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Innovative methods and applications in mucoadhesion research

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Abstract

The present review is aimed at elucidating relatively new aspects of mucoadhesions/mucus interaction and related phenomena that emerged from a Mucoadhesion workshop held in Munster on 2-3 September 2015 as a satellite event of the ICCC 13th –EUCHIS 12th. After a brief outline of the new issues, the focus is on mucus description, purification and mucus/mucin characterization, all steps that are pivotal to the understanding of mucus related phenomena and the choice of the correct mucosal model for in vitro and ex-vivo experiments, alternative bio/mucomimetic materials are also presented. Then a selection of preparative techniques and testing methods are described (at molecular as well as micro- and macroscale) that may support the pharmaceutical development of mucus-interactive-systems and assist formulators in the scale-up and industrialization steps. Recent applications of mucoadhesive systems (including medical devices) intended for different routes of administration (oral, gastro-intestinal, vaginal, nasal, ocular and intravesical) and for the treatment of difficult to treat pathologies or the alleviation of symptoms are described.
## Contents

1. Introduction ................................................................................................................................... 4

2. Mucoadhesion ...................................................................................................................................... 4

3. Mucous composition and properties as a function of location ................................................................. 6

4. Preparation of mucin or mucus ................................................................................................................. 8
   a. Purification of secreted mucins ............................................................................................................... 8
   b. Biomimetic approaches ............................................................................................................................ 10

5. Preparation of electrospun mucoadhesive formulations .............................................................................. 12

6. Methods for molecular scale testing of mucoadhesion ............................................................................. 15
   a. Spectroscopic studies ............................................................................................................................... 15
   b. Atomic Force Microscopy .......................................................................................................................... 19
   c. Scattering techniques (SAXS, SANS, SLS and DLS) .............................................................................. 21

7. Methods for macroscale testing of mucoadhesion .................................................................................... 26
   a. Rheology including polymer interaction in dilute solution ....................................................................... 28
   c. Inclined plane ........................................................................................................................................ 33
   d. Tensile testing ........................................................................................................................................ 38

8. Cellular methods ....................................................................................................................................... 38

9. Methods for characterising mucus permeability ..................................................................................... 40

10. Application specific requirement ........................................................................................................... 42
    a. Gastrointestinal drug delivery ................................................................................................................. 42
    b. Advances in the therapy of Helicobacter pylori .................................................................................... 43
    c. In the oral cavity ..................................................................................................................................... 48
    d. Colorectal drug delivery ........................................................................................................................ 49
    e. Vaginal drug delivery ............................................................................................................................... 53
    f. Nasal delivery ......................................................................................................................................... 54
    g. Ocular delivery ....................................................................................................................................... 60
    h. Intravesical drug delivery ....................................................................................................................... 64

12. Conclusion .............................................................................................................................................. 67

13. References ............................................................................................................................................ 68
1 1. Introduction

The present review stems from a Mucoadhesion workshop held in Munster on 2-3 September 2015 as a satellite event of the ICC 13th–EUCHIS 12th, held on 30 August–2 September. We are perfectly aware that there are a significant number of reviews and papers already available in the current literature on mucoadhesion and on the relevant pharmaceutical applications. Thus, in order to avoid duplications, the present review represents an update of the topic but with special focus on some new aspects of mucoadhesion/mucus interactions, taking inspiration and advantage of the multidisciplinary nature of the above conference which gathered biomedical as well as food technology and physical-chemistry experts.

2. Mucoadhesion

Definition of Mucoadhesion: It is common knowledge that mucoadhesion is a special case of bioadhesion, which is the ability of a material to adhere to a biological substrate. Thus in mucoadhesion the biological substrate is represented by mucosal tissue.

Opportunities and applications: The advantages are at least theoretically well understood. Mucoadhesive formulations are used to temporarily immobilize a delivery device on a specific site for targeted release and optimal drug delivery due to intimacy and duration of contact. Indeed it is no news that the short residence times of formulations (due to the various removal and dilution effects depending on the route of administration) at their intended site of action/absorption may result in reduced availability to the target tissue. Over the last 30 years, mucoadhesive polymers and formulations thereof have been developed for buccal, nasal, ocular, vaginal and oral applications. So far, a considerable number of papers focusing on the mucoadhesive properties of a wide range of polymeric materials have been published \(^{1-3}\). Such
a huge effort has not been paralleled by an increase of clinical applications which are still
limited to a two digit number \[4\].

Mechanisms: Concerning the mechanisms, it is recognised and experimentally proven that the
interaction between the mucus and mucoadhesive polymers is a result of physical entanglement
and secondary bonding, mainly H-bonding and van der Waals attraction, which according the
many authors, are mainly related to the following polymer properties: capability to create
strong H-bonding, high molecular weight, sufficient chain flexibility, and surface energy
properties favouring spreading onto mucus \[5\].

Testing methods: It has also to be recognized that a variety of in vitro tests have been developed
by different research groups with the aim of understanding the phenomenon at different length
scales, from observational (tensile testing, flow retention experiments) to molecular, using
sophisticated techniques from fluorescence and confocal microscopy to a variety of
spectroscopic techniques.

The new approaches-innovative aspects: In recent years, other concepts have emerged in the
literature in relation to mucoadhesion. The first observation is that in many physiological
situations the mucus layer is the main actor, and the focus should therefore be on its nature and
complexity/variability depending on the anatomical site and on its sensitivity to various physio-
pathological stimuli. It must also be recognized that mucins and mucus are quite different
substrates and their interactions with mucoadhesives are different and should be taken into
account when dealing with testing methods.

Mucus and food interactions: The comprehension of mucus interactions is also relevant in food
technology for food progression and nutrient digestion and absorption. There is a need to link
the knowledge acquired in this field to the problem of drug delivery.
Mucus penetration and mucoadhesion: Recently the focus has shifted to the mucus penetrating systems\cite{6, 7} and the study thereof not as an alternative but as a complementary opportunity to mucoadhesion. They may work together to assure the best results.

Mucomimetic approaches: The recent trend aimed at the development of mucomimetics substrates to formulate in vitro mucus or mucosae model for both testing and innovative products should be recognised.

Summary: In line with the ongoing research in the field, the review will illustrate the latest preparative and testing techniques that may support the pharmaceutical development of optimized systems, intended for the different routes of administration. This knowledge is the driving force for the pharmaceutical and related companies in the field. In addition, the review aims to elucidate the above relatively new aspects of mucus characterization, mucus penetration and mucomimetic phenomena that represent the basis for a science-based development of any technological, in vitro, ex–vivo test and for any sustainable formulation development.

3. Mucus composition and properties as a function of location

Mucus is a highly complex viscoelastic medium that provides a defensive barrier for many different epithelial surfaces including the respiratory, reproductive and gastrointestinal (GI) tracts. It performs a range of functions including lubrication, maintenance of a hydrated layer and it acts as a barrier to pathogens and toxic substances while facilitating the exchange of gases and nutrients with the underlying epithelium.\cite{8} The mucus layer comprises two different groups of mucins, secreted and membrane bound.\cite{9} Membrane bound mucins form the glycocalyx that provides an important link between the cell surface and the secreted gel layer. On the luminal side of the membrane, these membrane bound mucins have either SEA (self-
cleaving) -domains (MUC1, MUC3, MUC12, MUC13 and MUC17) or von Willebrand domains (MUC4). The membrane bound mucins play a role in both cellular protection and signalling\cite{10,11} through mechanisms such as the regulation of chemokine secretion.

The secreted mucins are produced by submucosal glands and goblet cells and are characterized by their high molecular weight and high proportion of O-linked carbohydrate.\cite{12} Mucus is continuously secreted with nearly 10L secreted into the adult GI tract alone.\cite{8} The composition of mucus varies in different parts of the body. The mucins secreted into saliva are primarily MUC5B and MUC7 and comprise about 16% of the total protein in saliva \cite{13}, whereas the primary secreted mucin in the stomach is MUC5AC but with lower concentrations of MUC5B and MUC6. It is possible that small amounts of MUC5B found in the stomach are pulmonary in origin as pulmonary mucins are expelled via the GI tract. Intestinal secreted mucin is predominantly MUC2 but again there are low concentrations of MUC6 and MUC11 in the small intestine and MUC5B, MUC11 and MUC12 in the large intestine. Pulmonary secreted mucins are primarily MUC5AC and MUC5B \cite{14,15}, both of which are considered to be gel forming. The secreted mucins of the female reproductive tract are primarily MUC5B but with lower concentrations of MUC5AC and MUC6.\cite{16} In addition to the mucins the mucus layer contains lipids, salts, proteins, macromolecules and cellular debris.\cite{17} In particular partially degraded cellular DNA provides a significant contribution to the viscosity of the mucus layer \cite{18}. Both secretory and transmembrane mucins have been detected in the eye, namely MUC2, MUC5AC, and MUC7, and MUC1, MUC4, MUC13, MUC15, MUC16, and MUC17, respectively \cite{19,20}. In turn, transmembrane MUC1 and MUC4 are the predominant mucins expressed in the normal human bladder \cite{21}.

The properties of the various secreted mucins can vary significantly depending on the location but they are still largely controlled by the basic properties of the mucins. Thus, they are generally of high molecular weight (in excess of 1MDa) and are primarily hydrophilic. The
extensive glycosylation means that mucins are stiff, extended polymers with a persistence length of 36 nm \(^{[22]}\) and having a negative charge, often associated with sialic acid groups or sulphate. The properties of mucins in solution very much depend on concentration and what other components are present in the local environment. The secreted mucins are normally considered to be gel forming.

In the GI tract the mucus layer varies widely in thickness. It is thickest in the colon and thinnest in the duodenum \(^{[23]}\). In the intestine the mucus barrier comprises two different regions, known as tightly adherent and loosely adherent.\(^{[24, 25]}\) In the large intestine, these regions are clearly delineated and under healthy conditions the tightly adherent layer provides a physical barrier to bacteria. However, in the small intestine this layer is much thinner and the loosely adherent layer dominates.\(^{[26]}\) Measurements of particulate diffusion through human cervical mucus has shown a network pore size of \(~100\) nm \(^{[27]}\) and AFM images of intestinal mucin have shown a similar pore size.\(^{[28]}\) Despite this data on intestinal mucus, as has already been stated, the small intestine is dominated by the loosely adherent layer, which is much more heterogeneous. This layer has been shown to allow the passage of even \(2\) µm particles provided that they carry a significant net negative charge.\(^{[18, 29]}\) This will be discussed in more detail in Section 9.

4. Preparation of mucin or mucus

a. Purification of secreted mucins

There are very good books that describe the preparation of mucins, especially one edited by McGuckin and Thornton \(^{[30]}\). As a starting point, we recommend that secreted mucus is removed by gently scraping the epithelial surface with a plastic scraper and then purified \(^{[28]}\). Because of the large size and complex structure of secreted mucins it is important to use an extraction buffer containing a strong chaotrope capable of disrupting hydrogen bonding.
network. For example, 4M guanadinium hydrochloride has been widely used. The resulting solution can then be purified using a two-step isopycnic density-gradient centrifugation, in which the first step removes proteins and the second step nucleic acid. Proceed by adjusting the sample to a density of 1.4 g/mL with CsCl and centrifuge (55K rpm at 10 °C for 62 h). The high degree of glycation leaves the mucin strongly Alcian blue positive and this can be used to identify the mucin containing fractions. Aliquots of fractions can be sampled, absorption at 280 nm measured and 2 μL of each fraction can be spotted and stained with Alcian blue. UV and Alcian blue-positive aliquots should then be pooled and diluted in extraction buffer lacking guanidinium hydrochloride (final guanidinium concentration 0.5 M), adjusted in density to 1.4 g/mL with CsCl, and centrifuged again (50K rpm at 10 °C for 96 h). Again aliquots can be sampled, measured at 280 nm and stained with Alcian blue. The fraction at 1.4−1.55 g/mL and strongly Alcian blue-positive but with weak absorption at 280 nm is identified as the mucin fraction. More detailed methods for the purification of specific mucins can be gathered from the literature. For example, MUC5B and MUC7 from saliva, MUC5B from respiratory- and cervical-tract secretions and MUC2 from intestinal mucus. Confirmation of the presence of mucin resulting from the purification should be undertaken using immunoreactions. There is now wide range of antibodies available against mucins from a range of animal sources indeed for many mucins it is possible to target specific regions of the molecule.

