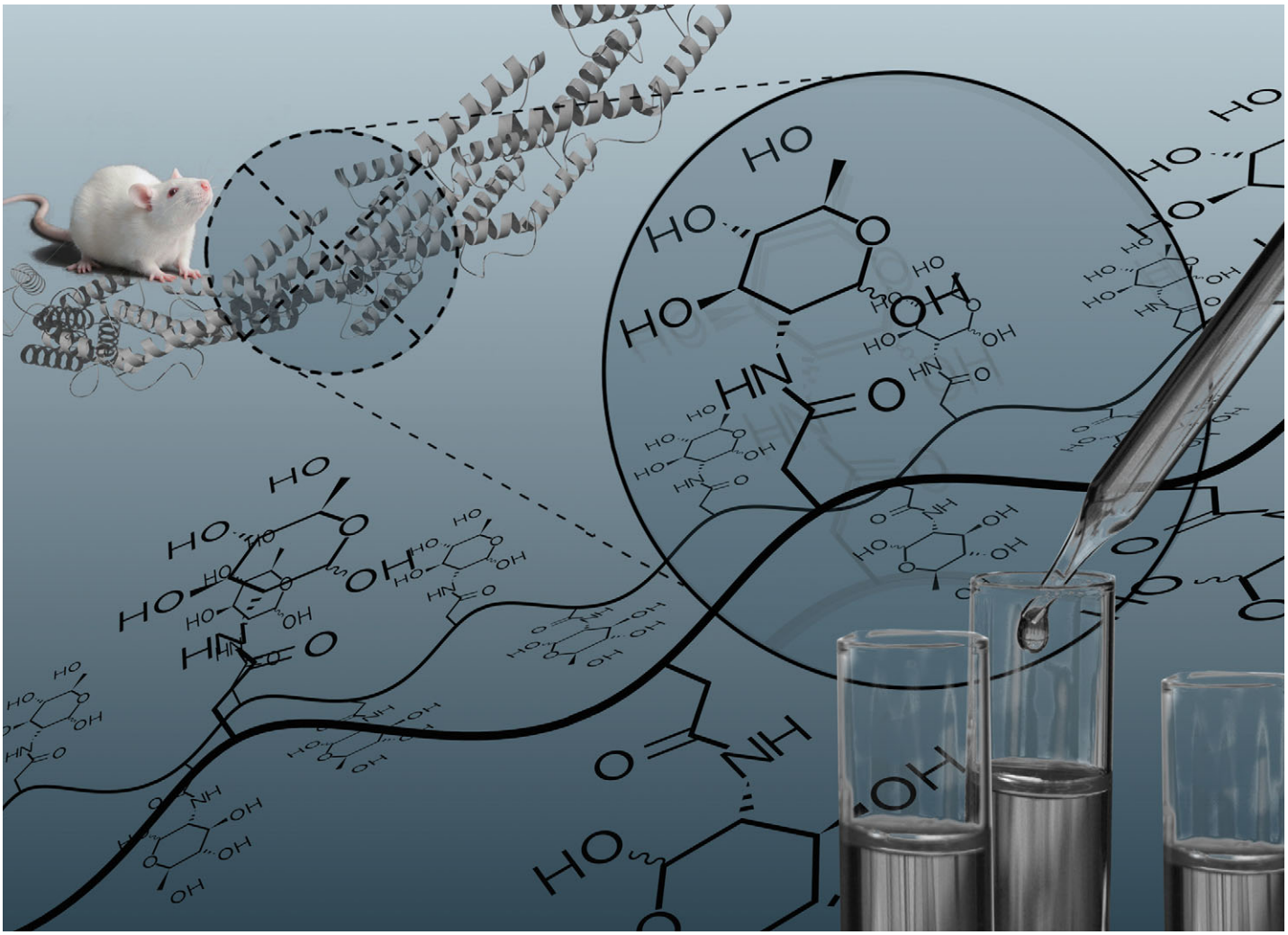
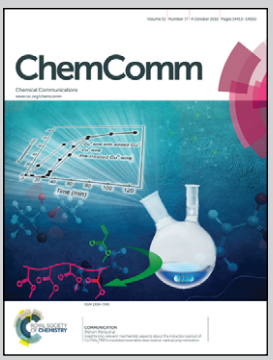


