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Absorption-promoting effects of chitosan in airway and intestinal cell lines: A comparative study

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ABSTRACT

This work explored the interaction of chitosan with Calu-3 and Caco-2 cell lines, as models of the airway and intestinal epithelium, respectively. The toxicity, tight junction opening and mucoadhesive effects of chitosan were compared in the two cell lines. Additionally, the role of mucus in the absorption-promoting activity of chitosan was studied systematically. Notably, chitosan exhibited a different degree of toxicity on the Calu-3 and Caco-2 cells. Chitosan's tight junction-opening effect, observed in terms of reduction of transepithelial electrical resistance and permeability enhancement, was apparent in both cell lines, though somewhat lower in Caco-2 compared to Calu-3 cell layers (though overall permeability was higher in the former). Tight junction opening and association of chitosan with the epithelial cell layers were more prominent in mucus-containing than in mucus-depleted Calu-3 cells and non mucus-excreting Caco-2 monolayers. Overall, the work suggests that chitosan exhibits a different level of toxicity in airway, as compared to intestinal cells and although absorption enhancement is apparent in both cell lines, enabling its potential use as an absorption-promoting excipient in both pulmonary and oral macromolecular delivery, the magnitude and the duration of the effect are dependent on the level of mucus present on the epithelial surfaces.

1. Introduction

Chitosan, a well-researched cationic polysaccharide produced by partial deacetylation of chitin, has been shown to considerably improve the absorption of macromolecules across mucosal surfaces (Illum, 1998; Illum et al., 1994; Thanou et al., 2001). Importantly, chitosan displays this interesting characteristic whilst being biocompatible, biodegradable and exhibiting low toxicity (Dornish et al., 1996; Grenha et al., 2007; Hirano et al., 1990), potentially making it a highly desirable excipient for use in the formulation of biotherapeutic dosage forms designed for mucosal delivery. The absorption-promoting effect of chitosan is thought to result from a combination of mucoadhesion and its ability to induce opening of the epithelial tight junctions, which otherwise present a strict structural barrier to permeability of macromolecules (Artursson et al., 1994; Dyer et al., 2002; Illum, 1998). The mucoadhesive properties of chitosan have been attributed mainly to an interaction between its positively charged amino groups with negatively charged sialic acid groups on mucus (Fiebrig et al., 1994). On the other hand, the capacity of chitosan to transiently open the tight junctions has been proposed to result from activation of the PKC-dependent signal transduction pathways (Smith et al., 2005).

The absorption-promoting potential of chitosan has been investigated and demonstrated in many in vitro cell culture studies, especially in Caco-2 cells (Artursson et al., 1994; Dodane et al., 1999; Kotzé et al., 1997; Lueßen et al., 1997; Ranaldi et al., 2002; Schipper et al., 1997, 1996, 1999; Smith et al., 2004). Comparison of interaction of chitosan with epithelial cells originating from different mucosal tissues is, however, sparse, despite the many publications on chitosan-induced mucosal absorption enhancement. Here we set out to compare the absorption-promoting effects of chitosan in widely used intestinal Caco-2 cells with those exhibited on somewhat less frequently utilised airway Calu-3 cells. Using the cell layers of Calu-3 and Caco-2 cultures the work investigated absorption enhancement-contributing effects of chitosan, including tight junction opening and mucoadhesion, in addition to measuring tissue-specific toxicity. Employing cell lines with a different capacity to produce mucus also enabled the study of the

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role of mucus on the absorption-promoting effects of chitosan as well as its interaction with the epithelium.

2. Materials and methods

2.1. Materials

Chitosan (hydrochloride salt) of average molecular weight 113 kDa and deacetylation degree 86% (commercially known as Protasan® UP Cl 113), referred to as 'chitosan' in this paper, was purchased from NovoMatrix, Norway. Calu-3 cells (passages 19–48) and Eagle's Minimal Essential Medium (EMEM) were obtained from the American Type Culture Collection (ATCC)–LGC Promochem (USA). Caco-2 cells (passages 44–58) were obtained from European Collection of Cell Cultures (ECACC). Dulbecco's Modified Eagle's Medium (DMEM), Hanks Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), N-acetyl cysteine (NAC), non-essential amino acids (100%), L-glutamine (200 mM), foetal bovine serum (FBS), antibiotic/antimycotic solution (10–12,000 U/ml penicillin, 10–12 mg/ml streptomycin, 25–30 μg/ml amphotericin B), trypsin–EDTA solution (2.5 mg/ml trypsin, 0.2 mg/ml EDTA), FITC-insulin and fluorescein isothiocyanate-labelled dextran (FITC-dextran) of approximate molecular weight of 4 kDa (FD4) were all supplied by Sigma–Aldrich (UK). Fluorescein isothiocyanate (FITC, Type I) was purchased from Molecular Probes (Paisley, UK). 4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid solution (HEPES) and 2-(N-morpholino)ethanesulphonic acid (MES) were obtained from Sigma–Aldrich (UK). Transwell® inserts (12 mm diameter, 0.4 μm pore size) were purchased from Corning (USA). ProLong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen (USA). Mouse, anti-human MUC-5A and goat, anti-mouse Alexa Fluor 594-IgG were obtained from Abcam (UK) and Invitrogen (USA), respectively. All other chemicals (reagent grade) were purchased form Sigma–Aldrich (UK).

2.2. Synthesis of FITC-g-chitosan

100 mg of chitosan was dissolved in 10 ml of deionised water. 20 ml of DMSO was then added to this solution, followed by the slow addition of 5 mg of FITC, previously dissolved in DMSO, under continuous stirring. The reaction was carried out overnight at room temperature in the dark. The resulting solution was poured in an excess of acetone and then centrifuged for 10 min at 3000 rpm. The pellet was washed several times with fresh acetone until the FITC fluorescence signal was no longer observed in the washing solution. The FITC-labelled polymer was then dissolved in water and dialysed against deionised water using a 1000 Da M_w cut-off membrane for three days, whilst protecting from light. The labelled product was then freeze-dried for a further three days, as previously described (Colonna et al., 2008). The structure of FITC-g-chitosan is shown in Fig. 1.

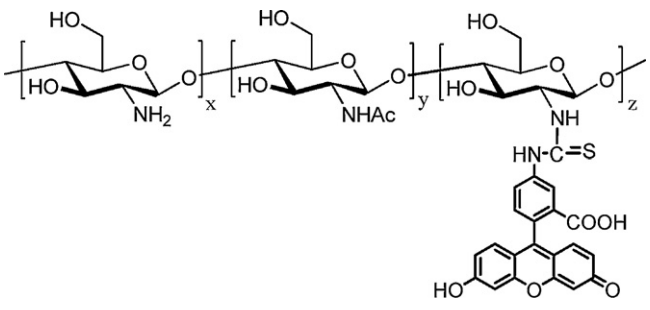

Fig. 1. Chemical structure of FITC-g-chitosan.

