THE EFFECTS OF POLYSORBATE SURFACTANTS ON THE STRUCTURE OF MUCUS GLYCOPROTEINS

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ABSTRACT

A dynamic oscillatory technique was used to assess the effect of polysorbate non-ionic surfactants on mucus rheology. Adherent mucus gel was scraped from the surface mucosa of pig stomachs and purified by gel exclusion chromatography followed by ultrafiltration and gelation. Rheological measurements of this gel were carried out on a Carri-Med Controlled Stress Rheometer. Appropriate volumes of surfactant solution were added to weighed samples of mucus gel so that a final concentration of 20 mM surfactant was achieved in a gel containing 8% w/w solids content. Polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60) and polysorbate 80 (PS80) all decreased both storage (elastic) modulus G' and loss (viscous) modulus G'' significantly at 10 Hz ($p<0.05$, ANOVA). The extent of rheological changes induced by the four polysorbates could be ranked as: PS80 > PS20 > PS60 > PS40. The mechanisms by which surfactants disturb the mucus structure are not fully understood, nonetheless, they could possibly affect the mucus gel properties by causing depletion of the glycoprotein constituents such as non-mucin proteins and mucin associated lipids. This might lead to the conclusion that polysorbates, by reducing the viscoelasticity of mucus gel could alleviate its barrier properties and facilitate the diffusion of concomitantly administered drugs via mucus gel.

Key words: Mucus gel, Rheology, Surfactant, Polysorbates, Glycoprotein, Mucin

INTRODUCTION

Mucus is a viscoelastic fluid lining the epithelium of the gastrointestinal, respiratory and reproductive tracts. Gastrointestinal mucus occurs as a water-insoluble gel adherent to the mucosal surfaces and as a viscous, mobile solution in the lumen (1). The mucus gel is composed of more than 95% water, 0.5-5% mucus glycoprotein (mucin), 0.5-1% free proteins, electrolytes and bacteria (2). Mucins are large glycoprotein molecules which are the major contributors to the rheological properties of mucus. The mucus gel layer (MGL), in addition to protection and lubrication, is a potential barrier to drug absorption (3, 4, 5, 6, 7). Gastrointestinal mucins are glycoproteins of large molecular size (~$10x10^3$ kDa), which are composed of more than 80% by weight carbohydrate (8) and carry a net negative charge due to ester sulphate and sialic acid moieties as the terminal sugars of the carbohydrate side chains (9). Apart from covalent S-S bonds, intra and inter-molecular non-covalent bonds are also believed to be responsible for the formation of the gel matrix (10). Reduction of the disulphide bonds by thiol reagents has been shown to result in solubilization of the mucus gel with loss of its viscoelastic properties (11, 12). This could lead to the elimination of the barrier properties and hence the protective role of the MGL as the reflux of bile from duodenum back to the stomach has been proposed as one potential cause of peptic ulcer due to the mucolytic effects of bile salts (13). While the effect of the anionic surfactant, sodium dodecyl sulphate (SDS), on mucus structure has also been reported (13), the effect of non-ionic surfactants on mucus structure appears to have been neglected as a topic of study despite their widespread use in pharmaceutical formulations.

Polyoxyethylene sorbitan esters (polysorbates or Tweens®) (Fig 1) are a group of non-ionic surfactants which are used as solubilizers, emulsifiers and wetting

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-spreading agents. They have shown little disrupting effects to biological membranes (14). In a recent study by Nani Roodi and Sajadi Tabassi (not published) all but PS20 induced little hemolysis to human erythrocytes at concentrations well above their cmc values.

The aim of the present study was to investigate the effect of polysorbates namely, polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60) and polysorbate 80 (PS80) on the structure of mucus as reflected by the changes in the rheological properties of the mucus glycoprotein gel.

MATERIALS AND METHODS

Materials:

Ethylenediaminetetraacetic acid (EDTA), PS20, PS40, PS60 and PS80 were from Aldrich Chemical Company (Gillingham, Dorset, UK), sodium chloride was from BDH (Poole, Dorset, UK), phenylmethylsulphonylflouride (PMSF) and sodium azide were from Sigma Chemical Company (Poole, Dorset, UK). All chemicals were used as supplied. The stomachs of freshly slaughtered pigs (*Suis scrofa domestica*) were collected in ice from Anglo-Dutch Meat (Kent, UK).

