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Permeation modulating properties of natural polymers – Effect of molecular weight and mucus

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ABSTRACT

The permeation modulating effects of 5 natural polymers; low-ester, amidated and high-ester pectin, as well as hyaluronic acid and chitosan were tested at two different molecular weights each. The model membrane was methotrexate treated HT29 cells grown for 2 or 3 weeks, respectively, thereby differing in the amount of goblet cells and hence mucus.

The pectins decreased the permeation of the paracellular marker carboxyfluorescein. Free acid groups and a high molecular weight increased this membrane protective effect. Chitosan displayed pronounced and hyaluronic acid modest permeation enhancing properties. In this case, a low molecular weight accentuated the effect. In all cases, the permeation modulating properties were reduced by mucus.

1. Introduction

The inclusion of permeation enhancers in a drug formulation can increase the absorption of poorly absorbed drugs, for example administered to the gastrointestinal tract. Many chemically different excipients have been tested either for para- or trans-cellular enhancement, or both. One group is chitosans and other mucoadhesive polymers. Negatively charged polymers, by chelating calcium, can affect tight-junctions, and thereby increase the paracellular transport of hydrophilic substances of large molecular weight like proteins and peptides (Aungst, 2000). Chitosan is an amine, and will be positively charged at lower pH values (pKa: 5.5–7.0). It is generally accepted that positively charged chitosan has shown some very promising abilities to increase the paracellular transport, and that this effect is limited by the higher pH of the small intestine, dramatically reducing the solubility of chitosan (Thanou et al., 2001).

It is not uncommon that the permeation enhancing effect is related to toxicity (Aungst, 2000). Natural polymers, like polysaccharides, are generally considered safer *in vivo*, and several of them are already in use as excipients in drug formulations. However, the molecular weight (Mw) is an important parameter, and can have an impact on their behavior. For many relevant properties, there is probably an optimal Mw.

Often, Caco-2 monolayers are used as model membranes for the initial screening of the permeation enhancing properties of excipients. Despite that fact that this well known and characterized model is widely accepted, there are some limitations. One of them is that Caco-2 cells do not produce mucus. *In vivo*, all mucus membranes are covered by a thick layer of mucus, and this barrier is often underestimated in *in vitro* studies. Even so, it has been suggested that the lower toxicity observed *in vivo* compared to *in vitro* was due to the mucus layer (Aungst, 2000). This is in line with the purpose of the viscoelastic mucus layer, i.e. protection in addition to lubrication. Mucus has been identified as a barrier to for example the hydrophobic drug testosterone (Behrens et al., 2001), although this effect was not seen by Pontier et al. (2001). Despite these conflicting results, there seem to be a consensus that mucus can be a barrier to larger particulates, like some bacteria and nanoparticles (Cone, 2009). Theoretically, mucus can also be a barrier to polymers.

The HT29 cell-line is, like Caco-2 cells, a human colon adenocarcinoma cell-line that spontaneously differentiate into monolayers of polarized enterocytes connected by tight-junctions, and is hence suitable for permeation studies. Methotrexate (MTX) treated clones have been shown to, postconfluently, differentiate into a mixed population of mucus-secreting goblet cells and enterocytes (Lesuffleur et al., 1990).

In this study, the permeation modulating properties of natural polymers were tested on HT29-MTX cells cultured for different period of times, and hence with different amount of goblet cells and mucus. In this way, the effect of mucus was tested. The 3 main commercially available types of pectin were tested, differ-

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ing in their amount of methoxylation and amidation. These pectins then contain a different amount of free acid groups which can crosslink calcium-ions, and hence have a potential effect on the tight-junctions. Two other well known permeation enhancing polymers were also tested; negatively charged hyaluronic acid and chitosan which carries amine groups. In all cases the testing was carried out at a physiological pH of 7.4. In addition to testing the effect of mucus, also the effect of reducing the molecular weight of the polymers was investigated. Cells were grown on Transwell filters, and carboxyfluorescein was used as a paracellular marker, in addition to measuring the transepithelial electrical resistance (TEER). Pictures were taken of the cell membrane to confirm the development of mucus. To my knowledge, this is the first time the permeation modulating effects of different types of pectin have been systematically tested.

