Assessment of Mucoadhesion Using Small Deformation Rheology Revisited

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ABSTRACT

This work revisits the commonly used approach to assess mucoadhesion in drug delivery by small deformation rheology. The results show that biosimilar mucus serves as a more predictive mucus model system when compared to mucin suspensions. Data is fitted including error propagation, different from previous studies only using calculated averages.

INTRODUCTION

Oral delivery of pharmaceuticals, and especially biopharmaceuticals such as insulin, is challenged by a low uptake from the gastrointestinal (GI) tract resulting in low bioavailability. This is mainly caused by degradation of the drug in the harsh intestinal environment caused by low pH and presence of proteolytic enzymes. Moreover, the large molecular size of biopharmaceuticals hampers successful penetration into and through the mucus layer protecting the epithelial membrane, and subsequently through the epithelial membrane before reaching the circulation in an intact form. One strategy to overcome those challenges is to protect the (bio)pharmaceutical from rapid degradation by loading it into tailored drug delivery systems (DDS). Additionally, the transit time of the DDS through the GI tract can beneficially be increased by maximizing the contact time between the DDS and the target site for absorption, thereby increasing drug absorption. This is done by including mucoadhesive components in the DDS design.

Until now, mucoadhesion of DDS and single excipient components hereof have been evaluated by measuring the change in apparent viscosity upon mixing a mucin suspension with the relevant test sample as compared to a mucin reference sample[1,2,3]. However, through the development of biosimilar mucus, we have shown that it is not only the mucin strains that are important for interactions with the DDS, but also the steric matrix of the mucus itself is crucial[4]. Especially the interactions between polyacrylic acid polymers of high molecular weight are shown to have an important role in mimicking the rheological behaviour of porcine intestinal mucus, due to chain entanglement and formation of secondary bonds[5]. Moreover, data obtained when using the biosimilar mucus to evaluate the interactive barrier properties have been shown to be more reproducible and less cytotoxic when compared to native porcine mucus[4,6]. Thus, we hypothesize, that applying biosimilar mucus matrices instead of mucin suspensions for assessment of mucoadhesion will provide a better model for prediction of mucoadhesion.

This hypothesis was addressed by evaluating three mucus model systems being mucin suspensions, biosimilar mucus, and porcine intestinal mucus (PIM). Further, a suspension of polyacrylic acid (PAA) was

used as a reference as it is shown to significantly contribute to the rheological behaviour of biosimilar mucus[5]. As test samples, the pharmaceutically relevant excipients alginate, hyaluronic acid, chitosan and poly(vinylpyrrolidinone) (PVP) was used. Chitosan was included as a positive reference for mucoadhesion and PVP as a negative[3]. Thus, a total of 16 combinations of excipients and model systems were investigated.

MATERIALS AND METHODS

Materials

Polyacrylic acid (PAA) (Carbopol® 974P NF) was purchased from Lubrizol (Brussels, Belgium), mucin from porcine stomach type II, bovine serum albumin (BSA) (P98%), cholesterol (>99%), polysorbate 80 (Tween 80), sodium alginate from brown algae, poly(vinylpyrrolidinone) (PVP) MW 40,000, chitosan MW 50,000-190,000, calcium chloride and magnesium sulfate from Sigma-Aldrich (Saint Louis, MO, USA). Phosphatidylcholine (purity 98%) was purchased from Lipoid (Ludwigshafen am Rhein, Germany), hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and acetic acid solution (3%) from Applichem (Darmstadt, Germany) and hyaluronic acid (Hyacare® 50) 50 kDa from Franken Chemie (Wendelstein, Germany).

Preparation of biosimilar mucus

The day prior to the experiment, biosimilar mucus was prepared as previously described[4]. Initially, 0.9% (w/v) polyacrylic acid was dissolved in 10 mM HEPES buffer, pH 7.4, containing calcium chloride (1.3 mM) and magnesium sulfate (1.0 mM) was added under constant magnetic stirring (subsequently denoted mucus buffer). Secondly, mucin was added (5% w/w) and pH re-adjusted to 7.4. Thirdly, a premix of polysorbate 80 (0.16% w/v), cholesterol (0.36% w/v), phosphatidyl choline (0.18% w/v) and BSA (3.1% w/v) was added, and pH adjusted again to 7.4. The prepared mucus was stored overnight at 4 °C.

