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Assessment of mucoadhesion by a resonant mirror biosensor

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Abstract

The aim of this study was to add knowledge to the existing theories of mucoadhesion and to review mucoadhesive polymers based on their ability to form non-covalent bonds with mucus glycoprotein. Resonant mirror biosensor was used to study the candidate mucoadhesive polymers hydroxypropyl methylcellulose, carboxymethylcellulose, Carbopol, hyaluronate, alginate and chitosan. Bovine submaxillary mucin was chosen as substrate, representing the major glycosylated protein in mucus. For comparison, non-glycosylated bovine serum albumin was used as an alternative substrate. The results of this study reveal that there is a clear correlation between the ionization state of the polymer, which is dependent on the pH of the surrounding environment, and its binding behavior. Ionizable polymers need to be in their unionized state to be able to form non-covalent bonds with mucus glycoprotein. Acidic polymers display binding behavior only at pH around or lower than their corresponding pK_a values and basic polymers vice versa. Chitosan was found to be the most mucoadhesive polymer. Unionizable polymers like hydroxypropyl methylcellulose did not display any affinity for mucus glycoprotein. Unionized amino- and carboxyl groups on polymers were found to be important structural feature of polymer for the formation of weak chemical bonds to mucus glycoproteins.
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1. Introduction

The concept of mucosal adhesives, or mucoadhesives, was introduced into the controlled drug delivery area in the early 1980s (Ahuja et al., 1997) and has gained much attention in the last two decades. Mucoadhesive polymers are able to interact with mucus which is secreted by the underlying tissue (Mathiowitz et al., 1999). More specifically, it is predicted that such polymers interact with mucus glycoprotein, called mucins, which are responsible for gel-type characteristics of the mucus. Mucoadhesive polymers can increase the contact time with the mucosal tissue and moreover also increase directly drug permeability across epithelial barriers (Robinson and Mlynek, 1995). Mucoadhesive polymers are applied in the field of local drug delivery, i.e. nasal, ocular, vaginal and intra-oral, and can increase the bioavailability dramatically.

Duchene et al. (1988) proposed the following three stages in mucoadhesion. Initially, an intimate contact (wetting) between the mucus gel and the swelling mucoadhesive polymer is required. This is followed by the penetration of the mucoadhesive polymer into the mucus gel network and entanglement of polymer and mucin chains. Third stage is the formation of weak chemical bonds between entangled chains. However, despite many studies in recent years, the phenomenon of mucoadhesion is not fully understood. Adhesion of certain polymers to mucus is a complex event and depends on the properties of the polymer, the biological substrate and the surrounding environment (Tamburic and Craig, 1997). Visualization studies of the mucoadhesive interface have questioned the second step in the mucoadhesion process (Lehr et al., 1992c). In this study, no evidence for intermixing between mucus and mucoadhesive hydrogel was found to occur in the μm-range. Interpenetration of free polymer chain ends, however, may still be possible in the nm-range.

Many papers have been published presenting slightly different theories and mechanisms of mucoadhesion. The reason for this disagreement is maybe not so surprising because there have been so many different *in vivo* and *in vitro* methods utilized to measure mucoadhesive properties of polymers, resulting in inconsistent results (Peppas and Buri, 1985; Saettone et al.,

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1989; Junginger, 1991; Lehr et al., 1992b; Mortazavi and Smart, 1994, 1995; Tamburic and Craig, 1997; Madsen et al., 1998a,b; Sigurdsson et al., 2002; Keely et al., 2005). Some viscosity enhancing polymers have also been considered as mucoadhesive. Although formulations containing these viscosity enhancing polymers may eventually also lead to a reduced clearance from the site of application (e.g. after ocular or nasal instillation), such effects must not be confused with "wet-on-wet" mucoadhesion, which should be only used to address the rather remarkable adhesion of a polymeric hydrogel to another one in the presence of excess liquid. Moreover, besides such "wet-on-wet" adhesion, there may also be some remarkable sticking of dry hydrophilic polymers when brought in contact to a wet or humid surface. Although this kind of "sticking" has been referred to as mucoadhesion by some authors as well (Mortazavi and Smart, 1995; Accili et al., 2004; Smart, 2005), binding forces typically decrease dramatically in the presence of excess amounts of water ("over hydration") (Henriksen et al., 1996). Such "dry-on-wet" adhesion probably involves quite different mechanisms (including, e.g. capillary attraction) and should therefore be strictly separated from the "wet-on-wet" adhesion of swollen mucoadhesive polymers to mucous surfaces, to which we are referring in this article.

