

Research Article

Effect of Thiolated Polymers to Textural and Mucoadhesive Properties of Vaginal Gel Formulations Prepared with Polycarbophil and Chitosan

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Abstract. The aim of this study was to design and evaluate of mucoadhesive gel formulations for the vaginal application of clomiphene citrate (CLM) for local treatment of human papilloma virus (HPV) infections. Chitosan (CHI) and polycarbophil (PC) were covalently modified using the thioglycolic acid and L-cysteine, respectively. The formation of thiol conjugates of chitosan (CHI-TG) and polycarbophil (PC-CYS) were confirmed by FT-IR analysis and PC-CYS and CHI-TG were found to have 148.42 ± 4.16 and 41.17 ± 2.34 μmol of thiol groups per gram of polymer, respectively. One percent CLM gels were prepared by combination of various concentrations of PC and CHI with thiolated conjugates of these polymers. Hardness, compressibility, elasticity, adhesiveness and cohesiveness of the gels were measured by Texture profile analysis and the vaginal mucoadhesion was investigated by mucoadhesion test. The increasing in the amount of the thiol conjugates was found to enhance the elasticity, cohesiveness, adhesiveness and mucoadhesion of the gel formulations but not their hardness and compressibility when compared to gels prepared using their respective parent formulations. Slower release rate of CLM from gels was achieved when the polymer concentrations were increased in the gel formulations. PC and its thiol conjugate were found to prolong the release of CLM longer than 70 h unlike gel formulations prepared using CHI and its thiol conjugate which were able to release CLM up to 12 h. Stability of CLM was preserved during the 3 month stability analysis under controlled room temperature and accelerated conditions.

KEYWORDS: chitosan–thioglycolic acid; clomiphene citrate; gel; human papilloma virus; polycarbophil–cysteine; texture profile analysis; vaginal mucoadhesion.

INTRODUCTION

Genital HPV infection is a sexually transmitted disease that is caused by human papilloma virus (HPV). HPV is a large family of viruses consisting of more than 100 strains and subtypes. Approximately 20 million people are currently infected with HPV (1,2). Infected patients usually display no symptoms. In some cases, visible venereal warts named as *Condylomata acuminata* or pre-cancerous changes in the cervix and vulva are observed (3). HPV can alter the genetic structure of cells and causes the proliferation of cells in a rapid and uncontrolled manner. Approximately 10 of the 30 identified genital HPV types can lead, in rare cases, to development of life-threatening cervical cancer and up to 87% of cervical carcinomas contain HPV DNA (4,5). In addition, HPV can be passed on from the pregnant woman to her baby. In very rare cases, the baby can develop warts in the throats or vocal cords (4).

Clomiphene citrate (CLM) is commonly used for infertility treatment due to its ability to initiate ovulation (6). Since HPV binds favourably to estrogen receptors, CLM has also found application in the treatment of HPV infection by competing with HPV for binding to the estrogen receptors (4). Hence, it is hypothesised that effective HPV treatment could be achieved locally by using mucoadhesive vaginal formulations of CLM.

Mucoadhesion is defined as the binding ability of a synthetic or natural polymer to the biological tissue (7). Thus far, mucoadhesive formulations have been prepared for various routes of administration such as rectal (8,9), vaginal (10–12), oral (13), buccal (14–16), nasal (17,18), and ocular (19,20) routes. Mucoadhesive drug delivery systems are capable of increasing the bioavailability of the active compounds by extending their retention time and subsequently controlling the release of the drug in the desired region (21,22). Importantly, a tight contact between the mucoadhesive material and biological tissue is crucial for mucoadhesion to occur. Due to the humidifying effect of the intermediate surface, polymer swells causing mutual interpenetration and formation of low energy chemical bonds between the mucus and the polymeric material (23,24). Examples of mucoadhesive polymers used in vaginal drug delivery systems include CHI, starch, collagen, gelatine, cellulose derivatives, sodium alginate and polyacrylic acids. Recently, polymers with

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abilities for covalently binding to mucus have been developed. The thiol conjugates of the polymers have an increased ability for interacting with mucus glycoproteins (25–27).

The aim of this study was to prepare CLM gel formulations with improved mechanical, mucoadhesion and sustained release properties for the local treatment of HPV infections. In this study, the anionic structured PC and cationic structured CHI and the thiol conjugates of these polymers were used to prepare the gel formulations. The formation of thiol conjugates were confirmed by FT-IR analysis and the amount of thiol groups conjugated per gram of polymer were calculated. Various experiments were performed to evaluate the mechanical properties (hardness, compressibility, elasticity, adhesiveness and cohesiveness) and the mucoadhesive ability of the prepared gel formulations. The release rate of CLM from gel formulations was also investigated via *in vitro* dissolution tests at an acidic pH (pH 5.0) corresponding to the pH of the vaginal mucosa. The stability of the drug in the gel formulations was determined on the basis of measuring the amount of drug in the defined storage conditions. It is hoped that by developing a sustained release CLM formulation for vaginal delivery, a safe and effective treatment of HPV infection could be realised.

