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A robust mucin-containing poly(vinyl alcohol) hydrogel model for the *in vitro* characterization of mucoadhesion of solid dosage forms

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ABSTRACT

Mucoadhesion testing at macroscopic scale needs a robust, convenient *in vitro* method as *ex vivo* methods suffer from poor reproducibility and ethical problems. Here we synthesized mucin-free poly(vinyl alcohol) (PVA) and mucin-containing PVA hydrogel substrates (Muc/PVA) to measure adhesion of polymer tablets. Freezing−thawing method was used for gelation to avoid chemical cross-linking and to preserve the functionality of mucin. The adhesion of first generation mucoadhesive polymers, poly(acrylic acid) (PAA) and hydroxypropylmethylcellulose (HPMC) was tested with outstanding reproducibility on individual batches of hydrogels and qualitative agreement with *ex vivo* literature data. Negatively charged PAA was less adhesive on Muc/PVA surface than on mucin-free PVA whereas HPMC as a neutral polymer displayed similar adhesion strength on both surfaces. Chitosan as a positively charged polymer showed enhanced adhesion on Muc/PVA substrate compared to mucin-free PVA. These results are corroborated by turbidimetric titration which indicated attractive electrostatic interactions between chitosan and mucin in contrast to the lack of attractive interactions for PAA and HPMC. These results prove the role of electronic theory in macroscopic mucoadhesion.

1. Introduction

Mucoadhesion is the interaction of materials, e.g., pharmaceutical formulations, and mucosal membranes potentially resulting in prolonged residence time at the specific sites of the body including gastrointestinal, respiratory or reproductive tracts as well as the eye surface[1,2]. The prolonged residence time of mucoadhesive formulations have multiple advantages such as increased bioavailability of the drug and reduced dosing frequency which finally resulting in improved patience compliance. In addition, systematic effects can also be reached by avoiding the first pass metabolism with transmucosal drug delivery [1–6]. Despite the fact that the application of mucoadhesive formulations dates back as far as 60 years [2,3,7,8], the complex mechanism of mucoadhesion is still not fully understood, and there is a strong demand to develop both novel formulations and characterization methods to reliably quantify the strength of mucoadhesion.

The complexity of mucoadhesion comes from the high variety of dosage forms in terms of morphology, consistence, chemical functionality and the diversity of multi-component mucus gel layer containing the high-molecular weight mucin proteins and also small molecular components such as salts and lipids. Mucin is a glycoprotein having a "bottle-brush"-like structure owing to its pendant oligosaccharide side chains terminated with sialic acid units (pK_a = 1.0 – 2.6) [1,8]. It is generally accepted that there is not a single theory to explain the whole process of mucoadhesion [1,2] but its strength is strongly affected by the chain entanglement, the electrostatic interactions and the hydrogen bonds between polymer excipients and the negatively charged mucin glycoproteins. Interactions are studied on different levels: molecular, colloidal and macroscopic ones [9–11]. Studies on shorter length scales provide clues on the possible interaction of polymers with mucin but data from macroscopic measurements might correlate better to the in vivo performance of dosage forms. Accordingly, here we are focusing on

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the macroscopic scale, particularly on the testing of adhesive strength of solid dosage forms. The adhesive strength is usually determined by tensile tests consisting of three phases [7]. First, the dosage form brought into intimate contact with the substrate to reach a pre-determined compressive force. Then, the contact force is kept constant and interfacial interactions develop in this stage. Finally, the dosage form is detached from the surface using a given deformation rate. The force is recorded as the function of displacement and either the force of detachment (the maximum of the force) or the work of adhesion (the absolute area under the curve) is used to characterize mucoadhesive strength [8]. These measurements can be performed under *ex vivo* and even in vivo conditions but the involvement of samples of biological origin suffer from the lack of reproducibility due the diversity of the samples but preparation and storing of the samples are also important issues. Furthermore, in the early stage of development it is discouraged to use animal tissues. Synthetic mucosa models are continuously developed to increase reproducibility, reduce costs, avoid ethical concerns regarding animal testing and promote sustainability [8,12].

In vitro monocomponent mucosa models consist only of mucin, which is the main component (~5%) of the mucus covering the underlying epithelial tissue in mucous membranes. Solid mucin tablet as a monocomponent model is used to characterize mucoadhesion ("mucin disk" method) [13–17]. The relevance of this model is that the role of mucin in mucoadhesion is considered very important due to its entanglement with the polymer excipients in dosage forms and also its large chemical variety allowing various secondary and even primary interactions with the components of the dosage form [2]. In some cases, the results of mucin disk measurements and data on *ex vivo* tissues are compared and some correlation is obtained but in other cases, *in vitro* and *ex vivo* measurements resulted in highly discrepant tendencies indicating the strongly limited performance of the model. These limitations stem from the extremely dissimilar viscoelastic behavior of the solid mucin tablet and the mucosa [15,18]. Synthetic hydrogels are also used as mucosa models to improve the predictive ability. The application of those hydrogels enables us to include a viscoelastic character into the model which is a determining factor in the strength of mucoadhesion [19]. Khutoryanskiy et al. developed a 2-hydroxyethylmethacrylate (HEMA) N-acryloyl glucosamine (AGA) copolymer hydrogel for mucoadhesive tensile strength tests. The AGA monomer was used to mimic the oligosaccharide side chains of mucins. They found quantitative agreement in the work of adhesion measured on their model surface and on buccal mucosa using tablets made of hydroxypropylmethylcellulose (HPMC) – cross-linked poly(acrylic acid) blends [20]. In further studies they elaborated testing methods for liquid [21] and thermogelling semi-solid dosage forms [22] utilizing the same HEMA-AGA hydrogel as a substrate. Eshel-Green et al. [12] developed poly(ethylene glycol) diacrylate hydrogels containing free thiols (PEGDA-QT) to serve as a tissue mimetic substrate. The free thiol groups are included to mimic the – SH groups of the cysteine rich subdomains of the mucin glycoproteins [23]. They found an increase in the work of adhesion of thiolated alginate compared to native alginate on PEGDA-QT substrate in agreement with the tendency measured on porcine small intestine [12]. Despite the promising results on synthetic hydrogel models, the complex chemical functionality of mucin glycoproteins is partially lost in the above-mentioned synthetic models. Some attempts have been made to develop semisynthetic multicomponent mucosa and mucus models in which mucin particles are immobilized in hydrogels [24] or mucin is converted into gels with additives [25,26]. Although, chemical cross-linking [26] or adding a polyanion to mucin [24] might alter its structure, functional groups or its surface charge. In either case, the strength of mucoadhesion was not characterized directly using these models, thus there is still a strong need for a robust model surface to study the adhesive joint in mechanical tests.

