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Evaluation of *in vitro* mucoadhesiveness and texture profile analysis of doxycycline *in situ* hydrogels

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Received July 13, 2019, accepted September 23, 2019

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Pharmazie 75: 7-12 (2020) *doi: 10.1691/ph.2020.9122*

Delivery of active ingredients to the oral mucosa from topically applied formulations reduces side effects from systemic administration and enhances the treatment efficiency. The challenge however, is to maintain the formulation at the administration site due to rapid salivary flow and mechanical movements of the mouth. Therefore, addition of mucoadhesive polymers could aid in enhancing the formulation residence time by increasing the mucoadhesion capacity but this effect is negligible especially if low ratio of mucoadhesive polymers are added to the formulation. Different mucoadhesive polymers at 0.5% w/w (either single or combination of two polymers) were added to the hydrogels and tested for mucoadhesion capacity, tensile strengths, adhesiveness, cohesiveness, compressibility and hardness. 0.5% povidone showed significantly highest work of mucoadhesion, 0.5% Carbopol formulation showed least cohesiveness and 0.5% HPMC showed highest adhesiveness, but a formulation containing a combination of 0.25% HPMC and 0.25% povidone showed the ideal parameters among all the mucoadhesive polymers tested. The effect of increase in concentration of HPMC (0.5, 1, 1.5, 2%) showed linear relationship for work of mucoadhesion and tensile strengths whereas for TPA the values were non-linear. The drug release from the optimized polymer matrices was found to follow zero-order release profile and the mechanism was found to be super case-II transport relaxation release. The results of this study indicate the mucoadhesive polymers do not impact the tensile strengths (p=0.05), but the texture properties and work of mucoadhesion of the formulations can be significantly (p<0.05) altered by the choice of mucoadhesive component at 0.5%w/w, though not for all the polymers tested. The study provides scope to predict *in vivo* performance and helps optimize for localized delivery.

1. Introduction

Oral mucosal conditions can potentially be treated with topical formulations rather than a systemic route. It's relatively high permeability also allows for systemic delivery of drug molecules and, thereby, prevents the first pass effect and pre-systemic elimination with in gastrointestinal tract (Madhav et al. 2009). However, there are also drawbacks, since it is difficult to maintain the formulation at the application site due to salivary wash out effect, mucus blanket layer and movements of mouth, that may dislodge the formulation. The mucus layer is constantly replaced and an unstirred mucus layer present above it (Shakya et al. 2011), that can be helpful in formation of non-covalent bonds like ionic interactions and hydrogel bonds or physical entanglements between the mucin and mucoadhesive polymers (Bernkop-Schnürch 2005). The mucoadhesive polymers are insufficient in most formulations in enhancing the residence time in gatrointestinal tract (Bernkop-Schnürch 2005) and especially if the ratio of mucoadhesive polymers is low. The mucoadhesive polymers that are compatible with the stability of the active component, doxycycline, were added and evaluated for their effect on mucoadhesion and texture properties of the formulation (Patlolla et al. 2019). By Prolonged topical delivery of doxycycline in matrix metalloproteinases (MMP) inhibitory concentrations, several oromucosal conditions could potentially be treated, such as aphthous ulcers (Skulason et al. 2009b), cold sores (in combination with monocaprin) (Skulason et al. 2012), oral lichen planus (Zhou et al. 2001), oral mucositis (Al-Azri et al. 2015), and periodontitis (Golub et al. 1995; Hannas et al. 2007). The MMPs present in dentine matrix or saliva (Tjaderhane et al. 1998) may also have a role in dentine matrix degradation in dentinal caries (Hannas et al. 2007; Martin-De Las Heras et al. 2000; Sulkala et al. 2002). Increased levels and activity of MMPs were also detected in saliva of patients with Sjögren's syndrome, the condition also linked to further promote caries. (Hannas et al. 2007; Konttinen et al. 1998).

Hydrogels are three-dimensional scaffolds of polymers which are actually insoluble but swollen polymeric matrices that are capable of imbibing large amounts of aqueous media or body fluids (Kopecek 2002; Semmling et al. 2013). Their water-absorbing capacity is due to the hydrophilic functional groups attached to the polymer backbone and their insolubility is due to the cross links between network chains. The hydrogels degree of flexibility is similar to natural tissue due to their high water content, which helps to mimic the natural environment at the application site (Ahmed 2015). Poloxamers are synthetic non-ionic triblock copolymers, comprised of long chains of poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO-PPO-PEO). These compounds show thermo-reversible properties in aqueous solutions and have capability to undergo reversible gelation at physiological temperature and pH. The addition of mucoadhesive polymer enhances the formulation residence time and thereby maximizes the therapeutic efficiency.

