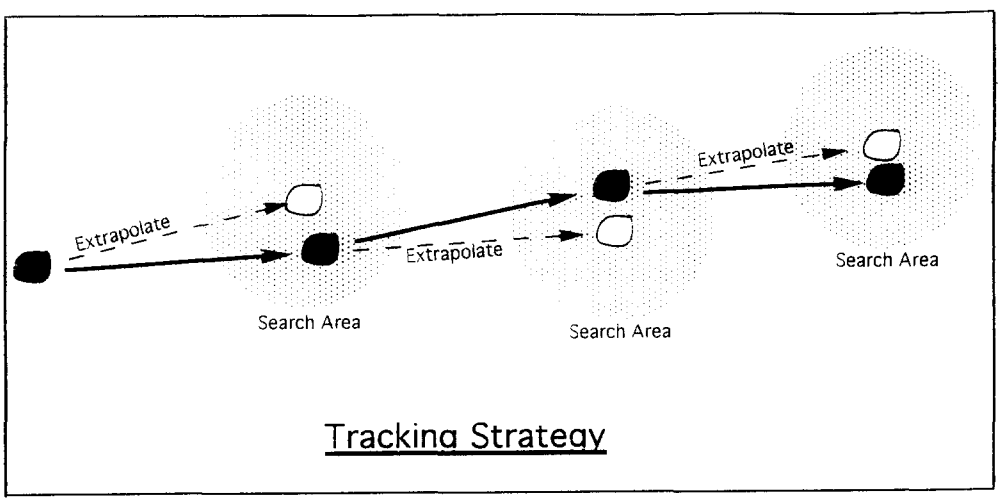
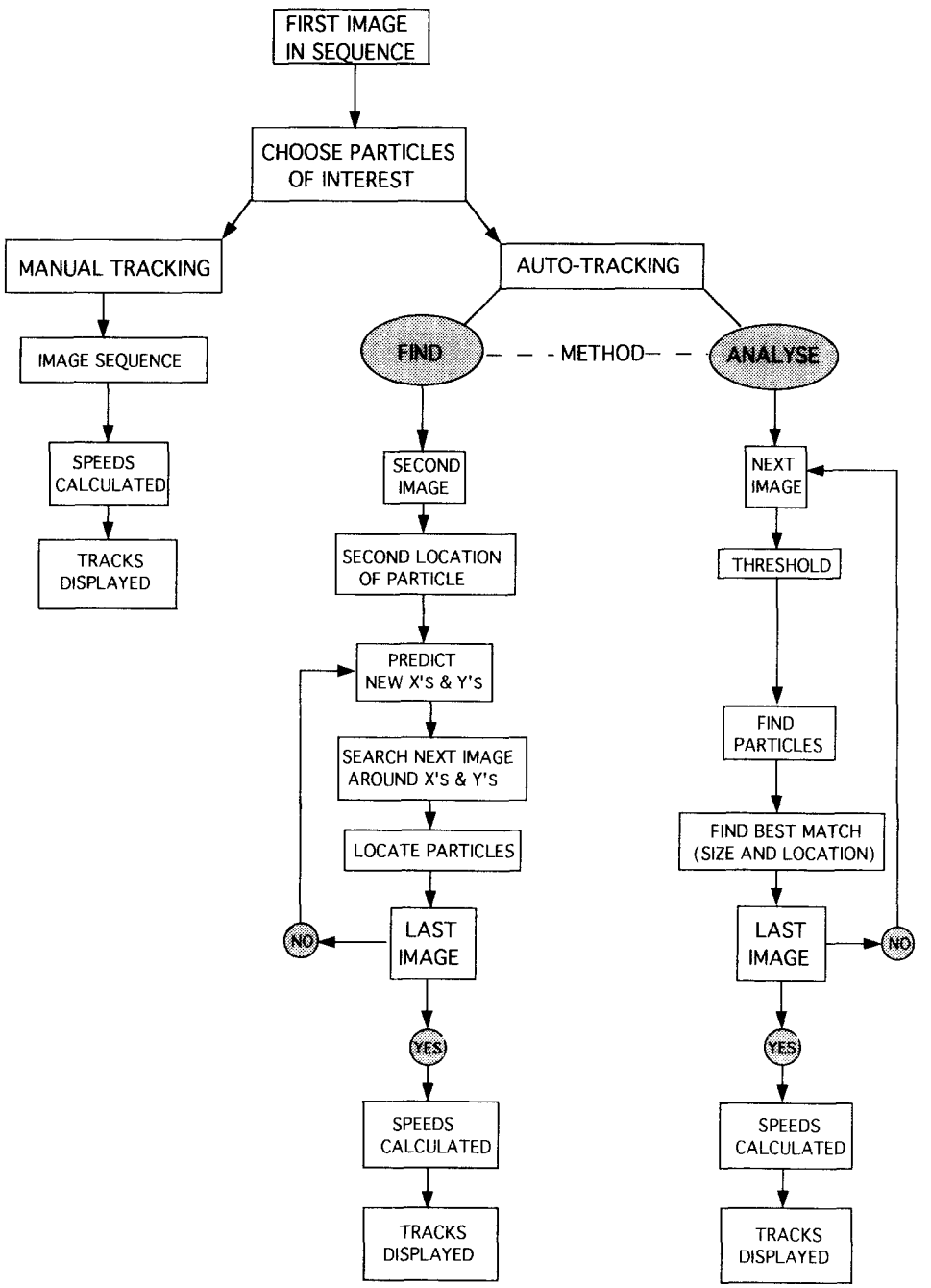