2.3. ^{1}H NMR characterisation

FITC-g-chitosan conjugate, chitosan and FITC were characterised by ^{1}H NMR using a Bruker Advance™ Ultra Shield 400 MHz spectrometer (Bruker, Germany) with D_2O/DCl (for FITC-g-chitosan and chitosan) or DMSO (for FITC) as solvents.

2.4. Cell culture

Calu-3 and Caco-2 cells were cultured to confluence in 75 cm^2 flasks at 5% CO_2, 37 °C. Once confluent, cells were detached from the flasks and seeded on permeable inserts (Transwell®) at 100,000 cells/cm^2. Calu-3 cells were maintained in EMEM supplemented with FBS (10%) antibiotic/antimycotic and L-glutamine, whilst Caco-2 cells were cultured using DMEM supplemented with FBS (10%), antibiotic/antimycotic, L-glutamine and pyruvate. Culture media were changed regularly (every other day). Unless otherwise stated, Calu-3 cells were grown on permeable inserts using air-interfaced culture (AIC), which was created on day 2 post-seeding. Where Calu-3 cultures having lower levels of mucus were desired in specific experiments, the cells were cultured on permeable inserts using liquid-covered culture (LCC). Cell confluence and tight junction integrity were assessed by transepithelial electrical resistance (TEER) measurements prior to further experiments. Calu-3 cell layers were typically used for TEER and permeability experiments on days 9–12 in culture, whilst Caco-2 cells were cultured for 21–23 days prior to their use as polarised monolayers.

2.5. Cell toxicity: MTS assay

The MTS colorimetric assay was performed to evaluate the effect of chitosan on cell viability (through measuring metabolic activity). Calu-3 and Caco-2 cells were seeded on 96-well plates at 10,000 cells/well and cultured for 24 h. Cells were incubated with chitosan at 0.0125%, 0.025%, 0.05% and 0.1% (w/v) in HBSS (MES-buffered, pH 6.0). Triton X-100 (0.1% (v/v) in HBSS, pH 6.0) and HBSS (pH 6.0) were used as a positive and negative control, respectively. Cells were incubated (at 37 °C, 5% CO_2) with chitosan solutions and controls for 2 h. At the end of the incubation interval, samples were removed and cells washed with PBS. The MTS reagent was subsequently applied to the cells and the assay conducted according to the manufacturer's instructions, with at least four repeats for each sample.

2.6. TEER studies

For the TEER studies, culture medium was removed from the cell layers and replaced with HBSS (pH 6.0 and 7.4 on the apical and basolateral sides, respectively). Cells were incubated (at 37 °C, 5% CO_2) in HBSS for ~45 min for equilibration, following which TEER was recorded: this was treated as the baseline TEER. Chitosan solutions were prepared by dissolving chitosan in MES-buffered HBSS (pH 6.0). Chitosan was applied to Calu-3 layers at 0.003% and 0.006% (w/v) and to Caco-2 monolayers at 0.003%, 0.006%, 0.0125% and 0.025% (w/v) for 2 h. The selection of these concentrations of chitosan was based on the results of the cell viability (MTS) assay. TEER was then recorded periodically, 0.5 h, 1 h, 1.5 h and 2 h in the presence of chitosan samples and 4 h and 24 h following the application of the samples (in the absence of chitosan samples; i.e. 2 h or 22 h following chitosan sample removal). Between TEER measurements cells were incubated at normal cell culture conditions. The change in TEER was presented as a percentage relative to baseline value. All experiments were performed in triplicates.

A separate experiment tested the effect of chitosan on TEER of Calu-3 cell layers cultured using AIC and LCC conditions. This

was conducted in an attempt to assess whether the different extent of mucus presence (more prominent in AIC compared to LCC cells) influences the tight junction opening effect of chitosan, as measured by its action on TEER. In this instance chitosan was applied to the cells at 0.003% (w/v) (in HBSS, pH 6.0) and TEER was measured periodically in a similar way to the experiment above.

2.7. Permeability enhancing effect of chitosan: comparison between Calu-3 and Caco-2 cells

Cell layers (AIC conditions for Calu-3 cells) were equilibrated in HBSS for approximately 45 min. Chitosan solution was then applied to the apical side of the cells in combination with the paracellular marker, FITC-dextran of approximately 4 kDa (FD4) in (MES-buffered) HBSS at pH 6.0. The final concentration of chitosan in the applied solution was 0.003% (w/v) for Calu-3 cells and 0.0125% (w/v) for Caco-2 cells; FD4 was used at a final concentration of 500 μl/ml. The concentration of chitosan used in the present experiment was chosen from the cell viability and TEER data, and reflects concentrations associated with a reversible TEER effect. FD4 permeability was determined by sampling the basolateral solution, undertaken periodically (every 30 min) for 3 h with replacement of the sampled volumes with fresh HBSS.

2.8. Effect of mucus on permeability enhancing capacity of chitosan

The effect of chitosan on FD4 permeability was tested in Calu-3 cells cultured using either AIC or LCC. Chitosan (0.003% (w/v) in HBSS, pH 6.0) was applied to the cells either in combination with FD4 (500 μg/ml) or the cell layers were treated with chitosan for 20 min followed by extensive cell washing (with PBS) and application of FD4. The latter experiment attempted to test whether the permeability-enhancing effect of chitosan remained following cell treatment and its removal from the cell layers. FD4 permeability was determined (in both instances) by sampling the basolateral solution periodically and its quantitation by fluorescence as described previously.

In a separate experiment, the permeability-enhancing effect of chitosan was compared with that of a non-mucoadhesive absorption enhancer, EDTA. In this instance FITC-insulin was used as a paracellular marker. Rather than applying chitosan in combination with the macromolecular solute, Calu-3 cells (cultured using AIC) were first incubated with either chitosan (0.003% (w/v) in HBSS, pH 6.0) or EDTA (0.125% (w/v) in HBSS) for 20 min. These solutions were then removed and cells washed extensively with PBS. FITC-insulin (in HBSS, pH 6.0) was applied to the cells at a final concentration of 80 μg/ml and its translocation across the cells determined by sampling (100 μl volumes) the basolateral solution at regular intervals for 3 h (with replacement of the sampled solution) and quantitation by fluorescence.

Another experiment assessing the impact of mucus on the permeability enhancing capacity of chitosan employed Calu-3 cell layers (cultured using AIC) treated with a mucolytic agent, N-acetyl cysteine (NAC), prior to permeability study. Cell layers were incubated with NAC (0.3% (w/v) in HBSS, pH 7.4) in three 20 min intervals, followed by cell washing. Chitosan and FD4 (0.003% (w/v) and 500 μg/ml, respectively; in HBSS, pH 6.0) were then applied and FD4 permeability measured as described previously. As a comparison, the experiment was also conducted on non mucus-producing Caco-2 cell monolayers using the same conditions.

All permeability experiments were conducted in triplicates and FD4 and FITC-insulin permeabilities were expressed as the apparent permeability coefficient (P_{app}), calculated using the following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t}\right) \times \left(\frac{1}{A \times C_0}\right)$$

where P_{app} is the apparent permeability in cm/s, $\Delta Q/\Delta t$ is the permeability rate (amount FD4 or FITC-insulin traversing the cell layers over time, as determined from the steady state accumulation of the solute in the receiver chamber over time), A is the surface area of the cell layer (1.1 cm^2) and C_0 is the initial FD or FITC-insulin concentration in the donor chamber.

