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An in vitro assessment of mucus/mucoadhesive interactions

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Abstract

One proposed mechanism of mucoadhesion involves the interpenetration of the mucus/mucoadhesive molecules, followed by the formation of secondary chemical bonds. In this study the nature of interactions between the mucus gel and the mucoadhesive macromolecule was investigated. A logarithmic frequency sweep between 10 and 0.002 Hz was used to investigate the nature of interactions between homogenised mucus gel and the model mucoadhesive Carbopol 934P (C934) at pH 6.2. The rheogram obtained was found to be intermediate between a physically entangled system and a cross-linked system, and was found to closely resemble that of a mixture of mucus glycoprotein (major structure forming component of the mucus gel) and C934 at pH 6.2. Furthermore, it was found that the addition of the hydrogen bond breaking agents urea and potassium thiocyanate (KCNS) to a mixture of homogenised mucus/C934 at pH 6.2 resulted in a reduction in the G' (storage modulus) as well as the G'' (loss modulus) of the mixture. The addition of the model monosaccharides L-fucose and D-galacturonic acid to the mucoadhesive poly(acrylic acid) (paa) shifted the paa carboxylic acid signals upfield and downfield, respectively, when tested using ^{13}C-NMR. The addition of urea and KCNS to the L-fucose/paa as well as the paa/water control mixtures at pH 6.2 resulted in a positional change in the chemical shift of the paa carboxylic acid signals, when examined using ^{13}C-NMR. Finally, the incorporation of urea into 50 mg C934 or poly(ethylene oxide) discs resulted in a reduction in their mucoadhesive strength in vitro. It is concluded that the mucoadhesive polymer could interact with the mucus glycoproteins by forming physical entanglements followed by hydrogen bonds with sugar residues on the oligosaccharide chains, resulting in the formation of a strengthened mucus gel network, which allows the mucoadhesive system to remain adhesive for an extended period of time. Disruption of hydrogen bonds could substantially reduce the adhesive strength of a mucoadhesive system, suggesting the importance of these bonds in mucoadhesion.

Keywords: Mucoadhesion; Bioadhesion; Mucus glycoprotein; Biorheology; Poly(acrylic acid); Carbopol 934P; ^{13}C-NMR; Hydrogen bonding; Tensiometer

1. Introduction

In recent years, considerable interest has been shown in the use of mucoadhesive dosage forms with regard to enhancing the local and systemic administration of peptides and other poorly absorbed drugs from the gastrointestinal tract.

The term 'bioadhesion' is used to define the attachment of synthetic or natural macromolecules to a biological substrate. When the

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substrate is 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; Jimenez-Castellanos et al., 1993).

Mucosal-adhesive materials have been investigated and identified in previous work (Chen and Cyr, 1970; Smart et al., 1984). These are generally hydrophilic macromolecules that contain numerous hydrogen bond forming groups (e.g., hydroxyl and carboxyl groups) and will hydrate and swell when placed in contact with water. In most cases these materials require wetting to become adhesive. However, over-hydration may result in the formation of a slippery mucilage and a loss of the adhesive properties.

The adhesive bond between a bioadhesive system 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. Furthermore, it is reasonable to suggest that an increase in the mechanical strength of the mucus layer by the mucoadhesive polymer could result in strong mucoadhesion.

Duchene et al. (1988) have proposed the following stages in the process of mucoadhesion. The first stage involves the establishment of an intimate contact between the mucoadhesive polymer and the mucus gel. Next, the mucoadhesive polymer could penetrate the mucus gel network, resulting in the formation of physical chain entanglements and secondary chemical bonds between the mucus gel and the mucoadhesive material.

