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Bioadhesive oesophageal bandages: protection against acid and pepsin injury

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Abstract

The rate of acid and pepsin diffusion through solutions of sodium alginate was measured using in vitro techniques. Previous work has demonstrated that solutions of alginate may adhere to the oesophagus for up to 60 min; this work measured their ability to protect the oesophageal epithelial surface from damage caused by refluxed acid and pepsin. Franz diffusion cells were used to measure the rate of acid and pepsin diffusion through an alginate layer. The effect of the type of alginate, alginate concentration and depth of alginate applied were investigated. The rate of both acid and pepsin diffusion was significantly reduced (ANOVA analysis; $P < 0.05$) in the presence of an alginate solution compared to the control. A 2% (w/v) alginate solution with a high guluronic acid component, in a layer of 0.44 mm depth, demonstrated the greatest reduction in acid diffusion with a permeation coefficient 14% than that of a control value. All three alginates demonstrated significant reductions in acid diffusion with both increasing depth and increasing concentration, as expected. Pepsin diffusion was also significantly reduced as the depth and concentration of applied alginate increased. This study demonstrates that an adhesive layer of alginate present within the oesophagus will limit the contact of refluxed acid and pepsin with the epithelial surface.

Keywords: Alginate; Oesophagus; Gastro-oesophageal reflux disease; Acid diffusion; Pepsin diffusion

1. Introduction

Gastro-oesophageal reflux disease (GORD) is caused by excessive reflux of acidic material from the stomach back into the oesophagus. Gastric reflux is a physiological event that occurs frequently in healthy individuals, however this process can become pathologic, leading to damage of the oesophageal mucosa (Orlando, 2000). The oesophagus, unlike the stomach and duodenum, has neither a well-defined mucus layer nor bicarbonate secreting cells, thus a comparative lack of native pre-epithelial defences against acid. It has been suggested that reflux symptoms may result from an imbalance between an excess exposure to acid and

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pepsins, and inadequate defence mechanisms (Brown and Rees, 1995).

Mucus present on the gastric epithelium provides a protective coating against the acidic environment. Mucin molecules undergo a sol–gel transition at low pH due to cross-linking of the molecules through hydrophobic interactions (Cao et al., 1999). This gel form of mucus is resistant to back-diffusion of secreted acid and maintains a pH gradient from pH 2 in the lumen to pH 7 at the apical cell surface (Khanvilkar et al., 2001). The thickness of the mucus layer in the human stomach has been reported to be 576 μm by Bickel and Kauffman (1981) whereas Allen (1989) reported a mean thickness of 192 μm for a continuous mucus layer. Mucus within the stomach provides an effective barrier to hydrogen ion diffusion; a study performed by Williams and Turnbery (1980) measured the permation coefficient of pig gastric mucus at a depth of 1 mm to be 1.75×10^{-5} cm^2 s^{-1} compared to 6.65×10^{-5} cm^2 s^{-1} for the control, a reduction to 26%. Slomiany et al. (1985) measured the permeation coefficient of porcine gastric mucus, also at a depth of 1 mm and reported a value of 6.51×10^{-6} cm^2 s^{-1} compared to 65.60×10^{-6} cm^2 s^{-1} for the control. The incorporation of sucralfate into the gastric mucin further reduced the permeation coefficeint providing eveidence that sucralfate strengthens mucus gels and aids in the retardation of acid diffusion (Slomiany et al., 1985). In both studies the presence of a mucus layer lead to a significant reduction in hydrogen ion diffusion compared to a control value. The diffusion coefficient of a drug through mucus depends upon the relative size of the drug molecule and the mesh size of the mucus gel formed by association of the mucin molecules. The relatively large size of pepsin, 35 kDa, indicates that mucus provides an effective barrier for pepsin diffusion (Allen et al., 1991).

