

3

Biopolymer Mucoadhesives

STEPHEN E. HARDING^{1*}, S.S. (BOB) DAVIS^{2,3}, MATTHEW P. DEACON^{1,2}
AND IMMO FIEBRIG^{1,2}

¹NCMH unit, University of Nottingham, School of Biological Sciences, Sutton Bonington, Leics., LE12 5RD, UK; ²School of Pharmacy, University of Nottingham, University Park, Nottingham, NG7 2RD, UK and ³DanBiosyst (UK) Ltd., Albert Einstein Centre, Highfields Science Park, Nottingham

Introduction

In this review we will consider how we can utilize the adhesive properties of certain types of biopolymer to increase the residence time of orally administered drugs as they pass through the stomach and small intestine. This utilization helps maximise the time window a drug spends near its site of optimum absorption. The review updates and builds on our earlier work (Fiebrig *et al.*, 1995a) which focussed more on the macroscopic aspects, and where an extensive table (Table 18.3) reviewed mucoadhesive performance; the reader is strongly recommended to cross-refer to that article. The current article will attempt to focus more on the molecular aspects of biopolymers interacting with mucus and its key macromolecular component – mucin. We will:

- Consider the principles of mucoadhesion and the strategy for the oral administration of drugs
- Consider absorption enhancement and the strategies used to delay gastrointestinal transit
- Look closely at the mucin substrate – on which there has been massive progress in our understanding of the molecular nature of this substance – and potential adhesive materials, particularly two types of polycationic polysaccharide and an unusual protein from the feet of mussels
- Consider the assay method: the ‘macroscopic’ mechanical/*in vivo* methods and the molecular mucin based methods
- Consider a ‘Case’ study on the molecular hydrodynamics in some detail: namely mucoadhesive interactions with gastric mucin
- Conclude on the most likely candidate mucoadhesive(s) and how they could be constructed into delivery systems.

*To whom correspondence may be addressed.

Bio-adhesion and mucoadhesion

'Adhesion' is defined by the physicist as 'the molecular force of attraction in the area of contact between unlike bodies that acts to hold them together' (Webster, 1989) and 'bioadhesion' is simply those adhesive phenomena where at least one of the adherents is biological (Kaelbe and Moacanin, 1977). The materials are attached to each other by interfacial forces for an 'extended' period of time (Gu, Robinson and Leung, 1988; Duchêne, Touchard and Peppas, 1988). Bioadhesive systems have been used for many years in the area of dentistry as denture adhesives (Wright, 1981; Hollingsbee and Timmins, 1990) with stoma based adhesives – such as karaya gum in Stomahesive® or synthetic polypectins (Winkler, 1986) – and also used for surgical applications such as the cyanoacrylates used as a 'surgical glue' (Wang, 1974, Harper and Ralston, 1983). Closely related to adhesion are film forming technologies such as the use of hydroxypropylmethyl cellulose for contact lens technology (see Silver *et al.*, 1994). Bacteria use polysaccharide adhesins to stick to surfaces, including the human gut. These adhesins are lectin-like, carbohydrate-binding molecules, expressed on the bacterial surface which bind specifically to sugar residues of mucins or other carbohydrates of the host cell surface (Beachey, 1980; Boedecker, 1984; Mergenhagen and Rosan, 1985; Hörstedt *et al.*, 1989). The term 'bioadhesion' has also been used to describe adhesive phenomena related to the ability of some non-biological macromolecules and hydrocolloids to adhere to biological tissues for therapeutic purposes in medicine (Kaelbe and Moacanin, 1977; Peppas and Buri, 1985).

'Mucoadhesion' is a term used for a bioadhesive phenomenon where the biological substrate is a mucosal surface (Robinson, 1990), and concerns how advantage can be made for therapeutic purposes, especially for the delivery of drug formulations taken through the mouth (Fiebrig *et al.*, 1995a) and delivered to the eye (see Saettone *et al.*, 1989).

Oral administration of drugs

The local treatment of diseases can be unsatisfactory, because the drug may not stay at the site of action long enough for the desired effect (typically eye, mouth or vaginal cavity). Similarly systemic treatment via the oral route can be hampered because the drug may not stay at the optimal site of absorption long enough. Despite the advances in alternative delivery systems (e.g., nasal, pulmonary), oral administration of drugs is the most popular route of administration for both medical profession and patients alike. The administration of dosage forms via the mouth can easily be undertaken and is generally safe. By contrast, invasive methods (e.g. injection) usually require the assistance of trained personnel and the procedure always involves certain risks.

Oral drug administration begins with ingestion of the dosage form through the mouth (*Figure 1*) and from there it passes down the oesophagus and into the stomach. Little drug can be absorbed from the stomach, largely because of its relatively small surface area, particularly in the case of cationic drugs which will be mainly ionised in the acidic conditions of the stomach. Nevertheless, the stomach may represent a site for local treatment. The major site of drug absorption is the small intestine. Its large surface area (~100 m² in a healthy adult) makes it very efficient for the uptake of solutes (Bowman and Rand, 1980). Theoretically, drug absorption can occur along the

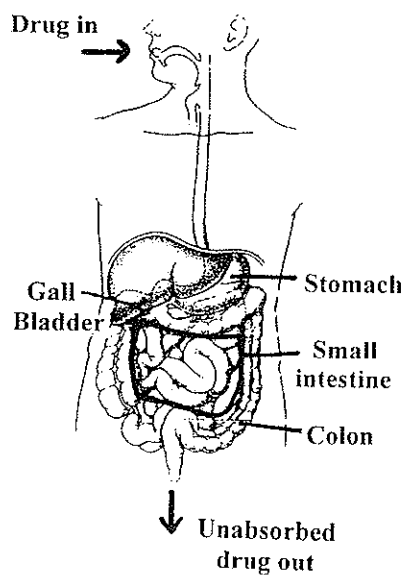


Figure 1. Drug administration through the gastrointestinal tract.

entire length of the small intestine, however the majority of drugs are actually absorbed from the proximal small intestine (Booth, 1967). 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 (Davis, 1989). The limited surface area of the large intestine can be compensated by a long transit time. More recent studies with a once-a-day preparation (e.g. theophylline) have shown that therapeutic drug levels can be maintained for periods of up to 24 h (Gruber *et al.*, 1987) even though these systems are expected to have emptied from a fasted stomach, passed through the small intestine and have arrived at the ileocaecal junction after 4 h (Davis, 1985). Oral drug delivery ceases eventually with the faecal excretion of any unabsorbed drug.

Absorption enhancement in oral drug delivery systems

Too rapid a transit, or low residence time in the stomach and small intestine is just one of three major problems in oral delivery. Low appearance of the drug in the systemic circulation (bioavailability) can be due to 1. rapid transit of the drug-containing delivery system past the ideal absorption site, 2. rapid degradation of the drug in the gastrointestinal tract once it has been released (this can be serious for peptide drugs) and, 3. low transmucosal permeability of the drug due to its size, ionisation, solubility or other characteristics of the drug molecule. Although this review concerns itself with addressing item 1., it turns out that the one of the best potential mucoadhesive materials (the polycationic polysaccharide *chitosan*) can also assist with solving the permeability problem, 3.

A satisfactory resolution of problem 1. will also be beneficial for polar drugs such as hydrochlorothiazide, which are only poorly absorbed from selected regions of the small intestine and whose bioavailability is believed to be dependent on the residence

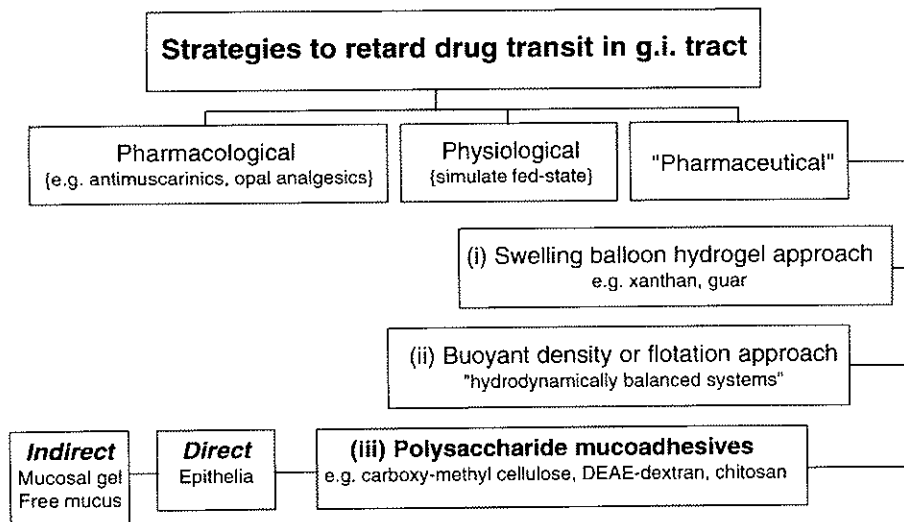


Figure 2. Strategies for retarding gastrointestinal drug transit.

time of the dosage form at or *upstream* of its small intestinal absorption window (Beermann *et al.*, 1976; Lynch *et al.*, 1987). Resolution of problem 1. will be particularly important in the case of controlled release drug delivery systems (DDS), designed to deliver drugs over extended periods of time (e.g. 12–24 h). Once in the colon, these DDS may be delivering a proportion of drug to a non-optimal site for absorption (Davis, 1985). An ideal oral sustained release dosage form should be comparable to an intravenous infusion, which continuously supplies the amount of drug needed to maintain constant plasma levels once a steady state is reached (Förster and Lippold, 1982)

Strategies for retarding drug transit through the gastrointestinal tract

The strategies for delaying drug transit through the gastrointestinal (g.i.) tract fall into one of three categories (*Figure 2*): *pharmacological*, *physiological* and *pharmaceutical*; the first two being less attractive than the last because of toxicity problems. (For another recent review of retentive systems see Hwang *et al.* (1998)). A pharmacological approach involves the co-administration or incorporation of a drug into the dosage form. This drug delays gastrointestinal emptying. Examples include antimuscarinics, e.g. propantheline, which are relaxants of the smooth muscle (Beermann and Groschinsky-Grind, 1978; Manninen *et al.*, 1973) or a drug that changes motility, e.g. opiate analgesics or derivatives such as loperamide (Minami and McCallum, 1984). However, the potential side effects that may arise from such treatments on a routine basis would not be acceptable for regulatory approval.

A physiological approach is the use of natural materials or fat derivatives such as triethanolamine myristate (Gröning and Heun, 1984, 1989), which stimulate the duodenal or jejunal receptors to slow gastric emptying. The use of large amounts of a 'volume filling' polymer such as polycarbophil (Harris *et al.*, 1990a,b) can also cause a 'slowing down' response in terms of gastrointestinal transit.

Pharmacological and physiological approaches thus set out to delay gastrointestinal

transit by modification of the rate of gastric emptying using 'passage-delaying agents'. By contrast, the *pharmaceutical strategies* attempt to achieve the same objective by actually retaining the dosage form at or upstream of its absorption site for as long as possible. This is achieved by a particular physical or physicochemical characteristic. Mucoadhesion is one strategy (*Figure 2*).

(i) *Swelling balloon hydrogel.* If large enough, the formulation will not be expelled from the fasted stomach even when the pyloric sphincter is in its non-contracted state. The size of such systems has to increase after ingestion to an extent that gastric emptying is totally inhibited (Moës, 1993). The size-related retention of a dosage form in the stomach has been studied with various systems to include systems such as swelling balloon hydrogels (Park and Park, 1987) or unfolding stratified medicated polymer sheets (BE Patent No. 867, 692) or non-erodible or erodible tetrahedron shaped devices (Cargill *et al.*, 1988, 1989). These have never passed beyond the experimental stage and clinical data are unavailable. In any case these gastric retention devices may not be safe. The hazard of lodging in the oesophagus (Kikendall *et al.*, 1983; Al-Dujaili *et al.*, 1983; Wilson, 1990) or permanent retention in the stomach with cumulative effects (Brahams, 1984; Vere, 1984) could lead to life-threatening problems.

Another approach uses dosage forms of moderately high density, based on the premise that high density formulations remain in the stomach longer than conventional formulations, since they would be localised in the lower part of the antrum provided the density exceeds that of the normal stomach contents, i.e. > 1.4 g/ml (Bechgaard and Ladefoged, 1978). The effectiveness of this approach has not been confirmed on a broad basis and the evidence remains controversial (Moës, 1993).

(ii) *Buoyant density/flotation approach.* This approach uses buoyant dosage forms which float on the gastric contents as a result of their relatively low density. Floating dosage forms have been discussed extensively by Moës (1993): The first floating dosage forms (F forms) (Sheth and Tossounian, 1984), also called 'hydrodynamically balanced systems' (HBS), were able to maintain their low density while a polymer hydrated and built a gelled barrier at the outer surface. Hoffmann-LaRoche produced patents for floating drug delivery systems and *in vivo* studies on diazepam HBS capsules such as Valium® CR and Valrelease® and the L-dopa plus benserazide containing formulation Madopar® HBS (Prolopa® HBS). Moës (1993) has attempted to clarify the conflicting views on the gastric retention capabilities of floating systems resulting from a number of *in vivo* trials by different authors (Müller-Lissner and Blum, 1981; Davis *et al.*, 1986; Timmermans and Moës, 1990; Timmermans, 1991; Kaus, 1987; Sangekar *et al.*, 1987; Lippold and Günther, 1991).

(iii) *Polymer mucoadhesion.* This involves attachment or encapsulation of the drug with a polymer which interacts with either the mucosal epithelia/glycocalyx lining of the gastrointestinal tract (this is called 'direct' mucoadhesion) or mucous surfaces (the gel and the sloughed mucus in the lumen) lining the gastrointestinal tract, hence providing a macromolecular 'brake' to the movement of the drug. A good challenge for mucoadhesion is the delivery of orally administered polar drugs (and possibly peptides and proteins). These materials have low absorption characteristics (and for peptides and proteins have a stability problem; enzymatic degradation and biotrans-

formation). A mucoadhesive alternative route to parenteral administration would be highly desirable (Junginger and Verhoef, 1992).

DIRECT VERSUS INDIRECT MUCOADHESION

If the polymer carrier can access and interact *directly* with the surface mucosal epithelium or *glycocalyx*, the decrease in diffusion path from the oral DDS to the absorbing biological membrane could be an additional advantage for improving absorption, particularly in intestinal delivery of peptide drugs, at the same time minimizing dilution and possible degradation in the luminal fluids (Hayton, 1980). The further addition of penetration enhancers to an adhering dosage form could enable alteration of membrane permeability and inclusion of specific enzyme inhibitors could prevent early degradation of the peptide (Wearly, 1991; Junginger and Verhoef, 1992) and consequently increase bioavailability. However, the epithelium may not be accessible: instead the *indirect route* of interaction with the ~40–450 µm thick mucosal surface/gel lining the gastrointestinal tract provides the most likely strategy (*Figure 3*). It is also worth noting that mucus is not a major barrier to absorption.

The adhesion of gastrointestinal retention dosage forms to the mucosa has been studied for over a decade, mainly by *in vitro* or *ex vivo* test with few *in situ* or *in vivo* studies and even fewer trials in man. Despite the fact that bioadhesion, or more specifically mucoadhesion, has led to some success in drug delivery for ocular, buccal, nasal, vaginal and cervical applications (Chen and Cyr, 1970; Schor *et al.*, 1983; Nagai *et al.*, 1984; Nagai, 1986; Duchêne *et al.*, 1988; Greaves and Wilson, 1993; Smart, 1993; Bouckaert, *et al.*, 1994), gastrointestinal mucoadhesive drug delivery systems have yet to be successfully established (see, e.g., Helliwell, 1993; Fiebrig *et al.*, 1995a).

The mucosal substrate: mucus and mucin

The last ten years has also seen a tremendous advance in our understanding of the structure and molecular biology of mucus, and in particular its major macromolecular component, *mucin*. Mucus is a viscoelastic substance with a characteristic stickiness and *Spinnbarkeit* (the ability to stretch into strands). By weight mucus is mostly water (95%–99.5%) and exists in gel or a viscous solution form. Its most important polymeric, gel-forming component is the mucus glycoprotein or mucin (0.5%–5%) (Harding, 1989; Carlstedt and Sheehan, 1988; Neutra and Forstner, 1987; Gibbons, 1972). The adherent mucus layer in the gastrointestinal tract is secreted by specialised cells. They are surface epithelial cells found mostly in the stomach but also in other parts of the gut and the goblet cells of the small and large intestine, as well as Brunner's glands in the duodenum (Neutra and Forstner, 1987; Allen, 1989; Ito, 1981). Unlike other gastrointestinal secretions, mucus adheres to the mucosal epithelial surfaces as a water insoluble gel (*Figure 3a*) until degradation and erosion takes place (Allen, 1989) leaving a mucin solution or slough on the lumen side of the gel.

For monitoring the thickness of the mucin a novel method has been developed which for the first time has enabled the preservation and visualization of the full thickness of the adherent gastric mucus layer and the underlying mucosa (Jordan *et al.*, 1998): this involves a modified periodic acid Schiff/Alcian Blue staining technique for

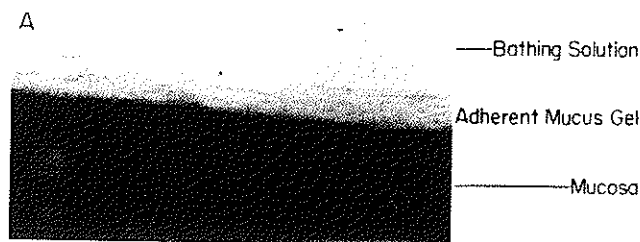


Figure 3a. Adherent gastric mucus viewed under bright field microscopy on a transverse section (1.6 mm thick) of rat gastric mucosa. Three distinct phases are seen: mucosa, mucus gel layer and the 'bathing' solution of free mucus. Reprinted with permission from Allen (1989) and Kerse *et al.* (1982).

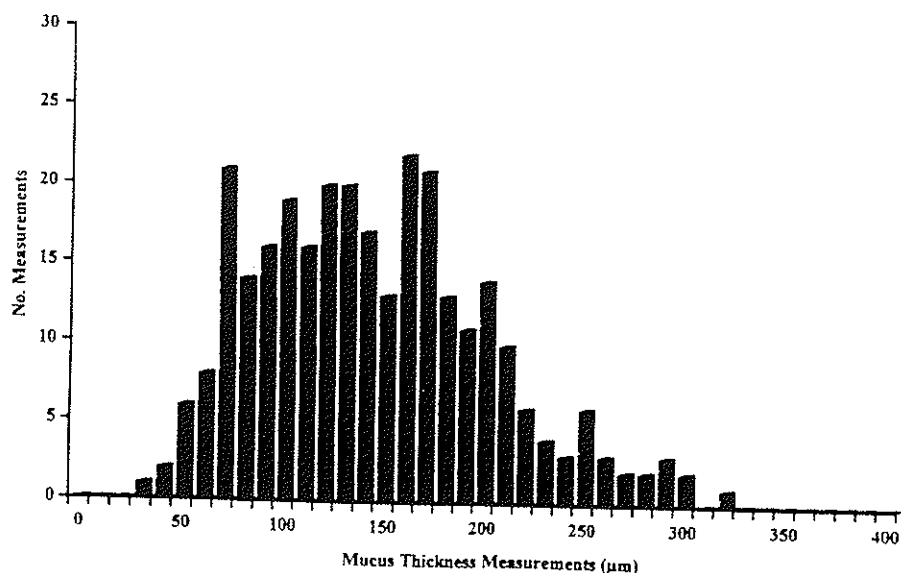


Figure 3b. Histogram of the mucus thickness from the antrum region of human stomachs. Overall mean (from 300 measurements) = 144 μm , standard deviation = 29 μm and the median = 160 μm . The Schiff/Alcian Blue staining technique on cryostat sections of gastric mucosa was used. Reprinted with permission from Jordan *et al.* (1998).

use on cryostat sections of gastric mucosa, and an example of the distribution of mucus layer thicknesses obtained for human gastric antrum mucus is shown in *Figure 3b*.

