Development and Rheological Profiling of Biosimilar Mucus

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ABSTRACT

This article presents the development of a biosimilar mucus mixture for assessment of the effect of mucus on drug absorption. The biosimilar mucus was optimized to represent a rheological profile similar to that of porcine intestinal mucus. This was obtained with a mixture of mucin, polyacrylic acid, lipids and BSA.

INTRODUCTION

The viscoelastic mucus layers covering the epithelial surfaces are likely to constitute a barrier to mucosal drug delivery[1]. The viscosity and the network structure of mucus can physically constitute a barrier to the diffusion of drugs and drug delivery systems and thereby decrease or prevent transmucosal drug absorption. Furthermore, the net negative charge and hydrophilicity of mucus and the interactions between mucus components and drugs amplify the barrier properties of mucus[2]. It is crucial to have suitable models to assess the effect of mucus on drug absorption.

Mucus is composed of ~95 % water, ~5 % mucin, small amounts of proteins, lipids, DNA and electrolytes[3]. Mucins are high molecular weight, heavily glycosylated proteins that are responsible for the rheological properties of mucus[4]. The selection of commercially available mucin is limited to mucin that is isolated from porcine stomach or bovine submaxillary glands and subjected to a purification process, this however reduces the molecular weight of the mucins[5]. It has been demonstrated that commercial porcine gastric mucin in itself does not represent the rheological properties of native mucus[6].

Mucus is a non-Newtonian viscoelastic gel with shear-thinning properties and dominant elastic behavior irrespective of its physiological origin[4,7]. However, the viscosity of mucus from different physiological locations appear to vary, which might be due to variation in pH and ionic strength that influence the viscosity[8] or it might correlate with differences in the detailed composition of mucus, but this has not yet been systematically investigated. Generally, the knowledge on variation in mucus between species, individuals, physiological sites and health/disease states with respect to content and rheological profile is sparse. This is mainly due to the difficulties in obtaining truly representative mucus[9] and in isolating the large, highly polydisperse and complex mucins for analysis[10]. The inaccessibility to obtaining native mucus have led to the development of several different simulated mucus mixtures and use of cell cultures as *e.g.* Calu-3 cells, which can secrete mucus, in contrast to the standard model for assessing intestinal absorption, the Caco-2 cells.

In rare cases, the simulated mucus mixture is based on a preceding analysis on

mucus content[11], and the results of the study by Larhed *et al.* were used as inspiration for the development of a biosimilar mucus mixture. Thus, the aim of the present study was to develop a biosimilar mucus mixture with rheological properties, which mimic those identified in porcine intestinal mucus. The intention is that this mucus can be used alone and/or in combination with cells as an *in vitro* mucosal model for assessment of the influence of mucus on oral drug absorption.

MATERIALS AND METHODS

Materials

Mucin from porcine stomach type II, bovine serum albumin (BSA) (> 98 %), linoleic acid (> 99 %), cholesterol (> 99 %), polysorbate 80 (Tween 80), Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, L-glutamine, non-essential amino acids, Hank's Balanced Salt Solution (HBSS), silicone oil (5 mPaS at 25 °C), $CaCl_2$, $MgSO_4$ and phenazine methosulfate (PMS) were obtained from Sigma Aldrich (Broendby, Denmark). Polyacrylic acid (PAA_{low}, 925 mPaS at 4 % (w/v)) was also from Sigma Aldrich, Polyacrylic acid (PAA_{high}, 34,900 mPaS at 0.5 % (w/v)) (Carbopol 974P NF) from Lubrizol (Brussels, Belgium) and sodium carboxymethylcellulose (NaCMC, 2921 mPaS at 2 % (w/v)) from Fagron (Copenhagen, Denmark). Phosphatidyl-choline (PC, > 98 %) was from Lipoid (Ludwigshafen, Germany), hydroxyethyl piperazineethane-sulfonic acid (HEPES) from AppliChem (Darmstadt, Germany), NaCl from Merck Chemicals (Darmstadt, Germany), fetal bovine serum (FBS) from Fisher Scientific (Slangerup, Denmark), and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega (Madison, WI, USA).

Isolation of porcine intestinal mucus

Pigs of approximately 3 months of age and 30 kg were fasted for 18-24 hours prior to surgery. Around one meter of the jejunum was removed from the anaesthetized pig. It was divided into smaller pieces and rinsed with isotonic 10 mM HEPES buffer containing 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 137 mM NaCl in order to remove undigested food. The mucus was gently scraped off the epithelial surface, aliquoted and stored at -20 °C until analysis.