MATERIALS AND METHODS

Materials

Clomiphene citrate was purchased from Koçak Pharma (Turkey), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDCI), ethylenediamine tetraacetic acid (EDTA), L-cysteine hydrochloride monohydrate and thioglycolic acid from Sigma (USA), polycarbophil (Noveon AA1) from BF Goodrich (USA), chitosan (medium molecular weight, 200–800 cP, deacetylation degree >75%) from Aldrich (Germany), hydrochloric acid (37%), 0.1 mM iodine solution, lactic acid from Merck (Germany). All chemicals used were of pharmaceutical grade and used without further purification.

Preparation of Conjugates

Polycarbophil–cysteine (PC-CYS)

PC-CYS conjugates were synthesized and purified according to previously described methods (25,27). These conjugates were prepared by formation of amide bonds between the carboxylic acid groups of the PC and the primary amino group of the CYS.

Firstly, PC was neutralised prior to the preparation of conjugates. 10 g of the polymer was slowly added to 4% methanolic sodium hydroxide solution (100 mL) with continuous stirring until neutralisation was achieved. Upon neutralisation, the polymers were precipitated and separated by filtration, washed with methanol until the pH 5 was reached and then dried in a ventilated oven at 35°C.

Two grams of neutralised PC were left to swell in 500 mL distilled water at room temperature for 24 h in order to stabilise the polymer dispersion. With the aim of activating the carboxylic acid moieties of PC, EDCI was added into the PC solution to form a final concentration of 50 mM. The pH value of the resultant solution was adjusted to pH 5 with 5N HCl

under a stream of nitrogen gas for 15 min to prevent the oxidation of sulphhydryl groups with atmospheric oxygen. 1 g L-cysteine hydrochloride monohydrate was added to the solution and dissolved by mixing. The conjugates formed were incubated under a stream of nitrogen gas for 3 h. The solution was dialysed in the dialysis tubing (MW cut-off 12–14 kDa, Mediacell International Ltd., UK) protected from light at 10°C against 1 mN HCl containing 2 µM EDTA, twice against 1 mN HCl containing 1% NaCl and finally against 0.5 mN HCl to remove EDCI and CYS from the conjugate solution. The pH value of the dialysed solutions was adjusted to pH 5 with 2 N NaOH. The solution was lyophilised and the conjugates kept at 4°C until needed for the preparation of gels.

Chitosan–thioglycolic acid (CHI-TG)

The conjugates were prepared by covalent modification of the CHI *via* formation of amide bonds between primary amino groups of the CHI with the carboxylic acid group of the TGA (28).

Briefly, 0.5 g CHI was hydrated in 4 mL of 1 N HCl for 2 h and diluted with distilled water to obtain a 1% CHI solution and kept at room temperature for 24 h to stabilise the polymer dispersion. EDCI in a final concentration of 50 mM and 0.5 g TGA was added into the solution and the pH value of the solution adjusted to 5 with 5 N HCl. The reaction mixtures were incubated for 3 h with permanent stirring. The resultant solution was dialysed twice in a dialysis tubing (MW cut-off 12–14 kDa) protected from light at 10°C against 5 mN HCl containing 1% NaCl and finally against 1 mN HCl to remove EDCI and TGA from the conjugate solution. The solution was lyophilised and the conjugates kept at 4°C until needed for the preparation of gels.

Characterisation of Conjugates

Fourier Transform Infrared (FT-IR) Analysis

The FT-IR spectrums of conjugates were measured in potassium bromide disks using a Perkin Elmer Model 1600 FT-IR spectrometer (USA) operating in transmission mode at a resolution of 4 cm⁻¹ in the range of 4,000–650 cm⁻¹. Twenty scans were co-added for each spectrum.

Amount of Thiol Groups

The amount of thiol groups on the conjugates was determined by iodometric microtitration (25). Three milligrams conjugate was hydrated in 1 mL of distilled water. The pH value was adjusted to pH 3 with 1 N HCl and 500 µL of 1% *m/v* starch solution was added. The solution was titrated with 1 mM iodine solution until the solution has turned into a clear light blue colour. The same process was repeated with the unmodified parent polymers as control groups. The amount of thiol groups in the conjugates determined using the equation below (Eq. 1):

$$\text{The amount of thiol groups (mg)} = M \times V \quad (1)$$

whereby *M*: The molarity of iodine solution used for titration; *V*: The volume of 1 mM iodine solution used for titration (mL) and 33 mg thiol group is equivalent to 1 mmol thiol.

Mucoadhesion Studies

TA-XTPlus Texture analyzer equipped with a 5 kg load cell was used for mucoadhesion studies (30,31). Freshly excised bovine vaginal mucosa was frozen at -30°C . A section of 2 mm thickness was taken from the inner part of the surface of the frozen vaginal mucosa and attached to the lower end of the probe (P0.5 Perspex, θ : 12.5 mm) of the instrument with cyanoacrylate glue. The mucosa was dipped into the cow vaginal mucus (frozen at -30°C just after the excision and adjusted to 37°C during the experiment) and kept for 10 min prior to the commencement of the experiment.

