

Transmission electron microscopy studies on pig gastric mucin and its interactions with chitosan

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Transmission electron microscopy has been employed to visualize the molecular structure and organization of a highly purified preparation of pig gastric mucin (molar mass $M \sim 9 \times 10^6$ g/mol, as determined by low speed sedimentation equilibrium), prepared for microscopy by two completely independent methods. Samples were prepared for imaging by air drying on mica (in the presence of 50% glycerol) as well as critical point drying in acetone/CO₂. The data appear consistent with the accepted linear model for mucins, and are consistent with regions of variable degrees of glycosylation along the polypeptide backbone chain, with the overall conformation that of a loose or random coil. The behaviour of this mucin in a dilute solution mixture with the potential mucoadhesive polymer chitosan ($M \sim 160,000$ g/mol) was then explored, and a clear interaction was demonstrated, consistent with dilute solution measurements using sedimentation velocity analysis in the analytical ultracentrifuge.

INTRODUCTION

The macrostructure of mucus glycoproteins or 'mucins' has been well described and appears to have a large number of common structural features, independent of whether they are gastrointestinal, cervical or tracheo-bronchial in source (Creeth, 1977; Allen, 1981, 1989; Carlstedt & Sheehan, 1984a, b; Silberberg, 1987; Sheehan & Carlstedt, 1989; Harding, 1989): mucins appear to have a very high molar mass, ranging from 0.5×10^6 to over 40×10^6 g/mol. Undegraded mucins are made up of multiples of a 'basic unit' of molar mass between 400,000 and 500,000 g/mol (Silberberg & Meyer, 1982) which are covalently linked together into linear arrays (Sheehan & Carlstedt, 1989). The basic unit is made from a single chain polypeptide backbone with two distinct regions: (i) a heavily glycosylated central protein core to which a large number of carbohydrate side chains are attached predominantly by *O*-glycosidic linkages via the serine and threonine amino acid residues; followed by (ii) one or two terminal peptide segments often referred to as 'naked proteins regions', because these regions are regions of low or even no glycosylation. These basic units are assembled linearly

into 'subunits' ($M \sim 2.5 \times 10^6$) and then further by disulphide bridging into the mucin macrostructure (Sheehan & Carlstedt, 1989).

The central glycosylated region ('T-domain' (Sheehan & Carlstedt, 1984)) is rich in serine, threonine and proline with many multi-branched oligosaccharides attached, ranging in length from 14 to 18 sugar residues. The carbohydrate chains may contain up to five different monosaccharides, namely D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid. The resulting structure has often been compared with a 'bottle brush', where the bristles represent the carbohydrate side chains and the central wire the protein core (Allen, 1978).

Unlike globular proteins, there is no evidence for a regularly folded 'secondary structure' for the basic unit in mucins or a stiff rod (Silberberg, 1987). A loosely coiled structure is much more likely for a number of reasons: (a) high levels of proline (producing bends and turns in polypeptides); (b) a loosely coiled structure being more efficient at engulfing and immobilizing local solvent than a stiff and extended form; (c) hydrodynamic data where values for the ratio of the sedimentation concentration regression coefficient, k_s , to the intrinsic viscosity $[\eta]$, and the so called Mark-Houwink sedimentation, diffusion and radius of gyra-

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tion coefficients are close to the value expected for a spheroidal or randomly coiled molecule (Creeth & Knight, 1965; Sheehan & Carlstedt, 1984; Harding, 1992).

