Progress in Colloid & Polymer Science Progr Colloid Polym Sci 94: 66–73 (1994)

Sedimentation analysis of potential interactions between mucins and a putative bioadhesive polymer

I. Fiebrig[1,2]), S. E. Harding[1]), and S. S. Davis[2])

University of Nottingham,
[1]) National Centre for Macromolecular Hydrodynamics, School of Agriculture, Sutton Bonington and
[2]) Department of Pharmaceutical Sciences, University Park, Nottingham, England

Abstract. A potentially fruitful but hitherto relatively unexplored application of the analytical ultracentrifuge is the investigation of interactions in pharmaceutical polymer drug carrier systems. In this study we investigate the interactions of the cationic material chitosan with gastric mucin: 1) The physicochemical properties of gastric mucin and chitosan (highly deacetylated chitosan "Sea Cure + 210") in solution are summarised; 2) We described how the gastric mucin (from pig) can be purified by a combination of preparative isopycnic density gradient ultracentrifugation and gel chromatography, and how its purity and structural integrity can be checked by analytical isopycnic density gradient ultracentrifugation (on a MSE Centriscan 75 Analytical Ultracentrifuge) and on line GPC/MALLS (gel permeation chromatography coupled on-line to a multi-angle laser light scattering photometer); 3) Using co-sedimentation experiments with the appropriate controls in an XL-A Ultracentrifuge (absorption optics) and an MSE Mk II Analytical Ultracentrifuge (Schlieren optics) a definite interaction between chitosan and pig gastric mucin was demonstrated, with complexes sedimenting faster than 1000 S, depending on the amount of the mucin used.

Key words: Drug delivery – bioadhesion – chitosan – mucin – analytical ultracentrifugation

Introduction

I. Sedimentation velocity and bioadhesion

Sedimentation analysis provides a potentially powerful tool for the investigation of many phenomena relevant to pharmaceutical sciences. One such application so far relatively unexplored is the evaluation of putative bioadhesive or mucoadhesive polymers as drug carriers for oral drug delivery systems [1]. The performance of such a mucoadhesive polymer in vivo will be dictated by its ability to "adhere" in a controllable way to the mucus lining of the stomach or small intestine, thus *increasing* the transit time of a dosage form and hence *enhancing* the drug absorption from the intestine [2].

Although there are many factors which can influence any possible interaction phenomena in vivo (such as pH, characteristics of the mucus layer, intestinal contents, motility), a fundamental study on potential interaction phenomena between a mucin (the polyanionic glycoprotein component of mucus which dictates its characteristic physical properties of high viscosity and viscoelasticity [3]) and the putative mucoadhesive in *dilute solution* is a required baseline for understanding the mechanism of interaction. However, it is appreciated that solute concentrations and environmental conditions can be quite different to the in vivo situation and the relevance of such basic physicochemical studies has yet to be ascertained.

For dilute solution interaction phenomena, the principle of *co-sedimentation* of component

macromolecules in a mixture is a particularly valuable one for assessing the strength of an interaction, and this principle is the cornerstone of our study here, in which we investigate the behaviour of mixtures of mucins with the cationic biopolymer chitosan; the latter molecule being of interest because of its opposite charge to the mucin [4]. Unfortunately small intestinal mucin is very difficult to harvest in amounts sufficient for a thorough investigation, so in this pilot study we investigate only the behaviour of mixtures of pig gastric mucin (as a model for human gastrointestinal mucin) and chitosan. The limitations of using pig gastric mucin (which has a lower charge density as opposed to small intestinal mucin in pigs as well as humans [5]) have to be taken into account.