It is believed that the adherent mucus layer plays a major role in protection of the delicate underlying epithelium against the various endogenous and exogenous insults, such as acidic pH (providing an 'unstirred boundary layer'), digestive enzymes (pepsin), pathogens (bacteria) and abrasion, while the soluble mucus may play an important role in acting as a lubricant for ingested food. The requirement for such a protective adherent gel layer becomes obvious since from a physiological point of view the luminal side of the gastrointestinal tract can still be considered as the outer side of the body. These and other aspects regarding the function of mucus have been

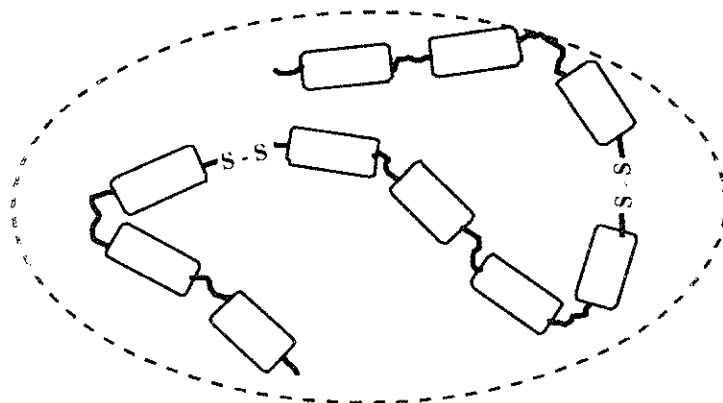


Figure 3c. Structural hierarchy of a mucin from the g.i. tract: a colonic mucin of the MUC2 type (from Jumel *et al.*, 1997). The general structural scheme of mucins from a variety of sources is similar. See text.

extensively described by various authors e.g. Allen (1981, 1983, 1989), Silberberg and Meyer (1982) and Bhaskar *et al.* (1992). Chemical analysis of the mucus gives evidence of a rather heterogeneous material which also contains small amounts of a variety of proteins, lipids, bacteria, sloughed-off epithelial cells and in some cases nucleic acids (Creeth, 1978). It becomes clear that mucoadhesion is a process that involves large amounts of water, or more vividly, it could be seen as 'adhesion to water in a semisolid form' where the mucins play a key role in maintaining the gel-like properties of the substrate for a potential drug delivery platform. The mucins themselves display considerable heterogeneity that has been well described (e.g. Carlstedt and Sheehan, 1984; Neutra and Forstner, 1987; Allen, 1989; Sheehan and Carlstedt, 1989; Harding, 1984, 1989).

Mucin

Mucins are large molecules with molecular weights ranging from 0.5×10^6 to over 20×10^6 g/mol. They contain large amounts of carbohydrate (for gastrointestinal mucins 70%–80% carbohydrate, 12%–25% protein and up to ~5% ester sulphate). Undegraded mucins are made up of multiples of a basic unit ($M \sim 400,000$ – $500,000$), linked together into the macroscopic mucin. Although originally thought to be arranged in a windmill type of structure (Allen, 1978), this model was shown to be incorrect: Instead the molecule is linked into linear arrays as shown by Creeth, Harding and coworkers (Harding *et al.*, 1983a,b) and by Carlstedt, Sheehan and coworkers (Carlstedt and Sheehan, 1984). Although linear, the mucin molecule in solution is loosely/randomly coiled into a spheroidal, highly swollen (by water imbibement) domain as confirmed by molecular hydrodynamics. Examples from electron microscopy clearly showing both these features are presented in *Figure 4: Figure 4a*, the linear 'secondary structure' and *Figure 4b* the overall spheroidal domain. The total architecture seems to be very similar for mucins from a variety of

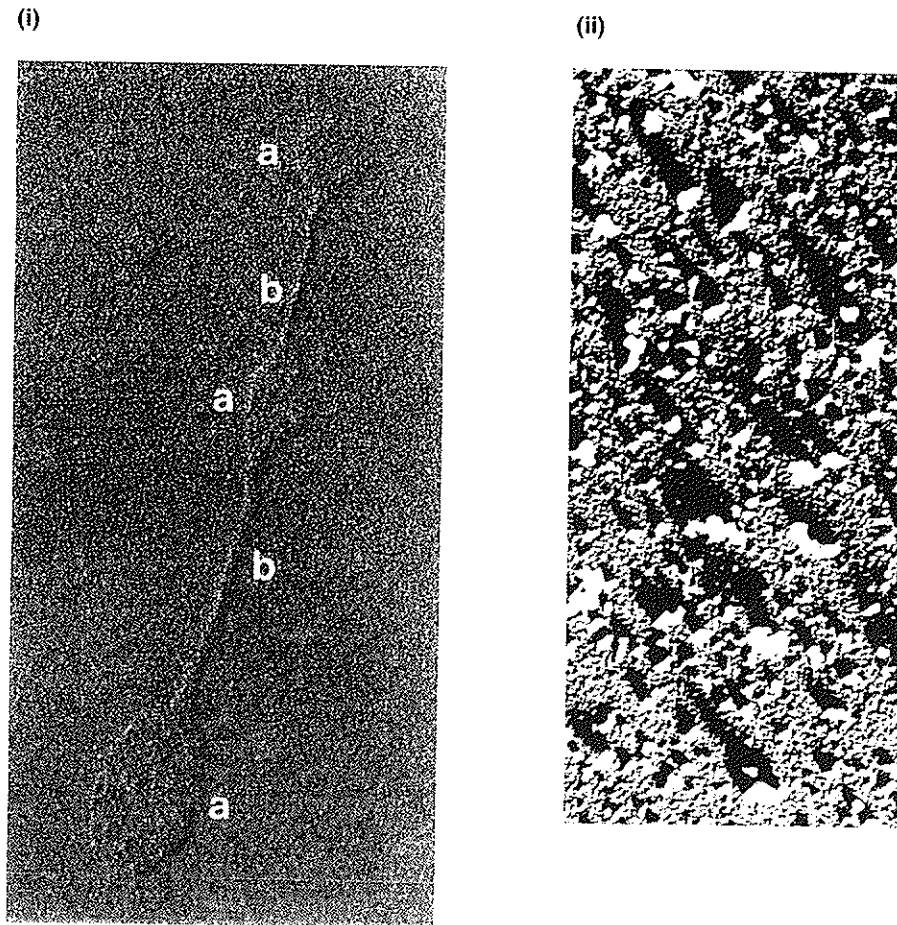


Figure 4. Electron microscopy of a bronchial mucins of the 'single subunit' type (molecular weight ($\sim 2\text{--}2.5 \times 10^6 \text{Da}$) prepared by air drying onto mica (i) or critical point drying (ii) and then linear shadowing with platinum. With (a) the mucins have been strung out by the large shearing and surface tension forces experienced during air drying onto mica, leaving clear flattened (a) and linear (b) regions visible. The flattened regions are likely regions of high glycosylation (Harding *et al.*, 1983). (i) and images similar to these clearly demonstrate a linear assembly for mucin molecules and not 'windmill' or 'star-like' structures as had been proposed earlier. With (ii) the critical point drying method has preserved the overall spheroidal domain (dark spherical regions) occupied by the randomly coiled molecule in solution (Hallett *et al.*, 1984). Magnification: (i) $\times 161000$, (ii) $\times 75000$.

sources (for example gastric, respiratory or cervical). The basic units are linked together by regions of low or no glycosylation (*Figure 4*) which are subject to trypsin digestion: the $\sim 400\text{--}500 \text{kDa}$ digestion products are thus commonly referred to as 'T-domains' (see Sheehan and Carlstedt, 1989). Every third or fourth T-domain is linked by a disulphide bridge; itself susceptible to reductive disruption by thiols. The thiol reduction products (of molecular weight 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 (*Figure 3c*) (Jumel *et al.*, 1997). 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 (mol. wt < 5 MDa) (Dodd *et al.*, 1998). 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 adhesion strategies.

PRIMARY STRUCTURE OF MUCINS

These advances in our understanding of the gross structure of mucins have been matched by similar advances that have occurred in the last ten years in our understanding of the primary structure of mucins. Although direct sequencing of the protein chain has been virtually impossible because of the insolubility of mucins stripped of their carbohydrate, eight different genes coding for mucin production have now been sequenced (see e.g., Hounsell *et al.*, 1997 and references therein). These are called 'MUC' genes and the ones known to date and the sources of mucin they code for are given in *Table 1*. It is seen that the key gene products as far as mucoadhesion are concerned are MUC2 and MUC3 in the small intestine and colon, and MUC5AC, MUC5B and MUC6 from the stomach.

The protein sequences emerging from elucidating these genes confirm the presence of large amounts of serine and threonine, sites for the O-glycosylation, and also the large amounts of proline – which has been known for years (Harding *et al.*, 1983a,b) to be assisting with the coiling of the mucin molecule. This knowledge of the genes has also revealed the concept of a tandem repeat of sequences of amino acid throughout the linear polypeptide backbone. What is also interesting has been the identification that certain of the selectin group of cell surface adhesion molecules have mucin-like repeat sequences (such as CD43) (Hounsell *et al.*, 1997).

The O-linked carbohydrate chains may contain up to five different monosaccharides; namely D-galactose, L-fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid (*Figure 5*). As multi-branched oligosaccharides they are covalently attached via O-glycosidic linkages from N-acetylgalactosamine to serine and threonine residues of the protein core. The absence of uronic acid and only trace amounts of mannose (<1%) distinguish mucin glycoproteins from the proteoglycans of connective tissue and serum glycoproteins, respectively. Sialic acid residues, which belong to a family of acidic sugars (Schauer, 1992) (in gastrointestinal mucins usually either N-acetyl or

Table 1. Characterized MUC genes (from Hounsell *et al.*, 1997)

<i>MUC</i> gene	Location
<i>MUC 1</i>	Breast and colon cell surface episialin
<i>MUC 2</i>	Colon and small intestine goblet cell secretion
<i>MUC 3</i>	Intestinal tissue
<i>MUC 4</i>	Tracheobronchial tract
<i>MUC 5AC</i>	Respiratory tract and goblet cell secretion
<i>MUC 5B</i>	Submaxillary gland secretion
<i>MUC 6</i>	Gastric gland secretion
<i>MUC 7</i>	Salivary gland secretion
<i>MUC 8</i>	Respiratory tract

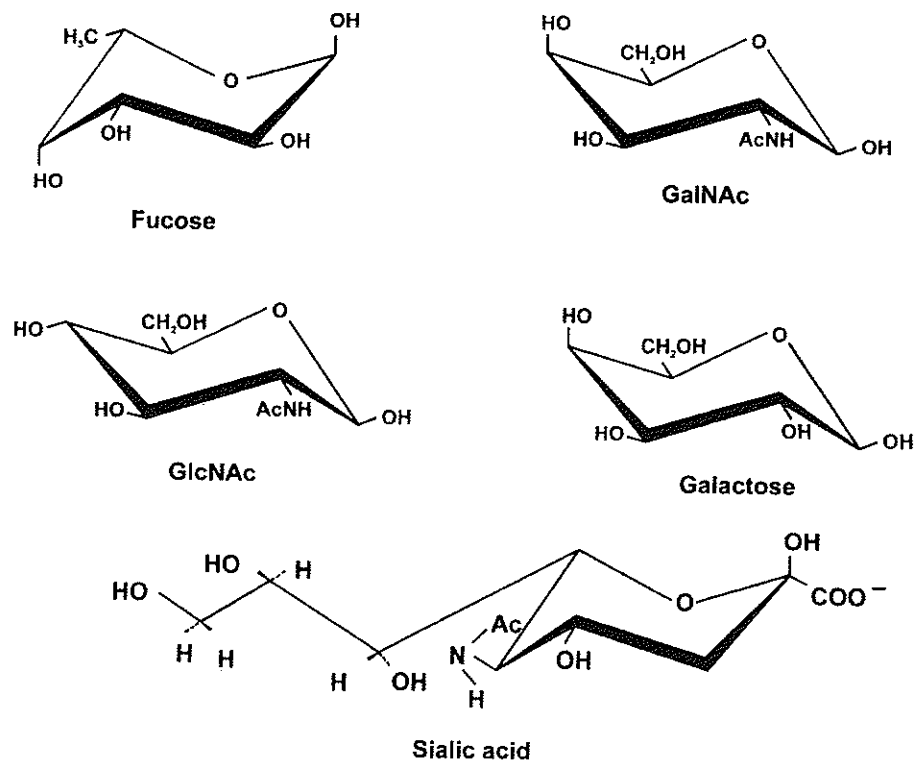


Figure 5. The principle sugars of gastrointestinal (also bronchial and cervical) mucins. The key ones, in terms of possible interaction sites for mucoadhesives are sialic acid ($-\text{COO}^-$ group for electrostatic interaction), N-acetyl glucosamine and N-acetyl galactosamine ($-\text{COCH}_3$ group, with the carbonyl for H-bonding and the hydrophobic methyl residue, and fucose ($-\text{CH}_3$ group).

N-glycolyl-neuraminic acid), are usually in a terminal position on the carbohydrate chain, whereas ester sulphate residues occur in a more internal position, e.g. as N-acetylglucosamine-6-sulphate in pig gastric mucus (Allen, 1978; Slomiany and Meyer, 1972). They both contribute in giving the molecule a net negative charge, thought to be of importance in interactions with polycationic materials (Lehr *et al.*, 1992b; Fiebrig *et al.*, 1994a, b). Other potential residues for mucoadhesive interaction are the carbonyl (hydrogen bonding) and methyl (hydrophobic bonding) groups on the N-acetyl residues (GalNAc, GlcNAc, sialic acid) and another methyl group in fucose.

Is mucus an appropriate target?

There are three physiological aspects which remain critical for the concept of gastrointestinal mucoadhesion: (i) turnover of the adherent mucus layer, (ii) interactions of the formulation with soluble, i.e. non-adherent mucus prior to adhesion and (iii) gastrointestinal motility.

TURNOVER OF THE ADHERENT MUCUS LAYER

Adherent mucus is continuously lost into the gastrointestinal lumen by proteolysis and mechanical sloughing (e.g. Allen, 1981; Allen and Carroll, 1985). The latter, caused by the ingested food and its digestion, is thought to be the major cause of the loss of gastric mucin (Waldron-Edward, 1977). A dynamic balance exists at the mucosal surface *in vivo*, between mucus secretion and mucus erosion. Mucus erosion, either by pepsin or by abrasion, must be replenished by the mucosal secretion of new material in order to maintain a protective function (Allen *et al.*, 1993). The difficulties in measuring mucus secretions *in vivo* have been outlined by Allen (1989). Studies on the turnover time of intestinal mucus gel layer in the rat *in situ* loop (Poelma and Tukker, 1987) by Lehr *et al.* (1991) have attempted to shed some light on the limitations to gastrointestinal mucoadhesion. The view of these authors is that the maximal residence time of a bioadhesive DDS at the site of adhesion would be limited by the time it takes for the mucus gel layer to be renewed as determined by the steady state of synthesis, secretion and degradation of the mucins (Allen, 1981). Although their estimate for the mucus turnover time is relatively crude (47–270 min), it is interesting to find that this time scale is similar to the mean residence time found for mucoadhesive microspheres (94 ± 18 min) in earlier experiments using the same animal model (rat). Furthermore it has been observed that stimulating the mucus output, by perfusion with 10 mM sodium taurocholate, led to a significant shortening of the mean residence time of microspheres. Of even greater interest is the observation that the microspheres did not become detached from dead mucosal tissue *in vitro* when the system was stirred for more than 18 h. This leads to a further consideration; that of choosing an appropriate model system. This will be discussed in more detail below. Although mucus turnover in an *in situ* isolated gut loop in the rat (which has undergone surgery and has been removed from its normal function) may be different from mucus turnover in healthy humans or patients, this physiological factor will limit potential adhesion to the adherent mucus in the gastrointestinal tract.

COMPETITIVE INHIBITORY INTERACTIONS WITH SOLUBLE MUCIN

Any formulation entering the gastrointestinal tract interacting with the mucus gel is likely also to interact with soluble mucins of the 'slough' or luminal material. This is an unavoidable complication which will reduce the efficiency of any adhesive system: that is any adhesive system targetted for groups on the mucus gel will also have the possibility to interact with the soluble mucus. Even if the epithelial cells are targetted, a 'competitive inhibition' for the mucoadhesive will recur as has been shown recently by Lehr *et al.* (1992d): Those authors used tomato lectin, a material that specifically binds to isolated pig enterocytes and monolayers of human Caco-2 cell cultures, and was proposed as a favourable candidate for specific bioadhesion to epithelial cells of the gastrointestinal tract. However, binding also occurred with crude pig gastric mucus. Thus, no mucoadhesive strategy can be 100% efficient! Other competitive inhibitors for mucoadhesion may also derive from other soluble components within the gastrointestinal tract, such as bile salts (Anderson, 1991).

GASTROINTESTINAL MOTILITY

Gastrointestinal motility patterns and in particular the so called 'housekeeper wave'

which involves strong gastrointestinal contractions, serves as a cleaning mechanism to clear all indigestible materials, including non-disintegrating dosage forms, from the stomach or proximal intestine (Code and Marlett, 1975; Grundy, 1985; Leung and Robinson, 1988). Thus, a good oral mucoadhesive drug delivery system also needs to resist the cleaning action of the 'housekeeper wave' and remain in the stomach or proximal small intestine.

TARGET FOR MUCOADHESIVES

Although the most appropriate target phase that would appear to give the best efficiency for a mucoadhesive system (if it were accessible) is the underlying mucosal glycocalyx, the target phase (in the stomach, small intestine and colon) most relevant to the concept of mucoadhesion is the water insoluble mucus gel lining the mucosa of the gastrointestinal tract. This mucus layer has a variable thickness, 50–450 μm , in man and about half that in the rat (Allen, 1978; Kerss *et al.*, 1982), with regional differences: For example (A. Allen, personal communication) mucus thickness in the stomach is variable but between a mean of 100–150 μm for the firm layer of adherent gel and another 100 μm of viscous mobile mucus on top of that under unstirred conditions. In the colon the adherent gel has a mean thickness $\sim 65 \mu\text{m}$ with something in the region of another 700 μm mobile viscous mucus that can be removed by suction. An important point is that in both cases the adherent gel barrier is continuous.

A variety of groups on the sugar residues on mucins provide potential sites for interaction of either an electrostatic, hydrogen bond or hydrophobic nature. The next question is: which is the appropriate mucoadhesive?

