DEVELOPMENT OF MUCOADHESIVE CELLULOSE DERIVATIVES BASED FILMS FOR THE TREATMENT OF VAGINAL CANDIDIASIS

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The development of easily administered targeted delivery for vaginal candidiasis is an area of active research. Challenges emerge from the specific conditions that may not permit enough time for the dosage form to reside on the infected area. Herein, we propose to develop films based on cellulose derivatives for the treatment of vaginal candidiasis. Gels of sodium carboxymethyl cellulose (Na-CMC) (F1), equal combination of Na-CMC and hydroxyethyl cellulose (HEC) (F2) and hydroxyethyl cellulose (HEC) (F3) were prepared and loaded with nystatin (NYS). The resultant gels were dried using solvent casting and characterized to detect glass transition temperature (T_g), mechanical properties, mucoadhesion, inhibition of candida growth toxicity on human embryonic kidney 293 cells (HEK) cells and drug release. T_g was affected by the polymer type and was found to be highest in F2, where equal ratios of HEC and Na-CMC were used. Mucoadhesion was highest in F1 (Na-CMC) films. The films showed moderate toxicity. The zone of inhibition was observed for the three formulations. Drug release was affected by the polymer type and was complete after 8 h in F2. The findings allowed concluding that the cellulose derivative based films were successfully prepared and were efficient in allowing the drug to elute and minimizing the growth of candida.

Keywords: hydroxyl ethyl cellulose, sodium carboxymethyl cellulose, candida, dried hydrogel, solvent casting, mucoadhesion

INTRODUCTION

Conventional vaginal dosage forms like gels, creams, vaginal suppositories, and vaginal films have been widely employed in the management of vaginal candidiasis.[1] The specific condition of the infected area makes films more practical than other pharmaceutical dosage forms.[2] For example, amorphous gels require the use of a special tool (applicator).[3] The use of vaginal pessaries may be considered inconvenient due to the size and thickness and the need to rest for a period of time.[4] Both gels and pessaries may cause leakage. The interesting properties of vaginal films compared to other dosage forms include their small size and thickness, ease of administration without the need for an applicator, bio-adhesiveness, comfort, formulation flexibility, reduced product leakage, and low cost.[5,6] Vaginal films were intended for contraceptive purposes.[7,8] Currently, there are three types of vaginal films available on the market: a scented vaginal film, a vaginal lubricant, and a contraceptive film.[8,9]

Traditional vaginal films are designed to dissolve quickly after administration, but additional research is being done to create vaginal films that dissolve more gradually or to manage drug diffusion to enable continuous drug release.[10-12]

When developing a pharmaceutical dosage form for vaginal administration, several criteria should be met:[13] firstly, the limitation of absorption attributed to the presence of four separate layers of cells in the vaginal mucosal cavity forming a barrier to drug absorption; secondly, the turnover of vaginal fluid and the mucus produced by cervical epithelial cells; and thirdly, cervicovaginal fluids form a physical barrier to the transportation of drugs and may interact with medications.[14] A healthy vagina has a pH ranging between 4.5 and 5, though several

disorders may change vaginal pH. The aforementioned limitations dictate the design of vaginal mucoadhesive films that can address those challenges. The film ingredients should ideally not affect this pH.[15,16] Those films would be prepared in the form of amorphous hydrogels, which would then be dried to produce films. Hydrogels are regarded as "smart delivery systems" due to the nature of the functional groups, they are sensitive to a range of environmental circumstances (temperature, pH, electrical current, and other similar parameters).[17]

In the therapy of vaginal candidiasis, there are a number of medications that are commonly prescribed by healthcare providers: fluconazole, boric acid and nystatin. Nystatin (NYS) is a polyene broad-spectrum antifungal medication used to treat cutaneous fungal infections caused by candida. Compared to other polyene antifungal medications, it has a broader antifungal activity.[18,19]

In order to use NYS in the local treatment of vaginal candidiasis, the drug needs to be incorporated in a dosage form that would adequately deliver the drug, would be adhesive, and not cause leakage. Hence, dried hydrogels were considered in this study. In order to prepare the mucoadhesive films, cellulose derivatives were used in this regard. Cellulose derivatives, such as sodium carboxymethyl cellulose (Na-CMC) and hydroxyethyl cellulose (HEC), are often employed in medical and industrial applications. Cellulose derivatives are biodegradable, non-toxic, and biocompatible.[20] Na-CMC has a hydroxyl group (-OH) and a carboxyl group (-COOH) as functional groups. Under basic conditions, the carboxyl group is almost completely ionized and becomes progressively less ionized as the pH decreases, altering the physicochemical properties of the compound.[21] HEC is frequently used as a grafting agent to improve the flexibility of CMC, as well as its mechanical properties.[18]