In this study we describe the basic solution properties of pig gastric mucin and chitosan and their admixture. We also describe the use of analytical density gradient sedimentation equilibrium to assess purity and GPC/MALLS (gel permeation chromatography on line with multi-angle laser light scattering) to assess structural integrity in terms of distribution of molecular weight of pig gastric mucin. The degree of interaction between pig gastric mucin and chitosan in dilute solution (*without* chromophore labelling) has been measured by comparing sedimentation rates of mucin absorbance boundaries scanned with UV absorption optics in the Optima XLA Ultracentrifuge and by comparing residual areas under Schlieren sedimentation diagrams (recorded in the MSE MkII analytical ultracentrifuge) for the chitosan in the presence and absence of mucin.

II. Mucus and mucins

The mucus layer which protects the underlying epithelium of the gastrointestinal tract, is composed, apart from ~ 95% water, mainly of mucus glycoprotein or "mucin" [6, 7]. It is the mucin component of mucus which dictates its physical properties (such as high viscosity, viscoelasticity and *spinnbarkeit*) and which might play an important role in bioadhesive drug delivery. Mucins from a wide variety of sources (besides gastrointestinal, for example, bronchial, cervical and ovarian) consist of a similar building block or "basic unit" (Fig. 1). This basic unit consists of a polypeptide backbone which is heavily glycosylated and has a molecular weight of

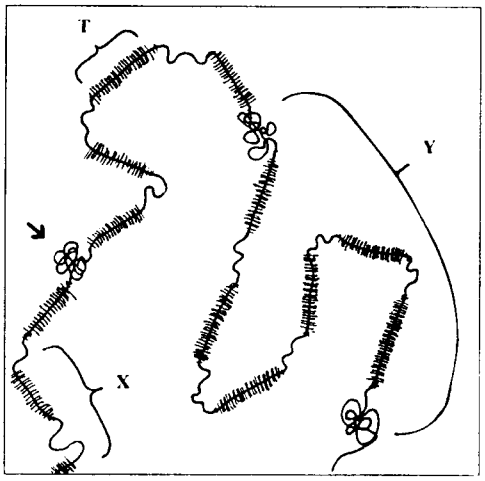

Fig. 1. Linear model for cervical mucin consisting of *basic units*: "T-domains" linked by naked peptide regions, some linked by disulfide bridging [16] (Arrowed region is susceptible to thiol attack) Key: T, "T-domain,"; X, "basic unit" and Y, "subunit"

~ 50 0000 g/mol. The carbohydrate side chains are built from 5–30 residues each [8–10]. The basic units are linked via naked (i.e. carbohydrate free) end regions into linear arrays, giving structures whose weight average molecular weights can range from 1×10^6 (e.g. ovarian cyst mucin) to over 40×10^6 (see, e.g. [11]). Thiol reduction gives rise to "subunits" consisting of 3–4 basic units. It is important to note that many of the carbohydrate chains contain sialic acid as their terminal sugar [12]. This rather acidic sugar is derived from neuraminic acid and has a pKa of 2.6. Fully dissociated under most physiological pH conditions, it gives the mucin molecule a net negative charge, allowing the possibility of ionic interaction with positively charged polymers.

III. Chitosan

In this study, chitosan, a derivative of chitin, was chosen as a possible mucoadhesive. It is particularly attractive because of its polycationic properties. It is composed of linear chains of *N*-acetyl glucosamine units with different degrees of deacetylation, upon which the charge density of a given chitosan preparation will depend [13] (Fig. 2).

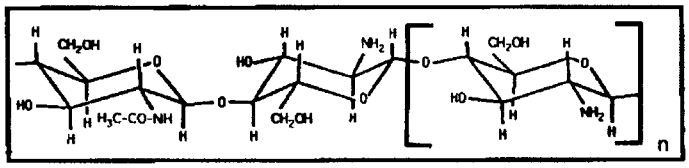

Fig. 2. Structure of chitosan, a derivative of chitin with different degrees of deacetylation of the basic *N*-acetyl glucosamine unit

Table 1. Characterization of Chitosan Sea Cure + 210 (SC + 210) [10]