The mucoadhesive

The most important requirement of a mucoadhesive is that it must be *non-toxic* with no undesirable physiological or pharmacological actions, and should *not be expensive*. To this end, biopolymers, and in particular *food grade polysaccharides* are particularly attractive candidates (see Tombs and Harding, 1998). Other important criteria are that the mucoadhesive should have *good wettability* (and *spreading ability*) and high drug loading and a suitable unloading capacity. The following molecular properties are important considerations: charge, hydrogen-bonding, hydrophobicity, flexibility (ability to overcome steric hindrance problems) and molecular weight/molecular weight distribution. The following molecular environmental factors are important: solubility, pH, ionic strength, presence of other salts (e.g. bile) and other macromolecules (antibodies, enzymes, polysaccharide etc.).

For bioadhesion to occur, an intimate contact between the adhesive and the substrate (mucus) is a prerequisite. Factors like good wettability as well as hydration are important (Huntsberger, 1967; Chen and Cyr, 1970; Peppas and Buri, 1985). During the establishment of the adhesive bond the total surface energy between the two materials is diminished, eliminating two free surfaces and creating a new interface. This first step is believed to be followed by physical or mechanical bond formation obtained by deposition and inclusion of the adhesive material in the crevices of the mucus and chain entanglement between polymer chains of both phases (also referred to as inter-diffusion) (Boddé, 1990; Jabbari *et al.*, 1993). Lehr *et al.* (1992c) have used electron microscopy in an attempt to visualize intermixing between a

polyacrylic acid derivative (polycarbophil) and mucus. They were unable to observe intermixing in the micron range but did not exclude this phenomenon for the nanometre range. Sufficient chain flexibility is required to form secondary chemical bonds such as van der Waals forces as well as hydrogen bonding (Leung and Robinson, 1988; Duchêne *et al.*, 1988). The formation of primary (covalent) chemical bonds is important in hard tissue adhesion in orthopaedics and dentistry. However, for mucoadhesion, chemical reactions of this type have not been considered so far, since a long term attachment is not required (Peppas and Buri, 1985).

POLYANIONIC AND NEUTRAL POLYMERS

Polymers with hydroxyl or carboxyl groups on their surface had been earlier claimed as the most desirable candidates for bioadhesion, rather than polymers with other functional groups or cationic moieties (Peppas and Buri, 1985). The synthetic polyacrylic acid derivatives known as polycarbophils (Carbopol® EX-55) and carbomer (Carbopol® 934) have to date been by far the most studied mucoadhesive polymers (Table 18.3 of Fiebrig *et al.*, 1995a). Both materials are polyanionic and interaction with mucus has largely been attributed to chain entanglement of the polymer chains with mucin as a result of swelling of the polymer in water and hydrogen bonding due to the carboxyl groups being in their unionised state at low pH (Robinson *et al.*, 1987; Leung and Robinson, 1988; Ponchel *et al.*, 1987a,b; Jabbari *et al.*, 1993). Polycarbophil is described as a water insoluble but swellable polymer of polyacrylic acid crosslinked with divinylglycol and used clinically in the treatment of diarrhoea and as a bulk laxative. Carbomer is a water soluble polymer of acrylic acid loosely crosslinked with allylsucrose. There have also been a wide range of polyanionic polysaccharides as possible biopolymer alternatives, such as alginate, pectin, carrageenan, xanthan and carboxy-methyl cellulose, but macroscopic (Lehr *et al.*, 1992b) and molecular studies (Anderson, 1991; Fiebrig, 1996) have yielded little or no mucoadhesion for these substances, possibly because both mucoadhesive and the mucin are polyanionic: the findings for polycarbophil are therefore rather surprising.

POLYCATIONS

According to Anderson *et al.* (1989), Anderson (1991) and later Lehr *et al.* (1992b), the need for hydrogen-bonding capabilities and negative charge in bioadhesive materials *should not be generalized*. These workers suggested that polycationic polymers might interact with the anionic sites on the mucins more favourably due to their opposite charges providing additional molecular attraction forces. For example, interactions between charged polymeric molecules have been employed in colloidal titration (Terayama, 1952; Senju, 1969). The method is based on the principle that positively charged macromolecules will react with negatively charged macromolecules. The neutralisation reaction will proceed stoichiometrically, allowing an estimation of either material if a standard colloid solution is used. Katayama *et al.* (1978) used the method for the titration of heparin using polydiallyldimethyl ammonium chloride as a standard polycation. Van Damme *et al.* (1992) measured the negative charge content in cartilage using polydiallyldimethyl ammonium chloride as well. Interactions between alginates and pectins with cationic polypeptides such as

poly(L-lysine) and poly(Lys-Lys-Ala) have been studied using circular dichroism (Bystricky *et al.*, 1990). Differences in interaction efficiency between the polymers were attributed to differences in conformational flexibility of the polyanionic chains in solution. Takahashi *et al.* (1990) studied the characteristics of polyion complexes of chitosan with sodium alginate and sodium polyacrylate using viscometry and Fourier transform infra-red spectroscopy (FT-IR). They found that chitosan and alginate reacted with a defined binding ratio which was found to be relatively constant in media of various pH values. In contrast, for polyacrylate-chitosan interactions the unit molecular binding ratio was greatly affected by the pH. (*n.b.* chitosans are generally poorly soluble above a pH ~6).

CHITOSANS

Chitosan appears to be an ideal candidate as a mucoadhesive polycationic polymer – it is produced on a large scale (Jeuniaux *et al.*, 1989; Alimuniar and Zainuddin, 1992). Although chitosan has not yet received regulatory approval by the Food and Drug Administration (FDA) for pharmaceutical use, chitosan containing material obtained from the treatment of the waste streams of food processing plants may be used as livestock feed in the USA so long as the level of chitosan does not exceed 0.1% (Weiner, 1992). It is known to interact with molecules containing N-acetylglucosamine, such as lysozyme (Cölfen *et al.*, 1996). Its properties are quite different from polyanionic chitin derivatives, such as carboxy-methyl chitin (Korneeva *et al.*, 1996).

Chitosan (Figure 6) has been approved as a food additive in Japan since 1983 (and also apparently in some European countries) and has been placed on the 'Japanese Natural Additive List'. It is used as a thickener and stabilizer (Weiner, 1992). It is a food ingredient in some dietary cookies and noodles from Hihon Kayaku Inc. and Tanami Foods Inc. as well as in vinegars of Nakano Inc., making use of its hypocholesterolaemic properties (Hirano, 1989). The food industry has also exploited the chelating properties of chitosan for the clarification of beverages such as apple and carrot juices (Imeri and Knorr, 1988; Soto Peralta *et al.*, 1989).

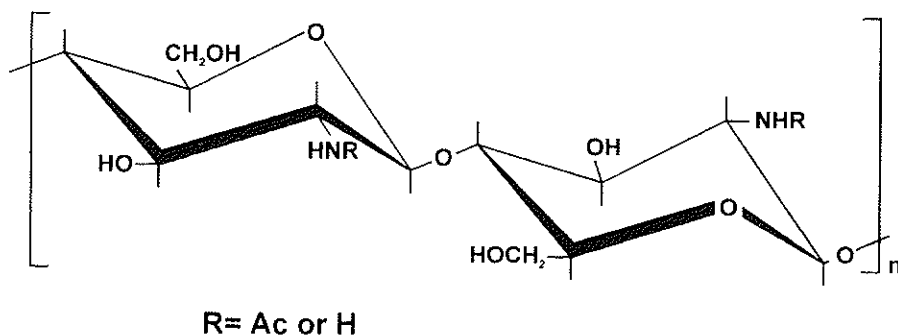


Figure 6. Chitosan. Pure poly-N-acetyl β -D-(1 \rightarrow 4) glucosamine is chitin and is insoluble. In chitosan, a proportion of the N-acetyl groups are deacetylated leaving a positive charge except at high pH (>6.5, where the molecule is again insoluble).

The lack of acute oral toxicity of chitosan has been supported by experiments in mice (Arai *et al.*, 1968) who determined an LD50 of > 10 g/kg. However the literature lacks adequate scientific studies on long term and widespread human exposure through food and pharmaceutical products (McCurdy, 1992).

Chitosan is a derivative of chitin; the insoluble structural exoskeletal polysaccharide of the shells of crabs and lobsters and can be harvested very cheaply (see Tombs and Harding, 1998); the chief producers are Norway, Japan, China and Russia. Like cellulose it is a $\beta(1\rightarrow4)$ -D-glucan. Unlike cellulose the residue on the number 2 carbon atom in the ring is N-acetylated (*Figure 6*). In native chitin these residues are fully acetylated. However, after extraction the chitin molecule can be deacetylated to varying degrees to give a polycationic molecule. The degree of acetylation is represented by the parameter F_A , with $F_A = 1$ (fully acetylated) corresponding to pure chitin and $F_A = 0$ to fully deacetylated chitosan.

Variations in molecular weight and degree of deacetylation together with the ability to form gels and films allow flexibility in formulation design (Acatürk, 1989; Miyayaki *et al.*, 1990; Errington *et al.*, 1993).

DEAE DEXTRAN

DEAE-dextran (*Figure 7*) is a polycationic derivative of dextran, obtained by reaction with 2-chlorotriethyl-amin-hydrochloride or chloroethyl-diethyl-aminochloride (see

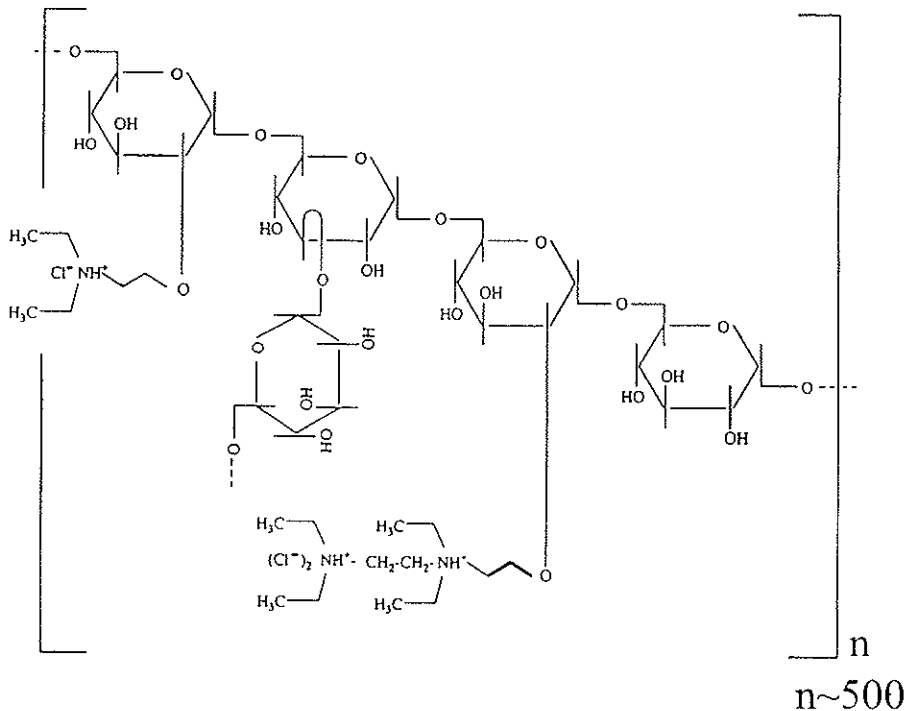


Figure 7. DEAE-dextran. DEAE: di-ethylaminoethyl. From Soldani *et al.* (1987).

Soldani *et al.*, 1987). Dextran itself is an $\alpha(1\rightarrow6)$ linked bacterial polysaccharide from *Leuconostoc mesenteroides*, with many branches of either an $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$ or $\alpha(1\rightarrow4)$ type. In most cases the length of the side chains is short and branched residues vary between 5 and 33 per cent. Partial hydrolysis and subsequent fractionation leads to polysaccharides of a particular desired range of molecular weight. DEAE dextran is used as a weak ion-exchange material for ion-exchange columns.

MUSSEL GLUE PROTEIN

The protein *mefp-1* is one of the major adhesive proteins used by marine mussels to bind strongly to underwater surfaces. This behaviour has been related to its strong surface active and adsorptive nature (Notter 1988; Olivieri *et al.*, 1992; Hansen *et al.*, 1994). This protein and related mussel adhesive proteins, are characterised by having high lysine contents and hydroxylated amino acids: *mefp-1* for example, consists of tandemly repeated decapeptides each containing two residues of lysine, one to two residues of Dopa (Waite, 1983; Laursen, 1992) one or two residues of *trans*-4-hydroxyproline and a single residue of *trans*-2,3,*cis*-3,4-dihydroxyproline (Taylor *et al.*, 1994). Several attempts have been made to make biomedical and commercial use of the adhesive properties of these substances (Baty *et al.*, 1997), for example, in experimental epikeroplasty and for cellular attachment (Robin *et al.*, 1988; Olivieri *et al.*, 1992) such as in the attachment of osteoblasts and epiphyseal cartilage cells to substrata (Fulkerson *et al.*, 1990). The strong adhesive properties have recently inspired a proposed use for these proteins as mucoadhesives for drug delivery

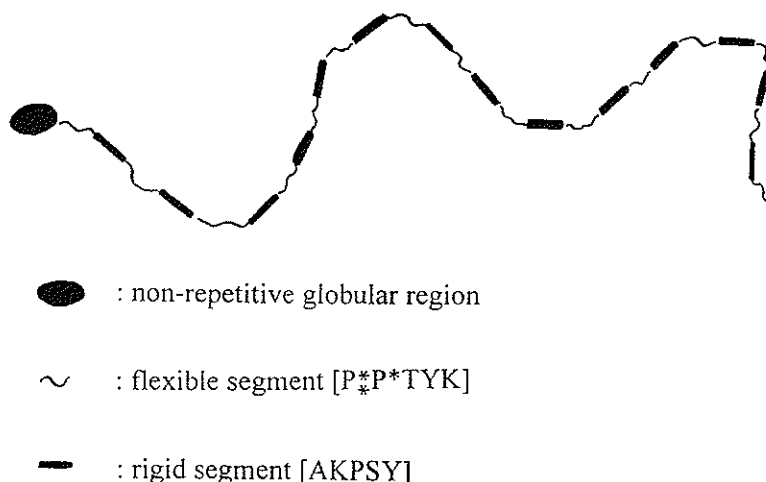


Figure 8. Consensus semi-flexible rod model for the mussel foot glue protein *mefp-1* from *Mytilus edulis*. This model takes into account the linear flexible properties consistent with molecular hydrodynamics by Deacon *et al.* (1998), earlier CD studies (Laursen, 1992) and the ability to adhere to surfaces (Baty *et al.*, 1997). The model consists of a globular region with a non-repetitive amino acid sequence and an extended region consisting of repeat sequences of amino acid with alternating stiff and flexible segments. Except at high pH (>7) and ionic strength, the chain will be relatively stiff due to electrostatic repulsion of segments. From Deacon *et al.* (1998).

(Schnurrer and Lehr, 1996) and these substances may offer an alternative to substances such as chitosans. However, applications have been hampered by the lack of knowledge on the solution structure and adhesive nature of the purified protein (Baty *et al.*, 1997) and until recently very little has been known of the oligomeric state or overall morphology of these molecules in solution (dilute or otherwise). Two recent studies have confirmed that the protein is monomeric in solution of molecular weight ~110 kDa (Deacon *et al.*, 1996) and has an extended conformation in solution (Deacon *et al.*, 1998a) as illustrated in *Figure 8*.

Direct and molecular strategies for studying mucoadhesion

Direct methods involve a study of a macroscopic interaction, usually involving whole mucus, whereas the molecular methods focus on the interactions involving the purified mucin component. Both strategies are highly complementary and should not be used in isolation.

The assay methods can either employ freshly excised tissue from various animals (frog, rat, rabbit, pig, cow, etc.), used either immediately as live or dead tissue or stored frozen and defrosted prior to use, or they use mucus or mucin at various degrees of degradation and purity either solubilised or as gel (usually from pig stomach or bovine submaxillary glands). Whatever model material is used, its relevance to the human mucus, whether in health or disease state, has to be considered (MacAdam, 1993). Dead mucosal tissue may well not produce any new mucus, while degradation of existing mucus will still take place. This will have a marked effect on the rheological characteristics of the substrate, considered to be highly relevant to adhesional phenomena. Mucus thickness may vary from species to species and intersubject, as well as intrasubject, variability of the mucosal tissue poses problems in terms of reproducibility. For the mucin based procedures, mucins, once extracted are subject to degradation by enzymes and mechanical disruption: they have to be handled with extreme care, and enzyme degradation must be kept to a minimum (e.g. by extraction in guanidine hydrochloride (Sheehan and Carlstedt, 1984) or with adequate protease inhibitors present). Mucin carbohydrate composition also varies within the gastrointestinal tract (Allen, 1989).

Small intestinal mucin is very difficult to solubilise and available usually in only small quantities. Gastric mucin from pigs appears to be an alternative since it is available in larger quantities and although its sialic acid content is low, its carbohydrate composition is comparable to human gastric mucin. Purification methods allow the removal of other components present in mucus in order to obtain purified mucin which still shows the gel-forming characteristics of native mucus (Sheehan and Carlstedt, 1989; Bell *et al.*, 1985; Allen, 1989).

Commercially available pig gastric mucins or mucus are somewhat different in the detail of their composition when compared with freshly prepared and purified material. They may be rather degraded or the freeze drying procedure may have altered the structure in such a way that it becomes difficult to redissolve them completely. Commercially available 'submaxillary' mucins are quite different from the mucins secreted in the gastrointestinal tract. They are secreted in a viscous soluble form rather than as water-insoluble gels (for a discussion of these differences see Gottschalk *et al.*, 1972). Nevertheless, highly purified mucins can give

more accurate information on the actual nature of the interaction of a putative mucoadhesive with the main mucin-forming component. The use of dilute mucin solutions also allows the study of mucin–bioadhesive polymer interactions on a fundamental level.

It has been recognized that the degree of hydration of the bioadhesive DDS, as well as the amount of water available, plays an important role in determining the strength of adhesion or whether adhesion can take place at all (Leung and Robinson, 1988; Chen and Cyr, 1970). The hydration aspect can be controlled in local applications such as mouth or vagina by drying excess water in the area immediately prior to application (Deasy and O'Neill, 1989). In the gastrointestinal tract, however, excess water at the site of adhesion as well as excess in the amount of surrounding liquid cannot be controlled. Lehr *et al.* (1992b) pointed out that numerous so-called mucoadhesive polymers adhere only under conditions where the amount of interstitial liquid is limited. This kind of dry-to-wet adhesion or 'blotting adhesion' is due to the capillary forces drawing liquid from the mucus into the delivery system (Huntsberger, 1967; Lehr *et al.*, 1992b; Mortazavi and Smart, 1993). If the polymer involved offers no intrinsic ability to form a bond with the substrate (e.g. some cellulose derivatives), the initial adhesive forces, although high at the beginning, may become negligible as soon as the material is fully hydrated (Junginger and Lehr, 1990). Therefore, adhesion measurements in fully hydrated systems and over a period of time are necessary to avoid attributing a high adhesive force erroneously to intrinsic mucoadhesive properties. The adhesion mechanism of capillary attraction between a dry, water-absorbing polymer and a wet, mucosal surface being dehydrated is quite different to the interactions between two hydrogels (polymer and mucus) in equilibrium with a third liquid phase (Mortazavi and Smart, 1993).