In this situation, mucoadhesive polymers must demonstrate significant interaction with the mucus glycoprotein even in fully hydrated state and in the presence of excess amount of water. The initial phase stage of mucoadhesive contact, penetration and subsequent binding, appears to be mainly governed by surface energy effects and spreading phenomena, as it was found that measuring surface energy and spreading coefficients can predict mucoadhesive performance (Lehr et al., 1992a, 1993). However, for optimal design of mucoadhesive drug delivery system, a better understanding of the molecular interactions between the glycoprotein and the polymer is needed, which most likely govern the later stages of mucoadhesive bonding after the surface energy initial contact and spreading.

In the last decade, the optical biosensor technique based on evanescent waves has become an established method of measuring molecular interactions (Rich and Myszka, 2000; Ward and Winzor, 2000). This technique allows monitoring any interaction between two molecules in real time without any label or tag as long as one of the molecules can be immobilized, with covalent or non-covalent bonds, on the surface of such system and the other stays in solution above the surface. Binding of molecules in solution to surface-immobilized molecules alters the refractive index of the medium near the surface and by utilizing a red laser which sweeps a range of incident angles, this tiny change in refractive index gives rise to a measurable signal. Great care must be taken to monitor all non-specific binding to the system surface. Common applications include ligand fishing, assay development and target identification (Cooper, 2002). To our knowledge, the resonant mirror biosensor has not been used before to quantify interactions between glycoprotein and polymers.

The aim of this study was to use an optical biosensor technique based on the resonant mirror principle to measure the interaction between several different polymers and mucus glycoprotein (step 3 in the mucoadhesion process defined by Duchene). This study should answer the following questions:

1. Which polymers are can form weak chemical bond to proteins and when?
2. What structural features of polymers are necessary for the formation of these bonds?

Bovine submaxillary mucin was chosen as a substrate, representing the major glycosylated protein in mucus. For comparison, non-glycosylated bovine serum albumin was also used as a substrate.

2. Materials and methods

2.1. Materials

Carboxymethylcellulose sodium salt (Tylopur C300P) (CMC) (Hoechst, Frankfurt, Germany), hydroxypropyl methylcellulose (Pharmacoat 606) (HPMC) (Shin-Etsu Chemical, Mühlheim, Germany), Carbopol® 934 (BF Goodrich, Chicago, USA), chitosan (Seacure 210+) (Pronova A/S, Drammen, Norge), chitosan (Protasan UP G) (Novamatrix, Oslo, Norway), Sodium alginate (Pronova UP) (Novamatrix, Oslo, Norway), and sodium hyaluronate pharma grade (Novamatrix, Oslo, Norway).

Bovine submaxillary mucin type I-S (BSM) and bovine serum albumin (BSA) were from Sigma (Taufkirchen, Germany). EDC/NHS coupling kit containing *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) from Affinity sensors (Cambridge, UK). Isotonic phosphate buffers (PBS) and isotonic acetate buffers were prepared from analytical grade compounds purchased from Merck (Germany).

2.2. Instrument

An IAsys instrument (Affinity sensors, Cambridge, UK) with double cell carboxylate cuvettes (FCC-5301), non-derivatised cuvettes (FCS-53001) or hydrophobic cuvettes (CD-5201) (Thermo ElectronGmbH, Dreieih, Germany) was used for the binding studies.

