Chitin Handbook, R.A.A. Muzzarelli and M.G. Peter, eds.,
European Chitin Society. 1997. ISBN 88-86889-01-1

Characterisation of chitosan-mucin complexes by sedimentation velocity analytical ultracentrifugation

Stephen E. Harding

University of Nottingham, Department of Applied Biochemistry and Food Science,
Sutton Bonington LE12 5RD, UK

There is increasing interest being expressed by the pharmaceutical industry in how chitosan molecules interact with the mucus and mucosal epithelia lining the gasterointestinal and tracheobronchial systems (Fiebrig *et al.*, 1995a). Interest in their use for drug delivery is principally because of their polycationic properties, biodegradability and lack of antigenicity. Three routes have been receiving considerable interest for the delivery of drugs, aided by chitosan: through mucosal epithelia in (i) the nose, (ii) the small intestine and (to a much lesser extent) (iii) the stomach. With (i) the main assistance of the chitosan is by enhancement of uptake through the epithelia themselves, whereas with (ii) and (iii) the main function of the chitosan is as a molecular "brake" or "parachute" for slowing down passage of the drug through the alimentary tract, giving an increased absorption time window as the drug passes through the stomach and then the small intestine.

The oral route is a popular method of administering drugs (Fiebrig *et al.*, 1995a). This requires passage through the alimentary tract and eventual absorption through the mucosal membrane, usually in the proximal small intestine. Unfortunately the amount of drug actually delivered, the "bioavailability", can be very much smaller than that ingested because of (i) too rapid a transit of the drug-containing system past the ideal absorption site (ii) rapid degradation of the drug in the gasterointestinal tract once it has been released and (iii) low transmucosal permeability due to the size, ionisation, solubility or other characteristics of the drug molecule. Chitosans provide an attractive mucoadhesive (or molecular brake) for a drug to be encapsulated because of their polycationic complementarity to the polyanionic mucins which constitute the key macromolecular component of mucus. Laboratory trials appear to have proven the success of chitosan as a mucoadhesive, using a range of macromolecular characterisation methods including co-sedimentation analysis and electron microscopy. Although the strong mucoadhesive ability of chitosan is well established, its ability to dock and undock a drug is still being explored.

Chitosans appear to have the interesting "bonus" effect of actually enhancing the ability of the mucosal membrane to absorb a drug molecules as large as insulin: this has been demonstrated for nasal epithelia, and a mechanism has been suggested where the chitosan reduces the beat frequency of the cilia assisting a transient opening of pericellular junctions.

The anionic mucin glycoprotein: key to mucoadhesion

Gasterointestinal, tracheobronchial and reproductive mucins appear to follow the same structural pattern shown in Fig. 1, the latest of these being discovered to conform to this pattern being colonic mucin (Jumel *et al.*, 1997): a linear backbone consisting of fundamental "monomer" units of M ~ 500,000 Da linked into a linear array. Each unit consists of a polypeptide backbone with a blanket of O-linked carbohydrate side chains of 3 - 30 residues in length. The two ends of the polypeptide are exposed and are linked covalently to give a large linear compound of molecular weights ranging from 2 - 50 x 10^6 Da. Every 3 or 4 of these links is by a disulfide rather than a peptide bond and can be broken by thiols. The thiol degradation products are known as "subunits" (Sheehan and Carlstedt, 1989). Several mucin genes have now been sequenced and tandem repeat sequences in the polypeptide chain have been discovered (Gum, 1992). The end saccharide residue of each *oligosaccharide* side chain is often N-acetylneuraminic acid (NaNA) commonly referred to as "sialic acid": mucins are thus polyanions.

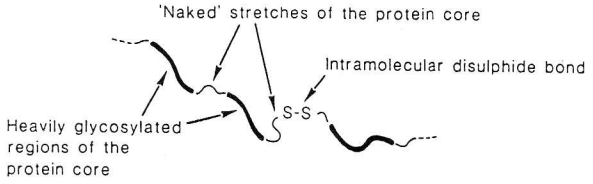

Figure 1. Part of mucin molecule. The glycosylated region are negatively charged, to an extent which depends on the number of sialic acid residues (Sheehan and Carlstedt, 1989).