Pre-epithelial defences within the oesophagus comprise an unstirred water layer that has a thickness in the range of 30–95 μm (Attwood, 1994; Sarosiek et al., 1983). This unstirred water layer can only support a pH gradient of approximately 1 pH unit (Orlando, 1994). Damage caused to the oesophagus by gastric contents appears to be the greatest with nocturnal reflux (Tobey et al., 1989); this suggests that oesophageal clearance mechanisms including salivation and peristalsis (that are reduced during sleep) are primarily responsible for protecting the oesophagus from acid reflux. Pepsin is an acid activated protease secreted within the stomach that has also been linked to oesophageal damage caused by gastric reflux (Gotley et al., 1991). Research has shown that greater damage is caused to the oesophageal epithelium by pepsin in combination with acid compared to acid exposure alone (Goldberg et al., 1969). Antacids and alginate containing formulations are usually indicated in the early treatment of GORD and both are available without prescription. Alginate based therapies (e.g. Gaviscon Advance®, Gastrocote® and Algicon®) form a raft that floats on the gastric contents forming a physical barrier against reflux; these treatments are not systemically absorbed and thus have limited drug–drug interactions, they are also suitable for use during pregnancy.

Alginates are natural polysaccharides derived from seaweed; they exist as block copolymers of two monomeric units, guluronic (G) and mannuronic (M) acid. In the presence of calcium ions, or at low pH (<3), alginates form gels; the strength of the gel formed is dependent upon the composition of monomers within the alginate chains with gel strength increasing with G content (Smidsrød and Draget, 1996). The acid gel is formed by hydrogen bonding between the acid and hydroxyl groups of the alginate at low pH values forming a cross-linked network. Long guluronate blocks are most important in this cross-linking and increased molecular weight has also been reported to enhance the strength of the gel formed (Draget et al., 1997). The rigid nature of poly-G blocks within an alginate chain means that the acid groups are exposed and can readily interact with hydroxyl groups leading to the formation of organised hydrogen bonds between two poly G blocks. This association is strong and provides a solid structure to a three dimensional gel, the number of poly-G–poly-G interactions determines the solid like nature of the gel formed and thus controls the mesh size within the gel, a greater number of associations leads to a smaller mesh size. Aslani and Kennedy (1996) measured the permeation coefficient of calcium or zinc alginate gels as approximately 1×10^{-7} cm^2 s^{-1} for acetaminophen. Exposure of these ionically cross-linked gels to simulated gastric fluid at a low pH resulted in the formation of alginic acid gels that demonstrated greater permeability than the corresponding calcium or zinc alginate gel (Aslani and Kennedy, 1996). The increased permeability is likely to be due to the looser mesh formed by hy-

drogen bonds in comparison to the ionic bonds formed by association with cations.

Potts et al. (2000) introduced the concept of oesophageal bandages as drug delivery systems with potential application in the treatment of GORD. Previous in vitro work has demonstrated that solutions of sodium alginate adhere to oesophageal tissue for up to 60 min (Batchelor et al., 2002). This study investigates whether these adhesive alginate oesophageal bandages provide pre-epithelial defences against both acid and pepsin. The diffusion of acid and pepsin (in an acidified solution) through alginate formulations was investigated. A reduction in the rate of acid reaching the epithelial cell layer indicates an enhanced pre-epithelial defence and may reduce the requirement for systemically administered therapies for GORD.

2. Materials and methods

2.1. Materials

Alginate chains are built, as randomised block copolymers, from two monomeric sugar units. The proportion and distribution of these monomers and their relative sequencing determines the chemical and physical properties of the alginate solutions and gels. Three sodium alginates were investigated in this study, whose properties are listed; H120L had a molecular weight (MW) of 416 kDa and a G fraction of 0.46; LF120 had a MW of 240 kDa and a G fraction of 0.44; LFR5/60 had a MW of 40 kDa and a G fraction of 0.64. The viscosities of these alginates measured at a shear rate of $10\,s^{-1}$ using a controlled stress rheometer (TA Instruments, AR1000N rheometer), were 4.12, 0.51 and 7.5×10^{-3} Pa s for H120, LF120 and LFR5/60 at 2% (w/v), respectively.