2.3. Methods

2.3.1. Covalent immobilization onto carboxylate surface (method A)

Immobilization of protein onto the carboxylate surface was performed according to the manufacturer's specifications. Briefly, after equilibration and obtaining a stable baseline with PBS, the carboxymethylated surface was activated with EDC/NHS for 10 min. BSM was covalently attached via primary amino groups onto activated surface in one cell of double cell cuvettes and BSA was immobilized on the surface in the other cell and served as a control. Unreacted sites on the surface were blocked with 1 M ethanolamine pH 8.5. Binding of the various polymers to BSM and BSA was measured in 50 μl (by adding 5 μl of polymer solution to 45 μl of buffer) of 0.01 M

Table 1
Structure and properties of the polymers tested in this study

	MW (kDa)	pK_a	Type	Structure
Carbopol 934P	3000	∼6.0	Acidic	
HPMC Pharmacoat 606	86	–	Neutral	R= H, CH3, iso-OH
CMC Tylopur C300P	140	∼3.5	Acidic	
Alginate Pronova LVM	75–200	∼3.5	Acidic	
Hyaluron	620–1150	∼3.2	Acidic	
Chitosan Seacure 210+	162	∼6.2	Basic	
Chitosan Protasan UP G 113	<200	∼6.2	Basic	

acetate buffers, pH 4.0 and 5.5, and phosphate buffers, pH 6.5, 7.4 and 8.2, at 20 °C. Concentration of the polymers ranged from 3.3×10^{-11} M (for $1 \times 10^{-}$, w/v Carbopol solution) to 1.1×10^{-6} M (for 1×10^{-2}, w/v alginate solution, see Table 1 for molecular weight of the polymers). After washing the surface with appropriate buffer and equilibrating for 5 min, binding was monitored for 10 min, followed by dissociation and regeneration of the surface by washing with mild acid or base for several minutes. Non-specfic binding was monitored by applying polymers to the same surface without the immoblized protein. Immobilized protein was prepared and used within 1 week. The concentration of polymer is well within its maximum solubility at all pH's.

2.3.2. Non-covalent immobilization onto hydrophobic or non-derivatised surface (method B)

After extensive washing, 30 μl of isotonic phosphate buffer pH 7.4 is pipetted into both cells of the cuvettes. 50 μl of the polymer (1 mg/ml in the same buffer) is added to both cells and the response is recorded for 10 min. The polymer covers the surface by hydrophobic and/or electrostatic interactions. Both cells are washed three times with buffer, leaving 30 μl of buffer in the cells. Total binding of polymer to the surface is recorded. 50 μl of BSM (4 mg/ml in buffer) is added to cell 1 and 50 μl of BSA (4 mg/ml in buffer) is added to cell 2. The response is recorded for 10 min. Both cells are washed three times with buffer, leaving 45 μl of buffer in the cells. The amount of immobilized protein on the surface can be calculated after this step.

Five microliters of polymer (1 mg/ml) is added to both cells and the response is recorded for 10 min. This signal is used to calculate how much polymer binds to the protein. Both cells are washed three times with buffer and the response is recorded. Both the polymer and the protein are removed from the surface by washing with 1 M HCl for 2 min and 1 M NaOH for 2 min. This washing cycle is repeated three times. Protein solutions were prepared and used within 10 h (intraday). The concentration of polymer is well within its maximun solubility at all pH's.

2.4. Calculations

Measured binding response in arc seconds is converted into mass of protein or polymer on the surface of a cuvette. It is not

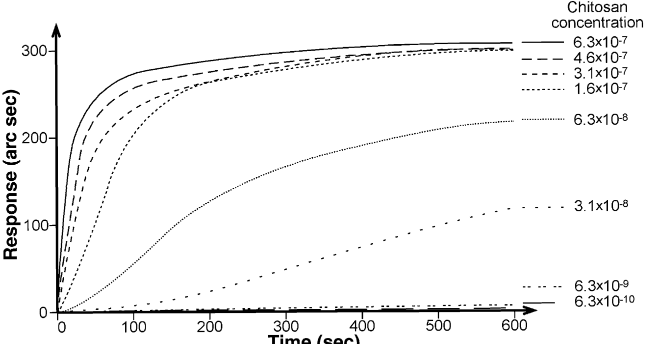

Fig. 1. Binding profile of chitosan (seacure 210+) to BSM at various polymer concentrations at pH 7.4 utilizing method A.

practical to compare the binding to the different protein on a molar scale since BSA is rather small (stokes radius of 3.7 nm), globular protein (Habeeb, 1966) and BSM is about 900 nm long rod (or shorter coil in saline solutions) (Bettelheim et al., 1962). Based on these facts and the sizes of the polymers, we assume that one molecule of BSA can only bind one or two molecules of polymer. In contrast, one molecule of BSM has been reported to bind many molecules of polymer, depending one the size of the polymer (Deacon et al., 2000). Although we may assume that in both cases the sensor surfaces are covered with the proteins it can be misleading to report the binding on a molar scale. Instead, binding of gram of polymer per gram of protein was used to quantify the interactions. The binding is visualized in real time and K_d values can be calculated but not compared because of the different binding capacities of the proteins. Kinetic calculations are therefore omitted.

