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A comparative study on the role played by mucus glycoproteins in the rheological behaviour of the mucoadhesive/mucosal interface

S.A. Mortazavi, B.G. Carpenter and J.D. Smart

Drug Delivery Research Unit, The School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth PO1 2DZ (UK)

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Summary

One proposed mechanism of mucoadhesion involves the interpenetration of the mucus/mucoadhesive molecules followed by the formation of non-covalent interactions. Mucus glycoproteins are believed to be the major structure forming component of mucus, giving rise to the cohesive and the viscoelastic nature of the mucus gel. The addition of a known mucoadhesive, the polyacrylic acid Carbopol 934P (paa), to purified mucus glycoprotein resulted in the formation of a strengthened gel network. This was indicated on visual examination, and also on rheological examination using mechanical spectroscopy. A large mean G' (the storage modulus, found between 10 and 0.1 Hz) was obtained for the glycoprotein/paa mix in comparison to when the glycoprotein and paa gels were tested separately at the same concentration. Similar results were obtained with crude and homogenised mucus samples. This gel strengthening phenomena was optimum at pH values around the pK_a of paa. It was concluded that it is the glycoprotein component of mucus which interacts with paa to produce gel strengthening, and this may increase the cohesive nature of the weakest component of a mucoadhesive joint, thus allowing prolonged mucosal adhesion. A rheological investigation of a pH 6.2 glycoprotein/paa mix between 10 and 0.002 Hz indicated that this gel strengthening effect resulted from both chain entanglement and the formation of secondary chemical bonds.

Introduction

The development of adhesive dosage forms for controlled drug delivery to or via mucous membranes is of interest with regard to local drug therapy and the systemic administration of peptides and other drugs poorly absorbed from the gastrointestinal tract.

Correspondence to: J.D. Smart, Drug Delivery Research Unit, The School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth PO1 2DZ, U.K.

Adhesion is believed to be an interfacial phenomenon which is influenced by surface energies. When an adhesive bond is established between two materials, the total surface energy of the system is diminished, destroying two free surfaces and creating a new interface. The attachment of synthetic or biological macromolecules to a biological tissue is referred to as 'bioadhesion'. When applied to a mucosal epithelium, a bioadhesive system adheres and presumably interacts primarily with the mucus layer, and this phenomenon is referred to as 'mucoadhesion' (Gu et al., 1988; Junginger, 1990).

Mucoadhesive materials have been identified as being hydrophilic macromolecules containing numerous hydrogen bond forming groups e.g. hydroxyl, and carboxyl groups (Chen and Cyr, 1970; Smart et al., 1984).

The adhesive bond between a polymer and mucus gel can be investigated in terms of the contribution of three regions: (i) the surface of the bioadhesive polymer; (ii) the interfacial layer between the bioadhesive material and mucosa; and (iii) the mucosal surface (Peppas and Mikos, 1990). The mechanically weakest component of the adhesive joint would be predicted to be the interfacial layer that consists, at least initially, of mucus.

Mucus is a weak viscoelastic gel that covers all the internal tracts of the body. It is a mixture containing up to 95% water and whose major structure forming component is glycoprotein (molecular mass $2\text{–}14 \times 10^6$ Da) (Marriott and Gregory, 1990). These glycoprotein molecules are capable of associating with each other by means of non-covalent interactions to form the gel matrix which is responsible for the rheological properties of the mucus. In addition, mucus glycoproteins are believed to be responsible for the interaction with the bioadhesive polymers and hence would be expected to be an important component in the process of mucoadhesion (Peppas and Mikos, 1990).

The process of mucoadhesion has been proposed to begin with the establishment of an intimate contact between the mucoadhesive polymer and the mucus gel (Duchene et al., 1988). The role of surface energy thermodynamics in mucoadhesion has been considered in other work (Lehr, 1991; Lehr et al., 1992) and this would be an important factor in establishing an intimate contact. The second stage involves the penetration of the mucoadhesive polymer into the mucus gel network, followed by the formation of secondary chemical bonds between the mucus and the mucoadhesive material.