2. Materials and methods

2.1. Materials

The test polymers were chitosan, hyaluronic acid and 3 types of pectin. The following information was provided by the manufacturers. Hyaluronic acid sodium salt (HA) from *Streptococcus equi*, batch 1420500, was purchased from Sigma–Aldrich and used as received. Mw: 1.63×10^3 kDa. Chitosan of medium molecular weight, 75–85% deacetylated, batch MKBC0060, was purchased from Sigma–Aldrich and used as received. Mw range: 190–310 kDa. The 3 types of pectin were derived from citrus and kindly provided by the manufacturer (CP Kelco, Denmark), and used as received. Low-ester pectin (LM pectin), brand LM-12 CG-Z, batch GR84468, had a degree of (amount of substituted sites/amount of monomers × 100 (%)) methoxylation of 33%. Amidated pectin, brand LM-102 AS-Z, batch S73081, had a degree of methoxylation of 30% and a degree of amidation of 19%. High-ester pectin (HM pectin), brand Type B Rapid Set-Z, batch: GR82670, had a degree of methoxylation of 70%. The paracellular marker was 5(6)-carboxyfluorescein (Sigma–Aldrich). Mw: 376.32 Da

The HT29-MTX cell-line was kindly provided by Dr. ThéclaLesuffleur (INSERM UMR S 938, Paris, France). These mucus-secreting cells have been adapted to and cultured for several passages in medium containing 10^{-6} M methotrexate (MTX) and reversed for several passages in drug-free medium (Lesuffleur et al., 1993). They do not need to be maintained in media containing MTX in order to differentiate after confluency.

Medium for cell growth: Dulbecco's Modified Eagle's Medium–high glucose (Sigma–Aldrich) supplemented with 3.7 g/L sodium bicarbonate, pH = 7.5, further supplemented with 10% inactivated fetal bovine serum (Sigma–Aldrich), penicillin (100 units/ml) + streptomycin (100 μg/ml) (Sigma–Aldrich) and 2 mM GlutaMAX™-I Supplement (Invitrogen).

Medium for permeation experiments: Hank's balanced salt solution (Sigma–Aldrich) supplemented with 1.26 mM $CaCl_2 \times 2\ H_2O$, 3.98 mM $MgSO_4 \times 7\ H_2O$ and 4.17 mM $NaHCO_3$, pH = 7.4 (HBSS++ buffer).

All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Degradation of polymers

Solutions of 5.0 wt.% of pectin and HA in 0.1 M HCl (pH = 1) were boiled for 3 h. After cooling, the evaporated water was replaced and the pH neutralized. The degraded samples were stored frozen until use.

5.0 wt.% of chitosan in 1 M HCl (pH = 0) was stirred for 18 h, followed by dialyzing against 0.001 M HCl (pH = 3). The molecular weight cut off for the SpectraPor® dialysis membrane was 8000 Da. The HCl solution was changed once a day for 7 days. The remains after dialyzing were dried using a rotary evaporator and final air drying.

2.2.2. Viscosity measurements

The specific viscosity η_{sp} was measured using an Ubbelohde viscometer (type 501 03, Schott, Germany) at 25 ± 0.2 °C. The concentrations (C) used varied from 0.005 to 0.500 wt.%. Pectins and HA were dissolved in pure water and the pH was adjusted to 7.0, while chitosan was dissolved in 0.1 M HCl. The intrinsic viscosity $[\eta]$ was obtained from a plot of the reduced viscosity $\eta_{red}(=\eta_{sp}/C)$, versus C. The Huggins' constant k', was determined from the equation $\eta_{sp}/C = [\eta] + k'[\eta]^2 C$.

