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Abstract

Mucosa-mimetic materials are synthetic substrates which aim to replace animal tissue in mucoadhesion experiments. One potential mucosa-mimetic material is a hydrogel comprised of N-acryloyl-D-glucosamine and 2-hydroxyethylmethacrylate, which has been investigated as a surrogate for animal mucosae in the mucoadhesion testing of tablets and solution formulations. This study aims to investigate the efficacy of this mucosa-mimetic material in the testing of thermogelling semi-solid formulations, which transition from solution to gel upon warming. Two methods for assessing mucoadhesion have been used; tensile testing and a flow-through system, which allow for investigation under dramatically different conditions. It was found that the mucosa-mimetic material

was a good surrogate for buccal mucosa using both testing methods. This material may be used to replace animal tissue in these experiments, potentially reducing the number of laboratory animals used in studies of this type.

1. Introduction

Many factors can be considered in the development of drug delivery systems. For topical drug delivery, a key parameter for evaluating dosage forms is their bioadhesion and, in the case of mucosal membranes, mucoadhesion; the adhesive interaction between a material and a mucosal membrane (Khutoryanskiy, 2011; Smart, 2005). Mucoadhesion can improve the retention of the formulation on the mucosal surface, increasing the retention time at the site of action, improving drug absorption, and thus bioavailability (Khutoryanskiy, 2014). Novel mucoadhesive systems are of interest in oral transmucosal (Khan et al., 2016; Shojaei and Li, 1997), nasal (Nakamura et al., 1996; Ugwoke et al., 2005), ocular (Hornof et al., 2003; Ludwig, 2005), vaginal (Andrews et al., 2009; Friedl et al., 2013), and rectal drug delivery (Değim et al., 2005), and these systems typically require evaluation on *ex vivo* animal mucosa (Cook and Khutoryanskiy, 2015; Ivarsson and Wahlgren, 2012).

One class of mucoadhesive formulations which is of interest is "thermogelling" materials, which transition from solution to gel upon warming from room to body temperature (de Araújo Pereira et al., 2013). Systems which gel *in situ* are easily administrated and can improve the retention of dosage forms at desired place, potentially increasing patient compliance to treatment (Van Tomme et al., 2008). Poloxamer P407 (also known as Pluronic F127), an ABA triblock copolymer of poly(ethylene glycol) – *b* – poly(propylene glycol – *b* – poly(ethylene glycol), is the

most commonly used thermogelling material (Nie, 2011), but has several drawbacks, such as weak gel strength and rapid dissolution (Wu et al., 2011). To attempt to enhance the mucoadhesion of poloxamer P407, bioadhesive polymers based on cross-linked poly(acylic acid) (PAA), such as Carbopol® 971P, Carbopol® 974P and polycarbophil, have been incorporated into poloxamer dispersions (Chang et al., 2002; De Souza Ferreira et al., 2015; Jones et al., 2009; Junqueira et al., 2016). Poly(acrylic acid) derivatives were previously reported as mucoadhesive (Jabbari et al., 1993), and the cross-linked forms, carbopol and polycarbophil, impart high viscosity to formulations, enhancing mucoadhesion.

Over the years, the mucoadhesion process has been widely studied (Iqbal et al., 2012; Peppas and Huang, 2004; Sogias et al., 2008; Sosnik et al., 2014), and in order to asses it, many different *in vitro* and *ex vivo* techniques have been developed (Bassi da Silva et al., 2017; Cave et al., 2012; Withers et al., 2013). Nevertheless, the force required to detach a dosage form from mucosal tissue is still the most commonly used technique (Carvalho et al., 2010; Cook and Khutoryanskiy, 2015; Nair et al., 2013). This is typically determined with the use of a texture analyser. According to the dosage form, some techniques prove to be more suitable than others. Solid dosage forms require detachment force method and cannot be measured by rheological methods like liquid dosage forms, for example. On the other hand, there is flow-through method which can be used for solid dosage forms (Patel et al., 2012) and liquid dosage forms (Cook et al., 2015; Irmukhametova et al., 2011).

Generally, most methods use *ex vivo* tissues to assess mucoadhesion, a large amount of which is sourced from laboratory animals slaughtered for that tissue (Cook et al., 2015)

Moreover, when used animal sources, there is a lower reproducibility of the method, considering the greater variation between these tissues. Therefore, with the aim of to reduce the number of animals killed for this tissue, this work aims to demonstrate the efficacy of a synthetic alternative to animal tissue in mimicking mucosa for the testing of mucoadhesive semi-solids. This mucosa-mimetic material provides a testing substrate which is inexpensive and homogenous compared to animal tissue. A substrate containing 20 mol% N-acryloylglucosamine (AGA) and 80 mol% 2-hydroxyethylmethacrylate (HEMA) was identified as being an effective mimic for pig buccal mucosa when testing the mucoadhesion of tablets and liquid dosage forms (Cook et al., 2015; Hall et al., 2011). This study investigates the efficacy of this "mucosa-mimetic" material when testing for the mucoadhesion of semi-solid dosage forms using two different methodologies. This is the first reported use of a mucosa-mimetic material in studying the mucoadhesion of semi-solids, with previous research investigating solid (Eshel-Green et al., 2016; Hall et al., 2011; Khutoryanskaya et al., 2010) or liquid (Cook et al., 2015; Eshel-Green et al., 2016) dosage forms. The mucoadhesion of semi-solid dosage forms is driven by non-covalent interactions of macromolecules with mucins, as for liquid and solid dosage forms, but also by the rheology of the formulation and its ability to wet a surface (Smart, 2005). For validation of this mucosa-mimetic material it is imperative that this class of materials be investigated.

2. Materials and Methods

2.1 Materials

Carbopol® 971P, Carbopol® 974P and polycarbophil were purchased from Lubrizol (Brazil). Triethanolamine, 2-hydroxyethyl methacrylate (HEMA), N,N'-methylenebisacrylamide (MBA), ammonium persulfate (APS), N,N,N',N'-

tetramethylethylenediamine (TMEDA), acetonitrile (ACN), fluorescein isothiocyanate-dextran (FITC, 10 kDa), and phosphate buffered saline (PBS) tablets were all purchased from Sigma-Aldrich (U.K.). 2.5 mL polypropylene vials, fitted with screw-on septa, having 8 mm internal diameter, were also purchased from Sigma-Aldrich (U.K.). Acryloyl glucosamine (AGA) was synthesised using a previously published procedure (Cook et al., 2015). Unless specified, all reagents were used without further purification. Porcine buccal mucosa was sourced from Wetlab-MedMeat (U.K.) and kept frozen at -80 °C.