Isolation of porcine intestinal mucus

Porcine intestinal mucus (PIM) was collected as previously described[6]. In brief, one to three meters of the proximal jejunum was collected from anesthetized pigs or immediately after euthanization. The pigs were ~3 months old, 30 kg and fasted 18-24 hr prior to surgery. The intestine was rinsed with mucus buffer, and the mucus gently collected. The samples were divided into aliquots and stored at -20 °C until further use. The animals were handled in strict compliance with the three R's and under license 2012-15-2934-00077 approved by the Danish Animal Experiments Inspectorate.

Sample preparation

Test samples of 20 mg/mL alginate, hyaluronic acid, chitosan and PVP were dissolved in mucus buffer and pH adjusted to 6.5-7.0. Due to the limited solubility of chitosan at this pH, it was dissolved in 3% (v/v) acetic acid and pH adjusted to 4.2, as precipitation occurred at higher pH values.

As one of the mucus model systems, the conventionally used mucin suspension of 50 mg/mL was prepared using mucus buffer[3], and pH adjusted to 7.4. This model system was compared to biosimilar mucus, prepared as described above. The PAA reference was a suspension of 9 mg/mL PAA in mucus buffer corresponding to the PAA concentration in the biosimilar mucus.

Small deformation rheology

The effect of mucus dilution on viscosity was first evaluated by mixing mucus with mucus buffer in 10% volume-ratio increments in the range 0:100 to 100:0 biosimilar mucus:buffer (v/v). Viscosity was measured using a steady state flow step, as further described below. Secondly, mucoadhesion was evaluated by mixing test samples with the respective mucus model

systems in ratios of 1:1 (v/v) for 15 min using magnetic stirring immediately before analysis. We have previously shown, that a mixing ratio of 1:1 reflects the mucoadhesive behaviour observed *in vivo,* when administering the test samples by oral gavage to conscious rats[7]. For reference measurements without mucus or without polymer as the test molecule, the test samples were mixed in ratios of 1:1 (v/v) with the mucus buffer and stirred for 15 min using magnetic stirring before analysis.

Mucoadhesion was evaluated using an ARES-G2 Rheometer (TA Instruments, New Castle, DE, USA). Due to limited sample volumes, a peltier plate and truncated cone (1°, 20 mm from TA Instruments, New Castle, DE, USA) were used, with the temperature set to 37 °C. A solvent trap cover was mounted to prevent evaporation during the measurement. The mixtures of biosimilar mucus and test samples were carefully mounted on the peltier plate, and a conditioning step was applied with a pre-shear set to 0.05 Pa followed by an equilibrium step of 5 min at 37 °C. Then, a steady state flow step was conducted, using a shear rate of 0.001 to 1000 s^{-1}, with three consecutive measurements of 10 sec each, allowing a maximum of 5 % variance[5], collecting 4 points per decade.

Statistical analysis

All experiments were run in triplicate (n=3), and error bars either plotted as standard deviation for average viscosity and error propagation for apparent viscosity (eq. 2).

EQUATIONS

Mucoadhesion is defined as an increase in the viscosity, where the apparent viscosity, $\eta_a > 0$. As previously described[2,3], η_a can be calculated by:

$$\eta_a = \eta_t - \eta_m - \eta_p \quad (1)$$

where η_t is the total viscosity of the mixed system, η_m the viscosity of the mucus model system and η_p the viscosity of the test sample. For $\eta_a > 0$, it is concluded that mucoadhesion occurs, whereas no mucoadhesion is detected for $\eta_a < 0$[3].