The gels were packed into 30 mm diameter tubes and centrifuged at 20,000 rpm for 10 min to remove the air bubbles from the gels and to ensure a smooth contact between the gels and the vaginal mucosa. The mucoadhesion studies were performed at 37°C . The probe holding the vaginal mucosa was lowered onto the surface of the gel with a constant speed of 0.1 mm s^{-1} and contact force of 0.5 N applied. After a contact period of 120 s, the probe was then moved vertically upwards at a constant speed of 0.1 mm s^{-1} . The area under the curve (AUC) was calculated from force–distance plot as the work of mucoadhesion. The formulation given below was used to calculate the work of mucoadhesion per square centimeter (mJ cm^{-2} ; Eq. 2). Each experiment was carried out in triplicate.

$$\text{Work of mucoadhesion } (\text{mJ cm}^{-2}) = \frac{\text{AUC}}{\pi r^2} \quad (2)$$

whereby πr^2 : Surface area of the vaginal mucosa which is in contact with the gel formulations.

In vitro Drug Release Studies

Five hundred milliliters pH 5 lactate buffer at $37 \pm 0.5^{\circ}\text{C}$ was used as the dissolution medium for the *in vitro* drug release studies. Five grams gel was put into a cylindrical vessel which has 3.8 cm diameter and 1 cm height. A 4 cm diameter sieve (θ : 30 mesh) was attached on the edge of the cylindrical vessel and located on the bottom of the dissolution vessel providing the edge remained at the upper surface (32). The study was held at 100 rpm using USP 24 paddle rotation method. Samples were taken at certain time intervals and the same amount of pH 5 lactate buffer was added to the medium. The absorbance of the solutions was read spectrophotometrically at 292 nm and the concentrations were calculated using the standard curve. The test was conducted in triplicate.

Stability of CLM in Gel Formulations

The gel formulations were filled into aluminium tubes and placed in the stability cabinets. The stability of CLM in gels was observed at $25 \pm 2^{\circ}\text{C}$, $60 \pm 5\%$ RH; $30 \pm 2^{\circ}\text{C}$, $65 \pm 5\%$ RH and $40 \pm 2^{\circ}\text{C}$, $75 \pm 5\%$ RH for 3 months. The samples taken after 0, 15, 30, 60, 90 days were weighed to control the loss of weight during the incubation periods and the CLM assay was performed using a high-performance liquid chromatography method described in USP 24. The experiment was performed in triplicate for each gel formulation.

Statistical Evaluation

Results of the mechanical and mucoadhesion properties of the formulations and *in vitro* drug release data were statistically evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. $p < 0.05$ was considered to be indicative of significant difference between the chosen formulations.

RESULTS AND DISCUSSION

Characterisation of PC-CYS and CHI-TG Conjugates

PC-CYS and CHI-TG conjugates were obtained by the covalent modification of the PC and CHI, respectively, *via* the formation of amide bonds. The suitable amount of EDCI for the activation of the carboxyl groups, the appropriate pH for the conjugate preparation and the reaction periods were selected according to previous studies.(25,27,32) FT-IR spectrums confirmed the formation of PC-CYS and CHI-TG conjugates (Fig. 1). According to the FT-IR spectrum of the PC-CYS conjugate (Fig. 1a), $-\text{SH}$ stretching peak at $2,569\text{ cm}^{-1}$ was related to the $-\text{SH}$ groups of CYS moiety; aliphatic carboxyl group at $1,698\text{ cm}^{-1}$ indicated the presence of free carboxyl groups of PC. The amide band $\text{C}=\text{O}$ stretching peak at $1,670\text{ cm}^{-1}$ and amide band $\text{N}-\text{H}$ incline at $1,633\text{ cm}^{-1}$ confirmed the formation of amide bond in the PC-CYS conjugates. According to spectrum of the CHI-TG conjugate (Fig. 1b), $-\text{OH}$ stretching peak at $3,519\text{ cm}^{-1}$ indicated the presence of hydroxyl groups of CHI; $-\text{SH}$ stretching peak at $2,601\text{ cm}^{-1}$ was related to $-\text{SH}$ groups of TG. The amide band $\text{C}=\text{O}$ stretching peak at $1,689\text{ cm}^{-1}$ and amide band $\text{N}-\text{H}$ incline at $1,636\text{ cm}^{-1}$ confirmed the formation of amide bond in the CHI-TG conjugates.

According to the determination of thiol groups of conjugates using the iodometric microtitration assay, PC-CYS and CHI-TG were found to have $148.42 \pm 4.16\text{ }\mu\text{mol}$ and $41.17 \pm 2.34\text{ }\mu\text{mol}$ of thiol groups per gram of polymer, respectively.

Mechanical Properties of Gel Formulations

A syringe or applicator is required for successful administration of semi-solid formulations *via* the vaginal route. Upon this application, dosage forms are rapidly removed by vaginal secretion. Hence, the drug release of vaginally administered dosage forms is subjected to variations and difficult to predict (33). In order to qualify as a mucoadhesive formulation, several criteria such as the ease of removal of the gel from the primary package, the ease of application of the product to the desired region and the retention at the application site without disintegration need to be accomplished (34). The vaginal semi-solid formulations should also have appropriate mechanical properties to boost clinical efficiency. The mechanical properties such as hardness, compressibility, elasticity, cohesiveness and adhesiveness of gel formulations were investigated using texture profile analysis (32, 34–36) (Table II).

The hardness test was performed to measure the required force to produce deformation of gels. The hardness influences the applicability of the gel to the desired site.