Preparation of biosimilar mucus

For preliminary studies, 8 % (w/v) mucin was dissolved in a 10 mM HEPES buffer under magnetic stirring with 0.56 % (w/v) PAA_{high}, 0.8 % (w/v) NaCMC or 0.8 % (w/v) PAA_{low} and the pH was adjusted to 7.4.

For optimization, the biosimilar mucus used was prepared with 0.3, 0.6 or 0.9 % (w/v) PAA_{high}, 5 % (w/v) mucin, 3.1 % (w/v) BSA and 0.65-3 % (w/v) of a lipid mixture (0.36 % cholesterol, 0.18 % phosphatidylcholine and 0.11-2.46 % linoleic acid) in 0.1625-0.75 % (w/v) polysorbate 80 in a 10 mM HEPES buffer containing 1.3 mM $CaCl_2$ and 1.0 mM $MgSO_4$ (pH 7.4).

The prepared mucus mixture was always stored overnight at 4 °C prior to analysis.

Rheological profiling

An AR-G2 cone and plate rheometer (TA Instruments-Waters, New Castle, DE, USA) was employed with a cone angle of 1° and a diameter of 40 mm. The plate temperature was kept at 37 °C and low-viscosity silicone oil was applied to a custom made solvent trap to prevent sample dehydration. Measurements were conducted in three steps: i) a stress sweep step where an appropriate torque was chosen (0.1-3 μN×m) ensuring measurements being within the linear viscoelastic range of the sample, ii) a frequency sweep step with angular frequencies (ω) of 0.1–100 rad/s was

conducted prior to iii) a steady state flow step with shear rates 1×10^{-3}–1000 1/s (three consecutive measurements of 10 sec resulted in < 5 % variance). TA Instruments Rheology Advantage Software (TA Instruments-Waters) collected the rheological data, *e.g.* the elastic/storage (G') and viscous/loss (G'') modulus, which were used to calculate the complex viscosity (η*) (Eq. 1).

$$|\eta * (\omega)| = \frac{\sqrt{G'(\omega)^2 + G''(\omega)^2}}{\omega} \quad (1)$$

Cell culture

Caco-2 and Calu-3 cells were obtained from was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and from American Type Culture Collection (ATCC) (Manassas, VA, USA), respectively. The cells were cultured at 37 °C, at an atmosphere of 5 % CO_2/95 % O_2 in DMEM supplemented with 10 % (v/v) FBS, penicillin/streptomycin (100 U/ml and 100 µg/ml), 1 % (v/v) L-glutamine and 1 % (v/v) non-essential amino acids.

Calu-3 cells were seeded at a density of 10^5 cells/cm^2 onto collagen coated (0.29 µg/ml, 200 µl/insert) 12-well polycarbonate Transwell® filter inserts (1.12 cm^2 growth area, 0.4 µm pore size) (Corning Costar, Sigma-Aldrich). After 24 hrs the apical medium was removed and subsequently the basolateral medium was replaced every other day, until the mucus was isolated from the apical compartment on day by gently scraping it off with a micro spoon 17.

Caco-2 cells were seeded in a 96-well plate (Nunc Transparent, Sigma-Aldrich) at a density of 0.9×10^5 cells/well and used approximately 24 hrs after seeding.

Cellular viability assay

The test solution, *i.e.* the complete mucus mixture or the mucus components separately, were diluted to different degrees using a 10 mM HEPES HBSS buffer. The solution of PAA and mucin was prepared as described for the biosimilar mucus, whereas the lipid solutions were prepared using a 10 mM HEPES isotonic buffer containing 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 137 mM NaCl.

The proliferating Caco-2 cells were incubated with 100 µl/well of test solution for 1 hr at 37 °C on a horizontal shaker (Edmund Bühler, Hechingen, Germany) at 90 rpm.

After gently washing the cells twice using the 10 mM HEPES HBSS buffer (pH 7.4), 100 µl of freshly prepared MTS/PMS solution was added to the cells. The absorbance was measured at 492 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) after 2.5 hrs of incubation at 37 °C on a horizontal shaker at 90 rpm. Buffer treated cells were used as the reference for expressing the relative viability.