Although the extraction and purification of mucins are well established methods, some disadvantages related to the short conservation time, lower yield of production and batch-to-batch variability lead frequently to the alternative use of commercial mucin. Commercial mucin the type from Sigma (Germany) or Orthana (Denmark) are purchased in lyophilized powder which then can be hydrated in ultrapure water or buffers for 3h at room temperature under
gentle stirring. An extensive dialysis allows removals of small ions or low-molecular-weight additive. Several treatments have been reported in the literature. Rossi et al. increased the solubility of mucin from Sigma by adding 2% (w/w) SDS to 12% w/w mucins dispersion \[35\]. SDS was then removed by 2 days dialysis against 10 volumes of 1M urea-1M NaCl, followed by other 2 days dialysis against 40 volumes water and finally against 0.1 M acetate buffer pH 4.5. Alternatively, the mucin dispersion can be centrifuged for 1 h at 25,000xg, the supernatant fraction collected, lyophilized and stored at 4°C until usage. The glycoprotein concentration can be measured by colorimetric method or absorbance reading at 280 nm and calculated on the basis of the difference before and after the treatment. Samples from Orthana have been characterized in terms of monosaccharides composition revealing a predominant presence of neutral O-linked oligosaccharides which confer high hydrophilicity and high solubility up to 200 mg/mL. Solution of this commercial mucin can be prepared by dispersion in water, extensive dialysis and finally lyophilized. Orthana as well Sigma mucins do not show the gelling properties upon lowering pH however rheological studies demonstrated the existence of a concentration-dependent variation of the viscosity from dilute to semi diluted to entangled state \[36\].

In addition to preparative methods such as those mentioned above, analytical methods such as agarose gel electrophoresis can also be used and separation monitored by lectin, immunochemical or histochemical staining. This method can be used to analyse minimally treated samples as long as they are protected from degradation. As the separation is based on the inherent charge of the mucin, it can be used to separate different mucins \[37\] or different glycated forms of the same mucin. \[38\]

b. Biomimetic approaches
In parallel with biological mucin and mucus, efforts to develop artificial mucus or mucus models have been put forth for long. Easily accessible mucus models or mimics are beneficial for all research disciplines requiring mucin, mucus or mucosa for a number of obvious reasons; biological mucus could be difficult to be accessed by some research groups, generally cumbersome to prepare, and presents ethical issue. Technically, biological mucus samples may reveal inconsistent structure and properties across studies due to differences between individual animal sources and/or preparation details. While some mucus models have been devised clearly in the context of mucoadhesion and drug delivery, some others have been developed for other purposes and thus may be considered for future mucoadhesion studies. Growing interests in mucus models are reflected in a few excellent review papers on this subject published in recent a couple of years, including by Groo and Lagrace and Authimoolam and Dziubla with a focus on artificial mucus, and by Cook and Khutoryanskiy with a focus on artificial mucosa, respectively. Briefly, mucus models can be classified into glycan micro-arrays, mucin layers, complexes of mucins with synthetic polymers, and synthetic polymers roughly according to the scale. Glycan micro-arrays have gained popularity for its specificity in probing glycan-binding receptors, antibodies, and enzymes and can be applicable to mucoadhesion too. Despite that micro-arrays with mucin-specific glycan arrays are also readily available, application in the context of mucoadhesion is rare probably due to the lack of three dimensional, mechanical barrier character in those systems. In fact, this is a common problem for all other types of two dimensional mucus models, such as various monolayers of mucins on substrates. Mucin-synthetic polymers complexes were motivated from that self-aggregated mucins, especially commercially available ones, in aqueous solvent even at physiological concentration or higher do not reproduce viscoelasticity comparable to that of native mucus. Thus, synthetic polymers, especially mucoadhesive polymers, are employed as crosslinker to enhance the
network forming capabilities of mucin aggregates. Representative polymers include guar
gum/borate,[53] alginate,[51] poly(acrylic acid),[52] and glutaraldehyde.[55]

Some hydrophilic and network-forming synthetic polymers, such as locust bean
gum/tetraborate,[56] poly(acrylic acid)/(hydroxypropyl)methyl cellulose,[59, 61, 62] poly(styrene)
sulfonate,[58] and poly(ethylene glycol)-block-poly(lactic acid),[57] N-acryloyl-D-glucosamine
(AGA)/2-hydroxyethylmethacrylate (HEMA),[63] poly(ethylene glycol diacrylate) (PEGDA)
[64] have been employed even without involving mucin molecules. The assessment of synthetic
polymeric systems or complexes of mucin and synthetic polymers as mucus models has
typically been conducted via characterization of rheological properties,[51-56] and adhesive
properties (detachment forces) against mucoadhesive drug tablets,[59] often in comparison with
biological mucus. These two properties represent mechanical integrity of mucus model and
their interfacial chemical properties against mucoadhesive polymers, respectively, in the
context of drug delivery researches. Nevertheless, no mucus model or mimic that can
universally replace biological mucus has emerged yet, presumably because of diverse and
complex properties required for mucoadhesion researches.

5. Preparation of electrospun mucoadhesive formulations

During the last two decades, electrospinning has gained increasing interest as a promising
technique for biomedical applications.[65-67] In drug delivery, nanofibers are appealing due to
their high encapsulation efficiency and flexible encapsulation capacity.[68] Moreover,
electrospun fibers allow for numerous delivery and encapsulation options; blend, core-shell,
particles combined with fibers, etc.[69, 70] Electrospun fibers have a large surface area that allows
for extensive interactions with the surrounding environment, which, depending on the
application, can be mucus or other biological components. Surprisingly, mucoadhesion of
nanofibers has not yet been extensively addressed. From the limited studies (examples from
the literature can be found in Table 5.1), it is evident that the mucoadhesive properties of nanofibers can be manipulated by changing nanofiber properties, such as the extent of cross-linking.\[^{71-73}\] Moreover, the inherent mucoadhesive properties of some biopolymers can be exploited when developing mucoadhesive nanofibers. Thus, biopolymers with known adhesive properties (such as alginate and chitosan) have been electrospun with increased bioadhesion of the nanofibers compared to those made from synthetic polymers.\[^{74, 75}\] However, the physicochemical properties of nanofibers does not necessarily correlate with those of the unprocessed material,\[^{76}\] for which reason mucoadhesion of biopolymeric nanofibers in general must be studied. Also, the effect of fiber morphology on the mucoadhesive properties, such as fiber diameter, is yet to be explored.

The oral mucosa is permeable and vascularized, and therefore an appealing delivery.\[^{77}\] The group of Yang developed a delivery system for the oral mucosa, based on a semi-interpenetrating network (sIPN) made from gelatin.\[^{78, 79}\] By cross-linking the fibers using polyethylene glycol diacrylate (PEG-DA) the authors obtained stable, mucoadhesive fibers. The mucoadhesion was affected by several factors: stability, porosity, swelling, and PEG composition of the scaffold.\[^{78}\] The sIPNs were used as a delivery system for insulin, and the authors found that the transbuccal permeability of the released insulin was larger than that of free insulin.\[^{78}\] Another delivery system targeting the oral cavity was developed by Tonglairoum et al., who developed polyvinylpyrrolidone/cyclodextrin/clotrimazole sandwich patches coated with chitosan (CS) or thiolated chitosan (CS-SH) for oral candidiasis.\[^{80}\] The authors studied the fiber’s mucoadhesion, and thus the ability to adhere to the oral mucus. It was shown that fibers coated with CS-SH exhibited a higher mucoadhesive strength compared to CS coated, which is in line with thiolated chitosan providing stronger interaction with the mucus.\[^{80}\] The mucoadhesive properties of nanofibers can also be controlled by adding
mucoadhesive small molecules. For instance, Wongsasulak et al. obtained increased mucoadhesion of zein–chitosan composite electrospun fibers by addition of alpha-tocopherol (α-TOC).[72, 81] Electrospun nanofibers have also been studied for vaginal drug delivery.[82, 83] In a study by Zong et al., polyethylene oxide (PEO)/polylactide composite electrospun nanofibers was developed and loaded with cisplatin for local chemotherapy. The mucoadhesive properties of the nanofibers caused the fibers to stay in the vagina and release the drug, whereas the gel leaked out. Accordingly, the nanofibers facilitated increased bioavailability of the drug as compared to a gel.[82] Electrospun fibers have shown promising results for mucosal drug delivery, however the full potential is still to be revealed.

Table 5.1 Examples of electrospun formulations for drug delivery.

<table>
<thead>
<tr>
<th>Mucosal target</th>
<th>Fiber material</th>
<th>Drug</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal mucosa</td>
<td>chitosan or thiolated chitosan/polyvinyl alcohol</td>
<td>Garcinia mangostana extract</td>
<td>[84]</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>polyvinyl alcohol</td>
<td>Diphenhydramine</td>
<td>[73]</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>polyvinyl alcohol</td>
<td>Docetaxel</td>
<td>[85]</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>Gelatin and photo-reactive polyethylene glycol diacrylate</td>
<td>Nystatin, insulin</td>
<td>[78, 79]</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>chitosan/polyvinyl alcohol</td>
<td>Clotrimazole</td>
<td>[86]</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>polyvinylpyrrolidone/cyclodextrin/clotrimazole and chitosan/polyvinyl alcohol</td>
<td>Clotrimazole</td>
<td>[80]</td>
</tr>
<tr>
<td>Sublingual mucosa</td>
<td>polyvinyl alcohol and sodium alginate/polyvinyl alcohol</td>
<td>Insulin</td>
<td>[71]</td>
</tr>
<tr>
<td>GI mucosa</td>
<td>polycaprolactone</td>
<td>Diclofenac sodium</td>
<td>[87]</td>
</tr>
</tbody>
</table>
6. Methods for molecular scale testing of mucoadhesion

a. Spectroscopic studies

Over the last 20 years a range of spectroscopic methods have been used for the in vitro analysis of the mucoadhesive behaviour of polymeric materials, and the determination of their affinity toward mucin at the molecular level.\textsuperscript{[90-92]} In particular, the interactions between glycopolymers\textbackslash mucins with mucoadhesive polymers have been investigated by \textsuperscript{1}H and/or \textsuperscript{13}C Nuclear Magnetic Resonance (NMR) spectroscopy or by NMR diffusion measurements.\textsuperscript{10} Analysis using NMR is advantageous, as no sample derivatization or pre-treatment is needed and due to the advantage of non-alteration of the normal bio-functionality of the biomolecules.\textsuperscript{11} Uccello-Barretta and co-workers have used proton selective relaxation rate NMR measurements for the determination of mucoadhesive properties of different polysaccharides.\textsuperscript{[93]} Mucoadhesivity can be determined by exploiting the possibility to detect changes of affinity to mucin of small probe molecules due to the mucin–polysaccharide interactions. They have demonstrated the affinity of ketotifen fumarate (KT) to mucin, and they
have used KT as an interaction probe to compare the bovine submaxillary mucin affinities of
tamarind-seed polysaccharide and larch arabinogalactan. Diclofenac sodium salt also has
high affinity for mucin (and low affinity for the polysaccharides), and was also employed as a
mucoadhesivity probe for polysaccharide mixtures containing tamarind seed polysaccharide
and hyaluronic acid. It has been shown that the selective relaxation rate of the ligand is a
more sensitive indicator of binding than the non-selective relaxation rate. Earlier studies using
\(^1\)H and \(^{13}\)C Nuclear Magnetic Resonance, recognised that the hydrogen bonds formed between
the carboxylic acid of poly(acrylic acid) and the glycoprotein component of mucus, play a
significant role in the process of mucoadhesion. Moreover, Griffiths et al., used pulsed-
gradient spin-echo (PGSE-NMR) diffusion measurements to study the interactions of various
model polymer therapeutics with mucin and to quantify their diffusion within mucin
solutions. A strong interaction with mucin was observed for a series of polyamidoamine
dendrimers and hyperbranched poly(ethylene imine), which displayed a characteristic pH-
dependent profile and led to significant reductions in their rates of diffusion.