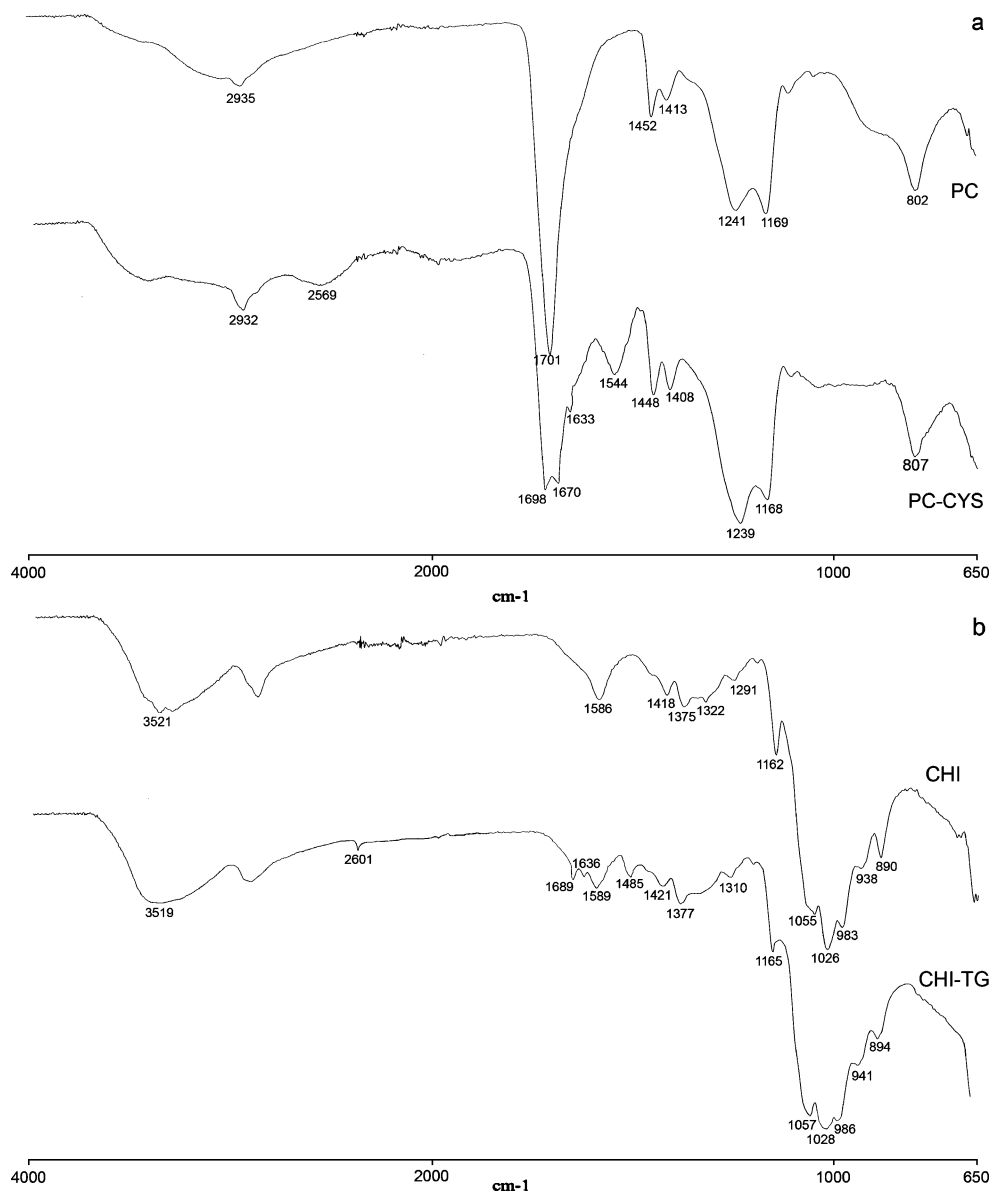


Fig. 1. FT-IR spectra of **a** polycarbophil and polycarbophil–cystein conjugate and **b** chitosan and chitosan–thioglycolic acid conjugate

Vaginal gels are required to have a low hardness value for ease of administration to the vagina (35). Ferrari *et al.* (37) described increased hardness of aqueous polymeric gels as a function of polymeric concentration. Jones *et al.* (38), reported that the product hardness were dependent on the concentration of polymers such as hydroxyethylcellulose (HEC), polyvinylpyrrolidone and PC in hydrogel formulations. It was also reported that the textural properties of gel formulations were significantly affected by the molecular weight, deacetylation degree and concentration of CHI (39).

Similar results were obtained in our study. We have found that the hardness values of the PC gels and CHI gels increased significantly by seven and four times, respectively, due to the increase in the polymer concentration from 2% to 4% for PC gels and from 2% to 3% for CHI gels ($p < 0.05$; Fig. 2). The addition of 0.2 g PC-CYS did not significantly change the hardness of gel formulations but when a higher amount of PC-CYS (0.5 g) was added, the hardness of PC

gels was slightly reduced ($p > 0.05$). However, the addition of CHI-TG conjugate did not significantly change the hardness of CHI gels ($p > 0.05$).

Compressibility is defined as the work that is required to achieve compaction of the product along a definite distance (29,40). A low compressibility value is required to enable ease of removal of the gel from the container and the ease of the spreadability of the gel on the application site (35,36). It was previously reported by several workers that increasing the concentration of each polymeric component significantly increased formulation compressibility (40–42). Jones *et al.* (43) also reported that the compressibility was affected by the concentration of HEC, carbopol and/or PC in gel formulations.

