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Buccal penetration enhancement properties of *N*-trimethyl chitosan: Influence of quaternization degree on absorption of a high molecular weight molecule

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Abstract

The aim was to evaluate the influence of the degree of quaternization of *N*-trimethyl chitosans (TMCs) on the mucoadhesive and penetration enhancement properties towards buccal mucosa. Fluorescein isothiocyanate dextran (MW 4400 Da) (FD4) was used as model molecule.

TMCs, obtained from chitosans of different MW (1460 and 580 kDa, respectively), were hydrated in distilled water and in pH 6.4 phosphate buffer (simulating the buccal fluid).

The polymer solutions were subjected to mucoadhesion measurements towards bovine submaxillary mucin dispersion and porcine buccal mucosa and to FD4 permeation tests through porcine cheek epithelium.

The trimethylation of chitosan allows maintenance or improvement of the mucoadhesive properties of the starting chitosans dependently on quaternization degree. In particular, the mucoadhesive properties increase on increasing degree of quaternization. The trimethylation does not produce any change in chitosan penetration enhancement properties when the medium is distilled water while if pH 6.4 buffer is used, the trimethylation produces an improvement in chitosan penetration enhancing effect.

TMC derived from the lower MW chitosan and characterized by the highest degree of quaternization shows the best mucoadhesive and penetration enhancement properties and is the most promising TMC to improve the bioavailability of hydrophilic and large MW molecules (like peptides and proteins) when administered via buccal route.

Keywords: Trimethyl chitosan; Quaternization degree; Mucoadhesive properties; Penetration enhancement properties

1. Introduction

Advances in biotechnology have made therapeutically active peptides and proteins readily available for

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therapeutic use. This has focused the attention on the problems related to delivery of such drugs. In particular the critical issue regards their poor bioavailability when administered via oral route. In fact, the rapid hydrolytic and enzymatic degradation that peptides and proteins meet in the gastrointestinal tract and the "first pass" elimination and the poor permeability of such drugs render oral administration impractical (Chien, 1992). The general approach to deliver peptides and proteins is the parenteral route, which is invasive and presents a poor compliance of the patients. Recently, a great deal of attention has been devoted in the pharmaceutical field to the development of drug delivery systems for administration of peptides and proteins through routes other than the oral one. The buccal route is a valid alternative to the oral one and offers many advantages (Shojaei, 1998). In particular, it improves the drug bioavailability due to the avoidance of first pass metabolism and of degradation in the gastrointestinal tract. Moreover buccal route is easily accessible and more acceptable to the patient with respect to the parenteral one. The permeability barrier, together with the small area available to drug absorption, still remains the main limitation to the protein or peptide delivery via buccal route (Sayani and Chien, 1996).

Chitosan is a naturally occurring, non-toxic, biocompatible and biodegradable polysaccharide (Paul and Sharma, 2000). For its good mucoadhesive properties (Lehr et al., 1992; Henriksen et al., 1996), chitosan has been employed in mucosal site-specific systems (Sinswat and Tengamnuay, 2003). Moreover, chitosan has been shown to be a potential penetration enhancer. In particular, chitosan showed penetration enhancement properties towards either monostratified or pluristratified epithelia. In particular, it is able to enhance drug absorption of hydrophilic high molecular weight molecules through intestinal (Artursson et al., 1994; Dodane et al., 1999) and nasal (Hamman et al., 2002) mucosae (monostratified and endowed with tight junctions), drug permeation across buccal (Senel et al., 2000; Rossi et al., 2003a; Sandri et al., 2004) and vaginal (Rossi et al., 2003b) mucosae (pluristratified and lacking tight junctions) and across corneal epithelium (Di Colo et al., 2004) (pluristratified and with tight junctions) (Yi et al., 2000; Reichl and Muller-Goymann, 2003). In those epithelia that are rich in tight junctions, the mechanism of penetration enhancement by chitosan is mainly due to a transient widening of the junctions between cells (Dodane et al., 1999), while the mechanism of penetration enhancement across pluristratified epithelia, which lack tight junctions, has still to be clarified (Kotzé et al., 1998; Senel et al., 2000; Senel and Hincal, 2001).