I. Carlstedt, J.K. Sheehan and coworkers (see e.g., Carlstedt *et al.*, 1985; Sheehan & Carlstedt, 1989) proposed a 'linear random coil' model for the macrostructure of the mucin in which the T-domains or 'basic units' (and the 3-4 basic unit forms called 'subunits') are linked together into a linear chain which has random coil properties in solution. This is essentially complementary to the 'swollen coil array' model (Harding *et al.*, 1983a, b; Harding, 1989; Sheehan & Carlstedt, 1989), where the basic units themselves were proposed, in addition, to adopt highly swollen (through solvent imbibement) loosely coiled spheroidal domains linked together by regions of low glycosylation which were much less swollen. The 'swollen coil array model' was based on hydrodynamic data supported by transmission electron microscopy measurements on a relatively low molar mass ($M \sim 2.0 \times 10^6$ g/mol) tracheobronchial mucin sample prepared for electron microscopy using air-drying onto mica (where the strong shearing forces on air-drying removed all tertiary structure but showed the apparent swollen domains (Harding *et al.*, 1983b)), and also critical point dried mucins which appeared to preserve the 3-dimensional integrity (Hallet *et al.*, 1984).

In this present work we (i) extend our earlier work to demonstrate these features for a higher molar mass gastrointestinal mucin, the highly purified pig gastric mucin (PGM), of molar mass $M \sim 9 \times 10^6$ g/mol. Although other preparations of this mucin have been previously intensively studied by Sheehan *et al.* (1986), Sheehan & Carlstedt (1990) and Stokke & Elgsaeter (1987), a joint study involving air-drying and critical point drying has, as far as we are aware, not yet been undertaken. We have also produced images of this mucin complexed with chitosan, a cationic polysaccharide of low degree of acetylation. These images support earlier hydrodynamic measurements (Fiebrig *et al.*, 1994) that high molecular weight complexes are formed. The consequence of this finding for the potential use of chitosans as pharmaceutical mucoadhesives is indicated.

MATERIALS AND METHODS

Pig gastric mucin (PGM) preparation

PGM was isolated from fresh pig gastric mucus according to a modified procedure of Hutton *et al.* (1990). The mucus gel was solubilized in an enzyme inhibitor cocktail by high shear homogenization (MSE, ATO Mix blender at high speed for 1 min) and freed from non-glycoprotein material by preparative caesium chloride isopycnic density gradient ultracentrifugation.

This was followed by gel permeation chromatography of the glycoprotein fraction on a Sepharose CL-2B column to separate high molecular weight material from degraded mucin. The totally excluded volumes were pooled and concentrated by ultrafiltration to a concentration of approximately 0.4 mg/ml, dialysed against distilled water and kept frozen at -20°C in 1 ml aliquots. The mucin solution was gently defrosted and dialysed into an acetate buffer (pH 4.5, $I=0.10$) prior to use. The mucin was checked as to its molecular integrity by sedimentation equilibrium analysis (molecular weight) and analytical isopycnic density gradient analysis (purity). Low speed sedimentation equilibrium following the procedure of Harding *et al.* (1992) demonstrated a weight average (apparent) molar mass, M_w of $(9.3 \pm 0.2) \times 10^6$ g/mol. Isopycnic density gradient analytical ultracentrifugation in Cs_2SO_4 was carried out according to the method described by Creeth *et al.* (1977). The mucin sample showed no detectable contamination neither by non-covalently bound protein nor nucleic acid. A clear single peak at a calculated density of 1.337 g/ml was obtained for the mucin (Fiebrig *et al.*, 1994), well in accordance with published values (Creeth & Denborough, 1970).

Chitosan

Chitosan is a linear polycationic macromolecule obtained from chitin via deacetylation of the amino group. The particular chitosan used in the present experiments (Sea Cure +210, Protan Ltd, Drammen, Norway) was previously well characterized (Errington *et al.*, 1993) showing a degree of acetylation of 11% and a M_w of $(162 \pm 10) \times 10^3$ g/mol.

Sample preparation for electron microscopy

Mucin

The material (~ 0.4 mg/ml), was defrosted and dialysed against acetate buffer as described above (pH 4.5, $I=0.1$).

Mucin/chitosan complex

Chitosan was dissolved in acetate buffer (pH 4.5, $I=0.1$) at a concentration of 2 mg/ml and mixed with equal volumes of the above mucin solution. The mixture was allowed to stand for 30 min at room temperature. The final concentration of mucin and chitosan in the mixture was 0.2 and 1 mg/ml, respectively.