Fig. 2. Schematic representation illustrating how the programme tracks a particle through successive images on the basis of its previous movements.

UK). The software was designed to track individual particles over a series of images, generating one pair of coordinates per particle per image. This then allows the speed of particles to be calculated. The problem is to find the new location of each particle in each new image. Because



Scheme 1. Flow diagram illustrating the processes involved in tracking particles using the three options available with the programme developed.

particles tend to move in a straight line with a relatively stable velocity, an estimate of the new location in the next image in the sequence can be made by extrapolating from the known location in the two preceding images. The actual new location is then obtained by searching in the immediate vicinity of the predicted location, as shown in Fig. 2. The white particles are the predicted locations and the black particles are the actual locations found by the programme.

Digitised images consist of a large array of pixels (picture elements) and every pixel has an associated number that represents the light intensity at that point; the graphite particles are dark and accordingly pixels covered by particles have low values. The software identifies particles as a group of pixels with low values. However, to measure accurately the distance travelled it is necessary to find the location of the same point on a particle.

The simplest strategy, that of choosing the location of the lowest value pixel, is not ideal because the orientation of the particles may alter and because of local variations in the illumination of the palate. A good approximation to the centre of a particle can be obtained by averaging the values of each pixel with the values in neighbouring pixels. When the area over which averaging is undertaken is similar to the area of the particle, the lowest pixel's value reliably provides the new location of the centre of the particle.

Overall, the strategy of searching around the predicted new location of each particle for the darkest area (lowest pixels value), produces an effective automated tracking system for particles with relatively regular velocities. Problems were only encountered when particles merged, disintegrated, or when stray particles moved into the search area. An outline of the programme is given in Scheme 1.

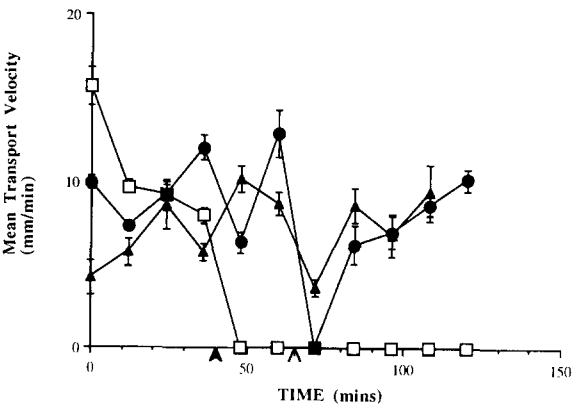


Fig. 3. Effect of various solutions on the MTV of frog palates. Palates treated with 1% Laureth 9 (□), cocaine (●) and phosphate buffer (▲). SE error bars shown. (∧) 1% Laureth 9 or 5% cocaine applied; (∧) 7.5% cocaine applied.