The effectiveness of chitosan as penetration enhancer is impaired by its insolubility at pH above 6 (for example, at the physiological pH of the buccal cavity). An attempt has been made to solve the chitosan solubility problems by synthesizing the partially quaternized derivative *N*-trimethyl chitosan chloride (TMC), which is soluble irrespective of pH and has proved a potent penetration enhancer of hydrophilic and/or high molecular weight molecules across the intestinal epithelium (Kotzé et al., 1997, 1998; Van Der Merwe et al., 2004), and also, of poorly water-soluble drugs, such as ofloxacin, across the cornea (Di Colo et al., 2004) in neutral environments. TMC acts on epithelial cell monolayers by means of the same mechanism shown by chitosan: opening the tight junctions between cells and favouring the paracellular drug transport. The TMC charge density, determined by the quaternization degree, and the molecular weight have been extensively studied as key factors to obtain both the mucoadhesion (Sinswat and Tengamnuay, 2003) and the penetration enhancement towards intestinal (Kotzé et al., 1997, 1998, 1999; Jonker et al., 2002), nasal (Hamman et al., 2002) and corneal (Di Colo et al., 2004) epithelia.

Until now no studies have been performed on buccal mucosa. Such a mucosa differs from the above mentioned epithelia for the reduced number of tight junctions and for the presence of a extracellular matrix rich in neutral and/or polar lipids that are the main barrier to drug absorption via paracellular route (Harris and Robinson, 1992). For these reasons, in the present work, it seemed interesting to evaluate the influence of the quaternization degree on the mucoadhesive and penetration enhancement properties of *N*-trimethyl chitosans towards buccal mucosa. The trimethyl chitosans were obtained from two chitosans characterized by different MWs. The chitosans were subjected to a methylation reaction to obtain two series of trimethyl chitosans called H and L. Both series were composed by three trimethyl chitosans that differed for the quaternization degree. The mucoadhesive and penetration enhancement properties of the polymers were tested upon hydration in distilled water, which allows the solubilization of all the polymers, and

in pH 6.4 phosphate buffer, which simulates the buccal fluid but not allows chitosan solubilization. Fluorescein isothiocyanate dextran, a hydrophilic molecule (MW 4400 Da) (FD4) having a MW comparable with that of peptides extensively used in therapeutics (such as insulin (5733 Da) and calcitonin (3417 Da)) was used as model molecule.

A rheological characterization of the polymer solutions prepared in the two different media was performed. Mucoadhesion measurements were carried out by means of a tensile stress tester using pig cheek mucosa as biological substrate. Bovine submaxillary mucin was also employed to verify if a commercial mucin (ready to use) could be a suitable substrate to investigate the mucoadhesive properties of the polymers studied. The polymer penetration enhancement properties were investigated by means of a Franz diffusion cell apparatus using pig cheek mucosa as model membrane. In order to verify the suitability of the in vitro/ex vivo model the permeability of fluorescein isothiocyanate dextran in aqueous solution was also tested.

2. Materials and methods

2.1. Materials

The trimethyl chitosans were obtained from two chitosans 90% deacetylated, one of higher MW (1460 kDa), from crab shells (ChH) (Sigma I), the other one of lower MW (580 kDa), from shrimps (ChL) (Chito-clear FG90, Primex, N) using procedures described in the literature (Sieval et al., 1998). Two series of polymers were obtained. The series called H, characterized by higher MW, included the starting chitosan hydrochloride ChH HCl and the three trimethyl chitosans that differed from quaternization degree (QD): TMChH1 (QD = 4%), TMChH2 (QD = 35%), TMChH3 (QD = 90%). In parallel the series called L, characterized by lower MW, included the starting chitosan hydrochloride ChL HCl and the three trimethyl chitosans: TMChL1 (QD = 3%), TMChL2 (QD = 46%), TMChL3 (QD = 78%). All the polymers were previously characterized by Di Colo et al. (2004).

Fluorescein isothiocyanate dextran (FD4) (Sigma, I), a hydrophilic and high MW molecule (MW 4400 Da) was used as model.

Submaxillary bovin mucin (Sigma, I) was used as ready to use biological substrate for mucoadhesion measurements.

2.2. Sample preparation

The polymers were hydrated in distilled water, medium which allowed solubilization of the starting chitosan salts and of the TMCs. The TMCs were also hydrated using pH 6.4 phosphate buffer (USP 25), to simulate the buccal environment. The starting chitosan salts were not soluble in such an environment. The polymer solutions (10 g) were prepared at a concentration equal to 4% (w/w) by gentle stirring at room temperature.