Previous studies on the mucoadhesive polymer poly(acrylic acid) (paa) have suggested the importance of unionised carboxylic acid groups in mucoadhesion (Ch'ng et al., 1985; Park and Robinson, 1985, 1987). Kerr et al. (1989), using ^{13}C-NMR spectroscopy, have reported interactions between the mucus glycoprotein (MG) and paa as a result of hydrogen bonding with paa carboxylic acid groups. Tobyn et al. (1992), using Fourier transform infrared spectroscopy, have also provided evidence of hydrogen bonding between the pig gastric MG, reconstituted in USP simulated gastric fluid, and the test mucoadhesive. A pronounced shift in the height ratio of the two adjacent MG peaks at 1100–900 cm^{-1} which was suggested to be due to the formation of hydrogen bonds with the mucoadhesive agent was reported. Recently, Mortazavi et al. (1993a) have suggested the formation of hydrogen bonds between the terminal sugar residues on the MG and the mucoadhesive paa, using infrared and ^{13}C-NMR. This study was set out to determine the nature of interactions (in particular hydrogen bonding) between the mucus gel and the model mucoadhesive paa. This was achieved using three separate techniques, which were dynamic oscillatory rheology, ^{13}C-NMR, and a tensiometer technique for measuring the adhesive strength of the test discs.

2. Materials and methods

2.1. Materials

Carbopol 934P (C934) was obtained as a gift from B.F. Goodrich (Hounslow, UK), KCNS, sodium azide, sodium chloride, sodium edetate (disodium salt), analar NaOH pellets, analar HCl, analar sodium dihydrogen orthophosphate (dihydrate), disodium hydrogen orthophosphate (dihydrate), and lactose were purchased from BDH Chemicals (Poole, UK), phenylmethylsulphonyl fluoride (PMSF) and analar urea were obtained from Sigma Chemical Co. Ltd (Poole, UK), Sepharose 4B (hydrated) was purchased from Pharmacia (Milton Keynes, UK), poly(acrylic acid) with a molecular mass of 2000 Da (paa), L-fucose, and poly(ethylene oxide) (PEO) with a molecular mass of 4000 kDa were obtained from Aldrich Chemical Co. Ltd (Gillingham, UK), deuterated water (D_2O) (99% deuterium) was purchased from Goss Scientific Instruments (Essex, UK), analar acetonitrile was obtained from CEN (Saclay, France), D-galacturonic acid was purchased from Fluka Chemical Ltd (Derbys, UK), and 13 mm diameter Whatman cellulose nitrate membrane filters with a pore size of 0.45 mm

were obtained from Fisons Scientific Equipment (Loughborough, UK).

2.2. Preparation of the crude mucus (CM)

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

2.3. Preparation of homogenised mucus gel (HM)

Batches of HM were prepared using the method described by Mortazavi et al. (1992). CM scraped from hog stomachs was mixed with an equal quantity of an isotonic PMSF containing solution, centrifuged at $2500 \times g$ for 1 h, the supernatant discarded and the lower gel layer taken. These were pooled, exhaustively dialysed for 24 h, homogenised by blending and the % w/w of solids present determined for each batch. If necessary the % w/w was adjusted with purified water to give a concentration of 30 mg g^{-1}.

2.4. Preparation of the mucus glycoprotein (MG)

Batches of MG were prepared using the method described by Mortazavi et al. (1993b). CM scraped from hog stomachs was mixed and homogenised by blending with an equal quantity of an isotonic solution containing PMSF as well as KCNS (to aid solubilization). The resulting mixture was stirred at 4° C for 6 h, centrifuged at $12000 \times g$ for 1 h, and the supernatant layer collected, pooled, and passed through glass wool to remove any particulate matter. 42 ml portions were then loaded onto a Sepharose 4B gel filtration column and eluted with a solution containing sodium chloride and sodium azide at a flow rate of 1.4 ml min^{-1}. The fractions containing the MG were detected at 280 nm, collected, pooled, exhaustively dialysed, their pH adjusted to the required value, and centrifuged at $25000 \times g$ for 24 h to obtain the MG. The % w/w of solids was determined as before and if necessary adjusted to give a concentration of 30 mg g^{-1}.