In this investigation, the second stage, the molecular interpenetration of the mucus/mucoadhesive macromolecule is studied, and the effect this has on the rheological and cohesive nature of the interfacial layer. Previous work by Allen et al. (1986) has shown a synergistic increase in the viscosity of gastric mucus glycoprotein by the addition of carbomer 934. Kerr et al. (1990) used mechanical spectroscopy to investigate the interaction between glycoprotein gels and polyacrylic acid and the effect of pH and polymer chain-length on this. More recently, Mortazavi et al. (1992) have observed significant mucus gel strengthening (using mechanical spectroscopy) on incorporating the polyacrylic acid, Carbopol 934P (paa). This study investigates the possible changes in the rheological behaviour of the major structure forming component of the mucus gel i.e. mucus glycoproteins, on the introduction of a known mucoadhesive polymer paa and whether the mucus gel strengthening phenomenon observed in our previous study (Mortazavi et al., 1992) could be due to the glycoprotein/paa interactions or involves other components of the mucus gel. The effect of pH on the rheological behaviour, along with the nature of the glycoprotein/paa interactions, were also studied using mechanical spectroscopy.

Materials and Methods

Materials

Carbopol 934P (paa) was obtained as a gift from B.F. Goodrich, Hounslow, U.K., Potassium thiocyanate, sodium azide, sodium chloride and sodium edetate (disodium salt) were purchased from BDH Chemicals, Poole, U.K., phenylmethylsulphonyl fluoride (PMSF) from Sigma Chemical Co. Ltd., Poole, U.K. and Sepharose 4B from Pharmacia, Milton Keynes, U.K.

Preparation of the crude mucus

Crude mucus was obtained by scraping hog stomachs obtained fresh from slaughter and was homogenised by gentle mixing. The % w/w of 'solids' was determined by leaving a small portion (0.5 g) in an open glass vial at 50°C for 48 h.

Preparation of homogenised mucus gels

Batches of homogenised mucus were prepared using the method described by Mortazavi et al. (1992). Crude mucus scraped from hog stomachs

was mixed with an equal quantity of an isotonic PMSF containing solution, centrifuged at 2500 × *g* for 1 h, the supernatent discarded and the lower gel layers taken. These were pooled, exhaustively dialysed for 24 h at 4°C, homogenised by blending and the % w/w of solids determined for each batch. If necessary the % w/w was adjusted with purified water to give a concentration of 30 mg g^{-1}.

Preparation of the mucus glycoprotein

Batches of glycoprotein were obtained by scraping hog stomachs which were subsequently homogenised by blending with an equal quantity of an isotonic solution containing PMSF (0.0175% w/v), sodium azide (0.02% w/v), sodium edetate (0.186% w/v), sodium chloride (0.9% w/v) and potassium thiocyanate (4.276% w/v). The latter component was added to aid mucus solubilization (Brown et al., 1981). The resulting mixture was then stirred at 4°C for 6 h and centrifuged at 12000 × *g* for 1 h at 4°C. The supernatant layers were then collected, pooled and passed through glass wool to remove any particulate matter. 42 ml portions were loaded onto a Sepharose 4B gel filtration column (height (35 cm) × diameter (6 cm)) and eluted with a solution containing sodium chloride (0.9% w/v) and sodium azide (0.02% w/v) at a flow rate of 1.4 ml min^{-1}. The fractions containing the glycoproteins were in the first significant absorbance peak at 280 nm and these were collected and pooled.

Exhaustive dialysis was carried out at 4°C and the pH of the dialysate adjusted to the required value. Finally, the dialysate was centrifuged at 25000 × *g* for 2 h at 4°C to obtain the glycoprotein gel. The % w/w of solids was determined as before and if necessary adjusted to give a concentration of 30 mg g^{-1}.

Characterisation of the mucus preparations

The mucus and the glycoprotein samples were analysed using SDS/polyacrylamide (7.5% w/v acrylamide/bisacrylamide) gel electrophoresis for the presence of glycoprotein and protein fractions, and the purity of the glycoprotein obtained, using a procedure similar to that described by Laemmli (1970). The samples were prepared for electrophoresis by mixing with a loading solution containing 2-mercaptoethanol, SDS and glycerol and heating to 100°C for 2 min in a similar manner to that described by Mantle and Allen (1981). After electrophoresis (23 mA/gel), the gels were stained with danzyl hydrazine for the presence of carbohydrate. The results confirmed the presence of glycoprotein (Mol. Wt > 205000) appearing near the top of the gel in all three samples tested (Fig. 1). The crude and the homogenised mucus samples also contained numerous other bands of carbohydrate residues whereas the purified glycoprotein only showed traces of these smaller molecules. Staining for protein with Coomassie brilliant blue, however, revealed the presence of small proteins, even in the purified glycoprotein sample.