Procedure 1: air drying onto mica

Glycerol was added to the samples to give a concentration of 50% and diluted to give a glycoprotein concentration of approximately 25 $\mu\text{g}/\text{ml}$. Fifty microlitres of the material were sprayed via a modified artist's airbrush onto freshly cleaved mica, transferred to a vacuum coating unit, evacuated to 1×10^{-5} mbar for

10 min, then rotary shadowed thermally with platinum at an angle of 7.5°. The replica was subsequently coated with carbon for stability and floated from the mica on water. Small pieces of replica were collected on square 300 mech. Cu grids.

Procedure 2: critical point drying

Two to three microlitres of undiluted sample were adsorbed onto freshly made glow-discharged Formvar[®]/carbon square 300 mech. Cu grids, vapour fixed for 60 s with glutaraldehyde and not allowed to dry. Grids were immersed in 50% acetone followed by 100% acetone, then critical point dried in CO₂ medium using a Balzers CPD030 critical point dryer. The grids were then rotary shadowed thermally with platinum at an angle of 7.5°.

After preparation, in both cases samples were imaged on a Siemens 102 TEM at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Pig gastric mucin

Essentially the images on the pig gastric mucin alone confirm previously published observations found for other preparations of mucins and show a difference depending on whether the samples are air-dried (Fig. 1a) or critical point-dried (Fig. 1b, 1c; Fig. 2),

each showing complementary features about the mucin. The images on Fig. 1b and c show two representative areas of the strikingly clear CPD (critical point dried) mucin sample and its 3-dimensional architecture. We can distinguish highly swollen regions of thicknesses between 50 and 150 nm, joined by nearly linear linker regions of approximate thickness 5–10 nm and lengths between 200 and 400 nm. The 'highly swollen' regions may correspond to the highly glycosylated regions (so-called 'bottle brush' regions) whereas the linker regions would represent peptide regions of low glycosylation. In contrast, Fig. 1a shows the somewhat different images for the air-dried mucin preparation. In this case, we essentially have a 2-dimensional flattened structure, the flattening being a direct result of the strong surface tension and shearing forces experienced by the mucin during the drying process. The highly glycosylated regions are not clearly distinguishable from the areas of low glycosylation. Similar to the findings of Sheehan *et al.* (1986) and Stokke & Elgsaeter (1987), the bottle brush-like structure cannot be resolved by electron microscopy using this preparation technique. Also, the side chains might be folded along the backbone, leading to a smoother overall appearance. The sample shows linear threads of an approximate thickness of 2–10 nm and also 'flattened globular' structures with many bends and a rather tangled appearance, threads with loops and kinks. Occasionally, complex structures, where filaments emanate from and return to a central core, were observed. These tangled filamentous core structures had