2.5. Rheological studies

1.5 g samples of HM were mixed with 1.5 g of a 5 mg g^{-1} C934 gel, pH adjusted to 6.2 with 0.1 M NaOH and the final weight made up to 4.5 g with purified water. When testing the effect of the hydrogen bond breaking agents, urea (0, 0.22, 0.89, and 1.78 M) and KCNS (0, and 0.22 M), on the HM (1.5 g)/C934 (1.5 g) mixtures, 1 ml samples of either urea or KCNS were added to the HM/C934 mixtures, pH adjusted to 6.2 with 0.1 M NaOH, and the total weight made up to 4.5 g with purified water.

As only small amounts of the MG 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 the required pH with 0.1 M HCl, 0.1 or 1 M NaOH, were mixed with 200 mg of a 5 mg g^{-1} C934 gel, previously adjusted to the required pH as before. Further mixtures containing 200 mg of a 5 mg g^{-1} C934 gel mixed with 200 mg purified water, both previously adjusted to the required pH, were also prepared.

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, UK) fitted with either a 2 cm 2° stainless-steel cone (for MG samples) or a 4 cm parallel plate with a 0.5 mm gap (for HM samples). Each sample was individually loaded and allowed to further equilibrate for 5 min. From initial studies a frequency sweep between 10 and 0.1 Hz was used to measure the mean storage modulus (G') and loss modulus (G'') of 15 data points. The G' and G'' values at 4 Hz, which was found to be the mid-point in the frequency sweep (10 0.1 Hz) conducted, were also recorded. A logarithmic frequency sweep between 10 and 0.002 Hz was also performed on a pH 6.2 HM/C934 (5 mg g^{-1}) sample.

2.6. ^{13}C-NMR studies

The NMR studies could only be conducted on viscous or 'liquid-like' samples. Unfortunately, due to the strong gelling properties of C934, it

was not possible to produce viscous samples at high pH values (pH 5.0 and above). Furthermore, preparation of 'liquid-like' C934 samples at low pH values (below 4.0) resulted in structural breakdown on the inclusion of MG, rendering sample unsuitable for analysis. Hence, the use of C934 as the model mucoadhesive had to be abandoned. It was thought that the use of a low molecular weight paa (poly(acrylic acid)) with poor gelling properties could solve the problem. However, it was found that the inclusion of paa into the MG resulted in visible gel breakdown, preventing the sample from analysis. As a result of the problems faced in using MG, further NMR studies involving MG were terminated. It was decided to use model neutral (L-fucose) and anionic (D-galacturonic acid (GAL)) monosaccharides, representing the terminal as well as the core sugar residues on the oligosaccharide chains of the MG. 2.0 g samples containing various concentrations of L-fucose (50, 60, 120, 200, and 300 mg g^{-1}) or GAL (50, 200, and 300 mg g^{-1}), mixed with paa (60 and 200 mg g^{-1}) were prepared by dissolving in purified water. 200 mg D_2O, used for the correct setting of the NMR spectrometer and the improvement of signal resolution, was added to the individual samples and the pH was adjusted to 6.2 with NaOH. The final weight of the sample was then adjusted to 2.0 g with purified water. Further mixtures containing either 60 or 200 mg g^{-1} paa in purified water were also prepared at pH 6.2 as described before. In addition 2.0 g samples of the following pH 6.2 mixtures in purified water were also prepared as before:

(i) paa (60 mg g^{-1})/urea (240 mg g^{-1})
(ii) paa (60 mg g^{-1})/L-fucose (60 mg g^{-1})/urea (240 mg g^{-1})
(iii) paa (60 mg g^{-1})/KCNS (60 mg g^{-1})
(iv) paa (60 mg g^{-1})/L-fucose (60 mg g^{-1})/KCNS (100 mg g^{-1})

Finally, as a control, 2.0 g samples of L-fucose (200 mg g^{-1}), KCNS (100 mg g^{-1}), urea (240 mg g^{-1}), and GAL (200 mg g^{-1}) were also prepared in purified water at pH 6.2. As before 200 mg D_2O was added to each sample and the final weight adjusted to 2.0 g with purified water.