The mucin glycoprotein dictates the properties of whole mucus, so to design an efficient mucoadhesive, the pharmaceutical scientist needs to understand the structure and mode of action of the interaction of any candidate mucoadhesive polymer with the mucin glycoprotein.

An electrostatic complex

When two biopolymers are mixed together one of three things can happen (Tolstoguzov, 1990; Harding *et al.*, 1995):

- nothing
- phase separation, due to thermodynamic incompatibility: i.e. molecules "pushing apart"
- the biopolymers interact non-covalently or covalently in either a reversible or nonreversible manner: ie. molecules "sticking together".

Thus, although many biopolymer mixtures are thermodynamically incompatible and "phase separate"; other mixtures however show a capacity for interaction to give soluble complexes in the correct pH, salt and temperature conditions. The situation can be further complicated because at low concentration, even thermodynamically incompatible systems can remain as a one phase system; further, "sticking together" attractive interactions can also lead to phase separation, particularly if the complex results in negligible net charge. Fig. 2 summarizes the four possibilities (A-D).

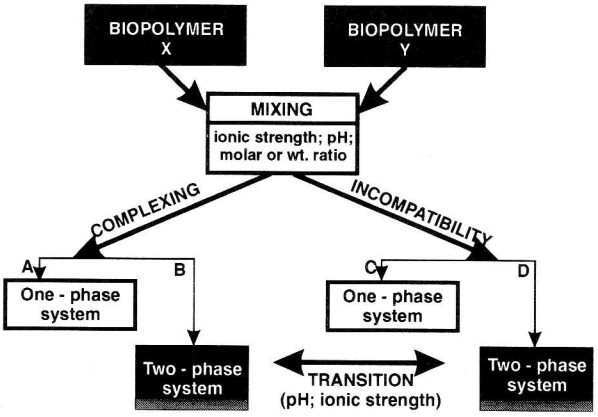

Figure 2. Tolstoguzov diagram representing the 4 possible consequences on mixing together two soluble biopolymers in an aqueous environment. The anionic mucin mixed with the cationic chitosan under mildly acidic conditions appears to correspond to possibilities A and B. Adapted from Tolstoguzov (1990).

Sedimentation velocity in the analytical ultracentrifuge

This is one of the simplest and most powerful methods that can be used for probing mucin-chitosan complexation. A recent simple description has been given by Harding (1994 a) and its application to mucin-chitosan complexes by Fiebrig *et al.* (1994) and Fiebrig (1995 a,b).

Commercial availability

Two types of analytical ultracentrifuge are currently available commercially, both from Beckman Instruments, Palo Alto, USA: the XL-A ultracentrifuge (Giebeler, 1992), with scanning absorption optics and the XL-I ultracentrifuge with integrated absorption and interference optics (Furst, 1997). Both come with full on-line data capture and analysis facilities. Unfortunately, neither have the Schlieren optical facility, which is a pity since this is the most useful for the study of large complexes, but an adaptation kit (Clewlow *et al.*, 1997) will hopefully soon be commercially available. Older instruments still in active use, such as the Beckman Model E or the MSE (Crawley, UK) MkII analytical, possess all three optical systems.

Preparation of sample

Mucin and chitosan are mixed together in the appropriate ratio under the appropriate solvent conditions. Because the interaction is electrostatic in nature, due attention has to be given to the temperature, pH and ionic strength of the solvent used. The latter two are critical since the solubility of chitosan is poor at neutral pH and even under acidic conditions, any neutralisation of the charge by complexation with the mucin needs to be taken into account in observing the behaviour of the mixture: complexes will be of the form "A" or "B" of Fig. 2. Cell filling is best not done via a syringe but via a gilson pipette with a special narrow tip, or via a regular tip and directly filling the solution channels whilst the upper window is off (see manufacturers manual): between 0.2 and 0.4 ml is used. The reference solvent is placed in the solvent channel of the cell (slightly more, say an additional 0.01 ml, than the solution in the solution sector) to ensure any optical signal from the meniscus in the solvent channel does not interfere with the signal from the solution channel.