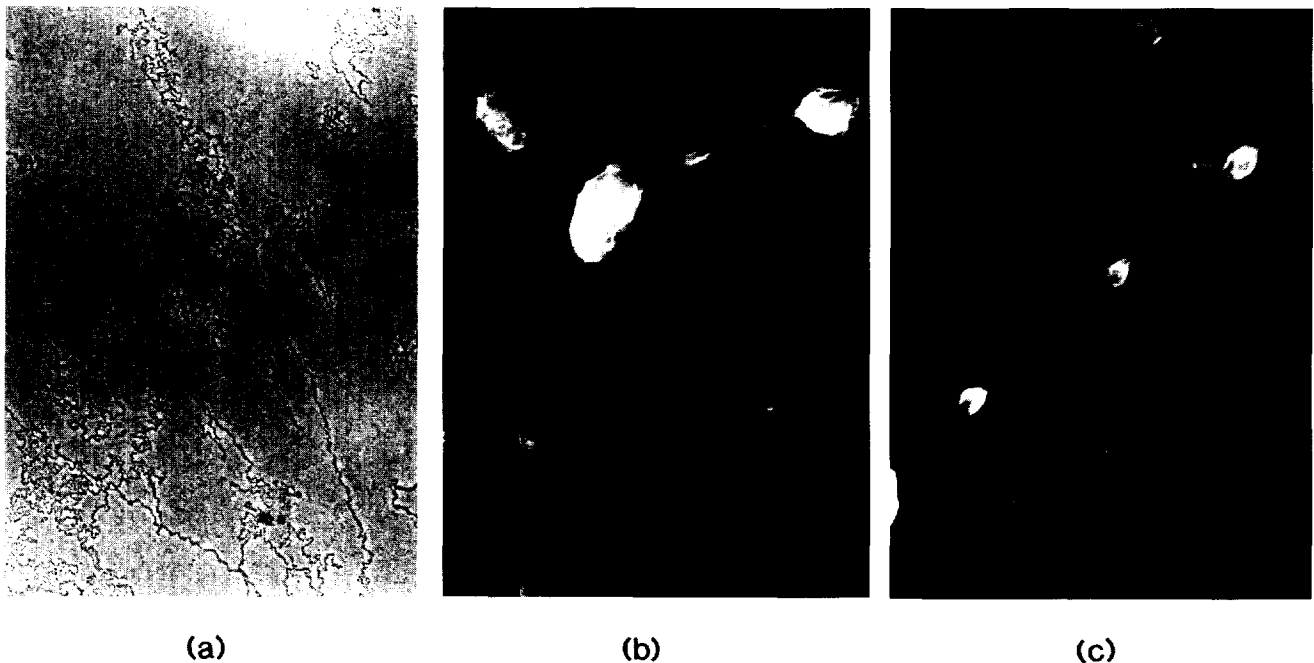


Fig. 1. Electron micrographs of gastric mucin prepared by (a) air-drying onto mica, $\times 29,800$; (b) and (c) critical point-drying, $\times 69,230$; followed in each case by rotary shadowing with platinum at an angle of 5°. In (a) a 2-dimensional 'plan' is seen of the protein backbone with its attached branched polysaccharides. Linker regions are clearly seen joining the presumptively heavily glycosylated HS-regions. In (b) and (c) the mucin is visualized with its 3-dimensional structure retained: the HS-regions are revealed as polyhedral entities of low asymmetry, joined by linker regions.