2.2.3. Cell culturing

The cells were kept in an incubator at 37 °C and 5% CO_2, and the medium was changed every day. For maintenance, the cells were passaged before 80% confluency with Trypsin-EDTA solution (Sigma–Aldrich). For the permeation experiments, cells of passage numbers 22 and 23 were used. The cells were counted using a hemacytometer and seeded at a density of 60,000 cells/cm^2 on uncoated polycarbonate Transwell filter inserts (Costar from Corning) with a 0.4 μm pore size and a cell growth area of 1.12 cm^2. The volume of the donor chamber was 0.5 ml, and the polystyrene acceptor chamber1.5 ml. Cells were seeded at the same density on polystyrene Nunclon™ cell culture dishes (Nunc A/S, Denmark) with a culturing area of 1.8 cm^2 for microscopic control.

2.2.4. Permeation studies

The cells were grown for 2 and 3 weeks, respectively, on Transwell filters before conducted to permeability studies. The TEER was higher than ~450 Ω cm^2 (filter: ~150 Ω cm^2). At the same time, pictures were taken of the control cells with a microscope (Olympus CKX41) equipped with a camera (Cool SNAP-Pro) to confirm development of mucus.

The permeation modulating properties of the polymers were tested at a concentration of 0.50 wt.% in HBSS++ buffer. The pH was adjusted to 7.4 again, if necessary. Un-degraded pectins and HA were dissolved in the buffer, while the degraded samples were thawed and diluted 1:10. Chitosan is not soluble at pH 7.4. The chitosan samples were therefore first dissolved in 0.1 M HCl at a concentration of 5.0 wt.%, before diluting 1:10 in HBSS++ buffer and adjusting the pH. Then, carboxyfluorescein was mixed with the polymer samples to a concentration of 20 μM of the paracellular marker.

The experimental design involved 3 parameters: polymer type (LM, amidated and HM pectin, HA and chitosan), Mw (2 levels: intact and degraded) and age of the cell monolayer (2 levels: 2 and 3 weeks), giving a total of 20 different experiments. In addition, for comparison, the permeation of 20 μM carboxyfluorescein in pure HBSS++ buffer without any polymer was used as control. Each experiment was performed in triplicate.

The filters with the cell-monolayers were washed and placed in wells containing 1.5 ml of preheated HBSS++ buffer in the acceptor chambers. 0.5 ml of sample was introduced to the donor chambers above the cell-monolayers, and the experiments were carried out in a shaking incubator at 37 °C and 70 rpm. After 30, 60, 105, 150, 195 and 240 min the filter was moved to a new well containing 1.5 ml of preheated HBSS++ buffer in the acceptor chamber. The amount of permeated carboxyfluorescein appearing in the acceptor chambers was measured as fluorescence using a plate reader (FLUOstar Omega from BMG Labtech) at λ_{ex} 490 ± 10 and λ_{em} 520 ± 10. At the end of the experiment, TEER was measured again using a meter and electrode (Millicell®-ERS (electrical resistance system) from Millipore).

Table 1
Results from viscosity measurements and estimated Mw.

	LM pectin	Amidated pectin	HM pectin	Hyaluronic acid	Chitosan
Intrinsic viscosity, [η] (dL/g)	3.35	4.31	5.36	29.49	9.30
Huggins' constant, k'	0.38	0.37	0.44	0.56	0.55
Estimated Mw (kDa)	72[α]	101[α]	137[α]	3788[β]	248[γ]
Degraded polymer					
	LM pectin	Amidated pectin	HM pectin	Hyaluronic acid	Chitosan
Intrinsic viscosity, [η] (dL/g)	0.26	1.24	0.63	0.31	7.02
Huggins' constant, k'	3.19	0.11	2.48	1.89	0.36
Estimated Mw (kDa)	2.2[α]	18[α]	7.3[α]	6[β]	171[γ]

[α] $K = 9.55 \times 10^{-2}$ (ml/g) $\alpha = 0.73$, dissolution medium: 0.09 M NaCl, 0.01 M NaF, 0.001 M Na_2EDTA, pH: 6.5(Anger and Berth, 1986).
[β] $K = 5.075 \times 10^{-2}$ (ml/g) $\alpha = 0.716$, dissolution medium: 0.2 M NaCl (Gura et al., 1998).
[γ] $K = 7.4 \times 10^{-2}$ (g/ml) $\alpha = 0.76$, dissolution medium: 0.2 M AcONa/0.3 M AcOH (Rinaudo et al., 1993).