RESULTS AND DISCUSSION

To obtain a highly viscous sample, 8 % (w/v) mucin was dissolved in a 10 mM HEPES buffer, as this was found to be the maximal mucin concentration in order to maintain the osmolality within a biocompatible range. The 8 % (w/v) mucin solution was subjected to rheological analysis and compared to the profile of mucus secreted from cultured Calu-3 cells. This cell line of bronchial origin displays significant mucus production and was employed as the end point sample for the preliminary studies. The mucin solution displayed dominant viscous behaviour, whereas Calu-3 mucus demonstrated dominant elastic behaviour (Fig. 1). Furthermore, the Calu-3 mucus demonstrated less frequency dependency of both the dynamic moduli. The mucin solution showed a more than 100-fold lower level of both apparent and complex viscosity, both of which did not decrease sufficiently with

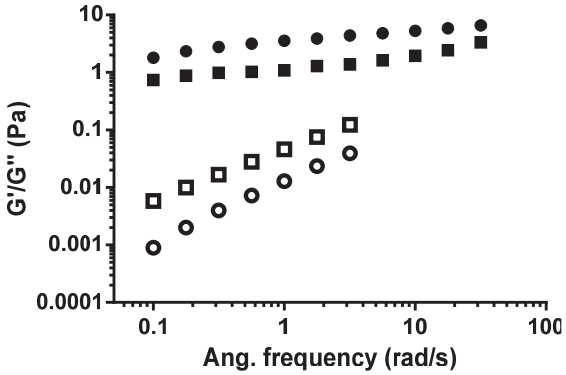

Figure 1. The elastic (G', circles) and viscous (G'', squares) modulus of 8 % (w/v) mucin (open) compared to mucus from cultured Calu-3 cells (filled).

increasing frequency and shear rate to match the profile of isolated Calu-3 mucus (Fig. 2).

Three polymers were thus added to the mucin solution in order to mimic the viscosity level of Calu-3 mucus. Polyacrylic acids (PAA) and sodium carboxy-methylcellulose (NaCMC) have a resemblance to mucin in being hydrophilic and anionic at neutral pH. Additionally, these polymers are widely used as pharmaceutical excipients. Inclusion of 0.8 % (w/v) of the PAA with the lowest molecular weight and lowest viscosity (PAA_{low}) in the mucin solution did not alter the complex viscosity of the mucin solution significantly, whereas the addition of 0.8 % (w/v) NaCMC caused a 2-fold increase in the complex viscosity (Fig. 2). This is consistent with an earlier study using these two polymer types together with 4 % (w/w) purified mucin. It was demonstrated that NaCMC interacted with mucin resulting in an increased complex viscosity compared to the polymer alone, whereas this was not the case for PAA_{low}, which is more sensitive to pH and ionic strength than NaCMC[12]. Thus, the polymer network of PAA_{low} is presumably broken down by the ions of the commercial mucin and the buffer[12].

The apparent viscosity of the mucin solution was found to increase in the presence of both NaCMC and PAA_{low} (although only above shear rates of 0.2 1/s for PAA_{low}). However, the actual values were >100-fold lower than the level of the Calu-3 mucus apparent viscosity at the lowest shear rates and ~10-fold too high at the highest shear rates (Fig. 2).

Addition of 0.56 % (w/v) high molecular weight and viscosity PAA (PAA_{high}) to mucin demonstrated a complex viscosity approaching that of Calu-3 mucus and also a more prominent shear-thinning behaviour (Fig. 2), *i.e.* the rate at which the interactions are destroyed exceeds their rate of replacement causing a decreasing viscosity[13]. The increased viscosity caused by PAA_{high} has also been observed in another study, where it was ascribed to chain entanglement and formation of secondary chemical bonds[14]. PAA_{high} was chosen for further studies.

It has been stated that mucus from cultured cells insufficiently mimic the characteristics of native mucus[15]. Thus, porcine intestinal mucus (PIM) was employed as the reference end point for the optimization studies for the mucus mixture. PIM demonstrated a higher level of viscoelasticity as compared to mucus from the Calu-3 cells (Fig. 3). The flow chart demonstrated that these differences were distinct at shear rates above 0.5 1/s and in particular above 10 1/s (Fig. 3). As the developed biosimilar mucus is intended as a model for assessing intestinal drug absorption and for use in the development of oral drug delivery systems, PIM is considered a more appropriate reference for optimization compared to mucus derived from a bronchial cell culture model.