Property		Units	Values
$\bar{v}$	partial specific volume	(mg/g)	0.580(± 0.011)
DA	degree of acetylation	(wt%)	11
M_w	weight average molecular weight	(g/mol)	$162(\pm 10)\times 10^3$
B	second virial coefficient	(ml mol g^{-2})	2.75×10^{-2}
BM_w		(ml g^{-1})	4455
$s^0_{20,w}$	sedimentation coefficient at zero concentration	(S)	1.41(± 0.05)
K_S	sedimentation coefficient concentration regression coefficient	(ml/g)	88.6(± 10.8)
$[\eta]$	intrinsic viscosity	(ml/g)	540(± 20)
$Dz_{20,w}$	translational diffusion coefficient	($cm^2 s^{-1}$)	$3.9(\pm 0.6)\times 10^{-9}$

Materials

Solutions

For all sedimentation velocity analyses an acetate buffer pH 4.0 I = 0.1 [14] was used. For the analytical isopycnic density gradient experiments on the mucin, a loading density of $\rho_e = 1.346$ g/ml was achieved by the addition of an appropriate amount of Cs_2SO_4 (analytical grade) to a mucin solution at a nominal concentration of 3 mg/ml.

Mucin

Fresh pig gastric mucus glycoprotein (PGM) was purified from fresh solubilized pig gastric mucus by preparative caesium chloride isopycnic density gradient ultracentrifugation in an enzyme inhibitor cocktail according to a modified procedure of Hutton et al. [15]. This was followed by gel permeation chromatography of the glycoprotein fraction on a Sepharose CL-2B column. The totally excluded volumes were pooled and concentrated by ultrafiltration, dialyzed against distilled water and fractions of 1ml solution kept frozen at −20 °C or freeze dried. The mucin preparation was gently defrosted and dialysed into the buffer or redissolved in buffer before use.

Chitosan

Sea Cure + 210, a glutamate salt of chitosan (Protan Ltd., Drammen, Norway) was used. This was an 11% acetylated preparation which had been previously well characterised in this laboratory. Table 1 summarises the relevant physical properties from earlier work performed by our group [16].

Experimental

Mucin purity-analytical isopycnic density gradient ultracentrifugation

This method was used to assess the purity of the pig gastric mucin following the procedures of Creeth et al. [17]. An MSE Centriscan 75 Analytical Ultracentrifuge was used, equipped with 280 nm absorption optics. The sample cell was loaded with the mucin/Cs_2SO_4 solution and run at a rotor speed of 50 000 rpm. A density gradient is built up by the caesium salt as a result of the centrifugal force, showing a lower density at the meniscus of the fluid column and an increasingly higher density towards the bottom of the cell. After reaching equilibrium approximately 48 h later, the glycoprotein fraction with a buoyant density of approximately 1.33 g/ml will concentrate at the centre of the fluid column, free protein will be

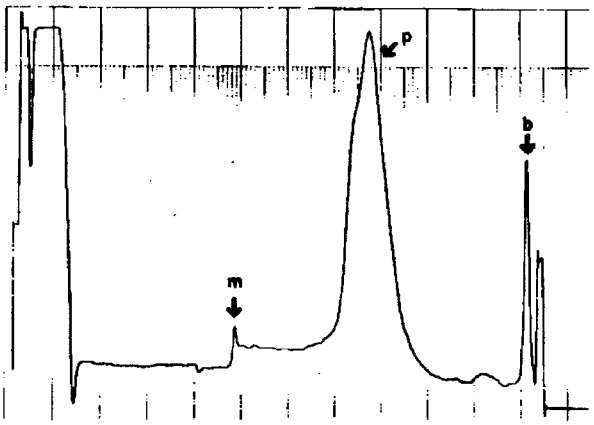

Fig. 3. Pig gastric mucus glycoprotein (PGM) in a density gradient of Cs_2SO_4 run on a MSE Centriscan 75 Analytical Ultracentrifuge at a rotor speed of 50000 rpm. Loading density $\rho_e = 1.346$g/ml. Key: m = solution meniscus; p = glycoprotein peak [calculated density at peak: $\rho_p = 1.337$g/ml ; b = cell base. The small shoulder on the lower density flank of the peak is due to density heterogeneity of the material which is not unusual for glycoprotein preparations of this kind [23, 24]