2.2 Hydrogel preparation

2.2.1 Mucoadhesive polymeric systems

Monopolymeric thermogelling systems were prepared by dispersing 20 % w/v poloxamer 407 in purified water at room temperature.

To produce the binary polymeric systems, Carbopol® 971P, Carbopol® 974P or polycarbophil (0.25, 0.20 and 0.25 %, w/w, respectively) were dispersed in purified water with stirring. The required amount of poloxamer 407 (20 %, 15 % and 15 %, w/w, respectively) was then added to this preparation and the mixture was stored at 4 ºC for 12 h, to ensure the complete polymer wetting. The polymeric systems were then stirred, to completely disperse the polymers. The preparations were then neutralized with q.s. triethanolamine, and kept at 4 ºC for at least 24 h before analysis (Bruschi et al., 2007; de Araújo Pereira et al., 2013; Fabri et al., 2011; Jones et al., 2009; Schmolka, 1972).

2.2.2 Synthesis of mucosa-mimetic hydrogels

As previously described by Hall et al (2011), purified water, MBA, APS, TMEDA and HEMA and AGA monomer(s) were added to glass vials (Table 1). The mixtures were vortexed until complete dissolution of all ingredients. Ethanol was then added before being mixed again and the mixtures were bubbled for 5 minutes with nitrogen. 2.0 mL aliquots of reaction mixture were then transferred to 2.5 mL polypropylene vials fitted with septa, which had been purged with nitrogen. The vials were then placed in a preheated water bath at 60 ºC, and reaction allowed to proceed for 3 h. The polymerisation was terminated by cooling the vial with cold water. The hydrogels were then purified by immersing samples in deionised water, which was changed daily, for two weeks to remove any unreacted chemicals.

Table 1. Composition of feed mixtures for synthesis of mucosa-mimetic hydrogels

Sample	HEMA:AGA ratio (mol %)	HEMA (g)	AGA (g)	MBA (g)	APS (g)	TMEDA (g)
HEMA hydrogel	100:0	4.3337	-	0.0052	0.0380	0.0116
20 mol% AGA hydrogel	80:20	3.4711	1.5393	0.0052	0.0380	0.0116

2.3 Equilibrium swelling degree measurements

Post-purification, a section of HEMA and AGA mucosa-mimetic hydrogels were removed by scalpel and the swollen samples were weighed. These samples were placed in small vials previously weighed and, allowed to dry in an oven at 50 ºC for at least 48 h, and reweighed. The equilibrium swelling degree (ESD) was then calculated according to equation 1:

$$ESD = (W_s - W_d)/W_d \quad (1)$$

where W_s and W_d are the weights of the swollen and dry sample, respectively. Each experiment was repeated at least 3 times for each hydrogel.

2.4 Density studies

The density of dry gels was calculated using the displaced volume of acetonitrile (ACN) in a 5 mL pycnometer (Sigma-Aldrich, U.K.). The empty pycnometer was weighed, filled with acetonitrile and reweighed, allowing for calculation of the pycnometer volume (V_{ACN+d}). After that, a known amount of dried hydrogel (W_d) was placed into a pycnometer, which was then filled with acetonitrile and weighed again (W_{ACN+d}). The density of the sample (ρ_x) may then be calculated using equations 2 and 3:

$$V_{ACN+d} = (W_{ACN+d} - W_d)/\rho_{ACN} \quad (2)$$

$$\rho_x = W_d / (V_{ACN+d} - V_{ACN}) \quad (3)$$

where ρ_{ACN} is the density of ACN (0.786 g/mL) and V_{ACN} is the volume of ACN.

2.5 Equilibrium swelling volume measurements

First, the hydrogel swelling ratio (Q_m) was calculated following the equation 4:

$$Q_m = W_s/W_d \quad (4)$$

where W_S and W_d are the weights of the swollen and dry sample, respectively. Then, the equilibrium swelling volume (ESV) was calculated by equation 5:(Thomas et al., 2016)

$$ESV = ((1 + \rho_x)/\rho_W). (Qm-1) \quad (5)$$

where ρ_W is the density of water.

2.6 Continuous shear (flow) rheometry

The continuous shear analysis of all thermogelling semisolid formulations without FITC-dex marker was performed at 37 ± 0.1 °C. In flow mode, a controlled stress rheometer (MARS II, Haake Thermo Fisher Scientific Inc., Germany) with parallel steel cone-plate geometry (60 mm, separated by a fixed distance of 0.052 mm) was used. Samples were carefully placed to the inferior plate, and allowed to equilibrate for at least 1 min prior to start the analysis. Flow curves were evaluated over shear rates ranged from 0 to 2000 s^{-1}. The flow properties of at least three replicates were measured, in each case.

The rheological properties of these formulations have been investigated previously using the following procedure, and are reported herein to discuss differences in formulations (Bruschi et al., 2007; de Araújo Pereira et al., 2013; Jones et al., 2009). The rheology of the formulations is a major factor in determining the retention, ease of application of the product and mucoadhesion of dosage forms, so it is important that the rheological properties of the materials be discussed. The ascending flow curves were fitted using the Power Law equation (equation 6).

$$\sigma = k.\gamma^n \quad (6)$$

where σ is the shear stress (Pa), *k* is the consistency index [(Pa.s)^n], γ is the rate of shear (s^{-1}), and *n* is the flow behavior index (dimensionless). The yield stress of the formulations was investigated by the following rheological models: Casson (equation 7) and Herschel–Buckley (equation 8) (Hemphill et al., 1993).

$$\tau = \sqrt[n]{\left(\tau_0^n + \left(\gamma n_p\right)\right)^n} \quad (7)$$

where τ is the shear stress (Pa), *n* is the flow behavior index (dimensionless), τ_0 is yield stress (Pa), γ is the rate of shear (s^{-1}) and n_p is Casson plastic viscosity.

$$\tau = \tau_0 + k.\gamma^n \quad (8)$$

where τ is the shear stress (Pa), τ_0 is yield stress (Pa), *k* is the consistency index [(Pa s)^n], γ is the rate of shear (s^{-1}) and *n* is the flow behaviour index (dimensionless). Then, the hysteresis area of each binary polymeric system was calculated using RheoWin 4.10.0000 (Haakes) software.