2.9. Association of FITC-g-chitosan with Calu-3 and Caco-2 cells

Cells were cultured on permeable supports as before. Calu-3 cells were cultured using AIC or LCC conditions. Both Calu-3 layers and Caco-2 monolayers were equilibrated in HBSS before sample addition. FITC-g-chitosan (0.003% (w/v) in MES-buffered HBSS, pH 6.0) was then applied to the cells for an incubation period of 2 h. Chitosan samples were then removed and cells washed (with PBS, pH 7.4) extensively. Cells were then fixed with paraformaldehyde and cell-containing filters excised and mounted (using DAPI-containing mounting medium) on glass slides for confocal imaging. Cells were imaged for the presence of FITC-chitosan using a Leica TCS SP2 system mounted on a Leica DMIRE2 inverted microscope.

2.10. Interaction of FITC-g-chitosan with mucus

Calu-3 layers (cultured using AIC) were incubated with FITC-g-chitosan (0.003% (w/v) in HBSS, pH 6.0) for 2 h. Cells were then washed extensively, fixed in paraformaldehyde and incubated with 1% (w/v) BSA in PBS for 1 h to block/minimise any non-specific antibody binding. BSA/PBS was then removed and cells incubated with mouse, anti-human MUC-5A antibody for 1 h. Cells were thereafter washed and incubated with goat, anti-mouse Alexa Fluor 596-IgG for 1 h. Following the incubation with the secondary antibody, cells were washed extensively and Transwell® filters ($n = 3$) excised and mounted on glass slides (using DAPI-containing mounting medium) for confocal imaging.

In a separate experiment, MUC-5A staining in Calu-3 cells was conducted following cell incubation with N-acetyl cysteine (NAC, 0.3% (w/v) in HBSS), which was conducted in 3×20 min steps. Cells were then fixed with paraformaldehyde, stained for MUC-5A and prepared for confocal imaging in the manner described above.

2.11. Statistical analysis

Statistical analysis on the data was conducted using Student's t-test, with $p < 0.05$ reported as statistically significant.

3. Results

3.1. Characterisation of FITC-g-chitosan

To allow visualisation of the interaction of chitosan with the epithelial cells, the first step was to chemically conjugate FITC to chitosan. Fig. 2 depicts the ^{1}H NMR spectrum of the synthesised derivative FITC-g-chitosan. The figure shows the characteristic peaks for chitosan at 2 ppm (due to the acetyl group), 3 ppm (H-2) and 3.5/4 ppm (H-3/6) for the ring structure of the sugar. The peaks related to and demonstrating the presence of FITC moieties in the material appear between 7 and 8.3 ppm, with the peaks not

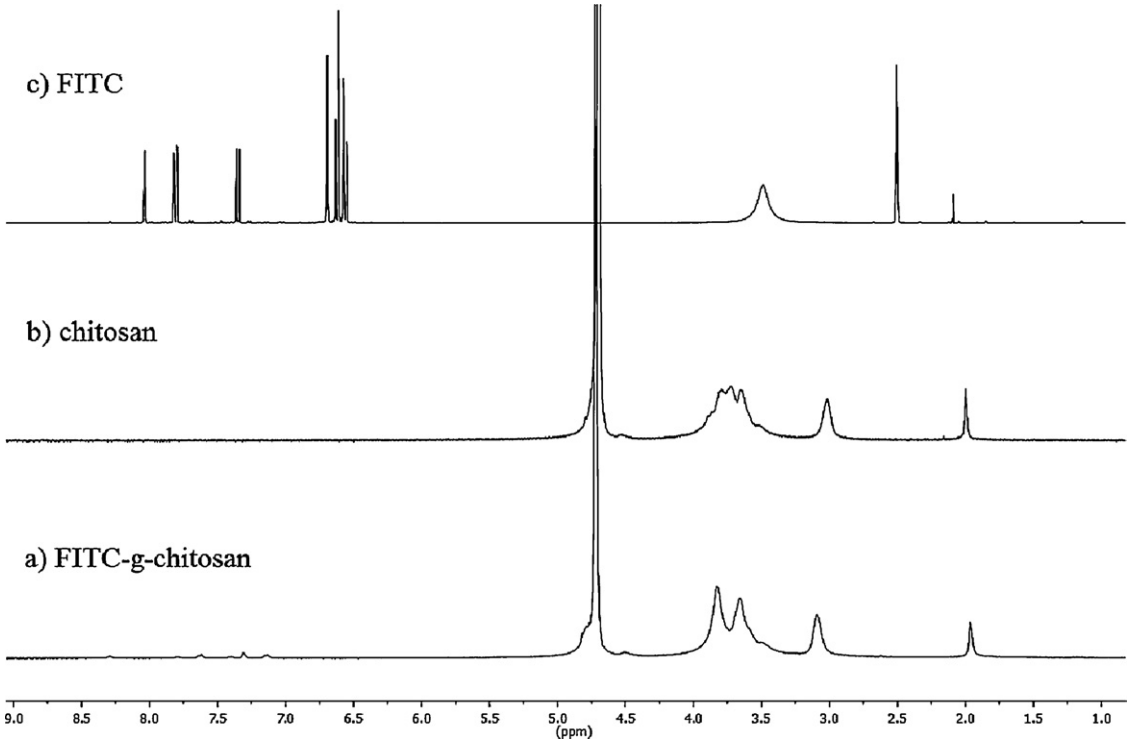

Fig. 2. ^{1}H NMR spectrum of FITC-g-chitosan (D_2O/DCl), chitosan (D_2O/DCl) and FITC (DMSO-d6).

being prominent due to the low content of FITC in each chitosan molecule. FITC molecule spectrum was recorded in DMSO-d6 due to its insolubility in aqueous solution.

3.2. Cell toxicity: MTS assay

Conducting a cytotoxicity assay in the initial phase of the study was important as it allowed identification of non-toxic concentrations of chitosan, which were subsequently used in further experiments. The effect of chitosan on the viability of Calu-3 and Caco-2 cells is shown in Fig. 3. A dose-dependent effect was especially noticeable in Caco-2 cells. It is clear from the figure that chitosan exhibited a markedly more prominent effect on the viability of Calu-3 cells compared to Caco-2 cells. Whilst all the tested concentrations of chitosan suppressed the relative Calu-3 viability by more than 80%, the reduction of Caco-2 viability ranged from 76% (with the highest tested concentration of 0.1% (w/v)) to 26% (with the lowest concentration of 0.0125% (w/v)).