From the plot of cumulated permeated carboxyfluorescein versus time, the slope (dQ/dt) can be found. The apparent permeability coefficient was then calculated: Papp (cm/s) = $dQ/dt * V/(A*C_0)$, where V = volume in receiver, A = area of cell-monolayer, C_0 = starting concentration in donor. The linear part of the slope was used ($R^2 > 0.98$), which included all time points for pectins and HA, as well as the control without polymer, while for the experiments involving chitosan, the slope was linear only after 105 min.

2.2.5. Diffusion of carboxyfluorescein

To test if the polymers had an effect on the diffusion of carboxyfluorescein, the above experiment (Section 2.2.3) was repeated with intact polymers (worst case), but where the cell monolayer was replaced by a piece of dialysis membrane (SpectraPor®, Mw cut off: 8000 Da).

2.2.6. Statistics

The results for the permeation experiments are presented as the mean and standard deviation (S.D.) of the 3 parallels. Each polymer system was compared to the control in pure buffer for statistically significant differences at $p < 0.05$.

3. Results and discussion

3.1. Degradation and characterization of polymers

The Mw is an important parameter when polymers are used as excipients. The manufacturer will, in most cases, give an estimate, but the Mw can vary from batch to batch. This is particularly the case for polymers of natural origin. Therefore, the intrinsic viscosities [η] of the different polymers were measured before and after degradation, and the results presented in Table 1. The [η] can be related to the Mw using the Mark–Houwinks relationship: $[\eta] = KM^{\alpha}$. A prerequisite is, however, that the exact values of K and α are known. This is rarely the case, and in most cases different values of K and α can be found in the literature. The values may also vary with dissolution medium, Mw etc. The values of Mw given in Table 1 are therefore estimates, but can be used to obtain a rank order of Mw for the different polymers.

Among the pectins, the Mw varied from about 70 to 140 kDa. The lowest Mw was found for LM pectin, the highest for HM pectin, with amidated pectin in between. These results are reasonable, as the manufacturing process used to manufacture LM pectin from HM pectin is also known to invoke hydrolysis of the pectin backbone. The estimated Mw of HA was more than two times higher than the value given by the manufacturer, but both the manufacturer's and the estimated value of Mw found here were substantially higher compared to the other polymers. The estimated Mw of chitosan was in line with the range given by the manufacturer, and in between the values of the pectins and HA.

All polymers were successfully degraded. Especially boiling the polymers in an acid solution was found to be very effective for reducing the Mw. The Mw was then reduced by a factor of ~6–30 for the pectins, and ~600 for HA. The ranking order of Mw for the pectins now changed in that amidated pectin had a higher Mw than HM pectin. The Mw was still lowest for LM pectin, and the Mw of HA was close to the values of HM pectin. It cannot be completely ruled out that the chemical structure of the polymers also changed to some degree. Especially the ester groups of pectin may be vulnerable to this treatment.

Chitosan melted during boiling, so instead, chitosan was degraded in a strong acidic solution. As shown in Table 1, the Mw was reduced during this treatment, but not to the same extent as for pectins and HA.

3.2. The cellular model

Monolayers of HT29-MTX cells have been used previously as a permeability model, for example for studying the effect of mucus on the permeation of drugs (Behrens et al., 2001). Pontier et al. (2001) reported that mucus was not a barrier to the 19 different drugs that were tested, and that the correlation of permeability between HT29-MTX and Caco-2 monolayers was very high. In fact, they suggested that the HT29-MTX monolayer can be used as an alternative model lacking P-glycoprotein.