Direct assay methods for mucoadhesion

By direct we mean 'whole mucus' assay procedures, and one of the simplest and most effective methods is tensiometry, which uses the force required to detach two surfaces, one coated with the mucus substrate, the other with mucoadhesive, as an index of mucoadhesion. Other direct methods (*Table 2*) include the 'flow through' technique, colloidal gold staining (adhesion number) and the *in-vivo* methods (endoscopy, radioisotope imaging)

TENSIOMETRY

The method employs putative mucoadhesive polymers that are usually in the form of tablets made by direct compression of the polymer or polymer coated surfaces from casting of polymer solutions. These are consequently put in contact with a mucus surface usually with a given force applied to the system for a given period of time after which the adhesive joint is destroyed by applying a vertical force in the opposite direction or a shear force in the horizontal direction. The force required to destroy the bond is taken as a qualitative and quantitative parameter for adhesion. If the experiment is done under full hydration of the polymer and in an aqueous environment

Table 2. Mucoadhesive assay methods

	Comment	References
'Direct' assays:		
Tensiometry	Force F required to dislodge two surfaces (one coated with mucus, the other mucoadhesive)	Lehr <i>et al.</i> (1992c)
Flow-through Colloidal gold staining	Flow rate, dV/dt required to dislodge two surfaces	Mikas and Peppas (1990); Junginger <i>et al.</i> (1990) Park (1989)
<i>In vivo</i>	Measures 'adhesion number'	Anderson (1991); Aoyagi <i>et al.</i> (1992)
Molecular mucin based assays:	Endoscopy, radioisotope imaging	
Viscometry and rheology	Intrinsic viscosity $[\eta]$ can be related to complex size via MHKS 'a' coefficient	Harding (1995, 1997a)
Dynamic light scattering	Diffusion coefficient, D, can be related to complex size via MHKS 'e' coefficient	Harding (1995, 1997a)
Turbidity/light scattering	'SEC MALLS' particularly useful for determining mol. wt. distribution of mucin.	Jumel <i>et al.</i> (1996, 1997); Fiebrig (1995)
Analytical ultracentrifugation	Turbidity: semi-quantitative indicator	
	Change in mol. wt (sedimentation equilibrium)	Harding (1995); Cölfen and Harding (1997)
	Sedimentation coefficient ratio of complex to mucin	Deacon <i>et al.</i> (1998a); Harding (1997b)
	Schlieren 'fingerprinting'	Deacon <i>et al.</i> (1998b)
Surface Plasmon Resonance	Needs mobile and immobile phase	see, e.g. Silkowski <i>et al.</i> (1997)
Imaging methods	Atomic Force Microscopy	Deacon <i>et al.</i> (1999)
	Transmission electron microscopy (conventional and gold labelled)	Fiebrig <i>et al.</i> (1995b, 1997)
	Scanning Tunneling Microscopy	Roberts <i>et al.</i> (1995)

the likelihood of mimicking *in vivo* conditions is higher, given that a potential formulation, which is usually swallowed with liquid would not arrive at the target site in a totally dry state. However, this method neglects the fact that a swallowed formulation does not make intimate contact with the mucus gel spontaneously. Furthermore, the cohesion of mucus is also related to its thickness and rheological features. The method does not distinguish adhesion and cohesion (Huntsberger, 1967; Lehr *et al.*, 1992c). Although information on the screening of polymers can easily be obtained, the method appears unsuitable for assessing adhesive behaviour of formulations intended for gastrointestinal application. Similar comments apply to the related 'Wilhelmy Plate' method used by e.g. Smart *et al.* (1984). Tensiometry seems more useful for buccal, vaginal or other applications where liquid is controllable and more limited.

Despite these limitations, Lehr and coworkers found a very interesting spectrum of results in terms of the mucoadhesive performance of a range of biopolymers (Lehr *et al.*, 1992b) and their results are summarised in *Table 3*. The disappointing performance of the neutral and anionic polysaccharides is clearly shown. By contrast the polycationic chitosans appear to give a very favourable interaction strongly indicative of the importance of electrostatic interactions, although polycationic dextran derivatives showed little adhesive potential. A possible explanation for this is the branching of the dextran which may shield off and provide steric blockage of any interaction with anionic groups on the mucin glycoprotein substrate. *Table 3* also shows the potential of the mussel foot glue protein (Schnurrer and Lehr, 1996). Lehr *et al.* (1992a) and Schnurrer and Lehr (1996) also obtained the rather surprising result of a very favourable interaction for the synthetic polyanionic polymer, polycarbophil. We will consider these results again later when we have dealt with the molecular hydrodynamics.

FLOW THROUGH SYSTEMS

Here mucoadhesive coated spheres are placed on a mucus gel surface and the shearing flow of fluid required to dislodge them is used as an index of adhesion: this seems to model the situation in the gastrointestinal tract better than tensiometry. A flow channel device was first described by Mikos and Peppas (1990). The channel had a length of approximately 30 cm, a width of 4 cm and a height of approximately 0.5 cm, and it was thermostatted by a jacket connected to a constant temperature water bath. A cavity inside the channel allowed placement of a mucin gel or the mucosal of a tissue and the placement of a single polymer microparticle on top of it. The channel was connected through a set of valves to a gas cylinder. The volumetric flow rate was gradually increased until the particle, which was observed by an optical microscope, was detached from the mucous surface. This particular system could be suitable as a model for studying nasal mucoadhesion. For gastrointestinal models a fluid has to be substituted for the air and live tissue has been used to monitor intestinal drug absorption at the same time (Junginger, *et al.*, 1990). The observation of adhesion of a formulation (which ought to be insoluble so as to avoid dilution and rapid wash-off as well as rapid drug leaching) from a flow of solution directly onto a mucus tissue would be most desirable. A swellable, but insoluble formulation is usually achieved by crosslinking of the polymer chains. This would lead to chain rigidity which in turn

Table 3. Mucoadhesive performance: Tensiometric analysis

Biopolymer	F (mN/cm ²)
Neutral polysaccharides:	
Hydroxy-propyl cellulose	-0 (2.8±2.8)
Hydroxy-ethyl starch	-0 (0.6±0.8)
Scleroglucan	-0
Anionic polysaccharides:	
Pectin	-0
Xanthan	-0
CMC (low viscosity)	1.8±1.1
CMC (medium)	-0 (0.3±0.3)
CMC (high viscosity)	1.3±1.0
Chitosans:	
Wella low viscosity	3.9±1.2
Wella high viscosity	6.7±0.7
Knapezyk	5.7±1.1
Daichitosan-H	8.0±5.7
Daichitosan-VH	9.5±2.4
Sea-cure 240	4.1±2.9
Sea-cure 210+	9.5±2.5
Sigma	6.6±3.0
Cationic dextrans:	
DEAE-dextran	-0
Amino-dextran	-0
Proteins:	
Mussel glue protein <i>mefp-1</i>	-9

Adapted from Lehr *et al.* (1992) and Schnurrer and Lehr (1996)

F: Force required for detachment of two surfaces in contact (mucoadhesive and mucus)

could limit mucoadhesion since the proposed 'interpenetration' or 'interdiffusion' mechanism would be restricted.

COLLOIDAL GOLD STAINING

Instead of measuring the adhesion strength or the duration of adhesion, the 'adhesion number' can be determined as a direct function of adhesion. In the method described by Park (1989) the adherent material (or substrate) consisted of colloidal gold particles of an approximate diameter of 18 nm with mucin adsorbed onto their surface (the particular mucin used was a solution of bovine submaxillary mucin Type I). Colloidal gold (cAu) sols were prepared by reducing H₂AuCl₄ with reducing agents like sodium citrate. Particle sizes varied depending on the reducing agent as well as on the preparation procedure.

The bioadhesive material used by Park was a copolymer made from acrylic acid and acrylamide cross-linked with N,N'-methylene-bis-acrylamide [P(AA-co-AM)]. The transparent hydrogels made of this material were cut into rectangular shapes of varying thickness. The polymer strips were incubated with the cAu-mucin conjugates and after a rinsing procedure, the absorbance of the strip was measured at a wavelength of 525 nm with a spectrophotometer using a transparent control polymer strip as a blank. The values obtained were a function of the amount of cAu adsorbed onto the surface. Alternatively the absorbance of the cAu-mucin preparation was measured

before and after incubation. In this case, the magnitude of the decrease in the absorbance value from an initial value was used as a quantitative parameter indicating an interaction between cAu-mucin and hydrogel. An image analyser was also used to quantify the intensity of red colour on the polymer surfaces. This approach was necessary for mucoadhesive polymers which were not transparent.

The cAu-mucin conjugates prepared by Park required the addition of albumin to stabilise the preparation further. Albumin molecules are believed to adsorb onto small bare spots on the cAu particle where mucin molecules do not cover (Horisberger and Rosset, 1977; De Mey, 1984). However, it can be argued whether any interaction phenomenon observed is due solely to the properties of the mucin. Moreover, if the affinity of albumin to the cAu is higher than that of mucin, a displacement of mucin from the cAu is also possible.

As outlined by Park (1989) an alternative approach using the colloidal gold staining technique is that of developing cAu-(bioadhesive polymer) conjugates instead of cAu-mucin conjugates. The polymer coated cAu particles acting as the adhesive this time could be directly applied to the surface of target tissues. In this case the cAu-polymer conjugate would act as a model drug delivery system. Chitosan-stabilised cAu has been successfully prepared by Horisberger and Clerc (1988) to use as a marker for anionic sites on various micro-organisms and by Fiebrig *et al.* (1994b, 1997) to visualize the sites of interaction of chitosan in a mucin-chitosan complex.

IN VIVO METHODS

These involve animal models, human volunteers or patients (see Table 18.3 of Fiebrig *et al.*, 1995a for an extensive comparison). For buccal, vaginal, cervical or nasal applications the residence time of the device can be inspected visually, while the subject can give direct information on aspects of tolerance (discomfort, usefulness, etc.) (Nagai, 1986; Bottenberg, *et al.*, 1991; Smid-Corbar *et al.*, 1991). Plasma levels of drug or pharmacodynamic effect (for e.g. delivery of insulin) can give indirect evidence. Aspects of intersubject variability and disease condition will need to be taken into account.

With regards to gastrointestinal bioadhesive DDS we are faced with major experimental difficulties. Once swallowed, the device has to reach the adherent mucus layer. As has been outlined earlier, the process of bioadhesion requires *intimate contact* in its first step. There is little experimental evidence for this prerequisite actually taking place in gastrointestinal bioadhesion. Lodged on the target surface a delivery system has to resist the dislodging forces of gastrointestinal motility. In humans this particular aspect has been examined for the first time in a double blind study by Anderson (1991) using coloured tablets made from DEAE-dextran and ethylcellulose (1:1) with ethylcellulose tablets as controls. Seven patients undergoing routine gastroscopy examination swallowed both tablets with approximately 20 ml of water immediately before endoscopy. Mucoadhesion, or the lack of it, was assessed using a finger controlled water jet attachment. The clinician sprayed the tablet with water, for a fixed time period and at a constant rate, and assessed adhesion in terms of the number of sprays required to dislodge the tablet. The results did not suggest any significant adhesion of the test formulation to the gastric mucosa as compared to the control. In 50% of the patients examined at longer time intervals (up until 65 min post dose)

neither the control nor the test tablet could be found in the stomach. For those patients where tablets could be observed, no significant differences in adhesive behaviour between control and test tablet with the gastric mucosa could be detected when judged using the finger controlled water jet attachment. There was no actual measurement of the water spray properties. However, the results illustrate the probable lack of adhesion for both tablet formulations as well as the intersubject variation in gastric emptying times for tablet formulations in the fasted state as observed by other authors.

The rat as an *in vivo* model has a mucus layer about half the thickness of that in man (Allen, 1978; Kerss *et al.*, 1982), while little is known about mucus turnover compared to man. It has been suggested that in the rat there is very little soluble gastric mucus when compared with the dog where there are considerable amounts of this material (Robinson *et al.*, 1987). Although the mucin of the pig gastrointestinal tract is similar to that of humans with regards to its carbohydrate and protein composition (Allen, 1989), gastric emptying in pigs has been shown to be slower than in man (Aoyagi *et al.*, 1992) and consequently this animal may not be the appropriate *in vivo* model. It is however possible to deliver directly to the intestine if required.

Molecular mucin-based assay methods

These methods focus on the interaction between the key macromolecular component of mucus, namely the mucin, and the mucoadhesive material (*Table 2*). The use of a standardised material throughout the experiments allows a comparison of results and avoids inter-sample variations. Such methods also allow for the study of the factors that may influence the interactions (ionic strength, bile salts, temperature, proteins etc.) and hence the elucidation of interaction mechanisms. The concentrations employed can be very low, as in the case of analytical ultracentrifugation and dynamic light scattering.

VISCOMETRY AND RHEOLOGY

These approaches involve the measurement of either the intrinsic viscosity $[\eta]$ of a dilute solution using for example simple capillary viscometers with proper thermal control (see, Harding, 1997a), or for more concentrated dispersions and gels the rheological parameters G'' (loss modulus) and G' (storage modulus) representing characteristic viscoelastic behaviour using e.g. cone-and plate type of viscometers (see Ross-Murphy, 1995). If the size of the complex is to be used as an index of mucoadhesive potential, the intrinsic viscosity can be related to the size of a complex between mucoadhesive and mucin via the Mark-Houwink-Kuhn-Sakurada (MHKS) 'a' coefficient (see Harding, 1995, 1997a) if an assumption is made about conformation (namely the conformation of the complex is the same as that of the mucin):

$$[\eta] \sim M^a \quad (1)$$

($a = 0, 0.4-0.5, 1.8$ for respectively a sphere, coil and rigid rod). However this method is complicated by the presence of unreacted mucin or mucoadhesive, and that measurement of $[\eta]$ normally requires extrapolation to zero concentration to avoid complications of non-ideality. In addition, if complexation is a reversible process, lowering the concentration may also cause dissociation.

DYNAMIC LIGHT SCATTERING (PHOTON CORRELATION SPECTROSCOPY)

This involves measuring the translational diffusion coefficient, D , and, where possible, its distribution, $g(D)$, using an autocorrelator, which correlates time fluctuations of scattered light through Brownian motion of the scattering macromolecules/complex. Like the intrinsic viscosity, the diffusion coefficient or $g(D)$ can be related to the size of a complex if assumptions are made about conformation. For example, in terms of molecular weight by another MHKS relation (see Harding, 1995):

$$D \sim M^{-\epsilon} \quad (2)$$

($\epsilon = 0.33, 0.5-0.6, 0.85$ for a sphere, coil and rod respectively), or (better) D is combined with the sedimentation coefficient from analytical ultracentrifugation (see below) to give M via a relation known as the Svedberg relation. Although the non-ideality complications are not as critical as for intrinsic viscosity, for non-spherical particles the autocorrelation function has to be extrapolated to zero angle because of complications of rotational diffusion, and like viscometry, the results will be complicated by the presence of unreacted mucin or mucoadhesive: although software can provide a distribution $g(D)$ and hence in principle resolve components of different D , results have to be considered very tentatively, since such results are from mathematical manipulation of data rather than a genuine mechanical separation as would be provided by chromatographic and sedimentation based procedures. The lack of mechanical separation also means samples have to be scrupulously clean from dust and large particulates that do not directly arise from the mucoadhesive-mucin complexation process.

TURBIDITY/LIGHT SCATTERING

Turbidimetric methods, although approximate, have been successfully applied to large supramolecular assemblies such as the T-even bacteriophages (see Bahls and Bloomfield, 1977; Harding, 1986) and have been recently used to study mucin-mucoadhesive complexes (see Fiebrig, 1997). A good quality spectrophotometer is required which measures only the loss of intensity of an incident beam as it passes through a suspension, and does not record appreciable amounts of scattered light. The loss of intensity has to be due to scattering and not absorption, so a wavelength is chosen away from any absorption bands.

Although simple, turbidimetry is only an approximate way of sizing a macromolecular assembly. More useful information is found if a light scattering photometer is used, which records the scattered intensity envelope away from the incident angle. From this and an accurate knowledge of the concentration of the scattering particles the molecular weight and radius of gyration can be measured (see Tanford, 1961). Care has to be taken with the analysis of very large particles ($M > \sim 50$ million Da), since application of the simpler 'Rayleigh-Gans-Debye' theory ceases to be valid. As with dynamic light scattering, samples and scattering vessels have to be scrupulously free of dust and other large contaminating particulates.

A revolutionary development has been the coupling on-line of light scattering

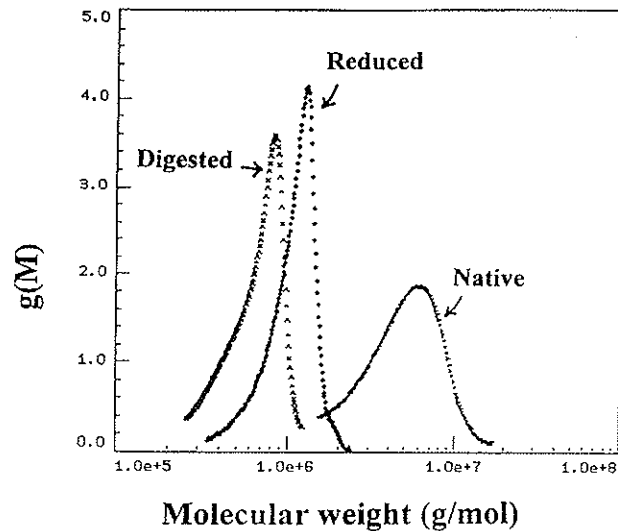


Figure 9. Molecular weight distributions evaluated from the technique of size-exclusion chromatography coupled to multi-angle laser light scattering, 'SEC-MALLS' for pig colonic mucin, in its native, thiol reduced (or 'subunit') and papain digested ('T-domain') forms. From Jumel *et al.* (1997).

photometers with size exclusion chromatography: this (i) provides an on-line clarification system, and (ii) provides a physical (as opposed to mathematical) separation of polydisperse systems (Wyatt, 1992). The method is ideal for checking the molecular integrity of a mucoadhesive and a mucin prior to mixing. The usefulness of size-exclusion chromatography coupled to multi-angle laser light scattering (SEC/MALLS) as a rapid assay for mucin molecular weight was first demonstrated by Jumel *et al.* (1995, 1997): *Figure 9* shows an example of a characterisation of pig gastric mucin (Jumel *et al.*, 1995) and colonic mucin, its subunits and T-domains (Jumel *et al.*, 1997). Beyond molecular weights of ~10 million, SEC systems generally fail to separate. However, other separation systems are now available, such as 'field flow fractionation' (FFF) and work is currently in progress to monitor the effectiveness of these systems for the characterisation of mucoadhesives (Deacon, 1999; Deacon *et al.*, 1999)

ANALYTICAL ULTRACENTRIFUGATION

In analytical ultracentrifugation the sedimentating boundary (sedimentation velocity) or equilibrium concentration distribution (sedimentation equilibrium) of a macromolecule or macromolecular complex in solution or suspension, under the influence of a centrifugal field, is recorded using absorption (uv or visible) or refractometric (Schlieren or Rayleigh interference) optics. Although the method is nearly 80 years old since its inception by T. Svedberg and coworkers, this technique has undergone a startling renaissance over the last decade, particularly in the protein biochemistry/molecular biology fields, where its power in characterising the stoichiometries, strengths and conformations of protein-protein interacting systems is now widely recognised (see Harding and Winzor, 1999). It has enormous potential for the study of

mucoadhesion. However, because of the generally much larger size and polydispersity of mucin, mucoadhesive (if it is not a protein) and complex, we have to apply ultracentrifugation as an assay for mucoadhesion in a slightly different way than used for the analysis of protein-protein interaction phenomena:

- *Change in molecular weight, M , as a measure of mucoadhesion.* Provided the molecular weight of the complex is within the range $\sim 1000 < M < 50$ million Da we can apply the absolute molecular weight probe of sedimentation equilibrium in the ultracentrifuge. The ratio of the molecular weight of the complex to that of the largest of the reactants (normally the mucin, of molecular weight typically between 2 and 10 million Da) can then be used as a measure of mucoadhesive properties.
- *Change in sedimentation coefficient, s .* For complex sizes within the range $10000 < M < 10^9$ Da we can apply sedimentation velocity in the ultracentrifuge to measure the sedimentation coefficient (sedimentation rate per unit centrifugal field). This will also be a measure of molecular weight and will also be influenced by conformation and hydration effects (see Pavlov *et al.*, 1997). We can either use the ratio of the sedimentation coefficients of the complex to the fastest sedimenting reactant {normally the mucin, of s typically between 20 and 60 Svedbergs (S)} as a measure of mucoadhesive potential, or we can assume a conformation for the complex (mucin appears to adopt a random coil conformation in solution) and convert the ratio of sedimentation coefficients to a ratio of molecular weights using the MHKS 'b' coefficient (see Harding, 1995, 1997b):

$$s \sim M^b \quad (3)$$

where $b = 0.667, 0.4-0.5$ and ~ 0.15 for a sphere, coil and rod, respectively.