Alginate solutions were prepared by slow addition of a measured mass of alginate powder to the designated volume of distilled water under vigorous stirring. 0.1 M hydrochloric acid was prepared by dilution with distilled water of a 5 M solution supplied by Sigma, UK. Porcine gastric pepsin (P7012), supplied by Sigma (UK) was prepared as a 0.3% (w/v) solution in 0.1 M hydrochloric acid. Synthetic membranes were used to evaluate the diffusion rate of acid and pepsin through the alginate solutions. Dialysis membrane (Sigma, UK) hydrated in water, with a molecular weight cut off value of 12–14 kDa was used in the acid diffusion study. Whatman glass microfibre (GF/C) filter paper was used to evaluate the diffusion of pepsin.

2.2. Diffusion apparatus

A common method used to measure the diffusion of drugs through mucus gels is via a Franz diffusion cell; this method was used to measure diffusion through alginate layers in this study. The diffusion studies investigated the rate of diffusion of both acid and pepsin from an upper donor chamber to a lower receptor probe coupled to a calibrated Sartorius pH meter. A UV Unicam Helios β spectrophotometer was used for the UV detection of pepsin in samples of fluid withdrawn at designated time points via a sample port. The diameter of the porthole between the two chambers was 17 mm; the volume of alginate applied was 0.1 mL unless stated otherwise. The area over which diffusion occurred was $227\,mm^2$.

2.3. Quantification of pepsin

The UV absorbance peak of the acidified pepsin solution (0.3%, w/v) was determined to be at $\lambda = 276$ nm. A series of concentrations of acidified pepsin solution were prepared to produce a calibration between concentration and absorbance, a linear regression of greater than 0.99 was found for the correlation. The assay was sensitive to pepsin from 0.001 to 0.1 % (w/v) over a linear range; with absorption values from 0.02 to 1.

2.4. Acid diffusion

Thirty millilitres of distilled water was dispensed into the receptor chamber and a magnetic stirring bar was placed on the bottom of the receptor. Hydrated dialysis membrane was secured to the base of the donor chamber and the edges were sealed using Parafilm® to prevent leaking. The donor chamber was then mounted on the receptor chamber and a clamp held the two chambers together. A set volume of aqueous sodium alginate was applied to the membrane surface in the donor chamber. A pH electrode was inserted into the solution through the sampling port to measure the pH over time. Twenty five millilitres of 0.1 M hydrochloric acid solution was gently poured into the donor chamber taking care to minimise disruption to the alginate layer. The pH value of the receptor solution was recorded at

set time points up to 30 min. A control experiment was performed without the aqueous alginate layer applied to the membrane. All experiments were performed at room temperature.

2.5. Pepsin diffusion

The method for pepsin diffusion was similar to that for acid diffusion but with the following alterations. The receptor chamber contained 0.1 M acid so that the diffusion rate of pepsin through the alginate layer could be investigated rather than diffusion due to differences between the donor and receptor media. Whatman glass microfibre filter paper was used in place of hydrated dialysis membrane to allow the passage of pepsin. The pepsin molecule is a much larger entity at 35 kDa compared to hydrogen ions so a membrane with a larger pore size was required. The donor chamber contained 0.3% (w/v) pepsin in 0.1 M hydrochloric acid solution. 1.5 mL samples were taken from the receptor at set time points up to 30 min. This volume was replaced using 0.1 M acid solution to maintain the volume and ensure continued contact at the membrane–solution interface. In calculating the concentration of pepsin in the receptor chamber over time the replacement of previous samples with fresh media was taken into account. Diffusion of pepsin was quantified according to the calibration described in Section 2.3.

2.6. Statistical analysis of results

Analysis of variance tests (ANOVA) were performed on the data collected with the significance level set at $P < 0.05$.

2.7. Manipulation of data

According to Fick's first law, the permeation through a layer is dependent upon both the permation coefficient of the diffusion barrier (P) and the thickness of the barrier (h) thus a thicker alginate layer should provide more resistance to diffusion. The permeability of the alginate layer can be calculated.

Fick's first law:

$$P = Jh/C$$

where J is the the flux of the diffusate through the layer, C the initial concentration of the drug in the donor and h the thickness of the applied layer. The thickness was calculated from division of the volume applied in mm^3 by the surface area (227 mm^2). The flux of the diffusate, J is defined as:

$$J = M/At$$

where M is the the mass of the diffusate present in the receptor at time, and t and A the area available for diffusion. In a graph of mass per area that has diffused against time the flux can be calculated as the gradient of the line. The initial concentration of hydrogen ions in the donor was 0.1 M (100 $\mu g\,cm^{-3}$) in all cases.