The error for η_a (∂) is calculated as error propagation by[8]:

$$\partial\eta_a = \sqrt{(\partial\eta_t)^2 + (\partial\eta_m)^2 + (\partial\eta_p)^2} \quad (2)$$

RESULTS AND DISCUSSION

It was first evaluated whether the viscosity of biosimilar mucus is affected by dilution with mucus buffer (Fig. 1). For the mixtures 100:0 to 30:70, a shear thinning behaviour is observed, whereas the viscosity of the mixtures from 20:80 to 0:100 are independent of shear rate. For all mixtures, a high reproducibility is observed. Moreover, a significant effect of dilution is detected, as the viscosity decreases with increasing fraction of mucus buffer in the mixture. This effect is most pronounced at low shear rates (0.1 s^{-1}), when the viscosity decreases 100-fold e.g. from 23 Pa.s to 0.23 Pa.s, when comparing the mixtures 100:0 with 30:70 biosimilar mucus:buffer (v/v) (Fig. 1, top). Contrary, at higher shear rates (5000 s^{-1}), the viscosity decreases 11-fold for 100:0 versus 30:70 from e.g. 5.1×10^{-2} Pa.s. to 4.5×10^{-3} Pa.s.

The viscosity at a shear rate of 100 s^{-1} is plotted as a function of mucus dilution (Fig. 1, bottom), showing that the viscosity is mostly affected when increasing the dilution from 50:50 to 0:100 (3.3×10^{-2} Pa.s. vs. 1.6×10^{-4} Pa.s.) when compared to the range from 100:0 to 50:50, where viscosity declines from 2.6×10^{-1} Pa.s. to 3.3×10^{-2} Pa.s.

This behaviour is most likely explained by the biosimilar mucus being capable of absorbing up to 50% (v/v) mucus buffer, without a dramatic effect on the viscosity. However, when reaching 70% (v/v) mucus

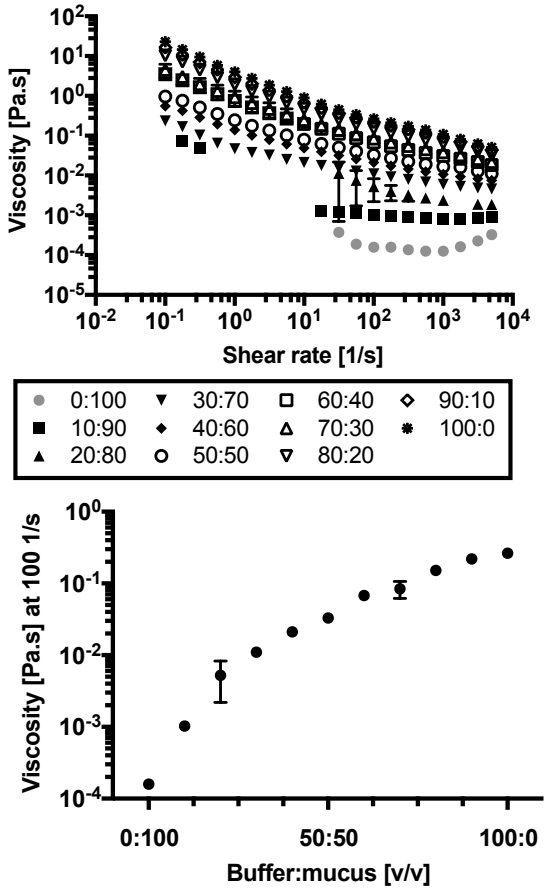

Figure 1. Top: viscosity as a function of shear rate for mixtures of biosimilar mucus:buffer (v/v). Bottom: relationship between dilution of mucus and viscosity at 100 1/s. Data is shown as n=3, error bars denote standard deviation.

buffer, the viscosity significantly decreases, suggesting that the microstructure of the biosimilar mucus is disrupted. It has previously been shown, that when decreasing the concentration of the high molecular weight PAA in biosimilar mucus from 0.9% to 0.6%, the viscosity decreases ~5-fold at shear rates of s^{-1} and ~100-fold when decreasing the concentration from 0.9% to 0.3% (w/v), using the exact same instrument, but equipped with a 40 mm cone[6]. The commonly used concentration of mucin in mucus mixtures used for mucoadhesion studies is 2 to 5% (w/w)[9], suggesting that a mucin gel network is already formed at concentrations of 2.5% (w/w) corresponding to the concentration in the 50:50 mixture. Thus, for the 0:100 to 30:70 mixtures, the mucin network may not be formed or is diluted, so that only very low viscosities is detected. The contribution from PAA in the biosimilar mucus is negligible, explaining the dramatic decrease in viscosity (Fig. 1, bottom) and the lack of shear thinning effect (Fig. 1, top). The latter behaviour can be ascribed to lack of mucin-mucin interactions present, resulting in no detectable change in viscosity when increasing the shear rate.