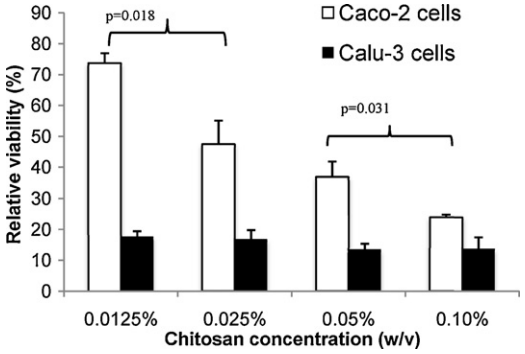

Fig. 3. Effect of chitosan on the viability of Caco-2 and Calu-3 cells. Viability was measured by the MTS assay following a 2 h incubation of the cells with chitosan at different concentrations. Results presented as % viability relative to controls (HBSS and Triton X-100; both at pH 6.0) and expressed as the mean ± SD ($n = 4$).

3.3. TEER studies

Application of different doses of chitosan to the cell layers was accompanied by a rapid and dramatic drop in TEER to levels as low as 4% of the baseline value (Fig. 4a), with TEER reduction persisting during the incubation period of cells with chitosan. These effects were common to both Calu-3 and Caco-2 cell layers, indicating a strong tight junction-opening effect in both cell lines. In Caco-2 cells a dose-dependent effect was apparent, with TEER at 2 h reaching significantly lower values with each increase in chitosan concentration ($p < 0.01$ in each case). The lowest tested concentration (0.003% (w/v)) produced a significantly larger effect in Calu-3 cell layers compared to Caco-2 monolayers (a drop to less than 5% of the baseline TEER versus 16%). Interestingly, application of chitosan to Calu-3 cells at 0.006% (w/v) led to an irreversible decrease in TEER, suggesting cell damage, whilst Caco-2 cells tolerated significantly larger concentrations of up to 0.025% (w/v) before TEER reversibility was somewhat compromised (in this instance TEER still recovered to 64% of the baseline value). This reflects the degree of sensitivity of cell lines towards the interaction with chitosan, as observed from the cell viability assay. The concentrations of chitosan producing a high level of TEER reversal (to >95% of the baseline value), following a potent initial effect on TEER, were 0.003% (w/v) and 0.0125% (w/v) in Calu-3 and Caco-2 cells, respectively. These concentrations were subsequently used in further experiments.

In testing the effect of mucus presence on chitosan-induced tight junction opening, the TEER experiments in Fig. 4b were conducted using Calu-3 cell layers grown under culture conditions that promote and limit the presence of mucus (air-interfaced culture, AIC and liquid-covered culture, LCC, respectively). A large drop in TEER ensuing following the application of chitosan is apparent in both culture conditions. This effect was somewhat delayed in the cells grown under AIC compared to LCC (TEER reduction to 34% and 23% of the baseline value, respectively, at 0.5 h measurement interval). However, recordings at later points revealed that the

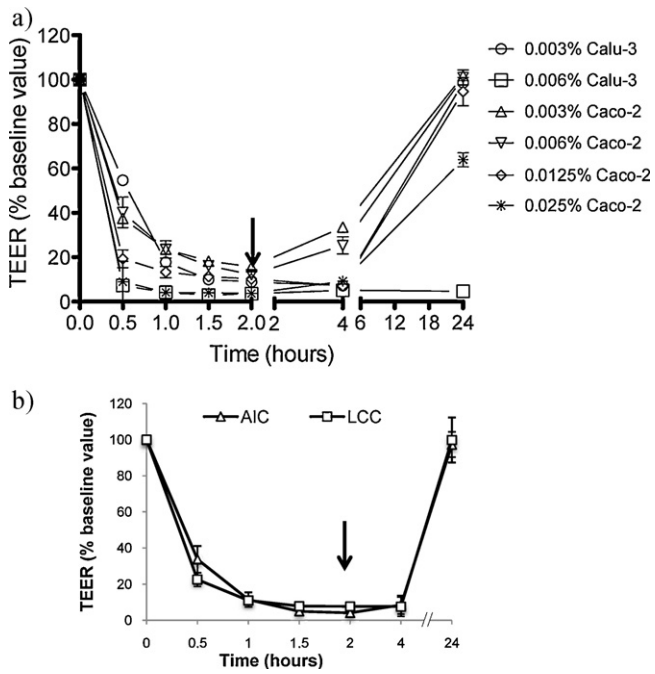

Fig. 4. Effect of chitosan on TEER. (a) Effect of chitosan on Calu-3 and Caco-2 cell layer TEER. Chitosan was applied (in HBSS; pH 6.0) to Calu-3 cells at concentrations of 0.003% and 0.006% (w/v) and to Caco-2 cells at 0.003%, 0.006%, 0.0125% and 0.025% (w/v). (b) Effect of chitosan on TEER of Calu-3 layers cultured using air-interfaced culture (AIC) or liquid-covered culture (LCC). TEER presented as % change compared to the baseline value where time 0 h represents baseline TEER. Arrows indicate sample removal and replacement with culture medium. Results presented as the mean ± SD ($n = 3$).

TEER-lowering effect of chitosan was of a larger magnitude (statistically significant at 2 h; $p = 0.045$) in AIC Calu-3 layers, reaching 4–5% of the baseline value, compared to LCC cell layers (TEER decrease to ~8% of the baseline figure). A large degree of TEER reversibility (to 97–99% of the baseline TEER) was observed in both tested conditions, suggesting lack of cell damage or compromised cell layer integrity.

3.4. Permeability enhancing effect of chitosan: comparison between Calu-3 and Caco-2 cells

Permeability experiments were conducted using chitosan at concentrations of 0.003% (w/v) and 0.0125% (w/v) in Calu-3 and Caco-2 cells, respectively, as dictated by the reversible TEER effect. The data (Fig. 5) demonstrates that in the presence of chitosan FD4 traversed the cell culture barriers to a significantly larger extent compared to controls (FD4 applied to cells in HBSS). This phenomenon was observed in both Calu-3 and Caco-2 cell layers, with the calculated apparent permeability (P_{app}) values revealing an increase in permeability of 11-fold and 7-fold compared to controls, respectively.

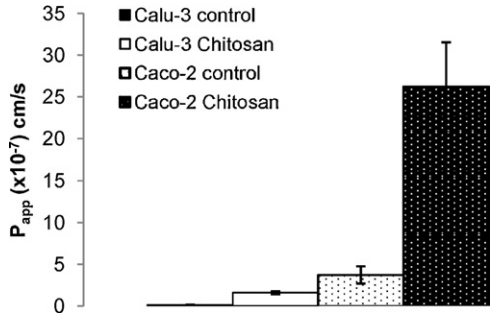

Fig. 5. Effect of chitosan on FD4 permeability across Calu-3 (a) and Caco-2 (b) cell layers. Data expressed as apparent permeability coefficient and presented as the mean ± SD ($n = 4$).

3.5. Effect of mucus on permeability enhancing capacity of chitosan

Fig. 6a compares the permeability enhancement of chitosan in Calu-3 cell layers cultured under AIC or LCC. A 9.9-fold enhancement in FD4 permeability was apparent in mucus-producing AIC cells, which is comparable to the data from the experiment in Fig. 5 (11-fold permeability enhancement). By contrast, in LCC cell layers the increase in FD4 permeability by the action of chitosan amounted to a factor of 5.3-fold.