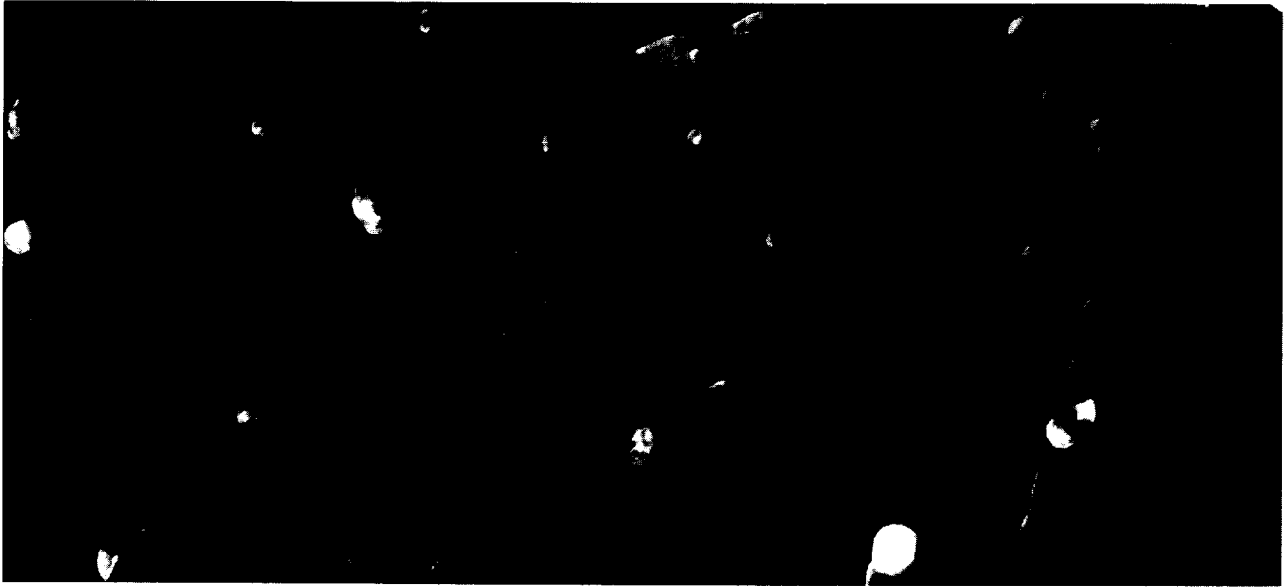


Fig. 2. Electron micrograph of a part of a large network of gastric mucin, prepared by critical point-drying followed by rotary shadowing (as in Fig. 1). Multiple HS-regions connected by linker regions are seen. Magnification $\times 59,400$.

already been described by Rose *et al.* (1984) in human tracheobronchial mucins.

PGM-chitosan complex

As a polycation, chitosan is already known on the basis of sedimentation velocity analysis in the analytical ultracentrifuge to interact strongly with mucin and its

sialic acid residues (Fiebrig *et al.*, 1994). In the present study, after complexing the mucin with chitosan, changes were evident both in plan and in structure (Fig. 3). In plan, spherical complexes of between 200 and 500 nm with a dense core from which mucins are emanating can be seen (Fig. 3a). In contrast, the 3-dimensional architecture shows the bottle brush structures to be heavily obscured, with many linker regions of mostly unmodi-