Summing up, those findings highlights that it is important to consider the effect of dilution on viscosity when applying Eq. 1. However, due to the impact of shear rate on the effect of dilutions, it is not straight-forward to derive a correction factor in Eq. 1.

Following the above studies, the hypothesis stating that using biosimilar mucus as a mucus model system instead of mucin suspensions better resembles the *in vivo* conditions was evaluated. As a control for *in vivo* relevance, PIM was included, along with a PAA suspension as PAA contributes to, but is not solely responsible for, the rheological profile of biosimilar mucus. The following depicted profiles (Fig. 2, 3, 4 and 5) for the mixtures where $\eta_a > 0$, imply that mucoadhesion occur.

Fig. 2 shows the apparent viscosity as a function of shear rate for mixtures of biosimilar mucus and test samples. As expected, significant mucoadhesion is detected for chitosan, whereas alginate and PVP show limited adhesion. For chitosan, the mucoadhesion can be ascribed to hydrogen bonding and hydrophobic interactions between the primary amino groups of the chitosan and the mucin chains[10]. PVP, however, was included as a negative reference for mucoadhesion based on a previous study[3]. Surprisingly, negligible mucoadhesion is observed for alginate. A previous study demonstrated a considerable degree of mucoadhesion for a

3% (w/v) alginate microsphere to sheep nasal mucosa *in vitro* and an increased adhesion when increasing the polymer concentration further[11]. In the present study, only 2% (w/v) solution was used and mixed 1:1 (v/v) with the mucus model system, which likely explains the limited degree of mucoadhesion. Also, mucoadhesion was expected to occur for hyaluronic acid, where hyaluronic acid of 202 kDa previously was shown to have better mucoadhesive performance to buccal, vaginal and intestinal tissue as compared to both 693 and 1878 kDa hyaluronic acid[12]. However, the mucoadhesive strength of polymers significantly increases with MW above 100,000[13], twice the MW compared to the hyaluronic acid used in the present study. Thus, the lack of mucoadhesive behaviour might be related to the relatively low MW, thus no detectable interactions are formed with the mucus matrix.

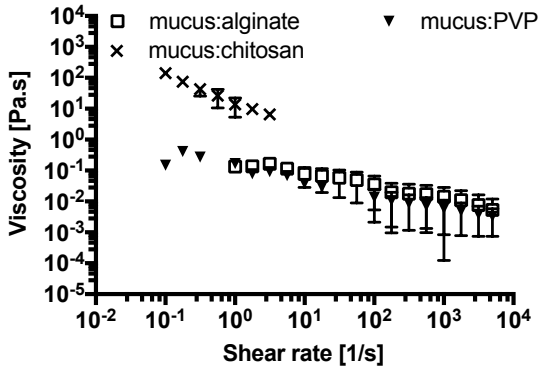

Figure 2. Apparent viscosity as a function of shear rate for mixtures of biosimilar mucus:test samples (v/v). Poly(vinylpyrrolidinone: PVP. Data is shown as n=3, error bars denotes error propagation.

With the PIM (Fig. 3) mucoadhesion is only observed for chitosan, being the most pronounced, and to some extent for hyaluronic acid. It is relevant to discuss, why no mucoadhesion is detected for alginate and PVP as observed when mixing with PIM. More studies are needed to further verify whether the adhesion observed in Fig. 2 is so limited that it is negligible and higher concentrations of alginate is needed together with high MW hyaluronic acid to induce mucoadhesion. Alternatively, the biosimilar mucus is more sensitive to interaction with polymer, and use of this model thus *may* overestimate the *in vivo* mucoadhesive behaviour of excipients. Inclusion of *in vivo* studies by e.g. using SPECT-CT would be highly relevant in order to follow the transit time of the selected excipients related to mucoadhesive behaviour. We suggest that the positive η_a observed for alginate and PVP in Fig. 2 is negligible, hence inclusion of higher concentrations of alginate and high MW hyaluronic acid would induce a $\eta_a >> 0$.