In the current work we synthesized a poly(vinyl alcohol) hydrogel containing 5 wt% mucin physically entrapped in the polymer network (Muc/PVA). The gelation was achieved using freezing−thawing method without any chemical reaction to preserve the functionality of mucin resulting in the formation of a physically cross-linked system. Chemical composition of Muc/PVA was confirmed measuring ATR-FTIR spectrum of freeze-dried gels. The particle-size distribution of mucin dispersions with and without PVA before gelation were analyzed looking for colloidal interplays. The morphology of the hydrogels was characterized by scanning electron microscopy. Oscillatory rheology was employed to characterize viscoelasticity of the hydrogels. Relaxation spectra were calculated for a deeper understanding of the effect of mucin on the relaxation processes in physically cross-linked PVA gels. We utilized Muc/PVA hydrogel as a substrate to measure the adhesion strength of polymer tablets using tensile testing method. To the best of our knowledge, there is no hydrogel system containing mucin described for tensile adhesion testing. We used three well-known polymers for adhesion testing: positively charged chitosan, negatively charged cross-linked poly(acrylic acid) (PAA) and neutral hydroxypropyl methylcellulose (HPMC). We hypothesized that the presence of electrostatic interactions between mucin and charged polymers affects the strength of mucoadhesion at macroscopic scale. We used mucin-containing and mucin-free hydrogels to confirm this hypothesis as the presence of mucin is expected to affect the strength of mucoadhesion for charged polymers if molecular interactions have a significant contribution in adhesion. For these three polymers we also studied interactions with mucin on the colloidal length scale using turbidimetry and zeta potential measurement to identify the origin of these interactions.

2. Materials and methods

2.1. Materials

PVA (M_w approx. 60 000 g/mol, degree of hydrolysis $\geq$ 98.0%), mucin (from porcine stomach, type II) were purchased from Merck. Carbopol® Ultrez 10 NF, a cross-linked poly(acrylic acid) derivative (PAA) from Lubrizol Advanced Materials Europe was kindly provided by Azelis Hungary Ltd., Hydroxypropylmethylcellulose (HPMC) Benecel™ K15M PH CR (M_w = 575 kDa) as a product of Ashland Inc. was a kind gift from ExtractumPharma Pharmaceutical Manufacturing, Marketing and Consulting Inc., Chitosan (M_w = 100 −300 kDa) was bought from Acros Organics. Phosphate-buffered saline (PBS) solution of pH = 7.4 was prepared by dissolving 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of Na_2HPO_4 2 H_2O and 0.12 g of KH_2PO_4 in 1 L of water, the pH being adjusted with 0.1 mol L^{-1} HCl. Ultrapure water ($\rho > 18.2$ MΩ cm, Millipore) was used for making PBS solution and for the preparation of the hydrogels. All experiments were performed at 25 °C unless otherwise stated.

2.2. Synthesis of hydrogels

Muc/PVA and PVA hydrogels were synthesized using freezing−thawing method. First, 20 wt% PVA solution was prepared by adding PVA into water and stirred continuously at 80 °C for 12 h. For the preparation of Muc/PVA hydrogels, 10 wt% mucin was dispersed in water and stirred for 2 h at room temperature. Then, the 20 wt% PVA solution was added to the mucin dispersion in 1:1 wt ratio and stirred for additional 4 h. PVA hydrogels were prepared by the dilution of the PVA stock solution (20 wt%) to 15 wt% by adding water and stirring at 80 °C for 4 h to form a clear solution. Finally, the mixtures were poured onto a glass plate bordered with a 4 mm thick silicone frame, covered with another glass plate and followed by a freezing at −20 °C for 18 h and thawing at room temperature (25 °C) for 6 h, in 3 consecutive cycles. Before Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) imaging, hydrogel samples were frozen at −50 °C and freeze dried. Prior to rheological characterization and adhesion measurements the excess of water was gently wiped from the surface of gels with a paper tissue.

2.3. Preparation of mucin dispersion and polymer solutions

For turbidimetric titration, mucin was dispersed in PBS buffer solution (pH = 7.4) at a concentration of 1 g L^{-1}, stirred for 2 h, sonicated for 10 min, centrifuged at 170 G for 5 min and the supernatant was used. HPMC and PAA were also dissolved/dispersed in PBS at a final concentration of 1 g L^{-1}. For PVA, the dissolution was done with heating (at 80 °C, for 2 h). In case of chitosan, 100 mg powder was dispersed in 80 mL water, pH was set to 4.0 with 1 mol L^{-1} HCl, after dissolution PBS were added to reach 100 mL volume. For zeta potential measurements 1 mmol L^{-1} KCl aqueous solution was used as solvent, concentrations were the same as for turbidimetry.

2.4. Turbidimetric titration

Turbidimetric titration was performed at 400 nm with an Agilent Cary 60 UV-Vis spectrophotometer. Each polymer solution was added stepwise into 2 mL of mucin dispersion. After each addition step the solution was mixed for 10 s before the turbidity (apparent absorbance) was measured. As the dispersions of mucin and PAA are turbid, we used the difference from additivity for comparison. The contribution of polymer-mucin interaction to the turbidity (turbidity contribution, *TC*) was defined as the difference of turbidity of polymer-mucin dispersion (*Tpm*) and the sum of turbidity of mucin dispersion (T*m*) and polymer solution/dispersion (*Tp*) at a given concentration Eq. (1).

$$TC = Tpm - (Tm + Tp) \quad (1)$$

2.5. Zeta potential measurements

The electrophoretic mobility of mucin and polymers in aqueous dispersion or solution was measured using a Brookhaven ZetaPALS instrument. Measurements were performed with 10 runs and 10 cycles and Smoluchowski model was used for calculation of zeta potential from electrophoretic mobility.