When the HT29-MTX cell-line is used to study the effect of mucus, a suitable reference model, without mucus, is necessary. One such reference model, used in the past, is Caco-2 cells (Behrens et al., 2001). This is reasonable, since the variation in lipid membrane composition between the two cell-lines is very small. Another strategy is to remove the mucus layer, either by N-acetyl–cysteine treatment (Meaney and O'Driscoll, 1999) or by agitation with buffer (Hilgendorf et al., 2000). This is not straightforward, however, and the actual removal of mucus should be documented, as well as the lack of any damage of the cell membrane. In this work, a new method for assessing the effect of mucus was suggested, where the control system was the same cell-line, but grown only for 2 weeks, and hence with less cells differentiated into mucus-producing goblet cells. The cell-monolayer grown for 2 and 3 weeks can be seen in Fig. 1(a) and (b), respectively. After 2 weeks, cells can still be seen (darker areas), while after 3 weeks, the whole area is covered by a lighter mucus layer. This gradual development of mucus on HT29-MTX cells was also visualized by Pontier et al. (2001) after 9, 16 and 23 days.

3.3. Permeation studies

The permeation modulating properties of the different types of pectin can be seen in Fig. 2, both as a function of Mw and age of cell-monolayer (i.e. amount of mucus present). The apparent permeability coefficient for carboxyfluorescein in pure HBSS++ buffer,

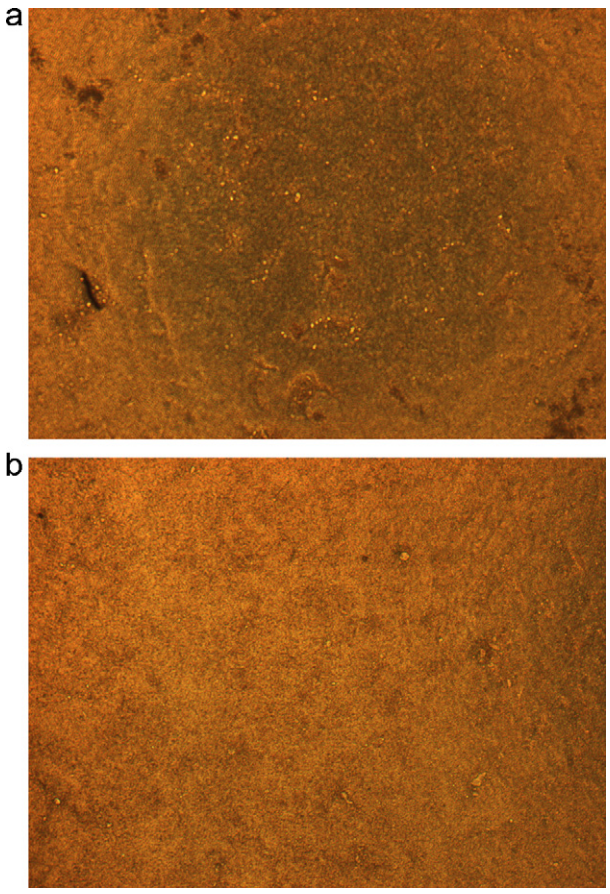

Fig. 1. The cell monolayer (area: 5 mm^2) after (a) 2 weeks and (b) 3 weeks.

without any polymer (the control), was higher after 3 weeks than after 2 weeks (about twice as high). That might seem like a contradiction, as the general conception is that the cell-monolayer integrity will increase with time. However, in this case, the increased number of mucus-producing goblet cells will probably render the membrane more permeable. This is in line with (Hilgendorf et al., 2000). Although goblet cells also form tight-junctions, the integrity is lower than for enterocytes.

As can be seen in Fig. 2, none of the pectins were permeation enhancing, despite their ability to bind calcium-ions. On the contrary, the pectins decreased the apparent permeability coefficient. This finding supports the general conception of pectin having a

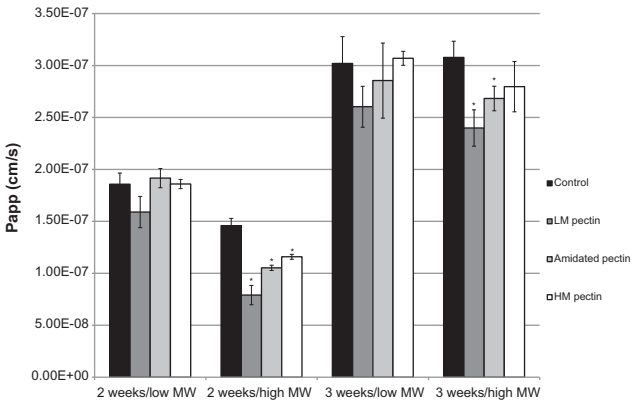

Fig. 2. The permeation modulating properties of LM, amidated and HM pectin. Results are expressed as the mean with the bar showing S.D. ($n = 3$). *Statistically different from control ($p < 0.05$).