In an experiment designed to test whether the permeability-enhancing effect of chitosan lasts following its removal from the cell layers, chitosan-treated Calu-3 cells, cultured using AIC or LCC, presented a lower barrier to FD4 permeability compared to control. The extent of permeability enhancement depended on the cell culture conditions and was significantly larger in AIC (9.7-fold) compared to LCC grown Calu-3 cell layers (6.2-fold) (Fig. 6b).

Next, we compared the permeability-enhancing effect of chitosan with that of a non-mucoadhesive absorption enhancer, EDTA, where the macromolecular solute (FITC-insulin) was applied separately after the permeability enhancers (and cell washing). The data (Fig. 6c) shows that pre-treatment of mucus-producing Calu-3 cells (AIC conditions) with either chitosan or EDTA increased the permeability of insulin, as compared to control (cells pre-treated with HBSS), with a significantly larger effect apparent in chitosan-treated cells (11.9-fold) compared to those exposed to EDTA (4.2-fold increase, though not statistically significant, $p = 0.0815$).

To exclude the possibility that the permeability-enhancing effect of chitosan was influenced by differences in the Calu-3 cell layer model other than mucus production following their culture under AIC or LCC, cells were cultured using AIC only and were treated with N-acetyl cysteine (NAC) for mucus removal prior to permeability assessment. A comparison is made with non mucus-producing Caco-2 cell monolayers. The data presented in Fig. 6d shows that in mucus-deprived Calu-3 cells chitosan induced a 6.4-fold increase in FD4 permeability. The permeability enhancement in Caco-2 monolayers previously treated with NAC amounted to 9.8-fold, which is somewhat larger than the increase in permeability with chitosan where treatment with NAC was omitted (7-fold, Fig. 5).

3.6. Association of FITC-g-chitosan with the cells

Fig. 7 shows confocal micrographs of Calu-3 layers (cultured under AIC or LCC conditions) and Caco-2 monolayers following their incubation with FITC-g-chitosan. In AIC Calu-3 cells, where the presence of mucus is promoted (Fiegel et al., 2003; Grainger et al., 2006), areas of intense fluorescence were clearly seen in some regions of the cell layer (Fig. 7a). In other areas, FITC-g-chitosan appeared as continuous 'rings' of fluorescence around individual cells. Fluorescence around the cell perimeter was also apparent in Calu-3 cells cultured using LCC where mucus is either absent or present in limited amounts (Fig. 7b), and in non-mucus producing Caco-2 monolayers (Fig. 7c). However, in contrast to AIC Calu-3 cells, areas of bright fluorescence were not observed in the latter two scenarios, suggesting a lower extent of FITC-g-chitosan association with the cells.

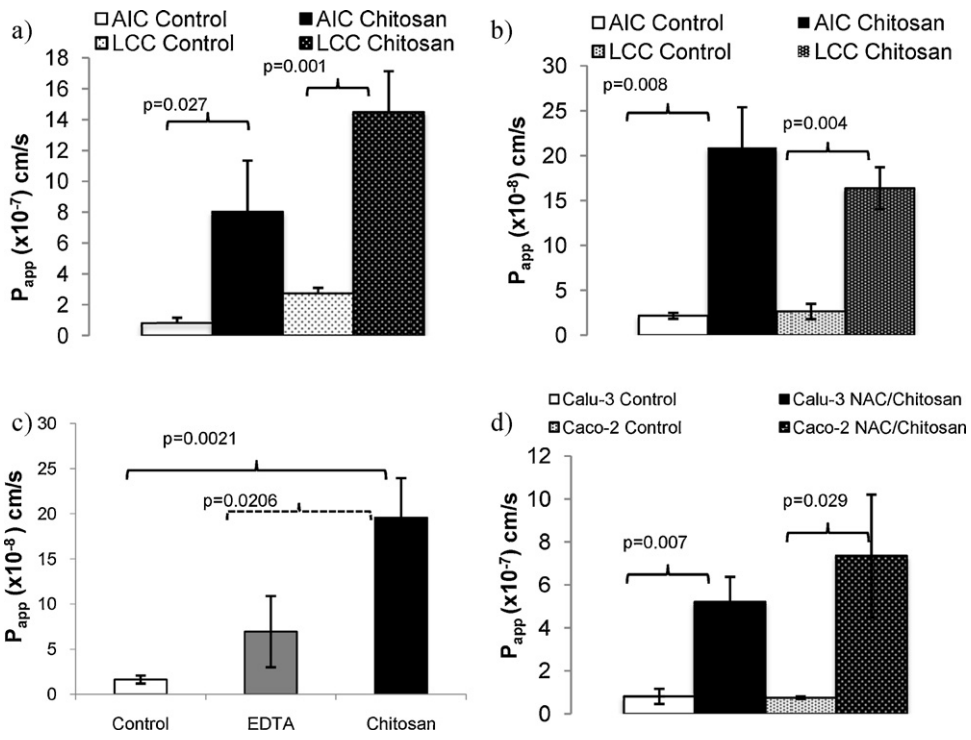

Fig. 6. Effect of mucus on permeability enhancing capacity of chitosan. (a) Effect of chitosan (0.003% (w/v) in HBSS, pH 6.0) on FD4 permeability across Calu-3 cell layers cultured using air-interfaced culture (AIC) or liquid-covered culture (LCC). (b) Effect of pre-treatment of cells with chitosan on FD4 permeability across AIC or LCC Calu-3 cells. Cells were incubated with chitosan for 20 min before cell washing and application of FD4. (c) Permeability of FITC-insulin in Calu-3 cells pre-treated with EDTA or chitosan. Cell layers were incubated with EDTA or chitosan at 0.125% (w/v) and 0.003% (w/v), respectively, for 20 min before cell washing and application of FITC-insulin. (e) Effect of chitosan on FD4 permeability across Calu-3 and Caco-2 cell layers subjected to N-acetyl cysteine treatment (NAC, 0.3% (w/v) in HBSS). Permeability expressed as apparent permeability coefficient (P_{app}, $\times 10^{-7}$ for subfigures (a) and (d) and $\times 10^{-8}$ for subfigures (b) and (c)). Data presented as the mean ± SD ($n = 4$) in all cases.

3.7. Mucoadhesion of FITC-g-chitosan

The present experiment, which also investigated the mucoadhesive property of chitosan, incorporated immunostaining of a secretory mucus protein (mucin), MUC-5A, to assess the interaction of chitosan with mucus. Confocal micrographs of representative Calu-3 cell layers incubated with FITC-g-chitosan, followed by immunostaining for MUC-5A, are shown in Fig. 8. The presence of green FITC-g-chitosan and red MUC-5A in the cell layer is clearly seen in the micrographs (Fig. 8ai and aii). Furthermore, FITC-g-chitosan and MUC-5A were predominantly localised in the same areas of the cell layer. This co-localisation is shown in the green and red channels as well as in the overlay image, where co-localisation of green and red fluorescence is shown in orange. Imaging the depth of the cell layer revealed that both FITC-g-chitosan and MUC-5A were largely present above the cells (Fig. 8aii).