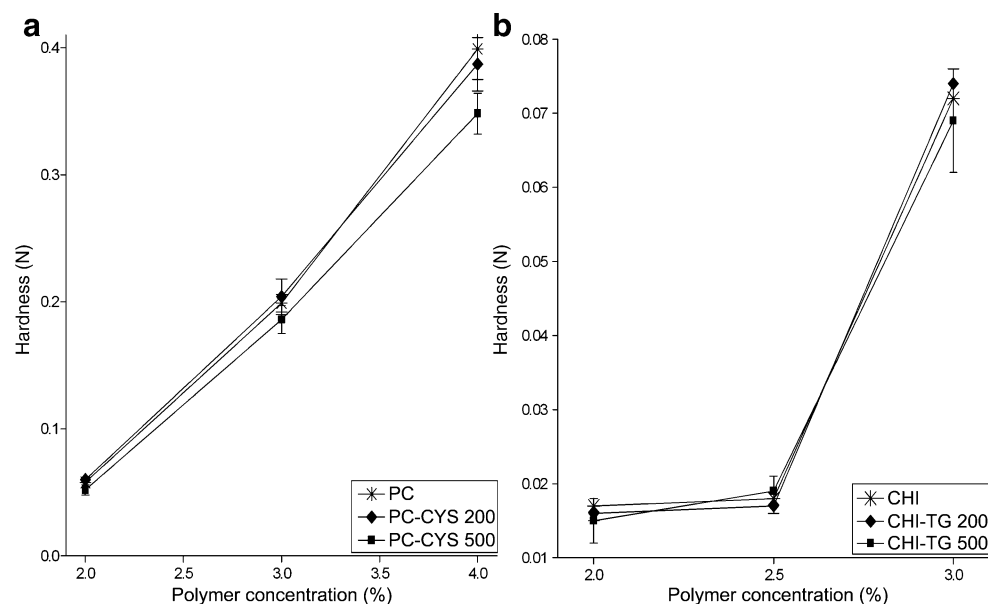
In this study, it was apparent that both concentration and type of polymer affected product compressibility. The results indicate that the compressibility of gels increased 6.5 times (PC1: 0.309 ± 0.028 Nmm; PC3: 2.032 ± 0.126 Nmm) and 21.5

Table II. Mechanical and Mucoadhesive Properties of Gel Formulations ($n=6$)

Formulation	Hardness $N \pm SD$	Compressibility (Nmm) $\pm SD$	Adhesiveness (Nmm) $\pm SD$	Cohesiveness $\pm SD$	Elasticity $\pm SD$	Work of Mucoadhesion ($mJ\ cm^{-2}$) $\pm SD$
PC1	0.058 \pm 0.004	0.309 \pm 0.028	0.182 \pm 0.011	0.919 \pm 0.035	0.590 \pm 0.034	0.063 \pm 0.003
PC2	0.199 \pm 0.007	1.235 \pm 0.107	0.428 \pm 0.052	0.865 \pm 0.030	0.609 \pm 0.045	0.074 \pm 0.007
PC3	0.399 \pm 0.024	2.032 \pm 0.126	0.666 \pm 0.048	0.856 \pm 0.024	0.665 \pm 0.040	0.092 \pm 0.007
PC-CYS1	0.060 \pm 0.002	0.312 \pm 0.034	0.219 \pm 0.018	0.922 \pm 0.039	0.602 \pm 0.048	0.082 \pm 0.005
PC-CYS2	0.204 \pm 0.014	1.225 \pm 0.132	0.472 \pm 0.059	0.868 \pm 0.026	0.605 \pm 0.053	0.098 \pm 0.005
PC-CYS3	0.387 \pm 0.021	2.005 \pm 0.130	0.790 \pm 0.054	0.859 \pm 0.029	0.676 \pm 0.036	0.118 \pm 0.009
PC-CYS4	0.052 \pm 0.007	0.285 \pm 0.016	0.234 \pm 0.012	1.034 \pm 0.022	0.575 \pm 0.026	0.110 \pm 0.008
PC-CYS5	0.186 \pm 0.011	1.112 \pm 0.059	0.519 \pm 0.043	1.018 \pm 0.015	0.582 \pm 0.021	0.136 \pm 0.011
PC-CYS6	0.348 \pm 0.016	1.834 \pm 0.112	0.856 \pm 0.051	1.002 \pm 0.026	0.625 \pm 0.032	0.209 \pm 0.010
CHI1	0.017 \pm 0.001	0.014 \pm 0.001	0.031 \pm 0.004	1.023 \pm 0.027	0.732 \pm 0.012	0.012 \pm 0.001
CHI2	0.018 \pm 0.001	0.022 \pm 0.006	0.041 \pm 0.011	1.012 \pm 0.014	0.744 \pm 0.016	0.017 \pm 0.001
CHI3	0.072 \pm 0.001	0.305 \pm 0.008	0.202 \pm 0.014	0.998 \pm 0.037	0.763 \pm 0.013	0.023 \pm 0.001
CHI-TG1	0.016 \pm 0.001	0.025 \pm 0.004	0.045 \pm 0.008	1.030 \pm 0.023	0.744 \pm 0.018	0.021 \pm 0.001
CHI-TG2	0.017 \pm 0.001	0.028 \pm 0.012	0.064 \pm 0.009	1.016 \pm 0.012	0.754 \pm 0.021	0.032 \pm 0.002
CHI-TG3	0.074 \pm 0.001	0.301 \pm 0.013	0.242 \pm 0.017	1.003 \pm 0.014	0.778 \pm 0.026	0.038 \pm 0.002
CHI-TG4	0.015 \pm 0.003	0.021 \pm 0.005	0.065 \pm 0.012	1.035 \pm 0.032	0.730 \pm 0.013	0.036 \pm 0.002
CHI-TG5	0.019 \pm 0.002	0.024 \pm 0.007	0.085 \pm 0.011	1.021 \pm 0.026	0.741 \pm 0.033	0.054 \pm 0.003
CHI-TG6	0.069 \pm 0.007	0.293 \pm 0.012	0.278 \pm 0.023	1.013 \pm 0.015	0.753 \pm 0.026	0.075 \pm 0.006