A mean MTV was determined from 10 randomly chosen, but uniformly distributed particles, within the specified area, from each run. The results were expressed as a percentage of the baseline MTV, taken as being the mean MTV recorded after the immediately preceding Locke Ringer rinse.

3. Results

As expected, the Locke Ringer and phosphate buffer controls had no effect on the transport velocity of the graphite particles on the frog palate. This can be seen from Fig. 3. Baseline MTVs varied widely between frogs but were in the range 5–20 mm/min. Application of the 1% Laureth 9 solution caused an irreversible halt of mucociliary transport on the frog palates as shown in Fig. 3, whereas aqueous cocaine solutions led to a reversible arrest of mucociliary transport at concentrations between 5.0 and 7.5%.

Table 2
Average initial percent decrease of MTV (±SE) after the application of 0.25% chitosans and 1% Laureth 9 for 10 min

Chitosan	Cl 113	Cl 211	Cl 210	Cl 313	Cl 411	Laureth 9
% decrease in MTV	62 ± 14	91 ± 12	20 ± 8	51 ± 12	35 ± 4	100 ± 0
% palates recovering within 180 min	100	100	100	89	72	–

Application of all the various chitosan solutions caused the mucociliary transport of the graphite particles to slow transiently or to halt. Apart from Seacure Cl 411 treated frogs, all palates regained their original MTV before the next application of chitosan. The initial decreases