- *Change in sedimentation concentration of reactants.* If the complexes are too large even for sedimentation velocity to pick up we can use a 'fingerprinting' assay whereby we determine the mucin or mucoadhesive loss (compared to a control) caused by complexation as a measure of mucoadhesive properties. This is particularly useful with the Schlieren refractometric optical system on the ultracentrifuge.

SURFACE PLASMON RESONANCE

This method which has had a significant impact in the study of protein-protein systems also has considerable potential for the study of mucoadhesion. It involves immobilising one phase and flowing over the second phase: in many ways it mimicks the 'flow through' technique described above in the 'Direct' assay procedures section. However, it has a serious disadvantage over the ultracentrifuge in that the method is not 'clean' in the sense that a third 'immobilising' phase is necessary. This is conventionally dextran, so assumptions over inertness have to be made. The greater the amount of material deposited from the mobile phase onto the immobile phase will affect the 'evanescent wave'. After certain assumptions, these affects can be related to a molar dissociation constant. If possible, experiments should be repeated with the mobile and immobile phases reversed, and the best application of this method is when used in conjunction with the analytical ultracentrifuge (see Silkowski *et al.*, 1997).

IMAGING METHODS

Another, but relatively new surface probe which 'images the surface' is Atomic Force Microscopy. The atomic force microscope can be used to investigate the interaction between mucin and mucoadhesive polymers. There are various modes of atomic force microscopy which include contact, non-contact and tapping, all of which may be performed in a liquid or a dry environment. In tapping mode the tip is brought into contact with the surface in a rapid intermittent fashion with the probe making very little contact with the sample. For standard forms of imaging (contact and non-contact) the picture is built up due to the collected topographical information caused by the deflection of a laser beam, targeted onto the reverse side of the cantilever, as it raster scans the surface. As tapping mode has intermittent contact with the surface it is ideal for soft samples, nearly achieving the resolution of contact mode with the non-invasive nature of non-contact modes of imaging. Using tapping mode it is possible to visualise mucin and polymers and study whether they are interacting without the harsh preparations needed in other methods. Two recent papers have indicated the usefulness of combining these measurements with those from surface plasmon resonance (Shakesheff *et al.*, 1995; Chen *et al.*, 1996).

Transmission electron microscopy has provided valuable information about the structure of mucins noted above (*Figure 4*). It has also been used to probe mucoadhesive complexes, in terms of conventional rotary shadowing of unlabelled material, and also on complexes where the mucoadhesive (chitosan) has been specifically labelled with colloidal gold or with gold tagged wheat germ agglutinin (Fiebrig *et al.*, 1997).

Case study: interaction of polycationic biopolymers with gastric mucin

We now illustrate the study of the molecular interactions between mucin and mucoadhesives by considering some of the work conducted by ourselves and others at Nottingham in conjunction with colleagues at Bristol, Lund, Trondheim and Oslo on the behaviour of mixtures of gastric mucin with DEAE-dextran, chitosan and mussel glue protein. The techniques that have been the cornerstone of our 'molecular' based approach have been primarily sedimentation velocity and electron microscopy. The sedimentation coefficient and molecular weight/molecular weight distributions (measured either by sedimentation equilibrium in the ultracentrifuge or using SEC-MALLS) has been routinely used by us as a measure of the structural integrity of the mucins (Jumel *et al.*, 1995, 1997).

1. DEAE DEXTRAN – GASTRIC MUCIN

We use the criterion of ratio of sedimentation coefficients, s , as an index of mucoadhesion between gastric mucin from pig stomachs. Two forms of the pig gastric mucin were used: a low-molecular weight 'single subunit-type' form ($M \sim 2$ million Da, s (at 20°C) ~ 17 Svedbergs, S, where $1S = 10^{-13}$ sec) and a 'whole mucin' form ($M \sim 8$ million, $s \sim 42S$). A Beckman (Palo Alto, USA) XL-A analytical ultracentrifuge has been used (Giebeler, 1992) with scanning uv-absorption optics. At 280 nm the mucin shows an absorption maximum whereas the DEAE-dextran is transparent and cannot be detected. Additionally, the DEAE dextran has a much smaller sedimentation

Table 4. Sedimentation velocity assay: pig gastric mucin and DEAE-dextran

mucin:DEAE-dextran ratio	Buffer and temperature	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
mucin subunits (M ~2 million Da):				
2.0:1.9 (mg/ml)	pH6.8, I=0.1, 20°C	17	19	1.1
	pH6.8, I=0.1, 37°C	17	20	1.2
1.8:3.2	pH6.8, I=0.1, 37°C	18	25	1.4
	whole mucins (M ~8 million Da):			
0.2:1.0	pH6.8, I=0.1, 20°C	35	65	1.9
	pH7.0, I=0.1, 20°C	42	55	1.3

UV absorption optics (using the MSE Centriscan and Beckman XL-A analytical ultracentrifuges). DEAE dextran control: sedimentation coefficient, $s \sim 2$ Svedbergs (S). For the subunit data, s values at 37°C have been normalised (for fluid density and viscosity) to 20°C (Anderson, 1991)

Table 5. Sedimentation velocity assay: pig gastric mucin and chitosans

chitosan	Buffer and temperature	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
sea-cure +210	pH4.5, I=0.1, 20°C	52	780	15
	pH4.5, I=0.1, 37°C	53	1990	38
KN50 Trondheim	pH4.5, I=0.1, 20°C	52	1630	31
	pH4.5, I=0.1, 37°C	53	2340	44

UV absorption optics (using the Beckman XL-A analytical ultracentrifuge).

Chitosan controls: sedimentation coefficient, $s \sim 1.5$ Svedbergs (S)

Mucin:chitosan ratio, 0.2 mg/ml: 1.0 mg/ml

Mucin M ~11 million Da

Sea-Cure +210 (Pro-Nova, Drammen, Norway): degree of acetylation, $F_A \sim 0.11$

KN50 (NTH-Trondheim): degree of acetylation, $F_A \sim 0.42$

coefficient ($\sim 2S$, as measured by a different analytical ultracentrifuge, the Beckman Model E with refractometric Schlieren optics) compared to the mucins (17-42S), so the ratio of s for the mixture to that of the mucin control can be used. An inspection of *Table 4* shows clearly that the interaction between the mucin and DEAE dextran is very modest for both 'subunits' and 'whole mucins' alike, with the maximum value of $s_{\text{mix}}/s_{\text{mucin}}$ being only 1.9. This is consistent with the results from macroscopic tensiometry analyses of Lehr *et al.* (1992c) (*Table 3*) and is probably a manifestation of steric shielding of the charged residues by the $\alpha(1 \rightarrow 3)$ or $\alpha(1 \rightarrow 4)$ branches in the dextran chain. This finding is also consistent with the lack of bioadhesive effect for coated tablets of DEAE-dextran in *in-vivo* trials (Anderson, 1991).

2. CHITOSANS – GASTRIC MUCIN

The very modest results for DEAE-dextran contrast dramatically with those for chitosans which demonstrate a huge interaction with pig gastric mucin. *Table 5* shows the results for two chitosans of different properties. Sea-Cure 210+ is a commercial high quality chitosan available from Pronova Ltd. (Drammen, Norway), and is highly positively charged with a low degree of acetylation of the C2 N-groups ($F_A \sim 0.11$): *Table 5* shows the formation of large complexes of $s_{\text{mix}}/s_{\text{mucin}} \sim 15-38$, with no residual unreacted mucin left, corresponding (if we assume a coiled conformation for the complex, i.e. $s \sim M^{0.5}$) to particles of mol wt $\sim 10^9 - 10^{10}$ Da, and consistent with a strong electrostatic interaction. The observations are consistent with the macroscopic

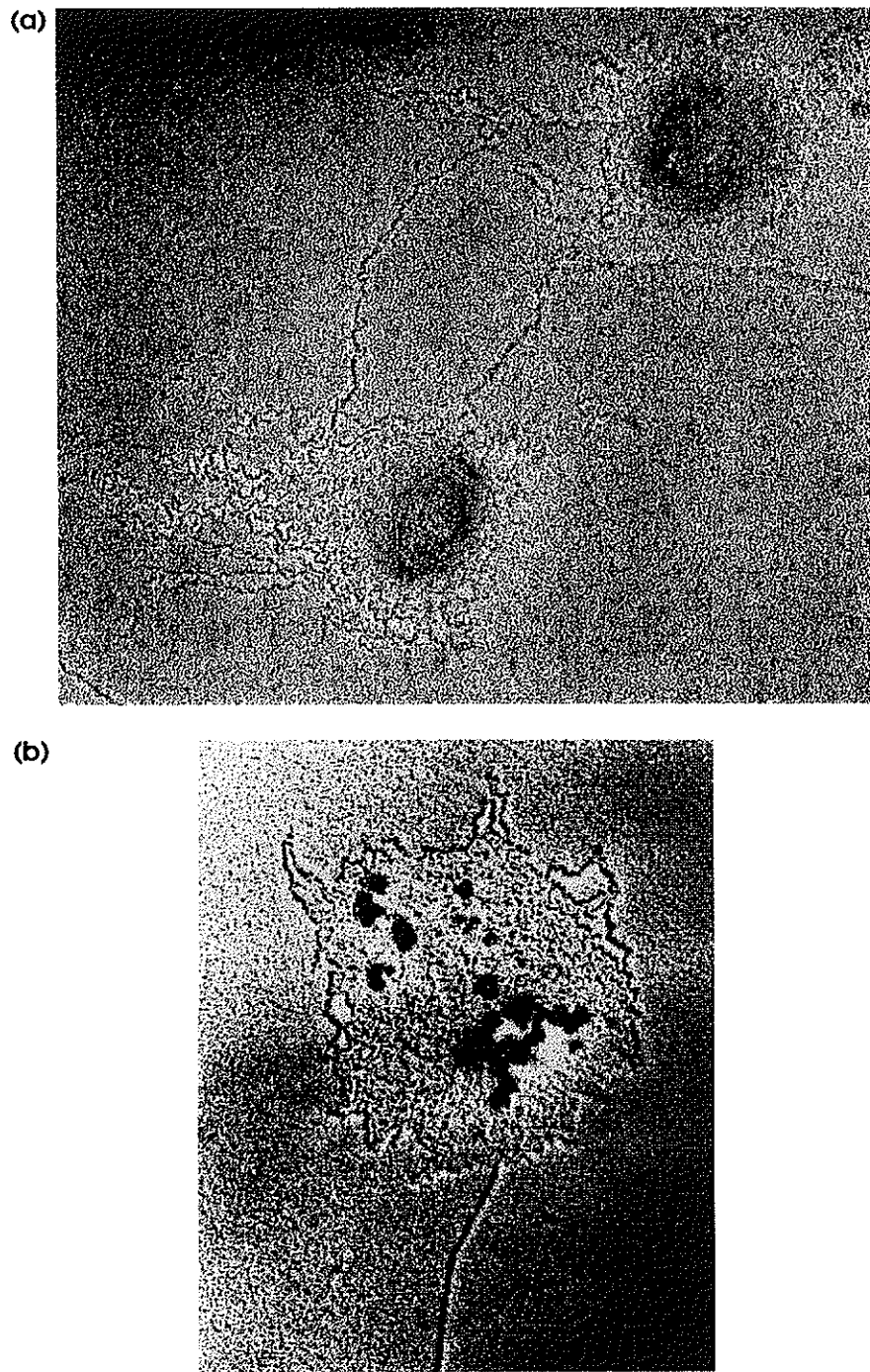


Figure 10. Transmission electron microscopy (both magnification $\times 75000$) of a chitosan-mucin complex. Chitosan: Sea Cure +210 ($F_A = 0.11$). Mucin: pig gastric mucin. (a) conventional rotary shadowing (b) complex formed after the chitosan had been conjugated with colloidal gold. From Fiebrig *et al.* (1997).

tensiometry results of Lehr *et al.* (1992c) and also from electron microscopy studies (Fiebrig *et al.*, 1995b, 1997) using rotary shadowing of the complexes conventionally prepared by air drying onto mica and where the chitosan had been labelled directly by colloidal gold (*Figure 10*) and by wheat-germ-agglutinin linked gold. With this labelling strategy, the chitosan is seen to be distributed throughout the complex with 'hotspots' clearly evident.

Significantly however, the chitosan of much lower charge (KN50 of $F_A \sim 0.42$) also shows a very strong interaction of comparable magnitude (*Table 5*). This would appear to indicate that substitution of a charged group on C2 with an acetyl group

Table 6. Sedimentation velocity assay: pig gastric mucin and chitosan (Sea Cure 210+) and bile salt

sodium tauro-cholate conc.	Temp	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
0mM	20°C	52	1085	21
	37°C	53	936	18
3mM	20°C	52	1375	26
	37°C	53	1877	35
6mM	20°C	52	973	19
	37°C	53	736	14

UV absorption optics (Beckman XL-A analytical ultracentrifuge).
Chitosan control: sedimentation coefficient, $s \sim 1.5$ Svedbergs (S)
Mucin:chitosan ratio, 0.2 mg/ml: 1.0 mg/ml. Bile salt: sodium taurocholate
Mucin M - 11 million Da

Table 7. Sedimentation velocity assay: pig gastric mucin and chitosan (Sea Cure 210+). *Effect of pH.*

pH	Temp	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
2.0	20°C	45	980	22
	37°C	132	1626	12
4.5	20°C	52	780	15
	37°C	53	1990	38
6.5	20°C	32	1524	48
	37°C	46	1580	34

UV absorption optics (Beckman XL-A analytical ultracentrifuge).
Chitosan controls: sedimentation coefficient, $s \sim 1.5$ Svedbergs (S)
Mucin:chitosan ratio, 0.2 mg/ml: 1.0 mg/ml.
Mucin M - 11 million Da

Table 8. Sedimentation velocity assay: chitosan (Sea Cure 210+) and different gastric mucins

mucin	mucin:chitosan ratio	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
PGM	0.2: 1.0 (mg/ml)	53	780	15
HGM	0.3: 1.0	11	222	20
OGM	1.0: 1.0	7	7*	1

UV absorption optics (Beckman XL-A analytical ultracentrifuge).
Chitosan control: sedimentation coefficient, $s \sim 1.5$ Svedbergs (S)
PGM: pig gastric mucin
HGM: human gastric mucin (Dr A. Corfield, University of Bristol)
OGM: Orthana (sialic acid free) pig gastric mucin (Orthana Ltd., Kastруп)
* A trace amount sedimenting at 415S was just visible

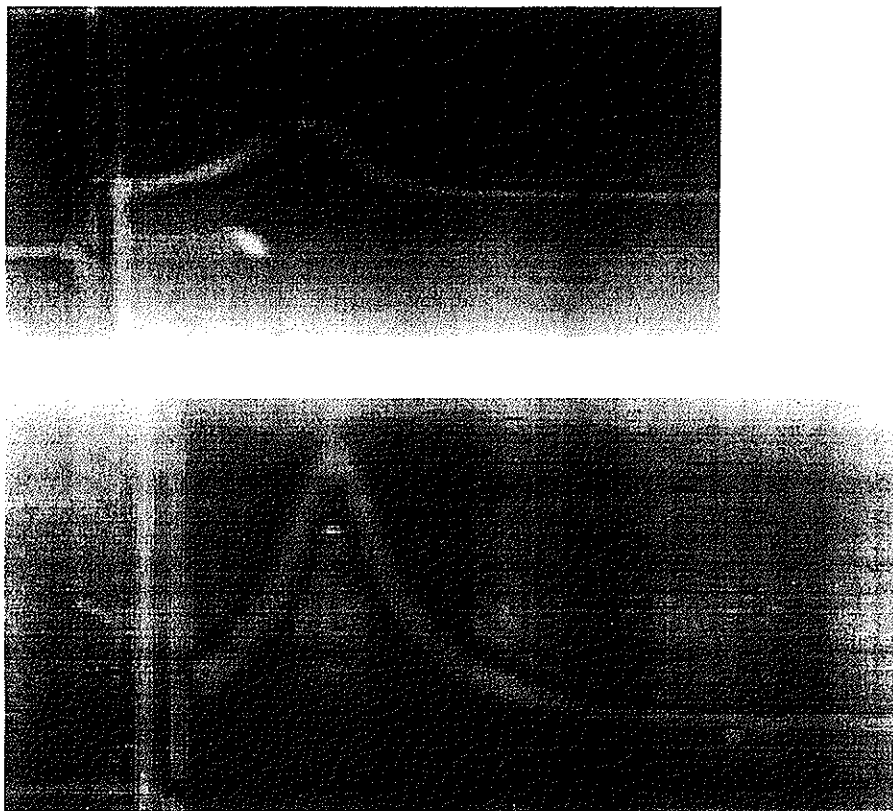


Figure 11. Sedimentation Schlieren 'fingerprinting' of mucoadhesive interaction. Top image: Schlieren image for residual chitosan (Sea Cure +210) left after the rest had complexed with pig gastric mucin at 0.1M ionic strength. Bottom image: chitosan control under the same conditions. Area under top Schlieren boundary = 247 pixels. Area under bottom Schlieren boundary = 1017 pixels. % chitosan interacted = 75.7%. Rotor speed = 35000 rev/min, temperature = 20°C. Schlieren data captured via a CCD camera onto a PowerMac computer. From Deacon *et al.* (1999).

(containing a carbonyl group for possible H-bonding and a hydrophobic methyl group) does not compromise the interaction. This suggests that non-electrostatic interactions may also be significant, or it may suggest there was still enough charge on the chitosan to interact sufficiently with all the mucin.