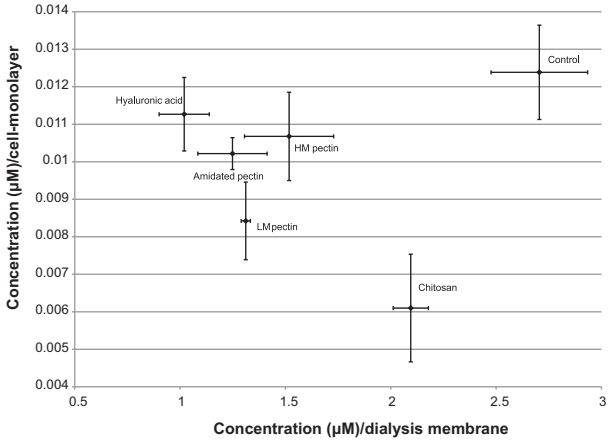

Fig. 3. The cumulated amount of permeated carboxyfluorescein after 1 h through a dialysis membrane and 3 weeks old cell-monolayer.

membrane protective effect (Bengmark and Jeppsson, 1995). This effect was generally favored by a high Mw of the polymers. But even more important was the amount of free carboxylic groups. Hence, the rank order of the membrane protective effect of the different types of pectin was: LM pectin > amidated pectin > HM pectin, despite the fact that the Mw was in fact lowest for LM pectin (Table 1). It is possible that the superior ability of LM pectin to engage in hydrogen bonding is responsible for this finding, either due to hydrogen bonding to the cell membrane or intramolecular hydrogen bonding between the polymer chains themselves, forming aggregates of a large Mw. The same rank order was mainly preserved also after degradation, indicating that the chemical differences between the different types of pectins were preserved at least to some extent. Mucus decreased the relative impact of the pectins on the membrane.

To test whether the polymers had a genuine effect of the cell membrane, or merely reduced the diffusion of carboxyfluorescein, the experiment was repeated for intact polymers (worst case), with the cell-monolayer substituted by a dialysis membrane. It was then assumed that any delayed or reduced permeation through the dialysis membrane was mainly due to a reduction of diffusion of carboxyfluorescein in the donor chamber due to an increase in viscosity caused by the polymers. With this set-up, no linear slope was obtained, due to a high permeation in the beginning, dramatically lowering the donor concentration of carboxyfluorescein. Therefore, the amount of cumulative permeated carboxyfluorescein after 1 h, both for the set-up with cells grown for 3 weeks, and without the cells, are compared in Fig. 3.

All the different types of polymers reduced the diffusion of carboxyfluorescein. However, the permeation through the dialysis membrane was ~100–300 times higher than through the cell-monolayer, indicating that diffusion towards the filter should not be the rate limiting stepin the cell-experiments. Also, there is no direct correlation between the amount permeated through the cell-monolayer and the diffusion. For example, the diffusion was almost the same for LM and amidated pectin, and not significantly different from HM pectin. Still, the permeation through the cell-monolayer was lower for LM pectin. This indicates that there is indeed a genuine effect on the cell membrane, accentuated by free acid groups on pectin.

Both HA and chitosan displayed permeation enhancing effects. Results are shown graphically in Figs. 4 and 5, respectively.