The aim was to study the role of different types of mucoadhesive polymers (Salamat-Miller et al. 2005) in the optimization of doxycycline hydrogel formulation residence time and patient compliance. Though the incorporated ratio of mucoadhesive polymer is small (0.5% w/w), its effect on formulation *in vitro* mucoadhesion, texture profile analysis (TPA) and viscosities was investigated. At present, there is a lack of oral mucosal-specific treatments or

formulations and some of the formulations in use are meant for dermatological application (Paderni et al. 2012; Sankar et al. 2011). The therapeutic efficiency of such products will be compromised, so the aim was to optimize the formulations parameters keeping them clinically relevant for the oral mucosa.

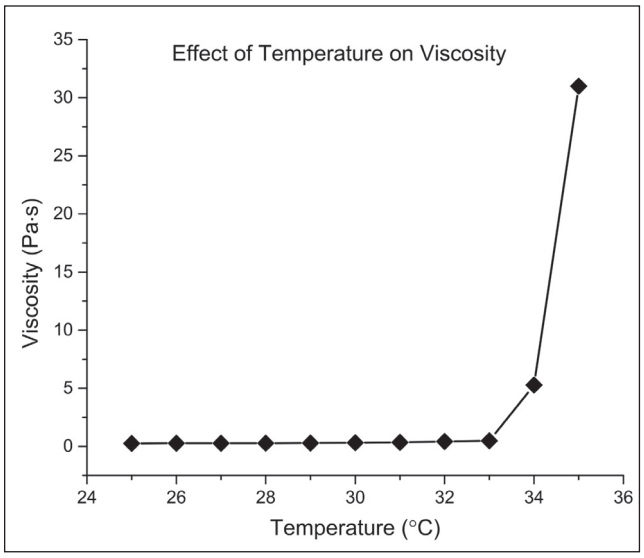
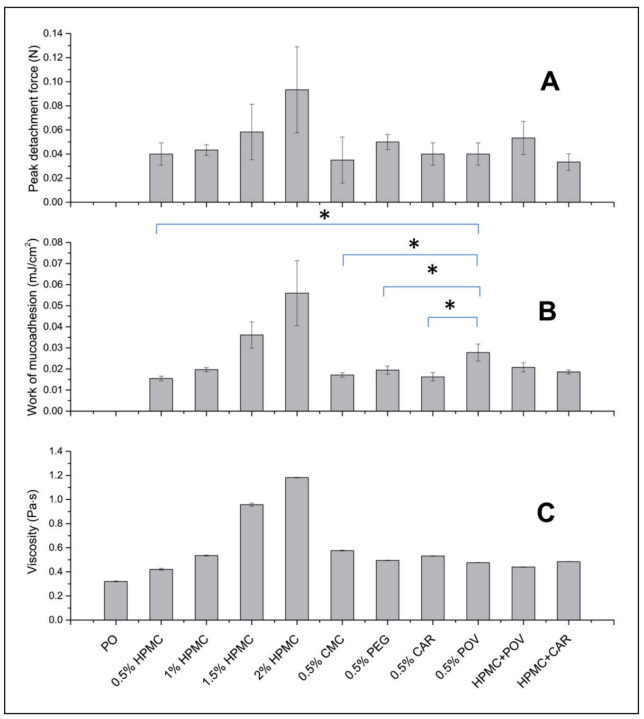


Fig. 1: Example of the effect of gelation temperature on a hydrogel viscosity



*p<0.05, One way ANOVA Turkey HSD *post hoc* test

Fig. 2: Comparison of peak detachment strength (tensile strength), work of mucoadhesion and viscosities 11 hydrogels containing different mucoadhesive polymers. PO = poloxamer 407 and188 only; HPMC = hydroxypropyl methyl cellulose; CMC = carboxymethyl cellulose, PEG = polyethylene glycol 6000, CAR = Carbopol 974P, POV = Povidone, HPMC+POV = 0.25% HPMC and 0.25% Povidone, HPMC+CAR = 0.25% HPMC and 0.25% Carbopol 974P