Moreover, the rheological analysis of formulations, with and without FITC-dex, was performed at 37 °C using an AR 1500 ex controlled stress/controlled rate rheometer (T.A. Instruments, UK), in flow mode, in conjunction with parallel steel plate geometry (40 mm, separated by a fixed distance of 600 μm). The samples were carefully applied to the lower plate of the rheometer, ensuring that formulation shearing was minimized, and allowed to equilibrate for at least 3 min prior to analysis. In continuous shear mode, upward flow curve for each formulation were measured over shear rates ranged from 0 to 500 s^{-1}. In each case, the continuous shear properties of at least three replicates were determined.

2.7 Oscillatory rheometry

With the aim of determining the viscoelastic properties of the samples, firstly, oscillatory rheometry of all poloxamer-based thermogelling formulations was performed in oscillation mode, using the controlled stress rheometer described above (MARS II, Haake Thermo Fisher Scientific Inc., Germany) and, the same cone-plate (60 mm, separated by a fixed distance of 0.052 mm), at 37 ± 0.1 °C. The samples were carefully applied to the plate, as already described. After linear viscoelastic region determination of each formulation, the frequency sweep analysis was evaluated from 0.1 to 10.0 Hz. Thus, the storage modulus (G') was calculated using RheoWin 4.10.0000 (Haakes) software. In each case, at least three replicates were evaluated (de Araújo Pereira et al., 2013; Jones et al., 2009).

Then, the possible interaction between poloxamer and Carbopol® 971P, Carbopol® 974P or polycarbophil was investigated by the difference between the dynamic modulus of the polymeric blends and the theoretical value of the modulus obtained by summation of the individual parts (Hemphill et al., 1993; Jones et al., 2009). Calculation of the interaction parameter for the binary mixtures was determined using the storage modulus values at 10.0 Hz of oscillatory frequency following the equation 9.

$$\Delta G' = G'_{\text{mixture}} - (G'_{\text{poloxamer 407}} + G'_{\text{carbomer or polycarbophil)}}) \quad (9)$$

2.8. Determination of gelation temperature ($T_{sol/gel}$)

Gelation temperatures of the thermogelling systems were determined as previously described (De Souza Ferreira et al., 2017). In oscillatory mode, with temperature ramp, using the same cone-plate previously described (60 mm). The determination of $T_{sol/gel}$ of

each formulation were performed after determination of the linear viscoelastic region at 5 °C and 60 °C. A temperature sweep analysis was performed over the temperature at 5–60 °C range with defined frequency (1.0 Hz), and rate of heating 10 °C/min using a controlled stress (resident within the linear viscoelastic region). G', G″, η' and tan δ were calculated using RheoWin 4.10.0000 (Haakes) software. The temperature at which the elastic modulus was halfway between the values for the solution, and for the gel was called $T_{sol/gel}$. $T_{sol/gel}$ was calculated for all binary system in which dynamic viscosity increased with increasing temperature and at least three replicate samples were evaluated in all cases (Andrews et al., 2009; Bruschi et al., 2007; de Araújo Pereira et al., 2013; Edsman et al., 1998).

2.8 Adhesion testing

2.8.1 Detachment test

The adhesive properties of the hydrogels were assessed using a TA.XT Plus texture analyser (Stable Micro Systems, UK). The hydrated 20 mol% AGA and HEMA hydrogels were kept immersed in deionised water (water bath) and equilibrated at 37 ±1 ºC for 0.5 h. Prior to measurements, the polypropylene vials containing hydrogels were cut away with a saw so that an 8 mm diameter cylinder of gel extended from the vial by approximately 2 mm. Poloxamer 407 20% (w/w) hydrogel and poloxamer 407/ Carbopol® 971P, Carbopol® 974P or polycarbophil thermogelling systems were kept immersed in a water bath at 37 ±1 ºC for 0.5 h and then, placed up to a hot plate which was equilibrated at 37 ±1 ºC on the texture analyser.

The synthetic hydrogels or the animal mucosal tissue were attached to a mobile probe (cylindrical, P/6) using double sided adhesive tape. The probe was lowered at a speed of

1 mm/s until it reached the mucoadhesive hydrogel surface with a determinate contact force. The contact force of 0.03 N was applied to the poloxamer 407 20% (w/w) and poloxamer 407/Carbopol® 971P formulations, while to poloxamer 407/Carbopol® 974P and poloxamer 407/polycarbophil formulations a contact force of 0.002 N was applied with the aim of to keep the substrate just in contact with the hydrogel surface. A force larger than this drove the sample into the system so that contact was made on two faces. Substrate and formulation where kept in contact for 30 seconds, then the probe was withdrawn at a rate of 10.0 mm/s until complete detachment of the mucoadhesive hydrogels from the synthetic hydrogels or animal mucosal tissue was observed. The maximum force of detachment and the work of adhesion (the area under the force/distance curve) were determined using Texture Exponent 32 software (Stable Micro Systems, UK). All measurements were performed at least 6 times and the adhesion parameters calculated as mean values ± standard deviation.

Adhesion of mucoadhesive hydrogels to animal mucosal tissues were studied using porcine buccal mucosa which were obtained from MedMeat (UK). These tissues were collected immediately after the slaughter of animals and were stored frozen at -20 ºC. Before testing, the mucosal tissues were defrosted in water at 35–37 ºC and the mucosa was excised from the cheek using a scalpel. In order to achieve the same surface area as the synthetic hydrogels during adhesion testing, mucosa was placed into a polypropylene sample vial and held in place with a screw-thread polypropylene cap with an 8mm diameter bore so that only an 8 mm diameter circle of mucosa came into contact with the thermogelling formulations. Detachment force and work adhesion was treated with two-way ANOVA (multiple comparisons), using Bonferroni post-hoc test. $P < 0.05$ was taken to be statistically significant.

2.8.2 Retention testing

Retention was studied using a flow-through system developed in-house (Cave et al, 2012). The system consists of a channel containing a testing substrate (either ex vivo mucosa, 'mucosa-mimetic' or PTFE), over which a syringe-pump washes PBS. This system is then maintained at 37 °C within an incubator. FITC-dextran was added to thermogelling preparations at 1 mg/g to allow for fluorescence imaging. FITC-dextran-labelled formulations (20 μL) were then pipetted onto the testing substrate and allowed to warm over 2 minutes. This time was sufficient to allow for gelation to occur, as tested by inversion of substrate. The testing substrate was then imaged using a Leica MZ10F fluorescence stereomicroscope, equipped with a GFP filter set and monochrome camera, using an exposure time of either 11, 40 or 211 μs for HEMA mucosa-mimetic hydrogel, mucosa tissue and AGA mucosa-mimetic hydrogel, respectively. The PBS buffer eluent was then flowed over the testing substrate (4 mL/min), and images were taken at 1, 5, 10, and 15 mL elution volume. The quantity of polymer remaining on the surface of the testing substrate was then assessed using ImageJ. Briefly, the region on which the fluorescent polymers were pipetted was selected, and the brightness of the pixels measured. This brightness was then measured at the remaining time points, and the % fluorescence calculated with respect to the starting brightness value. Retention data was treated with two-way ANOVA (multiple comparisons), using Bonferroni post-hoc test. $P < 0.05$ was taken to be statistically significant.