Following preparation, test samples were individually placed in sealed glass vials and stored at 4° C until use. 700 μl quantities of the test samples were individually placed in sealed 5 mm diameter NMR tubes and the ^{13}C spectra obtained at room temperature (22° C), using a JEOL GSX-270 high resolution Fourier transform spectrometer (JEOL, Japan) at 67.80 MHz. The deuterium signals of the D_2O present within the test

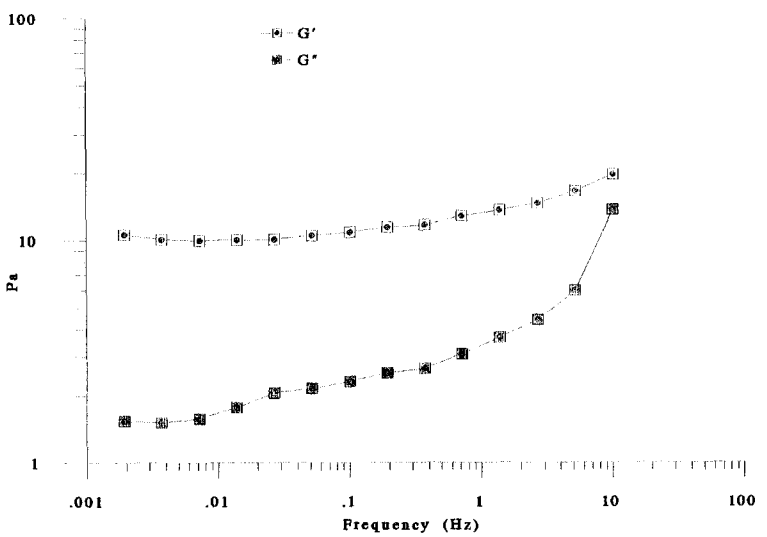

Fig. 1. Log frequency sweep (10–0.002 Hz) on the HM (30 mg/g)/C934 (5 mg/g) mixture at pH 6.5.

Table 1
Effect of urea and KCNS on the mean G' and G'' values as well as those at 4 Hz (shown in parentheses) of the HM/C934 mixtures at pH 6.2

Hydrogen bond breaker	Concentration (M)	G' (Pa)	G'' (Pa)
Control	0	9.0 (9.1)	7.1 (5.4)
Urea	0.22	3.8 (3.9)	5.0 (3.2)
Urea	0.89	2.3 (2.3)	4.4 (2.8)
Urea	1.78	2.6 (2.7)	4.7 (2.9)
KCNS	0.22	0.2 (0.3)	4.6 (1.6)

sample were used as the lock (i.e., for the correct setting of the instrument). 1–2 drops of acetonitrile was added to each sample as the internal standard.

2.7. Tensiometer studies

5.0 g powder blends of the test mucoadhesive (C934 or PEO) and urea in the ratio of 1:1 or 3:1 (3 parts mucoadhesive) were prepared. 50 mg samples of the test material were then compressed into 6.2 mm diameter flat-faced discs in a Specac infrared press, using a 1 tonne force for 5 s. As a control 50 mg discs containing lactose and the mucoadhesive material were prepared in exactly the same manner and ratios to that described above, except for replacing urea with lactose.

100 mg samples of the CM (8% w/w 'solids') were individually weighed, evenly spread over round Whatman cellulose nitrate membrane filters with a pore size of 0.45 mm and a diameter of 13 mm. The mucus coated membrane filters were then allowed to stand for 2 min, enabling the mucus gel to penetrate and cling onto the membrane filter. The mucus coated membrane filters were then individually mounted on a platform in a pH 6.0 isotonic phosphate buffer at 37° C and secured in place with a plastic cap to expose an 11 mm diameter circle of the mucus coated membrane filter. The 50 mg test discs were individually attached to a 1.5 g weight using a cyanoacrylate adhesive (Loctite superglue 3). This was suspended from an Oertling HC22 top pan balance (Oertling Ltd, Orpington, UK) and lowered onto the mucus coated membrane filter, which had been left in the pH 6.0 buffer for 1 min to equilibrate prior to testing. After 2 min the platform was lowered at a rate of 1 mm min^{-1}, until the test disc pulled clear of the membrane filter and the force at which adhesive bond failed (i.e., maximum detachment force) recorded.