'floating' at the meniscus with a buoyant density of 1.24 g/ml [18] and any nucleic acid will be concentrated at the cell bottom. The densities at each point of the fluid column can be calculated by the equations given by Creeth and Horton [19]. The mucin appears as one clear peak at a calculated density of 1.337 g/ml in accordance with published values by [20]. There was no free protein nor nucleic acid detectable (Fig. 3).

Mucin Purity and Structural Integrity – GPC/MALLS ~ (gel permeation chromatography on line with multi angle laser light scattering)

As a relatively rapid assay for the structural integrity of the mucin, a Dawn-F Gel Permeation Chromatography/Multi Angle Laser Light Scattering (GPC/MALLS) system was used [21]. For the GPC, two columns (packing material: Hydroxyethylmethacrylate crosslinked with ethylenglycol dimethacrylate) in series were used: one PSS Hema Bio linear followed by a PSS Hema Bio 40 (Mainz, FRG). The manufacturer's specified separation range of the column system for dextran was from *above* 1×10^6 to *below* 5000. Samples were injected at a nominal loading concentration of 1mg/ml. The high dilution during elution through the scattering cell (by approximately 10x) meant that correction for non-ideality effects was not necessary. After passing through the scattering cell, solute concentrations were recorded on-line by a Waters 410 differential refractive index detector. The MALLS chromatogram (Fig. 4) shows a very pure mucin material (symmetric peak), free of low molecular weight impurities.

Interaction studies–mucin detection

In the first set of experiments of mixture of defrosted solution of PGM (at a concentration of ~ 0.4 mg/ml and a weight average molecular weight of $M_W \approx (9.3 \pm 0.2) \times 10^6$) and a solution of SC + 210 (at a concentration of ~ 2 mg/ml) was run on a Beckman Optima XL-A Analytical

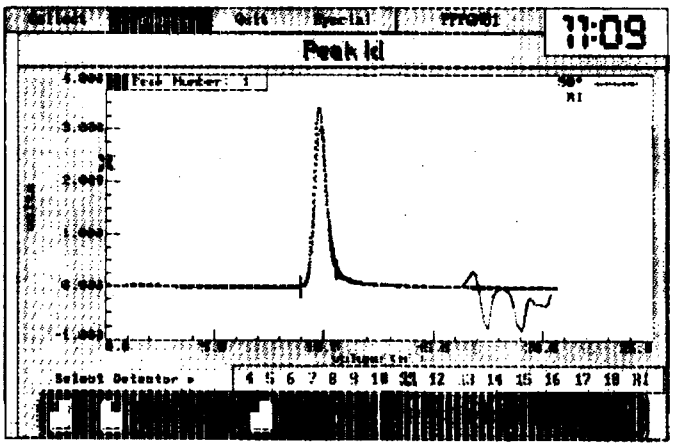

Fig. 4. Pig gastric mucus glycoprotein (PGM): Chromatogram from a Dawn-F Gel Permeation Chromatography/Multi-Angle Laser Light Scattering (GPC/MALLS) system. The trace of the refractive index detector overlays closely with the light scattering signal, the sample appears to be very pure and fairly homogeneous i.e. with no degraded mucin present. The noise on the far righthand side of the refractive index trace is due to small amounts of buffer salt eluting