To demonstrate the importance of the presence of mucus in the ability of chitosan to associate with the mucosal surfaces, Calu-3 cells were subjected to mucus removal treatment by incubation with NAC prior to their incubation with FITC-g-chitosan and immunostaining for MUC-5A. Fig. 8b shows micrographs of Calu-3 cells exposed to these procedures. It is obvious from the micrograph that the levels of association of both FITC-g-chitosan (green) and MUC-5A (red) with the cells was noticeably lower in this experimental scenario compared to cells not subjected to NAC treatment (Fig. 8a).

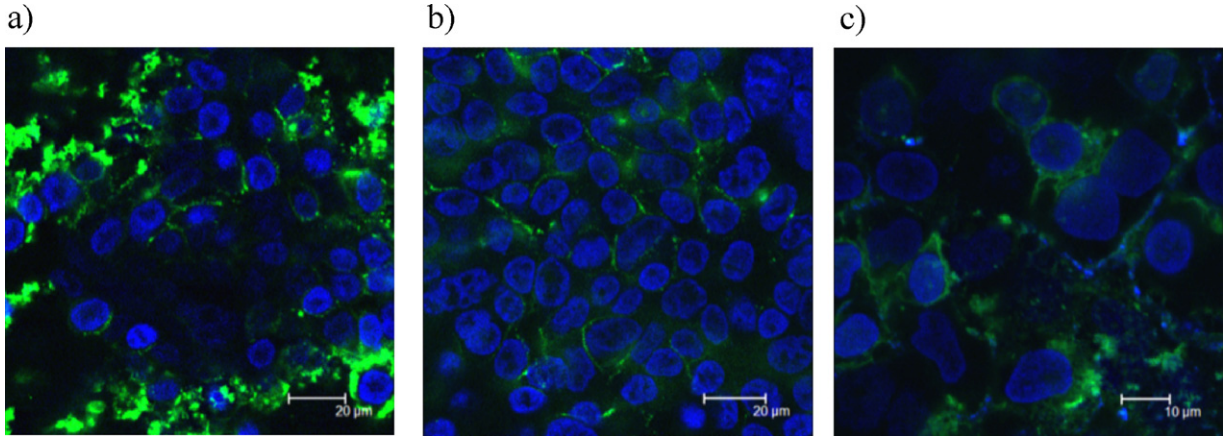

Fig. 7. Confocal micrographs depicting association of FITC-g-chitosan with Calu-3 and Caco-2 cells. (a) Calu-3 cells grown using air-interfaced culture, (b) Calu-3 cells grown using and liquid-covered culture, and (c) Caco-2 monolayers.

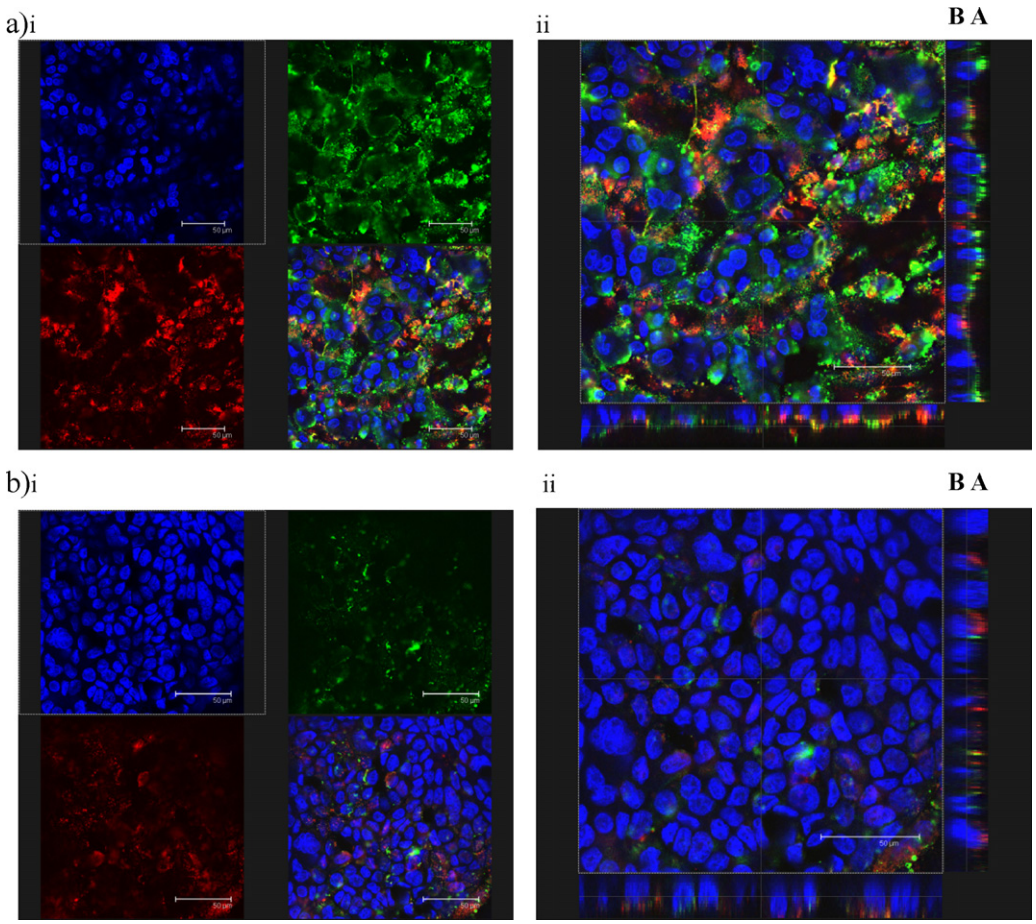

Fig. 8. Mucoadhesion of FITC-g-chitosan in Calu-3 cells. (a) Localisation of FITC-g-chitosan (green) and that of a secretory mucin, MUC-5A (red), where (i) depicts single section imaging and (ii) depicts imaging across the vertical axis. (b) Localisation of FITC-g-chitosan and MUC-5A following cell incubation with N-acetyl cysteine (i, single section; ii, vertical axis of the cell layer). Immunostaining was conducted using mouse, anti-human MUC-5A IgG (primary antibody) and goat, anti-mouse Alexa Fluor 594-IgG (secondary antibody). A = apical and B = basolateral side of the cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

The absorption-enhancing capacity of chitosan has been investigated for its potential use in formulations of biotherapeutics designed for mucosal delivery (Illum et al., 1994). Unlike certain classes of absorption enhancers, chitosan benefits from its proven lack of in vivo toxicity, which explains the existence of a large number of publications on absorption-enhancing properties of chitosan (Di Colo et al., 2008).