Basic principle of operation of the analytical ultracentrifuge

The ultracentrifuge cell containing the solution or dispersion of the macromolecule or macromolecular complex is placed in the rotor (4 or 8 hole), and balanced. With the 4 hole rotor, 3 cells (and one counterbalance) can be run simultaneously (with the 8 hole, 7 can). An xenon or mercury arc light source positioned below the rotor passes light via a monochromator or filter (order instruments) through the solution and then other optical components. With the interference system, the light source is usually a laser (without the monochromator). The moving boundary can then be recorded on photographic film or chart paper (order instruments) or as digital output (XL-A and XLI). The

shape of the sedimenting boundary provides information of the heterogeneity of the sample: the rate of movement of the boundary per unit centrifugal field provides the sedimentation coefficient, which depends on the shape and size of the sedimenting macromolecule or macromolecular complex (Harding, 1994 a).

Purity and homogeneity of the reactants

The purity and homogeneity of the mucin and, if appropriate, chitosan needs to be checked by SEC-MALLS analysis (Harding, 1994 b), sedimentation equilibrium (Harding, 1994 c) and sedimentation velocity in the ultracentrifuge. Analytical density gradient ultracentrifugation in caesium salts can be applied to check for the absence of nucleic acids and other contaminants (Fiebrig *et al.*, 1994).

Two stages to monitoring complexation:

(i) *Monitoring the complex directly by following the sedimentation properties of the complex.* Because the complex is likely to be very large, it is crucial to choose the rotor speed and scan intervals for the sedimenting boundary appropriately. Commence with a very low rotor speed (say ~ 2000 rev/min) and scan every few minutes to catch any fast moving boundary. Then steadily increase the speed say to ~ 10,000 rev/min, ~ 30000 rev/min and finally ~ 50000 rev/min to catch the slower moving species. If the complex is very large, due to the strong turbidity the absorption optical system on the XL-A should pick up the sedimenting boundary. The sedimentation coefficient of the boundary or boundaries can be obtained by using the Bridgman "g(s)" method now fully on-line to a computer (Staffora, 1992) or by an "off-line" method, i.e. downloading onto a printout (XL-A / XL-I) or photographic film (order ultracentrifuges) and locating the centre of the boundary(s) for each scan by eye, ruler and pencil, (often preferable!) and then re-digitising the centre-of boundary data using a graphics tablet (see Harding, 1994a). Computer programmes for both evaluate the sedimentation coefficient, s, from the rate of movement of the sedimenting boundary per unit centrifugal field. Although the normal practice in characterising macromolecules is to normalise sedimentation coefficienes to standard solvent conditions (the density and viscosity of water at 20 °C), since the temperature for complexation studies is body temperature, s values are most usefully determined and quoted at ~ 37 °C.

(ii) *Monitoring the complex indirectly by following the sedimentation properties of any residual uncomplexed chitosan or mucin.* By comparing the amount of chitosan or mucin "lost" through complexation, the stoichiometry of the complex

can be assessed. A popular way of recording the chitosan is using refractometric Schlieren optics. The mucin can be detected by either refractometric OR absorption optics, although the extinction coefficient for mucins is at least 5 times less than that of most proteins (because only ~ 20 % of the molecule is peptide). By comparing the area under a Schlieren peak (proportional to concentration) for chitosan in the mixture cell with that for a chitosan control the extent of complexation can be assessed. Fortunately the sedimentation coefficients of both reactants are quite different (chitosan, s ~ 2S; mucin s ~ 50S) so the chance of confusing one for the other is quite remote.