3. Results

The logarithmic frequency sweep (10–0.002 Hz) conducted on the HM/C934 mixture at pH 6.2 shows substantial G' (a measure of the resistance to elastic deformation and the extent of structuring within the sample) and G'' (a measure of the resistance to liquid flow) values being obtained throughout the frequency range, although these gradually decreased with a reduction in the frequency (Fig. 1).

The addition of urea to the HM/C934 gel mixtures resulted in a reduction in the G' and G'' values (Table 1). Compared with urea, KCNS resulted in a substantial decrease in the G' and to a lesser extent the G'' values of the HM/C934 mixture. The trend obtained for the G' and G'' values at 4 Hz was found to be similar to that of the frequency sweep conducted between 10 and

Table 2
Effect of pH on the G' and G'' values of the MG/C934 (5 mg g^{-1}), C934 (5 mg g^{-1})/water, and C934 (5 mg g^{-1} gel) samples at pH 6.2 and an experimental frequency of 4 Hz

Sample	pH	G' (Pa)	G'' (Pa)
MG/C934	4.3	84.6	29.2
	5.1	429.9	59.2
	6.2	325.0	68.5
	7.1	51.8	42.3
	8.2	14.3	18.2
C934/water	4.3	3.4	4.1
	5.1	26.3	26.3
	6.2	46.5	40.6
	7.1	13.1	16.4
	8.2	11.0	13.0
C934	4.3	29.7	24.8
	5.1	266.3	142.2
	6.2	406.0	179.3
	7.1	535.2	202.9
	8.2	319.0	165.4

0.1 Hz, justifying the validity of calculating the mean G' and G'' values as a mean of comparing the results obtained.

The rheological properties of the MG/C934 (5 mg g^{-1}), C934 (5 mg g^{-1})/water, and the C934 (5 mg g^{-1} gel) samples were found to be substantially influenced by the environmental pH (Table 2). The G' values were often found to be larger than the G'' values, confirming the formation of a structured and 'solid-like' gel network rather than a viscous liquid, in agreement with that reported in previous studies (Allen et al., 1986; Mortazavi et al., 1992, 1993b). With the MG/C934 mixtures an optimum pH of 5.1 was found to result in the strongest gel network, in agreement with that reported by Mortazavi et al. (1993b). In contrast, with the C934/water and the C934 samples the optimum gel strengthening effect, giving the largest G' and G'' values was observed at pH values between 6.2 and 7.1.

The region examined carefully during the ^{13}C-NMR studies was the chemical shift of the paa carboxylic acid groups, which are expected to occur around 180–185 ppm (Silverstein et al., 1981). The addition of the neutral sugar L-fucose to paa resulted in a small upfield shift of the paa carboxylic acid peaks, compared with the paa/water control sample at pH 6.2 (Table 3). An increase in the concentration of L-fucose in the fucose/paa mixture resulted in a further upfield shift of paa carboxylic acid groups. In contrast to L-fucose, the anionic GAL ($pK_a = 3.2$ (Mortazavi, 1993)) caused a downfield shift of paa carboxylic acid groups, compared with the paa/water control sample at pH 6.2.

Table 3
^{13}C-NMR chemical shifts (ppm) of paa carboxylic acid groups observed with paa/L-fucose, paa/GAL, and the paa/water control mixtures at pH 6.2

Sample	Chemical shift (ppm)
paa (60 mg g^{-1})/water (control)	184.019
paa (60 mg g^{-1})/L-fucose (60 mg g^{-1})	184.134
paa (60 mg g^{-1})/L-fucose (120 mg g^{-1})	183.970
paa (60 mg g^{-1})/L-fucose (300 mg g^{-1})	183.742
paa (60 mg g^{-1})/GAL (300 mg g^{-1})	184.575
paa (200 mg g^{-1})/water (control)	184.330
paa (200 mg g^{-1})/L-fucose (50 mg g^{-1})	184.362
paa (200 mg g^{-1})/L-fucose (200 mg g^{-1})	184.134
paa (200 mg g^{-1})/GAL (50 mg g^{-1})	184.526
paa (200 mg g^{-1})/GAL (200 mg g^{-1})	184.526