FD4 was added to each polymer solution under stirring at 0.2% (w/w) concentration.

2.3. Viscosity measurements

Each polymer solution was subjected to viscosity measurements by means of a rotational rheometer (Bohlin CS Rheometer, Bohlin Instrument Division, Metrics Group Ltd., Cirencester, UK). A cone plate combination (CP 4/20) was used as a measuring system. All measurements were carried out at 37 °C, after a rest time of 3 min.

The apparent viscosity was measured after application of 100 s^{-1} shear rate for 1 min.

2.4. Mucoadhesion measurements

The mucoadhesive properties of the polymer solutions were evaluated by means of a tensile stress tester (Ferrari et al., 1996). Cheek buccal mucosa and submaxillary bovin mucin (Sigma, I) were employed as biological substrates. Porcine cheek mucosa, obtained from a slaughterhouse, was deprived of the connective tissue with surgical scissors and stored at −20 °C before testing. The storage of mucosa does not affect the epithelial surface properties due to the glycoprotein molecules of mucin (such storage conditions are widely employed in literature (Park and Munday., 2002; Rossi et al., 2003a, 2003b; Sandri et al., 2004)). Moreover, no significant differences have been observed between the results obtained using freshly excised mucosa and mucosa stored at −20 °C.

The bovine submaxillary mucin was dispersed at 4% (w/w) concentration in pH 6.4 buffer.

Each polymer solution (100 mg) was layered on a filter paper disc (area = 2 cm^2) and fixed on the movable carriage of the apparatus.

The cheek mucosa was fixed, faced to the formulation, on the sample holder using a cyanoacrylate glue and hydrated with 100 μl pH 6.4 buffer.

The carriage was then moved until the contact between the sample holder and the movable carriage was established. A preload of 2500 mN was applied to allow the formation of the mucoadhesive joints. After a 3 min rest, the preload was removed and the movable carriage was moved forward at a constant speed (4 mm min^{-1}) up to the complete separation of the two surfaces. Both displacement of the movable carriage and force of detachment data were recorded and simultaneously collected on a personal computer.

The parameter work of adhesion (AUC) was calculated as the area under the force of detachment versus displacement curve by means of the trapezoidal rule (Ferrari et al., 1996).

The bovine submaxillary mucin (100 μl), dispersed at 4% (w/w) concentration in pH 6.4 buffer, was spread on a filter paper disc (area = 2 cm^2). The mucoadhesion measurements in presence of mucin were carried out in the same conditions as those used for measurements effected in presence of mucosa.

Blank measurements were also performed using a filter paper disc imbibed with 100 μl pH 6.4 buffer instead of the biological substrate (mucosa or mucin dispersion).

The normalized mucoadhesion parameter ΔAUC/AUC was calculated according to the following equation (Ferrari et al., 1997):

$$\frac{\Delta AUC}{AUC} = \frac{AUC_{bs} - AUC_{blank}}{AUC_{blank}}$$

where: AUC_{bs} = work of adhesion obtained in presence of the biological substrate (mucosa or mucin dispersion); AUC_{blank} = work of adhesion obtained from blank measurements.

Such a normalization allowed us to compare the mucoadhesive properties of polymer solutions characterized by different cohesive properties (viscosity) (Ferrari et al., 1997).

2.5. Permeation measurements through porcine cheek epithelium

Each polymer solution (100 mg) was subjected to permeation measurement by means of Franz diffusion cells (Permeager, USA) with a 20 mm diameter orifice (3.14 cm^2 area) thermostated at 37 °C. Fresh porcine cheek mucosa was dipped for 1 min in pH 7.4 saline isotonic solution (KH_2PO_4 1.0 g l^{-1}; Na_2HPO_4 8.10 g l^{-1}; NaCl 4.11 g l^{-1}) at 70 °C. Then the epithelium was peeled from the edges of the mucosa (Ganem-Quintanar et al., 2000). A circular epithelium membrane of 5 cm^2 area was placed between the donor and the receptor chamber of a Franz diffusion cell. Each polymer solution (100 mg) was applied on a portion of the epithelium membrane (3.14 cm^2) equal to the area of the Franz cell orifice. pH 6.4 buffer (2 ml) was added over the formulation in the donor chamber, to simulate the buccal environment, whereas pH 7.4 saline isotonic solution was used as acceptor phase.

