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Roles for dietary fibre in the upper GI tract: The importance of viscosity

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Abstract

Dietary fibre has long been recognized as healthy because of its prebiotic quality and a number of dietary fibres, especially beta glucan have been shown to lower levels of circulating LDL cholesterol. However, although EFSA allow health claims to be made for this, there is no fundamental understanding of the detailed mechanism involved. More recently dietary fibre has been shown to have a range of functionality in the upper GI tract. The presence of fibre can alter gastric emptying thus affecting fullness and satiety. These alterations are a result of differences in viscosity, nutrient release and nutrient sensing in the duodenum. The current proposed mechanisms for the cholesterol lowering effects involve disruption of the normal recycling of bile possibly by sequestering bile salts and fatty acids or by significantly decreasing the rate of absorption as a result of entanglement with intestinal mucus.

The use of quantitative confocal microscopy methods such as fluorescence recovery after photobleaching (FRAP) and multiple particle tracking has provided evidence that dietary fibre can combine with intestinal mucus and produce a layer that significantly delays the transport of lipid digestion products. We have also used similar methods in conjunction with more conventional rheology to show that DNA from the gut epithelium can contribute significantly to the barrier properties of the intestinal mucus layer.

The delay in the transport of nutrients to the gut epithelium has implications for the control of gastric emptying and through secretion of GI hormones such as CCK and thus for the satiating ability of foods. It may also have implications for the reabsorption of bile.

Introduction

Fibre has long been seen as part of a healthy diet that can reduce the risk of developing a wide range of disorders including cardiovascular disease and diabetes (Anderson et al., 2009). The definition of dietary fibre as food that is not digested in the upper GI tract has inevitably led to much of the research on fiber being concentrated on fermentation in the colon and only recently have the benefits to the gut more generally been discussed (Brownlee, 2011; Gidley, 2013). There have now been a number studies that show the health benefits of fibre in the diet and this fact has become sufficiently accepted to allow health claims to be made about specific types of fibre such as beta glucan (EFSA-NDA, 2011). However, despite the fact that health claims can be made based on clinical data, the detailed mechanism leading to the outcome may not be well understood (Lattimer & Haub, 2010). Such mechanisms are thought to include the increase in viscosity caused by the high molecular weight polymers in soluble fiber and the ability of the fiber to bind endogenous compounds such as enzymes or bile acids. In particular the increase in viscosity has been linked to lower calorie intake. For example, sodium alginate has been shown to reduce energy intake in a study where participants consumed a preload sodium alginate formulation, the daily premeal consumption of the polymer produced a significant 134.8 kcal (7%) reduction in mean daily energy intake (Paxman, Richardson, Dettmar, & Corfe, 2008).

Alginates are linear polysaccharides produced from marine algae. They are widely used in the food industry as a thickener (E401) and in the pharmaceutical industry for encapsulation. The linear alginate polymer is made from two types of saccharide building blocks. These are β-D-mannuronate and α-L-guluronate, known as M and G residues respectively. The M and G residues are 1-4 linked together in different blocks that can be comprised of G only (G-block), M only (M-block) or a mixture of M and G (MG-blocks). The ratio of M and G in the polymer has an impact on the rheological properties of the gels formed. Alginate solutions with a low M:G ratio (0.8) have been shown to have a higher gel strength than solutions with higher M:G ratios (1.3 and 2.5) (Jensen, Knudsen, Viereck, Kristensen, & Astrup, 2012). The gelation of alginate is almost independent of temperature but is induced by the presence of divalent cations such as Ca^{2+}, which is associated with the G-blocks and by reduction in pH.

Mucins are widely found in nature and provide a protective layer to a wide range of epithelial surfaces. Mucins are broadly separated into two categories, those that are membrane bound and those that are secreted. The viscoelastic properties of the secreted mucins are key to their functionality. In particular, gastrointestinal and pulmonary secreted mucins provide both lubrication and a barrier function to particulates whilst allowing the passage of small molecules (Corfield, 2015). In the small intestine on mammals, the secreted mucins such as MUC2 form a complex structure. After secretion from the goblet cells of the intestinal epithelium, the mucin granules unfold to form a tightly adherent mucus layer that comprises a hexagonal network with a mean pore size around 100 nm (Johansson, Sjövall, & Hansson, 2013; Round et al., 2012). This layer varies in thickness along the intestine, being thinnest in the proximal regions and thicker more distally. On the top of the tightly adherent layer is a loosely adherent layer that can be much more heterogeneous, especially in the small intestine.