times (CHI1 0.014 \pm 0.001 Nmm; CHI3: 0.305 \pm 0.008 Nmm) for PC and CHI parent polymer, respectively, upon increasing the polymer concentration in gel formulations ($p<0.05$; Fig. 3). While the addition of the PC-CYS conjugate was slightly reduced the compressibility of PC gels (PC-CYS3: 2.005 \pm 0.130 Nmm; PC-CYS6: 1.834 \pm 0.112 Nmm), the compressibility was not changed in formulations containing CHI-TG conjugate (CHI-TG3: 0.301 \pm 0.013 Nmm; CHI-TG6: 0.293 \pm 0.012 Nmm). It was evident that the gel formulations containing CHI and its conjugate showed less compressibility value than gels containing PC and its conjugate (Fig. 3). When these results were compared with previous studies (36), all formulations had suitable compressibility in the point of view of applicability to the vaginal site.

The elasticity is defined as the direction of re-construction of the gel after its deformation by compression in a defined period of time (44). The increase in the numerical value of elasticity obtained during texture profile analysis shows the decrease in the elasticity of gel (43). The basic physical mechanism of bioadhesion is related to the elasticity of the polymer chains whereby a high elasticity is required for strong bioadhesion between the polymer and the mucosal surface (45). It was noted that the elasticity of gels is decreased due to the increase in the polymer concentration in the gels (43). The results indicated that the elasticity slightly reduced for PC gels (PC1: 0.590 \pm 0.034; PC3: 0.665 \pm 0.040) and CHI gels (CHI1: 0.732 \pm 0.012; CHI3: 0.763 \pm 0.013) upon increasing the polymer concentration in gel formulations (Fig. 4). The type of polymer

**Fig. 2.** Hardness of the gel formulations ($n=6$)

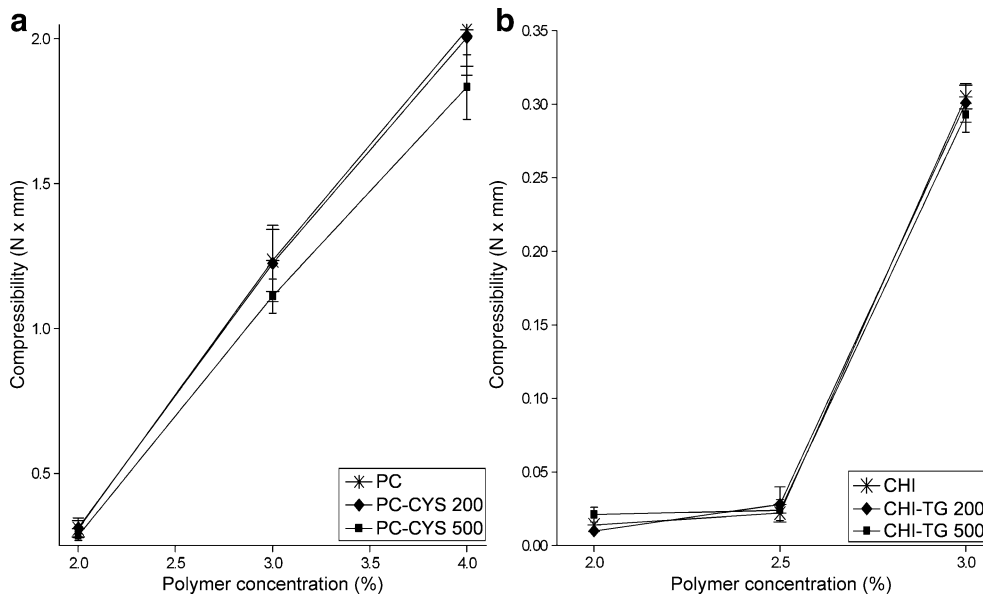


Fig. 3. Compressibility of the gel formulations (n=6)

also influenced the elasticity of gels. The elasticity of the PC and PC/PC-CYS gels were 1.24–1.35 times higher than that of CHI and CHI/CHI-TG gels. The addition of 0.5 g PC-CYS conjugate slightly increased the elasticity of gels at all concentrations of polymers tested (PC-CYS4: 0.575 ± 0.026 ; PC-CYS5: 0.582 ± 0.021 ; PC-CYS6: 0.625 ± 0.032). Interestingly, the addition of CHI-TG conjugates did not change the elasticity of CHI gels ($p > 0.05$; Fig. 4). It was seen that the elasticity of gel formulations containing PC (PC1-PC3) and its conjugate (PC-CYS1-PC-CYS6) was in more acceptable range (between 0.590 ± 0.034 and 0.676 ± 0.036) compared to that of gels (between 0.732 ± 0.012 and 0.778 ± 0.026) containing CHI (CHI1-CHI3) and its conjugate (CHI-TG1-CHI-TG6).