3. Results and discussion

3.1. 3.1.Effect of alginate used on hydrogen ion diffusion

The diffusion of acid was evaluated through 0.1 mL of 2% (w/v) solutions of each alginate and the results are shown in Fig. 1. The results show that the alginates provided significantly lower diffusion of acid over time compared to the control. The diffusion rate was calculated as the gradient of the line through the data points.

Both H120L and LF120 showed statistically similar profiles although LFR5/60 demonstrated the greatest potential in reducing the rate of acid diffusion. This result was interesting as LFR5/60 also demonstrates the lowest viscosity at 2% (w/v) aqueous solution. The rate of acid diffusion was inversly related to the G fraction of the alginate, a mathematical correlation was drawn and the correlation coefficient value was found to be >0.99. This is in agreement with work previously published by Smidsrød and Draget (1996) who suggested that the strength of an alginate gel is improved according to the number of G units present. However, this study uses only three alginates with a limited range of G content thus this work needs to be expanded over a broader range of G content to validate this trend. In addition the acid present decreases the pH of the alginate by a reduction in the protonation of the acid groups which promotes the formation of the hydrogen bonded three-deminsional gel. This data was converted from the concentration within the receiver to the mass diffused per surface area and this data was used to calculate the flux of the hydrogen ions, all plots had a linear fit with a regression of greater than 0.99. Table 1

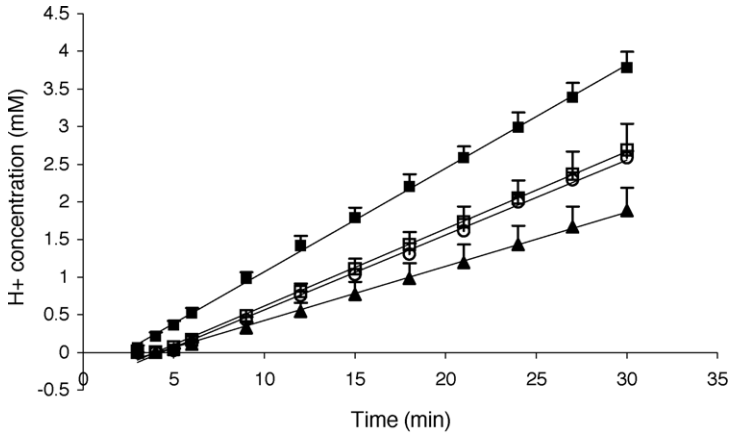

Fig. 1. Comparison of acid diffusion through a control or alginate layer. Control (■); 2% (w/v) LF120 (□); 2% (w/v) H120L (◯); 2% (w/v) LFR5/60 (▲). Mean data ± S.D. is shown; $n = 4$.

Table 1
The flux of hydrogen ions through alginate was used to calculate the permeation coefficient

Alginate	Flux ($\mu g\,cm^{-2}\,min^{-1}$)	Permeability coefficient ($\times 10^{-6}\,cm^2\,s^{-1}$)
Control	1.740	12.76
2% LFR5/60	0.236	1.73
2% LF120	1.040	7.63
2% H120	1.007	7.38

compares the flux of hydrogen ions and the permeation coefficients of the alginate layers from these diffusion experiments.

The flux of the hydrogen ions were calculated from the data shown in Fig. 1 and the results mirror those observed for the rate of diffusion, LFR5/60 shows significantly reduced flux and permeability coefficient compared to both LF120 and H120l whose values are similar in all cases. All alginates, at 2% (w/v), show significantly reduced flux and permeation coefficients compared to the control. The permeability coefficient of LFR5/60 was less than 14% of the control value, this is comparable to the permeation coefficient demonstrated by gastric mucus.

3.2. Effect of volume of alginate applied on hydrogen ion diffusion

Three different alginates were applied at three diffierent volumes: 0.05, 0.1 and 0.2 mL. This difference in volume applied resulted in the depth of the alginate layer being 0.22, 0.44 and 0.88 mm, respectively. The diffusion rates of hydrogen ions through these layers are shown in Table 2.