The use of attenuated total reflectance-Fourier transform infra-red spectral analysis (ATR-
FTIR) is another spectroscopy method to study the interfacial interaction/absorption, and the
diffuse phase across the interface of mucoadhesive polymers and mucin segments. Sriamornsak et al., studied the mechanisms of gastrointestinal mucoadhesion of different
pectin films in contact with mucin in different media. The diffusion of water was used as
an indirect measurement of any change resulting from the interpenetration of polymer–mucin
chains at the aqueous solution-polymer film interface. The ATR-FTIR data confirmed the
formation of hydrogen bonds and the changes resulting from the interpenetration of pectin–
mucin chains at the film interface. Furthermore, by using ATR–FTIR spectroscopy Xiang and
Li suggested that intra-polymer interactions, and inter-surface interactions played opposite
roles in the mucoadhesion performance of cationic polymers at the negatively charged buccal mucosa surface.\cite{102} The intra-polymer interactions can increase the crosslinking within the polymer and lead to the decrease of mucoadhesion, while the inter-surface interactions can promote mucoadhesion of the polymer. Optimal mucoadhesion can be achieved by balancing these two interactions. In a recent study, ATR-FTIR was used to investigate the molecular interactions between a chitosan hydrogel (consisting of non-ionic surfactant vesicles, niosomes, with chlorotoxin) and various cell lines for cancer therapy. The specific accumulation of mucoadhesive chitosan on the surface of ovarian epithelial carcinoma cells was confirmed, demonstrating chitosan’s specificity in targeting of mucin antigen overexpressing tumor cells.\cite{103}

Several of the mucoadhesive studies focus on bulk polymers, however, interest in the mucoadhesion at the nanoscale has been growing.\cite{104,105} In fact, the mucoadhesion ability of nanoparticulate systems is affected by their surface properties (hydrophobic, hydrophilic), surface charges and their size. To detect the mucoadhesive phenomena in the intestinal tract after oral administration of nanoparticulate systems, confocal laser scanning microscopy (CLSM) has been used.\cite{106,107} Chen et al., investigated the adhesion of chitosan-modified liposomes, (average diameter of ~200 nm) using CLSM and fluorophotometry with coumarin 6 as the fluorescent probe.\cite{108} Their studies indicated that the positively charged surface charge of the liposome particles played an important role in their interaction with the negatively charged mucin fibres. In another study, the in vivo mucoadhesion of pH-responsive thiolated chitosan nanoparticles for oral low-molecular weight heparin delivery was assessed using CLSM.\cite{109} Fluorescein-5-isothiocyanate (FITC)-labelled nanoparticles were prepared and the intensity of green fluorescence in the small intestine epithelium of rats after oral administration were evaluated. It is to note, that the CLSM method is sensitive to detect the organic dye-
labelled association of nanoparticles to the mucosal layer of the animal intestine, and does not
modify the properties of the developed formulations of the nanoparticles. Instead of organic
fluorescence materials, orally administered quantum dots (QDs, semiconductor nanocrystals
with diameters of 1–10 nm), could be used as fluorescence markers. Tahara and co-workers
have developed QD-loaded liposomes which had high biocompatibility and low toxicity in
Caco-2 cells.[110] By using CLSM, the fluorescent signal of QDs in the liposomes could be
detected in the intestinal mucosa after oral administration. Thus, QDs can be used for tracing
and detecting bioadhesion and uptake of liposomes in in vivo applications. The relaxation NMR
approach, using dexamethasone 21-phosphate as a mucoadhesivity probe, confirmed the in
vitro mucoadhesivity of nanoparticles obtained from quaternary ammonium chitosan
conjugates.[111] The high surface area of nanoparticulate aggregates significantly enhanced the
interactions with bovine submaxillary mucin. In addition to nanoparticles and liposomes, block
polymeric micelles were also tested for the development of mucoadhesive drug loaded
nanovehicles. The mucoadhesivity of solutions of micelles having acrylated end groups was
characterized by using $^1H$ NMR.[112] To quantify the extent of reaction, the decreased area
under the curve in the vinyl proton regime of the NMR spectra, (indicating interactions between
the acrylates and thiols present in cysteine residues of the mucin), was evaluated.

Overall, spectroscopic studies are very useful to investigate the interactions between polymers
or nanoparticles systems with mucus. The choice of the mucoadhesion spectroscopy method
affects the characterization of their bioadhesive\diffusion properties and the determination of
the mucoadhesive strength.
b. Atomic Force Microscopy

Atomic force microscopy (AFM) is another method that has been used in mucoadhesion measurements. The imaging mode can provide essential information about the amount and conformation of material adhering to the sample, while force spectroscopy enables sensitive adhesion measurements. In order to increase the surface contact area between the tip and the sample in force measurements, it is advantageous to prepare a so-called ‘colloidal probe’. As shown in Figure 6.1, a colloidal-sized particle is attached to the AFM cantilever using two component epoxy glue. The colloidal probe and the sample surface can be further functionalized with molecules of interest (mucin, APTES, -COOH, -NH₃, -OH groups, antibodies and others). Later on, the cantilever is moved towards the surface in the vertical direction. The deflection of the cantilever is measured during the approach and retracts of the probe; as a result, a force-distance profile is obtained. The maximum force of adhesion ($F_{adh}$) and the work of adhesion ($W_{adh}$) can be determined from the retract curve.
The colloidal probe approach has been used by Cleary et al. in order to measure the adhesion between a Pluronic-PAA modified glass bead and the mucous substrate\textsuperscript{[46]}. The mucoadhesion was studied in conditions of varying pH and ionic strength. It was also found that the time of contact between the probe and the sample affects the adhesive forces. Prolonged contact favors interdiffusion and interpenetration of polymer chains and mucin network, resulting in increased adhesive force. Pettersson and Dedinaite investigated the interactions between mica surface and silica particles coated with mucin and mucin-chitosan layers\textsuperscript{[113]}. In order to mimic the daily oral care procedure and its influence on mucous layers, the films were exposed to the anionic surfactant SDS. Another interesting approach to the colloidal probe method was
presented by Iijima et al., who have measured the interactions between mucin layers and
stimuli-responsive drug delivery vehicles \cite{48}. Instead of using the colloidal sized, glass or silica
particle attached to the AFM cantilever, the nanogel particles were freeze-dried and the
resulting granules were directly adhered to the tip by means of micromanipulation system.

Joergensen et al. used the image analysis of AFM scans in order to evaluate the mucoadhesive
properties of different pectins \cite{114}. Mucin coated mica was scanned in AFM liquid cell before
and after incubation with polymer solution, followed by comparison of the roughness
parameters extracted from the images. Siamornsak et al. investigated the structures of mucin,
pectin and their mixtures in acidic medium and deionized water, observing formation of large
aggregates in neutral pH conditions \cite{50}. Similar study by Deacon et al. assessed the interactions
between pig gastric mucin and chitosan \cite{115}.

AFM in mucoadhesion measurements presents both advantages and limitations. It allows
sensitive force measurements as a function of pH, ionic strength or time of contact, but it is
also time-consuming and can be affected by a choice of place in the case of heterogeneous
samples.

c. Scattering techniques (SAXS, SANS, SLS and DLS)

The detailed macromolecular structure of mucin has been addressed at molecular level using
high-resolution scattering techniques, namely, synchrotron SAXS \cite{116-118}, SANS \cite{117, 119} and
static and dynamic light scattering \cite{36}. This has allowed accounting for the properties of mucin
samples of different biological origin and methods of preparation. Thus, the cylindrical model,
and more recently, the double-globular (or “dumbbell”) comb model, has been used to describe
the complex mucin structure \cite{36,116,119}. The schematic structure of mucin at different length scales and its mechanical response at varying pH are represented in Figure 6.2.

**Figure 6.2.** Schematic representation of the biochemical structure of gel-forming mucin at different magnifications: A) entangled mucin network; B) mucin monomers cross-linked through disulfide bonds; C) mucin monomer with globular naked-protein regions and D) low scale representation of the bottle-brush highly glycosylated region of mucin (Sources: Modified from \cite{8,120}; mechanical spectra of pig’s gastric mucin (PGM) as a function of pH taken from Celli et al. \cite{121}; double-globular comb structural parameters taken from Di Cola et al. \cite{116} and corresponding to pharmaceutical mucin sample “Orthana” in aqueous medium in absence of salt). With permission of American Chemical Society and Elsevier.
Table S1 summarizes the results of biophysical studies, based on scattering techniques, namely, synchrotron SAXS, SANS, and SLS and DLS, that have addressed the structural properties of purified mucins of different biological origin. Fundamental parameters probed include the radius of gyration ($R_g$), the coil overlap concentration ($c^*$) and the slope of the intensity scattering curves (also known as the fractal dimension ($d_f$)). These parameters have been determined at varying conditions of pH, solvent, concentration and temperature. The wealth of documented studies has contributed to the elucidation of the mechanisms and molecular events that govern the properties of mucin that underlie its biological functions such as the formation of gel networks. Indeed, mucin participates in the formation of the gel which prevents the digestion of stomach epithelia caused by the acidic gastric juice. This is a function of pH, but also mucin concentration and ionic strength. At physiological conditions, the high concentration of mucin (> 20 mg/mL) and the high-molecular-weight of the molecules, favor the formation and stabilization of an entangled network which behaves as a weak reversible gel \cite{122}. On the other hand, mucin undergoes to sol-gel transition \cite{121,123} a low pH (pH < 4) due to a conformational change in which hydrophobic domains of the non-glycosylated cysteine-rich regions become exposed and the negative charges of the sugars residues responsible of maintaining the expanded structure get protonated. As observed in vitro for native mucin, this phenomenon is accompanied by increase of the size at pH ~2 \cite{124,125} due to aggregation of mucin by a combination of hydrophobic and electrostatic interaction and entanglement of the sugar chains resulting in an increase of the viscosity of the solutions \cite{121,126}. In support of the model proposed for mucin gelation, AFM images have shown that mucin is in an extended fiber-like shape at pH 6.0, whereas it forms well-defined clusters at pH 2.0 \cite{124}. Consequently, the different conformation of mucin throughout the mucus layer allows selective diffusion of HCl. At low concentration \cite{123}, in presence of high ionic strength \cite{126}, commercial mucin \cite{60}, does not gel. However, pH-dependent interactions, as shown by DLS
and CD-spectroscopy, are attributed to a conformational transition of mucin at pH < 4.0 \[^{127, 128}\] that imparts some fluidic viscoelasticity to the bulk sample \[^{121}\].

Recent studies using synchrotron SAXS have aimed to gain insight into the interaction between soluble commercial pig gastric mucin and alginites of high-molecular-weight (~ 400 kDa) and low-molecular-weight (~4 kDa) \[^{129}\]. Firstly, the structure of mucin alone (at 3 mg/mL), at three different values of pH, namely at 1.2, 2.5 and 4.0, was investigated. The scattering curves were characterized by a single fractal dimension, \(d_f = -1.6\) at pH 4.0, which at low-q range, increased to \(d_f = -2.6\) when lower pH were assessed. This observation is consistent with a pH-driven conformational transition in the mucin, in agreement with observations in other mucin samples differing in origin and preparation methods, as revealed from other techniques. The structure of mucin in three different concentration (namely, at 0.3, 1.5 and 3.0 mg/mL) was characterized by different scattering profile, being the one at lower concentration ideal to calculate the radius of gyration (\(R_g\)) that afforded a value of ~18 nm. Interestingly, when the more diluted mucin was mixed with two different types of alginites, different effects in the high-q range of the intensity scattering plot were observed. Indeed, the addition of the low-molecular-weight alginate produced a scattering profile in which the high-q range resembled the one of mucin solutions at high concentration (3 mg/mL). By contrast, this effect was less pronounced when adding the high-molecular-weight alginate, where the high-q region resembled more closely the behavior of mucin at low concentration. Based on this evidence, along with that from fluorescence quenching spectroscopy, viscosimetry and DLS studies, a general model was proposed to explain the interaction of soluble mucin with polyanions. This model accounts for the influence of molecular weight, charge and degree of chain contraction (Figure 6.3).

Although the overall net charge of mucin is negative, positively charged patches are expected to occur in the non-glycosylated protein globular regions of mucin due to the presence of
histidine, arginine and lysine. These positive patches represent sites for the interaction with negatively charged polysaccharides.

**Figure 6.3** Model of interaction between the mucin in its double-globular comb mucin structure and alginate as a function of alginate’s Mw (Alg 4 = 4 kDa; and Alg400 = 400 kDa) and chain flexibility. With permission of American Chemical Society.

Low-molecular-weight and stiff polyanions will interact mainly with the sites available on the globular regions without influencing the preferred conformation of mucin. Thus, minimal variation of the bulk properties such as size and viscosity are expected to occur. However, due to the small size, low-molecular-weight polyanions are able to penetrate in the globular structure inducing eventually rearrangement of the protein. On the other hand, high-molecular-weight and more flexible polyanions, due to the large size, might act as bridges between distant available sites thus influencing the initial conformation of mucin and favoring a reduction of the overall hydrodynamic volume.
7. Methods for macroscale testing of mucoadhesion

The methods to study mucoadhesion can be classified depending on the underlying physical phenomena involved and also depending on the type of formulation that can be tested. Table 7.1 and Table S2 summarize the investigative techniques available. In this Section, we focus on those that probe macroscale phenomena.

**Table 7.1.** In vitro methods used to study mucoadhesion as classified on the basis of the physical phenomena involved.