2.6. Particle size analysis

Apparent volumetric particle size distribution of mucin in aqueous dispersions was determined by a laser diffraction particle size analyzer (LA-950, Horiba). Spherical geometry of the particles was assumed and a refractive index of 1.42 for mucin was used [27].

2.7. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of the freeze dried gels, as well as the PVA and the mucin were recorded using a Bruker Tensor 27 FTIR spectrometer in attenuated total reflectance (ATR) mode. 32 scans were recorded from 4000 to 400 cm^{-1} with a resolution of 2 cm^{-1} for each sample.

2.8. Scanning electron microscopy (SEM) imaging

The morphology of the freeze dried gels was characterized by SEM (FEI Inspect S50, accelerating voltage: 20 kV). Solid specimens were coated with gold (Emitec K550x, 1,5 min, 35 mA) before taking images.

2.9. Rheological characterization

Rheological measurements were performed with an Anton Paar Physica MCR301 rheometer in oscillatory mode using a Peltier device to keep the temperature at 25.0 ± 0.1 °C. Solutions and dispersions were measured using a cone - plate geometry (CP-25–1; cone angle: 1°; sample gap: 0.054 mm) whereas for hydrogels a plate - plate geometry was used (PP-25; sample gap: 2.8 mm). Dynamic moduli (storage, G' and loss, G" modulus) were recorded at a strain (γ) of 1% in the angular frequency (ω) range of 500–0.5 rad s^{-1} with measuring 5 data points per decade.

2.10. Tablet preparation

Tablets for testing mucoadhesion were prepared by direct compression of polymer powders. The tablets were pressed using a Dott. Bonapace auto tablet press machine (CPR-6). During the preparation the compression force was recorded via inbuilt measurement cell of the tablet press. The crushing strength of the tablets was assessed using a tablet hardness tester (Dr. Schleuniger THP-4 M). The tablets have 7.0 mm diameter with biconvex geometry characterized by its highest (H) and smallest (L) thickness. Most important properties of the tablets summarized in Table S1, in which the successful preparation of tables is shown by hardness values of 50 N and higher for all compositions.

2.11. Adhesion testing

Adhesion between the tablets and hydrogel surfaces was measured using a mechanical tester (INSTRON 5566) with a circular holder for the gel-likesubstrates. From the 4 mm thick gel sheet a 25 mm diameter disk was cut and clamped into the circular holder which has a 15 mm diameter cavity in the upper mount. The tablet was attached to a cylindrical upper probe (10 mm diameter) with double sided adhesive tape. For testing a 10 N measurement cell was used, with the following program: compression at 0.01 mm s^{-1} speed, hold at 0.1 N force for 60 s and detachment at 0.05 mm s^{-1} speed. During the tests, force was recorded as a function of displacement. From these adhesion curves the work of adhesion (the absolute area under the curve) and the force of detachment (the maximum of the force) were determined.

3. Results

We successfully synthesized PVA hydrogels with and without mucin in a freezing−thawing process, which allowed us to avoid any chemical reaction possibly altering the mucin structure. This type of gelation is based on the formation of microcrystalline structures of PVA resulting in hydrogels with high mechanical integrity without using chemical cross-linkers[28]. A commercially available mucin (from porcine stomach, type II) was chosen as our goal was to develop a robust mucosa model which can be produced in large quantities and enables high-throughput measurements as an alternative of in vivo and *ex vivo* adhesion tests. The effect of mucin content on adhesive properties was studied by three polymers: 1) PAA as a strong mucoadhesive due to its cross-linked structure and despite its negative surface charge resulting in repulsive interaction with mucin, 2) HPMC with neutral character as a weaker adhesive compared to PAA [20] and 3) chitosan as a known cationic mucoadhesive [29]. Turbidimetric titration [30] was used to study the possible attractive interactions between the selected polymers and mucin in aqueous medium. The turbidity contribution for PAA and HPMC was negligible, while chitosan caused a marked increase in turbidity indicating polymer-mucin attractive interactions [29,30] as shown in Fig. 1. Such an increase of turbidity is explained by the polymer-induced aggregation of mucin particles. PAA has negative surface charge based on zeta potential measurements (Table 1), presumably causing repulsive ionic interactions with negatively charged mucin protein. The zeta potential of HPMC in solution was slightly negative possibly because of some hydroxide ion adsorption but no significant charge arises due to the neutral hydroxyl groups of this polymer. Nevertheless, hydroxyl groups are still capable to form hydrogen bonds with the hydroxyl or amide groups of mucin, but these hydrogen bonds are not strong enough to surpass the strength of water-HPMC and water-mucin interactions. A strongly positive zeta potential was detected for chitosan due to its protonated amine functionalities which can be the source of the attractive interactions with mucin seen on the results of turbidimetric titration. In addition to the polymers used later for tablet pressing, PVA as a matrix polymer was

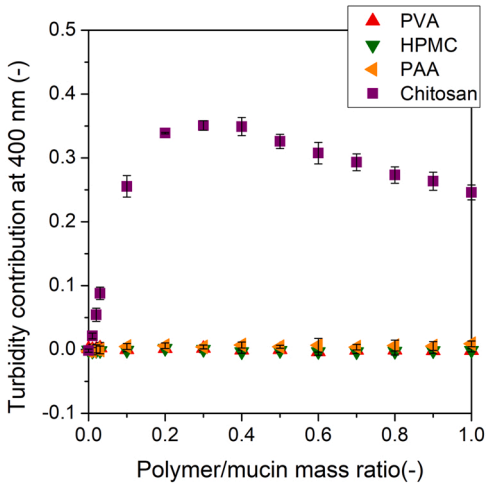

Fig. 1. Turbidimetric titration of of 1 g L^{-1} porcine mucin porcine mucin dispersion.

Table 1
Zeta potential of mucin dispersions and polymer solutions.