Preparation of purified mucus gel:

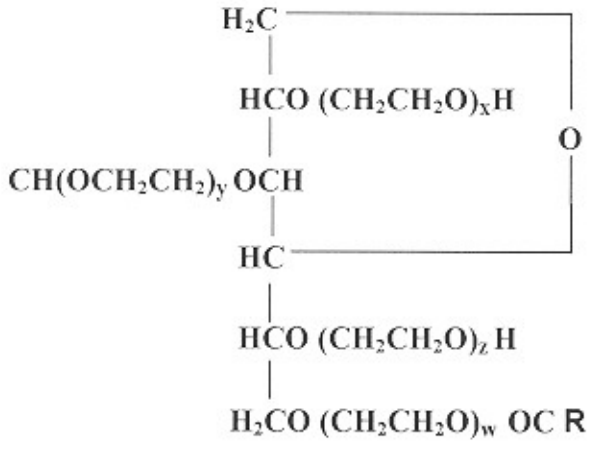
The adherent mucus which was gently scraped from the surface mucosa of pig stomach with the aid of a wooden spatula was homogenized with protease inhibiting solution (200 mM sodium chloride, 0.02% sodium azide, 5 mM EDTA and 1 mM PMSF). Care was taken to avoid scraping areas of the mucosa which were bile stained or ulcerated. Mucus from a batch of 60 stomachs was pooled and homogenized with five volumes of protease inhibiting solution in a domestic blender for one minute. The mixture was then centrifuged at 20,000xg for 1.5 h at 4°C to remove food and cell debris. The supernatant was filtered twice through glass wool to remove any remaining insoluble debris and stored at 4°C prior to gel filtration.

Mucin purification was carried out by gel exclusion chromatography in a glass column (40 cm height x 9.5 cm i.d.) filled with Sepharose CL-4B (Pharmacia) using protease inhibiting solution as the eluant. 200 ml aliquots of the filtered mucin solution were loaded onto each column at 4°C and eluted at a flow rate of 400 ml h^{-1} with the eluate being monitored spectrophotometrically at 280 nm, using a flow-through cell. Two peaks were monitored, the first excluded fraction (peak A) was collected while the second included fraction (peak B) was discarded. The solution from peak A was concentrated to 25% of its original volume by ultrafiltration in a Millipore Minisette system and then dialysed exhaustively against distilled water. The concentrated solution was further concentrated to a gel using an Amicon cell fitted with 1000 KD membrane (Omega). The total solid content of gels was determined by triplicate dry weight determinations. This was carried out by determining the dry weight of the gel by incubation of preweighed aliquots of the gel at 120°C for 2 h (or until constant weights were achieved).

Appropriate volumes of each surfactant solution were added to a known weight of the mucus gel so that a final concentration of 20 mM of each test compound was achieved in a gel sample containing 8% mucus glycoprotein. Distilled water was used as the control throughout the study. Samples were then mixed gently with the aid of a glass rod and left overnight at 4 °C to ensure the proper diffusion of the test compound into the gel and also to achieve a homogenous preparation.

Rheological testing:

A dynamic method of rheological measurement was carried out using a Carri-Med Controlled Stress Rheometer (CSL 100) on all control and test samples of mucus gel over a frequency range of 0.1-10 Hz. The instrument was employed in oscillatory mode using a steel parallel plate (4 cm diameter) as the upper member of the measuring system. The temperature of the lower plate was set at 20±0.1°C and the gap adjusted to 150μm. The required quantity of the sample was loaded centrally onto the lower parallel plate by means of a steel spatula. The lower plate was then raised gently to the pre-set distance from the upper plate. Sinusoidal deformation of the sample under test was produced by an electronically controlled system driving the measuring geometry. The amplitudes and frequencies used during the rheological assessment of the mucus samples were carefully selected by validation of the method. The frequency sweep was carried out with assumption that the collected data were produced within the linear viscoelastic region. The storage modulus G' (elastic component) and the loss modulus G'' (viscous component) were calculated by the oscillation software at a constant stress of 10 Pa at each frequency. Statistical analysis on all measurements



w+x+y+z = 20

PS20	R= C11H23	Polyoxyethylene (20) sorbitan monolaurate
PS40	R= C13H27	Polyoxyethylene (20) sorbitan monopalmitate
PS60	R= C17H35	Polyoxyethylene (20) sorbitan monostearate
PS80	R= C17H33	Polyoxyethylene (20) sorbitan monooleate

Figure 1. Chemical structure of polysorbates

Table 1. Percentage decrease in the storage and loss moduli (mean values of six measurements) of partially purified porcine gastric mucus (PGM) at three different frequencies treated with various polysorbates at 4°C compared to water as the control

Test Compound	Percent decrease in G'			Percent decrease in G''		
	0.1 Hz	4.5 Hz	10 Hz	0.1 Hz	4.5 Hz	10 Hz
PS20	-35.35[a]	-23.53[a]	-21.11[a]	-24.88[a]	-14.96[a]	-12.91[a]
PS40	-14.58[a]	-10.33[b]	-8.73[b]	-11.67[a]	-5.49[c]	-3.63[c]
PS60	-29.42[a]	-20.71[a]	-18.98[a]	-22.73[a]	-15.08[a]	-13.13[a]
PS80	-34.97[a]	-24.97[a]	-23.13[a]	-27.80[a]	-17.80[a]	-15.85[a]

a: $p<0.01$, b: $p<0.05$, c: Not significant ($p>0.05$)

regarding control and test samples was carried out using analysis of variance (ANOVA).