Table 4
Effect of urea (240 mg g^{-1}) and KCNS (100 mg g^{-1}) on the ^{13}C-NMR chemical shift (ppm) of paa carboxylic acid groups in the presence and absence of L-fucose at pH 6.2

Sample	Chemical shift (ppm)
paa (60 mg g^{-1})/water	184.019
paa (60 mg g^{-1})/water/urea	183.758
paa (60 mg g^{-1})/water/KCNS	184.346
paa (60 mg g^{-1})/L-fucose (60 mg g^{-1})	184.134
paa (60 mg g^{-1})/L-fucose (60 mg g^{-1}) /urea	183.627
paa (60 mg g^{-1})/L-fucose (60 mg g^{-1}) /KCNS	184.330

The addition of urea to both paa/water and paa/fucose samples at pH 6.2 resulted in an upfield shift of the paa carboxylic acid signals (Table 4). In contrast, the addition of KCNS to the paa/water and paa/fucose samples resulted in a downfield shift of the paa carboxylic acid signals.

As a control ^{13}C-NMR spectra of L-fucose, urea, KCNS, and GAL were also obtained. None of the samples produced signals at the experimental region (180–185 ppm), preventing interference with the paa carboxylic acid signals.

Incorporation of urea (one part) into the C934 and PEO discs at a ratio of 3:1 resulted in an insignificant ($p > 0.05$, Student's t-test) reduction in the mucoadhesive strength of C934 and PEO,

Table 5
Effect of urea on the adhesive strength of C934 and PEO test discs to mucus coated membrane filters in a pH 6.0 isotonic phosphate buffer ($n = 5$, S.D. = standard deviation)

Test disc	Ratio of test material/urea	Maximum detachment force (mN) (S.D.)
C934/lactose	3:1	109.64 (30.63)
C934/urea	3:1	70.10 (21.30)
C934/lactose	1:1	81.00 (25.39)
C934/urea	1:1	45.64 (5.75)
PEO/lactose	3:1	179.20 (37.50)
PEO/urea	3:1	142.30 (46.10)
PEO/lactose	1:1	117.20 (21.45)
PEO/urea	1:1	59.20 (20.05)

compared with the mucoadhesive/lactose control discs at the same ratio (Table 5). In contrast, when urea was mixed in a ratio of 1:1 with either C934 or PEO a significant ($p < 0.05$, Student's t-test) reduction in mucoadhesive strength of the test discs was observed, compared with the mucoadhesive/lactose control discs at the same ratio.

4. Discussion

Previous studies (Mortazavi et al., 1992, 1993b), using dynamic oscillatory rheology, have reported the formation of a strengthened mucus gel network on the addition of a 5 mg g^{-1} C934 gel to HM. In order to determine the nature of interactions between the HM and the C934 gel the logarithmic frequency sweep between 10 and 0.002 Hz on a HM/C934 mixture at pH 6.2 was conducted. The rheogram obtained (Fig. 1) was found to be intermediate between that seen with a physically entangled system and a cross-linked system (Lutz et al., 1973; Ross-Murphy and McEvoy, 1986; Clark and Ross-Murphy, 1987). In a physically entangled network at low frequencies macromolecules are given time to untangle and move past each other, resulting in a decline in G'. At higher frequencies polymer chains cannot get past each other and only show an elastic deformation. In contrast, in a cross-linked system the G' and G'' values are not influenced by the frequency of oscillation (i.e., the experimental time).