The effect of HA (Fig. 4) was observed only on 2 weeks old monolayer and only for degraded polymer. This finding is in line with Sandri et al. (2004) reporting increasing penetration enhancing properties of HA with decreasing Mw on Caco-2 cells, explained

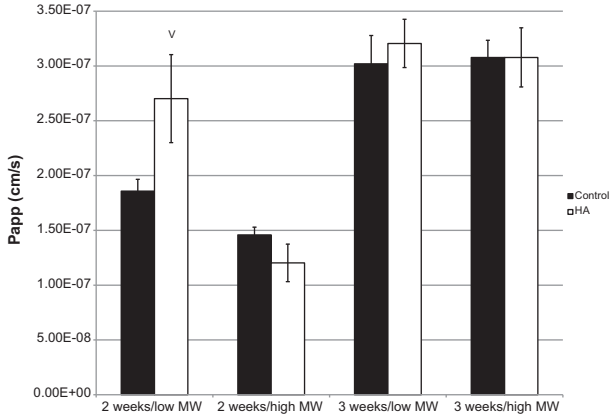

Fig. 4. The permeation modulating properties of hyaluronic acid. Results are expressed as the mean with the bar showing S.D ($n = 3$). ^{V}Statistically different from control ($p < 0.1$).

by an optimal length and tridimensional conformation to establish an intimate contact between the polymer chains and epithelial cells. Theoretically, the reduced diffusion coefficient of carboxyfluorescein in un-degraded HA, as seen in Fig. 3, can camouflage a hypothetic permeation enhancing effect of HA with a high Mw. However, this step of the permeation experiment was not considered to be rate limiting. Also the fact that the apparent permeability coefficient was similar both for intact and degraded polymers on 3 weeks old cells, indicates this.

No permeation enhancing properties were seen for HA on 3 weeks old cell-monolayers. An explanation for this may be the presence of more mucus, protecting the cell membrane. Solutions of HA have previously been reported to increase the nasal absorption of drugs *in vivo* in rats (Morimoto et al., 1991) and to display permeation enhancing properties on buccal and vaginal tissue *ex vivo* (Sandri et al., 2004). Despite this, no permeation enhancing properties were seen here for HA on 3 weeks old cell-monolayers, simulating the gastrointestinal mucosa. An explanation for this discrepancy may be the influence of other properties of HA, like mucoadhesion, on increasing the absorption *in vivo*, or possibly differences in the thickness of the mucosal layer among the different mucosal membranes.

Chitosan displayed pronounced penetration enhancing properties (Fig. 5), significant at both high and low Mw and on both 2 and 3 weeks old monolayers. The permeation enhancing effects of chitosan are well known, and have been reported many times

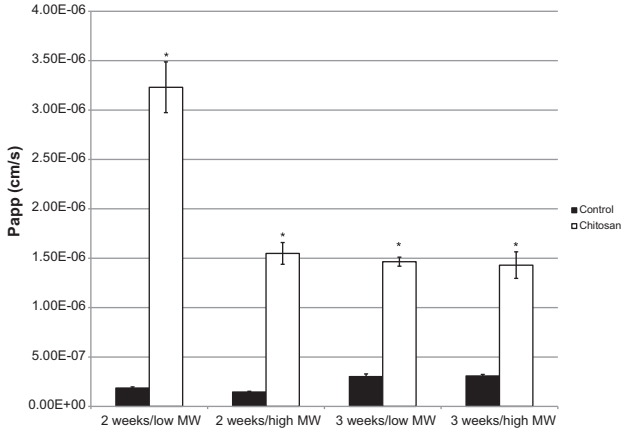

Fig. 5. The permeation modulating properties of chitosan. Results are expressed as the mean with the bar showing S.D. ($n = 3$). *Statistically different from control ($p < 0.05$).

before. However, here it was shown that chitosan is permeation enhancing even at higher pH, where chitosan is neither positively charged nor soluble. An explanation for this may be that chitosan was first dissolved at acidic pH, before the pH was adjusted, thereby precipitating chitosan as particulates. The sample then resembles for example chitosan nanoparticles prepared by ionic gelation, for which permeation enhancing properties have been demonstrated (Vllasaliu et al., 2010).