Sample	Zeta potential (mV)
Mucin	–5.3 ± 1.3
Mucin/PVA	–4.4 ± 0.9
PVA	–0.3 ± 0.6
PAA	–9.7 ± 2.1
HPMC	–2.1 ± 0.8
Chitosan	+11.1 ± 1.4

also tested with turbidimetry. Based on turbidimetry results, similarly to HPMC and PAA, PVA did not induce the aggregation of mucin particles suggesting the absence of strong interaction between PVA and mucin. Zeta potential measurements were also performed for mucin, PVA and mucin/PVA dispersions. For PVA no remarkable surface charge was detected. For mucin, a negative zeta potential was measured, which did not change in the presence of PVA (Mucin/PVA mass ratio =1/2) indicating the mucin in PVA matrix preserves its ability to form electrostatic interactions with other polymers.

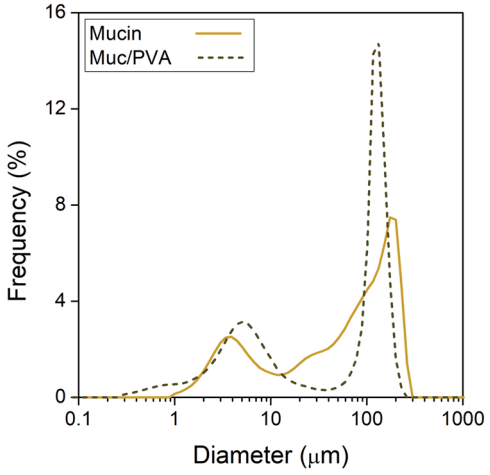

Fig. 2. Particle size distribution of porcine mucin and Muc/PVA aqueous dispersions.

Particle size analysis was performed for mucin and mucin/PVA aqueous dispersions. Mucin displayed one peak between 1 and 10 μm and multiple peaks between 10 and 300 μm diameter (Fig. 2). The latter might consist of 3 peaks, from which the largest is between 100 and 300 μm. Upon mixing the mucin with PVA in water the size distribution become bimodal with a slight size reduction of the peak above 100 μm and the disappearance of the peaks between 10 and 100 μm suggesting a coating effect of PVA on mucin particles.

As surface functionality determines the possible interaction during mucoadhesion, chemical composition was characterized by FTIR for the individual starting components (PVA and mucin) as well as freeze dried gels (PVA and Muc/PVA). Here we discuss only the most characteristic peaks for each sample. A broad band at around 3300 cm^{-1} appeared as the OH stretching in PVA (Fig. 3), while peaks at 2938 and 2909 cm^{-1} are attributed to the asymmetric and symmetric CH_2 stretching. A small peak at 1712 cm^{-1} might be due to C=O stretching of residual acetate groups of PVA. An intense peak at 1086 cm^{-1} belongs to C-O stretching. PVA hydrogels prepared by freezing–thawing method show a very similar spectrum to that of PVA having maximum 3 cm^{-1} shifts. Mucin has multiple functionalities due to its protein and polysaccharide structure, its complex IR spectrum discussed in several papers [31–33]. A broad peak above 3000 cm^{-1} is assigned to asymmetric and symmetric primary amine stretching vibrations, but OH stretching and amide NH stretching are also included in this region. Asymmetric and symmetric CH_2 stretching gives peaks at 2951 and 2922 cm^{-1}, respectively. The peak at 1629 cm^{-1} can be assigned to primary amine deformation with an overlapping amide I carbonyl stretch. Finally, the relatively intense peak around 1045 cm^{-1} might come from the stretching of the C-N bonds of primary amines. In Muc/PVA gels, the absorption bands of PVA dominate at higher wavenumbers (> 2900 cm^{-1}), due to the relatively high concentration of PVA, thus vibrations from methylene or amine groups of mucin cannot be distinguished from PVA signals. On the contrary, the C-N stretching of mucin around 1550 cm^{-1} clearly appears in Muc/PVA gels and even the carbonyl peak of PVA (1710 cm^{-1}) is surpassed by mucin's peak confirming the presence of mucin in the gels. Interestingly, an upward shift of the primary amine peak can be observed (at 1637 cm^{-1}) which might be due to the disruption of inter-particle interaction of mucin in the presence of PVA strengthening the

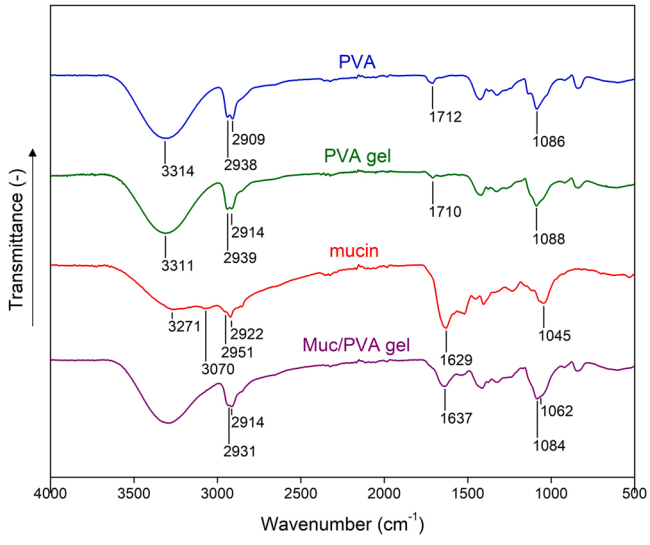

Fig. 3. ATR-FTIR spectra of PVA, freeze dried PVA gel, porcine gastric mucin and freeze dried Muc/PVA gel.