At fixed time intervals, the acceptor phase (500 μl) was withdrawn and replaced with fresh buffer. The model macromolecule FD4 was assayed by means of the HPLC method described in the following paragraph.

The permeation test was also effected using FD4 solutions (100 μl) prepared in the media employed to hydrate the polymers.

The parameter ER ("enhancement ratio") was calculated as follows:

$$ER = \frac{Q_p(FD4)}{Q(FD4)}$$

where: $Q_p(FD4)$ = amount of FD4 permeated through buccal epithelium in presence of the polymer solution at the end of the permeation experiment (6 h); $Q(FD4)$ = amount of FD4 permeated through buccal epithelium in absence of the polymer solution at the end of the permeation experiment (6 h).

This parameter is an index of polymer capability to increase the absorption of the model macromolecule FD4 through the buccal epithelium.

2.6. FD4 assay

FD4 was assayed by means of an HPLC method modified by Kamm et al. (2000). The HPLC apparatus (Perkin-Elmer, I) was equipped with a pump (binary

pump series 200), an autosampler (series 200) and a fluorimetric detector (series 200). A C_{18} column (Hyperbond (C18 10 μm, 300 mm × 3.9 mm), Hypersil, CPS Analitica, I) was the stationary phase. The mobile phase consisted of 2.5 mM KH_2PO_4 pH 5.0 (91.5%) and acetonitrile (8.5%). The flow rate was 1 ml/min and the injection volume was 20 μl. The detection wavelengths were: excitation λ = 490 nm and emission λ = 515 nm. Data acquisition and integration were carried out with Total Chrom software (Perkin-Elmer, I). A calibration curve was carried out using FD4 concentrations ranging from 0.5 to 20 μg ml^{-1}. The method was linear in the concentration range employed and the correlation coefficient R^2 was always greater than 0.9995.

2.7. Statistical evaluation

Statistical differences were determined using one-way ANOVA and post hoc Sheffe test for multiple comparisons (Siphar, Creteil, F). Differences between groups were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Viscosity properties

Fig. 1(a) and (b) shows the viscosity values (at 100 s^{-1} shear rate) measured for the polymers belonging to series H and L hydrated, respectively, in distilled water and in pH 6.4 buffer.

As expected, in both media the viscosity values of series H were higher than those of the series L. For both series the starting chitosans showed the highest viscosity values. The viscosity of TMC solutions decreased on increasing the quaternization degree. This could be due to a partial *O*-methylation, which occurred in the reaction conditions (Di Colo et al., 2004). Both *O*-methylation and *N*-trimethylation should cause a decrease in the TMC capability of forming hydrogen bonds with respect to starting chitosan salt (Hamman et al., 2002). Such a phenomenon implies an increase in polymer chain entanglement and a consequent decrease in viscosity properties. For all the polymers, the solutions prepared in distilled water are characterized by viscosity values higher than those observed for the solutions prepared in pH 6.4 buffer. This could be due to the shield effect of pH 6.4 buffer ions on the quaternized nitrogen: the decrease in the repulsion between the positively charged amino groups produces a decrease in viscosity.

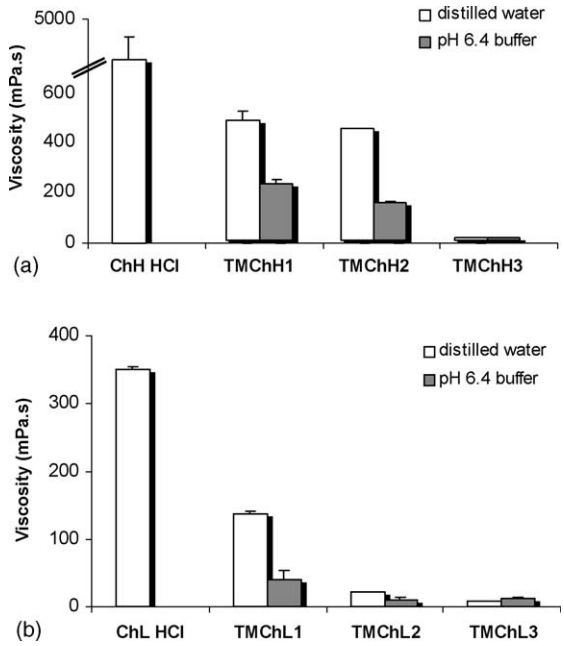

Fig. 1. Viscosity values of the series H (a) and L (b) (mean values ± S.D.; $n = 3$).