<table>
<thead>
<tr>
<th>Test method</th>
<th>Formulation</th>
<th>Mucosal surface/mucosa mimetic material/mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods based on the mechanical force determination [130]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texture Analyzer</td>
<td>Compressed polymers tablets [59], Polymers solutions [131], Cast polymer films [132, 133], Polymer gels [134-136], Compacted polymer microparticles into tablet [137]</td>
<td>Animal mucosal tissue [133, 135, 137], Mucosa-mimetic hydrogels [59], Mucin-coated (Sigma) filter papers [131, 132], Mucin (Sigma) disc [134], PGM (Sigma) gels [136]</td>
</tr>
<tr>
<td>Modified balance/modified surface tensiometer</td>
<td>Polymer coated glass [138], Compressed polymer [139, 140], Polymer cups [141]</td>
<td>Animal mucosal tissue [139, 142]</td>
</tr>
<tr>
<td>Tensile tester</td>
<td>Polymer paste [143]; Hydrogels [144]</td>
<td>Plexiglas® disk [143], gelled BSM [144]</td>
</tr>
<tr>
<td>Tensile stress tester</td>
<td>Composite hydrocolloids [145]</td>
<td>Filter paper [145]</td>
</tr>
<tr>
<td>Rotational cylinder</td>
<td>Compressed polymer tablets(^{[1,140]})</td>
<td>Animal mucosal tissue(^{[1,140]})</td>
</tr>
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<td>---------------------</td>
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<td>---------------------------------</td>
</tr>
<tr>
<td>Atomic Force Microscopy (AFM)</td>
<td>Polymer coated glass microsphere(^{[46]}); Polymer solution(^{[146]}); Mucin-polymer complexes(^{[50,115]})</td>
<td>Human buccal cells(^{[146]}); Freshly purified PGM(^{[115]}); PGM (Sigma)(^{[50]})</td>
</tr>
<tr>
<td>Methods based on mucoadhesive interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface Plasmon Resonance (BIACORE®)</td>
<td>Covalently-bound polymer on CM5 chip(^{[147]})</td>
<td>Submicron-sized commercial PGM suspension(^{[147]})</td>
</tr>
<tr>
<td>Dynamic light scattering (DLS)</td>
<td>Mucin-polymer complexes(^{[128,148]})</td>
<td></td>
</tr>
<tr>
<td>Turbidity</td>
<td>Mucin-polymer complexes(^{[128,149]})</td>
<td></td>
</tr>
<tr>
<td>IR-NMR</td>
<td>Freeze-dried mucin-polymer mixed solutions(^{[150]})</td>
<td>Crude homogenized porcine gastric mucus(^{[150]}); PGM solution(^{[150]});</td>
</tr>
<tr>
<td>Analytical ultracentrifuge</td>
<td>Polymer-mucin mixed solutions(^{[151-153]})</td>
<td>PGM from different gastric regions(^{[151]}); HGM(^{[152]})</td>
</tr>
<tr>
<td>Impedance crystal quartz microbalance (QCM)</td>
<td>Polymer solutions; Polymer-micelles(^{[154]})</td>
<td>BSM (Sigma) solution(^{[154]})</td>
</tr>
<tr>
<td>Method based on flow forces</td>
<td></td>
<td></td>
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<tr>
<td>Method based on fluorescent probes</td>
<td>Method based on rheological solution properties</td>
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<tr>
<td>Flow through systems</td>
<td>Fluorescence determination</td>
<td></td>
</tr>
<tr>
<td>Fluorescent labeled nanoparticles [155, 156]; Polymer microparticles [157]</td>
<td>Fluorescent labeled-nanoparticles [158]; Fluorescent labeled-polymer solutions [159, 160]; Polymer solutions [161]</td>
<td></td>
</tr>
<tr>
<td>Ocular tissue [155]; PGM (Sigma) solution [156]; Isolated small rat intestine [157];</td>
<td>Animal mucosal tissue [158-160]; Pyrene-labeled human conjunctival epithelial cells [161]</td>
<td></td>
</tr>
<tr>
<td>Method based on fluorescent probes</td>
<td>Multiple Particle Tracking</td>
<td></td>
</tr>
<tr>
<td>Fluorescence determination</td>
<td>Fluorescent particles [162]</td>
<td></td>
</tr>
<tr>
<td>Animal mucosal tissue [158-160]; Pyrene-labeled human conjunctival epithelial cells [161]</td>
<td>Purified PGM hydrogels [162]</td>
<td></td>
</tr>
<tr>
<td>Method based on rheological solution properties</td>
<td>Viscometer</td>
<td></td>
</tr>
<tr>
<td>Rheometer</td>
<td>Polymer-mucin mixed solution [129, 149, 163, 164]</td>
<td></td>
</tr>
<tr>
<td>Mucin (Sigma) solutions [129, 131, 149, 164-166]; Homogenised porcine gastric mucus [167, 168]</td>
<td></td>
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</tbody>
</table>

a. **Rheology including polymer interaction in dilute solution**

The interaction occurring between mucus and mucoadhesive polymers in mixed systems produces variation in the flow properties of the mixtures with respect to those of the single
components. Thus, the study of the rheological properties of mixtures of mucus or mucin in
solution with mucoadhesive polymers has been widely exploited. Steady-shear measurements
of viscosity, $\eta$ (defined as the resistance of a fluid to the imposed shearing force), and
oscillatory shear determinations of the mechanical viscoelastic moduli (namely, storage and
loss moduli, $G'$ and $G''$, respectively), have been used to study liquid and gel-like systems,
respectively [169]. In general, when two different macromolecular species (e.g., polysaccharide
and protein) are mixed in solution, either attractive or repulsive interactions can take place [170]
(Figure 7.1). Attractive interactions can result in the formation of a complex that either remains
as a soluble colloidal complex or precipitates as a coacervate. Repulsive interactions in turn,
depending on the concentration of the macromolecular species, can lead to phase separation or
cosolubility [170]. In the case of associative interactions, the bulk viscosity of dilute mixed
solutions is expected to decrease due to overall reduction of the hydrodynamic volume of the
macromolecules when they are combined. However, in some other cases, cooperative intra and
inter-polymer interaction can induce increase in viscosity which is higher than the expected
sum of the individual contribution, up to physical gelation. This “synergistic” interaction was
previously observed in xanthan and galactomannan or in plasma proteins and egg albumin
mixed system [169, 171]. In repulsive interactions, the viscosity of mixed solutions is expected to
remain similar to those of the individual stocks. However, if the conformation of one of the
molecules changes due to the exclusion into a segregated phase, then the viscosity of the
mixture can also deviate from the expected additive line. Viscosity synergism cannot
distinguish between binding interaction and exclusion effects [172], unless experimental criteria
are applied. In the experimental conditions in which polysaccharides and mucin solutions are
in the dilute regime ($\eta_{rel} \sim 2; \eta_{sp} \sim 1$), polymer exclusion effects are assumed to be negligible
[169] being the polymers well below the overlap coil concentration.
Figure 7.1 Schematic representation of the type of interaction that can occur in protein-polysaccharides blends in dilute solution mixtures (modified from [170]).

Mucus is a weak viscoelastic gel biological material which possesses both flow (viscosity) and deformation (elasticity) properties [120]. Such properties are regulated for example during peristaltic movement or copulation [8]. At higher concentration mucus is characterized by a shear thinning behavior (i.e. decrease in viscosity upon increase of the shear rate) typical of an entangled network. However, the soluble fraction of PGM (Sigma) at concentration of ~ 8 mg/mL (in 0.1 M TRIS pH 7.4) was found to behave as Newtonian fluid since any shear-dependence of the viscosity was observed [164]. The addition of human albumin produces an increase in viscosity due to association of albumin and mucin [164]. In the context of mucin and
polymer interactions, a rheological approach to screen the mucoadhesive properties of polymer
was described by Hassan and Gallo\textsuperscript{[163]}. The mucoadhesion strength of several polysaccharides
was evaluated by studying the viscosity enhancement occurring upon mixing solution of
polymers with commercial mucin sample using a viscosimeter Brookfield Model RTV
(Brookfield Engineering Laboratories, Stoughton, MD). The increase in viscosity (positive
synergism) respect to the sum of the individual viscosities of the two components measured in
the same conditions as the mixture (in terms of concentration, temperature, time and rate of
shear) but with an Ostwald capillary viscosimeter (Fisher Scientific Co., Pittsburgh, PA) was
attributed to physical entanglement between the two species and defined as component of
bioadhesion ($\eta_b$). For each polymer-mucin system, $\eta_b$ was calculated with the following
equation:

$$\eta_b = \eta - \eta_m - \eta_p$$

Eq. 1

where $\eta$ is the measured viscosity of the mixture and $\eta_p$ and $\eta_m$ the individual viscosity of the
polymer and mucin, respectively.

The $\eta_b$ values were found to be inverse proportional to the rate of shear per second ($\sigma$), thus,
the force of bioadhesion $F$, defined as intermolecular friction force per area unit was calculated
using the equation:

$$F = \eta_b \times \dot{\gamma}$$

Eq. 2

Based on this pioneering protocol, several subsequent studies aimed to test the mucoadhesion
of polymers. This procedure could distinguish between positive synergism (interaction), lack
of synergism (no-interaction) or negative synergism of the viscosity or of the mechanical
properties. Mortazavi et al.,\textsuperscript{[173]} reported the gel strengthening effect of poly-acrylic acid on
homogenized mucus and observed that was characterized by increased values of $G'$ (which
reflects the ability of a viscoelastic material to store the elastic energy and recover its initial
shape) and decrease in $G'$ (which reflects the loss of energy as liquid-like flow). Madsen et al., \cite{167} described the effect of mucoadhesive type and concentration on the profiles of the mechanical spectra of the mixtures in order to determine the type of gel formed. Some of the most relevant works based on rheological methods that have contributed to a systematic description of the mucoadhesive properties of polysaccharides are summarized in Tables S2 and S3. Sometimes, different outcomes have been observed for the same polymer-mucin mixture, such as in the case of chitosan-mucin \cite{149, 163} or cellulose derivative-mucin \cite{166, 167} depending on different experimental conditions, particularly the polymer concentration \cite{165} or mucin source, making direct comparisons and interpretations challenging \cite{174}.

Recent evidence \cite{175}, has shown that mixing two stock solutions of chitosan and mucin of matched $\eta_{rel} \sim 2.0$, at increasing f ratio (mass proportion of mucin respect the total mass in the mixture) a reduction in $\eta_{rel}$ to a minimum value ($f_{\text{min}}$) occurs beyond which, upon a subsequent increase in f, the $\eta_{rel}$ increases again to approach that of mucin stock solution. Such behavior describes a skewed U-shaped curve both in water and 0.1M NaCl (pH 4.5) as shown in Figure 7.2a and b, respectively, for a representative CS-mucin systems. This approach enables to determine, in a quantitative manner, the degree of interaction, given by the value of the area under the curve of the relative deviation from the theoretical additive line (or line of “no interaction”). Also, the method enables to determine the maximum stoichiometry of the interaction given by the f ratio of minimum $\eta_{rel}$ ($f_{\text{min}}$).

Mechanical force studies or rheological synergism are diagnostic of mucus (or mucin)-polymer interactions, however, no detailed information regarding the underlying molecular mechanisms of interaction can be deduced from these techniques. Table S3 offers a summary of the major rheological methods that have been used to study the interactions of polymers and proteins with mucin solutions and mucus gels.
Figure 7.2 Relative viscosity ($\eta_{\text{rel}}$) of chitosan–mucin mixtures of varying compositions expressed as the mass fraction of mucin ($f$) respect the total mass in a) water and b) 0.1 M NaCl (37°C, pH 4.5, inclination angle 50°). The red dotted line in a) and b) represents the calculated values of $\eta_{\text{rel}}$ of the mixtures assuming there is no interaction (additive line). The $\eta_{\text{rel}}$ values at $f = 0$ and 1 are the relative viscosities of the chitosan and mucin stock solutions, respectively.

The lower panels show the normalized data expressed as percentage deviation from the additive line in c) water and d) 0.1 M NaCl, both at pH 4.5 (mean values ± minimum and maximum, n=2). The blue shaded areas in plots c) and d) represent the integrated area under the curve calculated using a trapezoid approximation available in Origin 8.5 (Origin Lab Corp., Northampton, MA)\textsuperscript{[175]}.

c. Inclined plane
As pointed out in the introduction, a variety of methods can be used to study mucoadhesion and in Table 7.1 a classification of methods is given based on the physical phenomena involved. From a practical point of view it is useful to distinguish between mechanistic methods and functionality (or performance) test methods; the first ones (the most common are rheological and spectroscopic methods) give information on the events that occur at the mucoadhesive joint in order to prove the interaction mechanisms, whereas the second ones are aimed at evaluating the actual mucoadhesive properties/performance of formulations. In turn, they can be divided into mechanical tests (the most common are tensile testing and rotational cylinder) intended to measure the force needed to detach the formulation from the substrate and dynamic tests (among which flow through or flow retention methods) intended to mimic the physiological clearance mechanisms and to follow the fate of the formulation/loaded drug (retention on or removal from the mucosal substrate). Mechanical and dynamic methods are believed to provide information on the overall performance of the formulation as a delivery system.

The inclined plane method \cite{176, 177} can be classified as a special dynamic test that measures mucoadhesiveness as a function of the retention of the mucoadhesive material in contact with a mucosal substrate (mucin film or mucosal tissue). It has been devised to test liquid or semisolid formulations endowed with intrinsic flowing properties at test temperature. It is not applicable to solid formulations or very thick gels.