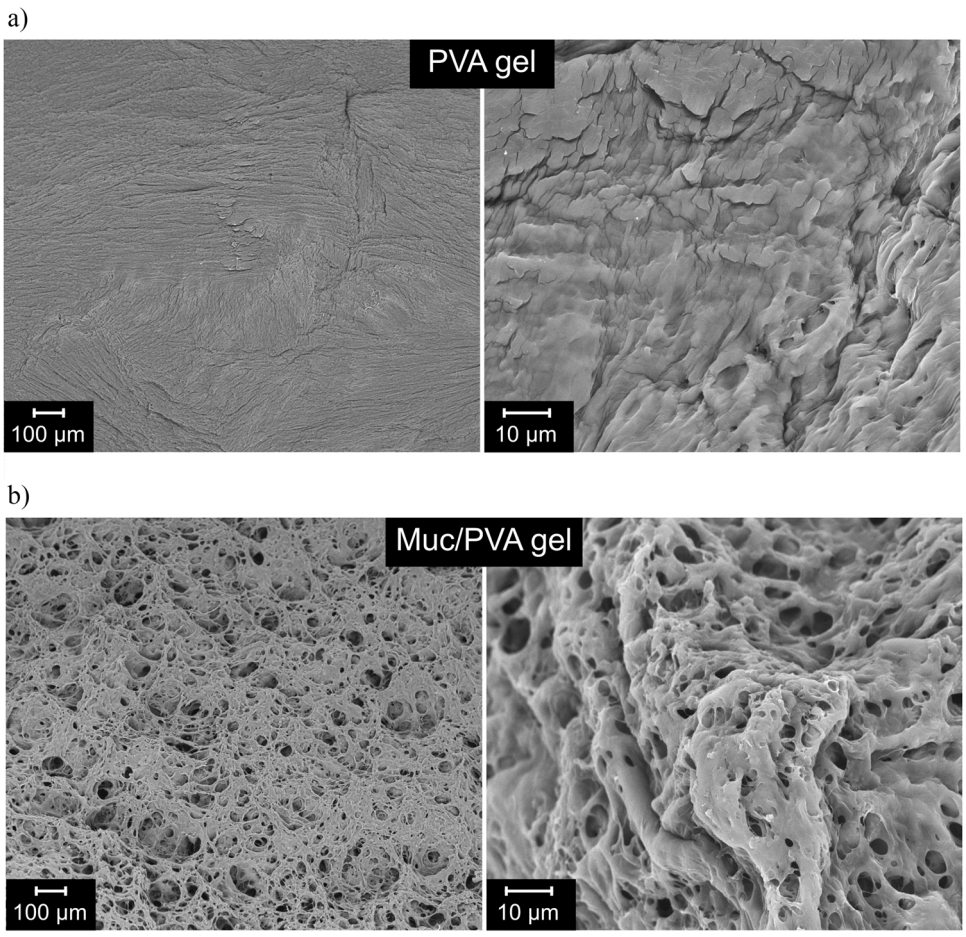

Fig. 4. Scanning electron micrographs of freeze-dried (a) PVA gel (without mucin) and (b) Muc/PVA (mucin-containing) gel at different magnifications.

theory of coating effect of PVA assumed for size distribution results. Finally, the C-O stretching of PVA also appear in Muc/PVA gels. All these results confirm the presence of mucin in PVA matrix.

The surface of the hydrogels was studied by SEM and the effect of the mucin on hydrogel morphology was studied. As it can be seen in Fig. 4a, a closely packed structure is observed for PVA gels and open pores were not detected even at micron scale (Fig. 4b). This is in agreement with Qi et al. [34] who observed a continuous surface without pores throughout the whole gel explained by the crystallization of PVA. In sharp contrast to reference gels, the Muc/PVA gels exhibited a strongly porous structure (Fig. 4c and d) with an even distribution of pores (pore sizes are generally below 5 μm). The morphology suggests that during the preparation of hydrogels the even distribution of mucin particles is ensured but the crystallization process of PVA might have been affected by the presence of mucin, similarly to Salecan/PVA gels whose pore size can be controlled by Salecan content [34]. In Muc/PVA gels, larger aggregates of mucin particles could not be observed (Fig. 4d) which might contribute to reproducible surface composition and reliable adhesion tests on the surface.

Various functions of the mucus gel layer including protection, lubrication, transport of nutrients etc. depend very much on its viscoelastic properties both on macro and microscale [25,26,35]. Viscosity and elastic nature of the mucus largely vary between organs and with age, diet or the pathological state of the individual. The accurate rheological characterization requires studies on various length and time scales. Although steady-state rotational shear measurements give valuable information on non-Newtonian behavior including the presence of yield stress and thixotropy but may irreversibly disrupt the structure. Thus, oscillatory shear is generally used to characterize the mucus of different origin in the linear viscoelastic region where the structure remains close to that at rest (small, reversible deformation). Different magnitudes of frequency can be tested in a single frequency sweep measurement to model different types of loads, e.g., to mimic deformation during breathing (small frequencies, 0.5 – 20 Hz) or coughing and blinking (high frequencies, up to 10^4 Hz) [35,36]. Accordingly, we used oscillatory shear experiments and all the measurements were done at a strain (γ) of 1% belonging to the linear viscoelastic region. Purified mucins lack several components compared to native mucin [37] and usually does not reproduce the viscoelastic properties of mucus gel possibly due to the cleavage of disulfide bridges during the extraction [25]. Contrary to the primarily elastic properties of native mucus with storage modulus dominating over loss modulus in a wide frequency range [36], the aqueous dispersion of purified mucins usually exhibits comparable storage and loss modulus, with loss modulus often being