The absorption-enhancing ability and the associated mechanism of action of chitosan have been extensively investigated in epithelial cell models in vitro, with studies predominantly conducted in Caco-2 cell cultures (Artursson et al., 1994; Dodane et al., 1999; Kotzé et al., 1997; Lueßen et al., 1997; Ranaldi et al., 2002; Schipper et al., 1997, 1996, 1999; Smith et al., 2004). Although Caco-2 cell monolayers are widely regarded as a very good in vitro model for intestinal absorption studies, they lack a mucus layer covering the epithelium (Chen et al., 2010). Studies incorporating mucus-producing cell lines to test the effects of chitosan are surprisingly rare, as are those comparing the interaction of chitosan with epithelial cells originating from different mucosal tissues (Kudsiova and Lawrence, 2008; Schipper et al., 1999). Our work addresses this by comparing the cell interaction and absorption promoting effects of chitosan in a mucus-producing bronchial cell line and a non mucus-producing intestinal cell line. A study of this nature is deemed imperative considering the suggested applications of chitosan as a mucosal absorption enhancer for both oral administration (Bowman and Leong, 2006; Lee et al., 2011; Qian et al., 2006) and delivery across the airways (Grenha et al., 2007, 2008). An investigation on the interaction of chitosan on both types of tissues with the view of highlighting any tissue-specific differences in its biological activity is clearly important. Furthermore employing mucus-producing cells makes the investigation of chitosan interaction with epithelial surfaces more relevant to the in vivo situation compared to studies conducted solely in Caco-2 monolayers, as previously has often been the case.

Routine work in our lab has repeatedly shown that airway Calu-3 cells are more sensitive to chitosan, compared to intestinal Caco-2 cells. This was clearly highlighted by the maximal concentration of chitosan that produced a reversible decrease in TEER (0.0125% (w/v) in Caco-2 cells, compared to 0.003% (w/v) in Calu-3 cells) (Fig. 4). This observation, which was reported previously by our group with chitosan nanoparticles (Vllasaliu et al., 2010), was reflected in the cytotoxicity study. A dose-dependent decline in relative cell viability was apparent in both cell lines following incubation with chitosan, however, the effect was significantly larger in Calu-3 cells. These observations were largely in agreement with the TEER data, suggesting that TEER measurement can be used as a reasonably accurate indication of cytotoxicity, in addition to testing tight junction modulation. The combination of cell viability and TEER data therefore suggests that the susceptibility of the employed airway and intestinal cell lines to chitosan is highly and significantly different.

It is generally accepted that the toxicity of chitosan in cell cultures is dependent upon numerous factors, including the degree of deacetylation, molecular weight and salt form (Schipper et al., 1996). A study by Kudsiova and Lawrence (2008) reported major cytotoxicity following the application of chitosan at pH 5.8 in Caco-2 cells; in bronchial 16HBE14o-cells, however, high-level toxicity was observed at the higher pH 6.5. Considering that chitosan exhibits a pH- or ionisation level-dependent toxicity (higher toxicity in more acidic environments), this study therefore indicates that the bronchial 16HBE14o-cell line is more susceptible to chitosan toxicity, which reflects our observation that an alternative airway-derived cell line is more sensitive towards chitosan than the intestinal Caco-2 cells. Other studies assessing the toxicity of chitosan in cell lines have reported variable outcomes (Silva et al., 2006; Dodane et al., 1999). Considering the toxicity of chitosan to pulmonary cell lines, it has been reported that application of chitosan solution at 1.5% (w/v) reduced the viability of Calu-3 cells to around 68% compared to control (Florea et al., 2006). Chitosan nanoparticles were shown to suppress the viability of A549 cells (human alveolar epithelial carcinoma) to around 70% at 0.1% (w/v) (Huang et al., 2004) and chitosan microparticles induced pro-inflammatory responses in rat lungs (Huang et al., 2005). However, other studies have reported a low toxicity of different formulations of chitosan in respiratory cell lines (Grenha et al., 2007; Smith et al., 2005). The variability in the toxicity data reported with chitosan can be explained by the differences in the experimental parameters between studies from different laboratories (and our work), making comparisons between different studies problematic. Despite the in vitro observations, in vivo studies testing the toxicity of chitosan (or its derivatives) have generally reported much lower, if any, detrimental effects (Banerjee et al., 2002; Gades and Stern, 2003; Rao and Sharma, 1997; Richardson et al., 1999; Sonaje et al., 2009; Zhang et al., 2008) and the general acceptance that chitosan is a safe material in the field of drug delivery is justified.

Application of chitosan produced a dramatic reduction in TEER in both cell lines, indicating a strong tight junction-opening effect (Fig. 4a). Tight junction opening was confirmed in permeability experiments where a significant increase in FD4 permeability was observed in both cell lines (Fig. 5), with the effect being somewhat larger in Calu-3 layers (11-fold) compared to Caco-2 monolayers (7-fold), despite the application of much lower concentration in the former scenario. It must be noted that although the increase in permeability with chitosan may be higher for Calu-3 cells compared to Caco-2, the final P_{app} values were considerably larger in Caco-2 monolayers due to higher baseline permeability displayed by Caco-2 cells.

To establish why chitosan exhibited a weaker permeability enhancing effect in Caco-2 compared to Calu-3 cells we tested the hypothesis that the presence of mucus in Calu-3 cell layers (but not in Caco-2 monolayers) contributed to the superior tight junction opening effect. In this regard, we compared chitosan-induced TEER changes in Calu-3 layers cultured under AIC conditions, which promote the presence of an intact layer of mucus, and cells grown using LCC, where mucus layer is less likely to be preserved. The data showed that although chitosan produced a large drop in TEER in both culture conditions, the magnitude of the effect was larger (statistically significant) in mucus producing (AIC) Calu-3 layers (Fig. 4b). Similarly, permeability enhancement was found to be superior in Calu-3 cells cultured using AIC, as compared to LCC cells (Fig. 6a), therefore indicating that the presence of mucus in Calu-3 cell layers enhances the tight junction modulating effect of chitosan.

Experiments assessing whether the increase in permeability with chitosan persists after its application to the cell layers, followed by washing, tested the effect of mucus on the time scale of the tight junction-opening effect of chitosan. The data (Fig. 6b) revealed that chitosan treatment (and washing prior to the application of the solute) led to a larger permeability enhancement in Calu-3 cell layers cultured using AIC compared to LCC, implying that the presence of mucus not only promotes the permeation-increasing effect of chitosan during its presence on the epithelial layer but also leads to a longer lasting effect following its removal from the cells. This is perhaps not surprising owing to the bioadhesive nature of chitosan, leading to sustainable tight junction-opening post-application. In another experiment assessing the sustainability of the permeability-enhancing effect of chitosan we compared the epithelial barrier altering property of chitosan with that of a classical, non-mucoadhesive absorption-enhancer, EDTA, in (mucus-producing) Calu-3 cells. The permeability enhancers were again applied separately and prior to the macromolecular solute (FITC-insulin), which minimised the possible problem of complexation between chitosan and insulin (Mao et al., 2006). The data (Fig. 6c) showed that chitosan-treated cells presented a lower barrier to FITC-insulin permeability compared to cells treated with EDTA. Although this may have arisen from the extent of the initial tight junction modulation being different for chitosan and EDTA, previous work in our group has shown that these two enhancers exert a similar permeability promoting effect (Vllasaliu, 2010). Therefore, the significantly larger level of permeability enhancement in cells preincubated with chitosan is likely to have resulted from its inefficient removal from the cells with washing, resulting in a proportion of the applied dose being present on the surface of mucus-producing cells, exhibiting a longer-lasting effect on the tight junctions.