RESULTS

The results of the rheological studies are expressed as plots of G' and G'' of control and samples as a function of frequency. In order to characterize the viscoelastic properties of the purified gel, G' and G'' measurements were made on an 8% mucus gel over a frequency range of 0.1-10 Hz and it was found that both G' and G'' increased by increasing frequency and the values of G' were always greater than the respective values of G'', showing the predominance of the elastic component over the viscous component of mucus gel (fig 2).

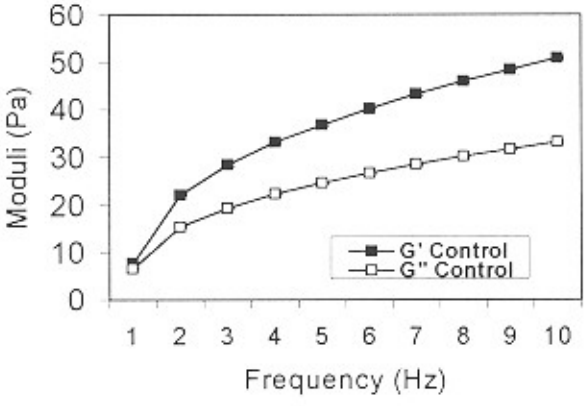


Fig. 2. Plots of mean storage and loss moduli of an 8% porcine gastric mucus (PGM) as a function of frequency measured by dynamic oscillation testing on a controlled stress rheometer (Carri-Med CLS 100) at 20±0.1 °C (n=6).

Figures 3, 4, 5 and 6 show the plots of G' and G'' for the mucus samples treated with the four polysorbates compared with the control samples. As table 1 shows, G' was decreased significantly by all polysorbates at frequencies 0.1 and 10 Hz ($p<0.05$) except for PS40 and PS60 at 0.1 Hz ($p>0.05$) and G'' decreased significantly at both 0.1 and 10 Hz by all polysorbates tested ($p<0.05$).

DISCUSSION

The results of this study showed that purified mucus glycoproteins from porcine stomachs can produce a viscoelastic gel in which the elastic response to deformation predominates over the viscous response even at the lowest tested frequency, i.e., 0.1 Hz (fig 2). The G' and G'' values showed a small frequency dependence especially at higher frequencies. These are characteristics of a weak, cross linked but stable viscoelastic gel which flows and anneal if it is sectioned. Partially purified porcine gastric mucus (PGM) has been used extensively in recent years as a model for human mucus in the rheological and biochemical studies of mucus (5, 15, 16, 17) due to the shortage of human mucus and by the fact that PGM has shown mechanical properties similar to those of human gastric mucus (10).

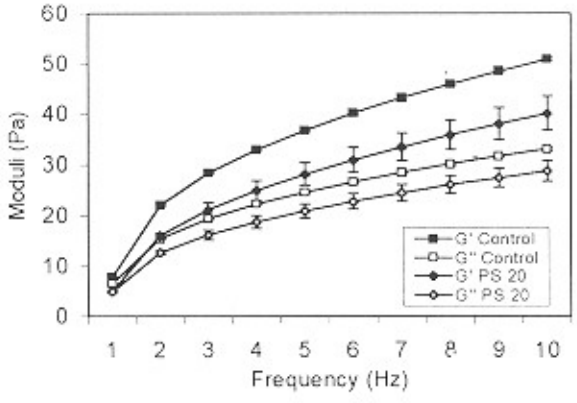


Fig. 3. Plots of mean storage and loss moduli of an 8% porcine gastric mucus (PGM) treated with 20 mM PS 20 as a function of frequency measured by dynamic oscillation testing on a controlled stress rheometer (Carri-Med CLS 100) at 20±0.1 °C (n=6).

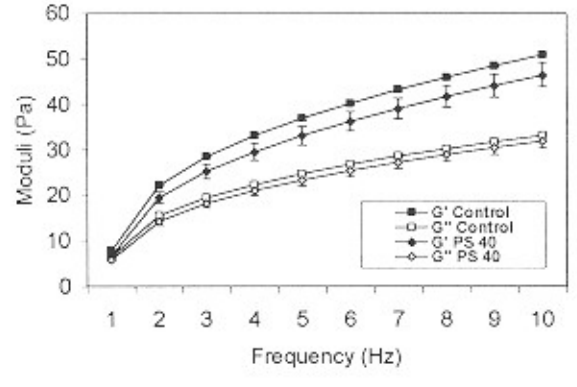


Fig. 4. Plots of mean storage and loss moduli of an 8% porcine gastric mucus (PGM) treated with 20 mM PS 40 as a function of frequency measured by dynamic oscillation testing on a controlled stress rheometer (Carri-Med CLS 100) at 20±0.1 °C (n=6).