3. Results

Examples of chitosan binding curves to BSM at various concentrations are shown in Fig. 1. Maximum binding capacity of the immobilized protein can be calculated from this data. At least six different concentrations of polymers were tested to calculate the maximum binding capacity (for method A).

Figs. 2 and 3 show relative binding (w/w) of the polymers tested to BSM and BSA utilizing method A and method B, respectively. Both methods gave similar results. HPMC and hyaluronate did not interact with the protein at all. Alginate, Carbopol and CMC show some binding behavior at low pH.

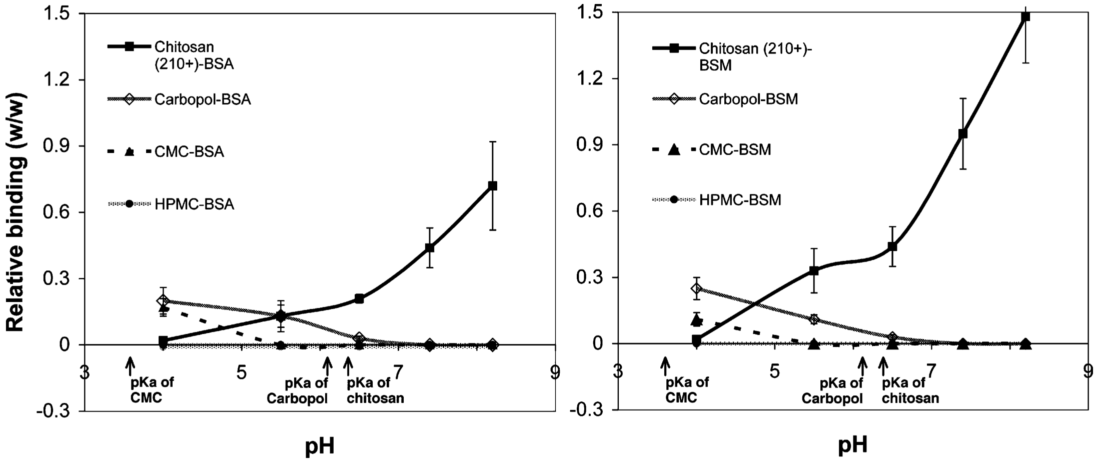

Fig. 2. Relative binding (w/w) of polymer to BSM or BSA measured with method A ($n = 6$) (±standard deviation).

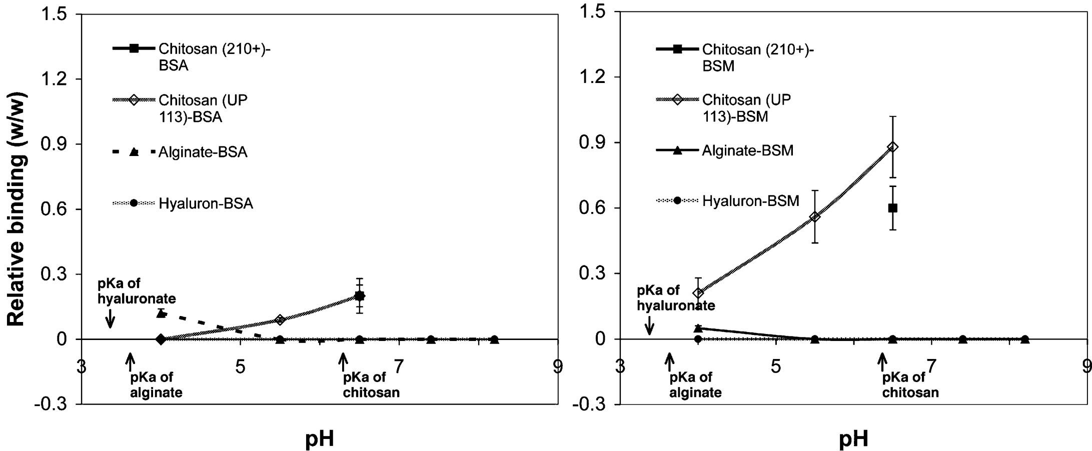

Fig. 3. Relative binding (w/w) of polymer to BSM or BSA measured with method B ($n = 4$) (±standard deviation). Binding of chitosan at pH value above 6.5 is not possible with method B due to limited solubility of chitosan.