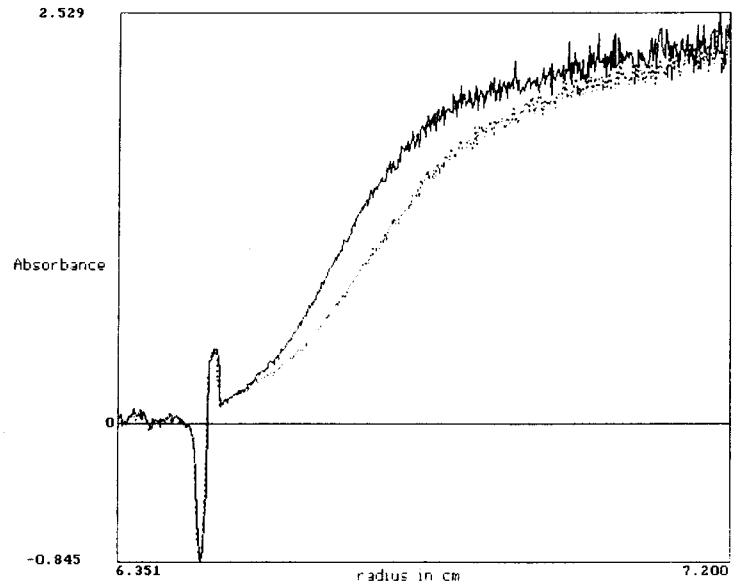

Fig. 5. PGM/Sea Cure + 210-complex in mixture cell, X-LA Analytical Ultracentrifuge, rotor speed 2000 rpm, scan interval 10 min, absorption optics at $\lambda = 230$ nm, sedimentation coefficient for complex: (1990.0 $\pm$ 18.0)S

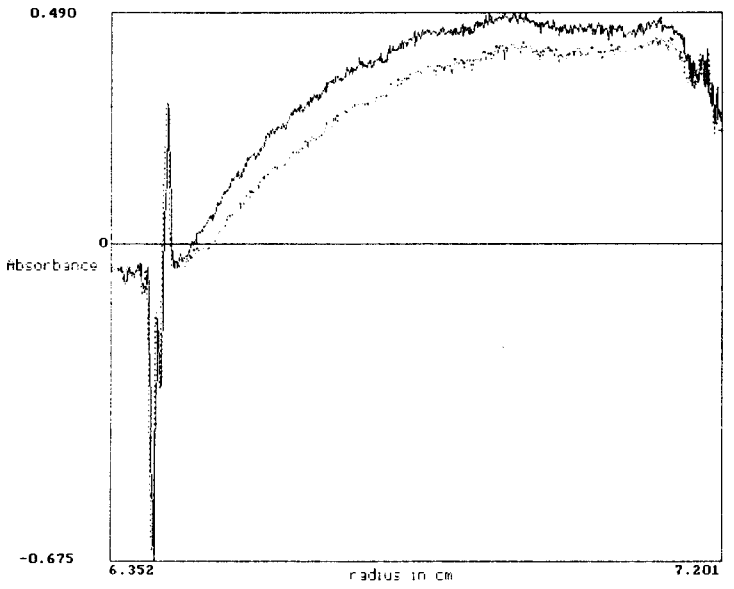

Fig. 6. PGM control cell, X-LA Analytical Ultracentrifuge, rotor speed 10000 rpm, scan interval 6 min, absorption optics at $\lambda = 230$ nm, sedimentation coefficient for mucin: (53.0 $\pm$ 2.8) S. Due to heterogeneity of the material the mucin boundary is quite shallow, very unlike the sharp sigmoidal boundaries typical for many proteins

Ultracentrifuge (equipped with UV/Vis absorption optics) against a mucin control diluted to an equal concentration. This centrifugation technique was used to follow the mucin and mucin/chitosan boundaries. Cells (12 mm pathlength) in a four-hole rotor were employed and the samples run at 37 °C at a rotor speed of 2000 rpm first (Fig. 5), to detect the sedimenting boundary of the mucin/chitosan mixture (advantage was taken here of the stability of the XL-A even at low rotor speeds), then speeded up to 10000 rpm to detect the fast moving boundary of the mucin control (Fig. 6). Sedimentation velocity traces were analysed on a digitizing pad connected to a computer equipped with software to generate sedimentation coefficients at concentrations corrected for radial dilution [22]. The value for the mixture cell was of (1990.0 $\pm$ 18.0) S, as opposed to a sedimentation coefficient of (53.0 $\pm$ 2.8) = S for the mucin control.