Description of the apparatus

The inclined plane apparatus basically consists of a plexiglas support whose angle of inclination with respect to the horizontal can be varied between 30° and 60°, thermostated at 37°C and placed above an electronic microbalance interfaced with a personal computer. An illustrated picture of the apparatus, including details of the plexiglass support (which is composed of a thermostated plate and an adapted substrate holder) is given in Figure 7.3.
The substrate holder (hosting two parallel channels) may be coated with a thin mucin film (prepared by casting) or covered with mucosal tissue. The surface area coated is normally 28 cm$^2$. The whole apparatus is placed in a transparent box allowing constant temperature to be maintained and avoiding disturbances during the measurements. An overall picture of the assembled apparatus is given in Figure 7.4.
**Figure 7.4** Overall picture of the assembled apparatus

Description of the operational procedure for measuring mucoadhesive properties

The substrate holder is coated with the mucin film and equilibrated. Porcine gastric mucin is normally used as biological substrate. Mucin films are prepared directly on the Plexiglas holder in the horizontal position, by pouring a measured volume of 8% w/w mucin dispersion in water then drying at 45°C for 45 min. A weighed amount of the formulation is placed on top of the substrate holder, still held horizontal and until equilibrated. The support plate is then inclined (at a given angle) and the amount of formulation dropped on the microbalance is recorded as a function of time. Blank measurements are performed in the absence of the mucin film on a weighed amount of sample using the same experimental conditions employed in the presence of mucin. The amount of formulation dropped down the inclined plate is recorded by means of suitable software as a function of time until a plateau is reached. The amount adhering to the inclined plate is calculated as the difference between the amount of formulation loaded and the amount dropped down from the balance (non-adherent) and expressed as a percentage (% adhered). An example is given in Figure 7.5.
Figure 7.5 Plots of the amount of sample dropped (non-adherent) on the balance as a function of time.

A normalized mucoadhesion parameter is calculated as follows: (% adhered mucin-% adhered blank)/% adhered blank and is equal to 56%. This parameter allows the mucoadhesive properties of a given formulation to be measured independently of the consistency of the sample, since the blank measurement allows for normalization [170].

Validation of the method

The inclination angle, quantity of mucin, length and width of the channels engraved on the sample holder, sample weight influence test results and their reliability and must be optimized to manage sample and testing variabilities. Recently these parameters have been the object of a validation exercise aimed at 1) evaluating the capability of the method to discriminate between different prototypes of a formulation intended for marketing and 2) assessing the precision and reproducibility of the method as well as the robustness with respect to operational parameters. This exercise could lead to the proposal of the method as a routine control method for the quality of the product.

Applications

The method has been profitably used to test the mucoadhesive properties of polymeric solutions, liquid or gel formulations (mouthwashes, vaginal washings, eye drops, buccal sprays, nasal washings, nasal sprays) [177] and even melted suppositories. The method has also been employed to test mucoadhesive systems characterized by in situ gelling properties, like swallowable gels intended for esophageal lining, or in situ gelling solutions used in diagnostic colonoscopy, since it enables evaluation of the contribution of gelation time to mucoadhesive performance [171].
d. Tensile testing

A texture analyzer can be used for the quantification of the tensile strength i.e. the force required to remove the formulation from a mucosal surface, which can be used as a measure of the mucoadhesive strength. In a generalized setup, the formulation is fixed on a probe which is subsequently lowered into a mucus sample. After incubation that ensures full contact between the formulation and the mucus, the mucoadhesive strength is measured as the force of detachment. Formulations such as tablets [178], films, [179, 180] hydrogels, [181] and fibers [80, 89] can be studied using this technique.

8. Cellular methods

Use of mucosal tissue of animal origin in mucoadhesion studies presents certain drawbacks, such as limited availability, time-consuming tissue preparation, small surface area, or significant variability of the obtained results [182]. In vitro study of mucoadhesion on cell cultures is an example of alternative method of measurements, based on the interactions between mucin and material of interest. Mucus-secreting HT29-MTX cell line is derived from human colon adenocarcinoma and often used as a model for human intestinal adsorption or buccal tissue in the oral cavity. Mucus secretion depends on the culture period and usually reaches maximum thickness after ca. 3 weeks of cell growth [183, 184]. Figure 8 presents the HT29-MTX cells 1 and 14 days after passaging, respectively.
There are several publications reporting use of cellular methods in mucoadhesion measurements. Jintapattanakit et al. assessed the mucoadhesion of trimethyl chitosan and PEGylated chitosan using HT29-MTX-E12 monolayers. Fluorescently labeled polymers were incubated with cells for 2 hours. Later on the cells were lysed and the uptake of polymers was determined as the amount of fluorescence per unit weight of cellular protein. The obtained results were similar when compared to mucin particle method. Chen et al. evaluated the mucoadhesion of probiotic alginate microcapsules by counting Lactobacillus reuteri strain released from the capsules adhering to the HT29-MTX after 1 hour of incubation. In another study, mucoadhesion of fluorescently labeled, non-coated and polymer-coated liposomes was measured after 2 hour of incubation with HT29-MTX cell monolayer. The amount of liposomes adhering to the mucus was determined by measuring the fluorescence intensity both directly on the cell monolayer and indirectly from the supernatant solution after incubation.
9. Methods for characterising mucus permeability

One of the primary functional roles of mucins is to provide a barrier to bacteria while at the same time allowing the passage of smaller components such as nutrients, bioactive agents or gasses depending on location. As a result the permeability of mucus and factors that can affect it are of great interest. The permeability of mucus depends on the pore size of the network, the size of the objects trying to pass through it and the interactions between the two. Ideally, it should be possible to mathematically model the diffusion through mucus in order to provide some degree of prediction and understanding of the phenomena involved [188]. A range of approaches are suitable to measure the diffusion of objects of different sizes ranging from single molecules to nano- and micro-particles. At the molecular level, the diffusion chamber has been used to determine the diffusion of molecules through mucus [189] but this has rather been superseded by other more relevant methods that can be applied directly on live animals in the gut [190] or nasal cavity [191].

Another commonly used method is NMR [192-194] and most recently the use of F-19 entrapped in different cyclodextrins was successfully used to probe the pore size of submaxillary and nasal mucins [193]. This method shows great promise for the future. There has been a significant amount of research on the use of particles as delivery systems through mucus and as a consequence a wide range of methods have been used many of which have been described in a recent review by Greissinger et al. [195]. The quartz crystal microbalance with dissipation (QCM-D) has been used to assess the combined properties between mucoadhesions and permeability of thiolated chitosans and their complexes with a layer of native porcine gastric mucin [196].
One of the most widely used approaches uses the tracking of particles of different sizes to determine mucus pore size \cite{27,197-199}. The advantage of using particles of a well-defined size is that the diffusion coefficient can be used to calculate the microviscosity of the mucus sample (Stokes viscosity). For example, a study on human cervicovaginal mucus using multiple particle tracking, revealed that the average pore size 340 +/- 70 nm and that the range was approximately 50–1800 nm \cite{199}. For smaller particles, fluorescence recovery after photobleaching (FRAP) has been used to determine diffusion coefficients in mucus \cite{27}. Using a combination of particle tracking and FRAP it was proposed that human cervical mucus had a pore size of 100nm. Particle tracking has also been used on particles as small as HIV virus \cite{200} showing that mucus has complex microrheological properties. This approach has also been extended to include gastric and intestinal mucus, MUC5AC and MUC2 respectively. In this case the diffusion of 500 nm latex beads was determined as a function of mucin concentration. The results showed that lowering pH caused both mucins to gel and the addition of a polyphenol (epigallocatechin gallate) to porcine gastric mucin caused a similar effect. A combination of FRAP and particle tracking has been used to show that soluble dietary fiber can decrease the permeability of porcine intestinal mucus \cite{201}. FRAP has also been used to characterize the superficial mucus layer and the periciliary fluid layer of the human bronchial system revealing hyperviscous airway pericilliary and mucous liquid layers in cystic fibrosis \cite{202}.

The disadvantage of both FRAP and particle tracking is that they rely on the passive diffusion of the probe in order to determine the physical properties of the microenvironment. Another approach that has been used is the use of optical tweezers to probe the microrheology and particularly, the rigidity of the mucus scaffold in a noninvasive way on the micrometer scale \cite{203}. All of these physical methods are in marked contrast to others who have tried to estimate intestinal mucus permeability based on structural parameters \cite{204}. This would indicate a pore size of about 1 micron for intestinal MUC2 mucus. Such a size is significantly larger than that
measured by AFM in reconstituted MUC2 mucin that suggested a distribution of pore sizes from 20 to 200 nm\textsuperscript{28}.

In addition to the pore size, the charge on mucus is a key factor in determining permeability\textsuperscript{205}. The net negative charge carried by the mucins under neutral pH conditions means that any positively charged particles or polymers tend to be mucoadhesive. This has led to the widespread use of cationic biopolymers such as chitosan for their mucoadhesive properties\textsuperscript{206}.

In the small intestine, it has been shown that 500 nm latex beads can diffuse through intestinal mucus when bile salts adsorbed to the surface while they were unable to do so in the absence of bile\textsuperscript{209}. In the same article the authors were able to show that E. coli were unable to diffuse through the same mucus regardless of the presence of bile. This suggested that a zeta potential of at least -20 mV was required for permeability of these particles. In addition to charge, hydrophobicity also plays a role in mucoadhesion and permeability\textsuperscript{128, 207}, and seems to play a role aggregation and structure formation by mucins as well as interactions with mucoadhesive polymers such as chitosans.

10. Application specific requirement

a. Gastrointestinal drug delivery

Oral delivery is the most commonly used and compliant form of drug administration. Moreover, the gastrointestinal tract (GI) is characterized by its highly absorptive surface which plays a role both for local and systemic effects. An important disadvantage of this administration route is however represented by the harsh conditions of the stomach which pose challenging goals for the development of carriers for the delivery of poorly stable drugs such as antibiotic and proteins and which goes towards the production of always more innovative and complex micro- and nanoformulations. In this context, the mucus layer lining the whole...
GI tract, with its variable thickness and composition, represents the major protective barrier against foreign particulate (e.g., bacteria but also micro-and nanoformulations). Nevertheless, this location also represents an important anchoring site for mucoadhesive drugs formulations which avoids their rapid clearance and improves their residence time in the GI.

Several diseases affect all of the GI tract including the accessory organs such as liver, pancreas and bladder. Gastrointestinal diseases cover acute, recurrent and chronic diseases including inflammatory bowel disease (IBD) and functional dyspepsia. Diseases of the upper part of the gastrointestinal tract are often associated with the hostile presence of Helicobacter pylori, the spiral-shaped Gram-negative bacteria which infect about half of the world population and establish life-lasting bacteria-host relationship \[^{208}\]. Because of the lack of symptoms in ~60% of the infected people, its presence as a commensal or pathogenic bacteria is still controversial \[^{209}\]. However, in a significant percentage of cases, the perpetuation of the inflammation of the gastric mucosa produces tissue damage that turns into more severe pathologies such as gastric and duodenal ulcer, adenocarcinoma of the distal stomach or MALT-lymphoma \[^{210}\].

b. Advances in the therapy of Helicobacter pylori

The first-line therapy for the management of H. pylori infection, based on the concomitant use of a proton-pump-inhibitor and a combination of two antibiotics (i.e. triple therapy), is facing failure in 20-30% of the cases. This daunting efficacy is due to the alarming increase in the antibiotic resistance in association with low patient compliance and different disease’s status, high bacterial load and polymorphism between strains and poor drug stability in the acidic environment \[^{211, 212}\]. Improving drug stability, gastroretention at pH 1.2 and release at H. pylori surviving condition (pH 6-7) and site-specific targeting of H. pylori are therefore the major requirements for new tailored and effective eradication therapy. Along with the preparation of dosage forms whose prolonged residence time in the stomach is due to their
ability to float in the gastric fluid as the consequence of their low density\textsuperscript{[213]}, or to unfold and expand as a result of their swelling properties\textsuperscript{[214, 215]}, great attempts have focused on the formulations of polymeric micro-or nanoparticles with enhanced mucoadhesion ability \textsuperscript{[216]}. Several research groups have demonstrated proof-of-principle of the superior antimicrobial activity of mucoadhesive micro- or nanoformulations both in vitro and in vivo with respect to the plain drug\textsuperscript{[217-219]}.

Besides the large portfolio of synthetic polymers such as poly-acrylic acid (PAA) and poly(lactic-co-glycol acid) (PLGA) and derivatives with proven mucoadhesive properties, proteins, and polysaccharides, used singularly or together like building blocks of drug delivery systems, also represent potential materials. Their advantages include biodegradability, biosafety, ubiquity, nutritional value but also amenability to being chemically manipulated\textsuperscript{[220]}. In this regard, nanoparticles comprising gelatin\textsuperscript{[221]} or gliadin (gluten-derived proteins) have been developed for the release of amoxicillin\textsuperscript{[219]}, clarithromycin-omeprazole\textsuperscript{[222]} or clarithromycin-amoxicillin-omeprazole\textsuperscript{[223]} for the treatment of H. pylori. While alginate, heparin or chitosan are the first choices for the formulation of mucoadhesive polysaccharide-based micro-and nanoformulations. The following section aims to review some strategies adopted to improve the performance of mucoadhesive polymer-based micro-and nanoformulations for gastric drug delivery.