3.2. Mucoadhesion properties

Fig. 2 shows the results of the mucoadhesion measurements performed on series H hydrated in distilled water using porcine buccal mucosa and bovine submaxillary mucin as biological substrates. When mucin was used as biological substrate, TMChH3 showed the

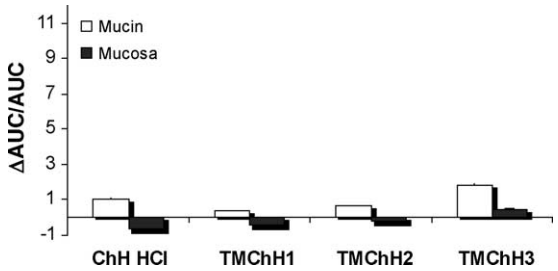

Fig. 2. ΔAUC/AUC values of the series H polymer hydrated in distilled water (mean values ± S.E.; $n = 8$. The error bars were calculated by means of the propagation error theory).

best mucoadhesive performance (as indicated by the highest value of ΔAUC/AUC), even better than that of the starting chitosan ChH HCl ($p < 0.001$). The mucoadhesive properties of TMCs increased on increasing the quaternization degree. This seems to be in disagreement with the results obtained by Snyman et al. (2003), who observed a decrease in maximum force of detachment on increasing quaternization degree. It is necessary to underline that the authors employ a parameter not normalized, which, then, takes into account also the intrinsic cohesive properties of the polymers. The different consistency of TMCs resulting from different quaternization degree could be in part responsible of the results obtained. The use of the normalized ΔAUC/AUC parameter allows to eliminate the contribution of sample consistency in the evaluation of the mucoadhesive potential.

In presence of mucosa as biological substrate, the starting ChH HCl showed a negative value of the normalized parameter ΔAUC/AUC, lower than those observed for TMCs. As observed in presence of mucin, the increase in quaternization degree produced an increase in ΔAUC/AUC values, even if only TMChH3 was characterized by a positive ΔAUC/AUC value.

Fig. 3 shows the results of the mucoadhesive measurements performed on series L hydrated in distilled water using porcine buccal mucosa and bovine submaxillary mucin as biological substrates. In presence of mucin, ChL HCl was characterized by the best mucoadhesive properties ($p < 0.001$), while TMCs presented comparable and positive values of the mucoadhesive parameter. When the mucoadhesive properties were evaluated in presence of buccal mucosa, ChL HCl showed the lowest value of the mucoadhesive parameter ($p < 0.05$). Analogously to that observed for series H, the mucoadhesive performance increased on increasing the quaternization degree.

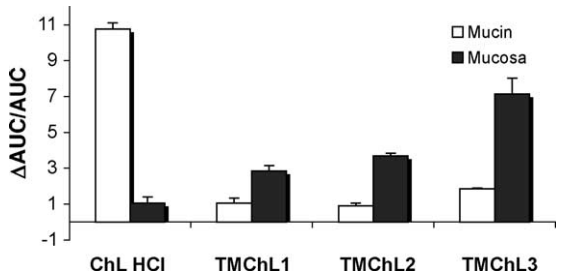

Fig. 3. ΔAUC/AUC values of the series L polymer hydrated in distilled water (mean values ± S.E.; $n = 8$. The error bars were calculated by means of the propagation error theory).

The differences in mucoadhesive properties observed for both the series in presence of the two substrates are probably due to the better buffering capability of the mucosa with respect to the mucin dispersion. The starting chitosans were always characterized by better mucoadhesive performance in presence of mucin: this is probably due to polymer poor solubility properties at the mucoadhesive interface when mucosa is used as substrate. The negative values observed for TMChH1 and TMChH2 are probably due to the same effect: the trimethylation degree, given the high molecular weight of the polymers, is not sufficient to increase the polymer solubility on the mucosal surface, moreover the high polymer molecular weight can be responsible for a lacking interpenetration between the polymer chains and the mucin. A similar trimethylation degree on lower molecular weight polymers (TMChL1 and TMChL2) results in positive values of the normalized mucoadhesive parameter.