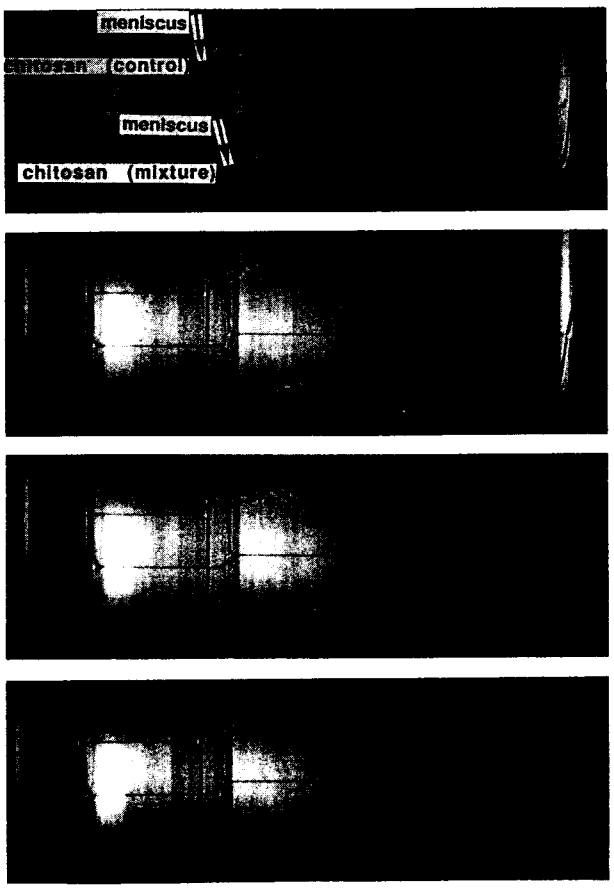

Fig. 7. Chitosan control [(2.1 ± 0.03) S] as well as residual chitosan in mixture cell [(2.5 ± 0.02) S], MSE Mk II Analytical Ultracentrifuge, rotor speed 35 000 rpm, Schlieren optics

Interaction studies–chitosan detection

Because the chitosan was "invisible" for UV detection at the wavelength used in the XLA studies above, it was important to detect the position and rate of movement of the chitosan boundary in the control, as well as to establish how much chitosan has been lost through the high molecular weight complex formation in the mixture. A required higher concentration of mucin was achieved by dissolving ~ 3 mg/ml of the freeze-dried PGM sample in buffer. Three 20 mm pathlength cells in a rotor were loaded onto the MSE Mk II Analytical Ultracentrifuge (equipped with conventional Schlieren optics): One cell containing a 1:1 mixture of 4 mg/ml SC + 210 solution and ~ 3 mg/ml mucin solution, weight average molecular weight of $M_W \approx (6.1 \pm 0.2) \times 10^6$. The other one containing the chitosan control and the third one containing the mucin control. The sedimentation velocity traces were analysed as mentioned above. Schlieren optics detected the sedimenting mucin control boundary which sedimented at (53.2 ± 1.0) S at a rotor speed of 15 000 and a temperature of 36 °C. The chitosan control sedimented at (2.1 ± 0.03) S. The mixture (mucin-chitosan complex sedimented too fast to be detected) left a residual amount of chitosan behind sedimenting at (2.5 ± 0.02) S (Fig. 7). The mixture was later run separately on the XL-A Ultracentrifuge at 37 °C, showing a sedimentation coefficient of (10 300.0 ± 250.0) S for the complex. This compares with the value of (1990.0 ± 18.0) S referred to above, which corresponds to approximately seven fold less mucin, indicating that the loading concentration of mucin in the mixture is a critical factor in governing the size of the complex. Due to the limited amount of highly purified mucin material further experiments on concentration dependence of the complex size were not possible. All given sedimentation coefficients were not corrected to standard conditions–differences are considered to be minor compared to the large changes observed through complex formation. The corrected sedimentation coefficient ($s^0_{20,w}$) for our chitosan control is in good agreement with the corresponding value given in Table 1.