Showcasing research by Michael T. Cook, Sarah L. Smith and Vitaliy V. Khutoryanskiy from the University of Reading and University of Hertfordshire, UK

Novel glycopolymer hydrogels as mucosa-mimetic materials to reduce animal testing

Novel synthetic glycopolymer hydrogels were designed to mimic animal mucosal tissues as reported. These materials could be used in testing of the retention of liquid pharmaceutical formulations on mucosal membranes. The development, structure and physicochemical properties of these mucosa-mimetic hydrogels and their applicability as substrates for retention of chitosan, pectin and dextran are reported.

As featured in:



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Novel glycopolymer hydrogels as mucosa-mimetic materials to reduce animal testing†

Michael T. Cook,[a] Sarah L. Smith[b] and Vitaliy V. Khutoryanskiy*[b]

Glycopolymer hydrogels capable of mimicking mucosal tissue in mucoadhesion testing have been designed. Liquid formulations containing mucoadhesive polymers were found to be retained on these tissues to the same extent as *ex vivo* gastric mucosa, when using a dynamic method of assessing mucoadhesion.

As materials chemistry has become more sophisticated, there has been increasing success in the design of biomimetic systems. In particular, hydrogels have great potential for use as mimics of biological systems and organs.[1] It is the aim of this study to design hydrogels which are able to mimic mucosal tissue, for use in mucoadhesion testing.

When drugs are administered by the oral, oromucosal, nasal, ocular, rectal and vaginal routes, they must cross a 'mucosal membrane' in the body in order to reach systemic circulation. These 'mucosal membranes' are the wet linings covering the gastrointestinal tract, airways, eyes, and reproductive tract, all of which bear either secretory mucous, or membrane-bound mucins.[2] Secretory mucous is comprised of water (<95%), mucins (<5%), inorganic salts (<1%), and other minor constituents.[3] Membrane-bound mucins differ in composition from those found in secretory mucous, and contain hydrophobic domains which anchor them to the epithelial surface.[4] Mucins are glycoproteins, bearing oligosaccharide side-chains, and have a carbohydrate content of around 80%.[5] The absorption of drugs across mucosal membranes is typically poor, reducing drug bioavailability.[6] One strategy to improve drug uptake is the use of 'mucoadhesive' polymeric materials, which improve the retention of a dosage form at the surface of the mucosa.[7] The assessment of mucoadhesive dosage forms is typically conducted by measuring their adhesion to or retention on *ex vivo* animal tissues. However, animal tissues may be difficult to source, can be highly heterogeneous, and often require the sacrifice of an animal specifically for this tissue.[8] A study conducted within our group (unpublished) of 348 papers on mucoadhesion published between 1998 and 2008 found that in 66% of the articles, laboratory animals were sacrificed specifically for their mucosal tissue. It is the aim of this study to produce a synthetic 'mucosa-mimetic' to be used as an alternative to *ex vivo* mucosal tissue, which will allow a reduction in the number of laboratory animals used in mucoadhesion testing, and provide an easy to handle, more homogenous substrate. These materials are based on glass-bound glycopolymer hydrogels, which may be able to mimic the oligosaccharide chains of mucin. These oligosaccharides are arranged in a 'bottle-brush' structure, which conceals the protein backbone.[3] Glycopolymers, polymers bearing pendant carbohydrate groups,[9] have been shown to mimic glycoproteins,[10,11] including mucin,[12,13] effectively.

The mechanism of mucoadhesion is complex, but is in part due to hydrogen bonding, electrostatics, and hydrophobic effects between mucoadhesive and mucosa.[14] Solid dosage forms, such as tablets, also adhere to mucosal membranes by wetting of the dosage form, partial dehydration of the tissue, polymer chain interpenetration,[15] and chemical interactions.[16] Liquid and semi-solid mucoadhesives lack the dosage form hydration and mucosa dehydration steps, and their adhesion to mucosal membranes is driven by chemical interactions, viscosity, and chain interpenetration.