The aim of this study was to prepare a new vaginal mucoadhesive delivery system in the form of a dried hydrogel to treat vaginal infections. The proposed polymeric films would release an effective concentration of NYS at the site of action. The films should be tested for mucoadhesion to overcome the acknowledged challenges of a wet hydrogel.

EXPERIMENTAL

Materials

The following materials were obtained from Sigma-Aldrich (Munich, Germany): sodium carboxymethyl cellulose (Na-CMC), hydroxyethyl cellulose (HEC), nystatin (NYS), and methanol.

Film preparation

The films were prepared using the solvent casting technique with modifications.[22] A known amount of NYS (equivalent to 50 mg) was dissolved in 50 mL of methanol. A homogeneous mixture of 4 g of HEC (and 2-3 drops of glycerine) was prepared by adding 100 mL of distilled water gradually with mixing at the rotation of 3000 rpm, using an IKA mixer (Werke GmbH, Germany). NYS solution was then added to the polymer and stirred well for 15 min till homogeneity was visually confirmed. The resultant gel was centrifuged for 15 min to eliminate air bubbles and left for 15 min to settle; then, it was poured into Petri dishes and allowed to dry in an oven overnight at 30 °C. The dried films were peeled the following day and weighed for 3 successive days to ensure consistent weight. The obtained films contained 1.25% NYS. The procedure was repeated using 4 g of Na-CMC and equal amounts of the two polymers, as illustrated in Table 1. The films were stored in plastic bags for characterization.

Thermal analysis

The glass transition temperature (T_g) of the prepared films was measured using an AQ800 DMTA (TA, NC, USA). The tensile mode was selected, and was employed at an oscillatory frequency of 1 Hz. A heating rate of 3 °C·min^{-1} was selected over a temperature starting from room temperature to 140 °C. Samples (n = 3) of rectangular forms (30 mm, 5 mm and 0.5 mm – length, width and thickness, respectively) were prepared. Glass transition temperature (T_g) was detected as the peak of *tan* δ as a perceptible reduction in the storage modulus taking place.[23,24]

Table 1
Formulations of the prepared films

Formulation	Na-CMC (g)	HEC (g)	NYS (mg)
F1	4		50
F2	2	2	50
F3		4	50

Mechanical analysis

A tensile analysis of the prepared films was implemented using data retrieved from a TA-XT Plus Texture analyzer (Stable Microsystems Goldaming, Surrey, UK). Five replicate samples of the dried films (30 mm length × 5 mm width x 0.5 mm thickness) were clamped between the static (lower) and moveable (upper) grips, ensuring that the length of the films under stress was constant (20 mm). The upper clamp was upstretched at a fixed rate (0.5 $mm.s^{-1}$) in anticipation of a fracture of the films. From the resultant stress-strain plot, the ultimate tensile strength (U.T.S) and % elongation were calculated.[25,26]

Mucoadhesion

The mucoadhesion properties were determined by an earlier described procedure with modification.[27] A TA-XT Plus Texture analyzer (Stable Microsystems Goldaming, Surrey, UK), equipped with a 5 kg load cell was used for measuring mucoadhesion. Fresh ewe vaginal mucosa was excised and frozen at -20 °C. A section with an approximate thickness of 2 mm from the inside region of the surface of the frozen vaginal mucosa was selected and mounted on the mucoadhesion test base. 50 mL of distilled water was applied to the surface of the tissue in order to rehydrate it before the experiment. Films (20 x 20 mm) were attached to the lower end of the cylindrical probe fitted with a circular base (from the extrusion device) with double-sided adhesive tape. The tests were carried out at room temperature. The probe was pulled down onto the surface of the tissue with a constant speed of 0.5 $mm.s^{-1}$ and a contact force of 1 N. A contact time of 2 min was allowed. The upper part was then moved up in a vertical manner at a constant speed of 0.5 mm. s^{-1}. The detachment force ($N.mm^{-2}$) was obtained from the force-distance plot.[27] Each experiment was carried out in 5 replicates.