The effect of naturally occurring surfactants, bile salts and lysophosphatidylcholine (LPC), on the rheolo-

gical properties of mucus has been studied by Martin *et al* (13) who found that all the bile salts and sodium dodecyl sulphate (SDS) decreased both the viscous and elastic parameters of mucus while phosphatidylcholine (PC) had little effect on the gel structure. El-Hariri (17), by the use of an oscillatory rheological method, demonstrated that bile salts were able to damage mucus structure by decreasing both the elasticity and the viscosity of the gel.

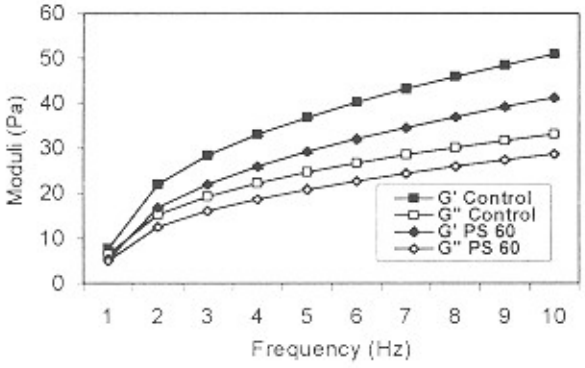


Fig. 5. Plots of mean storage and loss moduli of an 8% porcine gastric mucus (PGM) treated with 20 mM PS 60 as a function of frequency measured by dynamic oscillation testing on a controlled stress rheometer (Carri-Med CLS 100) at 20±0.1 °C (n=6).

Although the absolute values of G` and G`` were different amongst the various control batches of mucus that were used to determine the effects of surfactants, all the investigated polysorbates induced a decrease in G` and G`` over the range of frequencies that were investigated (table 1). The decrease in G` and G`` of the gel by the four tested polysorbates in terms of percentage reduction at 10 Hz could be ranked as PS80 > PS20 > PS60 > PS40 (table 1). The four polysorbates tested bear the same number of 20 oxyethylene units with different hydrocarbon chains and their hydrophobicity and molecular weight can be ranked as PS80 > PS60 > PS40 > PS20 indicating that no direct relation could be established between the surfactant structure and its effect on the gel viscoelasticity.

While the mechanisms by which surfactants disturb the mucus structure are not fully understood, it has been reported that bile salts might affect the mucus gel properties by causing depletion of the glycoprotein constituents such as non-mucin proteins and mucin associated lipids (18). These workers showed that associated lipids and covalently bound fatty acids impart a significant contribution to mucus viscosity and it was demonstrated that extraction of the associated lipids resulted in a 80-85% decrease in mucus viscosity and a further 39% decrease due to the removal of covalently bound fatty acids (18). Aono *et al.* (19) showed that bile salts are able to solubilize mucus components, including proteins and glycolipids. Since polysorbates like all surfactants are able to reduce the interfacial tension, it is conceivable that they could disrupt the electrostatic bonds between the associated lipids and mucus glycoproteins causing the extraction of lipids and consequently reducing the viscoelasticity of mucus. Alternatively, the interfacial activity of surfactants could also cause disruption of inter and intra-molecular non-covalent interactions of glycoprotein molecules especially any hydrophobic interaction and hydrogen bonds between carbohydrate side chains of the neighbouring mucus glycoproteins which results in the disruption of the gel network. It might be concluded that polysorbates, by reducing the viscoelasticity of mucus gel could alleviate its barrier properties and as a result facilitate the diffusion of concomitantly administered drugs via mucus gel.

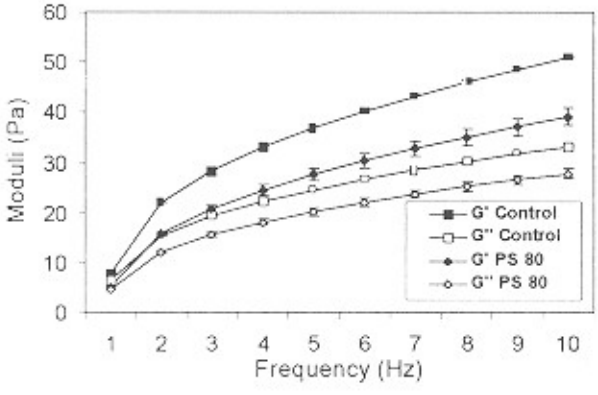


Fig. 6. Plots of mean storage and loss moduli of an 8% porcine gastric mucus (PGM) treated with 20 mM PS 80 as a function of frequency measured by dynamic oscillation testing on a controlled stress rheometer (Carri-Med CLS 100) at 20±0.1 °C (n=6).

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