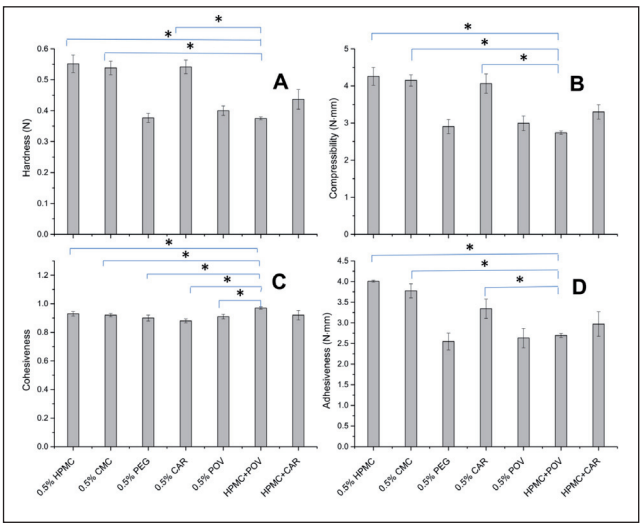
2. Investigations and results

2.1. Viscosity

The viscosities of the hydrogels were recorded as a function of shear rate and time. All the hydrogels were Newtonian i.e., the viscosity values did not tend to vary with shear stress rate or time. The effect of increase in temperature on viscosity for the hydrogel containing mucoadhesive polymers 0.25% HPMC + 0.25% Povidone was studied. The viscosity increased exponentially near to the gelation point as shown in Fig. 1. The effect of different mucoadhesive polymers on hydrogels (11 hydrogels, including hydrogel without any mucoadhesive component) viscosity at room temperature is shown in Fig. 2. The viscosity of hydrogels increased with the concentration of HPMC (Hydrogels A, B, C, D). Four hydrogel formulations containing 0.5% HPMC, 0.5% PEG, 0.5% povidone and 0.25% HPMC + 0.25% Povidone, showed viscosities around and below 0.4 Pa·s. Further hydrogel with 0.25%HPMC +0.25%Povidone was selected after comparing their tensile strengths and work of mucoadhesion values.

2.2. Mucoadhesion analysis

The increase in concentration of HPMC (Hydrogel A, B, C, D) showed good linearity for work of mucoadhesion (R^2=0.93) and tensile strengths (R^2=0.90). However, high concentration of HPMC (1.5 and 2%) did no longer behaved as *in situ* hydrogels, but gelled below room temperature. Seven hydrogels containing only 0.5% w/w of mucoadhesive component (single (Hydrogel A, E, F, G, I) or in combination (Hydrogel H, J)), were compared using ANOVA and Turkey HSD *post hoc* test and the ideal mucoadhesive polymers was selected after comparing their viscosities, area under the F-T curve (AUC), tensile strengths (peak detachment force) and work of mucoadhesion values. The 17% w/v crude mucin dissolved in water was equivalent to the native mucus layer concentration (Matthes et al. 1992). At p<0.05, 0.5% povidone showed significantly more work of mucoadhesion compared to hydrogels with single mucoadhesive component. 0.5% povidone hydrogel also showed higher area under F-T curve values (p<0.001) but relatively higher viscosity value and lower tensile strength, compared to 0.25% HPMC+ 0.25% povidone. Whereas for the tensile strength, 0.5% PEG-6000 apparently showed slightly higher force but the values were not significant (p=0.05). However, the mixture of 0.25% HPMC and 0.25% povidone apparently (Fig. 2) showed a synergy, having higher peak detachment force but the values were not significant (p=0.05). The hydrogel containing 0.25% HPMC + 0.25% Povidone, was selected from mucoadhesion and viscosity studies since it also showed less viscosity at room temperature and possessed high tensile strengths and optimal work of mucoadhesion values (Fig. 2).



*p<0.05, One way ANOVA Turkey HSD *post hoc* test

Fig. 3: Texture profile analysis where the comparison of hardness(A), compressibility (B), cohesiveness (C)and adhesiveness (D) are measured for hydrogels containing different mucoadhesive polymers: HPMC = hydroxypropyl methyl cellulose; CMC = carboxymethyl cellulose, PEG = polyethylene glycol 6000, CAR = Carbopol 974P, POV = Povidone, HPMC+POV = 0.25% HPMC and 0.25% Povidone, HPMC+CAR = 0.25% HPMC and 0.25% Carbopol 974P

Table: Poloxamers concentration and their effect on gelation temperature

S. No	Poloxamer 407 (%)	Poloxamer 188 (%)	Gelation temperature °C
1	18	10	No gelation up to 40 °C
2	19	10	38 ±1
3	20	10	36 ±1
4	21	10	33 ±1
5	21.25	10	32.3 ±1
6	22	10	31 ±1
7	22	11	32 ±1
8	22	3.5	20 ±1

2.3. Texture Profile Analysis (TPA)

The comparison of TPA of the 7 hydrogels (hydrogels, A, E, F, G, H, I, J) is presented in Fig. 3 for hardness, cohesiveness, compressibility and adhesiveness for five hydrogels containing 0.5% w/w mucoadhesive polymers and two hydrogels containing a mixture of two (also 0.5%). The formulation with 0.25% HPMC+ 0.25% povidone showed significantly lowest hardness (p<0.05) and compressibility (p<0.05) values. Increasing concentration of HPMC from 0.5% up to 2% showed an initial linear relationship but then behaved not linear, which might be due to increased viscosity from high concentrations of HPMC might have affected the gelation temperature of the poloxamers, which were supposed to undergo gelation near body temperature (Patlolla et al. 2019) (Table). The hydrogel containing 0.25% HPMC + 0.25% povidone showed significantly (p<0.05) highest cohesiveness value and the hydrogel with 0.5% Carbopol showed the least value. The hydrogel containing 0.5% HPMC showed significantly (p<0.001) highest adhesiveness, hardness and compressibility values.