Inhibition of candida growth

The study of the candida inhibition was carried out following a method described in the literature, with a few modifications.[28,29] An American Type Culture Collection (ATCC) strain of *Candida albicans* 10231 was employed to investigate the ability of the films to inhibit its growth. The optical density for *Candida albicans* was evaluated at $1x10^8$ $CFU.mL^{-1}$ (confirmed by viable cell count) and then swabbed. Polymers were cut into discs (n = 6, average thickness of 0.5 ± 0.02 mm and diameter of 2.8 mm), were planted on a Mueller Hinton Agar (MHA) plate and left for 48 h. The zone of inhibition was measured in (mm) and compared against the control (equivalent to 50 mg powder of NYS). The control was represented by cellulose discs immersed into NYS of the same concentration for three days, then removed, dried and used similarly to the polymeric films.

Film cytotoxicity tests

The cytotoxicity test for the films comprised the use of the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium assay and was conducted to determine the potential cytotoxicity, cell proliferation and viability by the MTT Cell Proliferation Assay (ATCC® 30-1010K).[30]

The procedure was carried out as prescribed in the literature.[31] The cells were seeded in a 96-well flat-bottom microtiter plate, containing 100 µL of medium at a density of $1×10^4$ cells/well, and allowed to adhere at 37 °C in a humidified 5% CO_2 incubator for 24 h. After that, the cells were treated with the prepared films (discs, n = 5, with the average thickness of 0.5 ± 0.02) for 24 h. Subsequently, 10 µL of MTT working solution was added to each well and the plate was subsequently placed in an incubator for 4 h. Then, 100 µL of detergent reagent was added to each well and the plate was left for 2 h. Finally, the intensity of the formazan crystals (purple color) was determined using the Synergy™ HTX Multi-Mode Microplate Reader at 570 nm (BioTek, Winooski, VT, USA).[32] Cell viability % was calculated as shown in Equation (1):

Cell viability (%) = (mean absorbance of treated cells/mean absorbance of control cells) × 100

***In vitro* drug release and kinetics**

The films were cut into a rectangular shape (10 x 30 mm), immersed in 10 mL of freshly warmed phosphate buffer pH = 5 (polysorbate, 5% *w/v* was added to enhance sink conditions), aliquot samples were withdrawn and replaced with fresh 10 mL of the same buffer at predetermined times. NYS amount was calculated with reference to a calibrated standard curve,[33] using the well acknowledged equation of the Beer-Lambert law. The standard calibration equation was an average of three trials, X = 44.85, (coefficient of determination, R^2 = 0.9951).

A mathematical model was applied to the percentage of drug released, as shown in Equation (2):[33]

Korsmeyer-Peppas: $Q_t = K_t n$ (2)

where Q_t (%) is the percentage of drug released at time t, K_t – release coefficient, and n – the diffusion release exponent.

RESULTS AND DISCUSSION

Thermal analysis

The glass transition started at a single temperature (T_g). This would indicate uniform physical dispersion.[34] The T_g of different polymers is presented in Figure 1. Formulation F3, which represents NYS loaded in the HEC film, had a T_g of 115.42 ± 5.22 °C, whereas F1, representing the CMC film, showed a transition of 96.27 ± 0.73 °C. Notably, the T_g of the blend F2 was found to be significantly increased to 131.79 ± 2.86 °C. One-way ANOVA followed by

Bonferroni multiple comparisons showed a statistically significant rise in T_g in F2 and F3, compared to F1 (p-value of 0.0001 and 0.01, respectively). A significant increase in T_g was also observed for F2, compared to F3 (p-value = 0.01). Both Na-CMC and HEC possess abundant OH groups that allow polymer interaction and H bond formation, minimizing the void volume, thus elevating the T_g, which justifies the value of F3.[32]

Mechanical analysis

The ultimate tensile strength (U.T.S) of different polymers is shown in Figure 2. One-way ANOVA followed by Bonferroni multiple comparisons exhibited significant increases in U.T.S in F2 compared to F1 (p-value = 0.01). Significant reduction was found in F2 compared to F3 (p-value = 0.003).