Examples

Figs. 3, 4 and 5 show examples for (i) the complex (Fig. 3), (ii) a mucin control (Fig. 4), and (iii) chitosan in a mixture cell and in a control cell (Fig. 5). These records were for a study on a highly deacetylated (degree of acetylation ~ 11 %) chitosan ("Sea-Cure + 210" from Pronova, Drammen, Norway) with pig gastric mucin, mixed in a weight concentration ratio of 1 : 5 (concentrations in the final mixture of ~ 0.4 mg/ml mucin and ~ 2 mg/ml chitosan). Fig. 3 (at 2000 rev/min) recorded using scanning absorption (in this case turbidity) optics revealed a very fast large turbidity boundary (at 230 nm) with a sedimentation coefficient, s = (1990 $\pm$ 18)S with nothing detectable following in its wake (i.e. no residual mucin, although any residual chitosan would remain undetected). The mucin control (at 10000 rev/min) is sedimenting much slower (Fig. 4) with an s of only (53.0 $\pm$ 2.8)S. To follow the chitosan control it is necessary to use the Schlieren optical system on an order ultracentrifuge (an MSE Mk II analytical). Fig. 5 (at 35000 rev/min) shows the Schlieren diagrams photographed at the start of the experiment for the chitosan control and residual unbound chitosan in the mixture cell. Accurate comparison of the area under the curves for chitosan in the control cell and unbound chitosan in the mixture cell (either off-line from the photographic records or on-line with a CCD camera linked to a PC (Clewlow *et al.*, 1997)) yields the stoichiometry on a weight : weight basis. Knowledge of the molecular weights of the reacting species (from light scattering or sedimentation equilibrium) allows calculation of the corresponding molar:molar basis. In the example of Fig. 5 (chitosan molecular weight ~ 160000 Da, mucin molecular weight ~ 9 x 10^6 Da) a molecular interaction ratio of chitosan : mucin is ~ 4 : 1 can be inferred. It is also possible to calculate the mean size of the complex from the sedimentation coefficient and molecular weight:

$$r_H = M(1 - \bar{v}\rho_o)/6\pi\eta_o s \qquad (1)$$

where M is the molecular weight of the complex, η and ρ the solvent density and viscosity. $\bar{v}$ is the partial specific volume (essentially the reciprocal of the anhydrous macromolecular density): strictly speaking a weighted average from the chitosan and mucin in the mixture needs to be used, but since the two v's are very close (~ 0.57 ml/g for chitosan and 0.63 ml/g for a mucin) a simple numerical mean of ~ 0.60 ml/g is adequate. Fiebrig et al. (1995 b) obtained a value of ~ 54 nm in good agreement with estimates from images obtained by electron microscopy where particles in the range of 25 - 75 nm radius were observed. A form of equation (1) can be given which is independent of M:

$$r_H = \{4.5(v + \delta/\rho)\eta s/(1 - \bar{v}\rho)\}^{1/2} \qquad (2)$$

but an estimate for the "hydration" δ (number of grams of water "bound" per gram of complex) is required. Although for globular proteins a value of 0.3 - 0.4 for δ has been justified (Zhou, 1995), for mucins, polysaccharides and especially for complexes of the two this will be considerably higher.

Other methods for studying complexes of this type

Sedimentation velocity in the analytical ultracentrifuge is one of a suite of methods for probing mucin-chitosan complexation phenomena. Because the complexes, with sedimentation coefficients in excess of 1000S appear to be at the top end of the technique in terms of particle sizes that can be comfortably handled, it is best used in conjunction with one or preferably more other independent methods. The simplest is turbidity (Tolstoguzov, 1990). Although this can in principle provide the size of the complexed particles (see Bahls & Bloomfield, 1977), it is used as a qualitative probe from visual inspection or from records of the optical density. Dynamic light scattering can be used to confirm estimates of the equivalent hydrodynamic radius from equations (1) or (2) and provide an estimate for the polydispersity (Harding, 1994 b). Static light scattering can also give similar information, particularly used on-line with size-exclusion chromatography (or field-flow fractionation) columns (Harding, 1994 c) so long as the columns (or fractionation membrane) do not interfere with the complex. Sedimentation equilibrium in the analytical ultracentrifuge (Harding, 1994 d) can also be used, but only up to a maximum particle molecular weight of ~ 20 x 10^6 Da. Electron microscopy has also been used to good effect - particularly on mucin-chitosan complexes (Fiebrig *et al.*, 1995 b) and by judicious labelling of the chitosan by colloidal gold / wheat germ agglutinin the distribution of chitosan within a complex can be visualized (Fiebrig *et al.*, 1997). Electron microscopy has been shown to give results consistent with those from ultracentrifugation on uncomplexed and complexed mucin (Fiebrig *et al.*, 1995

b). Rheological methods can also be used (see Ross-Murphy, 1995) although potentially the most powerful combination appears to be the ultracentrifuge combined with surface plasmon resonance (Silkowski *et al.*, 1997).