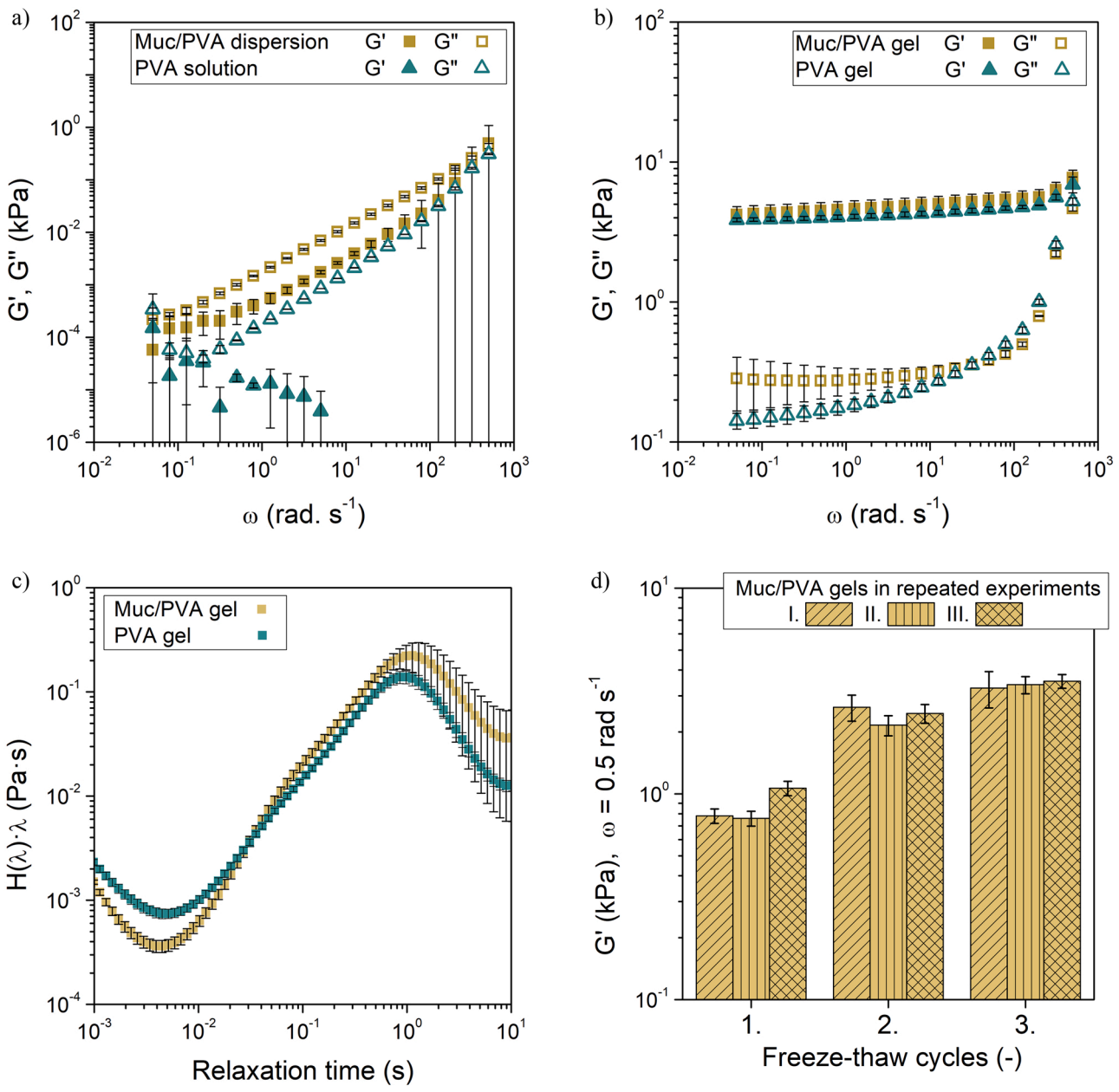

Fig. 5. Rheological characterization of PVA, muc/PVA aqueous dispersions and gels: (a) frequency sweep before freezing−thawing gelation, (b) after three freezing/thawing cycles of gelation, (c) relaxation spectra for gels, (d) storage modulus of the gels at a given frequency.

higher than storage modulus [38]. We measured the same frequency dependence for Muc/PVA aqueous dispersions as the storage modulus was lower than or only comparable to loss modulus over the whole frequency range studied (Fig. 5a). As a comparison, dynamic moduli of PVA solution are also shown. In the absence of mucin, loss modulus was significantly lower and storage modulus was negligible in this concentration range. Thus, the addition of purified mucin contributed to elastic properties even though a strong elastic response was not achieved. As a consequence, such a mucin dispersion cannot be used as a model surface for mucoadhesion as there is a strong correlation between viscoelastic properties and adhesion proposed by Zosel et al. [39] Furthermore, remarkable stiffness of the substrate is needed to avoid cohesive failure during tensile adhesion tests [19].

As the mucin usually loses its gelling ability during purification, PVA was used as a gelling agent to entrap mucin in a physically cross-linked polymer network. PVA forms mechanically stable hydrogels upon freezing-thawing cycles in aqueous solution as a microcrystalline structure forms with the increase of size of microgel particles with increasing number of cycles [28]. The advantage of such gelation is that mechanically stable hydrogels can be produced without cross-linker using three cycles as it is shown in Fig. 5b, after the three freezing-thawing cycles the frequency spectra of the samples completely