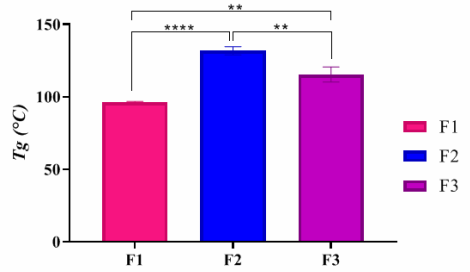

Figure 1: T_g values of the prepared films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC (data obtained from DMTA and presented as mean ± SD, (n = 3); **p-value < 0.01, ****p-value < 0.0001 significant difference among the films)

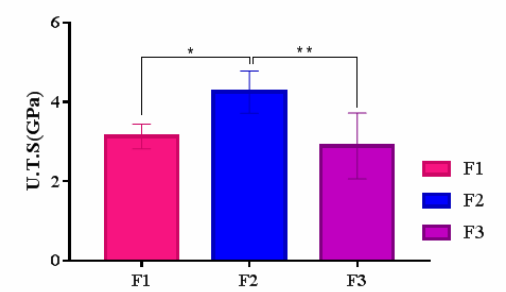

Figure 2: U.T.S values of polymer films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; (data presented as mean ± SD, (n = 3); *p-value < 0.05, **p-value < 0.01 significant difference among the films)

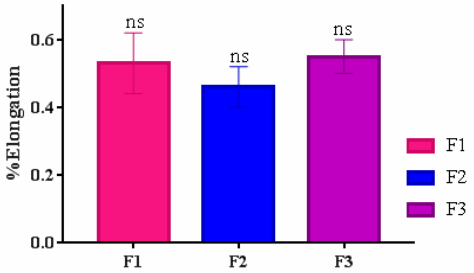

Figure 3: % Elongation values of polymer films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; (data presented as mean ± SD, (n = 3); non-significant (ns) difference among the films (p-value > 0.07))

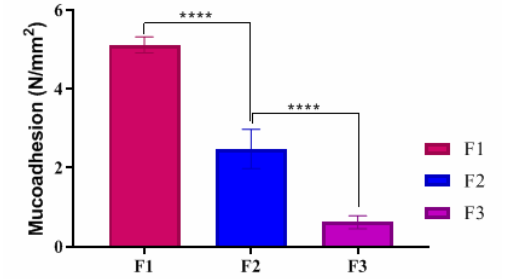

Figure 4: Mucoadhesion results of the prepared films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; (data presented as mean ± SD, (n = 4); ****p-value < 0.0001, significant difference among the films)

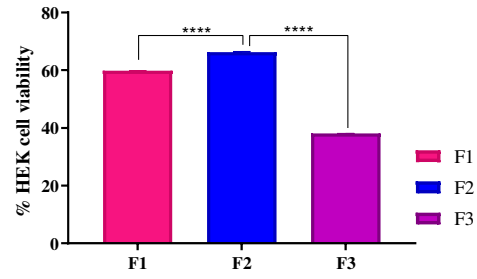

Figure 5: % HEK cell viability after application of the films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; (data presented as mean ± SD, (n = 4); ****p-value < 0.0001, significant difference among the films

The U.T.S was the highest in F2: 4.25 ± 0.53 GPa, corresponding to the highest value of T_g.[25] The highest T_g indicates that the entanglement between the polymer chains is significant, subsequently, the void volume is diminished, and therefore, the exertion of greater force is required to obtain a fracture.[35]

The % Elongation of different polymers is shown in Figure 3. One-way ANOVA followed by Bonferroni multiple comparisons revealed non-significant changes in % Elongation among the prepared films (p-value > 0.09).

Mucoadhesion

The mucoadhesion process comprising a polymeric drug delivery platform is a series of complex events that requires wetting, adsorption, and interpenetration of polymer chains, amongst various other processes.[36] A mucoadhesion polymeric platform offers the advantage of longer residence time, which may lead to lower administration frequency,[36] and improved bioavailability.[37] The mucoadhesion results of the tested polymeric films are shown in Figure 4. One-way ANOVA, followed by Bonferroni multiple comparisons, showed a significant reduction in mucoadhesion between F2 and F3, compared to F1 (p-value < 0.0001), where it was 5.12±0.20 N/mm^2 for F1, 2.48± 0.49 for F2, and 0.62±0.16 for F3, respectively. A significant decrease was also found for F3 compared to F2 (p-value < 0.0001). Mucoadhesion typically represents the force required to detach two surfaces.[38] Therefore, a high value signifies better adhesion and subsequent retention at the site of treatment.[38] However, the required force should be designed to consider the site of application, variation in human strength due to age, and pathological conditions. The excised samples were treated properly, namely, the films were immersed into an adequate volume of distilled water (50 mL) and were allowed to contact the tissues for 2 min. The observed high value of mucoadhesion in F1, which contains merely Na CMC as a polymer is attributed to its anionic nature. The negatively charged polymer is capable of forming hydrogen bonds between its carboxylic and hydroxyl groups of the mucus glycoproteins.[38]