Fick's law states that the rate of diffusion through a layer is inversly proportional to the thickness of the layer, thus as expected a deeper layer leads to slower diffusion. As anticipated the greater is the volume of alginate applied the greater is the reduction in the diffusion rate compared to the control value. Statistically significant reductions in the rate of diffusion were noted for both LF120 and H120L alginates as the depth applied increased from 0.22 to 0.88 mm (ANOVA anal-

Table 2
The effect of the volume of alginate applied on the diffusion rates of H^+ ions through the alginate layer (mean ± S.D. is shown; $n = 4$)

Volume applied (mL)	Rate of diffusion of acid ($mM\,min^{-1}$)		
	2% (w/v) LFR5/60	2% (w/v) LF120	2% (w/v) H120L
0—control	0.1367 ± 0.0065		
0.05	0.071 ± 0.007	0.092 ± 0.009	0.087 ± 0.008
0.1	0.065 ± 0.005	0.080 ± 0.004	0.078 ± 0.004
0.2	0.059 ± 0.006	0.046 ± 0.003	0.049 ± 0.004

ysis; $P < 0.05$). LFR5/60 showed statistically similar rates of diffusion for all three depths of alginate applied (ANOVA analysis; $P > 0.05$); these were significantly lower that the other alginates at 0.22 and 0.44 mm however, the rate of diffusion at 0.88 mm was greater than expected. The results from this study show that thick layers of alginate can reduce the diffusion rate by up to 33% of the control value, indicating that alginate, like mucus, can retard the diffusion of hydrogen ions. The oesophagus has a surface area of approximately 200 cm^2 (Washington et al., 2001); thus a dose of 4.4 mL would form an alginate depth of 0.22 mm (the thinnest layer measured in Table 2) if distributed evenly over the entire epithelial surface.

3.3. Effect of alginate concentration on hydrogen ion diffusion

Different concentrations of each alginate were evaluated to reduce the rate of acid diffusion. The relationship between concentration and diffusion rate is shown in Fig. 2.

A 10% solution of LFR5/60 reduced the diffusion rate to one-tenth of the control value, yet this solution is pourable and could be used as the basis of a liquid formulation designed to coat the oesophagus to reduce damage caused by gastric reflux. No significant differences were seen in the rate of diffusion between the two alginates, LF120 and H120L, examined at each concentration value. However the presence of alginate even at the lowest concentration significantly reduced the rate of diffusion of acid ($P < 0.05$) compared to the control. Both alginates (LF120 and H120L) demonstrated significantly greater diffusion rates at concentrations of 1% (w/v) compared to 4% (w/v). Alginate LFR5/60 was examined at 2, 5 and 10% (w/v) solutions and demonstrated significantly reduced rates of diffusion at each concentration examined. The concentration of alginate present will increase the number of sites available to cross-link, which leads to the formation of a denser network. Studies on mucus have suggested that in a mucus gel 95% of the mass of the gel is water (Allen, 1989), similarly with these alginates the majority of the mass present is water. As the concentration of alginate increases there is more solid structure present to form cross-links and to reduce the mesh size within the three-dimensional gel produced. The greatest effects are noted with LFR5/60, which has the greatest G content, once again suggesting that G units are most important in the formation of alginic acid based gels.

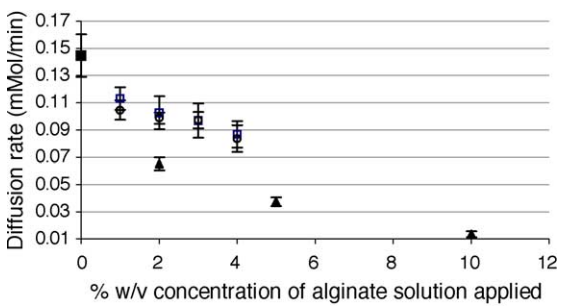

Fig. 2. Diffusion of hydrogen ions through alginate decreased with increasing alginate concentration. (■) Control; (□) LF120; (○) H120L; (▲) LFR5/60 ($n = 4$, ±S.D.). Mean data ± S.D. is shown; $n = 4$.