Micro- and nanoformulation with improved gastroretention

Among mucoadhesive polysaccharides, chitosan, the semi-synthetic cationic aminopolysaccharide derived by partial deacetylation of chitin, remains the most adopted mucoadhesive biopolymer for the preparation of matrix-type or core-shell micro- and nanosystems for the delivery of low-molecular-weight drugs such as antibiotics. But chitosan is also a unique building block of galenic formulations due to its adjuvant properties and
antimicrobial activity toward pathogenic bacteria like \( \text{H. pylori} \). Chitosan-based micro- or nanoparticles can be prepared by ionotropic gelation with tripolyphosphate (TPP). The high solubility of chitosan at low pH and the high porosity of chitosan-microspheres, however, restrict its applications in controlled release devices in the gastric compartment. Manipulating the crosslinking properties or the solubility of chitosan represents, therefore, a strategy to overcome this limitation.

Exposing chitosan microsphere to reacetylation with acetic anhydride can modulate the release of amoxicillin or metronidazole with respect the un-reactacetylated formulation but can decrease the encapsulation efficiency of metronidazole and also the antimicrobial activity if the exposure time is too high. Also, chemical crosslinking of chitosan microsphere with glutaraldehyde or with genipin, the low cytotoxic agent derived from hydrolysis of geniposide, can prevent their rapid dissolution in simulated gastric fluid. Nevertheless, this procedure can adversely influence the mucoadhesive properties of the microsphere if the crosslinking is superior to optimal time. Zhu et al. solved the inconsistency between mucoadhesives and controlled release by encapsulating a model drug into Eudragit® cores into chitosan/gelatin microsphere only slightly crosslinked with TPP. In this study, the authors evaluated the effect of type and density of crosslinking regard swelling properties, mucin adsorption on the surface and in situ retention. To protect chitosan-glutamic acid nanoparticles from rapid dissolution at low gastric pH, Chang et al. proposed an original approach. They included the nanoparticles in a pH-sensitive gel comprising alginate-Ca\(^{2+}\)-gelatin which would adhere first to the gastric mucosa, would shrink at pH 1.2 and protect the nanoparticle from rapid dissolution, swell up to 50% at pH 4.5, and then collapse at pH 7 allowing 80% release amoxicillin-nanoparticles. Besides chitosan, improved mucoadhesive properties can be achieved using other polymer mixtures such as dextran derivatives (e.g., dextran sulfate) and...
cellulose acetate \cite{235}, cholestyramine and cellulose acetate butyrate \cite{236} or ethylcellulose and carbopol-934P \cite{237}.

pH sensitive formulations

Due to its almost unique property among the biopolymers of bearing positive charges along its chain, chitosan is often used in combination with other mucoadhesive negatively charged polymers such as alginate (Arora et al 2011) or heparin \cite{238, 239} to form polyelectrolyte complexes. Because of the formation of polyelectrolyte complexes is often enthalpically driven, (i.e., they are formed mainly by electrostatic interactions or hydrogen bonding), they are more promising as stimuli-responsive materials \cite{240}. Beside the fact that heparin has shown to accelerate gastric ulcer healing, chitosan-heparin nanocomplex for the delivery of berberine, a natural isoquinoline used in traditional Eastern medicine to treat gastro-enteritis, showed a pH-dependent drug release which was up to 19\% of the initial amount at pH 1.2, ~10\% at pH 6 and ~50\% at pH 7 due to collapse of the complex \cite{239}. Chitosan-gold nanoparticles of size below 50 nm were used to stabilize the surface of negatively charged liposomes comprising L-\(\alpha\)-phosphatidylcholine and 1,2-dioleoyl-sn-glycero-3-phosphate and prevent rapid liposome aggregation and fusion \cite{241}. Thus, the coated liposomes were able to release only 10\% of doxycycline at pH 1.2 and a release up to 90\% at pH 7.4 within 24 h and to fuse with the H. pylori only at pH 7.4. Moreover, only the coated doxycycline-loaded liposomes were able to inhibit the bacterial growth completely respect to the plain drug or the empty coated liposomes \cite{241}.

Site-specific drug targeting

Formulations of higher complexity comprise a functionalization of the surface which allows specific interaction directly with the surface of H. pylori. With this purpose, Umamaheshwari
et al.\textsuperscript{[242]} anchored a lipid bilayer of phosphatidylethanolamine (PE) on the surface of polyvinyl alcohol beads for the release of acetohydroxamic acid with the aim of plug-and-seal specific receptor on H. pylori surface. Besides their ability to inhibit the bacterial growth completely and to be more stable than normal liposomes, they also appeared to prevent the adhesion of H. pylori to a cell monolayer and gastric tissue section\textsuperscript{[242]}. The concept of plug-and-seal as an approach to prevent bacterial infection is the topic of intense research of discovery of new potential inhibitors (e.g., polysaccharide) and their usage as anti-adhesive preparation\textsuperscript{[243]}. Due to the presence of adhesines on H. pylori surface able to recognize fucose-bearing antigens on the epithelial/mucosal surface, fucose has been introduced in nanoformulation as targeting moiety. Ramteke et al. used a carbodiimide method to covalently conjugate fucose to chitosan\textsuperscript{[244]}. This conjugate was used to prepare chitosan-glutamic acid nanoparticles for the concomitant delivery of amoxicillin, clarithromycin and omeprazole which were able to eradicate H. pylori from Swiss albino mice respect the unconjugated chitosan-glutamate nanoparticles or plain drugs\textsuperscript{[244]}. Lin et al.\textsuperscript{[218]} combined the fucose-conjugated chitosan and genipin-crosslinking technology to formulate a chitosan-heparin nanocomplex for the delivery of amoxicillin. Such a formulation was obviously more effective in eradicating H. pylori from infected mice than plain amoxicillin due to the most efficient interaction with bacterial receptor recognizing fucose and also to reduce the H. pylori-associated gastric inflammation as concluded by histological inspection\textsuperscript{[218]}. More recently, a site-specific chitosan/TPP nanoparticle loaded with amoxicillin was produced by conjugating chitosan with the ureidododecanoid acid, introducing, therefore, a moiety recognized by the urease-transporter protein present on H. pylori surface\textsuperscript{[245]}. The ureido-modified nanoparticles were superior in inhibiting the bacterial growth respect plain amoxicillin and unmodified chitosan/TPP ones. Moreover, such inhibition of the growth was
reduced by the addition of competitive substrate urea suggesting that the antibacterial activity is due to a direct delivery of amoxicillin on the bacterial surface as evidenced by flow cytometry analysis, CLSM imaging and OD measurements of bacterial growth \cite{245}.

c. **In the oral cavity**

The oral cavity includes different structures, most important being the lips, the cheeks, the palate, the floor of the mouth and the tongue. The inner surface of the oral cavity is protected by a mucous membrane. The secretion of saliva moisturizes the mucus membrane and forms the acquired enamel pellicle at the teeth and is thus very important in order to have a good oral health \cite{246}. Saliva is constantly produced from three major glands and is composed of inorganic ions such as phosphate and calcium as well as organic constituents such as proteins, carbohydrates and lipids. The microflora of the oral cavity is rich and more than 700 different bacteria species can be found here \cite{247}. The pH of a normal healthy mouth is around 6.5-7.5 \cite{248}.

The oral cavity can be used for both local drug delivery for treating different infections of the oral mucosa and diseases connected to the teeth such as dental caries and periodontitis in addition to systemic drug delivery via the buccal route.

The greatest challenge when aiming for drug delivery to the oral cavity is the secretion of saliva which could be as high as up to 7 ml/min \cite{249}. Saliva will efficiently flush any foreign substances, also drugs, away. Also the gingival crevicular fluid (GCV) dilutes and flush away substances placed in the periodontal pockets \cite{250}. A mucoadhesive formulation has therefore been proposed in order to prolong the residence time in the oral cavity. However, the mucus layer of the oral cavity has a turnover rate of 12-24 hours \cite{248}. This implies that the residence time in the oral cavity can never be longer than this period of time. In addition, eating, drinking, swallowing and chewing lower this period of time even further. Many new formulations have
lately been approved for treating periodontitis\textsuperscript{[251, 252]}. The success of these formulations is due
to the use of mucoadhesive polymers enabling the formulation to stay as a reservoir in the
periodontal pockets for an extended period of time. However, the periodontal pockets can
perhaps be seen as an easier target than the oral mucosa; when the formulation is placed in the
pocket the environmental challenge is more predictable.

Different studies have revealed positively charged materials such as chitosan and positively
charged lipids to exert the highest mucoadhesive/bioadhesive properties in the oral cavity\textsuperscript{[253]}. However, toxicity studies have shown that positively charged formulations seem to be more
toxic than their negative counterparts\textsuperscript{[254]}. This complicates the picture since the formulations
with the highest degree of mucoadhesion also seem to give the highest toxicity.

A formulation, especially a nano- or micro formulation, placed in the oral cavity should also
be non-reactive towards saliva. Saliva is composed of globular proteins that can react with
the formulation. A study by Nguyen et al. showed that positively charged liposomes reacted
strongly with saliva. Also some of the negatively charged liposome reacted dependent on the
type of negatively charged lipid used\textsuperscript{[255]}. However, when the liposomes were coated with
the biopolymer pectin, the interaction disappeared\textsuperscript{[256]}.

d. Colorectal drug delivery

The colorectal mucosa can be regarded as an optimal site for drug delivery, following oral or
rectal (e.g. suppositories, enemas etc.) administration. For instance, the colonic mucosa
contains less digestive enzymes and therefore harbors reduced proteolytic activity than the
mucosa of the stomach or small intestine\textsuperscript{[257]}. Thus, especially small peptides or proteins can
be absorbed in higher concentrations due to less degradation. Furthermore, colonic bacteria can
be exploited for the metabolism of prodrugs into effective metabolites\textsuperscript{[258]}.
These characteristics rendered the colorectal mucosa an important target for systemic drug
delivery. Furthermore, local administration of various drugs poses an important basis for the
treatment of various colorectal diseases such as infectious colitis, inflammatory bowel diseases
(IBD) or colorectal cancer. In this regard, it is challenging to optimize the bioavailability of
drugs with a maximum concentration at the absorbing/inflamed site for a prolonged time
together with minimal systemic side effects. Micro-and nanoformulations can be designed
either to increase the stability of drugs, to optimize the ratio between the loaded amount and
the loading volume, and to perform a passive or active delivery in the colorectal mucosa.\textsuperscript{[259]}

Charged biocompatible polymers (e.g. polysaccharides) are able to interact with both healthy
and inflamed mucosa by virtue of their numerous charges, high molecular weight and chain
flexibility. In addition, some of them are characterized by pH-dependent properties (e.g
solubility), which render them optimal materials for the generation of micro-and
nanoformulations with mucoadhesive properties. This further enables a prolonged contact with
the mucosa and favors cellular uptake. Additionally, pH sensitivity protects the drug from the
acidic environment of the stomach and allows its release in lower GI regions.\textsuperscript{[260]}

Thus, mucoadhesive strategies have been exploited to improve systemic or local drug
administration in various ways. In the subsequent sections, we will discuss challenges and
strategies for colorectal targeting of mucoadhesive formulations and possible medical
indications.

Micro- and nanoformulations for colorectal delivery

Regarding the design of micro-and nanoformulation for colorectal application, large attention
has been posed to the use of polysaccharides due to numerous advantages namely i) their
susceptibility to degradation by glycosidases produced by the intestinal microflora, which
avoids their accumulation and favor drug release, ii) their ability to interact with the mucosa,
which favors prolonged contact with the absorption site, and in special cases iii) to function as absorption enhancer or as promoter of wound healing \cite{261}.

Almost three decades ago, the ability of chitosan to promote drug absorption through the intestinal epithelium and also other mucosa such as the nasal one was observed in models of cell monolayers \cite{262}. Since then, chitosan has been intensively used to generate challenging formulations for the oral delivery of drugs, e.g. insulin and others, in form of TPP-crosslinked nanoparticles \cite{263} alone or entrapped into a liposome structure \cite{264}, in combination with alginate \cite{265, 266}, gum arabic \cite{267}, hyaluronic acid \cite{268}, lecithin \cite{269} among many others. Chitosan-based formulations can be tailored for specific purposes addressing for instance pH-sensitivity by using a multi-ion crosslinking strategy based on TPP, SO$_{4}^{2-}$ and Mg$^{2+}$ as recently reported by Lin et al.,\cite{270} or adding selective interaction with globet cells by chemically modifying N-trimethyl chitosan chloride with a CSK targeting peptide \cite{271}. Other authors use chitosan and albumin to coat pH-sensitive insulin-loaded alginate/dextran sulfate nanoparticles and investigated the delivery of insulin in presence or absence of inhibitors of permeability \cite{272}. Mucoadhesive formulations have not been proposed only for incorporation and delivery of drugs for already established treatment (e.g insulin, steroids, IBD therapeutics \cite{273}) as will be discussed in the following section, but also for new therapeutic strategies such as delivery of antisense RNA sequences (e.g siRNA technology)\cite{274}.