There is already evidence that certain types of alginate can have a significant effect on the rheological properties of mucus (Taylor-Nordgard, Nonstad, Olderoy, Espevik, & Draget, 2014). Taylor-Norgard et al. showed that the addition of low molecular weight G-block

oligomers were able to decrease the rheological properties of sputum from cystic fibrosis patients. The same group have also shown the ability of high molecular weight alginate to increase the rheological properties of gastric mucus (Taylor, Pearson, Draget, Dettmar, & Smidsrod, 2005). In this study we have attempted to build on this earlier work by showing that alginate may decrease the permeability of intestinal mucus by increasing the local viscosity. Thus, we hypothesise that soluble dietary fibre, in this case sodium alginate interacts with intestinal mucus in a way that decreases its permeability.

Materials and Methods

Materials
Alginates with a range of mannuronic/guluronic (M/G) ratios were kindly donated by Danisco (DK-8220 Brabrand). The sample chosen had an M/G ratio of 35/65 and a quoted molecular weight of 315 kDa. Subsequent measurement of the molecular weight yielded a weight average of 280 kDa. The ex vivo porcine mucus was prepared as described previously (Macierzanka et al., 2011). Briefly, fresh porcine small intestine was obtained from a local abattoir. The gut was rinsed through with ice cold phosphate buffer (10 mM phosphate pH 6.5, 5 mM EDTA, 0.5 mM Pefabloc (AEBSF)). The gut was then opened out flat and mucus was collected by gently scraping. Samples were frozen and stored at -20 ℃ for furthe r use.

Fluorescent labelling
Samples of alginate were fluorescently labelled using DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein (Life Technologies Ltd, Paisley, UK). The alginate was dissolved at 10mg/ml in 50mM sodium bicarbonate and adjusted to pH 9.0 with 1.0M NaOH. This was mixed overnight at 1:0.4 v/v with a solution of DTAF (10mg/mL in DMSO) at room temperature. The reaction mixture was dialysed in 10kDa cut-off dialysis tubing against PBS until no residual DTAF could be detected in the dialysate by UV absorbance at 490nm.

Rheology
Rheological properties of the ex vivo mucus containing increasing concentrations of sodium alginate were investigated using a controlled strain AR2000 rheometer (TA Instruments, Crawley, West Sussex, UK) equipped with a cone and plate geometry (aluminium cone; 6°/20 mm cone angle/diameter, truncation gap 12 µm). A viscosity ramp test was conducted using a shear rate range from 0.01 to 500 s^{-1} over 15 min and the temperature was held at 37℃. All experiments were done in triplicate and the data shown as mean values.

Emulsion and digestion
A protein stabilised emulsion was produced using the method described in Mackie et al. (Mackie, Macierzanka, Aarak, Ridout, & Bajka, 2015). Briefly, 3.0 mg/mL sodium caseinate solution in 150 mm NaCl pH 6.5 stabilised emulsion containing 18% triglyceride (sunflower oil) was prepared by passing a premix of oil and Na-Cas for a total of 6 times at 20,000 psi through a Microfluidiser (Microfluidics, Massachusetts, USA). The emulsion was digested using the standardised in vitro digestion protocol recommended by the Infogest COST Action (Minekus et al., 2014), involving 2 hours gastric followed by 2 hours of small intestinal simulation. The mean size ($D_{3,2}$) of the original emulsion was 1.0 µm. The mean size of the emulsion after digestion was 725 nm and the size distributions can be seen in a supplementary figure (Figure S1) to (Mackie et al., 2015)

