Mucoadhesive interactions

S.E. Harding[1]
National Centre for Macromolecular Hydrodynamics, University of Nottingham, School of Biosciences, Sutton Bonington LE12 5RD, U.K.

Abstract
The adhesive properties of certain types of biopolymer can be used to increase the residence time of orally or nasally administered drugs. A fuller understanding of the molecular processes underpinning such 'mucoadhesive' phenomena will help in the optimal design of delivery systems. The interactions involved are, however, less well defined compared with those often encountered in protein-recognition phenomena: mucoadhesive interaction products can be very large and polydisperse, so to probe them we need to adopt a different strategy to those used by protein biochemists. Reviewed herein is some of the recent work at physiological or near-physiological solution conditions involving molecular hydrodynamics – with analytical ultracentrifugation and SEC-MALLs (size-exclusion chromatography coupled to multi-angle laser light scattering) as the cornerstones – reinforced by viscometry and the imaging probes of electron microscopy and atomic force microscopy. These clearly demonstrate the mucoadhesive properties of both an unusual cationic protein [Deacon, Davis, Waite and Harding (1998) Biochemistry **37**, 14108–14112] and more significantly chitosan polysaccharides of varying degrees of charge/acetylation as a function of solution conditions, and are providing the platform for the construction of stable formulations.

Introduction
The route for the delivery of drugs that is still the most popular with medical staff and patients alike is through the mouth and down the alimentary tract: the oral route. The major site for drug absorption by this route is the small intestine which offers ≈100 m^2 of surface epithelia across which transfer can at least in principle take place [1]. If the drug is poorly soluble, or is in the form of a controlled release dosage form, significant absorption of the drug may also occur in the large intestine [2]. However, the clearance time through the whole alimentary tract is generally too short (4–12 h), rendering oral drug administration a very inefficient process, with much of the drug unabsorbed. In fact this is one of three major problems with the oral route: (i) too rapid a transit past the ideal absorption site, (ii) rapid degradation of peptide-based drugs in the gastrointestinal tract once the drug has been released and (iii) low transmucosal permeability. In this article I am just going to focus on how we can deal with problem (i) – the problem of too rapid transit – by using biopolymers with the appropriate characteristics as 'macromolecular brakes', focusing on the underlying biophysical principles and intermolecular interactions involved. It turns out that one of these biopolymers, namely chitosan, also appears to help address problem (iii) – the mucosal permeability problem – and this additional feature is proving particularly advantageous with nasal delivery systems.

A fuller understanding of the molecular processes underpinning such 'mucoadhesive' phenomena will help in the optimal design of delivery systems, but to do this it is important to consider the nature of the mucus-based substrate, and in particular its mucin component which, along with the water component, dictates the properties of mucus, and in particular its characteristic viscoelastic and protective properties.

Key words: chitosan, glue protein, micron size complex, mucin, ultracentrifuge.
Abbreviations used: SEC-MALLs, size-exclusion chromatography coupled to multi-angle laser light scattering; M_w, weight-average molecular mass; M_z, z-average molecular mass; s, sedimentation coefficient.
[1]e-mail steve.harding@nottingham.ac.uk

The substrate: adherent mucus and free mucins
The adherent mucus gel lining the alimentary tract has a minimum thickness of ≈40–50 μm and a maximum thickness of ≈300 μm (see e.g. [3]) depending on the individual and the region of the alimentary tract. Although most of mucus is water (≈95–99% by weight) the key macromolecular components are a class of glycoprotein known as mucins (1–5%).