To confirm that the difference in the level of chitosan-induced permeability enhancement in AIC and LCC grown Calu-3 cell layers is solely due to the presence of mucus and not other differences in cell characteristics resulting from non identical culture conditions, we treated the AIC Calu-3 cells with a mucolytic agent, NAC, for mucus removal prior to the application of chitosan. In NAC-treated (mucus deprived) cells chitosan enhanced FD4 permeability in a less efficient manner (Fig. 6d) compared to mucus-preserved cells. Together the data therefore suggests that the presence of mucus on the epithelial surface may facilitate the permeability promoting effect of chitosan. Our findings do not agree with those reported by Schipper et al. (1996) where the presence of an intact mucus layer in HT29-H cells was reported to reduce the absorption-enhancing effect of chitosans, a phenomenon explained to occur through charge interaction between chitosan and mucus (with the positive charge of chitosan being essential for absorption enhancement), or mucus possibly presenting a diffusion and enzymatic barrier, preventing contact of chitosan with epithelial cells. The difference in observations between our work and the study above could be related to the extent of mucus presence in the epithelial cell models. Whilst Schipper et al. (1996) observed that a layer of mucus 40–60 μm thick almost completely covered the HT29-H cell layer, we show that the distribution of the secretory mucin (MUC-5A) in Calu-3 cell layers was patchy and did not completely cover the cells (Fig. 8ai). It is possible that the conditions in Calu-3 cell layers provide a favourable environment for the action of chitosan on the tight junctions, with the thin and incomplete layer of mucus promoting an intimate interaction of chitosan with the epithelial cells (through mucoadhesion), whilst preserving the positive charge (due to low mucus levels) which is important for the effect of chitosan on the tight junctions. This is shown schematically in Fig. 9. It must also be noted here that administration of peptide formulations containing chitosans nasally in vivo (in sheep) and in situ intestinal administration in rats was associated with remarkably improved peptide absorption compared to controls (Illum et al., 1994; Lueßen et al., 1996), therefore demonstrating that the presence of mucus in absorption sites in these experiments did not affect the absorption enhancing effect of chitosan.

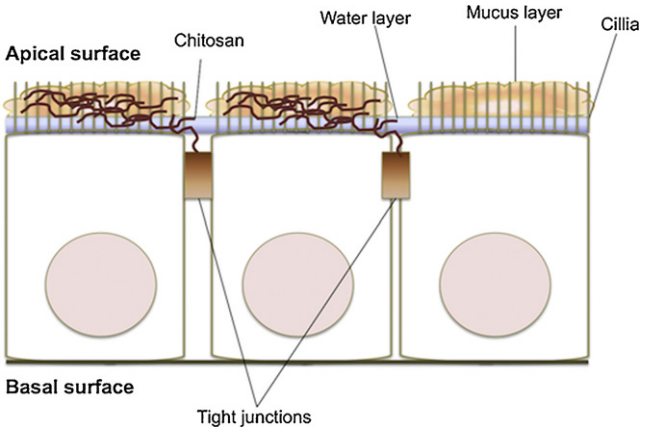

Fig. 9. Schematic representation of the perceived interaction of chitosan with mucus-producing Calu-3 layers. Chitosan is strongly mucoadhesive and binds to mucus present on the apical side of the cells. The distribution of mucus as a thin and incomplete layer 'covering' the cells leads to chitosan localisation at close proximity to the plasma membranes and the tight junctions, where it exerts an effect on epithelial tight junctions, altering the epithelial barrier.

The role of mucus on cell association of chitosan was examined following the application of FITC-g-chitosan to the cell layers with a different extent of mucus. Fluorescence was clearly apparent in all scenarios, even after extensive washing following the exposure of the cells to fluorescent chitosan. However, larger and brighter areas of fluorescence, indicating a greater content of fluorescent material, were seen in Calu-3 cells cultured under conditions that facilitate the presence of mucus in cell cultures (Grainger et al., 2006) compared to those limiting mucus presence and non mucus-producing Caco-2 cells (Fig. 7). This can be explained by the larger levels of mucus leading to a greater retainment of chitosan on the apical surface of the cells, as reported previously for chitosan nanoparticles (Behrens et al., 2002). Studying the interaction of chitosan with a mucus component, MUC-5A secretory mucus protein (mucin), we demonstrated strong association of FITC-g-chitosan with the cells in the areas of the cell layer where MUC-5A presence was also clearly evident (Fig. 8a), hence demonstrating a direct contact with a key mucus component. In Calu-3 cells subjected to treatment with NAC prior to incubation with FITC-g-chitosan a dramatically reduced level of association of FITC-g-chitosan with the cells was clearly evident, presumably due to diminished presence of MUC-5A in the cell layer, which was obvious (Fig. 8b). This confirms the notion that the association of chitosan with the cells is largely due to its mucoadhesive properties (Fiebrig et al., 1994).

Overall our work demonstrates interesting differences in the way chitosan interacts with different epithelial cell lines. In mucus-producing cells modelling the airways, chitosan exerts its potent tight junction-opening effects at remarkably low concentrations – significantly lower compared to the dose applied in non-mucus producing cells of the intestinal origin. However, the same trend applies to toxicity, with Calu-3 cells exhibiting high susceptibility at concentrations as low as 0.006% (w/v). We suggest that these phenomena may partly be explained by the capacity of the cells to produce mucus. In Calu-3 cells, where the presence of a mucin was clearly demonstrated, positively charged chitosan strongly interacted with and adhered to the negatively charged mucus. This strong interaction with mucus could in turn mean that chitosan was in close vicinity to the epithelial cell components, including the tight junctions, allowing exertion of tight junction-opening effects. The same principle may apply to cell toxicity, with strong mucoadhesion producing local high concentrations of the polycation, in addition to promoting localisation of chitosan in the close proximity to the plasma membranes, which could lead to increased points of contact and enhanced interaction with the epithelial plasma membranes or other cell components, producing toxic effects.

5. Conclusion

Calu-3 and Caco-2 cultures displayed a significantly different sensitivity towards chitosan. However, chitosan produced a powerful effect on the tight junctions of both cell lines, despite the cytotoxicity-determined dosage adjustment for each cell line. The interaction of chitosan with the cells and its absorption-promoting capacity are strongly dictated by the presence of mucus on the epithelial surface, with data suggesting that mucus-rich epithelial surfaces are more sensitive to the tight junction opening effects of chitosan. The application of chitosan as an excipient intended to improve the absorption of therapeutic macromolecules across the mucosal surfaces may therefore require dosage optimisation (for optimal absorption enhancement without toxic effects) depending on the nature (including the extent of mucus presence) of the mucosal surface.

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