Chitosan, on the other hand, shows greater binding at high pH and has the greatest affinity for the mucus glycoprotein of all the polymers. Alginate, Carbopol and CMC displayed similar affinity for glycosylated protein (BSM) and non-glycosylated protein (BSA). Chitosan had much greater affinity to BSM than to BSA. Chitosan did not bind to BSM or BSA if the protein was pre-treated with glucosamine, a monomer of chitosan. Both methods gave similar results

4. Discussion

4.1. About the methods

Method A has two drawbacks when studying glycoprotein–polymer interaction. The glycoprotein is very large molecule and therefore is unlikely to completely cover the surface of the cuvette when immobilized (because of spatial hindrance). This opens up the possibility for the polymer to bind non-specifically to the bare surface of the cuvette, even though all the unreacted sites have been blocked with ethanolamine.

Mucoadhesive polymers bind very strongly to the glycoprotein. Some acid or base is then needed to regenerate the surface for the next binding cycle. Not all polymer is removed from the glycoprotein when using too dilute acid or base. On the other hand, the sugar residues may be cleaved off from the glycoprotein when using to strong acid or base and the protein degrades (Downs and Pigman, 1970). Both situations results in decreased observed binding in the next binding cycle. However, this restriction probably only applies to very large glycoprotein that has limited stability in solutions.

The major advantage of method B is that non-specific binding of polymer to the surface of the cuvette is eliminated. It is also a big advantage that freshly prepared glycoprotein can be used for each binding cycle since it is frequently degraded in solution. The major disadvantage is however that this method is much more time consuming that when doing experiments with protein covalently immobilized to the surface.

4.2. Polymer-protein binding

Some of the results obtained by the resonant mirror technique are very intriguing, because they are not in line with some previous studies. This holds in particular for the allegedly mucoadhesive polymers HPMC and CMC (Smart, 1991; Madsen et al., 1998b; Han et al., 1999; Accili et al., 2004). To discuss the results, it is necessary to review the structure and the properties of the polymers, see Table 1.

All the polymers, except Carbopol, are cellulose-like polymers but have different functional groups. Each polymer can be classified as an acidic, neutral or basic polymer. Hyaluronate, alginate, CMC and Carbopol are acidic (carboxyl groups) with estimated pK_a values about 3.1, 3.5, 3.5 and around 6.0 (±0.5), respectively, and are therefore unionized at pH values lower than about 2.5. At pH 4.0 (lowest value measured in this study), hyaluronate is almost completely ionized, alginate and CMC are partially ionized and Carbopol is unionized. There is a clear correlation between the state of ionization and binding behavior. Hyaluronate does not bind to the protein at pH 4 but alginate, CMC and Carbopol show some affinity for the protein. Only Carbopol shows affinity for the protein at pH 5.5, all the other acidic polymers are fully ionized and do not interact at all with the protein. No interaction occurs between the acidic polymers and the proteins at pH 6.5 or at higher pH values (7.4 and 8.2). The acidic polymers show similar affinity for glycosylated protein (BSM) and non-glycosylated protein (BSA). It can be speculated that hyaluronate would display binding behavior at pH value lower than 4 and Saettone et al. (1989) have reported that hydrated hyaluronate matrixes display mucoadhesive properties at pH 3.5.

The basic polymer chitosan shows reverse binding behavior pattern, i.e. strong binding at the higher pH values and almost no binding at the lower pH values. Chitosan has pK_a about 6.2 and, analogue to the acidic polymers, the unionized form possesses stronger mucoadhesive properties than the ionized form. However, chitosan displays two noticeable differences. First, it has much more affinity for the glycoprotein than BSA. Second, it has some affinity for the glycoprotein at pH 4.0 where the polymer is fully ionized (measured with method B).