The vagina is an ideal site for the application of systemic and local drug administration due to its large surface area for drug absorption. The main disadvantage of the vaginal dosage

forms is their rapid removal from the vaginal system (33). The retention of most formulations in the vaginal site is limited due to the presence of vaginal secretions and weak cohesive forces (46). The cohesiveness of the gel is important to determine the reconstruction ability of the gel after application (40,46). The high cohesiveness value increases the performance of the product at the application site by providing full structural recovery following gel application (36). It was observed the greater reduction in cohesiveness of gel formulations containing the higher concentrations of each polymer such as HEC, carbopol or PC due to the proportionally greater semi-solid character of gels (40). In this study, the cohesiveness of PC gels (PC1: 0.919 ± 0.035 ; PC3: 0.856 ± 0.024) and CHI gels (CHI1: 1.023 ± 0.027 ; CHI3: 0.998 ± 0.037) was decreased slightly upon increasing the polymer concentration as previously described (40) (Fig. 5). The addition of 0.5 g PC-CYS

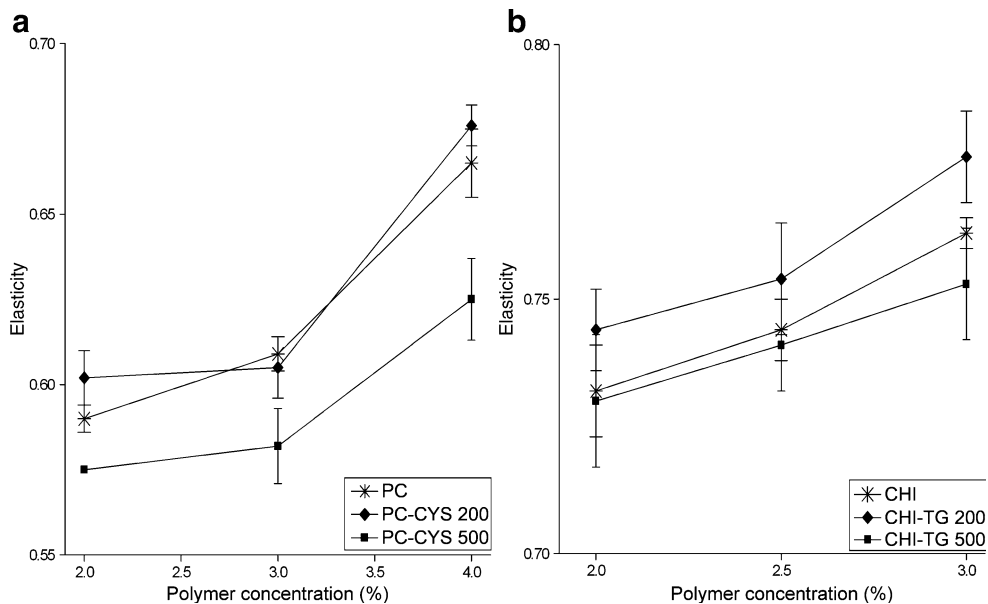


Fig. 4. Elasticity of the gel formulations (n=6)

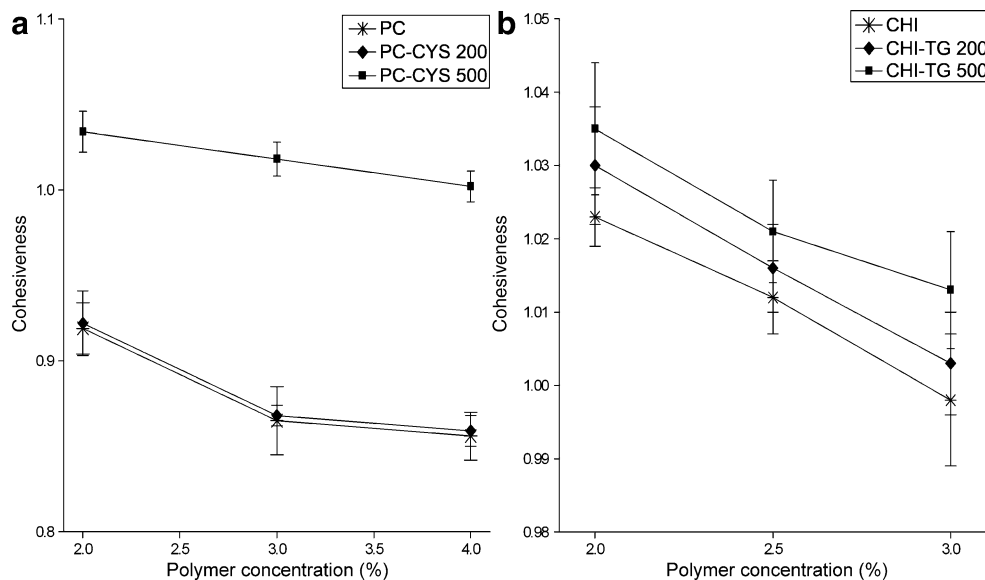


Fig. 5. Cohesiveness of the gel formulations ($n=6$)