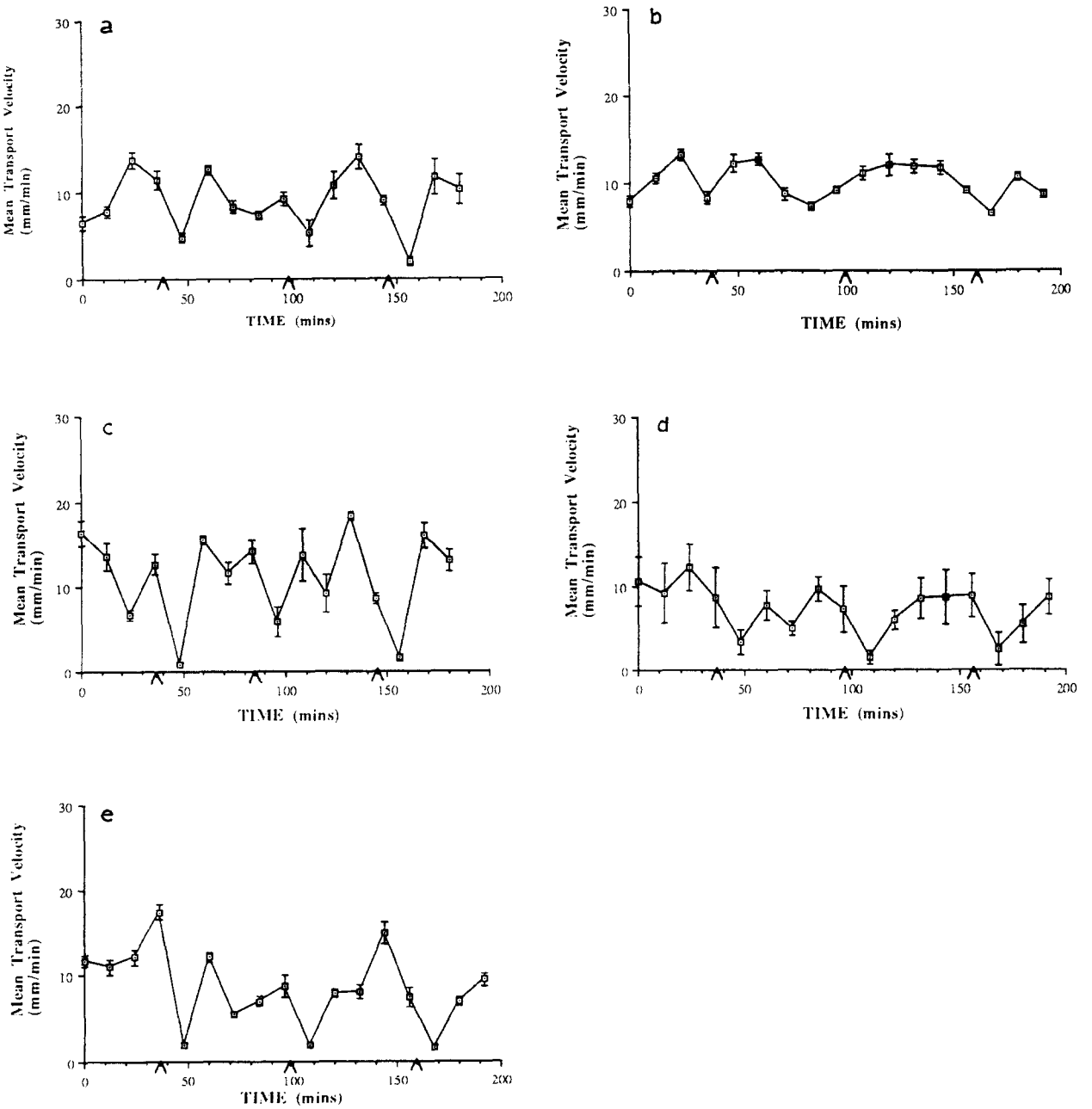


Fig. 4. Effect of a 0.25% solution of chitosan (a) 113 Cl, (b) 211 Cl, (c) 210 G, (d) 313 Cl and (e) 411 Cl on the mucociliary transport velocity of a frog palate. (∧) Chitosan applied.

in MTV caused by Seacure Cl 113 and G 210 were completely reversible after the first Ringer wash.

Table 2 shows the mean initial decreases in MTV caused by the chitosans tested. Seacure G 210 caused the greatest initial mean decrease in the transport velocity. Fig. 4a–e demonstrate one example of the results obtained for each chitosan. Seacure Cl 313 caused a reversible decrease in MTV on four of the palates and a slight increase in MTV on two of the palates.

Seacure Cl 211 caused the least initial mean interference to the transport velocity of any of the chitosan solutions tested. No effect on MTV was recorded on any of the six palates.

All six palates tested showed a decrease in MTV on application of Seacure Cl 411. Although the palates began to recover after the first wash, complete recovery to initial MTV seldom occurred.

For each palate the transport velocity of the graphite particles recorded after chitosan exposure followed by three washes with Locke Ringer, were compared using paired Student's t-tests, to determine whether the mean transport velocity decreased after each successive chitosan application.

No significant decreases ($p < 0.01$) in the transport velocities were found for Seacure Cl 113, 211 and G 210. Chitosans Seacure Cl 313 and 411 showed two and five cases out of 18, respectively, where significant decreases in transport velocities were found between the successive runs (Table 2).

4. Discussion and conclusions

The method of data analysis used in this study is a significant improvement on the manual techniques employed previously (Braga, 1981; Batts et al., 1990). The advantages of this system include the simultaneous tracking of many particles widely distributed over the palate, and accuracy, since simply timing a particle's movement between two points assumes that a linear path is taken. In addition, changes in both particle speed and direction throughout the recording period are identified which can be displayed as a final image of the palate using a colour scale to denote speed. The data are also expressed as a set of average particle speeds. Operator fatigue due to the lengthy and laborious procedures involved in manual tracking is also avoided, making the results obtained more reliable.