3. Results and Discussion

3.1 Synthesis and characterization of mucosa-mimetic hydrogels

Hydrogels of HEMA and 20 mol% AGA were produced in hydroalcoholic solution using free-radical polymerisation with a water-soluble cross-linker, MBA. ATR-FTIR

spectroscopy demonstrated that the HEMA hydrogel contained characteristic absorbances related to the HEMA monomer, such as the ester carbonyl stretch at 1700 cm^{-1} and broad alcohol vibration at ~ 3420 cm^{-1}, with no residual monomer, as evidenced by the absence of a C=C absorbance at ~1640 cm^{-1}. In addition to the HEMA absorbances, AGA had peaks at 1650 and 1560 cm^{-1}, related to the amine linking the sugar ring to the polymer backbone. These spectra are in accordance with those previously reported (Cook et al., 2015). In this study, 20 mol% AGA was produced as a reported "mucosa-mimetic" material, whilst HEMA will act as a control to indicate whether interactions with semi-solid dosage forms are identical for all hydrogels.

[FIGURE 1 HERE]

Figure 1. ATR-FTIR spectra of 100 % HEMA (green) and HEMA:AGA (80:20 mol%) (blue) hydrogels after drying.

In addition to spectroscopic analysis, the swelling properties of the two hydrogels were measured. ESD values are an indicator of the magnitude by which the hydrogel's weight increases upon contact with water, ESV values indicate volume changes associated with swelling until equilibrium. In order to calculate ESV values, dried samples of hydrogels were analysed by pycnometry, which gives values of density. ESD values presented in table 2 are comparable to previously published data (Hall et al., 2011), ESV values indicate that 20 mol% AGA hydrogel swelled to a greater extent than 100 mol % HEMA hydrogels. This is possibly a result of the large number of hydrogen-bonding groups on AGA, which may solvate to a greater extent than the HEMA pendant groups. The differences in equilibrium swelling volumes are likely to result in differences in entanglements and interaction between dosage form and gel due

to different mesh sizes and polymer volume fractions. Mesh size increase with ESV, whilst polymer volume fractions decrease (Thomas et al., 2016).

Table 2. Swelling parameters and densities of 20 mol% AGA and HEMA hydrogels.

Sample	Parameters		
	Equilibrium swelling degree	Density, g/mL[a]	Equilibrium swelling volume
100 mol% HEMA	3.35 ± 0.59	1.79 ± 0.03	6.12 ± 0.38
20 mol% AGA[b]	3.80 ± 0.02	1.64 ± 0.16	7.25 ± 0.02

[a] of dried mass

[b] Difference in values for 20 mol% AGA and 100 mol% HEMA are statistically significant using two-tailed T-testing ($p < 0.01$).

3.2 Production of thermogelling formulations

In order to determine whether 20 mol% AGA was capable of mimicking buccal mucosa in the mucoadhesion testing of semi-solids, four thermogelling formulations were prepared. These formulations were based on poloxamer P407, which undergoes a sol-gel transition upon warming. Cross-linked poly(acrylic acid) derivatives were incorporated into poloxamer P407 with the intention of producing a range of formulations with different rheological and chemical properties. These formulations are based on previously reported thermogelling systems (De Souza Ferreira et al., 2015; Jones et al., 2009). The composition and rheological properties of the thermogelling formulations are shown in table 3. The first formulation is 20 % poloxamer P407, which

undergoes a sol-gel transition at 29.7 ± 0.6 °C, as determined by rheological method. Formulations F1, F2, and F3 include cross-linked poly(acrylic acids), giving a diverse range of rheological properties.

The rheological properties of these formulations have been investigated previously, and are reported herein to discuss differences in formulations (Bruschi et al., 2007; de Araújo Pereira et al., 2013; Jones et al., 2009). At all formulations, a shear-thinning behaviour (pseudoplastic flow), with yield value and hysteresis area was observed. In the most of cases the hysteresis area was characteristic of rheopectic material, which have the down-curve coming back above the up-curve and, this profile is quite common in binary polymeric systems at 37 °C (De Souza Ferreira et al., 2015; Jones et al., 2009). The addition of poly(acrylic acid) derivative decreased the consistency index of the systems when compared to the formulation containing just poloxamer (Table 3). The F2 demonstrated higher consistency index, since this poly(acrylic acid) derived – Carbopol® 974P – has cross-link density larger than the others cross-linked poly(acrylic acid) type. On the other hand, the Carbopol® 971P has lower cross-linking density, therefore, it showed a low consistency index. Moreover, in flow rheology, greater yield values were detected for most of formulations, at 37 °C, as expected. Commonly, the yield value of the carbomers, has indicated an improvement of retention time of the blends containing bioadhesive and thermoresponsive polymers in the application site (De Souza Ferreira et al., 2015).

According to the magnitude of the elastic moduli, in the oscillatory rheometry, the interaction parameter was derived. The rheological synergy, between poloxamer and poly(acrylic acid)s derived, provides evidence of adhesive interactions between them. In this sense, F1, F2 and F3 formulations, demonstrated strong interaction between the two

polymers. Thus, evidencing, beyond secondary bonds, the hydrogen bonds between carboxyl groups, which are widely distributed in acrylic acid chain, and hydroxyl groups of poloxamer (Jones et al., 2009). As already observed, in these concentrations (20/0.20; 15/0.25 and 15/0.25 %) the formulations containing respectively Carbopol® 971P, Carbopol® 974P and polycarbophil, have demonstrated better interaction parameter (at 37 °C). This is consistent with the greater amount of poloxamer in micellar form, which can be available to form hydrogen bonds with carboxylic groups of poly(acrylic acid) (De Souza Ferreira et al., 2015; Khutoryanskiy and Staikos, 2009).