The method for collecting the PIM included rinsing the intestine with buffer prior to the mucus isolation and freezing of the mucus before analysis. Therefore, the influence of these procedures on the rheological profile was examined, and both were found to cause

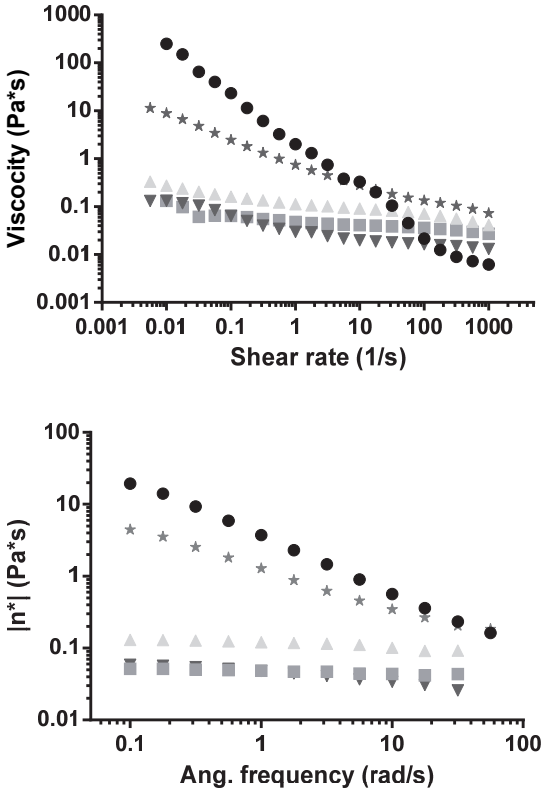

Figure 2. The apparent viscosity as a function of shear rate (upper) and the complex viscosity as function of angular frequency (lower) of 8 % (w/v) mucin in the absence (inverted triangles) and in the presence of 0.8 % (w/v) PAA_{low} (squares), 0.8 % (w/v) NaCMC (triangles) or 0.56 % (w/v) PAA_{high} (stars) compared to mucus isolated from cultured Calu-3 cells (circles).

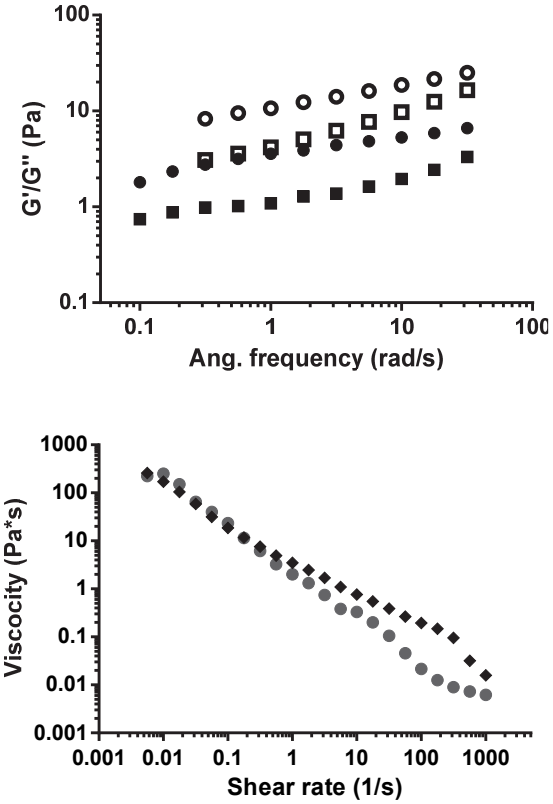

Figure 3. The elastic (G', circles) and viscous (G'', squares) modulus of mucus from cultured Calu-3 cells (filled) compared to mucus isolated from porcine intestine PIM (open) (upper).
The apparent viscosity as a function of shear rate of PIM (diamonds) and mucus from Calu-3 cells (circles) (lower).

a decrease in the viscosity of the mucus (Fig. 4). Freezing mucus at -20 °C has previously been demonstrated to decrease the complex viscosity of human cervical mucus[8], presumably due to mechanical damage as a result of ice crystal growth[13]. Rinsing the intestine increases the hydration of mucus, which causes the spacing between the fibers to increase[16] resulting in a decreased viscosity.