Some attempts to use non-animal materials to study mucoadhesion were focused on testing solid dosage forms by tablet detachment from their surface (tensile testing). These studies tested glass surfaces,[17] polypropylene,[18] and tanned leather[19] with limited success. Previously we have demonstrated that hydrogels are able to mimic porcine buccal mucosa in the assessment of solid dosage forms (tablets) using tensile testing. This ability of hydrogels to mimic buccal tissues correlated well with the equilibrium swelling degree (ESD) of the materials, but was also dependent on their chemical structure.[8] Liquid and semi-solid dosage forms containing mucoadhesive polymers are

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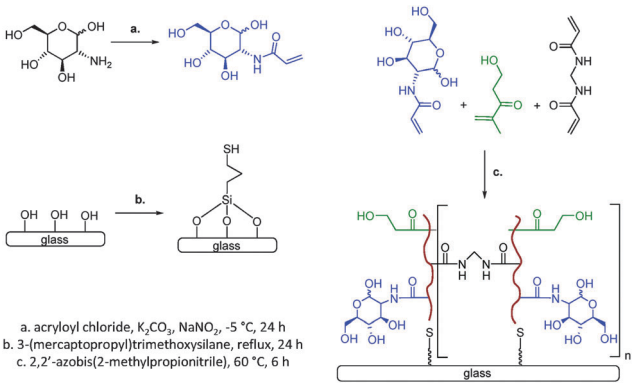
† Electronic supplementary information (ESI) available: Experimental details, SEM images of materials and mucosal surfaces, mechanical testing and IR spectra. See DOI: 10.1039/c5cc02428e

more commonly used for drug delivery to the eye (*e.g.* eye drops), airways (nasal sprays) and some gastrointestinal formulations (*e.g.* Gaviscon®). Tang *et al.*[20] reported the use of a dialysis membrane to mimic a mucosal surface during the retention of liquid and semi-solid peptide formulations; however, they did not validate this substrate against biological mucosa. There is a clear need in the development of artificial materials that could be used as substrates to test retention of these formulations in place of animal mucosal tissues. This study is the first report of the successful design of hydrogel substrates to test liquid and semi-solid mucoadhesive dosage forms.

In order to mimic the neutral sugars found in the side-chains of mucins, a glycomonomer, *N*-acryloyl-*D*-glucosamine (AGA), was synthesised by modification of an existing procedure.[12] The structure of the product was confirmed by ^{1}H NMR, ^{13}C NMR, FTIR, and mass spectroscopy. AGA can be copolymerised with 2-hydroxyethylmethacrylate (HEMA) by thermally-initiated radical polymerization in the presence of a cross-linker to produce glycopolymer hydrogels. If this polymerization is conducted in the presence of glass silanized so that it bears thiol groups on its surface, then the gel can be covalently bound to the surface of the glass (Scheme 1).[21] This is due to the thiol acting as a chain-transfer agent, allowing propagation of polymer chains from the surface of the glass.[22] Glass-bound glycopolymer hydrogels were synthesised (Fig. 1b), composed of 20 mol% AGA and 30 mol% AGA, with the remainder of the hydrogel consisting of HEMA. 100 mol% HEMA hydrogels were also synthesised as a control. The internal structure of the glycopolymer materials was highly porous (Fig. 1a), however, the surface had no discernible porosity (Fig. S1, ESI†).

HR-MAS ^{1}H NMR experiments on glycopolymer hydrogels (Fig. 1c) confirmed the presence of both AGA and HEMA in the hydrogel, and the absence of monomers. FTIR also confirmed the presence of AGA in glycopolymer hydrogels, by the appearance of amide carbonyl and C–N stretches at 1646 and 1544 cm^{-1} (Fig. S2, ESI†). Elemental analysis revealed that the 20 mol% and 30 mol% AGA hydrogels had a final AGA content of 9.5 ± 0.4 mol% and 21.5 ± 1.0 mol% AGA (Table 1). The deviation of final hydrogel composition from the feed mixture is consistent with reactivity ratios of HEMA and acrylamide found by Kucharski and Lubczak,[23] which indicate that in bulk, HEMA



Scheme 1 Synthetic route to glass-bound glycopolymer hydrogels.