Moreover, using the increase of viscosity and elastic moduli (G'), in oscillatory rheology analysis, the gelation transition temperature was determined. As known, poloxamer P407 is a thermoresponsive polymer and monopolymeric systems exhibit gelation temperature ($T_{sol/gel}$) (Jones et al., 2009). Furthermore, when Carbopol® 974P (F2) and polycarbophil (F3) were used, the $T_{sol/gel}$ increases, since the addition of other polymers can interfere in the micelles formation and change the gelation temperature. However, considering the suitable range of $T_{sol/gel}$ from 25 °C to 37 °C, all formulations proved to be appropriate to mucosal application, becoming gel at body temperature (Bruschi et al., 2007; Gratieri et al., 2010; Yun Chang et al., 2002). These formulations also represent a diverse group of semi-solids with different viscosities, consistencies, flow behaviours and gelation temperatures. This diversity is important in validating the mucosa-mimetic material.

Table 3. Composition and rheological properties of thermogelling formulations.

Sample ID	Poloxamer P407 conc. (% w/w)	PAA type, conc. (% w/w)	Viscosity at 32 s^{-1} [d] (Pa.s)	Consistency index (k)[b,d] (Pa.s)	Flow behavior index (n)[b,d]	Yield stress[b,d] (Pa)	Hysteresis area[b,d] (Pa.s)	Tsol/gel (°C)	Interaction parameter[b,c] (Pa)
Poloxamer[a]	20	N/A	12.530 ± 0.062	196.10 ± 12.27	0.14 ± 0.01	220.35 ± 4.05	-22991.67 ± 2464.22	28.67 ± 0.58	N/A
F1[a]	20	Carbopol 971P, 0.20	26.277 ± 2.908	43.15 ± 0.50	0.85 ± 0.00	836.77 ± 5.25	857500.00 ± 7424.62	27.88 ± 0.06	1944.73 ± 381.93
F2	15	Carbopol 974P, 0.25	6.171 ± 0.331	139.40 ± 6.01	0.256 ± 0.005	120.61 ± 0.12	-17520.00 ± 8553.99	36.04 ± 0.06	2509.33 ± 215.85
F3[b]	15	Polycarbophil, 0.25	6.935 ± 0.257	60.963 ± 3.307	0.359 ± 0.006	230.067 ± 4.484	-35990.00 ± 5507.62	36.42 ± 0.02	1927.03 ± 93.85

[a](De Souza Ferreira et al., 2015)

[b](De Souza Ferreira et al., 2017)

[c]Interaction parameter between poloxamer and cross-linked poly(acrylic acid) by storage modulus (G') at 10 Hz

[d] at 37°C

Flow rheograms for each formulation are shown in Figure 2. All formulations show responses to shear which are typical for pseudoplastic semi-solids. This is also reflected in their reported flow-behaviour indices (Table 3), which all had values lower than one, calculated to fitting to a power-law model. Marked differences in viscosities are apparent between the formulations (Figure S1, ESI).

Formulations were also prepared with 1 mg/g FITC-dextran (10 kDa) incorporated, in order to conduct flow-through experiments (Figure 2, orange). With the aim of observing the structural changes or marker interactions with the binary systems, the flow rheology was studied. There were no apparent changes in flow rheology profile when FITC-dextran was incorporated into pluronic and F1 formulations, but there were observable increases in viscosity for formulations F2 and F3. This is consistent with increased physical cross-linking in these systems, which are more influenced by the presence of the marker. Despite an increase in the viscosity may modify the retention

time of the formulations during the flow-through experiments, the viscosity has not been greatly changed between formulations with and without FITC-dextran and, interferences were not observed in the results.

[FIGURE 2 HERE]

Figure 2. Flow rheograms for a) pluronic, b) F1, c) F2, and d) F3 formulations with (orange circles) and without (black circles) 1 mg/g FITC-dextran (10 kDa). Data presented as mean ± standard deviation (N = 3).

3.3 Mucoadhesion testing

The adhesion of thermogelling formulations was determined using two methods, texture analysis and a flow-through system. Texture analysis measures the force required, or work needed, to remove a dosage form from a substrate (e.g. mucosal membrane), and is the standard method of testing the mucoadhesion of solid dosage forms. In order to measure adhesion of semi-solid dosage forms by this method, modification had to be made to standard procedures. 100 mol% HEMA and 20 mol% AGA hydrogels were formed in polypropylene vials, which were then cut back so that the hydrogel extended by approximately 2 mm from the end of the vial. This gave a flat surface of hydrogel on which to measure adhesion. The vial was then attached to the probe of a texture analyser (Figure 3a). This allowed the thermogelling systems to be maintained on a hot-plate at the base of the texture analyser whilst the adhesion of hydrogels to their surface was determined (Figure 3b). This gave a force-distance relationship from which either the maximum force required to remove the dosage form or the area under the force-time curve could be determined, giving the force and work of adhesion, respectively (figure 2c).

[FIGURE 3 HERE]

Figure 3. Modifications made to a texture analyser allow for testing substrates (hydrogels, mucosa, and polypropylene) to be pressed against thermogelling semisolid formulations (a). Removal of the testing substrate from the formulations (b) gives a force-time curve from which values of force and work of adhesion can be measured (c).

The adhesion of thermogelling formulations to hydrogels, mucosa, and a control of polypropylene is shown in Figure 4. The mucosa-mimetic 20 mol% AGA hydrogel and buccal mucosa gave mean values of adhesion which were closest to buccal mucosa, giving values of adhesion which were not statistically significant in two out of four formulations, for work and force of adhesion, as determined by two-way ANOVA. Kruskal–Wallis testing gave no significant differences between 20 mol% AGA and mucosa, but experimental replicates were not sufficient to determine conclusively whether non-parametric statistics were required. Generally, values of adhesion were higher in the control hydrogel, 100 mol% HEMA, than the mucosa-mimetic 20 mol% AGA hydrogel and the buccal mucosa. Average % deviations from mean mucosa values across all formulations for buccal mucosa, 20 mol% AGA, 100 mol% HEMA, and polypropylene, were 5 %, 23 %, 79 %, and 52 %, respectively.

[FIGURE 4 HERE]

Figure 4. Force (a) and work (b) of adhesion of thermogelling formulations to hydrogels, buccal mucosa, and polypropylene, as determined by texture analysis. Data represented as mean ± standard deviation (N = 6). “ns” designates no statistical significance, * indicates $p < 0.05$, and *** indicates $p < 0.001$, using two-way ANOVA with Bonferroni post-hoc.