The acrylic acid based C934 ($(\text{-}CH_2CHCOOH)_n$) with a pK_a value between 5.35 and 7.2 (Park and Robinson, 1987) can form hydrogen bonds in the unionised state with proton accepting groups, e.g., hydroxyl or oxyethylene groups (Tsuchida and Abe, 1982; Bednar et al., 1984). Therefore, it is possible that C934 could form hydrogen bonds with the negatively charged mucus gel (Gu et al., 1988), following the formation of physical entanglements. The addition of the hydrogen bond breaking agents urea and KCNS (Hamaguchi and Geiduschek, 1962; Hatefi and Hanstein, 1969) to the HM/C934 gel mixtures at pH 6.2 reduced both the G' and G'' values (Table 1). However, at the same concentration of 0.22 M KCNS was found to disrupt the gel strength far more than urea, suggesting its greater potency. The formation of hydrogen bonds between the mucus gel and C934 is therefore suggested to be important in the formation of a strengthened mucus gel network, which is required to keep the mucoadhesive dosage form in place.

MG (molecular mass $2\text{–}14 \times 10^3$ kDa) (Marriott and Gregory, 1990) is the major structure forming component of the mucus gel which is capable of forming non-covalent interactions with the other MG molecules to form the gel matrix and is also responsible for the formation of a strengthened mucus gel network, on the addition of the mucoadhesive polymer (Mortazavi et al., 1993b). In fact the rheogram obtained from the logarithmic frequency sweep (10–0.002 Hz) conducted on the HM/C934 mixture at pH 6.2 (Fig. 1) was found to closely match that of the MG/C934 mixture (Mortazavi et al., 1993b), further suggesting the importance of MG in mucoadhesion.

With the C934 (5 mg g^{-1}) gel alone the optimum pH, giving the largest G' and G'' values was found to be at pH 7.1 (Table 2). At this pH the majority of the C934 ($pK_a = 5.79$ (Mortazavi, 1993)) carboxylic acid groups are expected to be in their ionised form, repelling each other and resulting in the formation of an expanded polymer network and hence the large G' and G'' values obtained. Beyond pH 7.1 the build up of sodium counterions result in some polymer chain coiling by shielding the ionised carboxylic acid groups and hence reducing the resistance to flow and deformation, i.e., G' and G'' values (Glavis, 1962).

In contrast to the C934 alone samples, a pH of 6.2 was found to result in the optimum resistance to deformation with the C934 (5 mg g^{-1})/water mixtures. In the C934 (5 mg g^{-1})/water mixture the final concentration of C934 would be 2.5 mg g^{-1}, in contrast to the 5 mg g^{-1} concentration within the C934 sample when tested alone. The extra water available within the C934 (5 mg g^{-1})/water mixture at pH 7.1 could result in the over-hydration and presence of an insufficient number of polymer chains to interact and form a

gel, i.e., the C934 concentration falls below its gelling point. At pH 6.2 the C934 carboxylic acid groups are less ionised than pH 7.1, resulting in the formation of a less expanded C934 network within the C934 (5 mg g^{-1})/water mixture. This smaller degree of ionisation would ensure that the C934 network becomes sufficiently expanded without actually being over-hydrated, resulting in the formation of an optimum gel strength.

With the MG/C934 (5 mg g^{-1}) gel mixtures the optimum gel strength was seen at pH 5.1. This finding strongly suggests the possibility of interactions between C934 and the MG at pH 5.1, which would not have been expected if the rheology of the C934/MG mixture was to follow that of C934. Furthermore, this finding suggests that the presence of unionised carboxylic acid groups within C934 is critical in the formation of strong interactions with the MG molecule. These interactions could be resulted from the formation of hydrogen bonds between the C934 carboxylic acid groups and the proton accepting groups (e.g., hydroxyl) within the MG. These interactions are unlikely to be due to electrostatic interactions, since at the experimental pH of 6.2 the mucus gel carries a net negative charge (Gu et al., 1988) due to the presence of sialic acid and sulphate residues on the oligosaccharide chains of the MG.

^{13}C-NMR spectroscopy was employed to further examine the possibility of hydrogen bonding between the carboxylic acid groups of the model mucoadhesive paa and the model monosaccharide L-fucose and GAL.

The occurrence of the neutral monosaccharide L-fucose and the anionic sialic acid ($pK_a = 2.6$) on the terminal position of the MG oligosaccharide chains, in addition to other neutral and sulphated sugar residues present along the oligosaccharide chains have been reported (Clamp et al., 1978). Hence, it was decided to use the neutral sugar L-fucose and the anionic GAL as model monosaccharides representing the terminal as well as other sugar residues on the oligosaccharide chains of the MG.