Contrary to that observed for series H, TMCs of series L are characterized by better mucoadhesive properties in presence of mucosa than in presence of mucin: in absence of solubility problems at the mucoadhesive interface, the buccal mucosa provides a more complete substrate for interaction than mucin dispersion due to mucin glycoprotein types and concentrations: these features are also able to confer a proper pH on the mucosa/polymer interface.

Moreover, polymers of series L always showed greater values of the normalized mucoadhesive parameter: this behaviour probably results from a deep interaction between the polymer chains and the mucin glycoprotein of mucosa. The mucoadhesive joint is, in fact, favoured by chain flexibility, which allows the polymer to better interpenetrate the mucin chains.

Figs. 4 and 5 show the results of the mucoadhesive measurements carried out on H and L series, respectively, upon hydration in pH 6.4 buffer. Also at pH 6.4 the increase in quaternization degree produced better mucoadhesive properties for both series independently of the substrate used (mucin dispersion or mucosa). While the change in hydration medium did not affect the mucoadhesive performance of TMCs of series H, TMCs of series L were characterized by a better mucoadhesive performance when distilled water was used. This is due to the fact that TMCs of series H had poor

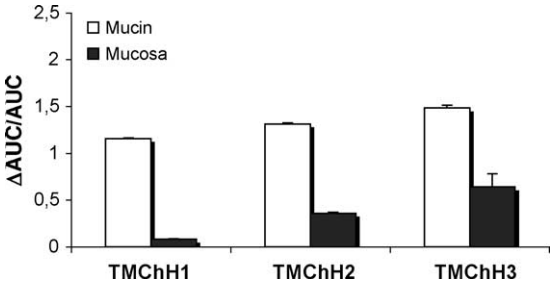

Fig. 4. ΔAUC/AUC values of the series H polymer hydrated in pH 6.4 (mean values ± S.E.; $n = 8$. The error bars were calculated by means of the propagation error theory).

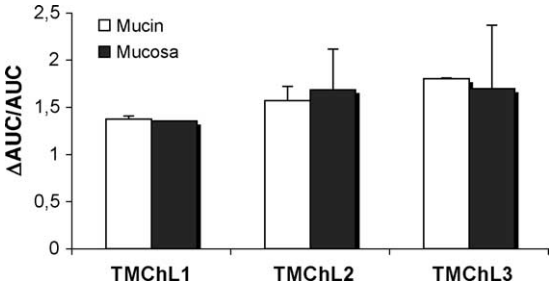

Fig. 5. ΔAUC/AUC values of the series L hydrated in pH 6.4 buffer (mean values ± S.E.; $n = 8$. The error bars were calculated by means of the propagation error theory).

solubility at the mucoadhesive interface also when hydrated in distilled water.

3.3. Permeation measurements through porcine cheek epithelium

Figs. 6 and 7 show FD4 permeation profiles of polymers of series H and L, respectively, hydrated in distilled water in comparison with a FD4 solution hydrated in the same medium.

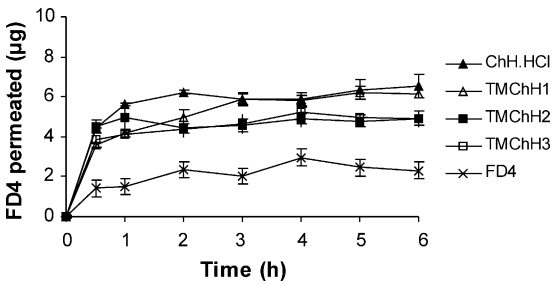

Fig. 6. FD4 amount (μg) permeated across buccal epithelium vs. time profiles of the series H and the FD4 solution hydrated in distilled water (mean values ± S.E.; $n = 6$).

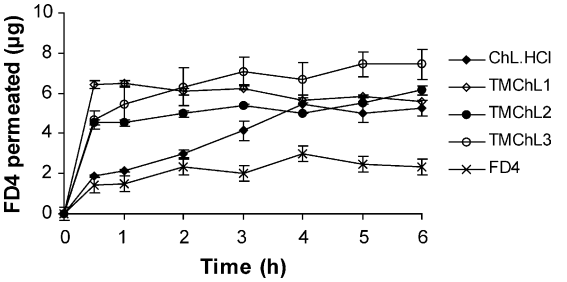

Fig. 7. FD4 amount (μg) permeated across buccal epithelium vs. time profiles of the series L and the FD4 solution hydrated in distilled water (mean values ± S.E.; $n = 6$).