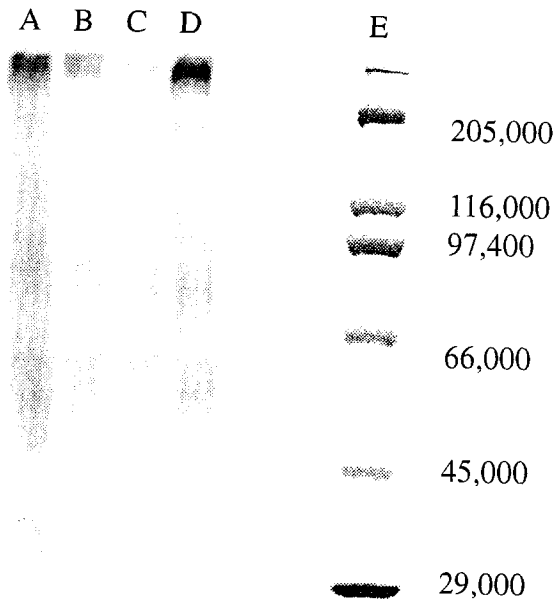

Fig. 1. SDS/PAGE using a danzyl hydrazine stain (the image has been imported into a computer programme and the shades inverted) of crude mucus (A), homogenised mucus (B), the supernatent after centrifugation (C) and purified glycoprotein (D) (also showing protein markers (E) with their molecular weights).

Experimental procedure

Preparation of test samples 1.5 g samples of either crude mucus or homogenised mucus were mixed with an equal quantity of paa gel (5 mg

g^{-1}) and the pH was adjusted to the required value (initially 6.20) using either 0.1 M NaOH or 0.1 M HCl. The final weight of the sample was then adjusted to 4.5 g using purified water. Further mixtures containing 1.5 g of either crude mucus or homogenised mucus alone and 1.5 g paa alone were adjusted to pH 6.20 and made up to 4.5 g with purified water. Other samples were prepared for investigation by making mucus/paa mixtures at various pH values between 4 and 8.

As only small amounts of glycoprotein were available from the purification procedure the experimental technique was modified to minimise the quantities used. 200 mg samples of the glycoprotein gel, previously adjusted to pH 6.20, were mixed with 200 mg of a 5 mg g^{-1}, pH 6.20 paa gel. Further mixtures containing 200 mg glycoprotein gel with 200 mg water, and 200 mg paa with 200 mg water were also prepared. Using a similar procedure, the effect of pH was investigated by preparing further glycoprotein/paa mixtures at various pH values between 4 and 8.

Rheological studies All samples were allowed to equilibrate at 4°C overnight, prior to testing at 15°C using a Carri-Med CSL 100 Rheometer (Carri-Med Ltd, Dorking, U.K.) fitted with either a 2 cm stainless-steel cone (for glycoprotein samples) or a 4 cm parallel plate with a 0.5 mm gap (for crude and homogenised mucus samples). Each sample was individually loaded, allowed to further equilibrate for 5 min, then tested using a frequency sweep between 10 and 0.1 Hz and the mean storage modulus (G') and loss modulus (G'') calculated.

A logarithmic frequency sweep between 10 and 0.002 Hz was also performed on a pH 6.20 glycoprotein/paa sample.

Results

The crude mucus was visibly non-homogeneous, therefore only limited studies were attempted with this sample.

Addition of paa to the crude and homogenised mucus as well as the glycoprotein gels resulted in visible gel strengthening within the sample vials at room temperature. In contrast, the mucus/water, glycoprotein/water and paa/water mixtures were found to behave more like low (to medium) viscosity liquids. This was confirmed when examined using mechanical spectroscopy

TABLE 1

Comparative rheological assessment of the various mixtures of mucus and paa at pH 6.20 ($n = 3$ except for the crude mucus containing preparations)

Sample	G' (Pa) (S.D)	G'' (Pa) (S.D)
Crude mucus/paa	6.20	4.47
Crude mucus/water	1.90	8.10
paa/water	1.41	5.70
Homogenised mucus/paa	18.95 (6.00)	8.76 (1.51)
Homogenised mucus/water	0.44 (0.01)	1.53 (1.93)
paa/water	2.57 (1.01)	5.40 (0.69)
Glycoprotein/paa	306.80 (86.40)	68.90 (18.70)
Glycoprotein/water	5.04 (2.47)	2.10 (1.20)
paa/water	37.50 (4.37)	35.10 (2.54)