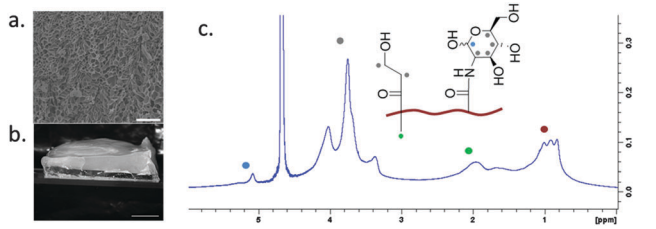


Fig. 1 (a) Glass-bound hydrogel's internal structure by SEM (scale: 100 μm) and (b) the whole sample (scale: 5 mm); (c) HR-MAS ^{1}H NMR spectrum of a 30 mol% AGA hydrogel.

Table 1 Hydrogel composition and characteristics

I.D.	% AGA	Thickness (mm)	Elastic modulus (kPa)	ESD
100% HEMA	N/A	2.0 ± 0.2	24.3	2.24 ± 0.21
20% AGA	9.5 ± 0.4	3.5 ± 0.5	13.7	4.94 ± 0.02
30% AGA	21.5 ± 1.0	3.9 ± 0.3	6.7	13.1 ± 2.70

radicals react preferentially with HEMA monomers rather than acrylamide. The presence of increasing AGA in the hydrogels led to an increased ESD of the gels (Table 1), with an associated increase in thickness. A reduction in mechanical strength was also observed (Fig. S3, ESI†), reflected in the reduction of the elastic modulus of the materials (Table 1). The larger number of hydrogen-bonding groups is the likely reason for this increase in ESD, and reduction in strength. Furthermore, increasing AGA concentration up to 40 mol% did not yield free-standing hydrogels, but a viscous solution.

The rationale for testing if the glycomonomer hydrogels were 'mucosa-mimetics' or not was to see whether mucoadhesive polymers were retained on their surface similarly to *ex vivo* mucosa. In order to measure this, a flow-through system adapted from Cave *et al.*[24] was developed. Briefly, either a mucosal membrane or hydrogel (the 'testing substrate') was placed onto a channel within an incubator at 37 °C (Fig. 2ai). Then, mucoadhesive polymers labelled with fluorescein were pipetted onto the testing substrate (Fig. 2aii). An eluent of either PBS or a suitable simulated bodily fluid was then passed over the testing substrate (Fig. 2aiii), washing the mucoadhesive polymer from the surface. At defined volumes, images were taken using a fluorescence stereomicroscope (Fig. 2aiv). From these images, the fluorescence remaining on the testing substrate can be measured, using the pixel intensity.

The tissues used in this study were porcine gastric mucosa and bovine cornea, which represent mucosal membranes bearing secretory mucous and membrane-bound mucins, respectively. The mucoadhesive polymers chosen were chitosan and pectin, which are commonly-used mucoadhesive agents.[25,26] These polymers were also chosen because they are basic and acidic, respectively, so represent two different classes of mucoadhesive materials. Due to the different functional groups present, two different methods had to be developed to label these polymers. Chitosan was labelled with fluorescein isothiocyanate, as previously described,[27] whilst pectin was labelled with fluoresceinamine using a developed protocol (ESI†). The eluents chosen to test