Discussion

A clear interaction between isolated and purified pig gastric mucin (major component of gastric mucus) and a polycationic derivative of chitin (as potential bioadhesive drug carrier) was found, evidenced by a significant increase of the sedimentation coefficient of the mucin in combination with chitosan. The mucin control sedimented at (53.2 ± 1.0) S [$M_W \approx (6.1 \pm 0.2) \times 10^6$], 1.5 mg/ml, and (53.0 ± 2.8) S [$M_W \approx (9.3 \pm 0.2) \times 10^6$], 0.2 mg/ml. The chitosan control sedimented at (2.1 ± 0.03) S. For the mixture we obtained values of 10 300.0 ± 250.0) S and (1990 ± 18.0) S respectively, for mucin concentrations of 3.0 mg/ml and 0.4 mg/ml.

As expected in an excess of chitosan the mucin/chitosan complex sediments first, leaving residual chitosan behind. Comparisons of the

areas under the curve, between the chitosan control (AUC: chitosan = 1) and the residual chitosan in the mixture cell (AUC: chitosan in mixture = 0.69) shows by subtraction an approximate chitosan/mucin ratio of 1 : 2 (w/w ratio) and 3.75 : 1 (molecular ratio). The increased sedimentation coefficient of chiotsan in the mixture [(2.5 ± 0.02) S compared with the chitosan control [(2.1 ± 0.03) S] is probably due to a lower viscosity of the solution as a result of the decreased concentration of chitosan. The sensitivity of the size of the complex to the amount of mucin could have implications for the in vivo situation, where the mucin concentrations (in gel/sol form) are likely to be much higher.

In conclusion, chitosan would appear to have some potential as a bioadhesive. However, we have only examined mucin/chitosan interactions at very low concentrations and at a single pH. In vivo not only water and mucin, but also proteins, lipids etc. may be present in the mucus layer and pH values can vary from as low as pH 1.5 in the stomach up to over pH 7.5 in the small intestine. These results nevertheless should form the firm basis of experiments more relevant to in vivo conditions. As to the actual mechanisms of the interaction, this will require further detailed study such as the effects of pH, ionic strength and other solvent conditions (e.g. the presence or absence of bile salts) and this will be subject of a further study.

Finally this is yet another example of how a combination of sedimentation methods can be used together to assay for an interaction between molecules at high dilution.

Acknowledgements

We would like to thank Prof. A. Allen and F. Fogg from the Department of Physioloical Sciences, Newcastle, for their valuable help and information on the preparation of mucin as well as K. Jumel for the GPC/MALLS analyses and P. Mistry for technical assistance. The University of Nottingham would like to thank Hoechst U.K.Ltd for their financial support.