The metric by which adhesion is measured from force-displacement curves during texture analysis impacted on results. Whilst poloxamer samples gave comparable rank-orders of adhesion using either work or force, F1-3 gave different rank orders depending on which value was used. The force of adhesion reflects the force required to overcome the adhesive forces between testing substrate and the thermogelling formulations, as fracture always occurred at the interface between the two materials. The work of adhesion is often the preferred metric for measuring mucoadhesion, as it is seen as being more relevant to the "bedside" application. When determining the work of adhesion, adhesive interactions are confounded by cohesive forces within testing substrate and formulation, and thus the elasticity and plasticity of the materials will be reflected in this value.

Focusing on the values of force of adhesion allows for discussion of the adhesive forces alone. The adhesion of 100 mol% HEMA hydrogel to all formulations is higher than, or equal to, the adhesion of those formulations to the mucosa-mimetic 20 mol% AGA hydrogel. This can be rationalized by consideration of the physicochemical properties of the hydrogels. 100 mol% HEMA contains fewer functional groups capable of hydrogen bonding than the 20 mol% AGA hydrogel per monomer unit, and has lower degrees of swelling (Table 2). The mucoadhesion of poloxamer and PAA is often attributed to the formation of hydrogen bonds between polymer and secretory mucins on the surface of tissue. As adhesion to 100 mol% HEMA is higher than 20 mol% AGA it is not likely that adhesion can be simply attributed to hydrogen bonding; there may be additionally complementary chemical interactions, such as van der Waals forces or the so-called "hydrophobic effect", wherein the poor solvation of hydrophobic moieties promotes

their interaction (Smart, 2005). An additional factor affecting adhesion to the hydrogels is the water content, reflected in equilibrium swelling values. Differences in swelling values likely reflect differences in hydrogel mesh sizes, which in turn will affect the interpenetration and entanglement of polymer across the formulation-hydrogel interface (Sahlin and Peppas, 1997). Additionally, the high water content of the hydrogels may have a lubricative effect, leading to a reduction in adhesion to the more swollen 20 mol% AGA hydrogel. This would also effectively reduce the concentration of polymer in the hydrogel, lowering number of monomer units with which interaction can occur per unit area. It is believed that 20 mol% AGA is able to mimic buccal mucosa due to the chemical similarity of the AGA monomer with oligosaccharide side-chains adorning the mucin glycoproteins secreted on the mucosal tissue. It is likely that physical properties, such as swelling degrees, also play a role in this mimicry (Hall et al., 2011).

Adhesion of formulations to polypropylene was generally equal to or greater than adhesion to 20 mol% AGA and buccal mucosa. Formulations cannot adhere to polypropylene via entanglement or by polar interactions, so it is believed that this adhesion is a result of the absence of water from the polypropylene. The formulations are able to wet the surface of polypropylene sufficiently to allow adhesion without the lubricative effect of water. Polypropylene has been used to evaluate the mucoadhesion of wetted polymer films, but was not found to be a good mimic of mucosa in this study (Choi et al., 1999).

The retention of thermogelling semi-solids on HEMA, 20 mol% AGA, buccal mucosa, and PTFE, a “non-stick” control, was also determined using a flow-through method previously developed (Figure 5) (Cave et al., 2012; Cook et al., 2015; Irmukhametova et

al., 2011; Withers et al., 2013). There was no statistically significant difference in retention values between 20 mol% AGA and buccal mucosa for the poloxamer, F2, and F3 formulations at any washing volume (Figure 4a, 4c, and 4d, respectively). However, there were significant differences at 1 and 5 mL volumes, using formulation F1. All formulations were retained most poorly on PTFE, indicating that the retention is not simply the result of the rheology of the semi-solids, dissolution of the gel, or release of FITC-dextran from the formulation.

[FIGURE 5 HERE]

Figure 5. The retention of FITC-dextran labelled pluronic (a), F1 (b), F2 (c), and F3 (d) formulations on 100 mol% HEMA, 20 mol% AGA, buccal mucosa, and PTFE, using a flow-through method. Data presented as mean ± standard deviation (N = 3). "ns" designates no statistical significance, * indicates $p < 0.05$, and ** indicates $p < 0.01$, using two-way ANOVA with Bonferroni post-hoc.

The adhesion of semi-solids to surfaces is a complex phenomenon, arising from several different interactions (Figure 6) (Smart, 2005). Polymer-solvent interactions dictate, in part, dissolution of the dosage form, as well as the favourability of interaction with the surface. Cohesive interactions within the dosage form affect its rheology, which in turn modulates retention. Polymer-substrate interactions allow for adhesive bonding between gel and surface, and stabilize polymer-polymer entanglements where polymer mobility is possible. In the flow-through model (figure 5), formulations have greater adhesion to hydrogel and mucosa than to PTFE. This is likely a result of polymer entanglement with the surfaces, and concomitant formation of non-covalent bonds. Both 20 mol% AGA and 100 mol% HEMA are good mimics of buccal tissue using this method, with 20 mol% AGA performing marginally better. Adhesion to 20 mol% AGA is consistently

lower than to 100 mol% HEMA, which may be attributed to greater swelling degrees, or an increased hydrophilicity modulating formulation-hydrogel interactions. It is conceivable that the lower hydrophilicity of 100 mol% HEMA indicates that hydrophobic interactions improve mucoadhesion, but this is confounded by factors such as competition for hydrogen-bonding groups with water.

[FIGURE 6 HERE]

Figure 6. An overview of the factors dictating the mucoadhesion of semi-solids.

4. Concluding remarks

Reducing the use of animals in research is a key goal of many researchers worldwide. The development of mucoadhesive formulations typically requires the use of *ex vivo* animal tissue, which could be reduced were there a validated synthetic substrate capable of mimicking mucosa. The adhesion of thermogelling semi-solid formulations to 20 mol% AGA, a potential mucosa-mimetic material, 100 mol% HEMA, buccal mucosa and controls has been studied with the aim of supporting the use of 20 mol% AGA hydrogels as mucosa-mimetic materials. A 20 mol% AGA hydrogel was shown to be a good surrogate for buccal mucosa using two methods of studying mucoadhesion. Controls also allow for study of mucoadhesive interactions, indicating that mucoadhesion occurs in these dosage forms largely as a result of polymer entanglement and polymer-surface interactions, and is not simply governed by the rheology of the dosage forms.

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Figure 1. ATR-FTIR spectra of 100 % HEMA (green) and HEMA:AGA (80:20 mol%) (blue) hydrogels after drying.