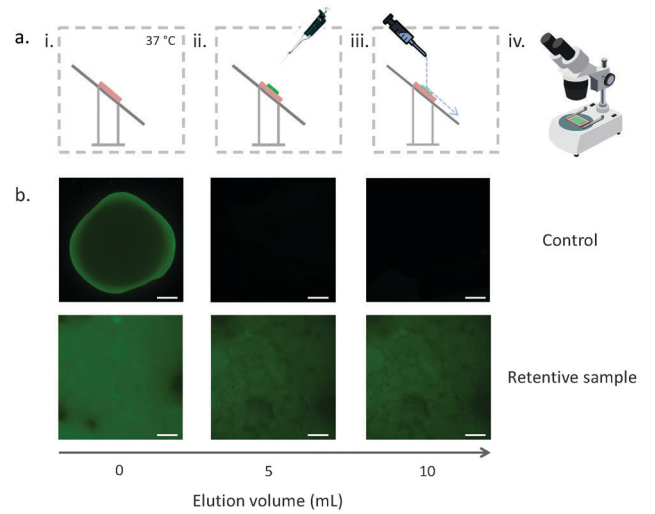


Fig. 2 (a) Schematic diagram of retention testing rig, showing testing substrate placement (i), application of mucoadhesive (ii), washing of surface with syringe pump (iii) and microscopic analysis (iv); (b) exemplar micrographs taken from retention experiments, showing a control (PTFE) with low retention, and an example retentive sample (20 mol% AGA hydrogel); scale: 1 mm. Please note that the fluorescence micrographs have been cropped and brightened by 50% for clarity.

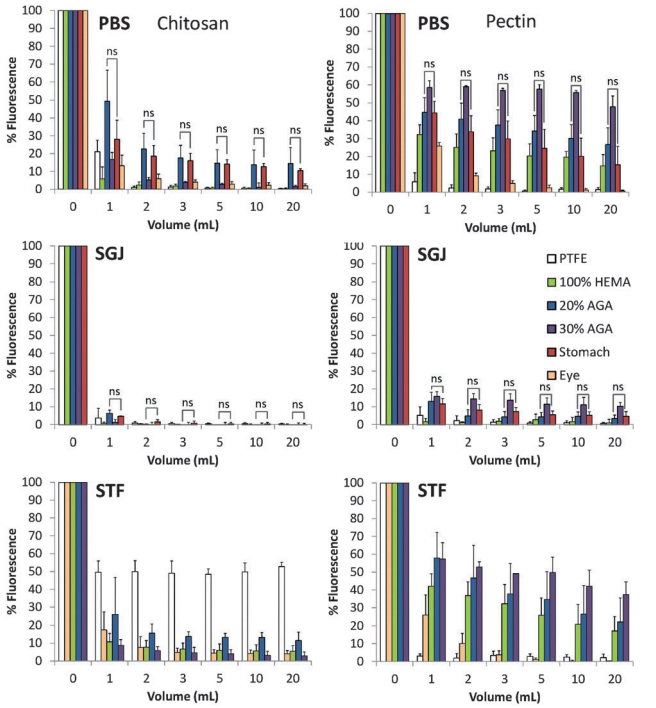


Fig. 3 Retention profiles of either chitosan (left) or pectin (right) on testing substrates during washing with either PBS, simulated gastric juice (SGJ), or simulated tear fluid (STF). Data presented as mean ± standard deviation, $n = 3$; n.s. indicates no significant difference by *t*-testing, two-way ANOVA has also been conducted (in text). Larger version in Fig. S6 (ESI†).