The FD4 solution showed the lowest permeation profile: only 2 μg of FD4 permeated across the epithelium in 6 h. This indicates that, as expected, the permeation of the model macromolecule through the buccal epithelium was poor and difficult in absence of a penetration enhancer. The TMCs and the starting chitosan of series H showed comparable permeation profiles (Fig. 6). This indicates that when distilled water is used as hydration medium, the trimethylation does not produce any significant effects on the penetration enhancement properties of the starting chitosan.

TMCs of series L were characterized by FD4 permeated amount after 6 h comparable to that observed for the starting chitosan (Fig. 7). However, the permeation profiles of TMCs were characterized by a faster induction of the penetration enhancement effect than the starting chitosan: in particular the TMCs reached the maximum in the penetration enhancing effect within 1 h while the starting chitosan took 4 h to produce the same effect.

Figs. 8 and 9 show FD4 permeation profiles of TMCs of series H and L, respectively, hydrated in pH 6.4 phosphate buffer, in comparison with a FD4 solution hydrated in the same medium.

Also in pH 6.4 buffer, FD4 solution showed the lowest permeation profile; this confirms that the model macromolecule poorly crossed the buccal epithelium in absence of a penetration enhancer independently of the hydration medium employed.

For both series H and L, the increase in quaternization degree produced an increase in the penetration enhancement properties of TMCs: TMChH3

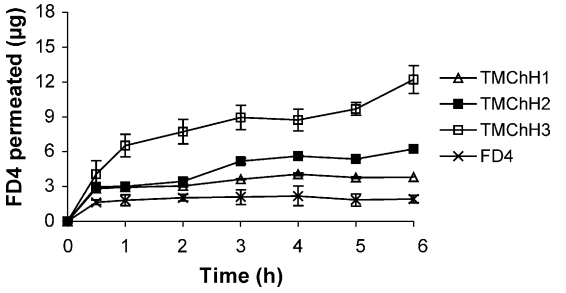

Fig. 8. FD4 amount (μg) permeated across buccal epithelium vs. time profiles of the series H and the FD4 solution hydrated in pH 6.4 buffer (mean values ± S.E.; $n = 6$).

and TMChL3 presented the highest FD4 permeation profiles.

The different profiles of FD4 permeated versus time obtained for the polymer solutions are probably due to the different dismantling action of chitosans and TMCs on the cell multilayer of the buccal epithelium. These profiles which level off in the first hour, suggest that the effect has a limited duration: in particular the interference of the polymer with the deeper cell layers is probably transient and the basal cells of the epithelium can partially recover their structure if their exposure to the polymer is prolonged. Only the most effective polymers (TMChH3 and TMChL3) are capable of sustaining the penetration enhancement effect throughout the experiment duration time.

Fig. 10 shows the values of the ER parameter calculated for series H (a) and the L (b) hydrated either in distilled water or in pH 6.4 buffer.

The polymers belonging to both series, independently of the hydration medium, showed ER values greater than 2 indicating the capability of the polymers

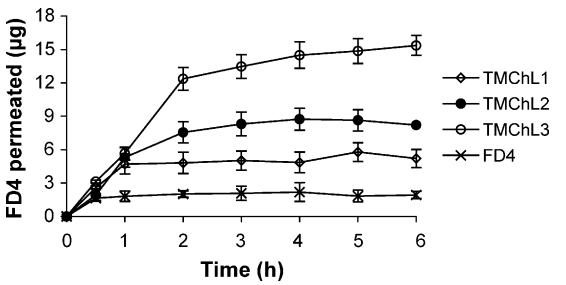

Fig. 9. FD4 amount (μg) permeated across buccal epithelium vs. time profiles of the series L and the FD4 solution hydrated in pH 6.4 buffer (mean values ± S.E.; $n = 6$).