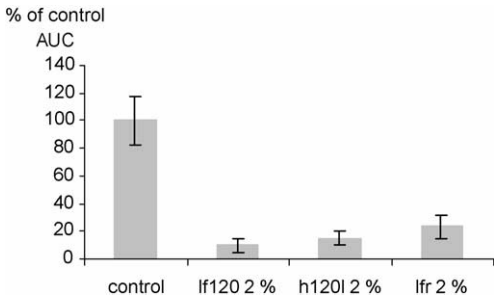

Fig. 3. Comparison of relative pepsin diffusion; a control compared to alginate ($n = 4$, ±S.D.).

3.4. Pepsin diffusion through alginate

The rate of pepsin diffusion through alginate layers was examined. 0.1 mL of each alginate at 2% (w/v) was used as test solutions and the rate of pepsin diffusion was measured. The rate of pepsin diffusion was not a linear phenomenon over 30 min, so the area under the

Table 3
The flux of pepsin through alginate was used to calculate the permeation coefficient

Alginate	Flux ($mg\,cm^{-2}\,min^{-1}$)	Permeability coefficient ($\times 10^{-6}\,cm^2\,s^{-1}$)
Control	0.0464	11.34
2% LFR5/60	0.0114	2.79
2% LF120	0.0068	1.66
2% H120	0.0071	1.73

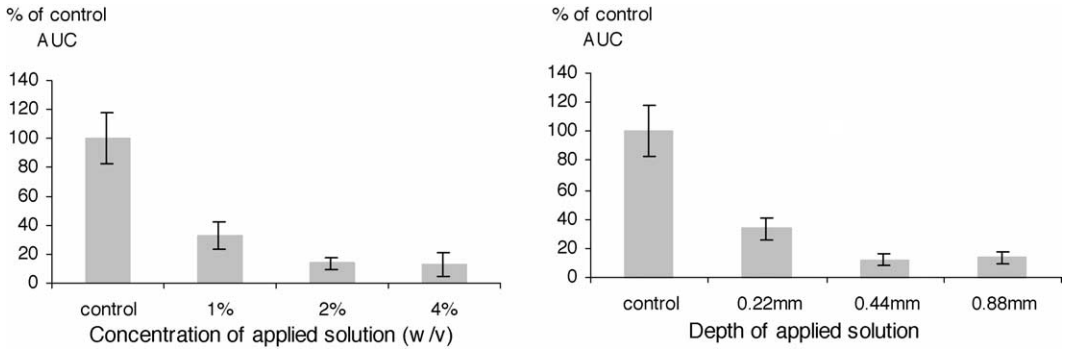

Fig. 4. A comparison of the reduction in pepsin diffusion compared to the control, according to the concentration and depth of alginate layer applied ($n = 4$, ±S.D.).

curve was used to compare the extent of pepsin diffusion for the test solutions. The results show that all alginates demonstrated some resistance to pepsin diffusion compared to the control. Fig. 3 shows a comparison of the area under the curve for all alginates examined, the control value was normalised to 100% and the relative areas are shown as a percentage of this control value.

The results show that all the alginates demonstrated significantly reduced pepsin diffusion ($P < 0.05$), however, there were no significant differences between the three alginates tested ($P > 0.05$). The permeation coefficient of alginates to pepsin diffusion was calculated in a similar manner to the permeation coeffeicients for acid. However, pepsin studies used a linear portion of the line from 5 to 30 min to calculate the flux. In plots of mass diffused per unit area against time the gradient was calculated as J, the flux. All plots gave linear regression values greater than 0.8. Table 3 compares the flux and permeation coefficient of the alginates compared to the control.

All alginates showed significantly reduced permeation coefficients compared to the control. In contrast to the trend noted for acid diffusion, LFR5/60 had the highest permeation coefficient. Diffusion of pepsin is likely to be simpler than diffusion of hydrogen ions; the size of the pepsin molecule is the limiting factor in its diffusion thus the mesh size of the alginate gel will dictate the diffusion pathway.