Mucoadhesive formulations for the treatment of colorectal inflammation and cancer

As previously discussed, mucoadhesive formulations have been evaluated in order to optimize the local treatment of colorectal diseases. Currently available studies were mainly aiming at an improved treatment of infectious colitis, inflammatory bowel diseases (IBD) such as Crohn’s disease (CD) and ulcerative colitis (UC) and colorectal cancer (CRC).
Regarding infectious colitis, mucoadhesive formulations have been developed for the treatment of Clostridium difficile infection (CDI). Clostridium difficile is a toxin-producing, gram-positive bacterium that causes mild to severe colitis, frequently following previous treatment with antibiotics. Symptoms range from asymptomatic carriage to severe disease with toxic megacolon and standard treatment includes antibiotics such as vancomycin or metronidazole. As infection reoccurs in about 10 to 40 percent of cases following initially successful therapy, there is a huge demand for improved therapeutics. In order to increase colonic delivery of vancomycin for the treatment of CDI, Bigucci et al. created vancomycin-containing chitosan/pectin polyelectrolyte complexes. These complexes show pH-dependent swelling and drug release together with colonic mucoadhesion, which suggest superior drug delivery in comparison to standard formulations. However, in vivo data supporting this concept are missing so far.

In addition to infectious colitis, IBD also pose an interesting target for mucoadhesive drug formulations. Both CD and UC result in a chronic relapsing inflammation of the gastrointestinal tract that leads to severe complaints including diarrhea, abdominal pain, fever and rectal bleeding in affected patients. Whereas UC is restricted to the large intestine, CD can affect every part of the gastrointestinal tract including the large intestine. Treatment of IBD includes various immunomodulatory approaches including salicylates, steroids, immunosuppressives and biologicals such as anti-TNF therapeutics depending on the severity of disease activity. Especially mild active disease is frequently treated with locally administrated drugs such as salicylates and steroids as oral formulations, enemas or suppositories. Oral drug delivery especially, harbors the challenge of selective targeting of the colorectal region in patients with Crohn’s colitis or ulcerative colitis. In this regard, mucoadhesive strategies have been evaluated to improve colorectal drug delivery for IBD treatment. Similar to colorectal drug targeting for systemic therapy, mucoadhesive
formulations containing chitosan and/or alginate particles have been exploited to deliver
standard IBD therapeutics such as 5-aminosalicylic acid \[^{276}\] or prednisolone to the large
intestine \[^{277, 278}\]. Interestingly, negatively charged liposomes show an improved drug delivery
in comparison to the free drug solution in experimental models of colitis, possibly due to an
increased adhesion of negatively charged liposomes to the inflamed mucosa. Again, most of
these data have been generated either in vitro or in vivo with preclinical models of intestinal
inflammation. Thus, a proof for a transfer of these strategies for the treatment of human
diseases is still missing.

e. **Vaginal drug delivery**

The vaginal tract has a relatively large surface area of 60 cm\(^2\) and a rich blood supply. The pH
of the vaginal tract is controlled by the bacteria Lactobacillus converting glycogen and
carbohydrates to lactic acid. The pH varies between 4 and 5 depending on the menstrual cycle
\[^{279}\]. There are no secreting glands in the vagina and the amount of fluid is therefore sparse i.e.
around 6 g \[^{280}\]. Only small amount of additional liquid can be held without starting to leak out.
The vaginal fluid consists of inorganic and organic salts, mucin, proteins, carbohydrates, urea
and fatty acids. The vaginal fluid acts as a protecting barrier and consists of different
antimicrobial substances \[^{281}\]. The surface of the vaginal tract is covered by a mucous
membrane. The mucin-layer consists of two different types of mucins; cell-associated mucin
and secreted mucin forming the outer layer \[^{282}\]. The secreted mucin has a rapid turnover and
can trap foreign particles which will then be efficiently cleared away.
The vaginal tract can in principle be used for both systemic and local delivery of drugs, where
local drug delivery for combating for instance fungal, bacterial or viral infections has been the
interest of many studies.

There are many challenges related to achieving local drug delivery to the vaginal tract. The
formulation must be able withstand changes in the pH, release the drug in the small amount of
fluid present and also be able to penetrate the mucus layer before being cleared away by the self-cleaning action of vagina. In addition, the formulation must not leak out of the vagina\textsuperscript{[282]}. A mucoadhesive formulation will have the possibility of enhancing the time the formulation stays in the vagina, however; often the formulation also needs to penetrate the mucus layer in order to achieve the desired effect. This could be complicated if the formulation sticks too well to the mucus layer. In addition, mucoadhesive particles may change the protective properties of the mucin-layer, letting both the drug and pathogens permeate the mucus barrier\textsuperscript{[283]}. The size of the particle seems to be important in order to be able to penetrate the mucus-layer. Nanoparticles have therefore been proposed to be a promising formulation in order to achieve local vaginal drug delivery\textsuperscript{[284]}. A new approach for obtaining local vaginal drug delivery is muco-resistant nanoparticles such as polyethylene glycol (PEG) coated particles\textsuperscript{[285]}. These particles can diffuse faster through the mucus-layer than mucoadhesive particles. The size of the particles should be between 200-500 nm.

f. Nasal delivery

Since the pioneering studies on chitosan as a nasal penetration enhancer\textsuperscript{[286]}, the nasal route has become an attractive option for transmucosal drug delivery, especially for protein/peptide drugs. The nasal mucosa consists of epithelial cells underlined with rich vascularity that provides direct entry of the drug into systemic circulation via passive diffusion and eventual rapid onset of the pharmacological effect. The area available for drug absorption is relatively large but still limited and not easily available. Similarly to the intestinal mucosa, nasal mucosa is monostratified and characterized by the presence of tight junctions and by an abundant mucus secretion. Approximately 1.5–2.0 litres of mucus are secreted daily by goblet cells and serous glands within the nasal cavity, known to contain ca. 1% of proteins, including several proteases\textsuperscript{[287]}. Moreover, mucociliary clearance is a local defence mechanism with regard to
respiratory airways that tends to remove foreign bodies (namely bacteria) including formulations from the mucosal site.

Due to the above anatomical and physiological constraints, administration of drugs with the aim of systemic absorption inevitably requires the use of absorption promoters, typically chemical penetration enhancers, but also of enzyme inhibitors, to escape peptidase action and the use of mucoadhesive formulations to increase the duration and intimacy of contact with the nasal mucosa. Finally, a prerequisite for nasally applied formulations is that it does not interfere with normal nasal functioning. Since the nasal mucosa is prone to damage by penetration enhancers, which would impair its functions, special attention should be given to the cytotoxicity of absorption promoters and the reversibility of their effect on the nasal membrane should be ascertained.

Besides the vulnerability and the above limitations, the nasal route can be easily managed with an appropriate mucoadhesive formulation for the administration of poorly absorbable peptide/protein drugs or by avoiding hepatic first-pass effect. The use of chitosan and chitosan derivatives, well-known multifunctional polymers, certainly has represented a valid strategy to overcome such limitations. The mucoadhesion properties of chitosan can be exploited also in nasal administration of vaccines. Due to the rapid entry to blood circulation, the nasal route is also promising in the management of crisis situations and intense acute pain, such as heart attack, hypoglycaemia, seizure, severe nausea and vomiting, or breakthrough cancer pain.

Finally, nasal mucosa is also the target for locally applied, locally acting products intended for the treatment of various upper respiratory syndromes such as flu-like symptoms (common cold)
and allergic rhinitis. These pathological conditions are characterised by an abundant mucus production accompanied by headache and discomfort. Those symptoms might be caused by viral/bacterial infections that are common pathologies affecting both adults and, more frequently, children of school age. They might also be caused by acute and chronic reactions to allergens.

Pharmacological treatment requires oral administration of anti-inflammatory drugs (aspirin, paracetamol, NSAIDs) and antibiotics as well as local treatment, through sprays or aerosol, with corticosteroids and antihistamines, which is likely to produce side effects such as mucosal dryness and secondary fungal infections. Given that mucus overproduction is one of the most fastidious symptoms, likely to be severe in children, a valuable approach could be to exploit the well-known interaction between chitosan and mucins to obtain a mucolytic effect, thus counteracting the mucus excess. The supposed mechanism of action is the physical interaction between the negatively charged mucin macromolecules and the positively charged side groups of chitosan. The approach has led to the development of a liquid nasal spray, having low viscosity to ensure sprayability, intended to reduce or eliminate the excess of mucous liquid production in rhino-faryngeal diseases (Figure 10.1).

Figure 10.1 Nasal spray
The commercial formulation, developed as a medical device and named Captomucil® contains a specially devised chitosan grade sourced from fungi having an average MW of 15000 Da and a deacetylation degree of 70%, soluble at neutral pH (6-7) and lacking any irritancy towards nasal mucosa (Patent App. N. MI2014A000825) and likely to produce a mucolytic effect even at very low concentrations. To assess the functionality of the medical device, a rheological approach was used, based on two different techniques [165, 292] to measure the interaction between chitosan and mucin. Both techniques require the use of highly-purified mucin, sourced either from the submascillary cavity of cows or from the stomach of pigs. Two sets of formulations were tested, that differed for the salt contents, since it is known that tonicity has an effect on mucus rheology and on nasal mucociliary clearance [293]. In the first sets of experiment, performed according Rossi S. et al. [165], a hypotonic Captomucil® formulation (i.e., without salt addition, with chitosan concentration ranging between 0.15-0.16 w/v), was examined for its capability of reducing the viscosity of the submascillary bovine mucin solution in the concentration range 0.5-6% w/w of mucin. The liquid formulation was mixed with mucin solution at different concentrations (see Figure 10.2) in a volumetric ratio mimicking the physiological one. Blank samples (mucin or formulation) were also prepared by diluting with distilled water appropriate volumes of either mucin dispersion (mucin blank) or liquid formulation (formulation blank).

Rheological measurements were made at 37°C on each and every set of samples (mucin blank, formulation blank and mucin-formulation mixture) with a rotational rheometer. The rheological interaction between mucin and chitosan was quantified by means of the rheological synergism parameter $\Delta\Pi$ (mPa.s), calculated with equation 3.

$$\Delta\Pi= \Pi_{mix} - (\Pi_f + \Pi_{muc})$$

Eq. 3

Where: $\Pi_{mix}$=viscosity of the formulation and mucin mixture, $\Pi_f$=viscosity of formulation blank and $\Pi_{muc}$=viscosity of mucin blank. The results (Figure 10.2) showed positive rheological
synergism values at very low mucin concentration (0.5-% w/w) and low shear rates, whereas at the highest mucin concentration (4-6% w/w) definite negative rheological synergism values were recorded. Since the synergism values observed at low values (0.5-2% w/v) and at low shear rates were affected by a high standard deviation, it was suggested that the method were not sensitive enough at very low concentrations.

Figure 10.2 Rheological synergism as a function of shear rate evaluated for five formulation-mucin mixtures (mean values +/- standard deviation; n=3)

By contrast, the definite negative synergism values observed at high concentrations (4-6% w/v) indicated that, in those conditions, chitosan produces the maximum interaction with mucin macromolecules. In other words, a massive precipitation of mucus should occur when in presence of a very viscous mucus. To deal with the limited sensitivity of the method at low concentrations, in the second sets of experiments performed according to Menchicchi et al. [292], three Captpmucil® formulations having different contents in chitosan and sodium chloride (chitosan concentration was 0.25, 0.45 and 0.16% w/v, NaCl concentration was 2.2, 2.2 and
0.40 w/v%, respectively) were examined for dynamic viscosity using a falling sphere viscometer.

Using this method it is possible to work at very low shear rate with precision and probe differences in rheological behavior with a very high sensitivity. The deviation of the viscosity of the mixture mucin/formulation from the sum of the viscosity of mucin solution plus the viscosity of the formulation represents the rheological synergism that may be either positive or negative depending on the sign of the deviation. The method allowed to explore mucin/formulation ratio much lower than the other method ranging between 0.1 to 1.0 The results obtained for the 3 formulation tested are given in Figure 10.3.

Figure 10.3 Relative deviation (in %) from the additive line (i.e., of no interaction) of the relative viscosity ($\eta_{rel}$) of chitosan-based Captomucil® formulations (as in label) in the presence of pig gastric mucin as a function of mucin/formulation mass ratio $f$ (37 °C).
A negative deviation is observed within the whole range of mucin/captomucil ratios tested and the maximum negative deviation, meaning the maximum interaction and mucus precipitation thereof, occurs in the whole range of mucin/formulation ratio for the mixture 1c (green symbols) having the lower Captomucil® concentration and with the lower salt concentration (slightly hypertonic formulation) whereas higher concentrations of Captomucil® and salt in 1a and 1b (black and red symbols) (hypertonic formulation) caused a weaker interaction specifically not in the whole range of ratios examined.