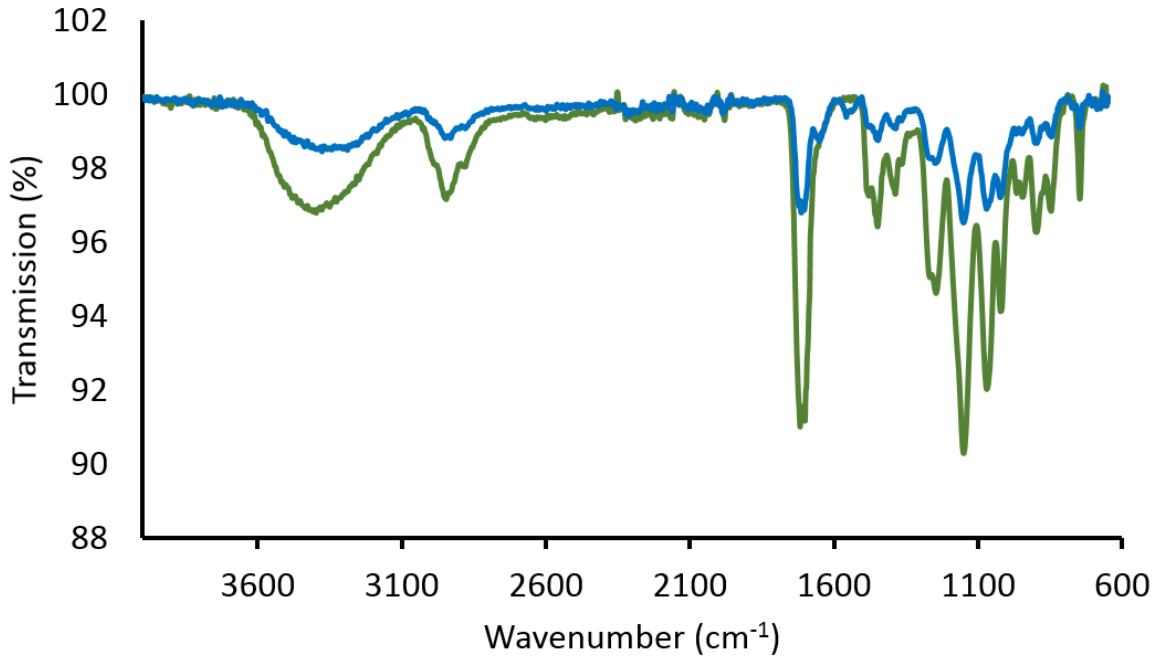
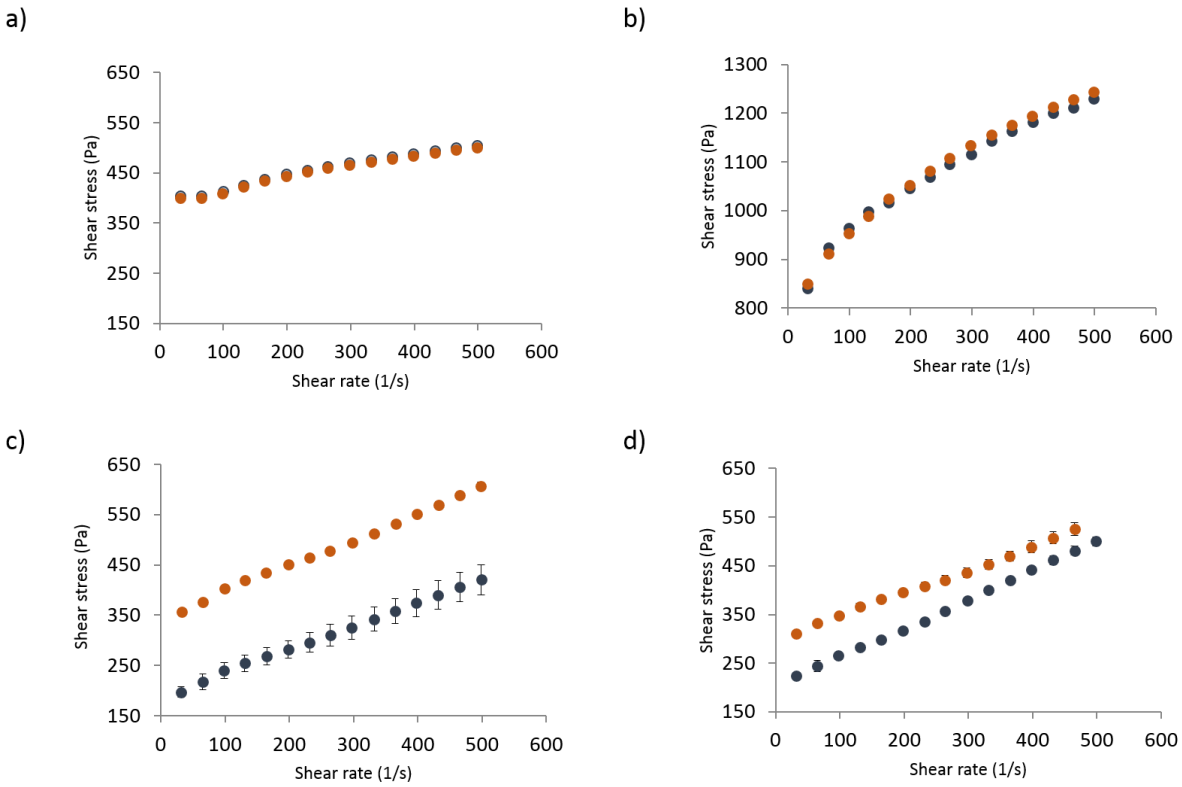


Figure 2. Flow rheograms for a) pluronic, b) F1, c) F2, and d) F3 formulations with (orange circles) and without (black circles) 1 mg/g FITC-dextran (10 kDa). Data presented as mean ± standard deviation (N = 3).