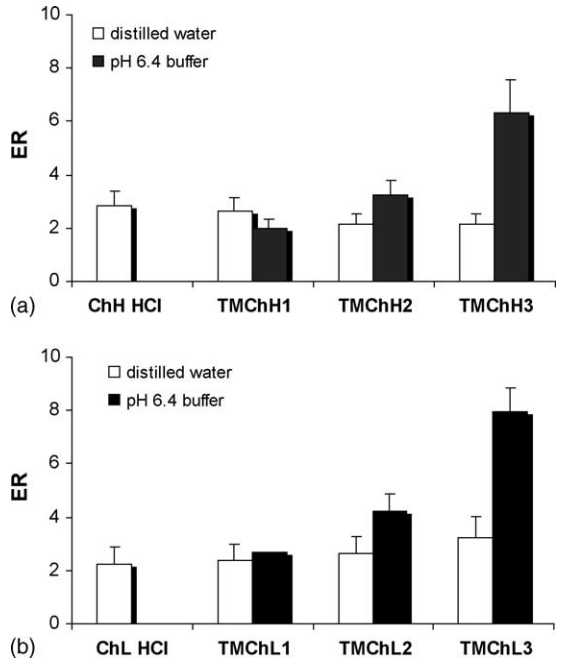

Fig. 10. ER parameter values calculated for the H (a) and L (b) series and FD4 solution (mean values ± S.E. $n = 6$). The error bars calculated by means of the propagation error theory).

to increase more than twofold the FD4 amount that crossed the buccal epithelium.

Polymers of series H and L showed comparable ER values when distilled water was used as hydration medium. The increase in quaternization degree does not produce any significant change in ER value when the polymers were hydrated in distilled water. When pH 6.4 buffer was used, ER increased on increasing quaternization degree independently of the series considered. The TMCs (TMChH3 and TMChL3) with the maximum trimethylation degree presented the highest ER values ($p < 0.05$). In particular TMChL3 was characterized by an ER value equal to about 8, greater than that observed for TMChH3.

4. Conclusions

The starting chitosans were characterized by higher viscosity than those of TMCs; in particular the TMC viscosity decreased on increasing the quaternization degree independently of the hydration medium employed (distilled water or pH 6.4 phosphate buffer).

The *O*-methylation and the *N*-trimethylation probably produce a decrease in TMC capability of forming hydrogen bonds (Snyman et al., 2003). This results in a decrease in the viscosity properties. Each TMC hydrated in distilled water showed viscosity higher than the same polymer hydrated in pH 6.4 buffer. The shield effect of pH 6.4 buffer ions on the quaternized nitrogen probably produces a decrease in the repulsion between the positively charged amino groups causing a decrease in the viscosity.

The trimethylation of chitosan allowed the overcoming of the poor solubility properties of the starting chitosans at pH close to neutrality and the preservation or the improvement of the mucoadhesive properties of chitosan dependently on the TMC quaternization degree. In both media and independently of the biological substrate used, the mucoadhesive performance increased on increasing the quaternization degree. The differences observed in the mucoadhesion parameter, when the two different substrates are used, indicate that commercial mucin does not always represent a suitable alternative to mucosa, especially when polymers characterized by a pH dependent solubility are considered.

Moreover, the *N*-trimethylation did not produce any changes in the penetration enhancement properties of FD4, the model macromolecule, when distilled water was used as hydration medium, while it improved the penetration enhancement capability when pH 6.4 buffer was used. Since chitosan already presents a high affinity for water, it is likely that the trimethylation does not produce an increase in solvent affinity and consequently an improvement in penetration enhancing properties. On the contrary, at pH 6.4 the chitosan trimethylation produces an improvement in polymer solubility, which results in better penetration enhancing properties.

The series L was characterized by better mucoadhesion and penetration enhancement properties than those of series H. In particular, the TMC characterized by the lower MW and by the highest quaternization degree presented the best mucoadhesive and penetration enhancement properties. The charge density and the space conformation of TMChL3 are likely to be optimal for a deep interaction with the buccal mucosa and with the epithelial structures, the main barriers against the drug permeation (Hamman et al., 2003). The mucoadhesion seems to play a key role to enhance the drug absorption; the polymer mucin interactions responsible for the formation of the mucoadhesive joint are likely to be involved in penetration enhancement mechanism. The polymer/mucin interpenetration probably weakens the epithelium barrier, perhaps partially dismantling the structure of the extracellular matrix and the intercellular joint.

TMChL3 seems to be the most promising polymer for the development of a drug delivery system intended for a mucosal buccal application to enhance the buccal absorption of hydrophilic macromolecule such as peptides with MW lower or close to that of FD4 (insulin 5733 Da, calcitonin 3417 Da, buserelin 1239 Da, leuprolide 1209 Da).

Further investigations are in progress to study the mechanism of TMC penetration enhancement and the epithelium structures involved in the enhancement effect.

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