The addition of bile salts (*Table 6*) does not appear to compromise the interaction significantly, except at ~6mM, but still significant complex sizes were present ($s_{\text{mix}}/s_{\text{mucin}} \sim 14-18$). Lowering the pH from 6.5 towards and below the pKa of the sialic acid residues on the mucin appears to reduce the complex size ($s_{\text{mix}}/s_{\text{mucin}}$ drops from ~48 to ~22 at 20°C and from ~34 to ~12 at 37°C), but the interaction is still significant (*Table 7*). The observations of *Tables 6* and *7* are therefore supportive of the view that there may be a significant contribution from non-electrostatic as well as electrostatic types of interaction in the chitosan-mucin system. *Table 8* compares different types of gastric mucin, and although the preparation of the human gastric mucin is of much smaller molecular weight ($s \sim 11S$ corresponds to

a molecular weight between that of a T-domain and subunit), the interaction is not compromised. However, a much smaller pig gastric mucin preparation that is sialic acid free (from Orthana Ltd., Kastrup, Denmark) appears to give very little interaction.

Building on this investigation of the performance of mucins from different sources, Deacon *et al.* (1998b) have very recently investigated the mucoadhesive performance of chitosan with highly specific mucins from different regions of the stomach: corpus,

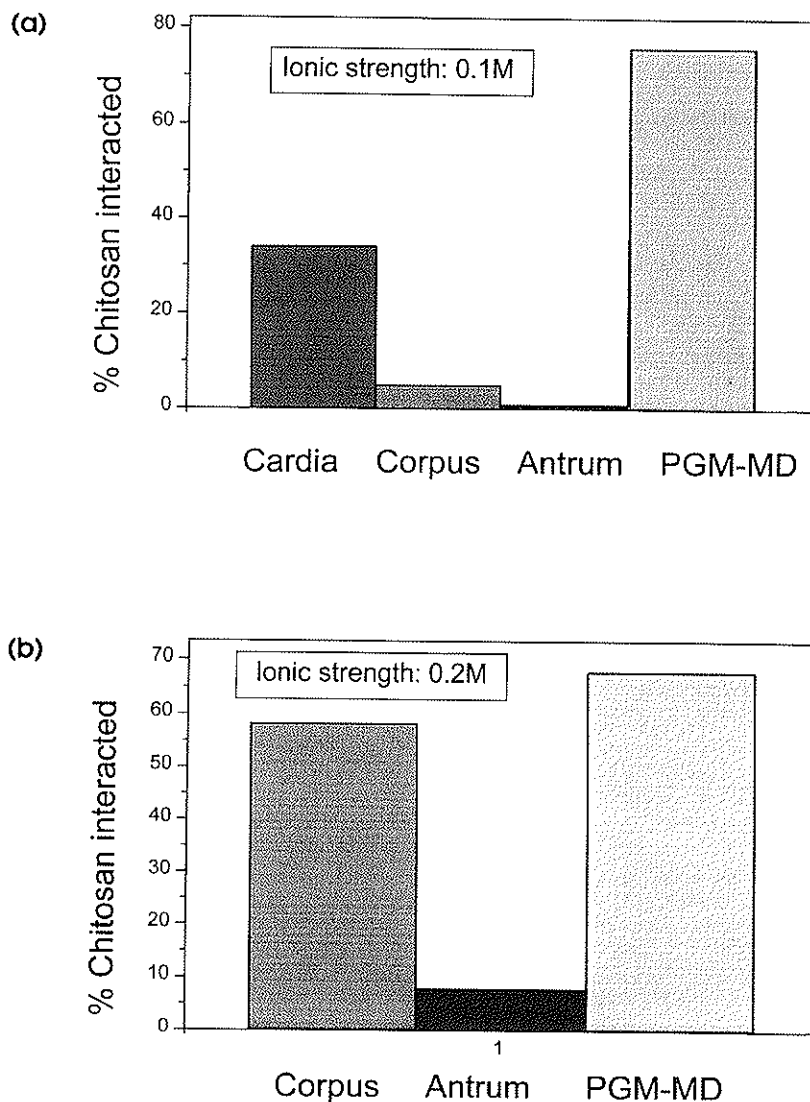


Figure 12. Comparison of amount of chitosan (Sea-Cure +210) interacted with pig gastric mucin for native pig gastric mucin prepared at Nottingham (PGM-MD) with three highly specific mucins from different regions of the stomach prepared at Lund (Cardia, Corpus and Antrum), using the Schlieren fingerprinting procedure (a) ionic strength 0.1M; (b) ionic strength = 0.2M. Other conditions as Figure 11.

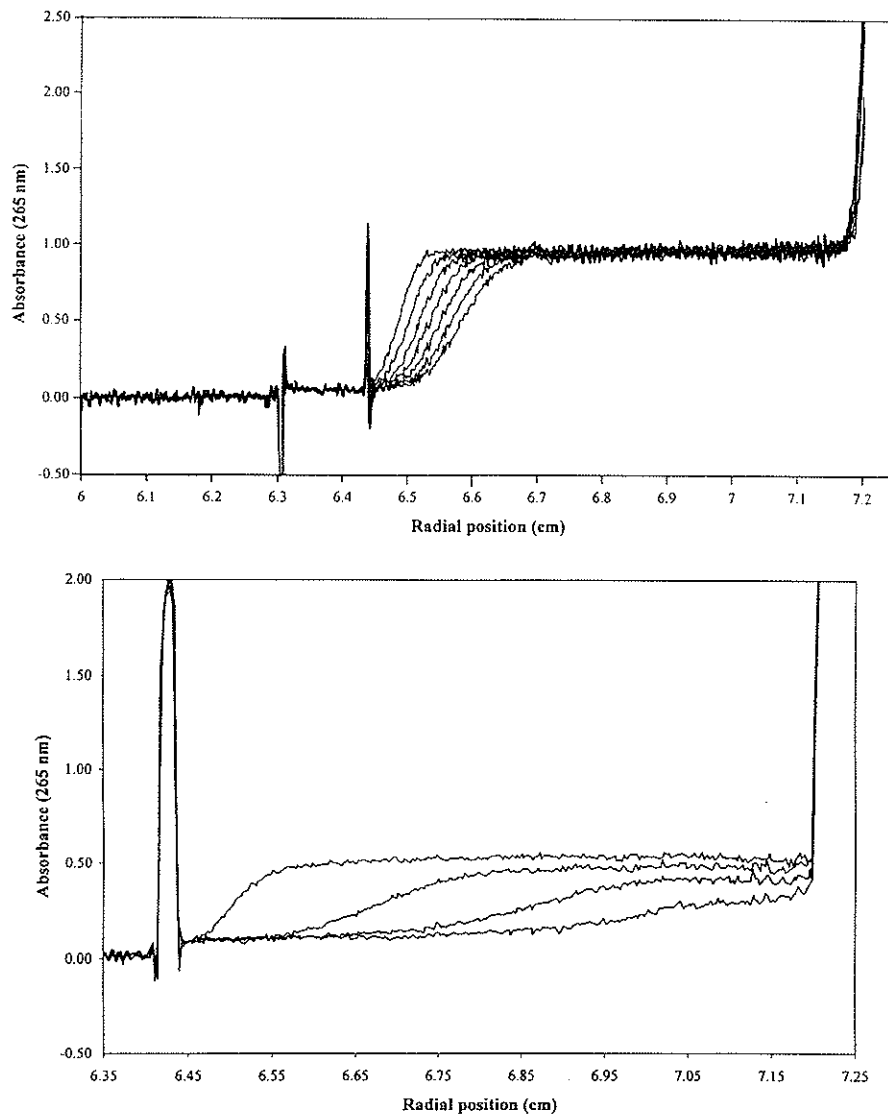


Figure 13. Sedimentation velocity analysis demonstration of mucoadhesion of the mussel glue protein *mefp-1* with pig gastric mucin. XL-A analytical ultracentrifuge used equipped with scanning uv-absorption optics (a) *mefp-1* control. Rotor speed = 40,000 rev/min, temperature = 20.0°C, scan interval = 10 min, loading concentration = 0.8mg/ml. Sedimentation coefficient, $s_{20} = (2.34 \pm 0.17)S$. (b) *mefp-1*: mucin complex (Concentration of mucin after mixing = 0.1 mg/ml; concentration of *mefp-1* after mixing = 0.4 mg/ml. Rotor speed = 2,000 rev/min, temperature = 20.0°C, scan interval = 10 min, $s_{20} \sim 7000S$. (From Deacon *et al.*, 1998).

antrum and cardia, and compared them with the performance of the 'whole' pig gastric mucin. Because of scarcity of material, the sedimentation 'fingerprinting' technique (Figure 11) had to be employed (see page 67). This involved the use of the criterion of 'amount of chitosan interacted', as determined from the areas under refractive index gradient curves (Schlieren optics on the analytical ultracentrifuge, linked on-line via

a CCD camera to a PowerMac computer). *Figure 12* shows a clear difference (under 0.1M ionic strength solvent conditions) of the interaction strength in the order cardia>corpus>antrum. Although at 0.2M insufficient cardia material was available, interestingly the corpus and antrum showed an increase in interaction strength, again consistent with the hypothesis that non-electrostatic types of interaction play a role in mucin-chitosan binding.

3. MUSSEL GLUE PROTEIN – GASTRIC MUCIN

The highly positively charged 'glue protein' *mefp-1* from the feet of *Mytilus edulis*, has to date given the strongest demonstration of an interaction with mucin, at least on the basis of molecular hydrodynamics (Deacon *et al.*, 1998). *Figure 13* compares the sedimentation velocity profile for native *mefp-1* and the mixture of *mefp-1* with mucin. The sedimentation coefficient for the native *mefp-1*, $s_{20,w}$ of 2.3S is consistent with an extended conformation for this protein (model shown in *Figure 8*) of molecular weight $M \sim 110,000$ Da which has been shown by sedimentation equilibrium in the ultracentrifuge to be monomeric in solution. However, when mixed with mucin, the *mefp-1* is completely reacted, and moves down at an $s \sim 7000$ S, and corresponds to an $s_{\text{mix}}/s_{\text{mucin}}$ of ~ 130 (*Table 9*).

From molecular hydrodynamics to dosage form

The next problem is constructing an adequate delivery system out of the mucoadhesive polymer. The best strategy would appear to be encapsulation *via* microspheres, and indeed similar encapsulation strategies have been invoked also for intravenous systems. The delivery system could be typically microspheres using e.g. alginate beads for the encapsulation of insulin producing islet of langerhans cells for the treatment of diabetics (Soon-Shiong *et al.*, 1994), or for mimicking secretory granule encapsulation systems (Siegel, 1998)

He *et al.* (1998a) have recently investigated the use of chitosan microspheres for gastroretention. Non-crosslinked and crosslinked chitosan microspheres were prepared by a spray drying method. The microspheres so prepared were spherical in shape and positively charged. The particle sizes ranged from 2 to 10 μm . The size and zeta potential of the particles were influenced by the level of crosslinking. With decreasing amount of crosslinking agent (either glutaraldehyde or formaldehyde), both particle size and zeta potential were increased. He *et al.* (1998a), found that the preparation conditions also had an influence on the particle size. DSC studies revealed that drugs incorporated in the microspheres (H2 antagonist drugs cimetidine, and famotidine) were molecularly in the form of a solid solution. The release of model drugs

Table 9. Sedimentation velocity assay: pig gastric mucin and mussel glue protein *mefp-1*

mucin: <i>mefp-1</i> ratio	Buffer and temperature	s_{mucin} (S) control	s_{mix} (S) complex	$s_{\text{mix}}/s_{\text{mucin}}$
0.1 mg/ml: 0.4 mg/ml	pH6.8, I=0.1M 20°C	53	7000	130

UV absorption optics (Beckman XL-A analytical ultracentrifuge).

Mefp-1 controls: sedimentation coefficient, $s \sim 2.3$ S

Mucin $M \sim 11$ million Da

Mefp-1 $M \sim 110000$ Da

(cimetidine, famotidine and nizatidine) from these microspheres was fast, and accompanied by a burst effect.

A modified spray drying method in the form of a novel w/o/w emulsion-spray drying technique was developed by He *et al.* (1998 b) to prepare chitosan microspheres with a sustained drug release pattern. The release of the model drugs (cimetidine, famotidine), from the microspheres prepared by the emulsion-spray drying method, was greatly retarded with release lasting for several hours, compared with drug loaded microspheres prepared by conventional-spray drying or emulsion methods where drug release was almost instantaneous. The slow release of the drug was partly due to the poor wetting ability of the microspheres, which floated on the surface of the dissolution medium. The addition of a wetting agent increased the release rate significantly. The coating of the microspheres with gelatin decreased the rate of release of drug in the presence of wetting agents.

The mucoadhesive properties of chitosan and chitosan microspheres has been evaluated by He *et al.* (1998c) studying the interaction between mucin and chitosan in aqueous solution by turbidimetric measurements and the measurement of mucin adsorbed on the microspheres. A strong interaction between chitosan microspheres and mucin was found. Adsorption studies were carried out for the adsorption of mucin to chitosan microspheres with different crosslinking levels. The adsorption of type III mucin (1% sialic acid content), to chitosan microspheres followed Freundlich or Langmuir adsorption isotherms. When the content of sialic acid was increased (i.e. type I-S mucin, 12% sialic acid content), the adsorption type followed more closely an electrostatic attraction type of isotherm. The heat of the adsorption was found to be 13–23 kJ/mol. A salt-bridge interaction was proposed for the interaction of the negatively charged mucus glycoprotein with chitosan microspheres. The level of mucin adsorption was found to be proportional to the absolute values of the positive

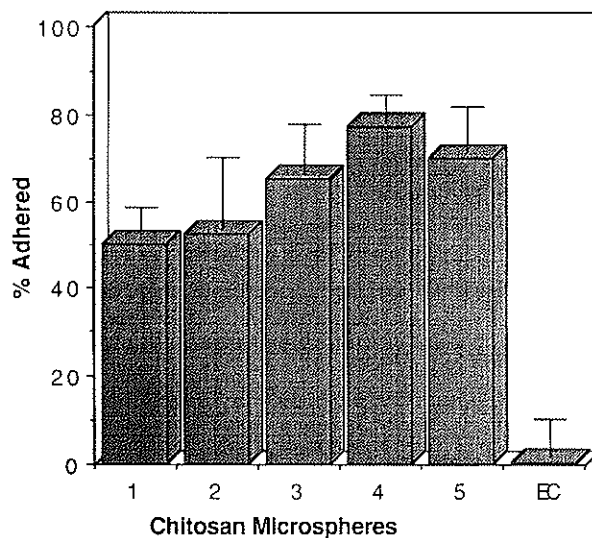


Figure 14. Mucoadhesive measurement of chitosan microspheres on rat small intestine by a particle counting technique. (EC – Ethyl cellulose microsphere).

zeta potential of chitosan microspheres and negative 'zeta potential' of mucin glycoprotein. Factors leading to a reduction or a reversal of these absolute values (e.g. different crosslinking levels of chitosan microspheres, different types of mucin, different pH, or ionic strength of the medium used) led to a reduction in the amount absorbed.

Biological studies have shown that chitosan microspheres were retained by a biological tissue; rat small intestine (*Figure 14*). Non-adhesive microsphere of the same size prepared from ethyl cellulose (EC) were used as a control. More than 50% of the administered chitosan microspheres were absorbed to the tissue, whereas but few of the EC microspheres were bound.

Further experiments are now in progress to include phase I clinical studies in human subjects, where the retention of the microspheres can be followed in a non-invasive manner through a process of labelling of the microspheres with a gamma emitting radionuclide (Indium-111)

Processes involving the coating of chitosan onto delivery systems can also be used to achieve mucoadhesion. Takeuchi *et al.* (1996) have developed a chitosan coated liposome system. The liposomes were loaded with insulin. Preferential adsorption of the coated liposomes in the rat small intestine was demonstrated. The blood glucose levels were reported to be reduced significantly after one administration of the system to rats.

Acknowledgement

We thank Professor A. Allen and Dr Peter Dettmar for their helpful comments.

References

- ACATÜRK, F. (1989). Preparation of a prolonged-release tablet formulation of diclofenac sodium. Part 1: Using chitosan. *Pharmazie* **44**, 547–549.
- AL-DUJAILI, H., SALOLE, E.G. AND FLORENCE, A.T. (1983). Drug formulation and oesophageal injury. *Adverse Drug Reactions and Acute Poisoning Reviews* **2**, 235–256.
- ALIMUNIAR, A. AND ZAINUDDIN, R. (1992). An economical technique for producing chitosan. In *Advances in Chitin and Chitosan*. Eds. C.J. Brine, P.A. Sandford and J.P. Zikakis, pp 627–632. London: Elsevier Applied Science.
- ALLEN, A. (1978). Structure of gastrointestinal mucus glycoproteins and the viscous and gel-forming properties of mucus. *British Medical Bulletin* **34**, 28–33.
- ALLEN, A. (1981). Structure and function of gastrointestinal mucus. In *Physiology of the Gastrointestinal Tract*. Ed. L.R. Johnson, pp 617–639. New York: Raven Press.
- ALLEN, A. (1983). Mucus – a protective secretion of complexity. *Trends in Biochemical Sciences* **8**, 169–173.
- ALLEN, A. (1989). Gastrointestinal mucus. In *Handbook of Physiology – The Gastrointestinal Physiology. Salivary, Gastric and Hepatobiliary Secretions*. Ed. S.G. Schultz and G.M. Makhlof, Section 6, Vol. III, pp 359–382. USA: American Physiological Society.
- ALLEN, A. AND CAROLL, N.J.H. (1985). Adherent and soluble mucus in the stomach and duodenum. *Digestive Diseases and Sciences* **30**, 55S–62S.
- ALLEN, A., FLEMSTRÖM, G., GARNER, A. AND KIVILAAKSO, E. (1993). Gastroduodenal mucosal protection. *Physiological Reviews* **73**, 823–857.
- ANDERSON, M.T., HARDING, S.E. AND DAVIS, S.S. (1989). On the interaction in solution of a candidate mucoadhesive polymer, diethylaminoethyl-dextran, with pig gastric mucus glycoprotein. *Biochemical Society Transactions*, 63rd meeting Guildford, **17**, pp 1101–1102.