Also for chitosan, the general picture observed for HA, was confirmed: the penetration enhancing effect was significantly higher at low Mw and on the 2 weeks old monolayer, presumably providing the best conditions for an intimate contact between the polymer and cell membrane. These finding are in line with earlier work conducted with positively charged chitosan at pH 5.5 (Schipper et al., 1999). It should also be noted that for chitosan, the curve was linear only after 105 min (see Section 2.2.4 Permeation studies), and in fact, the permeation was very low for the first 1 h (Fig. 3). But in the case of a low Mw and low amount of mucus, the curve was linear already after 60 min, indicating a faster access to the tight-junctions.

At the end of each experiment, the TEER was measured. Generally, permeation of carboxyfluorescein is a more sensitive indicator of paracellular integrity than TEER, and the TEER values did not provide any additional information.

In general, before advocating *in vivo* use of certain permeation enhancers, the toxicity should also be monitored. However, this was not the main focus of this work, which was to illustrate the influence of Mw and mucus on polymers' diffusion towards and effect on the cell surface. The concentrations chosen are within normally used limits (Sandri et al., 2004). However, although natural polysaccharides are generally accepted to display low toxicity, especially chitosan has been suspected of toxicity. A recent study showed a complete TEER recovery of Caco-2 cells only at a concentration of chitosan nanoparticles as low as 0.0125 w/v % and a partial recovery (29% of baseline values) with a concentration of 0.1 w/v % (Vllasaliu et al., 2010).

In summary, this paper has demonstrated some permeation modulating properties of different types of natural polysaccharides. The 3 main commercially available types of pectin, LM, amidated and HM pectin, were all found to display some membrane protective effects. Free acid groups accentuated this property. This was despite the fact that free acid groups on pectin are well known calcium chelators, previously reported as a mechanism to transiently open tight-junctions. HA displayed modest and chitosan pronounced permeation enhancing properties. Hence, the well known permeation enhancing properties of chitosan were preserved even as a dispersion at a pH close to neutrality, where chitosan is no longer positively charged. From this work, charge does not seem to be a dominant parameter for predicting the effect of a polymer on a cell membrane. Uncharged chitosan displayed permeation enhancing properties, and the two negatively charged polymer types, pectins and HA, displayed opposite effects in pectins being membrane protective and HA being permeation enhancing. Consequently, an explanation for the results may be found in the chemical structure of the polymers. Chitosan is a linear copolymer consisting of glucosamine and N-acetyl glucosamine linked together randomly, HA is a polymer of the disaccharide glucuronic acid and N-acetyl glucosamine, while for pectin the dominant feature is galacturonic acid units with different types and degree of functionalization, as well as complex sugar chains attached. It is therefore possible that for example N-acetyl glucosamine is favorable in relation to increasing the permeation enhancing properties of a polymer.

Two different Mw were tested for each polymer. The effect of Mw was not systematically investigated, and independent studies are needed to identify the optimal Mw, which is most likely

somewhere in between the values reported here. Nevertheless, Mw was found to have an impact on the results. Generally, a high Mw was found to increase the membrane protective effects, and a low Mw increased the permeation enhancement. Since the permeation of carboxyfluorescein was not directly correlated to its diffusion coefficient, the results can probably be identified as a direct effect on the cell membrane. The fact that mucus reduced this effect, as shown for 3 weeks old compared to 2 weeks old cell-monolayers, supports this hypothesis. Even though mucus was not identified as a diffusional barrier to the paracellular marker carboxyfluorescein itself, it may be a barrier to the permeation modulating effect of excipients. This effect has previously been reported for a mixed micellar system consisting of sodium taurocholate and linoleic acid as an absorption enhancer for dexotropropxyphene (Meaney and O'Driscoll, 1999). Theoretically, there is also another explanation for the observations; that the tight-junctions formed by goblet cells are harder to manipulate than the tight-junctions formed by enterocytes. In either case, as this paper has shown, the inclusion of mucus-secreting goblet cells in the model membrane can give different results. This was especially the case for hyaluronic acid, where the permeation enhancing effect was no longer present. This is an important finding, as the membranes found *in vivo*, also express goblet cells.

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