Inhibition of candida growth

Vaginal candidiasis is a common condition caused mainly by *Candida albicans*. It affects 70-75% of women in their life.[39] The inclusion of NYS as an antifungal agent in vaginal films was reported in the literature.[40] NYS is a polyene antibiotic that exhibits a broad spectrum of activity against fungi.[40] To detect its efficacy in the formulation, the zone of inhibition was measured. The zone of inhibition for the different polymers is shown in Table 2. One-way ANOVA, followed by Bonferroni multiple comparisons, showed a significant increase for F1, F2, and F3, compared to the control (p-value = 0.0001). A significant increase in the zone of inhibition was found in F2 and F3, compared to F1 (p-value = 0.0001 and 0.001, respectively).

Film cytotoxicity tests

After the treatment with the prepared films, the % HEK cell viability was lower than 80%, as shown in Figure 5. One-way ANOVA, followed by Bonferroni multiple comparisons, showed a significant decrease in cytotoxicity in F1 and F3, compared to F2 (p-value < 0.0001). A significant decrease was also found in F3, compared to F2 (p-value < 0.0001). In the evaluation of cytotoxicity, the prepared films have % cell viability between 80-60% and can be considered weakly cytotoxic.[14]

Table 2
Zone of inhibition values (in mm) of the prepared films against *Candida albicans*

Time (day)	Control	F1	F2	F3
1	9.50 ± 0.55	24.33 ± 0.82	23.50 ± 1.38	20.17 ± 1.17
2	21.50 ± 0.55	17.50 ± 1.76	23.00 ± 1.41	20.83 ± 1.33

Control: cellulose discs, F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; (data presented as mean ± SD, (n = 6))

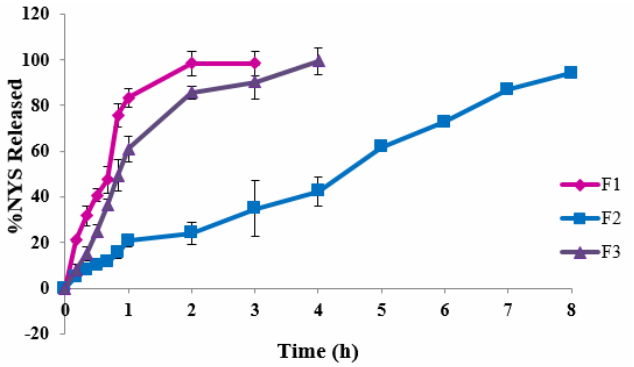

Figure 6: % NYS release of the prepared films, where F1 – NYS loaded in CMC, F2 – NYS loaded in CMC:HEC, and F3 – NYS loaded in HEC; data presented as mean ± SD, (n = 3)

***In-vitro* drug release**

The release of NYS from the prepared films was studied and the data are shown in Figure 6. The release rate was significantly faster in F1 (NYS+Na-CMC), where it was 21.11 ± 0.72% at 10 min and was complete after 3 h. The release was significantly slower in F2, which contained equal amounts of Na-CMC and HEC, and for which, at the same time points, the release was 5.12 ± 1.13% and 8.00 ± 2.4%, respectively, and was complete after 8 h. However, for F3 the release was complete after 4 h. F1 contains Na-CMC, which has an acid dissociation constant (pK_a) of 3.9,[41] and tends to deprotonate at pH 5.[42]

The release exponent from the Korsmeyer-Peppas equation, (n), was detected.[43] The equation is specifically applicable when a release percentage is less than 60, and for the inclusion of no less than 4 determination points. The release exponent (n) was 0.58, 0.59, and 1.05 for F1, F2 and F3, respectively (*i.e.* $0.5 < n < 1$), which means that, under elevated pH, an anomalous non-Fickian transport mechanism is observed. According to the Korsmeyer-Peppas equation (Eq. 2), the drug release mechanism is governed by swelling, erosion, and diffusion.[44]