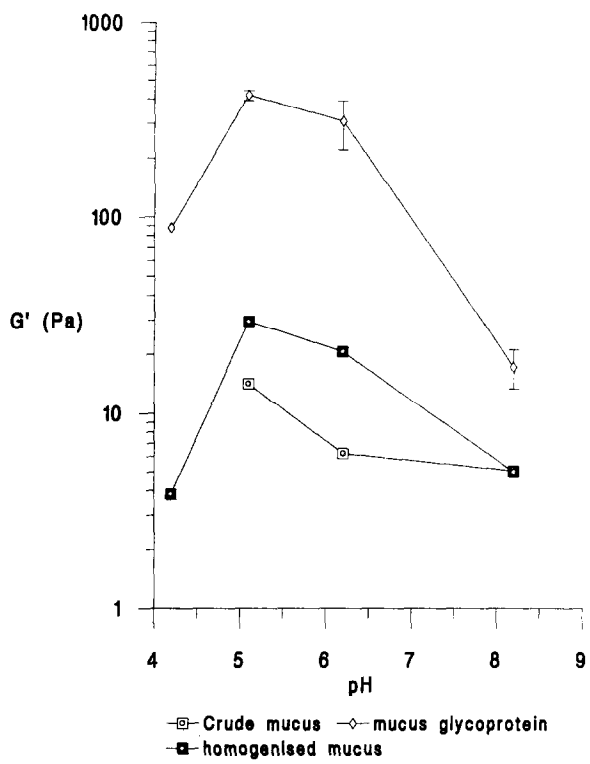

Fig. 2. The effect of pH on G' for glycoprotein/paa, crude mucus/paa and homogenised mucus/paa mixtures (for glycoprotein/mucus, the mean value given with $n = 3$, S.D. bars).

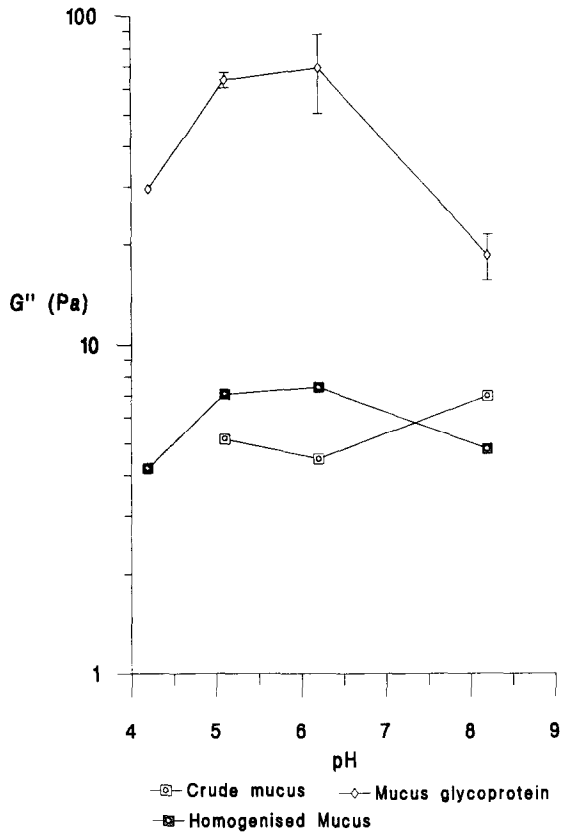

Fig. 3. The effect of pH on G'' for glycoprotein/paa, crude mucus/paa and homogenised mucus/paa mixtures (for glycoprotein/paa mixtures $n = 3$, S.D. bars)

(Table 1). A large G' (a measure of the resistance to elastic deformation and representative of the extent of structuring within the sample) was found for all the mixtures compared to the paa and mucus samples alone, confirming the formation of a strong gel network as reported in previous work (Allen et al., 1986; Mortazavi et al., 1992). The G' value was substantially greater than that obtained when the constituents of each mix were tested separately at the same concentration. The G'' (a measure of the resistance to liquid flow) was smaller than G' for the mucus/paa samples, suggesting the mix was structured and 'solid-like' rather than a viscous liquid.

The pH was found to affect this gelling phenomenon (Fig. 2) as indicated by the substantial change in G'. Similar, but smaller, changes were observed with the G'' values (Fig. 3). Visible signs of gel breakdown were observed at pH 4.2 and analysis of the crude mucus/paa mixture was not possible due to the complete disruption of the gel structure.