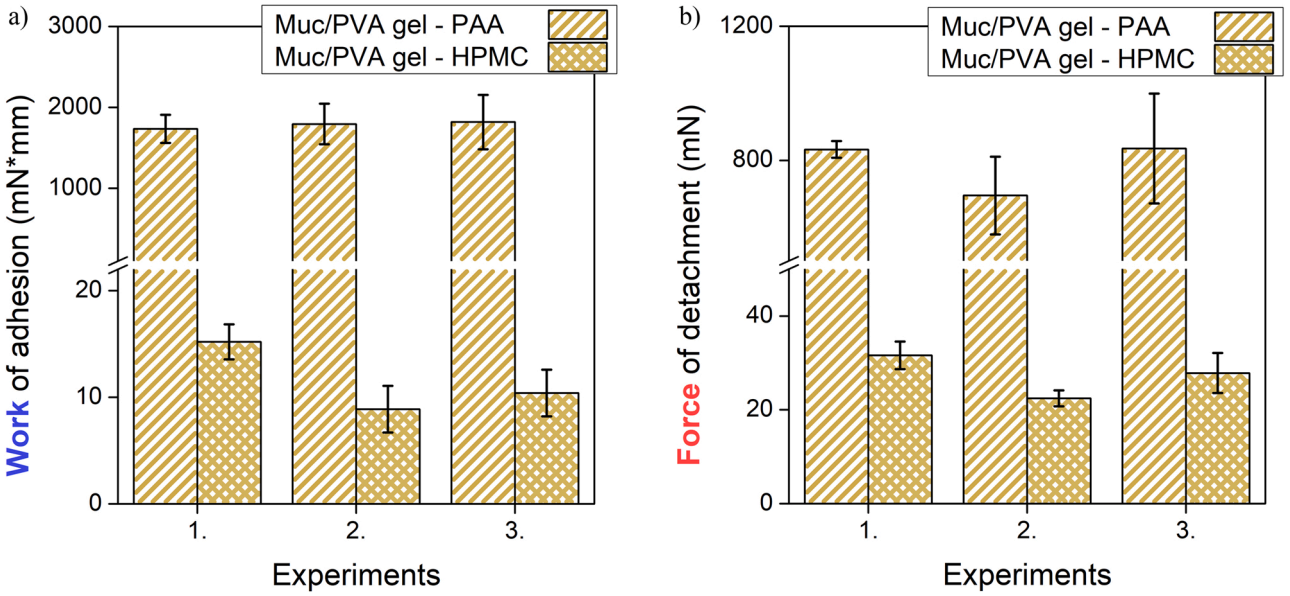

Fig. 6. Adhesion measurements on different Muc/PVA hydrogel batches (a) work of adhesion and (b) force of detachment.

changed. Storage modulus became larger at almost all frequencies than loss modulus for both – PVA and Muc/PVA – hydrogels, and the moduli are comparable only at very high frequencies ($> 2{\bullet}10^2$ rad s^{-1}) which can be explained by the persistence of physical entanglements longer than the timescale of the deformation. The hydrogels dynamic behavior is slightly different at low frequencies. Loss modulus of PVA gels gradually approaches zero as the frequency decreases while Muc/PVA gels show a plateau or even a very slight increase of loss modulus at low frequencies suggesting an imperfect network structure with non-cross-linked branches in the presence of mucin. Nevertheless, the storage modulus reaches a plateau for both gels at low frequencies, from which a stable hydrogel structure is expected over long time scale. It is important to point out that the loss modulus is not negligible at either frequency which is important for adhesion as purely elastic materials display poor adhesive properties due to the lack of energy dissipation processes upon separation of surfaces. The upper limit of sufficient adhesion is generally dictated by the so-called Dahlquist criterion which considers 100 kPa as the upper limit of compressive modulus [39]. This requirement is met by the gels studied taking into account that the shear modulus (approx. 3–4 kPa for PVA and muc/PVA gels) is in principle the third part of Young's modulus (33 kPa). At the same time, a sufficient stiffness is required to avoid cohesive failure during adhesion which might be ensured by the cross-linked structure of these gels.

For a deeper analysis of relaxation processes in hydrogels, their relaxation time spectra were calculated from frequency-dependent dynamic moduli (Fig. 5c) using the built-in method of the Rheoplus V3.40 software. Here, only hydrogels are discussed as spectra could not be calculated for suspensions and solutions. It must be emphasized that it is very difficult to accurately determine the relaxation spectra even for hydrogels from experimental data due to the limited number of data points to avoid sample evaporation and also the limited frequency range. Nonetheless, the main conclusions can still be drawn for the relaxation behavior of PVA and Muc/PVA hydrogels. In both cases, a complex spectrum was obtained rather than a single, narrow relaxation peak. A main peak was observed at around 1 s, but it spans over the whole time range with a small shoulder for both gels below 0.1 s. At short times, transition region appears possibly due to the chain entanglements. In the medium range, gels are in their rubbery state, while at longer times (above 1 s) slightly different relaxation behavior can be seen although the existence of other relaxation processes cannot be confirmed due to the limit in measurement frequency. Nevertheless, the viscoelastic properties of PVA hydrogels were not altered significantly by the addition of mucin suspension, thus we could prepare a PVA-based, mechanically stable mucosa model with the functionalities of mucin protein. Repeated preparation of Muc/PVA hydrogels with at least half year between each batch shows reproducible stiffness after each cycle (Fig. 5d) proving the robustness of the preparation method, and thus reproducible adhesion tests were expected on these gel surfaces.

The adhesion on the synthesized Muc/PVA hydrogel as substrates was studied by tensile tests using polymer tablets. Likewise Khutoryanskiy et al. did in their pioneering work [20] we also used a weak and a strong mucoadhesive: HPMC and PAA. PAA has a highly hydrophilic character and a cross-linked structure, which explains its strong adhesion on the hydrogel surface due its possible water uptake from the hydrogel and chain entanglement effects [40]. In terms work of adhesion PAA displays much higher (by two orders of magnitude) values compared to HPMC (Fig. 6a), while the force of detachment of PAA was roughly one order of magnitude higher than that of HPMC (Fig. 6b). These are in qualitative agreement with the previous results of Khutoryanskiy et al. measured on *ex vivo* substrates [20]. To address one of the main disadvantages of *ex vivo* measurements, reproducibility of our mucoadhesion measurements was tested. As shown in Fig. 6a, b, each measurement had relatively low standard deviation with negligible differences in both the adhesive force and work between tests carried out at different times and on different batches of the hydrogel substrates. These results indicate the robustness of adhesion measurements on hydrogel surfaces for PAA and HPMC tablets.

After testing the robustness of the methods on Muc/PVA hydrogel substrates, the number of polymers was extended with cationic chitosan, whose interaction with mucin confirmed by our turbidity and zeta potential measurements. We hypothesized that these second-order