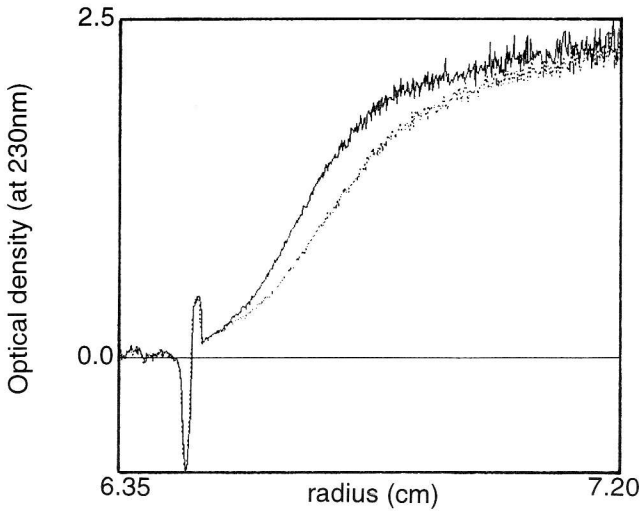

Figure 3. Sedimentation velocity boundaries (at an interval of 10 min) of a pig gastric mucin / 210-chitosan complex, recorded in the XL-A ultracentrifuge, rotor speed = 2000 rev/min, temp = 37 °C. s = (1990 $\pm$ 18)S. From Fiebrig et al. (1994). Direction of sedimentation is from left to right.

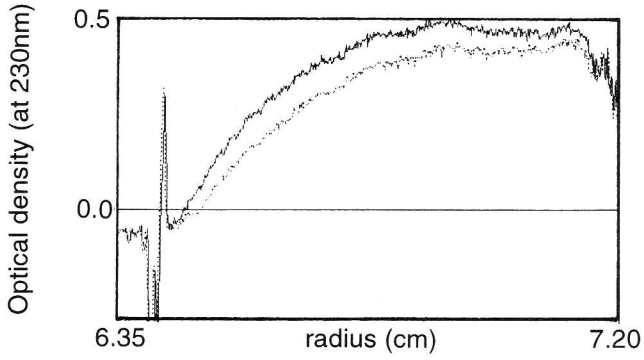

Figure 4. As Fig. 3 but for the mucin control (at the same mucin loading concentration). Scan interval = 6 min. Rotor speed = 10000 rev/min. s = (53.0 $\pm$ 2.8)S.

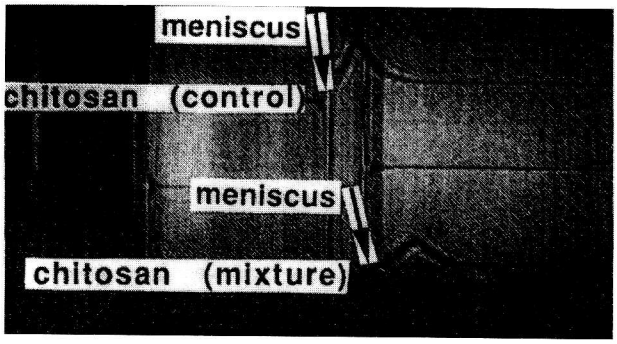

Fig. 5. As Figs 3 and 4, but run in an MSE Mk II analytical ultracentrifuge (35000 rev/min) with photographic Schlieren optics. Initial scans for chitosan in the control cell (top) and mixture (at the same chitosan loading concentration). Area under Schlieren peak for chitosan in mixture cell ~ 0.7 times that in control cell.

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