Diffusion

The diffusion coefficients were determined using three different methods. The ability of the sodium alginate to penetrate into porcine mucus was determined using time-lapse microscopy. Ex vivo porcine mucus and fluorescein-labelled alginate were layered in a 9 mm diameter by 0.9 mm depth perfusion well (CoverWellTM, Sigma, Poole. UK). Laser scanning confocal microscopy was used to follow the diffusion of the alginate into the mucus for 90 minutes using a Leica SP1 with a 20x objective (Leica Microsystems, Mannheim, Germany). Linear fluorescent intensity profiles were generated from the time-lapse images and the diffusion coefficient was calculated. The diffusion of alginate in mucus was also assessed using fluorescence recovery after photobleaching (FRAP). Briefly, fluorescein-labelled alginate was mixed into mucus samples at concentrations between 0 and 0.1%. Samples were gently mixed and then loaded onto glass slides using 9 mm x 120 μm SecureSeal spacers (Sigma, Poole, UK). The FRAP measurements were made using a Leica SP5 (II) Laser scanning confocal microscope. A bleach spot of 50 μm diameter was used with an initial post bleach of 3.7 s at 37 ms/frame, followed by 25 s at 250 ms/frame. Diffusion coefficients were calculated from the fluorescence signal using nonlinear least-square fitting as described by (Ladha et al., 1996).

The final method for determining diffusion coefficients of probe particles was particle tracking, again using a confocal microscope. The Stokes viscosity was calculated from ensemble data from particle tracking of 500 nm latex beads. The mean square displacement (MSD) of 500 nm latex beads was determined over 50 frames at 2 frames/s using multiple particle tracking as outlined in more detail previously (Macierzanka et al., 2011). The diffusion coefficient (D) was calculated from the MSD using the relation $D = MSD/4t$, where t is the timescale over which the displacement has occurred. The viscosity was then calculated from the diffusion coefficient via the Stokes Einstein relation.

Results and Discussion

In this article we describe a set of experiments to assess the ability of sodium alginate to decrease the diffusion of the products of digestion from the intestinal lumen to the site of absorption. As outlined above, one of the primary effects of soluble fibre in the small intestine is thought to be its ability to increase the viscosity of luminal contents and thus decrease the diffusion of nutrients from the site of hydrolysis to the intestinal enterocytes. Thus our first measurement was to determine the minimum concentration required to increase viscosity under conditions simulating the small intestinal environment. In particular, we determined the viscosity of sodium alginate as a function of concentration over a shear rate range between 10 to 500 s^{-1}. Over the specified range there was no significant difference in viscosity as a function of shear rate, therefore the mean value was used. The results are shown in Figure 1 and indicate that in the intestinal environment, local concentrations of the alginate would need to be greater than 0.1% in order to increase the viscosity much above that of water and that concentrations close to 1% would be required to have a really significant effect on viscosity. This data is consistent with previous assessment of the critical overlap concentration (c*) for alginate of 0.2-0.4% wt/wt depending on conditions (Nickerson & Paulson, 2004). Although this study showed that c* decreases as the ionic strength increases, it is still likely that concentrations in the intestine would need to

be in excess of 0.4% in order to provide sufficient viscosity to have an effect of nutrient and enzyme diffusion. Given the extent dilution of food/chyme due to gastrointestinal secretion as it passes down the gut, it is likely that this would require concentrations in excess of 3% in the original food (Minekus et al., 2014).
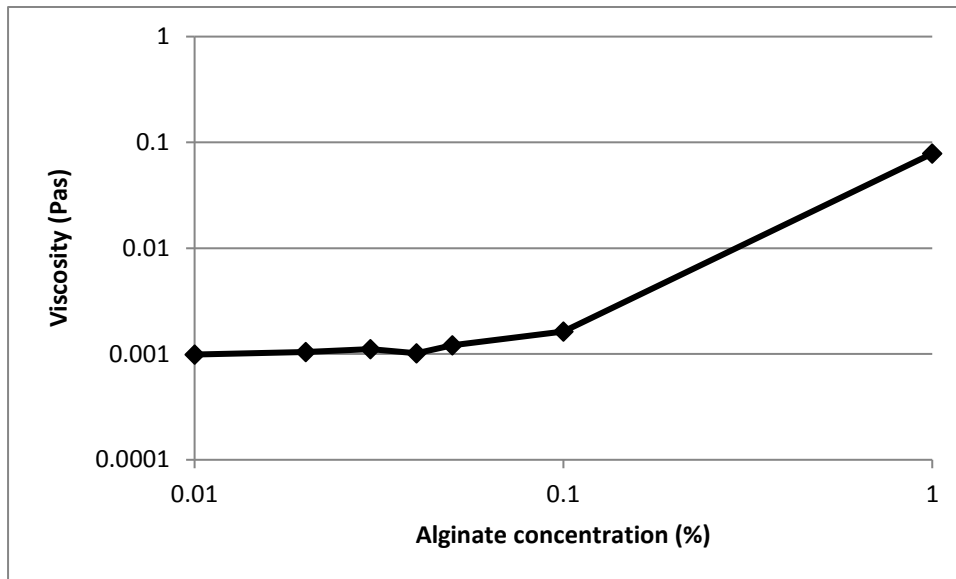

Figure 1 The change in viscosity of sodium alginate solutions as a function of concentration determined at 37℃.