Mucins are large molecules with molecular masses ranging from 0.5 to over 20 MDa. They contain large amounts of carbohydrate (for gastrointestinal mucins 70–80% carbohydrate, 12–25% protein and up to ≈5% ester sulphate). Undegraded mucins from a variety of sources are made up of multiples of a basic unit (≈400–500 kDa), linked together into linear arrays as shown first in 1983 by Creeth and co-workers [4,5] to give the macroscopic mucins with molecular masses claimed to be as high as ≈50 MDa [6]. The basic units are linked together by regions of low or no glycosylation which are subject to trypsin digestion: the ≈400 kDa digestion products are thus commonly referred to as T-domains (see [7]). Every third or fourth T-domain is linked by a disulphide bridge and these are susceptible to reductive disruption by thiols. The thiol reduction products (of molecular mass between 1.5 and 2.5 MDa) are commonly referred to as subunits. One of the most recent examples of such architecture in a mucin is that of colonic mucin [8]. Mucins are characterized not only by large molecular

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masses but by large molecular-mass distributions, as seen by analytical ultracentrifugation [9,10], and by the powerful technique of SEC-MALLs (size-exclusion chromatography coupled to multi-angle laser light scattering) for the first time by Jumel and co-workers [8,11]. Even mucins produced externally by cell lines appear to adopt this architecture, although they appear to be only up to one or two subunits in length (<5 MDa) [12]. In solution mucins adopt a random-coil conformation (see [7]), occupying a time-averaged spheroidal domain as shown by hydrodynamics and critical-point-drying electron microscopy [13]. Mucins which are different are the submaxillary mucins, with a lower carbohydrate content and different structure, but these are not so relevant in terms of gastrointestinal or nasal adhesion strategies.

Although direct sequencing of the protein chain has been virtually impossible because of the insolubility of mucins stripped of their carbohydrate, several *MUC* genes coding for mucin production have now been sequenced (see e.g. [14,15] and references cited therein): the key ones as far as mucoadhesion are concerned are *MUC2* and *MUC3* which code for mucins secreted into the small intestine and colon, and *MUC5AC*, *MUC5B* and *MUC6* into the stomach. The characteristic features are tandem repeats (e.g. 22 residues for *MUC2*), and high levels of serine, threonine and proline. Serine and threonine are the sites for O-glycosylation and the high levels of proline help to coil the molecule up [4,5]. The key sites for mucoadhesive interactions appear to be on the carbohydrate residues, either of the electrostatic type through terminal sialic acid residues or any sulphonated residues, or of the hydrophobic type through possible clusters of fucose residues which possess a methyl group.

The mucoadhesive and tests for ‘adhesiveness’

The mucoadhesive should be non-toxic, not expensive, with a high drug-loading capability. The toxicity issue means that, compared with synthetic polymers, *polysaccharides* are a very attractive option, since these, or at least the vast majority of these, are non-toxic; indeed many are used widely in food products as thickeners and stabilizers. It needs of course to be adhesive towards the mucus layer, and also not only to have a high drug-loading capacity but also high *unloading* capacity in the small intestine or thereabouts (see [16,17]).

The simplest ‘macroscopic’ test for adhesiveness is to perform a *tensiometry* experiment which involves the force required to detach two surfaces. Researchers at the Universities of Saarbrucken and Ghent coated one surface with mucus and the other with a selection of candidate mucoadhesive polysaccharides [18]. In this way they were able to show that neutral polysaccharides such as HP-cellulose, HE-starch and scleroglucan, and polyanionic polysaccharides such as pectin, xanthan and carboxymethyl cellulose gave virtually no interaction. A series of polycationic chitosans showed strong interactions whereas two other polycationics, namely DEAE-dextran and amino-dextran showed no significant interaction. Other macroscopic probes using mucus, such as flow-through methods (flow rate required to dislodge a mucoadhesive-coated sphere), colloidal gold staining (measurement of the so-called ‘adhesion number’) and *in vivo* methods (endoscopy and radioisotope imaging), have also proved useful [17].