As a result of the problems faced with the use of C934 it was decided to use a low molecular mass (2000 Da) paa ($(-CH_2CHCOOH-)_n$) as the model mucoadhesive. The anionic polymer paa with a pK_a value of 5.98 (Mortazavi, 1993) contains numerous carboxylic acid groups, and could form hydrogen bonds with the hydroxyl groups of L-fucose and GAL as well as the carboxylic acid groups of GAL ($pK_a = 3.2$) in its unionised state. Moreover, ionised carboxylic acid groups of GAL could still form hydrogen bonds with the unionised carboxylic acid groups of paa. The interactions formed between paa and L-fucose are unlikely to be due to the formation of electrostatic interactions, since L-fucose is not charged. The formation of electrostatic interactions between GAL and paa is also highly unlikely, since they are not oppositely charged at any pH value. The results obtained from this study (Table 3) showed a downfield chemical shift of the paa carboxylic acid groups in the presence of GAL, compared with the paa/water control sample. In contrast, the carboxylic acid groups of paa were shifted upfield in the presence of L-fucose, compared with the paa/water control mixture. Based on the results obtained, it is proposed that the addition of both the neutral and anionic monosaccharides to paa results in the formation of hydrogen bonds. However, the hydrogen bonds formed between the anionic monosaccharide and paa are suggested to be stronger than those formed between paa and the neutral monosaccharide.

The addition of urea to both paa/L-fucose and paa/water control mixtures at pH 6.2 resulted in an upfield shift of paa carboxylic acid groups (Table 4). Urea is suggested to form hydrogen bonds with the paa carboxylic acid groups, and by doing so can disrupt the hydrogen bonds formed in paa/L-fucose and paa/water mixtures. The strength of the hydrogen bonds formed between urea and paa is proposed to be weaker than those formed between paa and L-fucose as well as those between the paa carboxylic acid groups, hence the upfield shift of the paa carboxylic acid groups observed.

In contrast to urea, KCNS, which is thought to indirectly disrupt hydrogen bonds (Hamaguchi and Geiduschek, 1962; Hatefi and Hanstein, 1969), was found to cause a downfield shift of the carboxylic acid signals of paa in paa/L-fucose as well as paa/water mixtures at pH 6.2 (Table 4).

As with urea, KCNS is proposed to form hydrogen bonds with the carboxylic acid groups of paa, hence disrupting the paa/L-fucose and paa/paa hydrogen bonds formed. However, it is proposed that the strength of the hydrogen bonds formed between KCNS and paa is greater than those formed between paa and L-fucose as well as those between the carboxylic acid groups of paa. This could explain the stronger deshielding of the carbon atoms of paa carboxylic acid groups and the resulting downfield shifts observed.

The results obtained from the tensiometer studies suggest a significant reduction in the mucoadhesive strength of the neutral polymer PEO as well as the anionic C934 in the presence of a high concentration of urea (Table 5). This finding would further suggest the importance of hydrogen bonds in mucoadhesion.

In conclusion it is suggested that a mucoadhesive macromolecule could interact with the glycoprotein component of the mucus gel by forming physical entanglements and secondary chemical bonds, in particular hydrogen bonds. This could result in the formation of a strengthened mucus gel network, capable of resisting deformation, allowing the mucoadhesive system to remain adhesive for an extended period of time. It is further proposed that the mucoadhesive polymer is likely to form hydrogen bonds with the terminal sugar residues on the oligosaccharide chains of the MG. Anionic sugar residues are suggested to form stronger hydrogen bonds with the mucoadhesive macromolecule, compared with the neutral sugar residues. It was found that the destruction of hydrogen bonds formed between the mucus gel and the mucoadhesive system could substantially reduce the mucoadhesive strength of the test system. Hence, the formation of hydrogen bonds between the mucoadhesive polymer and the MG is suggested to be an important component in mucoadhesion.

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