The viscosity of chyme in the lumen of the small intestine is likely to have an impact on rates of nutrient diffusion as well as the diffusion of other endogenous compounds such as digestive enzymes, with higher viscosity generally leading to lower diffusion. However, the intestine is already surrounded by a viscous polymeric mucus layer. But it is not clear whether such a layer could have its permeability affected by the presence of dietary fibre, in this case alginate. As already described in the introduction, intestinal mucus is primarily composed of a large heavily glycated protein called MUC2 but also contains a wide range of other components that might potentially affect viscosity. The compounds include DNA in various states of degradation that are present as a direct result of cell shedding from the villus tips. Intestinal mucus is continuously secreted and maintains a viscoelastic barrier to bacteria and other particles. Thus in order to maximise changes in permeability, it would be necessary for the soluble fibre to diffuse through the mucus in order to avoid being trapped in the quickly degrading outer layers. With this in mind we measured the ability of fluorescently labelled sodium alginate to diffuse into a layer of ex-vivo porcine mucus. The fluorescent labelling of the alginate was undertaken as described above. Using confocal microscopy the fluorescence within the mucus layer was measured as a function of the distance from the mucus boundary as a function of time. The data from a typical experiment is shown in Figure 2.
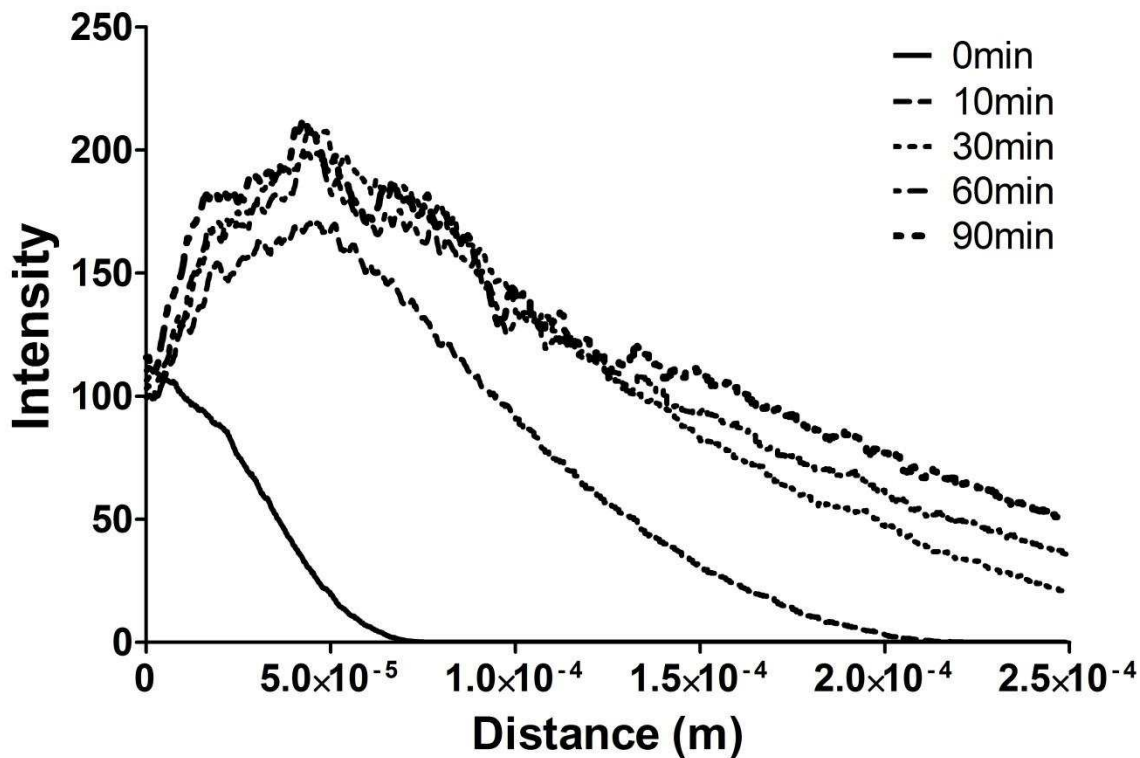
Figure 2 Fluorescence intensity from fluorescently labelled sodium alginate diffusing into porcine intestinal mucus plotted as a function of distance from the mucus boundary. The fluorescence profiles were measured at regular times over a period of 90 minutes. All measurements were made at 37 ℃.