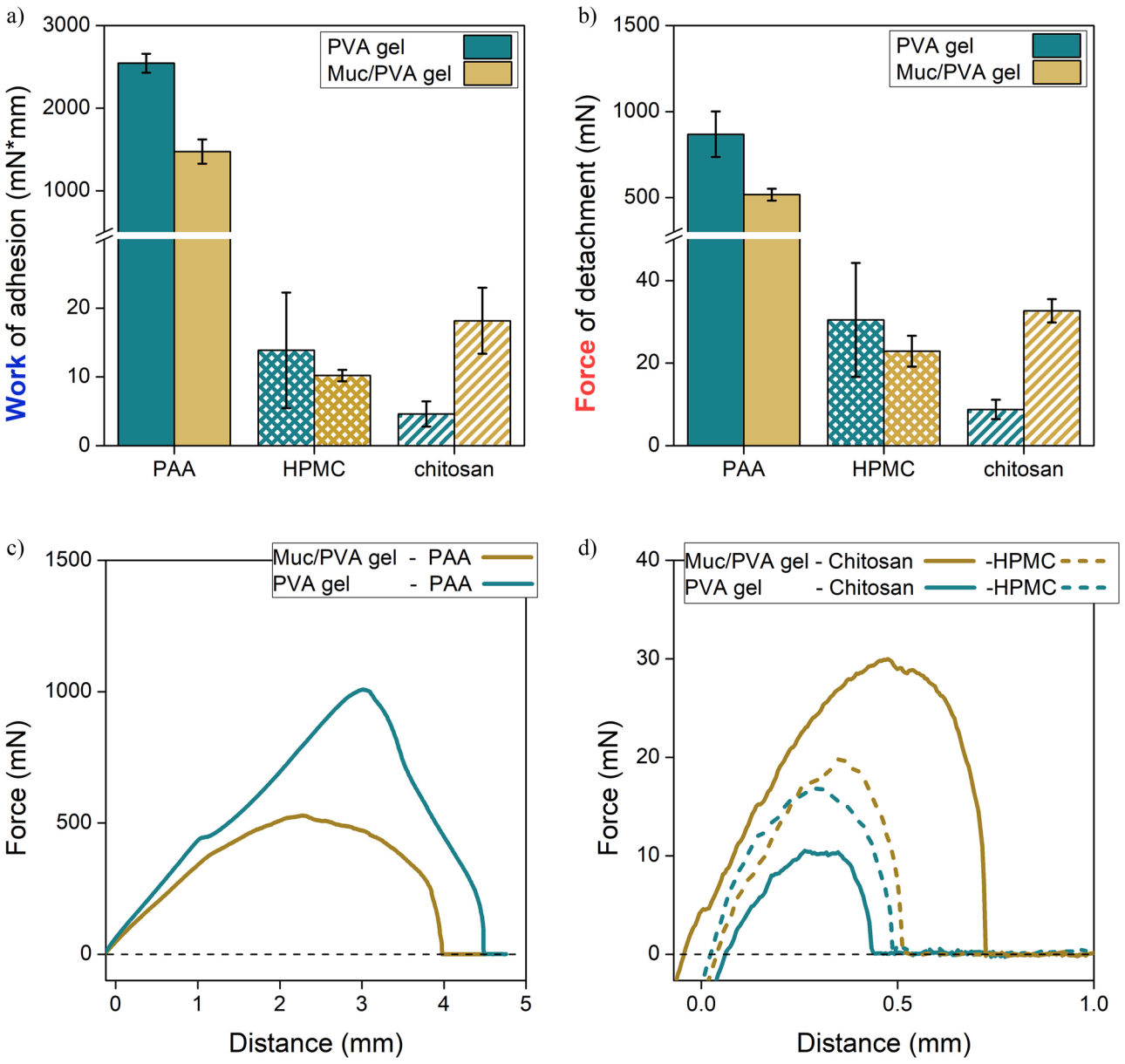

Fig. 7. Adhesion measurements comparing Muc/PVA and mucin-free PVA hydrogel (a) work of adhesion, (b) force of detachment (c) typical adhesion curves using PAA, (d) chitosan and HPMC tablets,.

attractive interactions might be strong enough to detect a difference in macroscopic mucoadhesion on mucin-containing hydrogel substrate (Muc/PVA) and mucin-free PVA hydrogel. According to our results chitosan tablets show significantly stronger adhesion (Fig. 7a, b) on mucin-containing PVA substrates compared to the tests on pure PVA gels. HPMC shows no significant differences compared the two different substrates, but PAA displays higher adhesion on pure PVA surfaces. Comparing adhesion curves measured with the same type of polymer tablet on Muc/PVA versus mucin-free PVA hydrogel substrates has very similar trail and all the curves are typical for purely adhesive debonding process (Fig. 7c, d), thus the difference in adhesive force and work can be explained by interfacial interactions. Zeta potential tests could provide a possible explanation for the adhesion test's results. Mucin dispersion showed a reasonable negative potential value, even upon mixing with PVA (Mucin/PVA aqueous dispersion) while pure PVA has no significant zeta potential, PAA bears the most negative, HPMC has a slightly negative and chitosan has a strongly positive zeta potential. So, according to the electronic theory [4] it is reasonable why the positive chitosan shows higher, negative PAA weaker and the neutral HPMC similar adhesion strength on the negative Muc/PVA hydrogel compared to the neutral PVA hydrogel. These results indicate that polymer-mucin electrostatic interactions seen at colloidal level could cause significant difference in mucoadhesion at macroscopic scale.

4. Conclusion

In the current work mucin-containing PVA hydrogel (Muc/PVA) was successfully synthesized for the robust characterization of mucoadhesion of solid polymer tablets with tensile testing method. Chemical cross-linking was avoided by using freezing–thawing method for the gelation. Particle size distribution suggested a coating effect of PVA, which ensured the even distribution of mucin particles in the matrix. The presence of mucin was confirmed also by FTIR measurements. Mechanical properties of mucin-containing and mucin-free PVA hydrogels were similar according to oscillatory rheology measurements. First, adhesion properties of the Muc/PVA hydrogel substrate were

tested using PAA and HPMC tablets. The results showed qualitative agreement with *ex vivo* literature data and outstanding reproducibility on different batches of hydrogel substrate. Based on turbidimetric titration results, chitosan showed attractive interaction with mucin, while interaction of mucin with HPMC and PAA was not observed. On anionic Muc/PVA hydrogel higher adhesion was measured for cationic chitosan compared to the adhesion on mucin-free PVA gel, for neutral HPMC there was no significant difference, whereas anionic PAA showed stronger adhesion on pure PVA. These results could be a proof of electronic mucoadhesion theory and could strongly emphasize the need of mucin in mucosa mimetic materials.

CRediT authorship contribution statement

Benjámin Gyarmati: Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition. **Gergely Stankovits:** Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Barnabás Áron Szilágyi:** Methodology, Formal analysis. **Dorián László Galata:** Writing – original draft, Investigation. **Péter Gordon:** Resources, Writing – original draft. **András Szilágyi:** Supervision, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2022.112406.

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