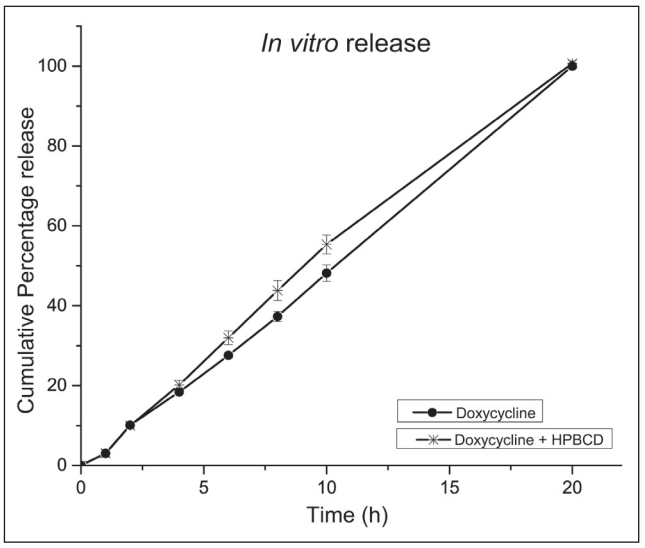


Fig. 4: Release of doxycycline alone or incorporated in (2-Hydroxypropyl)-β-cyclodextrin from a mucoadhesive polymer matrices containing 21% Poloxamer 407, 10% Poloxamer 188, 0.25% HPMC and 0.25% Povidone.

2.4. In vitro release studies

Doxycycline release profile alone and when incorporated in HPβCD, and formulated in a hydrogel manufactured from Poloxamer 407 (21%), Poloxamer 188 (10%) and 0,25% HPMC + 0,25% povidone are presented in Fig. 4. The release was significantly faster in the presence of HPβCD, although the overall release time was 20 h. From Fig. 5, it is evident that the hydrogels followed zero order kinetics in the presence and absence of HPβCD (R^2=0.99). Further the values were fitted in Korsmeyer-Peppas and good correlation values (R^2=0.99) were obtained. The (release exponent) n>1, for both the hydrogels (n=1.14 for doxycycline hydrogel and n=1.09 for doxycycline in combination with HPβCD) suggested that the drug release occurred through, super case-II transport relaxational release (Ofokansi et al. 2007).

3. Discussion

In situ hydrogels should attach to the oral mucosa with adequate attachment force and for this purpose suitable and clinically-relevant mucoadhesive polymers were incorporated into several hydrogels and tested. As the concentration of HPMC was increased in the hydrogels their tensile strengths also increased but the hydrogels lost their *in situ* gelling capacity, but gelled instead below room temperature. The polymers were also preferably selected from non-ionic, which were another important considered, keeping in view, the stability of the active compound, doxycycline (Patlolla e*t al.* 2019). As the hydrogels were *in situ* forming in nature, for the ease of administration and also to improve the patient compliance, they should ideally have low viscosity values at room temperature. The mechanical properties of the hydrogels (Jones et al. 1996a; Jones et al. 1996b) help in understanding the ease of applicability, improving the patient compatibility and improving spreading characteristics, product performance and the residence times of the formulations at the application site. These parameters may , therefore, predict the outcomes beforehand with a scope for improvements (Jones et al. 1997b). Conducting texture profile analysis and classification of parameters from such measurements were started by Szczesniak et al. (Bourne 1978; Szczesniak 1963; Szczesniak 1975), although TPA of hydrogels in their original form was first introduced by Jones et al. (Hurler et al. 2012; Jones et al. 1996a) and refined by several authors (Hurler et al. 2012; Oliveira Cardoso et al. 2017; Tan et al. 2000).

Ideally, the buccal formulations should possess low hardness and compressibility and yet high cohesiveness and adhesiveness (Tan et al. 2000). A high hardness decreases the residence time of the formulation at the site of application, so the formulations should be rightly balanced (Cevher et al. 2008; Sezer et al. 2008). Low hardness and compressibility value indicates minimum work required to transfer the formulation from the container on to the site of application or the oral mucosa (Tan et al. 2000). Here the formulations are *in situ* thermosensitive gels, so they will be liquid at room temperature. The TPA properties were only studied for these hydrogels at 37 °C. One formulation, containing a mixture of 0.25% HPMC+ 0.25% povidone showed the lowest hardness and compressibility indicating that once after gelation at the site of application, it would easily attach to the inflamed mucosal region without causing further exacerbation during application or spreading. This hydrogel also showed good internal gel strength to regain its structure during the mechanical dislodgement forces in the oral cavity. So, if a hydrogel is desired to have good residence times even after moderate dislodgement movements in the oral cavity then 0.25% HPMC+ 0.25% povidone would be a good choice.