The first thing that can be seen from Figure 2 is that indeed the fluorescent alginate was able to diffuse into the layer of porcine intestinal mucus. However, in addition to this it is clear that the intensity of the fluorescence gradually increases in the boundary region. This is most likely because the alginate becomes "clogged up" with the alginate. This entrapment of alginate in the boundary layer effectively prevented all of the fluorescent alginate from diffusing through the mucus. In addition, the peak in fluorescence in the boundary layer suggest that there was some attractive interaction between the alginate and the mucus leading to higher local concentrations in the boundary region but allowing unbound alginate to diffuse further into the mucus. These facts have been determined from a merely qualitative analysis of the data but in addition we can make use of the fact that the fluorescence is a more or less direct measure of the local concentration of polymer. As a result we can make use of Fick's law describing the flux of the polymer and derive a formula giving the diffusion coefficient of the alginate through the mucus. In this case a more useful form can be derived in which the fluorescence intensity F(x,t) is given by the following equation

1. $F(x,t) = a.erfc(\frac{x}{\sqrt{4Dt}})$

where x is the distance from the mucus boundary, t is the elapsed time, a is a scaler, erfc is the complimentary error function and D is the diffusion coefficient. When all the fluorescence profiles such as those given in Figure 2 were analysed using equation 1, the diffusion coefficient in all cases was seen to decrease over time. This already suggests that there was

a change in permeability of the mucus.

It should perhaps be stressed that the attractive interaction seen between the mucus and the alginate was not replicated in the purified mucin system. The ability of MUC2 mucin to bind alginate was tested in QCM experiments published elsewhere (Mackie et al., 2015). In these experiments mucin that had previously been adsorbed to a QCM chip was exposed to a solution of alginate. No increase of mass on the chip was observed suggesting that the alginate did not interact with the adsorbed mucin.

As already indicated above the apparent diffusion coefficients derived from equation 1 generally decreased as a function of time. This is demonstrated in Figure 3 in which the diffusion coefficients after 90 minutes are shown to be consistently less than the diffusion coefficients after 10 minutes, regardless of the concentration of alginate being used.
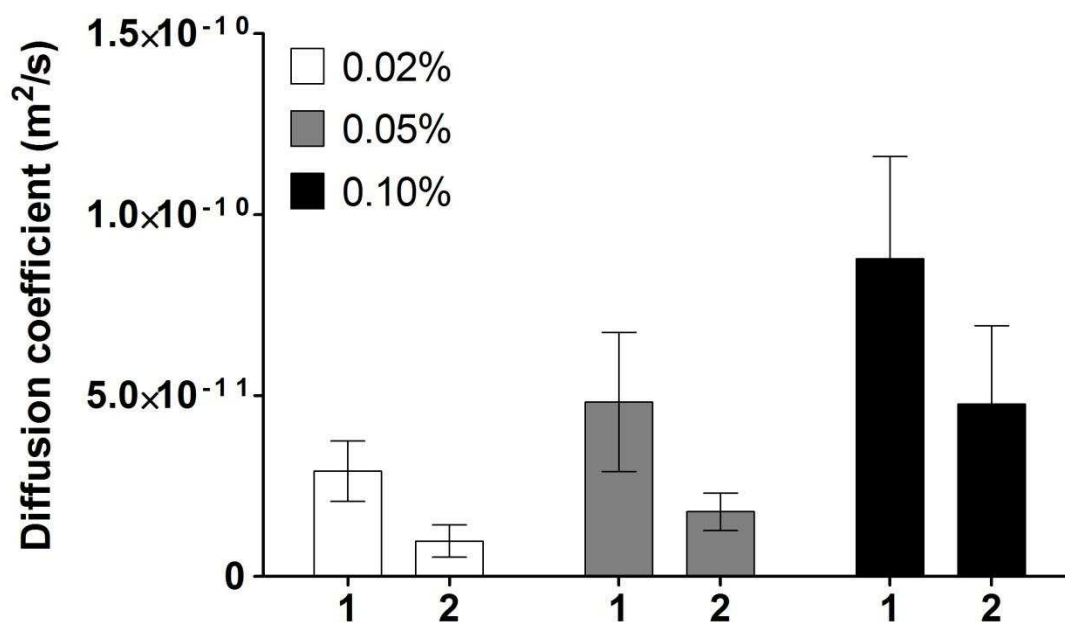