References

1. Anderson MT (1991) The Interaction of Mucous Glycoproteins with Polymeric Materials, Ph.D. thesis, University of Nottingham, England
2. Robinson JR (1990) Rationale of bioadhesion/mucoadhesion. In: Gurny R, Junginger HE (eds) Bioadhesion: Possibilities and Future Trends. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, pp 13–15
3. Allen A (1981) Structure and function of gastrointestinal mucus. In: Johnson LR (ed) Physiology of the Gastrointestinal Tract by Raven Press, NY, pp 617–639
4. Lehr C-M, Bouwstra JA, Schacht EH, Junginger HE, (1992) In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharmaceutics 78:43–48
5. Allen A (1989) Gastrointestinal mucus. In: Schultz SG, Forte JG, Rauner BB (eds) Physiology of the Gastrointestinal Tract – The GI-System III, American Physiological Society, Bethesda, Maryland, pp 359–382
6. Carstedt I and Sheehan JK (1988) Structure and macromolecular properties of mucus glycoproteins. Monogr. Allergy. Karger Basel, 24:16–24
7. Neutra MR and Forstner JF (1987) Gastrointestinal mucus: Synthesis, secretion and function. In: Johnson LR (ed) Physiology of the Gastrointestinal Tract, 2nd ed. Raven Press, NY, pp 975–1009
8. Silberberg A (1987) A model for mucus glycoprotein structure Biorheology 24:605–614
9. Carlstedt I, Sheehan JK, Corfield AP and Gallagher JT (1985) Mucus glycoproteins. In: A Gel of a Problem, Essays in Biochemistry 20:40–76
10. Allen A (1978) Structure of gastrointestinal mucus glycoprotein and the viscous and gel forming properties of mucus. Br Med Bull 34:28–33
11. Harding SE (1989) The macrostructure of mucus glycoproteins in solution. Adv Carb Chem Biochem 47:345–383
12. Schauer R. (1992) Sialinsäurereiche Schleime als bioaktive Schmierstoffe. Nachr Chem Tech Lab 40, Nr 11:1227–1231
13. Sandford, PA (1989) Chitosan: Commercial uses and potential applications. In: Skjåk-Braek G, Anthonsen T, Sandford P (eds) Chitin & Chitosan, pp 51–69
14. Dawson MC, Elliot DC, Elliot WH, Jones KM (1986) Data for Biochemical Research, Clarendon Press Oxford, p. 429
15. Hutton DA, Pearson JP, Allen A, Foster SNE (1990) Mucolysis of the colonic mucus barrier by faecal proteinases: Inhibition by interacting polyacrylate. Clinical Science 78:265–271
16. Errington N, Harding SE, Vårum KM, Illum L (1993) Hydrodynamic characterization of chitosans varying in degree of acetylation. Int J Biol Macromol 15:113–117
17. Creeth JM, Bhaskar KR, Horton JR (1977) The separation and characterization of bronchial glycoproteins by density-gradient methods. Biochem J 167:557–569
18. Ifft JB, Vinograd J (1966) The buoyant behaviour of bovine serum mercaptalbumin in salt solutions at equilibrium in the untracentrifuge. II. Net hydration ion binding and solvated molecular weight in various salt solutions. J Phys Chem 70:2814–2822
19. Creeth JM, Horton JR (1977) Macromolecular distribution near the limits of density-gradient columns, Biochem J 161:449–463
20. Creeth JM, Denborough MA (1970) The use of equilibrium-density-gradient methods for the preparation and

characterization of blood-group-specific glycoproteins. Biochem J 117:879–891
21. Wyatt PJ (1992) Combined differential light scattering with various liquid chromatography separation techniques In: Harding SE, Sattelle DB, Bloomfield VA (eds) Laser Light Scattering in Biochemistry. The Royal Society of Chemistry, London, pp 35–58
22. King DJ, Byron OD, Mountain A, Waeir N, Harvey A, Iawson ADG, Proudfoot KA, Baldock D, Harding SE, Yarranton GT, Owens RJ (1993) Expressions, purification and characterization of B72.3 Fv fragments. Biochem J 290:723–729
23. Creeth JM, Bhaskar KR, Horton JR (1977) The separation and characterization of bronchial glycoproteins by density gradient methods. Biochem J 167:557–569
24. Harding SE, Creeth JM (1983) Polyelectrolyte behaviour in mucus glycoproteins. Biochim et Biophys Acta 746:114–119

Received June 18, 1993;
accepted October 13, 1993

Authors' address:

Mr. Immo Fiebrig
Department of Pharmaceutical Sciences
University Park
Nottingham NG7 2RD, United Kingdom