- ANDERSON, M.T. (1991). The interaction of mucous glycoproteins with polymeric materials. *Ph.D. thesis*, University of Nottingham, UK.
- AOYAGI, N., OGATA, H., KANIWA, N., UCHIYAMA, M., YASUDA, Y. AND TANIOKA, Y. (1992). Gastric emptying of tablets and granules in humans, dogs, pigs, and stomach-emptying-controlled rabbits. *Journal of Pharmaceutical Sciences* **81**, 1170–1174.
- ARAI, K., KINUMAKI, T. AND FUJITA, T. (1968). Toxicity of chitosan. *Bulletin of the Tokai Regional Fisheries Research Laboratory* **56**, 89–94.
- BAHLS, D.M. AND BLOOMFIELD, V.A. (1977). Turbidimetric determination of bacteriophage molecular weights. *Biopolymers* **16**, 2797–2799.
- BATY, A.M., LEAVITT, P.K., SIEDLECKI, C.A., TYLER, B.J., SUCI, P.A., MARCHANT, R.E. AND GEESEY, G.G. (1997). Adsorption of adhesive proteins from the marine mussel, *Mytilus edulis*, on polymer films in the hydrated state using angle dependent X-ray photoelectron spectroscopy and atomic force microscopy. *Langmuir* **13**, 5702–5710.
- BEACHEY, E.H. (1980). *Bacterial Adherence*, Series B, Volume 6, London and New York: Chapman and Hall.
- BECHGAARD, H. AND LADEFOGED, K. (1978). Distribution of pellets in the gastrointestinal tract. The influence on transit time exerted by the density and diameter of pellets. *Journal of Pharmacy and Pharmacology* **30**, 690–692.
- BEERMANN, B., GROSCHINSKY-GRIND, M. AND ROSEN, A. (1976). Absorption, metabolism and excretion of hydrochlorothiazide. *Clinical Pharmacology and Therapeutics* **19**, 718–721.
- BEERMANN, B. AND GROSCHINSKY-GRIND, M. (1978). Enhancement of the gastrointestinal absorption of hydrochlorothiazide by propantheline. *European Journal of Clinical Pharmacology* **13**, 385–387.
- BELL, A.E., SELLERS, L.A., ALLEN, A., MORRIS, E., ROSS-MURPHY, S. (1985). Properties of gastric and duodenal mucus: effects of proteolysis, disulphide reduction, bile, acid, ethanol and hypertonicity on mucus gel structure. *Gastroenterology* **88**, 269–280.
- BHASKAR, K.R., GARIK, P., TURNER, B.S., BRADLEY, J.D., BANSIL, R., STANLEY, H.E. AND LAMONT, J.T. (1992). Viscous fingering of HCl through gastric mucin. *Nature* **360**, 458–461.
- BODDÉ, H.E. (1990). Principles of Bioadhesion. In *Bioadhesion – Possibilities and Future Trends*. Eds. R. Gurny and H.E. Junginger, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, APV, Vol. 25, pp 44–64.
- BOEDECKER, E.C. (1984). *Attachment of Organisms to the Gut Mucosa*. Volumes I and II. Boca Raton, Florida: CRC Press.
- BOOTH, C.C. (1967). Sites of absorption in the small intestine. *Federation Proceedings* **28**, 1583–1588.
- BOTTENBERG, P., CLEYMAET, R., DE MUYNCK, C., REMON, J.P., COOMANS, D., MICHOTTE, Y. AND SLOP, D. (1991). Development and testing of bioadhesive, fluoride-containing slow-release tablets for oral use. *Journal of Pharmacy and Pharmacology* **43**, 457–464.
- BOUCKAERT, S., TEMMERMAN, M., DHONT, M. AND REMON, J.P. (1994). The treatment of bacterial vaginosis with a bioadhesive vaginal slow-release tablet with metronidazole. *Proceedings of the International Symposium on Controlled Release of Bioactive Materials* **21**, 585–586.
- BOWMAN, W.C. AND RAND, M.J. (1980). *Textbook of Pharmacology*. Oxford: Blackwell Scientific Publications, Chapter 25.
- BRAHAMS, D. (1984). Death of a patient participating in trial of oral morphine for relief of postoperative pain. *Lancet* **1**, 1083–1084.
- BYSTRICKY, S., MALOVÍKOVÁ, A. AND STICZAY, T. (1990). Interaction of alginates and pectins with cationic polypeptides. *Carbohydrate Polymers* **13**, 283–294.
- CARGILL, R., CALDWELL, I.J., ENGLE, K., FIX, J.A., PORTER, P.A. AND GARDNER, C.R. (1988). Controlled gastric emptying. I. Effects of physical properties on gastric residence times of non-disintegrating geometric shapes in beagle dogs. *Pharmaceutical Research* **5**, 533–536.
- CARGILL, R., ENGLE, K., GARDNER, C.R., PORTER, P., SPARER, R.V. AND FIX, J.A. (1989). Controlled gastric emptying. II. *In vitro* erosion and gastric residence times of an erodible device in beagle dogs. *Pharmaceutical Research* **6**, 506–509.

- CARLSTEDT, I. AND SHEEHAN, J.K. (1984). Is the macromolecular architecture of cervical, respiratory and gastric mucins the same? *Biochemical Society Transactions* **12**, 615–617.
- CARLSTEDT, I. AND SHEEHAN, J.K. (1988). Structure and macromolecular properties of mucus glycoproteins. *Monographs in Allergy*, Basel, **24**, 16–24.
- CHEN, J.L. AND CYR, G.N. (1970). Compositions producing adhesion through hydration. In *Adhesion in Biological Systems*. Ed. R.S. Manly, pp 163–181. New York: Academic Press.
- CHEN, X., DAVIES, M.C., ROBERTS, C.J., SHAKESHEFF, K.M., TENDLER, S.J.B. AND WILLIAMS, P.M. (1996). Combined surface plasmon resonance and scanning force microscope instrument. *Journal of Vacuum Science Technology* **B14**, 1582–1586.
- CODE, C.F. AND MARLETT, J.A. (1975). The interdigestive myo-electric complex of the stomach and small bowel of dogs. *Journal of Physiology* **246**, 289–309.
- CÖLFEN, H. AND HARDING, S.E. (1997). MSTARA and MSTARI: interactive PC algorithms for simple, model independent evaluation of sedimentation equilibrium data. *Eur. Biophys. J.* **25**, 333–346.
- CÖLFEN, H., HARDING, S.E., VÅRUM, K.M., AND WINZOR, D.J. (1996). A study by analytical ultracentrifugation on the interaction between lysozyme and extensively deacetylated chitin (chitosan). *Carbohydr. Polym.* **30**, 45–53.
- CREETH, J.M. (1978). Constituents of mucus and their separation. *British Medical Bulletin* **34**, 17–24.
- DAVIS, S.S. (1985). The design and evaluation of controlled release systems for the gastrointestinal tract. *Journal of Controlled Release* **2**, 27–38.
- DAVIS, S.S., STOCKWELL, A.F., TAYLOR, M.J., HARDY, J.G., WHALLEY, D.R., WILSON, C.G., BECHGAARD, H. AND CHRISTENSEN, F.N. (1986). The effect of density on gastric emptying of single- and multiple-unit dosage forms. *Pharmaceutical Research* **3**, 208–213.
- DAVIS, S.S. (1989). Small intestine transit. In *Drug Delivery to the Gastrointestinal Tract*. Eds. J.G. Hardy, S.S. Davis and C.G. Wilson, pp 49–61. Chichester: Ellis Horwood Ltd.
- DEACON, M.P. (1999). PhD Dissertation, University of Nottingham, England.
- DEACON, M.P., DAVIS, S.S., WHITE, R., WAITE, J.H. AND HARDING, S.E. (1996). Monomeric behaviour of *Mytilus edulis* (mussel) glue protein in dilute solution. *Biochem. Soc. Trans.* **25**, 422S.
- DEACON, M.P., DAVIS, S.S., WAITE, J.H. AND HARDING, S.E. (1998). Structure and mucoadhesion of mussel glue protein in dilute solution. *Biochemistry* (in press).
- DEACON, M.P., DAVIS, S.S., WHITE, R.J., NORDMAN, H., CARLSTEDT, I., ERRINGTON, N., ROWE, A.J. AND HARDING, S.E. (1999). Are chitosan-mucin interactions specific to different regions of the stomach? Sedimentation velocity in the ultracentrifuge can give us a clue. *Carbohydrate Polymers* (in press).
- DEASY, P.B. AND O'NEILL, C.T. (1989). Bioadhesive dosage form for peroral administration of timolol base. *Pharmaceutica Acta Helveticae* **64**, 231–235.
- DE MEY, J. (1984). Colloidal gold as marker and tracer in light and electron microscopy. *Electron Microscopy Society of America* **14**, 54–66.
- DODD, S., PLACE, G.A., HALL, R.L. AND HARDING, S.E. (1998). Hydrodynamic properties of mucins secreted by primary cultures of Guinea-pig tracheal epithelial cells: determination of diffusion coefficients by analytical ultracentrifugation and kinetic analysis of mucus gel hydration and dissolution. *European Biophysical Journal* **28**, 38–47.
- DUCHÈNE, D., TOUCHARD, F. AND PEPPAS, N.A. (1988). Pharmaceutical and medical aspects of bioadhesive systems for drug administration. *Drug Development and Industrial Pharmacy* **14**, 283–381.
- ERRINGTON, N., HARDING, S.E., VÅRUM, K.M., ILLUM, L. (1993). Hydrodynamic characterization of chitosans varying in degree of acetylation. *International Journal of Biological Macromolecules* **15**, 113–117.
- FIEBRIG, I., HARDING, S.E. AND DAVIS, S.S. (1994a). Sedimentation analysis of potential interactions between mucins and a putative bioadhesive polymer. *Progress in Colloid and Polymer Science* **93**, 66–73.
- FIEBRIG, I., HARDING, S.E., STOKKE, B.T., VÅRUM, K.M., JORDAN, D. AND DAVIS, S.S. (1994b). The potential of chitosan as mucoadhesive drug carrier: studies on its interaction with pig gastric mucin on a molecular level. *European Journal of Pharmaceutical Sciences* **2**, 185.

- FIEBRIG, I., DAVIS, S.S. AND HARDING, S.E. (1995a). Methods used to develop mucoadhesive drug delivery systems: bioadhesion in the gastrointestinal tract. In *Biopolymer Mixtures*. Eds. S.E. Harding, S.E. Hill and J.R. Mitchell, Chapter 18. Nottingham: Nottingham University Press.
- FIEBRIG, I., HARDING, S.E., ROWE, A.J., HYMAN, S.C. AND DAVIS, S.S. (1995b). Transmission electron microscopy studies on pig gastric mucin and its interactions with chitosan. *Carbohydr. Polymers* **28**, 239-244.
- FIEBRIG, I., VÄRUM, K.M., HARDING, S.E., DAVIS, S.S. AND STOKKE, B.T. (1997). Colloidal gold and colloidal gold labelled wheat germ agglutinin as molecular probes for identification in mucin/chitosan complexes. *Carbohydrate Polymers* **33**, 91-99.
- FÖRSTER, H. AND LIPPOLD, B.C. (1982). Conception of peroral sustained-release dosage forms: calculation of initial and maintenance dose with consideration of accumulation. *Pharmaceutica Acta Helveticae* **57**, 345-349.
- FULKERSON, J.P., NORTON, L.A., GRONOWICZ, G., PICCIANO, P., MASSICOTTE, J.M. AND NISSEN, C.W. (1990). Attachment of epiphyseal cartilage cells and 17/28 rat osteosarcoma osteoblasts using mussle adhesive protein. *Journal of Orthopaedic Research* **8**, 793-798.
- GIBBONS, R.A. (1972). Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins. In *Glycoproteins: Their Composition, Structure and Function*. Ed. A. Gottschalk, pp 31-141. Amsterdam: Elsevier Publishing Company.
- GIEBELER, R. (1992). The Optima XL-A: A new analytical ultracentrifuge with a novel precision absorption optical system. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. Eds. S.E. Harding, A.J. Rowe and J.C. Horton, pp 16-25. Cambridge: Royal Society of Chemistry.
- GOTTSCHALK, A., BHARGAVA, A.S. AND MURTY, V.L.N. (1972). Submaxillary gland glycoproteins. In *Glycoproteins: Their Composition, Structure and Function*. Ed. A. Gottschalk, pp 810-829. Amsterdam: Elsevier Publishing Company.
- GREAVES, J.L. AND WILSON, C.G. (1993). Treatment of diseases of the eye with mucoadhesive delivery systems. *Advanced Drug Delivery Reviews* **11**, 349-383.
- GRÖNING, R. AND HEUN, G. (1984). Oral dosage forms with controlled gastrointestinal transit. *Drug Development and Industrial Pharmacy* **10**, 527-539.
- GRÖNING, R. AND HEUN, G. (1989). Dosage forms with controlled gastrointestinal passage - studies on the absorption of nitrofurantoin. *International Journal of Pharmaceutics* **56**, 111-116.
- GRUBER, P., LONGER, M.A. AND ROBINSON, J.R. (1987). Some biological issues in oral controlled drug delivery. *Advanced Drug Delivery Reviews* **1**, 1-18.
- GRUNDY, D. (1985). *Gastrointestinal Motility*. Lancaster: MTP Press Ltd.
- GU, J.-M., ROBINSON, J.R. AND LEUNG, S.-H.S. (1988). Binding of acrylic polymers to mucin/epithelial surfaces: structure-property relationships. *Critical Reviews in Therapeutic Drug Carrier Systems* **5**, 21-67.
- HALLETT, P., ROWE, A.J. AND HARDING, S.E. (1984). A highly expanded spheroidal conformation for a mucin from a cystic fibrosis patient: new evidence from electron microscopy. *Transactions of the Biochemical Society* **12**, 878-879.
- HANSEN, D.C., LUTHER, G.W. AND WAITE, J.H. (1994). The adsorption of the adhesive protein of the blue mussel *Mytilus-edulis-L* onto type 304L stainless steel. *Journal of Colloid and Interface Science* **168**, 206-216.
- HARDING, S.E. (1989). The macrostructure of mucus glycoproteins in solution. *Advances in Carbohydrate Chemistry and Biochemistry* **47**, 345-381.
- HARDING, S.E. (1984). An analysis of the heterogeneity of mucins. No evidence for a self-association. *Biochemical Journal* **219**, 1061-1064.
- HARDING, S.E. (1986). Applications of light scattering in microbiology. *Biotechnology and Applied Biochemistry* **8**, 489-509.
- HARDING, S.E. (1989). The macrostructure of mucus glycoproteins in solution. *Advances in Carbohydrate Chemistry and Biochemistry* **47**, 345-381.
- HARDING, S.E. (1995). On the hydrodynamic analysis of macromolecular conformation. *Biophysical Chemistry* **55**, 69-93.
- HARDING, S.E. (1997a). The intrinsic viscosity of biological macromolecules. Progress in

- measurement, interpretation and application to structure in dilute solution. *Progress in Biophysics and Molecular Biology*. Ed. T.L. Blundell, **68**, 207–262.
- HARDING, S.E. (1997b). Chitosan-Mucin Complexes. Characterisation by sedimentation velocity analytical ultracentrifugation. In *Chitin Handbook*. Eds. R.A.A. Muzzarelli and M.G. Peter, pp 457–466. European Chitin Society.
- HARDING, S.E., ROWE, A.J., AND CREETH, J.M. (1983a). Further evidence for a flexible and highly expanded model for mucus glycoproteins in solution. *Biochemical Journal* **209**, 893–896.
- HARDING, S.E., CREETH, J.M. AND ROWE, A.J. (1983b). Modelling the conformation of mucus glycoproteins in solution. In *Proceedings of the 7th International Glucoconjugates Conference*. Eds. A. Chester, D. Heinegard, A. Lundblad and S. Svensson. pp 558–559. Sweden: Olsson-Reklambyra.
- HARDING, S.E. AND WINZOR, D.J. (1999). Analytical Ultracentrifugation. In *Protein-Ligand Interactions: A Practical Approach*. Eds. S.E. Harding and P.Z. Chowdhry, Vol. 1, Chap. 8. Oxford: Oxford University Press.
- HARPER, C.M. AND RALSTON, M. (1983). Isobutyl 2-cyanoacrylate as an osseous adhesive in the repair of osteochondral fractures. *Journal of Biomedical Materials Research* **17**, 167–177.
- HARRIS, D., FELL, J.T., SHARMA, H.L. AND TAYLOR, D.C. (1990a). GI transit of potential bioadhesive formulations in man: a scintigraphic study. *Journal of Controlled Release* **12**, 45–53.
- HARRIS, D., FELL, J.T., TAYLOR, D.C., LYNCH, J. AND SHARMA, H.L. (1990b). GI transit of potential bioadhesive systems in the rat. *Journal of Controlled Release* **12**, 55–65.
- HAYTON, W.L. (1980). Rate-limiting barriers to intestinal drug absorption: A review. *Journal of Pharmacology and Biopharmaceutics* **8**, 321–334.
- HE, P., DAVIS, S.S. AND ILLUM, L. (1998a). Chitosan microspheres prepared by spray drying. *International Journal of Pharmaceutics* (in press).
- HE, P., DAVIS, S.S. AND ILLUM, L. (1998b). Sustained release chitosan microspheres prepared by novel spray-drying methods. *Journal of Microencapsulation* (in press).
- HE, P., DAVIS, S.S. AND ILLUM, L. (1998c). *In vitro* evaluation of the microadhesive properties of chitosan microspheres. *International Journal of Pharmaceutics* **166**, 75–68.
- HELLIWELL, M. (1993). The use of bioadhesives in targeted delivery within the gastrointestinal tract. *Advanced Drug Delivery Reviews* **11**, 221–251.
- HIRANO, S. (1989). Production and application of chitin and chitosan in Japan. In *Chitin and Chitosan*. Ed. G. Skjåk-Bræk, T. Anthonsen, T. and P. Sandford, pp 37–43. London: Elsevier Applied Science.
- HOLLINGSBEE, D.A. AND TIMMINS, P. (1990). Topical adhesive systems. In *Bioadhesion – Possibilities and Future Trends*. Eds. R. Gurny and H.E. Junginger, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, APV, Vol. 25, pp 140–164.
- HORISBERGER, H. AND ROSSET, J. (1977). Colloidal gold, a useful marker for transmission and scanning electron microscopy. *Journal of Histochemistry and Cytochemistry* **25**, 295–305.
- HORISBERGER, M. AND CLERC, M.-F. (1988). Chitosan-colloidal gold complexes as polycationic probes for the detection of anionic sites by transmission and scanning electron microscopy. *Histochemistry* **90**, 165–175.
- HÖRSTEDT, P., DANIELSSON, A., NYHLIN, H., STENLING, R. AND SUHR, O. (1989). Adhesion of bacteria to the human small-intestinal mucosa. *Scandinavian Journal of Gastroenterology* **24**, 877–885.
- HOUNSELL, YOUNG, M. AND DAVIES, M.J. (1997). Glycoprotein changes in tumours: a renaissance in clinical applications. *Clinical Science* **93**, 287–293.
- HUNTSBERGER, J.R. (1967). Mechanisms of adhesion. *Journal of Paint Technology* **36**, 199–211.
- HWANG, S.-J., PARK, H. AND PARK, K. (1998). Gastric retentive drug-delivery systems. *Critical Reviews in Therapeutic Drug Carrier Systems* **15**, 243–284.
- IMERI, A.G. AND KNORR, D. (1988). Effects of chitosan on yield and compositional data of carrot and apple juice. *Journal of Food Science* **53**, 1707–1709.