The results show that the types of chitosan tested only briefly affected the frog palate mucus clearance, and can therefore be considered to have no adverse effect on the mucociliary clearance mechanism. Indeed, the transient decrease in MTV caused by the majority of chitosans is advantageous because the contact time of therapeutic drugs with the nasal mucosa is extended which will increase bioavailability.

Mucociliary clearance is the result of interactions between cilia, mucus and periciliary fluid, which all have the capacity to alter transport rates. Measuring ex vivo mucociliary clearance rates instead of monitoring the change in a single parameter provides a more relevant toxicity test for the effects of substances on nasal clearance in vivo.

This toxicity model, however, gives no definite information as to whether interference or damage to the cilia, alteration of mucus properties or damage to the epithelial surface is causing the observed decrease in MTV. The speed of recovery and high dependence of molecular weight on slowing the transport velocity, however, initially suggest a physical cause.

The mucoadhesive properties of chitosan have already been reported by Fiebrig et al. (1993) who used ultracentrifugation techniques to determine an interaction between Seacure G 210 and pig gastric mucus. Lehr et al. (1992) and Rentel et al. (1993) used viscosity techniques to determine an interaction between chitosan and rat intestinal mucus.

Mucins from all sources are present in mucus in very low quantities, 1–3%, but are wholly responsible for the gel structure of mucus. Hassan and Gallo (1989) showed that chitosan at pH 5.5 significantly interacts with mucin. Mucins from different sources possess a similar basic unit, a protein core surrounded by carbohydrate side chains constituting up to 70% by weight of the

molecule, usually ~ 500 000 g/mol. The terminal sugar of many of the carbohydrate chains is sialic acid which, having a pK_a value of 2.6, is negatively charged in acidic conditions.

In dilute acidic solutions chitosan molecules exist as stable moderately flexible, cationic, worm-like chains (Kienzle-Sterzer et al., 1984). This is due to the stiff nature of the glucosamine ring and the limited rotation of the ß1–4 glucosidic linkage caused by various intramolecular interactions. Therefore, the chitosan solutions tested can easily penetrate the random coil configuration of the negatively charged mucin network where they adhere to the negatively charged sialic acid groups. Any such interactions will alter the crucial viscoelastic properties of the mucus. Depending on the extent of this interaction, mucus transportability will decrease and the recorded MTV will be slower.

The higher the molecular weight of the chitosan, the slower the chitosan was found to clear from the palate. This is possibly due to the longer chitosan chains forming denser, more entangled, more viscous and therefore more adhesive complexes with mucus. This causes increased resistance to cilia movement, the cilia beat frequency therefore decreases and mucociliary clearance is slowed. Once the chitosan has been removed, either by washing or by the clearance system itself, the MTV returns to normal suggesting no permanent damage to the nasal system.

The retardation of particle transport recorded was not uniform over the whole surface of the palate. This could be due to the chitosan draining from, or adhering to, the palate in different areas, or may simply reflect inherent differences in mucus stream speeds. The degree of chitosan deacetylation appeared to have no effect on the degree of MTV retardation recorded in this study.

Many other effective nasal enhancers have been shown to be toxic when applied to nasal tissue. Chandler et al. (1991a,1991b showed that Laureth 9, sodium taurodihydrofusidate, and lysophosphatidylcholine all caused histological damage to the nasal epithelium of the rat, when applied in concentrations known to be effective as absorption enhancers. Contrary to this, Illum et al. (1994) showed very little or no effect on the membrane of Chitosan Seacure G 210 when applied to the nasal cavity of the rat. Hermens et al. (1990) showed that Laureth 9, sodium taurodihydrofusidate and various bile salts decreased ciliary activity in human nasal tissue and Pritchard et al. (1993) demonstrated that sodium deoxycholate and egg lysophosphatidylcholine slowed frog palate mucociliary transport rates.

Therefore, the results of this study using this potential nasal delivery system are encouraging. Further studies are currently underway to augment this initial toxicity study and to investigate the mode of action of chitosan.

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