Increase in concentration of HPMC (0.5, 1, 1.5 and 2%), showed linear relationship in mucoadhesion study whereas, for TPA study the relationship was non-linear and decreased with increase in concentration. The mucoadhesion study was a surface test where the probe touches the surface of hydrogel and measures the detachment force whereas in TPA the probe travels into the hydrogel and factors like increase in osmolality from increased polymer concentration, increase in viscosity and decrease in *in situ* gelling temperature might play role in this effect. Interestingly, the cohesiveness was not affected with the increasing concentrations of HPMC, which may be due to the low gelation temperature or increased viscosity. Though 0.5% HPMC showed highest adhesiveness, it showed the highest values for hardness and low cohesiveness. Having low viscosity, 0.5% HPMC did also possess high adhesiveness which may benefit several mucosal disease conditions where there is decreased mucin production, which in

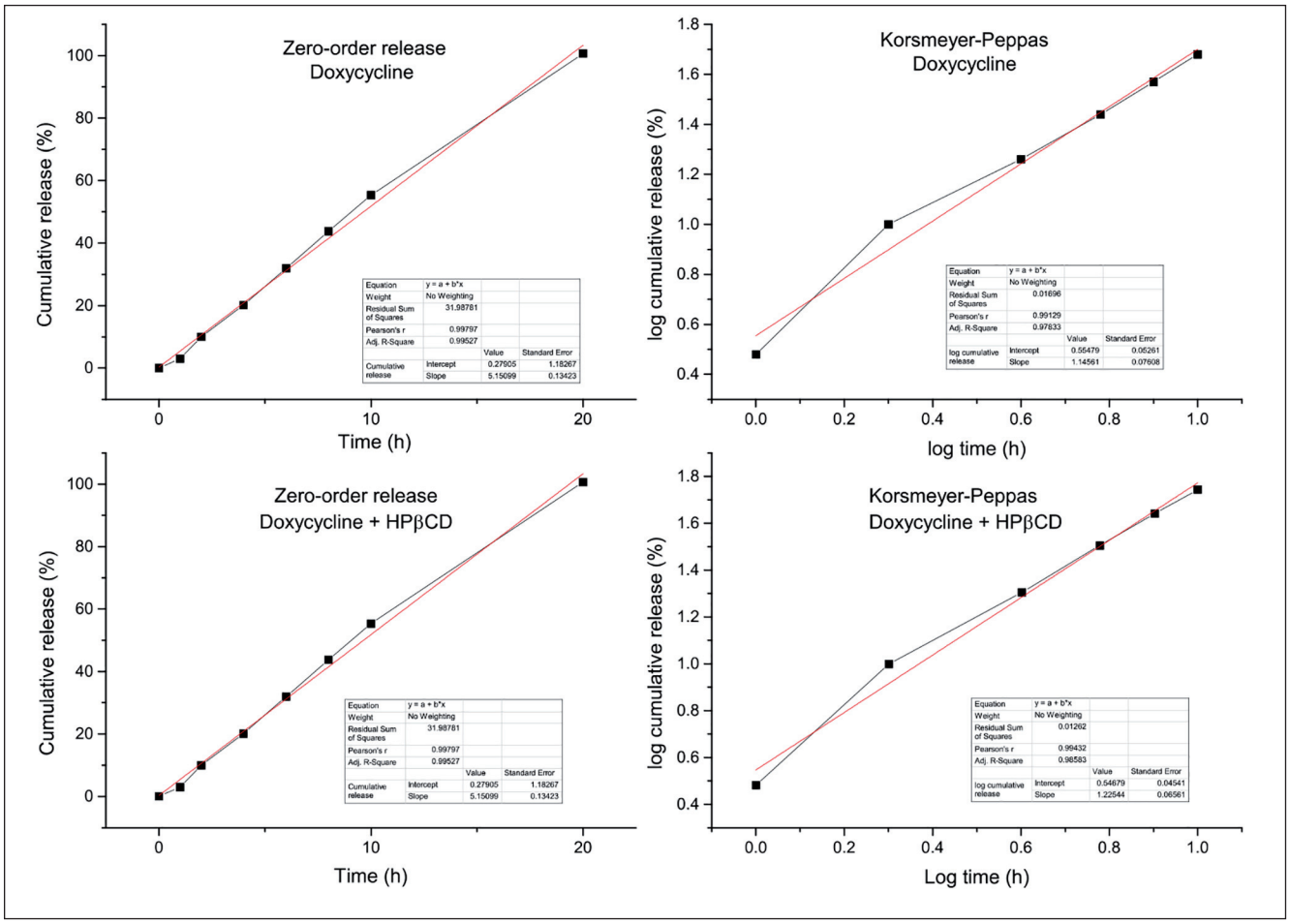


Fig. 5: Kinetic model fitting for drug release from hydrogel containing only doxycycline and doxycycline and (2-Hydroxypropyl)-β-cyclodextrin complex.