The correlation between ionization and binding behavior (lack of binding) indicates that polymer needs to be in its unionized form to bind to protein or glycoprotein. HPMC is the only polymer that is neutral and unionized at all pH values but it does not bind at all to either protein. This is a striking example of how experimental condition can affect the results. HPMC have been reported as mucoadhesive when measured under "dry-on-wet" conditions (Han et al., 1999; Accili et al., 2004) but has been reported to lose this mucoadhesive property when shifted to wet-on-wet conditions (Henriksen et al., 1996). In other words, if the mucoadhesion process is a three-step process as defined by Duchene (Duchene and Ponchel, 1992) (contact, interpenetration and formation of weak chemical bond), then HPMC can be classified as an apparent mucoadhesive. In contrast to HPMC, prolonged hydration periods (wet-on-wet conditions) did not decrease the mucoadhesive properties of chitosan (Henriksen et al., 1996), which is then be classified as a "wet-on-wet" mucoadhesive and is supported by this study. Therefore, it is likely that different functional groups of the polymers are responsible for the formation of weak chemical bonds (the third step in mucoadhesion) and "wet-on-wet" adhesion to mucous surfaces.

Ionized polymers have different conformation in solution because of electrostatic repulsion between approaching segments of same charge. Unionized polymers will adopt a more folded conformation relative to the ionized molecule. In theory this should diminish the penetration of the mucoadhesive polymer into the mucus gel network and entanglement of polymer and mucin chains (step 2 in the mucoadhesion process). This study focuses on step number three with single layer of glycoprotein forming the mucus surface (formation of weak chemical bonds) and it cannot tell if polymer conformation plays as important part there as in step number 2.

The results of this study suggest that hydroxyl groups are not essential structural features for glycoprotein binding nor are strong anionic charges, while unionized carboxyl groups and

unionized amino groups are. However, this does not mean that those structural features (hydroxyl groups and anionic charge) are not important for the first two steps in the mucoadhesion process. By using resonant mirror biosensor, the formation of weak chemical bonds in the mucoadhesion process can be estimated (the third step) without undergoing the first two steps, i.e. the intimate contact and interpenetration.

Unionized carboxyl groups have similar affinity for glycosylated and non-glycosylated protein, However, unionized amino groups have more affinity for the glycosylated protein suggesting that basic mucoadhesive polymers have more affinity to the sugar residues than to the amino acid residues. Amino acid make up for about 36% of the weight of BSM, sialic acids about 30%, and hexosamine and neutral sugars make up the rest (Bettelheim and Dey, 1965). BSM is dissociated and hence negatively charged at pH 3 or higher, which might explain why anionic polymers do not interact with the protein under such conditions. In the same way, at low pH cationic chitosan does not interact with the glycoprotein either. Other factors, such as surface energy effects or interpenetration, are of course also important (Lehr et al., 1992b; Harding, 2003; Peppas and Huang, 2004), and it is important to realize that only binding of the polymers to the mucin, not whole mucus, was examined in this study.

The results of this study confirm the need for a clear definition of the mucoadhesion concept or approval of authorized definition of this process. The results indicate that merely viscosity enhancing polymers, which do not display favorable surface energy properties, do not interpenetrate mucus layers and most important, do not interact with glycoprotein, should not be considered as mucoadhesives. As those polymers show the same non-specific adhesion to mucus surface as to other biological or non-biological surfaces, the binding strength being much dependent on the degree of swelling/hydratation, they are only apparent mucoadhesives.

The resonant mirror biosensor appears as an interesting new technique to better understand the physicochemical mechanism and nature of the mucin-polymer interaction and to allow a fast screening of new candidate mucoadhesive polymers requiring only minimal amounts of polymers (about 10 mg).

5. Conclusion

The use of an optical biosensor technique based on a resonant mirror has not been used before to quantify the interaction between mucin and candidate mucoadhesive polymers. The ranking of mucoadhesive binding strength obtained by resonant mirror technique corroborates with the outcome of earlier studies by other techniques except those performed under "dry-on-wet" conditions. Candidate mucoadhesive polymers must feature ionizable functional groups to be able to from weak chemical bonds with mucus glycoprotein which is the last step of three in the mucoadhesion process.

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