with were PBS, simulated gastric juice and simulated tear fluid (ESI†). This testing rig allowed clear visualization of samples that were either non-retentive, and those that retained mucoadhesives on their surface (Fig. 2b). The retention of the two mucoadhesive polymers on a control surface (PTFE), porcine gastric mucosa, bovine cornea, or glass-bound hydrogels is shown in Fig. 3. It can be seen that the retention of a mucoadhesive is dependent on the type of polymer used, the eluent, and the substrate onto which the mucoadhesive is placed. It was found that the inclusion of AGA at different ratios into the hydrogels modulated the retention of polymers thereon. A 20 mol% AGA glass-bound hydrogel was able to mimic porcine gastric tissue in these tests with both PBS and simulated gastric solution, showing no statistically significant differences from animal mucosa using two-way ANOVA with Bonferroni *post hoc* test (multiple comparisons, $p < 0.05$). The retention of a non-mucoadhesive control, FITC-dextran, on 20 mol% AGA had no statistically significant difference ($p > 0.05$) to gastric mucosa using both PBS and SGJ (Fig. S4, ESI†). There was no correlation found between any of the glass-bound hydrogels and bovine cornea, including FITC-dextran control. It was found that 20 mol% AGA mimicked bovine cornea when testing with simulated tear fluid and chitosan ($p > 0.05$), but not in other conditions ($p < 0.05$). 100 mol% HEMA and 30 mol% AGA were able to mimic bovine cornea when testing in PBS with chitosan ($p > 0.05$), but not in other conditions ($p < 0.05$). Comparison of hydrogels and mucosal membranes to control experiments (PTFE) showed that in every case the retention of mucoadhesives was governed by more than rheological or solubility-based effects, which are cited as mechanisms by which mucoadhesives adhere to mucosal membranes.[28] It is likely that the greater retention of chitosan on PTFE with STF is the result of the poor solubility of chitosan in this eluent enhancing hydrophobic interactions. In all non-control substrates and eluents used, pectin appeared to be retained better than chitosan, consistent with a study measuring their adhesion to porcine intestinal mucosa.[29] SEM images of hydrogels (Fig. S1, ESI†) and mucosal membranes (Fig. S5, ESI†) revealed no similarity in topology between the mucosa-mimetic 20 mol% AGA hydrogel and porcine gastric mucosa surface features; the hydrogel had no visible surface porosity at the magnifications used, and was flat, whilst the mucosa was porous and highly irregular. Mechanical testing (Fig. S3, ESI†) showed that the mucosa-mimetic 20 mol% AGA hydrogel was stronger, with an elastic modulus of 13.7 kPa, than porcine gastric mucosa, which has an elastic modulus of only 1.5 kPa. Though this difference in mechanical properties has not adversely affected the 20 mol% AGAs performance as a mucosa-mimetic in testing liquid mucoadhesives, it must be considered if these materials were to be used as a model in different experiments. Due to the differences in topology and mechanical properties of the testing substrates, and non-dependence of rheological and solubility effects, it is likely that the reason that 20 mol% AGA hydrogels are good mucosa-mimics for porcine gastric mucosa is similarity of chemical interactions, which are the result of the incorporation of a glycomonomer into the hydrogel. These interactions are likely hydrogen-bonding and hydrophobic interactions, as has been investigated in the literature,[26] though ion-dipole interactions and electrostatics may also play a part.[3] The presence of

glycomonomer pendant groups in the hydrogel may mimic the oligosaccharides adorning the mucin glycoproteins present in secretory mucin. *N*-Acetylglucosamine residues found in these oligosaccharides[4] bear particular similarity to AGA. In addition to chemical interactions, physical entanglement aids mucoadhesion,[6] thus the network structure also plays a role in determining retention. 30 mol% AGA hydrogels were found to be poorer mimics of the mucosae studied. Increasing AGA concentration increased swelling degree, lowering the polymer volume fraction, and loosening the network structure. It appears that there is a careful balance needed between the chemical interactions possible with the hydrogel, and the network structure present which 30 mol% AGA does not meet. This is consistent with a study of the adhesion of tablets to these materials,[8] which also used 10 and 15 mol% AGA hydrogels, and found that ESD increased with increasing AGA, and that 20 mol% AGA had the best mucosa-mimetic properties – using concentrations lower or higher than this was detrimental, though this adhesion process is also driven by dosage form hydration. Additionally, higher water content has been associated with poor adhesion due to overhydration of dosage forms.[7] Retention of mucoadhesive polymers on bovine cornea was lower than on porcine gastric mucosa. This is likely the result of the lack of a secretory mucous layer on the surface of the cornea. In gastric mucosa bearing secretory mucous layers up to 450 μm thick,[6] diffusion of mucoadhesive into this mucous layer occurs, leading to chain interpenetration, associated with improved mucoadhesion.[30] The lack of a secretory mucus layer is likely to be the reason why no mimic of the relatively hydrophobic cornea was found.

In conclusion, a material has been developed which is able to mimic porcine gastric mucosa in mucoadhesion testing experiments. This is based on a glass-bound hydrogel consisting of 20 mol% AGA, and 80 mol% HEMA. The retention of mucoadhesive polymers on the material was not significantly different from *ex vivo* mucosal membranes sourced from an abattoir. Due to lack of similarity with topology and mechanical properties, it is believed that the ability to mimic mucosal tissue is due to the presence of specific groups which are able to mimic the large oligosaccharide content of the mucin glycoproteins present in secretory mucous. This material could be used in the pre-screening of mucoadhesive dosage forms, thus reducing the number of lab animals used in mucoadhesion research.

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