turn reduces the capability for mucoadhesive bonds between the polymer and mucin. High adhesiveness generally increases the stickiness of the formulation and hence has chances for attachment even in the absence of mucoadhesive bonds. High cohesiveness implies the good ability for structural reformation after the application which affects the product performance (Jones et al. 1997b, 1996b), but again the formulations here are *in situ* forming hydrogels which form after the thermosetting at the application site. This also indicates that they may better withstand the mechanical movements with in the oral cavity. Cohesiveness is dimensionless (Bassi da Silva et al. 2018; Jones et al. 1997a; Lau et al. 2000; Oliveira Cardoso et al. 2017; Sezer et al. 2008; Tan et al. 2000).

High adhesiveness indicates prolonged residence times at the application site (Tan et al. 2000) but might not correlate to the mucoadhesion values as they tend to be greatly influenced by the presence of mucoadhesive bonds (Jones et al. 1997b). The AUC values obtained for adhesiveness are negative (Huang et al. 2007; Hurler et al. 2012; Sezer et al. 2008) , which are sometimes presented on a negative scale (Huang et al. 2007; Hurler et al. 2012). Higher AUC values indicates higher adhesiveness (Jones et al. 1997a; Sezer et al. 2008) where it indicates the ability of hydrogel to adhere to any other material (Oliveira Cardoso et al. 2017) and this might be due to combined effects of adhesive and cohesive forces, viscosity and viscoelastic properties of the hydrogel (Adhikari et al. 2001). The mucoadhesive test is, therefore, more clinically relevant in the presence of artificial mucus and artificial membrane, since the values were greatly influenced in the presence of mucoadhesive bonds (Jones et al. 1997b).

The study indicates that the texture properties and mucoadhesiveness of the hydrogels can be optimized/modified significantly for TPA and mucoadhesion (except for tensile strengths) by the choice of mucoadhesive polymer at 0.5% w/w either single (Hydrogel A, E, F, G, I) or in combination (Hydrogel H, J) and also by slightly increasing the ratio of mucoadhesive component (Hydrogel A, B, C, D). The formulation mucoadhesive components can be utilized to increase residence time, ease of applicability, spreadability and to better withstand the forces with in the oral cavity. Though hydrogel containing 0.25% HPMC and 0.25% povidone was not ideal in every test, it showed optimal work of mucoadhesion, hardness, compressibility and cohesiveness, among all the mucoadhesive polymers tested. Also, the formulation was *in situ* forming hydrogel, where it should have good flow-ability for easy of spraying/application. Therefore, a formulation with minimum viscosity was considered. Overall, the study of texture properties helps in improving the patient compliance, as it is immanently linked to patient's sensory anticipation and mucoadhesion tests help in improving the drug residence time.

4. Experimental

4.1. Materials

Doxycycline hyclate analytical standard, povidone, carboxymethyl cellulose (CMC), chitosan, Polyethylene glycol (PEG) 6000, polyvinyl alcohol and crude mucin Type II were purchased from Sigma-Aldrich (Darmstadt, Hesse, Germany). Doxycycline hyclate used in manufacture of hydrogels was obtained from Hovione (Taipa, Macau). Hydroxypropyl-β-cyclodextrin (HPβCD) was a kindly provided from Roquette pharmaceuticals (Lestrem, Hauts-de-France, France). Poloxamer 407 and poloxamer 188 were obtained from BASF (Ludwigshafen, Rhineland-Palatinate, Germany). Hydroxypropyl methylcellulose (HPMC) was obtained from Norsk Medisinaldepot (Oslo, Eastern Norway, Norway) and carbopol 974P NF was obtained from Lubrizol (Cleveland, Ohio, United States).