Many strategies and approaches have been investigated to deliver antifungal medications to the vagina, including vaginal gels,[45] pessaries, and films.[12] Adhesive films offer many benefits, specifically, they provide easier patient cooperation, due to ease of product usage and minimum product leakage, and could provide longer periods of contact with the infected tissue, which may allow better use of the medication. The films may be fabricated from a wide variety of polymers, such as gellan gum,[42] chitosan,[40] HPMC, zein,[8] and xanthan.[46] HEC was used as a rapid dissolving film.[47] Also, Na-CMC was used as gel.[48] Herein, blend films of HEC and Na-CMC were employed in the fabrication of vaginal films and were loaded with NYS for the treatment of vaginal candidiasis. The films were successfully prepared as gels, then poured and cast to dry for further characterization. The films exhibited good suppression of candida growth, presented as the zone of inhibition (Fig. 6) and were affected by polymer type that subsequently influenced drug release. The release was tested at pH 5 to simulate physiological conditions.

Drug release is a complex multi-factorial process that is governed by several constraints, including polymer properties, drug properties, and medium conditions, for instance, the selected temperature, pH, medium constituents, volume, and finally time of the experiment. Therefore, it is difficult to correlate the release profile to one parameter only. Polymer properties are mainly exemplified by chemical structure, T_g, swelling, erosion, and hydrophobicity. If the experiment was conducted under the dynamic conditions, the release rate could be slower due to the lack of proper contact time between the medium and the films. The release of the films was complete in all formulations, but the rate was significantly affected by the polymer type. The fastest drug release was observed in F1, which contains Na-CMC. The anionic polymer was expected to dissolve at pH 5 and therefore to allow faster departure of the drug, compared to F3 and F2. Moreover, the pK_a of Na-CMC is lower than the tested pH of drug release (and swelling), the swelling experiment showed that F1 and F2 dissociated at pH 5 into an amorphous structure and the platform started to erode after 10 min, thus, swelling values could not be recorded. The release was also reflected by T_g (Fig. 1). As illustrated, the lowest T_g was observed in F1,

followed by F3, and was found to be the highest in F2, which contained an equal ratio of the two polymers. It is not uncommon for a T_g of a blend to be higher than those of the separate polymers. This is attributed to the formation of hydrogen bonding between the two polymers, which will subsequently minimize the void volume uttering the films required to elevate temperature to transfer it to rubbery state.[34] Moreover, a higher T_g indicates more brittle films that will require longer time till wetting, this was reflected in the case of F3, which lasted for 8 h before dissolving into an amorphous gel as per our observation. The thermal properties affected mechanical properties as well. Higher T_g would signify a low void volume in the polymer, making the force required for the film to break to be higher (data are presented by the U.T.S., Fig. 2) Another important feature in the fabrication of the mucoadhesive films is their ability to adhere to the tissue. The conducted bio-adhesion experiment was performed on excised tissue and was prepared as described in the literature.[27] The adhesion was affected by the polymer type, where the anionic polymer (Na-CMC) formed attraction to mucin, which decreased in correspondence with the decrease in Na-CMC content.

The films showed moderate toxicity upon investigation on HEK cells. This could be attributed to the low oxygen permeation, as the prepared films were dried and then throughout the experiment were transferred to amorphous films, leading to low oxygen permeability and cellular death. However, those films were used in many pharmaceutical preparations and the designated tissue will take its oxygen from blood circulation. Nevertheless, the use of a cross-linker may minimize the toxicity of the films.

CONCLUSION

In the present work, hydrogels were prepared successfully from cellulose derivative polymers HEC and Na-CMC, and their combination, and were loaded successfully with NYS. The film that contained the combination of polymers (F2) showed higher T_g. value of 131.79 ± 2.86 °C. The highest mucoadhesion property – of 5.12±0.20 N/mm^2 – was found in F1, which contained Na-CMC. The films showed the ability to release the drug within proper time, which varied according to the polymer type, from 3 h in F1 to 8 h in F2. The release mechanism was governed by swelling, erosion and diffusion. F2 exhibited the highest tensile strength – of 4.25 ± 0.53 GPa. The zone of inhibition was greater than for the control in all three formulations, and exceeded 20 mm in F2 and F3 on the second day of the experiment. The three films showed moderate toxicity towards HEK cells.

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