Methods involving raw mucus are, however, not rigorous because of its variable nature from batch to batch and also with time because of the presence of degradative enzymes: in the alimentary tract mucins are constantly being degraded and replenished. In order to understand the fundamental molecular processes involved complementary molecular mucin-based analyses are needed. A selection of potential probes are available: the hydrodynamic-based techniques of viscometry/rheology, surface plasmon resonance, dynamic light scattering, turbidity, SEC-MALLs and the analytical ultracentrifuge, together with the imaging techniques of electron microscopy and atomic force microscopy. At Nottingham we have found analytical ultracentrifugation particularly useful because of the huge range of particle sizes analysable (from a small sucrose molecule of 342 Da to particles of $\approx 10^9$ Da) and its ability to separate and analyse solutions of macromolecules without the need for separation media or inherent assumptions of inertness. Electron microscopy and atomic force microscopy have proved powerful complementary probes. SEC-MALLs has proved particularly useful for checking the molecular integrity of the mucins and mucoadhesives and viscometry for assaying the stability of mucoadhesive formulations.

Checking the molecular integrity of the mucin and mucoadhesive

Mucins and mucoadhesives – particularly polysaccharides, are very different from the protein systems which have been the main focus of this meeting, in that they are much larger and often very polydisperse. For an assay procedure to be rigorous it is essential that the intergrity of both mucin and mucoadhesive are assessed before an experiment with the two mixed together is performed. We routinely perform (i) an SEC-MALLs analysis on both, which provides an assay of the purity, molecular mass (principally the weight average, but also the molecular-mass distribution) and polydispersity (ratio of the *z*-average molecular mass, M_z, to the weight-average molecular mass, M_w, or the ratio of M_w to the number average, M_n), and (ii) a sedimentation velocity experiment on both, which provides also an estimate of purity via a so-called g(s*) plot or distribution of sedimentation coefficients (from analysis of the change with time of the whole concentration distribution in the centrifuge cell) and the (weight average) sedimentation coefficient. We can also perform as a back up a sedimentation equilibrium analysis using the MSTAR procedure [19,20] to provide an independent check on M_w, and M_z via an analogous procedure [21]. Attempts to investigate an interaction without checking integrity are of limited use. Excellent modern instrumentation for both SEC-MALLs and analytical ultracentrifugation are readily

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available and centres like the National Centre for Macromolecular Hydrodynamics at the University of Nottingham provide a facility for researchers in laboratories without access to these.

Choice of mucin

Unfortunately mucin of the required purity from human alimentary tract is very difficult to obtain in any useful quantity. We therefore use pig gastric mucin as our model mucin system, although we also have managed to perform mucoadhesive assays on highly purified mucin from different regions of the human stomach as we will consider below. Unless otherwise stated, the mucin used is highly purified pig gastric mucin of $M_w \approx 10$ MDa, depending on the success of the purification procedure.

Analytical ultracentrifuge criteria for mucoadhesion

There are two principal approaches. We could assay for change in molecular mass using sedimentation equilibrium, but this has an upper limit of ≈50 MDa (unless we start turning the rotor by hand!). Since complexes can be very large, a more sensible assay procedure (using the same equipment) is to use sedimentation velocity (which can cope, as we have already noted, with complexes as large as 10^9 Da) with change in sedimentation coefficient, s, as our marker for mucoadhesion. If we so wish we can then convert this to a change in molecular mass if we assume a conformation ($s \approx M_w{}^b$ where b is 0.67, 0.15 or 0.4–0.5 for a sphere, rod or coil respectively). We choose though simply to use s directly as our size criterion (as used in, for example, ribosome size representations, 30 S, 50 S etc., or in seed globulins, the 7 S, 11 S soya bean globulins etc.). Where a mucin is available in only miniscule amounts (e.g. from different regions of the human stomach), we can use a special procedure known as *Sedimentation Fingerprinting* where we assay for its effect on the mucoadhesive.