4.2. Manufacture of hydrogels

Ten hydrogels were prepared by cold method as described previously (Patlolla et al. 2019) and one control with Poloxamers alone. Poloxamer 407 (21% w/w) and

Poloxamer 188 (10% w/w) were added to the refrigerated solvent and then the mucoadhesive polymers were added (Patlolla et al. 2019). The pH was adjusted to 6.55 with 1M HCl or 1M NaOH solutions. Doxycycline or doxycycline-HPβCD complex were added in the final step and the final weight was made up by adding deionized water. The preparation of doxycycline- HPβCD complex is describe in our previous work (Patlolla et al. 2019). Following this, mucoadhesive polymers and hydrogels were made: *Hydrogel A-D* contained hydroxypropyl methyl cellulose in 0.5%, 1%, 1.5% and 2% (w/w) concentrations, respectively. *Hydrogel E* contained 0.5% carboxymethyl cellulose, *Hydrogel F* contained 0.5% polyethylene glycol 6000, *Hydrogel G* contained 0.5% Carbopol 974P, *Hydrogel H* contained 0.25% HPMC and 0.25% povidone, *Hydrogel I* contained 0.5% povidone, and *Hydrogel J* contained 0.25% HPMC and 0.25% Carbopol 974P.

4.3. Gelation temperature

Gelation temperature was adjusted by a slight modification of a technique described by Pisal et al. (2004). *In situ* hydrogel (2 g) was transferred into a test tube and sealed with Parafilm® to prevent solvent evaporation. Temperature of the water bath was increased in 2°C increments until 25°C and 1°C increments thereafter until gelation occurred, which was measured by tilting the test tube.

4.4. Quantitative analysis using HPLC

The HPLC method for doxycycline hyclate was carried out as described in the Ph. Eur. (Ph. Eur. 2014). A reverse-phase HPLC system from Dionex Softron (Germering, Bayern, Germany) was used for quantification. The Ultimate 3000 series consisted of p680 pump with a DG-1210 degasser, an ASI-100 autosampler and a VWD- 3400 UV-Vis detector. The stationary phase used was a PLRP-S styrene-divinyl benzene copolymer column (250 mm x 4.60 mm) with pore size, 8 µm from Agilent (Santa Clara, California, United States) maintained at 60 °C and flow rate 1 ml/min.

4.5. Viscosity

Viscosity measurements were carried out using a cone and plate geometry viscometer (Brookfield DV-I+, Middleboro, Michigan, United States). The temperature at the sample cup of viscometer was maintained at 25±0.1 °C, using a recirculating bath (Polystat). 0.5 mL of the sample was introduced on to the center of plate and allowed to equilibrate for 5 min. The values were recorded from 25 °C until the gelation occurred with 1 °C increments and the values were stable for 30 min at each temperature. The values were recorded as a function of time (0-60 min) and shear rates for each sample and triplicate values were recorded.

4.6. In vitro mucoadhesion

The mucoadhesive properties of the hydrogels were evaluated with a Texture Analyzer TA-XT2i (Stable Microsystems, Godalming, Surrey, United Kingdom) equipped with a 5 Kg load cell. A 5 ml beaker containing 2.5 g of hydrogel was adhered under the moving probe, with the help of double sided adhesive tape. The beaker containing hydrogel was equilibrated at 37 °C to induce gelation before each measurement. An artificial membrane (hydrocolloid membrane made of carboxymethylcellulose, gelatin and pectin) (DuoDERM, ConvaTec, Reading, Berkshire, United Kingdom) coated with mucin (17% w/v of crude mucin stirred in water overnight at room temperature, pH was adjusted to 6.0 and viscosity, 0.039 (±0.002) Pa·s), known to show best simulation (Skulason June 2009; Skulason et al. 2009a), was adhered on to the bottom of the moving probe (cylindrical graphite probe (P/10), with a 10 mm diameter).

During the test, the probe attached with artificial mucus membrane was lowered onto the surface of the hydrogel with a contact speed of 0.1 mm/s and as soon as it touches the surface it applies a constant force of 0.005 N for 90 s, allowing the artificial membrane to make mucoadhesive interactions with the mucoadhesive polymers present in the hydrogel. The trigger force was 0.003 N. After 90 s, the probe was pulled vertically up with a speed of 0.1 mm/s until 10 mm height was reached. The force required to detach the artificial mucus membrane from the surface of hydrogel was recorded as a function of elongation and both maximum strength and area under force-time curve were obtained. The AUC values obtained from force-time curve were converted to force-distance. Each experiment was repeated at least 6 times.

$$\text{Work of mucoadhesion } \frac{AUC}{\pi r^2} = \frac{N \cdot mm}{cm^2} = \left(\frac{mJ}{cm^2}\right) \qquad \text{(Eq.1) (Amasya et al. 2012)}$$

Tensile strength was measured as the peak detachment force required to detach the test hydrogel from the mucosa. Generally, the greater the peak detachment force the greater is the mucoadhesion strength.

Work of mucoadhesion was measured as work done to detach the hydrogel from the mucus membrane and generally, the greater the work of mucoadhesion, the greater is the mucoadhesive strength.