The log frequency sweep between 10 and 0.002 Hz, using the glycoprotein/paa sample at pH 6.20, shows substantial G' and G'' values being obtained throughout the frequency range, although these gradually decreased with a reduction in the frequency (Fig. 4).

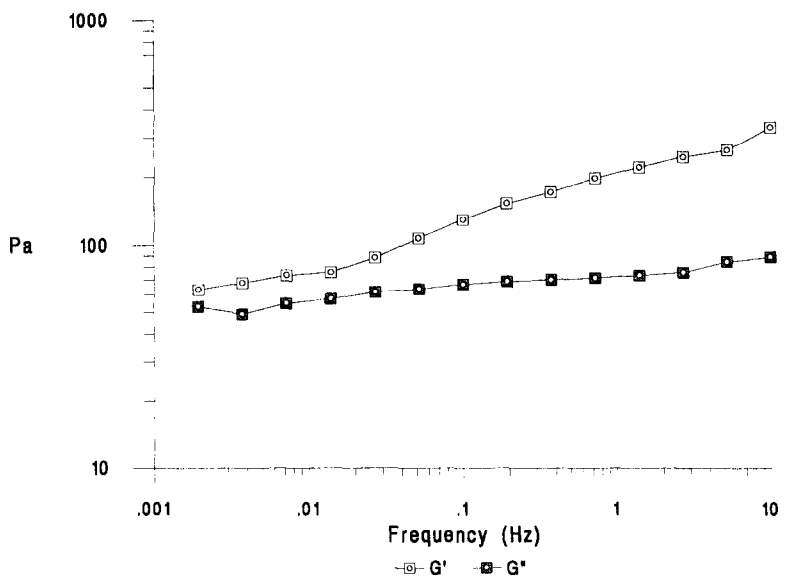

Fig. 4. The effect of frequency on the storage modulus (G') and loss modulus (G'') of the glycoprotein/paa mix at pH 6.20.

Discussion

The results obtained with purified glycoprotein are similar to those observed using homogenised mucus and crude mucus (Table 1). The differing values of G' and G'' between the three mucus preparations are due to the differing concentrations of glycoprotein within each sample, and the varying degrees of damage caused during the preparation procedures. The formation of a greatly strengthened gel network on the inclusion of the known mucoadhesive polymer paa at acid to neutral pH values occurs in all cases. The paa/mucus mixtures are visibly much more gel like than the paa and mucus samples evaluated separately at the same concentration. The formation of a strong gel network is therefore due to the interaction of paa with the mucus glycoprotein component. The optimum pH for the gel strengthening phenomenon was found to be in the weakly acidic to neutral region, around the pK_a of the paa (Figs 2 and 3). This indicates the need for the presence of a certain number of unionised carboxyl groups within paa for optimum interaction with the glycoprotein. Similar findings have been reported in other studies of mucoadhesion (Park and Robinson, 1987). The gel breakdown observed at (and below) pH 4.2 was not reported by other workers (Kerr et al., 1990). This breakdown was observed with the glycoprotein sample and might, as suggested in previous work (Mortazavi et al., 1992), be due to the presence of the low molecular weight proteins detected by gel electrophoresis.

The log frequency sweep on the glycoprotein/paa mixture (Fig. 4) is intermediate between that seen with the physically entangled systems and cross-linked systems, as described by Ross-Murphy and McEvoy (1986). The G' and G'' values for a cross-linked gel would not be influenced by the frequency of oscillation (i.e., the experimental time), while a physically entangled gel network would show a substantial decline in G' at low frequencies. This is because in physically entangled systems macromolecules are given time to untangle and move past each other at low frequencies so that the material behaves more like a viscous liquid, while at higher frequencies they are only able to show elastic deformation. This intermediate behaviour suggests that the gel formed on mixing these macromolecules may be both an entangled and weakly cross-linked system held together by secondary chemical bonds (e.g., hydrogen bonds).

In conclusion, it can be suggested that the mucoadhesive polymer paa interacts with the glycoprotein component of the mucus gel to form the strengthened gel network. If molecular interpenetration occurs during mucoadhesion then this would strengthen the interfacial layer of the mucoadhesive joint which would help to retain the dosage form at its site of application.

In addition, the gel strengthening phenomenon observed may be due to the formation of an entangled network which is further strengthened by the formation of secondary (non-covalent) chemical bonds between the mucoadhesive polymer chains and the glycoprotein network present within the mucus gel.

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