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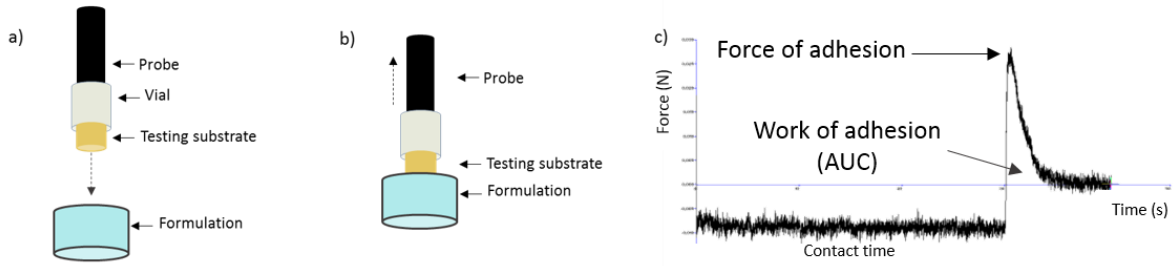

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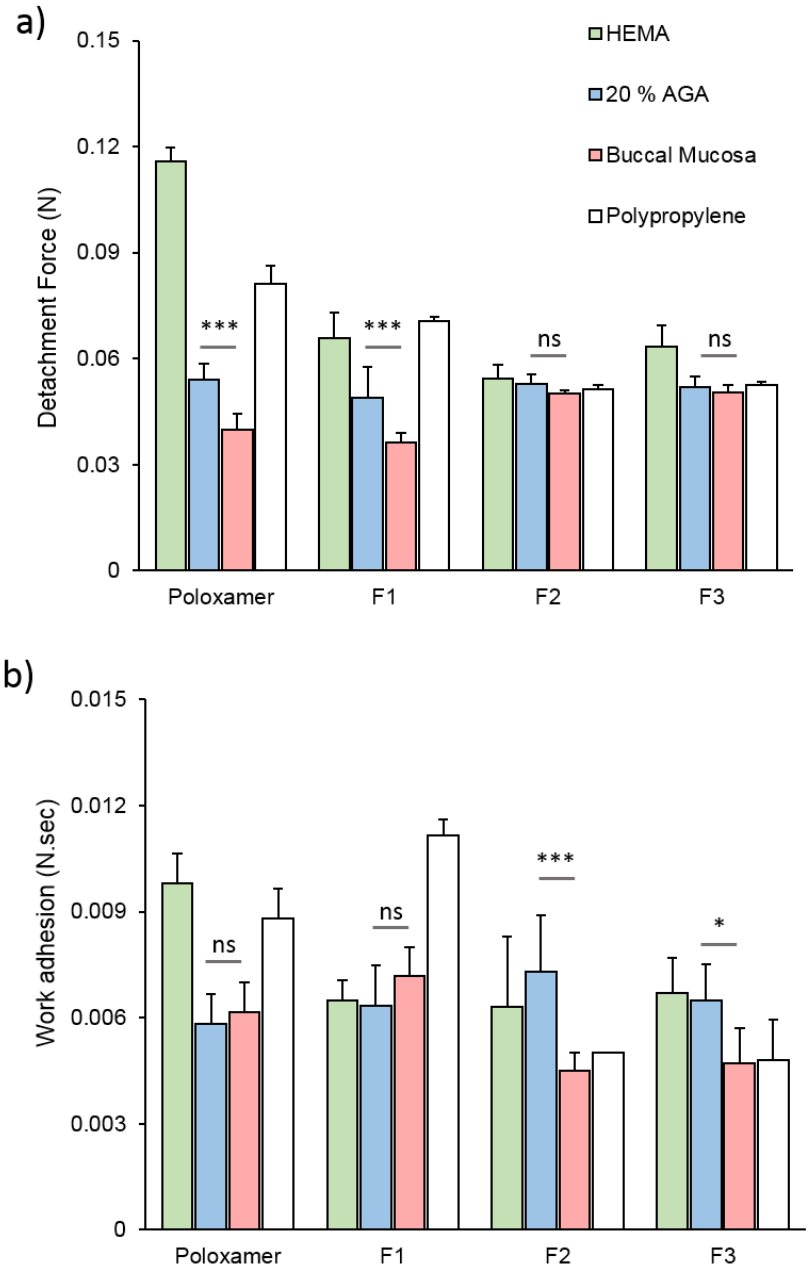

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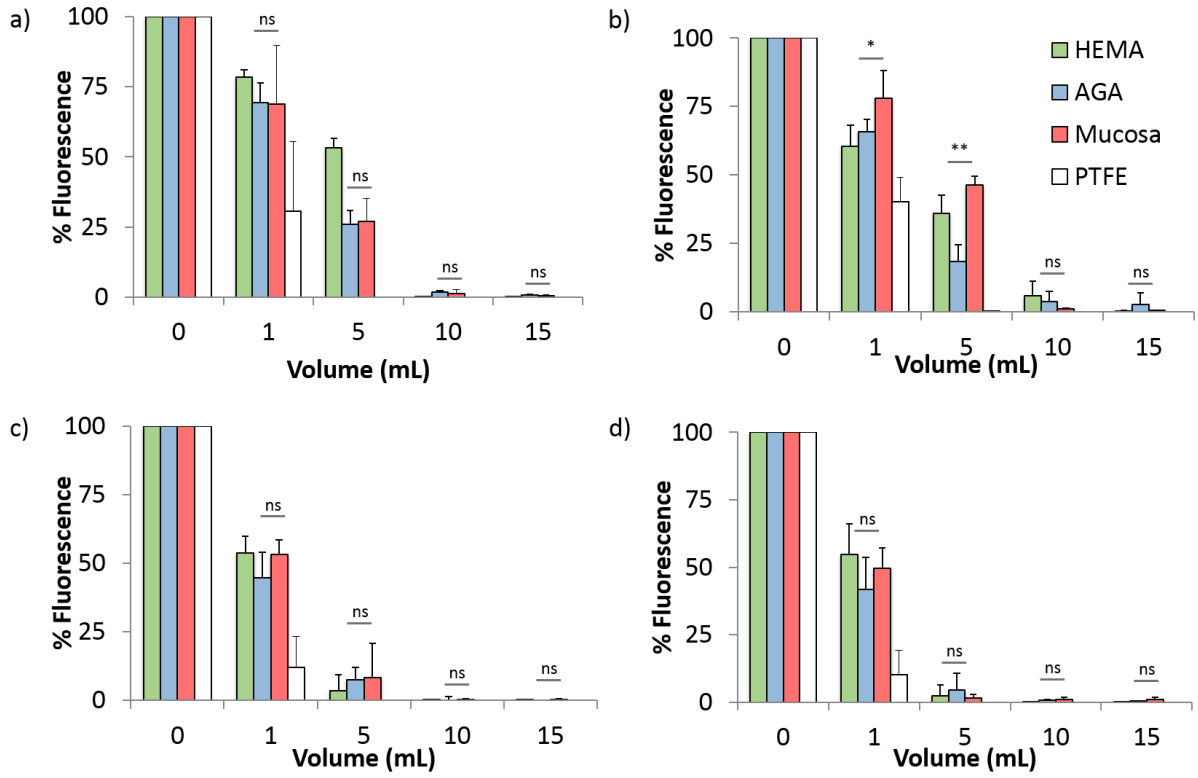

Figure 6. An overview of the factors dictating the mucoadhesion of semi-solids.