4.7. Mechanical properties of the hydrogels

The mechanical properties or texture properties or texture profile analysis (TPA), of the hydrogels were analyzed using the Texture Analyzer TA-XT2i equipped with a 5 kg load cell and operating in TPA2 mode and analyzed for hardness, compressibility, cohesiveness and adhesiveness. The test was carried out after the hydrogels were allowed to gel in an oven at 37 °C for least 20 min prior to each test and throughout the test period (< 4 min), remain gelled. Test speed was set at 0.5 mm/s, pre-test and post-test speeds set at 0.1 mm/s. The time interval of 5 s was set between the 2 compression cycles and the penetration depth was 10 mm for 2 cycles. The trigger force for the probe was 0.010 N and the probe used was a cylindrical graphite probe (P/10), with 10 mm diameter.

The plot was obtained for force-time from the texture analyzer software which later either were converted to Force-distance (N·mm) values as the test speed of the probe is known or the X-axis (time) was changed to distance (Jones et al. 1997a) to obtain the values in units of N·mm. Same test conditions were used for all the measurements (Hurler et al. 2012; Jones et al. 1997a). At least six repeated measurements were recorded for each hydrogel formulation.

Hardness (Bourne 1978; Szczesniak 1975) was measured as the maximum force required to achieve a deformation (Jones et al. 1997a; Oliveira Cardoso et al. 2017) of the hydrogel or defined as maximum peak force in the first cycle of compression (Sezer et al. 2008).

Cohesiveness was measured as internal structural strength, which maintains strong interconnections with certain level of resistance to rupture (Lau et al. 2000; Oliveira Cardoso et al. 2017). Cohesiveness was calculated from the ratio of area under curve (AUC) under force-time curve for second compression cycle to the AUC obtained from the first compression cycle (Bourne 1978; Jones et al. 1997a; Szczesniak 1975).

Compressibility was measured as work done to deform the hydrogel during the first cycle of compression (Jones et al. 1997a).

Adhesiveness was measured as the work required to overcome the adhesive forces between the entire surface of probe and the entire surface of gel which comes in contact with the probe, while the probe is in retraction mode after the compressibility cycle (Jones et al. 1997a) or can be measured as negative region of force-time AUC after the first cycle of compression (Bourne 1978; Sezer et al. 2008; Szczesniak 1975).

4.8. In vitro release studies

The drug release from the *in-situ* hydrogels was studied using a membrane-less model (Radivojsa et al. 2013; Zhang et al. 2002). The drug used was doxycycline (0.1% w/w) and doxycycline (0.1% w/w) complexed with HPβCD (2.4% w/w)(Patlolla et al. 2019). 1.8 g of poloxamer solution was carefully introduced onto the bottom of test tube (10 mm diameter) with the help of a syringe and care was taken to prevent bubble formation and was placed in an oven at 37 °C for 5-10 min to induce gelation. A phosphate buffer solution at pH 6.6 was used as a release medium to simulate the oral environment. 10 ml release medium pre-equilibrated at 37 °C was carefully added from the sides of the test tube containing semisolid hydrogel. Then the test tube was placed in a thermostatic shaker (100 rpm, 37 °C). At predetermined time intervals, the release medium was totally replaced by 10 mL fresh medium. The release medium was diluted to 10 times with 0.01M hydrochloric acid solution and analyzed for doxycycline. Triplicate measurements were performed and the samples were filtered with 0.45 µm membrane filters for HPLC (Phenex PTFE filters, Phenomenex, UK). Doxycycline release profile from the hydrogel matrices was analyzed using zero order and Korsmeyer-Peppas kinetic models (Bagyalakshmi J et al. 2011).

Zero order:

$$M_t = M_0 + k_0 t \qquad \text{(Eq.2) (Abdelbary and Aburahma 2015)}$$

M_t is the amount of drug released at time *t;* K_0 is the zero-order release constant and M_0 is the amount of drug released into the solution at time t=0, usually M_0=0

$$\frac{M_t}{M_\infty} = Kt^n \qquad \text{(Eq.3) (Singhvi and Singh 2011)}$$

Korsrneyer-Peppas (power law model):

where M_t / M_∞ is the fraction of drug released at time "*t*". *K* is the release rate constant incorporating structural and geometrical characteristics of the device and n is the release exponent characteristic of release mechanism. The release exponent "*n*" value was calculated from the initial 60% of the drug release curve, which was plotted as log cumulative percentage drug release *versus* log time.

4.9. Statistics

RStudio (Version 1.1.463 RStudio, Inc. Boston, Massachusetts) was used for the calculation of statistical significance using one-way analysis of variance (ANOVA) followed by Turkey HSD *post hoc* test and $p<0.05$ was considered statistically significant.

Acknowledgements: This work was supported by a research grant from the University of Iceland (Rannsóknarsjóður Háskóla Íslands).

Conflicts of interest: None declared.

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