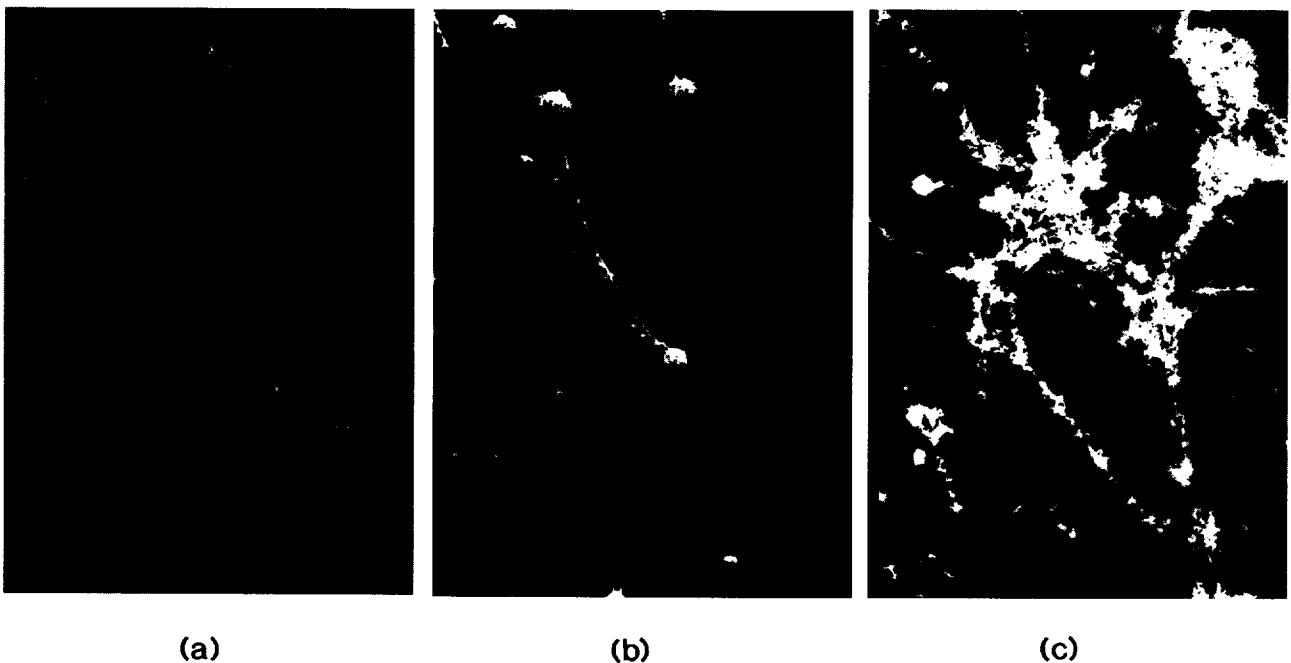


Fig. 3. Electron micrographs of gastric mucin with bound chitosan, prepared by (a) air-drying followed by rotary shadowing with platinum at 5° , $\times 59,650$; (b) and (c) by critical point-drying followed by unidirectional shadowing with platinum at 5° . In (a) it is seen that the detail of the course of the mucin has been largely obscured, forming a spherical agglomerate with a dense core; in (b) and (c) however the corresponding HS-regions are of similar overall size, whilst the linker regions remain visible, with only minimal evidence of thickening. Magnifications: (b) $\times 19,160$; (c) $\times 29,800$.

fied thickness being present (Fig. 3b, 3c). The HS highly swollen regions are in most cases of similar size, although sometimes their separation from the linker domains is less apparent. We can thus conclude that chitosan predominantly binds *internally* to the HS-regions, whilst leaving the linker regions largely untouched.

Such an internal binding would have the effect of considerably increasing the mass of the mucin polymer without any significant concomitant increase in friction. From this we can predict an anomalously large rise in sedimentation coefficient (sedimentation rate per unit centrifugal field) of mucin after binding. The sedimentation coefficient of gastric mucin has been determined by analytical ultracentrifugation at 37.0°C and at a concentration of ~0.2 mg/ml, and estimated as $(53.2 \pm 2.8) S$; for chitosan by itself at ~2.0 mg/ml, a value of $(2.20 \pm 0.03) S$ was found. For the complex a value of $(1990.0 \pm 19.0) S$ was determined. The sedimentation coefficient of mucin, therefore, shows a very large increase indeed after binding of chitosan (in an approximately 0.2 to 1 mass ratio). However, the electron micrographs suggest that the binding occurs mostly internally to the HS-regions of the mucin, with only a minor amount attaching to the linear structures. The hydrodynamics of so complex a structure as mucin must itself be complex, but we can offer a description which is at least semi-quantitative in the following terms. The HS-regions in the uncomplexed mucin are joined by very thin and extended linker regions. The total structure may, therefore, be treated as an elastically linked oligomer (ELO) as defined by Rowe (1989), from which it is predicted that in the limit of linker flexibility, the sedimentation coefficient of the mucin will approximate to that of the individual HS-regions (Rowe, 1989; equation 1). To test this hypothesis, we can compute from the sedimentation coefficient, molar mass (above) and a partial specific volume of 0.64 ml/g (Snary *et al.*, 1971) that the mucin has a frictional ratio of ~4.1 and a Stokes' radius of ~53.7 nm. This latter value compares well with the size of the structures seen after critical point drying (Fig. 1), in the range of 50–150 nm diameter. If the binding of chitosan to the linker regions is supposed to be sufficient to increase the rigidity of those structures, then a large increase in the sedimentation coefficient will result from the loss of elasticity, which will cause the whole mucin structure including the extra mass from bound chitosan to migrate as a single hydrodynamic structure (Rowe, 1989). An exact prediction of the magnitude of the effect cannot be made: as noted (Rowe, 1989) the basic theory of ELOs has yet to be applied to multiple linkages. However, approximate calculations suggest that at least 20–30 HS-regions would need to be linked together to account for the observed sedimentation coefficient of the complex. This estimate is compatible with the evidence from electron microscopy.

From this it is predictable that the overall properties

of the mucin, which enable it to fulfil its physiological function, would be only minimally affected by such binding. Whilst it cannot yet be concluded that targeted drug delivery via chitosan or a similar polymeric drug carrier is achievable clinically, the particular properties revealed in our study, in which HS-regions are apparently capable of acting as localized 'applicator sponges' held close to the epithelial surface with minimal interference with normal physiological functions, are consistent with at least some of the principal logical requirements of such a targeted bioadhesive drug delivery system.

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