Mucoadhesion involving a protein: the mussel glue protein *mefp1*

Before we look at polysaccharide mucoadhesion, as a simple illustration consider the mussel glue protein *mefp1*. This is an unusual protein of ≈110 kDa that consists of a globular region with a long protruding tail of alternating flexible and rigid regions [22]. This tail is rich in lysine – the molecule at neutral pH and below therefore behaves as a polycation. In free solution at 20°C *mefp1* sediments at ≈2.3 S, as shown by a sedimentation velocity experiment in an XL-A ultracentrifuge (using UV absorption optics as the detection system) at 40 000 rev./min at a protein concentration of 0.8 mg/ml. This is quite typical for a highly asymmetric protein of ≈110 kDa. If we repeat the experiment but with the protein in the presence of some highly purified mucin (at 0.1 mg/ml, too low to be picked up by the absorption optical system), the effect on the protein is spectacular, with the whole quantity of *mefp1* now sedimenting at ≈7000 S. Despite this impressive demonstration *mefp1* would be of limited practical use since protein-based mucoadhesives would be rapidly eaten away by the enzymes of the digestive tract. 7000 S also represents too strong an interaction with little opportunity for control. However, this provides the stepping stone for consideration of the use of polycationic polysaccharides that are not attacked by the digestive system and can be readily manipulated to control the extent of complexation.

Mucoadhesive experiments on polysaccharides

UV absorption optics are used as the optical decetion system. However, in this case the mucoadhesive is invisible (most polysaccharides do not absorb in the near-UV, ≈280 nm), but the pig gastric mucin at the concentrations normally employed is visible. The sedimentation ratio ($s_{complex}/s_{mucin}$), the ratio of the sedimentation coefficient of any complex involving the mucin to that of pure mucin itself, is used as the measure for mucoadhesion.

Mucoadhesion experiments involving guar, alginate, carboxymethyl cellulose, xanthan and DEAE-dextran

Experiments on a series of neutral and polyanionic polysaccharides were performed. No significant change in the sedimentation coefficient of the mucin was seen (sedimentation ratio, $s_{complex}/s_{mucin}$, ≈1), reinforcing the macroscopic observations using tensiometry that were considered above (see Table 1). The polycationic dextran derivative DEAE-dextran gave sedimentation ratios of 1.1–1.9 depending

Table 1 | **Sedimentation coefficient ratio ($s_{complex}/s_{mucin}$) as an index of adhesiveness**
Based on data from [16,17,23–26].

Mucoadhesive	$s_{complex}/s_{mucin}$	Conditions
DEAE-dextran	1.1–1.9*	pH 6.8, 20°C
	1.2–1.4*	pH 6.8, 37°C
Chitosan ($F_A \approx 0.11$)	48	pH 6.5, 20°C
	34	pH 6.5, 37°C
	15	pH 4.5, 20°C
	38	pH 4.5, 37°C
	22	pH 2.0, 20°C
	12	pH 2.0, 37°C
	26	pH 4.5, 20°C + 3 mM bile salt
	35	pH 4.5, 37°C + 3 mM bile salt
	18	pH 4.5, 20°C + 6 mM bile salt
	14	pH 4.5, 37°C + 6 mM bile salt
Chitosan ($F_A \approx 0.42$)	31	pH 4.5, 20°C
	44	pH 4.5, 37°C

*Depending on the mixing ratio.

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on the mixing ratio and temperature [17,23,24]. This is extremely modest considering the high charge density on the polymer with lots of potential sites for interaction with the fully deionized sialic acid groups on the mucin. This disappointment also reflects the disappointing result from the tensiometry analyses [18]. The $\alpha(1\rightarrow3)$ branches of the dextran appear to be responsible for considerable steric hindrance, preventing access to the charged mucin groups.