Studies have suggested that the protein and lipid content of mucus contribute to its rheological behaviour[8,17]. Furthermore, as lipids and proteins of mucus have been found to reduce the diffusion of several drugs[11,18], their inclusion in mucus seemed to be crucial for the preparation of a biosimilar mucus. Thus, with inspiration from Larhed *et al.*[11], a lipid mixture of linoleic acid, cholesterol and phosphatidyl-choline and bovine serum albumin was included in the mucin mixture. Furthermore, the mucin content was reduced to 5 % (w/v), as this is closer to the denoted *in vivo* values[3] and resulted in an osmolality of this more complex mixture within the biocompatible range. The divalent cations Ca^{2+} and Mg^{2+}, which are important for the tight junction integrity, were included to improve the biocompatibility of this mucus

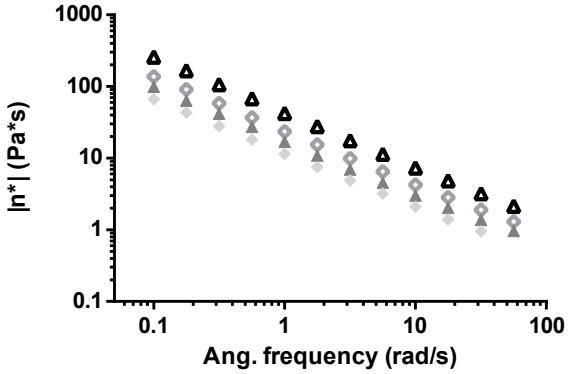

Figure 4. The complex viscosity as a function of angular frequency of porcine intestinal mucus (PIM). PIM was isolated from unrised intestine (triangles) and rinsed intestine (diamonds) and was analysed prior to (open) or after freezing (filled).

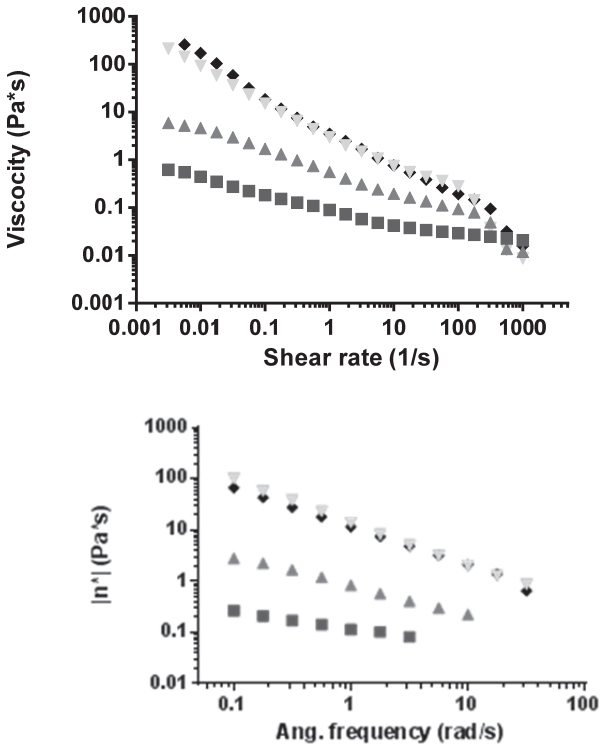

Figure 5. The apparent viscosity as a function of shear rate (upper) of the biosimilar mucus with varying content of polyacrylic acid (PAA_{high}) compared to porcine intestinal mucus (diamonds). 0.3 % (squares), 0.6 % (triangles) and 0.9 % (w/v) (inverted triangles) PAA_{high} was included in a mucus mixture containing 5 % (w/v) mucin, 3.1 % (w/v) BSA and 3 % (w/v) of a lipid mixture in 0.75 % (w/v) polysorbate 80.

mixture. PAA_{high} was then titrated into this more *in vivo* like mucus mixture (Fig. 5). It was found that 0.9 % (w/v) of PAA_{high} matched the profile of PIM and this concentration was used for further studies.

The cellular compatibility of the biosimilar mucus was assessed by applying dilutions of the complete mixture as well as of solutions of the different components to proliferating Caco-2 cells of intestinal origin. The high content of linoleic acid in the lipid mixture was found to reduce the viability of cells when exposed to the mucus (Fig. 6). The other lipids together with polysorbate 80 and the mixture of PAA_{high} and mucin were found to be compatible with the cells at all tested concentrations (Fig. 6).

Thus in order to achieve cellular compatibility of the biosimilar mucus it was necessary to decrease the linoleic acid content of the mucus to a level of 0.11 % (w/v) (data not shown). Within the tested concentration range, it was found that altering the lipid composition did not affect the rheological profile (Fig. 7). This seems to be in contradiction with the general understanding[7], however it can be questioned whether earlier studies *e.g.* illustrating decreased viscosity upon removal of mucin associated lipids from dog gastric mucin solely assess the effect of the presence or absence of lipids17. Furthermore, to the authors' knowledge, no studies have been based on rheological profiling using lipids in combination with commercial mucin.