These results allowed to choose the best concentration of chitosan and salts for the final formulation. Moreover, the results obtained demonstrate that the rheological approaches, appropriately used according to physiological conditions; can be profitably used to measure the precipitation capacity of mucolytic formulations.

g. **Ocular delivery**

To avoid physical and biochemical insults, the eye is isolated from the rest of the body by a number of barriers. It is protected by the blood-retinal and blood-anterior chamber barriers. Also, to meet its complex metabolic demands, the eye is supplied by an efficient microvasculature that enables the supply of nutrients and oxygen, and the removal of waste products at high fluxes. Due to this physiological and anatomical constraints, drug delivery to the eye poses important challenges. Among these include to overcome physical and metabolic barriers. Namely, structural barriers on the periphery of the retina comprised by tear secretion and blinking, the conjunctival lining, connective tissue barriers of sclera and cornea, and blood retinal barriers. Other factors that limit the transport of drugs to the eye include transporter expression, melanin binding, and the physical state of the vitreous humour. Altogether, these
factors result in a short residence time, drug drainage, and need for frequent instillation. Typically, drops instilled onto the corneal surface would be removed within 1-2 min. Also, in general, only a negligible fraction of systemically administered drugs reach the ocular tissues. Hence, increasing the bioavailability of topically administered drugs to the eye, has been a major challenge. The structure of the surface of the eye is very complex. A pocket formed by the eyelids fold over the cornea and conjunctiva. This pocket contains the tears (ca. 7 µL) that sit on a mucus layer that coats the lining of the pocket. Two important tissues in this region are the conjunctiva and the cornea. The conjunctiva has a high density of mucus-producing goblet cells, while the cornea has no goblet cells. Hence, the mucin that is secreted by the conjunctival goblet cells stretches across and loosely attaches to the cornea. Hence, any mucoadhesive formulation needs to attach to the conjunctival mucus and presumably not to the corneal mucus, given its loose attachment to the underlying tissue. Besides, binding of any material to the cornea might interfere adversely with the vision process. Eye mucus is produced by goblet cells in the conjunctiva. More specifically, these goblet cells are the crypts of Henle in the conjunctival surface of the upper and lower tarsus and the glands of Manz on the limbal conjunctiva. Mucus-producing goblet cells are contained in the nonkeratinized columnar epithelium. The mucus is wiped over the surface of the cornea by the windshield-wiper-like movement of the upper eyelid. The human tear film is approximately 3.0 µm thick as determined by reflectance spectroscopy and optical coherence tomography. The mucin’s turnover rate is ca. 15-20 h. The innermost mucus layer has been estimated to be 0.05 to 1.5 µm thick. The occurring transmembrane and secretory mucins outlined in Section 3, play several crucial roles in increasing the wettability of the ocular surface glycocalyx, mediating intracellular signal transduction, as well as in modulating the viscoelastic properties and surface tension of the tear film.
Given that a number of eye pathologies are “silent” diseases with no major symptoms other than the gradual loss of vision (e.g., glaucoma or cataracts), patient-friendly prolonged release formulations are essential to comply with the dosing regimen. Drug delivery to the eye can be either through the corneal or the non-corneal (scleral and conjunctival) route. Mucoadhesive polymers have been extensively evaluated either as solutions, inserts, in situ gelling systems or as colloidal particulate systems to enhance the drug penetration across the cornea epithelium for ocular drug delivery. Examples include polyacrylic acid complexed to pilocarpine \[^{297}\]; polyacrylic acid crosslinked with divinyl alcohol for the delivery of progesterone \[^{298}\]; polyvinyl alcohol inserts loaded with antibiotics, sulfonamide and atropine \[^{299}\]. Also, natural polymers such as polysaccharides have been used as ocular delivery systems, namely pullulan, hyaluronic acid and chitosan \[^{300}\].

Colloidal delivery systems such as liposomes \[^{301}\], biodegradable nanoparticles \[^{302}\] and nanocapsules \[^{303}\] offer special features as compared to other alternatives. Ex vivo studies \[^{304}\] demonstrated that the corneal penetration of encapsulated indomethacin in the form of nanoparticles, nanocapsules and nanoemulsions was 3-fold higher than the commercial formulation (Indocollyere®). No differences in drug absorption were noticed among the three different systems, thus suggesting that the colloidal nature was the key factor, regardless of the composition of the system. Independently, it was realized that a greater spreading coefficient and lower contact angle was attained between an excised corneal tissue and a drop of a cationic nanoemulsion, as compared to an anionic one \[^{305}\]. This study already pointed to the importance of particles with capacity to interact with the negatively charged surface of the cornea that would prolong the residence time and drug absorption. In studies led by the group of Prof. Maria J. Alonso \[^{306}\], they determined the ocular drug disposition after instillation of \(^{14}\)C-
indomethacin-loaded chitosan-based nanocapsules to conscious rabbits. The results showed that chitosan nanocapsules increased the drug levels in cornea and aqueous humor to a significantly greater extent than the commercial drug preparation or the drug loaded uncoated systems. Later, it was shown that ionically crosslinked nanoparticles comprised by chitosan-tripolyphosphate (TPP) were effective for the ocular delivery the hydrophobic immunosuppressive peptide cyclosporin A to conscious rabbits, as a promising therapy for management of external inflammatory/autoimmune ocular diseases, such as keratoconjunctivitis sicca or dry eye disease. These studies led to the conclusion that chitosan nanoparticles adhere to the ocular surface and some of them enter the conjunctival and the corneal epithelia. It was also found that the retention of the nanoparticles was more important in the conjunctiva than in the cornea. Overall, these results indicated that the affinity of chitosan for the ocular surface (either cornea or conjunctiva) is greater when it is in a particulate form than when in solution. Similar conclusions were reached in studies with stearylamine positively charged liposomes for the ocular administration of acyclovir to rabbits \[307\]. These liposomes would bind intimately on the cornea surface to lead to an increase of residence time and to the complete coating of the corneal surface. In turn, the negatively charged liposomes were expelled by the cornea surface and the drug concentration in the cornea after 2.5 h of administration was half that of the positively charged liposomes.

More recently, mucoadhesive nanoparticles comprised by chitosan and hyaluronic acid have been found with very low cytotoxicity and with the ability to enter the corneal epithelial cells and to be taken up by CD44 receptor-mediated endocytosis \[308\]. These new type of particles were found effective for the delivery of DNA into both the corneal epithelium (HCE) and conjunctival cells (NHC) with high levels of expression of the CD44 receptor. Another study, showed that polyethylenimine (PEI)/DNA polyplexes coated with low molecular weight
hyaluronic acid were effective for transfecting human corneal epithelial (HCE) cells. More studies, however, are necessary to understand in greater depth at the sub-cellular level the interplay between drug targeting, trafficking in the corneal epithelium. The role of mucoadhesion in these systems needs to be elucidated in further detail. Particularly, aiming to correlate in vitro studies with their in vivo counterparts.

h. Intravesical drug delivery

The bladder is a hollow organ that comprises multiple layers of tissue. From the luminal to the outermost surface the layers are: urothelium, detrusor muscle and adventitia. The urothelium is the innermost layer and it serves as a permeability barrier. In fact, the water tight barrier between blood and urine formed by urothelium is the toughest known barrier to drug delivery. It has exceptionally high transepithelial resistance ranging from 10 000 to >75 000 Ω/cm² owing to paracellular resistance of tight junctions pooled with apical plasma membrane transcellular resistance. The urothelium is composed of three different cell types (from the detrusor to the apical side), namely, basal, intermediate and umbrella cells (named after their characteristic shape). The umbrella cells comprise a water impermeable barrier that is armored by multiple rigid-looking plaques in consort with tight junctions joining its apical surface and uroplakins further enhanced by a layer of mucin and other glycosylaminoglycans (GAGs) (Figure 10.4). Membrane-associated MUC1 and MUC4 are the predominant mucins of the glycocalyx, while heparan sulfate, chondroitin sulfate and dermatan sulfate, have been identified among the main GAGs. The primary role of the mucin/GAG layer may be more in line with an antibacterial adherence function rather than its role as a barrier [314]. The GAG layer may also be important for the formation and attachment of particulates to the urothelium and stone formation [315].
Figure 10.4. Structure of the urinary bladder and the urothelium. Abbreviation: GAG — glycosaminoglycan\textsuperscript{[316]}.

The mucin layer is thought to act as an anti-adherent and prevents adhesion of foreign particles. However, it also prevents effective diffusion of drug instillations into the bladder wall. Hence, the delivery of drug formulations into the urinary bladder wall needs to overcome these barriers. The most common dysfunctions are bladder cancer, interstitial cystis (IC), bladder pain syndrome, and overactive bladder (OAB). Recent comprehensive reviews on novel drug delivery systems for bladder dysfunction therapy have been recently been published\textsuperscript{[310, 316, 317]}.

Intravesical drug delivery (IDD) involves the administration of drug into the bladder using a catheter. The advantages of IDD include overcoming systemic adverse effects and shortcomings of oral therapy such as drug or formulation specific vagaries in absorption, metabolism and renal excretion; as well as the greater exposure of the tissue to the drug. By
contrast, there is substantial drug dilution during urine voiding, there is low urothelial drug
permeability, and the need for repeated catheterizations. To counteract the limitations
associated with low drug permeability, mucoadhesive formulations offer great promise. IDD
approach is amenable to modulating the release and absorption characteristics of instilled drugs
through coupling them to novel carriers, such as liposomes, microspheres, nanoparticles and in
situ gelling systems.

Dysfunction of the GAG/mucin layer is the currently prevailing hypothesis for IC. Intravesical
administration of hyaluronic acid, as surrogate of the GAGs of the urothelium has been reported
[318-320]. This approach was found to result in inhibition of leukocyte aggregation, cell migration
and promoted adherence of immune complexes to polymorphonuclear cells. Also, it has been
shown that hyaluronic acid suppresses the secretion of pro-inflammatory interleukins IL-6 and
IL-8, and increases the synthesis of sulphated GAGs.

The main challenge in IDD is to increase drug transport. This can be achieved either by physical
or chemical methods. We will only address here the latter. Mucoadhesive formulations for IDD
must fulfill three basic criteria, namely that the carrier should have rapid attachment or
adhesion to the bladder wall after instillation into the bladder, must not obstruct the flow of
urine or any of the normal functions of the bladder, and it should be able to stay attached to the
affected site for a number of hours even after voiding of urine [310]. A number of mucoadhesive
IDD formulations have been researched. These include the use of polymers of both natural and
synthetic type, such as chitosan, carbomers, polycarbophil (PC) gelatin, polyethylene glycol,
poly(methylidene malonate-2.1.2), and cellulose derivatives [309]. In a comparative study,
chitosan was found to exhibit greater mucoadhesion to the bladder wall than CMC and
polycarbophyl, thus resulting in a slower drug release and prolonged residence time [321].
In a different study, chitosan-thioglycholic acid (TGA) nanoparticles loaded with trimethoprim have been used for IDD in an in vitro study using porcine urinary bladders. It was found that the thiol groups and disulfide bonds introduced to chitosan-TGA conferred greater stability, superior mucoadhesion and more sustained and controlled release than the corresponding unmodified chitosan nanoparticles. The adhesion time of chitosan-TGA NP was around 14-fold increased, while unmodified chitosan NP were washed out after three hours and six micturitions.

Downregulation of sensory nerves by using neurotoxins like capsaicin, resiniferatoxin (RFX) or botulinum toxin has proven itself a viable approach in urology, particularly for urinary contingency and OAB. Formulations based on liposomes and thermosensitive hydrogels loaded with these drugs have also been investigated in the therapy of bladder dysfunction. Capsaicin-loaded chitosan-based nanocapsules have been found to increase of paracellular drug permeability in various epithelial cell lines (namely, MDCK and Caco-2).

Knowing that these nanocapsules have also proven to be mucoadhesive (see previous section on ocular delivery), it would be reasonable to expect that these formulations may prove to be highly effective in IDD in future studies.

12. Conclusion

In summary, throughout this review, we present an updated overview of the recent progress on the experimental methods and applications in mucoadhesion research. As illustrated with reference to specific cases, the method of choice to examine the mucoadhesive properties of a given biomaterial (namely a medical device or pharmaceutical formulation) can vary widely. A sounder understanding of the phenomena at play that influence the molecular level interactions of mucin and mucus with a large diversity of materials is likely to contribute to the development of innovative applications on a more rational basis. Novel methods currently
under development and that have not been addressed in this review include the use of in vitro
cell culture or biomicrofluidics approaches.

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