Mucoadhesion experiments involving chitosans

A contrasting picture is seen for chitosans. Chitosans are derivatives of chitin (after an alkali-extraction procedure) and hence are available in large quantities from the shells of crabs, lobsters and other crustaceans. Pure chitin is poly-*N*-acetyl glucosamine. The N-acetyl groups are de-acetylated in chitosan to an extent represented by the degree of acetylation F_A, with $F_A = 1$ being pure chitin and $F_A = 0$–0.6 representing the range of soluble chitosans. We stress here that chitosans are only readily soluble at pH values of 6.5 or less, and this factor has to be borne in mind in the formulation of any mucoadhesive product involving these substances. Interestingly, whereas mucins present two types of residue for potential mucoadhesive interaction (the charged acidic groups on sialic acid and any sulphonated residues, and the hydrophobic methyl groups on fucose residues) chitosans present similar opportunity (the charged NH_3^+ groups on deacetylated N-acetyl groups and also the hydrophobic acetyls on non-deacetylated residues). The results are quite spectacular [16,17,25,26]. A highly charged chitosan ('sea-cure' 210+) of $F_A \approx 0.11$ has impressive sedimentation ratios of 15–38 depending on the temperature. Interestingly for a lower-charged chitosan of $F_A \approx 0.42$, values of 31–44 were returned, reinforcing the view that both electrostatic and hydrophobic effects are important.

The demonstration of large-size interaction products by the analytical ultracentrifuge used in this manner is reinforced by images from the powerful imaging techniques of electron microscopy and atomic force microscopy. Conventional transmission electron microscopy clearly demonstrates large complexes of the order of ≈ 1 μm in size [27], and if we label the chitosan with gold we can see that the chitosan is distributed throughout the complex with 'hot spots' in the interior [28]. Images from atomic force microscopy, visualized in topographic and phase modes, again shows complexes of this size. Control experiments show a loose coiled structure for pig gastric mucin and a shorter, stiffer conformation for the chitosan, consistent with solution measurements [29].

Effect of environment on the extent of mucoadhesion

The beauty of the analytical ultracentrifuge is that since it is a pure solution technique with no columns or membranes it allows us to easily alter the surrounding solvent conditions. For example [17], if we vary the pH we see that the sedimentation ratio is $\approx$34–48 at pH $\approx$6.5 but is still significant at pH 2.0 ($s_{complex}/s_{mucin} \approx 12$–$22$; Table 1), well below the pK_a of the sialic groups on the mucin – which not only suggest the importance of the electrostatic contribution but again strongly indicate the existence of significant hydrophobic types of interaction. Attempts to investigate the effects of bile salts and differing ionic strengths down the alimentary tract also yield very much the same picture. At 0 mM bile salt we observe $s_{complex}/s_{mucin} \approx 18$–$21$, whereas at 6 mM the interaction is still significant, with $s_{complex}/s_{mucin} \approx 14$–$18$.

Sedimentation fingerprinting: assay of chitosan mucoadhesiveness on human mucins

We would dearly love to perform these types of experiments on human small intestinal mucin if we could only get them in sufficient quantities in purified form. We have, however, been successful in performing experiments on human mucin extracted from different parts of the stomach, namely the cardia, corpus and antrum regions. Although available in miniscule quantities we can assay mucoadhesiveness of chitosan on these by using a modification of the approach using the analytical ultracentrifuge described above, called sedimentation fingerprinting. In this method, introduced 4 years ago [30], we use the Schlieren optical system on the older Model E ultracentrifuge (unfortunately at the time of writing this optical system is not yet available on the commercial XL-A or XL-I analyticals). The Schlieren optical system gives the refractive index gradient (related to the concentration gradient) as a function of radial position in the ultracentrifuge cell. The area under a 'Schlieren peak' provides a measure of the sedimenting concentration. Although the mucins from human stomach are at too low a concentration to be detected we can assay for interaction from the loss of area under the chitosan peak caused by interaction. In this way it was possible to demonstrate significant differences between different regions of the stomach (Figure 1).