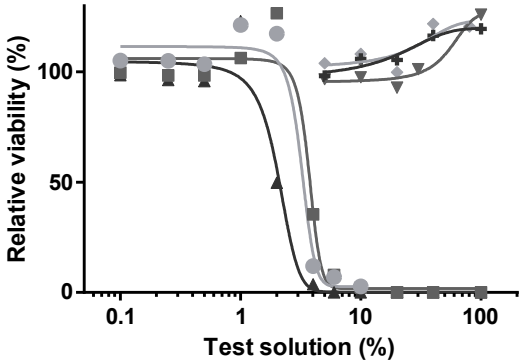

Figure 6. The relative viability of Caco-2 cells incubated with different concentrations of the complete biosimilar mucus mixture (circles) and its components (w/v): lipid mixture with 2.46 % linoleic acid, 0.36 % cholesterol, 0.18 % phosphatidylcholine and polysorbate 80 (squares); 2.46 % linoleic acid and polysorbate 80 (triangles); 0.18 % phosphatidylcholine and polysorbate 80 (pluses); 0.36 % cholesterol and polysorbate 80 (diamonds) or 0.9 % PAA_{high} and 5 % mucin (inverted triangles). The data were fitted in Graphpad Prism using a hillslope constant of -1 or 0.03. n=3-6.

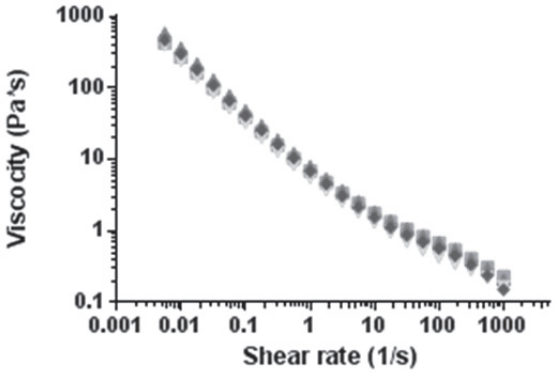

Figure 7. The apparent viscosity as a function of shear rate of biosimilar mucus with varying content of lipid and linoleic acid in particular. Represented are profiles for the mucus mixture without lipid, mucus with 0 %, 0.11 %, 0.26 % and 2.24 % (w/v) linoleic acid with fixed content of cholesterol and phosphatidylcholine at 0.36 % and 0.18 % (w/v).

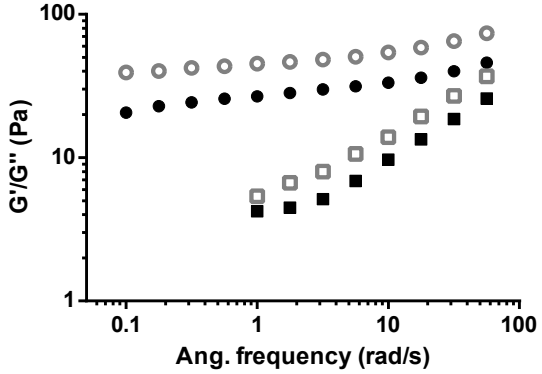

Figure 8. The elastic (G', circles) and viscous (G'', squares) modulus of biosimilar mucus with (filled) or without (open) bovine serum albumin (BSA).

In contrast to the evaluation of the lipid content, it was demonstrated that the inclusion of BSA decreased the viscosity of the biosimilar mucus mixture (Fig. 8). A previous study reported that there was no rheological contribution of serum proteins to the viscosity of mucus isolated from dog trachea[8], whereas another study showed a concentration dependent increase in viscosity when 0.5-2 % (w/v) BSA was added to a 0.8 % (w/v) mucin solution[19]. However, no reports on the effect of protein in more concentrated or complex mucin mixtures have been found in the scientific literature.

CONCLUSION

The addition of a polyacrylic acid polymer of high molecular weight to a solution of mucin was found to be necessary in order to obtain a mucus mixture with a rheological profile comparable to porcine intestinal mucus, *i.e.* shear-thinning dominant elastic behaviour at a comparable viscosity level. The final biosimilar mucus contained 5 % (w/v) mucin, 0.9 % (w/v) polyacrylic acid, 3.1 % (w/v) BSA, 0.65 % (w/v) of a lipid mixture (0.36 % cholesterol. 0.18 % PC and 0.11 % linoleic acid) with 0.16 % polysorbate 80. This mixture was found to be compatible with proliferating

Caco-2 cells. When applied to cell monolayers, such a combined model may constitute a representative intestinal mucosal model applicable for predicting the effect of the mucus on drug absorption.

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