Fig. 4 compares the relative area under the curve of pepsin diffusion versus time for alginate LF120 of different concentrations and depths. The depth was 0.44 mm and concentration of 2% (w/v) was used unless otherwise stated.

An increase in alginate concentration from 1 to 2% (w/v) lead to a significant reduction in pepsin diffusion ($P < 0.05$). However a further increase in alginate concentration did not reduce pepsin diffusion. Increasing the depth of the alginate layer applied had a similar effect; doubling the depth of the layer from 0.22 to 0.44 mm lead to a significant reduction in pepsin diffusion however a further increase did not significantly affect pepsin diffusion ($P > 0.05$). Pepsin diffusion through alginate is controlled by the mesh size of the alginate network, greater interactions within the alginate will lead to a denser network and thus reduced pepsin diffusion. These results indicate that above 2 % (w/v) the alginate concentration does not affect the mesh size within the three-dimensional network, likewise an increase in depth of alginate above 0.44 mm will not additionally stengthen the barrier provided.

4. Conclusions

An adhered alginate layer present on the oesophageal epithelial surface may prevent damage caused by gastric reflux in a similar manner to the natural protective coat of mucus present within the stomach. The thickness of an overlying mucus layer has been reported to significantly influence the rate of drug entry into underlying tissues (Khanvilkar et al., 2001); likewise the thickness of an adhesive alginate layer will influence the transfer of acid and pepsin to the cellular surface within the oesophagus. However, a limiting thickness was determined for pepsin diffusion. The rapid turnover time of mucus within the gut will

also slow the diffusion of molecules through the mucus layer. This study models a worst-case scenario with acid permanently in contact with an alginate layer; in reality acid and pepsin exposure from gastric reflux occurs for short periods of time and is removed effectively by both peristalsis and saliva flow. The in vitro retention study (Batchelor et al., 2002) demonstrated that the retention of alginates can withstand rapid saliva flow and thus the acid that is not cleared will not make direct contact with the oesophageal epithelium but will diffuse into the adhered alginate.

Solutions of sodium alginate have similarities with mucus gel: both have high water content; both have a net negative charge and in acidic conditions both form a cross-linked three-dimensional structure. The three-dimensional structure of both alginate and mucus represents a balance between polymer–polymer interactions and polymer–solvent interactions to build up an extensively hydrated, yet cohesive, cross-linked network. The retardation of hydrogen ion diffusion through alginate and mucus gel is likely to be due to a combination of factors. Both substances have high water content; mucus has 95% water (Allen, 1989) and the alginate solutions were prepared at concentrations ranging from 90 to 99% water by weight. In 1958, Heatly proposed that a major factor in the retardation of hydrogen ion transfer through mucus was due to unstirred water held within the three-dimensional gel that is not available for diffusion (Heatly, 1959). This theory can also explain one mechanism by which alginate retards the diffusion of hydrogen ions. Both mucus and alginate possess a net negative charge, thus cations may become associated with the alginate or mucus molecule restricting their diffusion, although this effect would be small. The viscous nature of both alginate and mucus due to the formation of a three-dimensional gel mesh provides a sieve that prevents the free motion of large macromolecules including pepsin (Edwards, 1978). It would be of great interest to measure the pH within this three-dimensional network to monitor the buffering effect of the alginate.

Alginate LFR5/60 demonstrated the best potential to minimise the diffusion of acid, it was also able to significantly reduce pepsin diffusion compared to the control. The greater is the concentration of alginate as well as the volume applied lead to enhanced reduction in the rate of diffusion of both acid and pepsin, although this results was anticipated from theoretical assumptions. Preliminary work suggests that alginates with a high G content were better at reducing the rate of acid diffusion. High G content leads to alginate structures that are better able to gel under acidic conditions forming strong, rigid gels (Smidsrød and Draget, 1996). This factor is likely to enhance the adhesion of the alginates at lesion sites in vivo as their acidic nature will enable the alginate to form a gel cap over the surface of the lesion with a higher permeation coefficient that may reduce further contact of acid or pepsin with the lesion. Further studies will be performed to utilise the adhesive layer as a means to deliver drugs that aid in the overall oesophageal defence against injury caused by reflux.

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