- ITO, S. (1981). Functional gastric morphology. In *Physiology of the Gastrointestinal Tract*. Ed. L.R. Johnson, Vol. 1, pp 517–555. New York: Raven Press.
- JABBARI, E., WISNIEWSKI, N. AND PEPPAS, N.A. (1993). Evidence of mucoadhesion by chain interpenetration at a poly(acrylic acid)/mucin interface using ATR-FTIR spectroscopy. *Journal of Controlled Release* **26**, 99–108.
- JEUNIAUX, C., VOSS-FOUCHART, M.-F., POULICEK, M. AND BUSSERS, J.-C. (1989). Sources of chitin, estimated from new data on chitin biomass and production. In *Chitin and Chitosan*. Eds. G. Skjåk-Bræk, T. Anthonsen and P. Sandford, pp 3–11. London: Elsevier Applied Science.
- JORDAN, N., NEWTON, J., PEARSON, J. AND ALLEN, A. (1998). A novel method for the visualization of the *in situ* mucus layer in rat and man. *Clinical Science* **95**, 97–106.
- JUMEL, K., FIEBRIG, I. AND HARDING, S.E. (1995). Rapid size distribution and purity analysis of gastric mucus glycoproteins by size exclusion chromatography/ multi angle laser light scattering. *International Journal of Biological Macromolecules* **18**, 133–139.
- JUMEL, K., FOGG, F.J.J., HUTTON, D.A., PEARSON, J.P., ALLEN, A. AND HARDING, S.E. (1997). A polydisperse linear random coil model for the quaternary structure of pig colonic. *European Biophysical Journal* **25**, 477–480.
- JUNGINGER, H.E., LEHR, C.-M., BOUWSTRA, J.A., TUKKER, J.J. AND VERHOEF, J. (1990). Site specific intestinal absorption using bioadhesives: Improved oral delivery of peptide drugs by means of bioadhesive polymers. In *Bioadhesion – Possibilities and Future Trends*. Eds. R. Gurny and H.E. Junginger, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, APV, Vol. 25, pp 177–190.
- JUNGINGER, H.E. AND LEHR, C.-M. (1990). Bioadhäsive Arzneistoffabgabesysteme und Arzneiformen für perorale und rektale Anwendung. *Deutsche Apotheker Zeitung* **130**, 791–801.
- JUNGINGER, H.E. AND VERHOEF, J.C. (1992). Perorale Applikation von Peptiden und Proteinen. *Deutsche Apotheker Zeitung* **132**, 1279–1290.
- KAELBE, D.H. AND MOACANIN, J. (1977). A surface energy analysis of bioadhesion. *Polymer* **18**, 475–481.
- KATAYAMA, T., TAKAI, K.-I., KARIYAMA, R. AND KANEMASA, Y. (1978). Colloid titration of heparin using cat-floc (polydiallyldimethyl ammonium chloride) as standard polycation. *Analytical Biochemistry* **88**, 382–387.
- KAUS, L.C. (1987). The effect of density on the gastric emptying and intestinal transit of solid dosage forms: comments on the article by Davis *et al.* *Pharmaceutical Research* **4**, 78.
- KERSS, S., ALLEN, A., GARNER, A. (1982). A simple method for measuring thickness of the mucus gel layer adherent to rat, frog and human gastric mucosa: influence of feeding, prostaglandin, N-acetylcysteine and other agents. *Clinical Science* **63**, 187–195.
- KIKENDALL, J.W., FRIEDMAN, A.C., OYEWOLE, M.A., FLEISCHER, D. AND JOHNSON, L.F. (1983). Pill-induced esophageal injury. *Digestive Diseases and Sciences* **28**, 174–181.
- KORNEEVA, G.A., VICHOREVA, G.A., HARDING, S.E. AND PAVLOV, G.M. (1996). Hydrodynamic study of carboxymethyl chitin. *Abstracts American Chemical Society* **212** (part 1) 75-cell.
- LAURSEN, R.A. (1992). In *Results and Problems in Cell Differentiation*, vol 19, Biopolymers. Ed. S.T. Case, pp 55–74. Berlin: Springer Verlag.
- LEHR, C.-M., BOUWSTRA, J.A., TUKKER, J.J. AND JUNGINGER, H.E. (1990). Intestinal transit of bioadhesive microspheres in an *in situ* loop in the rat. A comparative study with copolymers and blends based on poly(acrylic acid). *Journal of Controlled Release* **13**, 51–62.
- LEHR, C.-M., POELMA, F.G.J., JUNGINGER, H.E. AND TUKKER, J. (1991). An estimate of turnover time of intestinal mucus gel layer in the rat *in situ* loop. *International Journal of Pharmaceutics* **70**, 235–240.
- LEHR, C.-M., BOUWSTRA, J.A., KOK, W., DE BOER, A.G., TUKKER, J.J., VERHOEF, J.C., BREIMER, D.D. AND JUNGINGER, H.E. (1992a). Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. *Journal of Pharmacy and Pharmacology* **44**, 402–407.
- LEHR, C.-M., BOUWSTRA, J.A., SCHACHT, E.H. AND JUNGINGER, H.E. (1992b). *In vitro*

- evaluation of mucoadhesive properties of chitosan and some other natural polymers. *International Journal of Pharmaceutics* **78**, 43–48.
- LEHR, C.-M., BOUWSTRA, J.A., SPEIS, F., ONDERWATER, J., VAN HET NOORDEINDE, J., VERMEIJ-KEERS, C., VAN MUNSTEREN, C.J. AND JUNGINGER, H.E. (1992c). Visualization studies of the mucoadhesive interface. *Journal of Controlled Release* **18**, 249–260.
- LEHR, C.-M., BOUWSTRA, J.A., KOK, W., NOACH, A.B.J., DE BOER, A.G. AND JUNGINGER, H.E. (1992d). Bioadhesion by means of specific binding of tomato lectin. *Pharmaceutical Research* **9**, 547–553.
- LEUNG, S.S.-H. AND ROBINSON, J.R. (1988). The contribution of anionic polymer structural features to mucoadhesion. *Journal of Controlled Release* **5**, 223–231.
- LIPPOLD, B.C. AND GÜNTHER, J. (1991). *In vivo* Prüfung einer multipartikulären Retard-Schwimmzartneiform. *European Journal of Pharmacy and Biopharmaceutics* **37**, 254–261.
- LYNCH, J., POWNALL, R.E. AND TAYLOR, D.C. (1987). Site-specific absorption of hydrochlorothiazide in the rat intestine. *Journal of Pharmacy and Pharmacology* **39**, 55P.
- MACADAM, A. (1993). The effect of gastro-intestinal mucus on drug absorption. *Advanced Drug Delivery Reviews* **11**, 201–220.
- MANNINEN, V., APAJALAHTI, A., MELIN, J. AND KARESOJA, M. (1973). Altered absorption of digoxin in patients given propantheline and metoclopramide. *Lancet* **1**, 398–400.
- MCCURDY, J.D. (1992). FDA and the use of chitin and chitosan derivatives. In *Advances in Chitin and Chitosan*. Eds. C.J. Brine, P.A. Sandford and J.P. Zikakis, pp 659–662. London: Elsevier Applied Science.
- MERGENHAGEN, S.E. AND ROSAN, B. (1985). *Molecular Basis of Oral Microbial Adhesion*. American Society for Microbiology, Washington, DC.
- MIKOS, A.G. AND PEPPAS, N.A. (1990). Bioadhesive analysis of controlled-release systems. IV. An experimental method for testing the adhesion of microparticles with mucus. *Journal of Controlled Release* **12**, 31–37.
- MINAMI, H. AND MCCALLUM, R.W. (1984). The physiology and pathophysiology of gastric emptying in humans. *Gastroenterology* **86**, 1592–1610.
- MIYAYAKI, S., YAMAGUCHI, H., TAKADA, M., HOU, W.-M., TAKEICHI, Y. AND YASABUCHI, H. (1990). Pharmaceutical application of biomedical polymers. *Acta Pharmaceutica Nordica* **2**, 401–406.
- MOËS, A.J. (1993). Gastroretentive dosage forms. *Critical Reviews in Therapeutic Drug Carrier Systems* **10**, 143–195.
- MORTAZAVI, S.A., CARPENTER, B.G. AND SMART, J.D. (1993). A comparative study on the role played by mucus glycoproteins in the rheological behaviour of the mucoadhesive/mucosal interface. *International Journal of Pharmaceutics* **94**, 195–201.
- MORTAZAVI, S.A. AND SMART, J.D. (1993). An investigation into the role of water movement and mucus gel dehydration in mucoadhesion. *Journal of Controlled Release* **25**, 197–203.
- MÜLLER-LISSNER, S.A. AND BLUM, A.L. (1981). The effect of specific gravity and eating on gastric emptying of slow-release capsules. *New England Journal of Medicine* **304**, 1365–1366.
- NAGAI, T., NISHIMOTO, Y., NAMBU, N., SUZUKI, Y., SEKINE, K. (1984). Powder dosage form of insulin for nasal administration. *Journal of Controlled Release* **1**, 15–22.
- NAGAI, T. (1986). Topical mucosal adhesive dosage forms. *Medicinal Research Reviews* **6**, 227–242.
- NAGAI, T., MACHIDA, Y., SUZUKI, Y. AND IKURA, H. (1983). *Japan Pat.* 1,177,734, May 13, 1983.
- NEUTRA, M.R. AND FORSTNER, J.F. (1987). Gastrointestinal mucus: Synthesis, secretion and function. In *Physiology of the Gastrointestinal Tract*. Ed. L.R. Johnson, pp 975–1009. New York: Raven Press.
- NOTTER, M.F.D. (1988). Selective attachment of neural cells to specific substrates including cell-tak, a new cellular adhesive. *Exp. Cell Res.* **177**, 237–246.
- OLIVIERI, M.P., BAIER, R.E. AND LOOMIS, R.E. (1992). Surface properties of mussel adhesive protein-component films. *Biomaterials* **13**, 1000–1008.
- OLIVIERI, M.P., RITTLE, K.H., TWEDEN, K.S. AND LOOMIS, R.E. (1992). Comparative

- biophysical study of adsorbed calf serum, fetal bovine serum and mussel adhesive protein-component films. *Biomaterials* **13**, 201–208.
- PARK, K. AND PARK, H. (1987). Enzyme-digestible balloon hydrogels for long-term oral drug delivery: synthesis and characterization. *Proceedings of the International Symposium on Controlled Release of Bioactive Materials* **14**, 41–42.
- PARK, K. (1989). A new approach to study mucoadhesion: colloidal gold staining. *International Journal of Pharmaceutics* **53**, 209–217.
- PAVLOV, G.M., ROWE, A.J. AND HARDING, S.E. (1997). Conformation zoning of large molecules using the analytical ultracentrifuge. *Trends in Analytical Chemistry* **16**, 401–405.
- PEPPAS, N.A. AND BURI, P.A. (1985). Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues. *Journal of Controlled Release* **2**, 257–275.
- PIMIENTA, C., LENAERTS, V., CADIEUX, C., RAYMOND, P., JUHASZ, J., SIMARD, M.A. AND JOLICOEUR, C. (1990). Mucoadhesion of hydroxypropylmethacrylate nanoparticles to rat intestinal ileal segments *in vitro*. *Pharmaceutical Research* **7**, 49–53.
- PIMIENTA, C., CHOUINARD, F., LABIB, A. AND LENAERTS, V. (1992). Effect of various poloxamer coatings on *in vitro* adhesion of isohexylcyanoacrylate nanospheres to rat ileal segments under liquid flow. *International Journal of Pharmaceutics* **80**, 1–8.
- POELMA, F.G.J. AND TUKKER, J.J. (1987). Evaluation of a chronically isolated internal loop in the rat for the study of drug absorption kinetics. *Journal of Pharmaceutical Sciences* **76**, 433–436.
- PONCHEL, G.F., TOUCHARD, F., DUCHÊNE, D. AND PEPPAS, N.A. (1987a). Bioadhesive analysis of controlled-release systems. I. Fracture and interpenetration analysis in poly(acrylic acid)-containing systems. *Journal of Controlled Release* **5**, 129–141.
- PONCHEL, G.F., TOUCHARD, F., WOUESSIDJEW, D., DUCHÊNE, D. AND PEPPAS, N.A. (1987b). Bioadhesive analysis of controlled-release systems. III. Bioadhesive and release behaviour of metronidazole-containing poly(acrylic acid)-hydroxypropyl methylcellulose systems. *International Journal of Pharmaceutics* **38**, 65–70.
- ROBERTS, C.J., SHIVJI, A., DAVIES, M.C., DAVIS, S.S., FIEBRIG, I., HARDING, S.E., TENDLER, S.J.B., WILLIAMS, P.M. (1995). A study of highly purified pig gastric mucin by scanning tunneling microscopy. *Protein Peptide Letters* **2**, 409–414.
- ROBIN, J.B., PICCIANO, P., KUSLEIKA, R.S., SALAZAR, J. AND BENEDICT, C. (1988). Preliminary evaluation of the use of mussel adhesive protein in experimental epikeratoplasty. *Archives of Ophthalmology* **106**, 973–977.
- ROBINSON, R., LONGER, M.A., VEILLARD, M. (1987). Bioadhesive polymers for controlled drug delivery. In *Annals New York Academy of Science*. Ed. R.L. Juliano, Vol. 507, pp 307–314.
- ROBINSON, J.R. (1990). Rationale of bioadhesion/mucoadhesion. In *Bioadhesion – Possibilities and Future Trends*. Eds. R. Gurny and H.E. Junginger, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, APV, Vol. 25, pp 13–28.
- ROSS-MURPHY, S.B. (1995). Small deformation rheological behaviour of biopolymer mixtures. In *Biopolymer Mixtures*. Eds. S.E. Harding, S.E. Hill and J.R. Mitchell, pp 85–98. Nottingham: Nottingham University Press.
- SAETTON, M.F., CHETONI, P., TORRACCA, M.T., BURGALASSI, S. AND GIANNACCINI, B. (1989). Evaluation of muco-adhesive properties and *in vivo* activity of ophthalmic vehicles based on hyaluronic acid. *International Journal of Pharmaceutics* **51**, 203–212.
- SANGEKAR, S., VADINO, W.A., CHAUDRY, I., PARR, A., BEIHN, G. AND DIGENIS, G. (1987). Evaluation of the effect of food and specific gravity of tablets on gastric retention time. *International Journal of Pharmaceutics* **35**, 187–191.
- SCHAUER, R. (1992). Sialinsäurereiche Schleime als bioaktive Schmierstoffe. *Nachrichten aus Chemie Technik und Laboratorium* **40**, 1227–1232.
- SCHNURRER, J. AND LEHR, C.-M. (1996). Mucoadhesive properties of the mussel adhesive protein. *Int. J. Pharmaceutics* **141**, 251–256.
- SCHOR, J.M., DAVIS, S.S., NIGALAYE, A. AND BOLTON, S. (1983). Susadrin transmucosal tablets (nitroglycerin in synchro controlled release base). *Drug Development and Industrial Pharmacy* **9**, 1359–1377.
- SENIJU, R. (1969). *Koroido Tekireiho [Colloid Titration]*, Tokyo: Nankodo.

- SHAKESHEFF, K.M., CHEN, X., DAVIES, M.C., DOMB, A., ROBERTS, C.J., TENDLER, S.J.B. AND WILLIAMS, P.M. (1995). Relating the phase morphology of a biodegradable polymer blend to erosion kinetics using simultaneous *in-situ* atomic-force microscopy and surface-plasmon resonance analysis. *Langmuir* **11**, 3921–3927.
- SHEEHAN, J.K. AND CARLSTEDT, I. (1989). Models for the macromolecular structure of mucus glycoproteins. In *Dynamic Properties of Biomolecular Assemblies*. Eds. S.E. Harding and A.J. Rowe, pp 256–275. Cambridge: Royal Society of Chemistry.
- SHETH, P.R. AND TOSSOUNIAN, J. (1984). The hydrodynamically balanced system (HBS®): a novel drug delivery system for oral use. *Drug Development and Industrial Pharmacy* **10**, 313–339.
- SIEGEL, R.A. (1998). A lesson from secretory granules. *Nature* **394**, 427–428.
- SILBERBERG, A. AND MEYER, F.A. (1982). Structure and function of mucus. In *Mucus in Health and Disease-II*. Eds. E.N. Chantler, J.B. Elder and M. Elstein, pp 53–74. New York: Plenum Press.
- SILVER, F.H., LIBRIZZI, J., PINS, G., WANG, M.-C. AND BENEDETTO, D. (1994). Physical properties of hyaluronic acid and hydroxypropylmethylcellulose in solution: Evaluation of coating ability. *Journal of Applied Biomaterials* **5**, 89–98.
- SILKOWSKI, H., DAVIS, S.J., BARCLAY, A.N., ROWE, A.J., HARDING, S.E. AND BYRON, O. (1997). Characterisation of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation. *European Biophysical Journal* **25**, 455–462.
- SLOMIANY, B.L. AND MEYER, K. (1972). Isolation and structural studies of sulfated glycoproteins of hog gastric mucosa. *Journal of Biological Chemistry* **247**, 5062–5070.
- SMART, J.D., KELLAWAY, I.W. AND WORTHINGTON, H.E.C. (1984). An *in-vitro* investigation of mucosa-adhesive materials for use in controlled drug delivery. *Journal of Pharmacy and Pharmacology* **36**, 295–299.
- SMART, J.D. (1991). An *in vitro* assessment of some mucosa-adhesive dosage forms. *International Journal of Pharmaceutics* **73**, 69–74.
- SMART, J.D. (1993). Drug delivery using buccal-adhesive systems. *Advanced Drug Delivery Reviews* **11**, 253–270.
- SMID-KORBAR, J., KRISTL, J., COP, L. AND GROSELJ, D. (1991). Formulation and evaluation of oral mucoadhesive films containing metronidazole. *Acta Pharmaceutica Jugoslavica* **41**, 251–258.
- SOLDANI, H., MACCHERONI, M., MARTELLI, F., MENGOZZI, G. AND CARDINI, G. (1987). Biochemistry and pharmacology of Diethylaminoethyl-dextran (DEAE-D). *Int. J. of Obesity* **11**, 201–207.
- SOON-SHIONG, P., HEINTZ, P., MERIDETH, N., YAO, Q.X., YAO, Z., ZENG, T., MURPHY, M., MALONY, M.K., SCHMEHL, M., HARRIS, M., MENDEZ, R. AND SANDFORD, P. (1994). Insulin independence in a Type I diabetic patient after encapsulated islet transplantation. *Lancet* **343**, 950–951.
- SOTO PERALTA, N.V., MÜLLER, H. AND KNORR, D. (1989). Effects of chitosan treatments on the clarity and colour of apple juice. *Journal of Food Science* **54**, 495–496.
- TAKAHASHI, T., TAKAYAMA, K., MACHIDA, Y. AND NAGAI, T. (1990). Characteristics of polyion complexes of chitosan with sodium alginate and sodium polyacrylate. *International Journal of Pharmaceutics* **61**, 35–41.
- TAKEUCHI, H., YAMAMOTO, H., NIWA, T., HINO, T., KAWASHIMA, Y. (1996). Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm. Res.* **13**, 896–901.
- TANFORD, C. (1961). *Physical Chemistry of Macromolecules*. Chap. 5. New York: John Wiley and Sons.
- TAYLOR, S.W., WAITE, J.H., ROSS, M.M., SHABANOWITZ, J. AND HUNT, D.F. (1994). *Trans-2,3-cis-3,4-dihydroxyproline*, a new naturally-occurring amino-acid, is the 6th residue in the tandemly repeated consensus decapeptides of an adhesive protein from *Mytilus edulis*. *Journal of the American Chemical Society* **116**, 10803–10804.
- TERAYAMA, H. (1952). Method of colloid titration (a new titration between polymer ions). *Journal of Polymer Science* **8**, 243–253.