Figure 3 Diffusion coefficients of fluorescent sodium alginate through porcine intestinal mucin after 10 minutes (1) and 90 minutes (2) and at three different concentrations.

Although the alginate diffusion indicates the plausibility of the idea that soluble dietary fibre might decrease the permeability of intestinal mucus, this was not the primary result we were interested in. The next step was to show that the presence of the soluble fibre could decrease the permeability of intestinal mucus to digestion products. In order to do this we passed a protein stabilised emulsion through our standardised in vitro digestion protocol (Minekus et al., 2014). The resulting digesta was fluorescently labelled using Nile Red and the diffusion coefficient through mucus of this fluorescently labelled material was determined by FRAP. The results, which are shown in Figure 4, show a decrease in diffusion of the digesta by a factor of two in the presence of 0.1% sodium alginate. It is clear that even at the relatively low concentration of 0.1%, which generated only a very slight increase in viscosity in Figure 1, the ability of lipid digestion products to diffuse through ex vivo mucus was diminished. In fact it is not simple to interpret this data as the size of the diffusing objects was not well defined and was further complicated by particulates from sources such as the pancreatin.
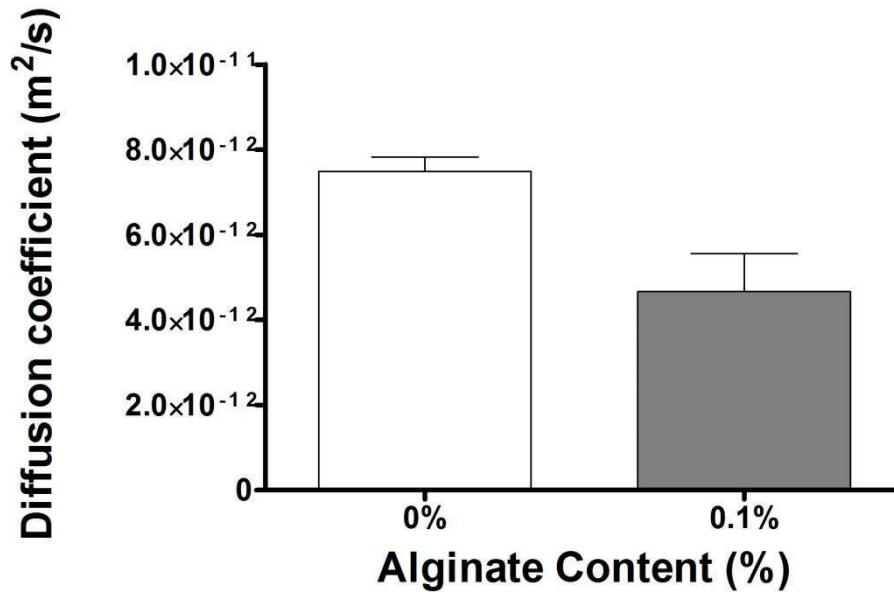
Figure 4 The diffusion coefficient of fluorescently labelled digesta through porcine intestinal mucus in the presence or absence of 0.1% sodium alginate. The diffusion was determined by FRAP experiments conducted at 37℃.