The alimentary tract versus the nose

Despite the clear demonstration of the mucoadhesivenss of chitosan we are still a long way off producing successful delivery formulations. Encapsulation systems involving chitosan have been attempted and there is a huge literature in pharmaceutical journals on this. An example is the use of tripolyphosphate to cross-link chitosan into a sphere [31]: if this is done in the presence of a drug, the drug can be encapsulated. Tripolyphosphate-linked chitosans have been shown to give good mucoadhesion [32]. The existence of a wide range of environmental conditions down the alimentary tract is still a tough task to deal with. To bypass the problem the nasal route offers a most attractive alternative. The benefits of mucoadhesion of chitosan can be employed

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Figure 1 | Comparative adhesiveness of a chitosan (sea cure 210+, $F_A \approx 0.11$) to mucins from different parts of the stomach, as assayed by Sedimentation Fingerprinting

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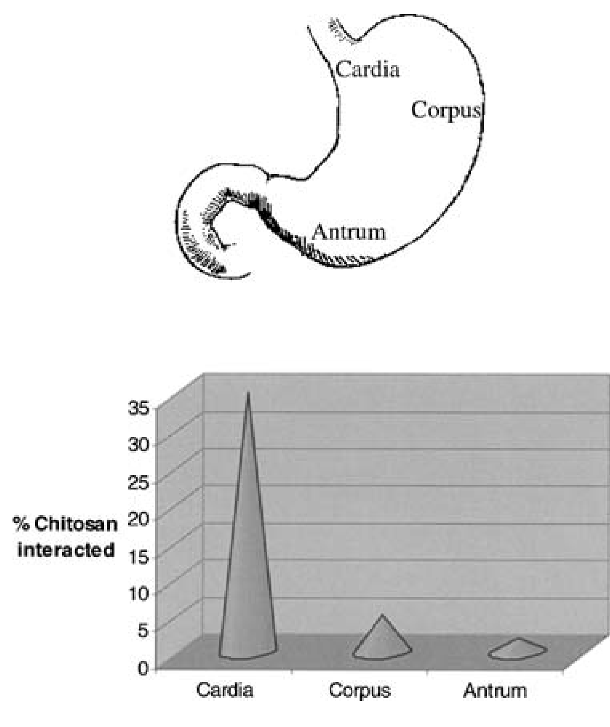

(without the need for a complicated encapsulation system) and chitosans offer the added benefit of apparently enhancing the absorption of drug through the surface epithelia. Illum [33] and her group have shown the mucoadhesiveness of chitosan solutions by demonstrating longer clearance times from the nose compared with normal saline. Chitosan powder formulations are even more impressive. Illum has also shown enhanced delivery of insulin from chitosan solutions and especially chitosan powder formulations [33].

Current work

This is focused on the generation of stable encapsulation (oral delivery) and nasal delivery forms [34]. I have already alluded to the problems through the oral route. Although the nasal route using chitosan is extremely attractive, there are some problems here. The production of acceptable delivery systems requires a thorough and rigorous investigation of the stability of chitosan-based systems. Chitosans are only readily soluble below pH 6.5. Derivatization can render them soluble at higher pH values, but are they still viable? Will a chitosan formulation remain stable on the shelf of a pharmacist's shop in tropical conditions or will it need to be kept in a refrigerator? Here use of viscometry is proving a particularly valuable probe. Another barrier that hasn't been crossed at the time of writing is that chitosans are still not approved by the US Food and Drug Administration, even though they are used in dietary products. It is hoped that our increased understanding of the fundamental behaviour of these substances in free solution and in mucoadhesive situations will accelerate this approval.

This outline review has focused on the biophysical principles and molecular interactions underpinning mucoadhesion. A fuller review of the pharmaceutical aspects can be found in [17].

The work described in this outline review is the culmination of a collaboration with a large group of researchers: principally Professor S.S. (Bob) Davis and Professor Lisbeth Illum together with Dr Clive Roberts, Dr Conny Jumel and Professor Arthur Rowe of the University of Nottingham, Professor Adrian Allen at the University of Newcastle, Professor Ingemar Carlstedt at the University of Lund, Professor Bjorn T. Stokke at the University of Trondheim, Dr Alan Smith of West Pharmaceuticals, and the following University of Nottingham PhD students: Morag Anderson, Immo Fiebrig, Matt Deacon and Monica Fee. The support of Hoechst Pharmaceuticals, Optokem Ltd. and presently the BBSRC and West Pharmaceutical Services during this period is warmly appreciated.

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