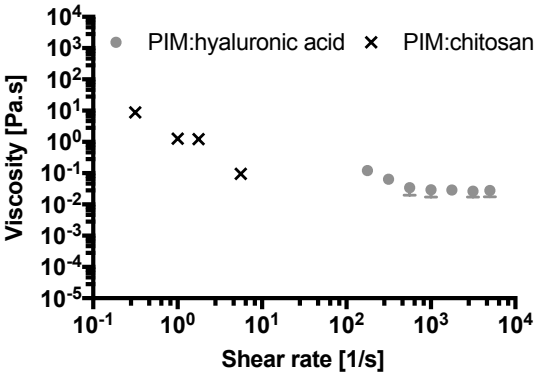

Figure 3. Apparent viscosity as a function of shear rate for mixtures of porcine intestinal mucus (PIM): test sample (v/v). Data is shown as n=3, error bars denotes error propagation.

In literature, simple mucin suspensions are the most commonly accepted model systems used to study mucoadhesion using small deformation rheology[3]. Thus, this model was included and data is shown in Fig. 4. With this model, only very limited mucoadhesion is observed for alginate and hyaluronic acid. Surprisingly, no interaction with chitosan is detected, although it shows the most significant adhesion of all tested excipient to biosimilar mucus and PIM. This finding confirms our hypothesis, that it is

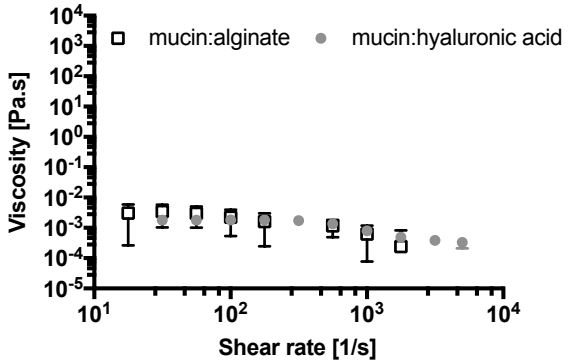

Figure 4. Apparent viscosity as a function of shear rate for mixtures of mucin:test samples (v/v). Data is shown as n=3, error bars denotes error propagation.

not only the mucin chains that are important for mucoadhesion, but also steric interactions with the full mucus matrix.

PAA was included as a control (Fig. 5), as it significantly contributes to the rheological behaviour of biosimilar mucus[5]. Interestingly, PVP showed a marked adhesion to PAA, despite no interactions observed for the other excipients tested. The interaction between the two polymers PAA and PVP is explained by strong

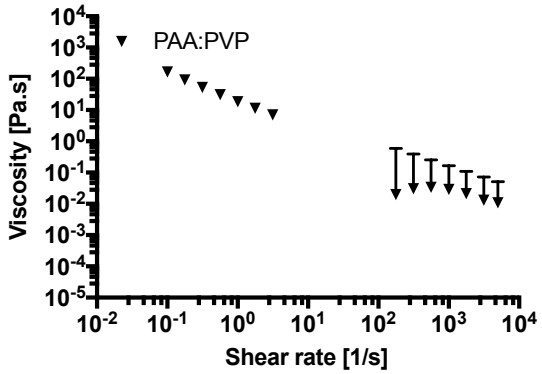

Figure 5. Apparent viscosity as a function of shear rate for mixtures of polyacrylic acid (PAA):test samples (v/v). Poly(vinylpyrrolidinone): PVP. Data is shown as n=3, error bars denotes error propagation.

hydrogen bonding forming hydrophobic regions[14]. However, as no adhesion is observed for chitosan, as is the case for biosimilar mucus (Fig. 2), it is suggested that when included in the matrix of the biosimilar mucus such interactions are not occurring or are only negligible as seen in Fig. 2.

CONCLUSION

Mucoadhesion of four pharmaceutically relevant excipients; alginate, chitosan, hyaluronic acid and PVP were evaluated using small deformation rheology. PIM, biosimilar mucus, and mucin suspension comprised the model systems and PAA solution was included as a control. It was shown, that the interactions with biosimilar mucus resembles the interactions observed with PIM, whereas the conventionally used mucin suspensions did not reveal any significant mucoadhesion for the excipients tested. Thus, we confirm the hypothesis that biosimilar mucus serves as a better model system to study mucoadhesion when compared to mucin suspensions.

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