The porcine ex vivo mucus was prepared as described above and was then used to provide samples of purified MUC2 mucin and DNA that were prepared as described previously (Macierzanka et al., 2014; Macierzanka et al., 2011). The motion of the 500 nm latex beads was followed using particle tracking to yield an ensemble mean square displacement. From these values the average diffusion coefficients and thus the average Stokes viscosities were calculated as a function of concentration for the ex vivo mucus, MUC2 mucin and DNA. From the data in Figure 5 it is apparent that the ex vivo mucus used in the experiments described above needed the highest concentration to increase the viscosity significantly, while the mucin and in particular the DNA were able to generate significant increases in viscosity at much lower concentrations. It can be assumed that the scraped porcine small intestinal mucus contained many other components apart from mucin and DNA that did not contribute to the viscosity and thus more material was required. However, it is not entirely clear why the purified mucin and DNA were so different as their molecular weights would lead one to expect the mucin to be more viscous as its molecular weight was $>4 \times 10^6$ as opposed to 3×10^5 for the DNA. The most likely explanation lies in the different structural forms adopted by the two polymers.
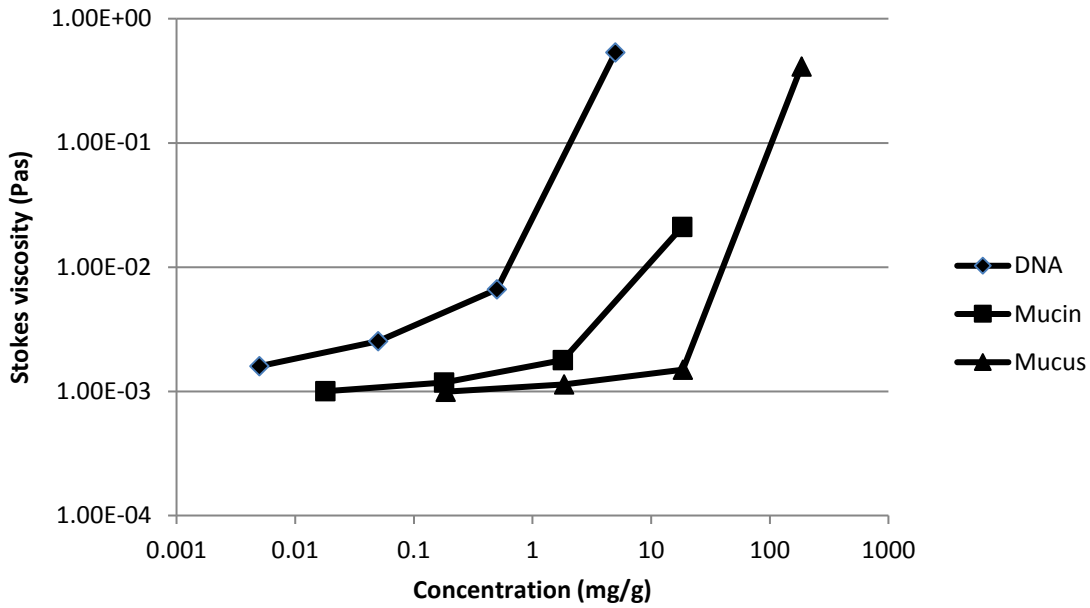
Figure 5 Stoke viscosities calculated from the diffusion of 500 nm latex beads plotted as a function of polymer concentration. The diffusion was calculated from particle tracking experiments conducted at 37°C.

The same method was used to assess the increase in Stokes viscosity of the ex vivo mucus as a function of increasing alginate concentration. In this case there was a linear increase in viscosity with added alginate, which again suggests that there were no specific interactions between the mucus and alginate. The linear increase also confirms that these low concentrations of less than 0.1% are still below c* and so are unlikely to be sufficient to have a significant effect on the diffusion of either enzymes or digestion products even in the presence of mucus.
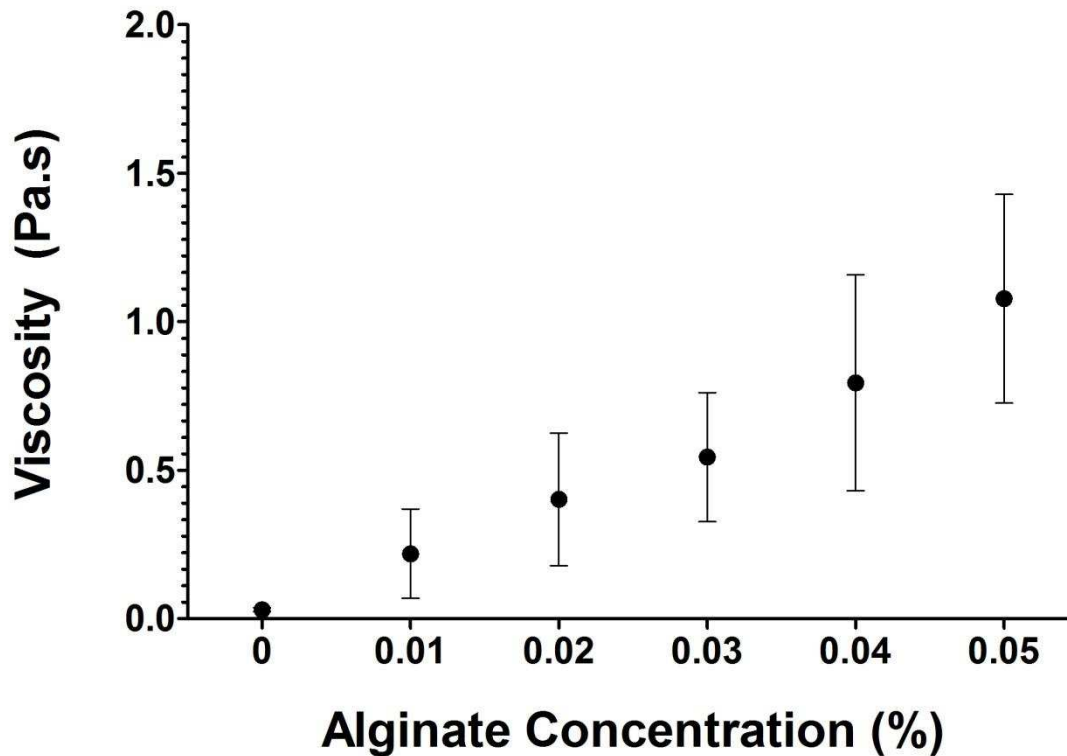
Figure 6 Stokes viscosity of ex vivo mucus as a function of increasing concentrations of sodium alginate, calculated from the diffusion of 500 nm latex beads

The heterogeneous nature of the small intestinal mucus layer is carried over into the ex vivo mucus used in these experiments. This leads to differences in local concentrations of mucus where the diffusion of beads and polymer will be highest. However, assuming that the polymer reached equilibrium and that there was little interaction between the mucus and alginate, it should be at a similar concentration everywhere. This in turn will lead to variations in the ratio of alginate to mucus and so locally it might be that the permeability of the mucus layer is governed largely by the alginate concentration.

As dietary fibres such as alginates that can decrease diffusion across the mucus layer, this could significantly influence rates of nutrient absorption. Changes in intestinal nutrient sensing and subsequent endocrine response through the secretion of GI hormones such as CCK in the proximal small intestine and GLP-1 more distally could alter gastric motility and alter feelings of satiety (Camilleri, 2015). This may have that added benefit of improving glycaemic control (Parker, Gribble, & Reimann, 2014). In addition, altering lipid absorption could also result in changes in bile acid reabsorption and recycling, indeed this is the purported mechanism of cereal β-glucan's cholesterol lowering ability (Othman, Moghadasian, & Jones, 2011). While further in vivo studies are required, the inclusion of dietary fibres in foods has the potential as a nutritional strategy to aid in the prevention and treatment of metabolic diseases such as obesity and type-2 diabetes.

Conclusions

In this article we have used the dietary fibre sodium alginate to test the idea that intestinal mucus permeability can be decreased as a result of entanglement. We have shown that sodium alginate is able to diffuse into scraped porcine intestinal mucus and that interaction between the two increases the alginate concentration in the boundary region. As a result of the entanglement the apparent diffusion of the alginate through the mucin was gradually decreased over time. This suggests that the permeability of the mucus to large polymers was decreased but we were subsequently able to show that the permeability to lipid digestion products was also decreased by almost a factor of two. This has potential importance for understanding the role of soluble dietary fibre in decreasing rates of nutrient absorption. By using 500 nm latex beads as probe particles we also demonstrated the importance of DNA in maintaining the high viscosity of the mucus layer and we were also able to show that the addition of low concentrations of alginate linearly increased the local viscosity of the mucus layer at the micron scale.

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