conjugate increased the cohesiveness of gels at all concentrations of polymers tested (PC-CYS4: 1.034 ± 0.022 ; PC-CYS5: 1.018 ± 0.015 ; PC-CYS6: 1.002 ± 0.026). Similar results were obtained with gels containing 0.5 g CHI-TG conjugate (CHI-TG4: 1.035 ± 0.032 ; CHI-TG5: 1.021 ± 0.026 ; CHI-TG6: 1.013 ± 0.015) (Fig. 4). According to results, it was seen that the gel formulations containing highest amounts of PC-CYS conjugate (PC-CYS4 - PC-CYS6) and highest amounts of CHI-TG conjugate (CHI-TG4–CHI-TG6) showed highest and acceptable cohesiveness.

A product designed for topical application to mucous membranes should preferably possess adhesive properties, as these will enhance the time of location at the site of application and hence improved clinical efficiency (29). Adhesiveness in texture profile analysis is commonly defined as the work required to overcome the attractive forces between the surface of the sample and the probe surface (32). It was reported that the increase in gel adhesiveness

caused by an increase in polyacrylic acid concentration might be attributed to the greater ability of polyacrylic acid to chemically interact with the probe (36).

In our study, the type of polymer used in the preparation of gels influenced the adhesiveness of the resultant gel. PC gels were at least four times more adhesive than CHI gels when compared to gels prepared with same amount of polymer ($p < 0.05$). The adhesiveness of gels increased upon increasing the polymer concentration from 2% to 4% for PC gels (PC1: 0.182 ± 0.011 Nmm; PC3: 0.666 ± 0.048 Nmm) and from 2% to 3% for CHI gels (CHI1: 0.031 ± 0.004 Nmm; CHI3: 0.202 ± 0.014 Nmm). Addition of the PC-CYS conjugates at increasing amounts were found to increase the adhesiveness of PC gels, particularly significant improvement in adhesion was observed when the highest concentration of polymer (4%) were used whereby the adhesion results were 0.666 ± 0.048 Nmm and 0.856 ± 0.051 Nmm for PC6 and PC-CYS6 formulations, respectively ($p < 0.05$; Fig. 6). There was no

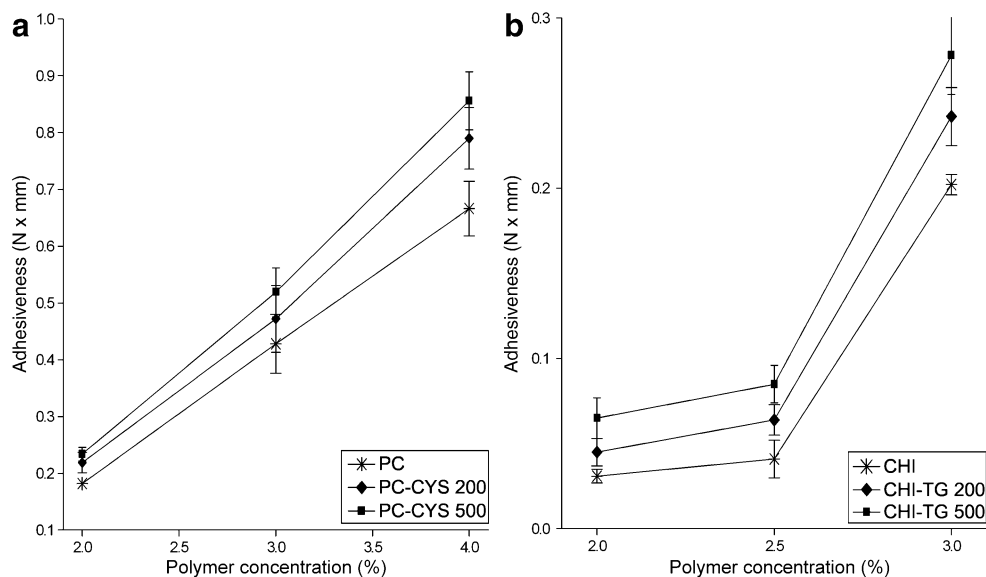


Fig. 6. Adhesiveness of the gel formulations ($n=6$)

significant difference in adhesiveness of CHI gels prepared using 2% (CH1: 0.031 ± 0.004 Nmm) and 2.5% (CH2: 0.041 ± 0.011 Nmm) polymer ($p > 0.05$), but adhesiveness of gels was significantly increased when 3% polymer was used (CH3: 0.202 ± 0.014 Nmm) ($p < 0.05$). Addition of 0.5 g CHI-TG conjugate increased the adhesiveness of gel formulations at all concentrations of polymers tested ($p < 0.05$; Fig. 6). While the lowest adhesiveness was seen for CHI1 (0.031 ± 0.004 Nmm), PC-CYS3 (0.790 ± 0.054 Nmm) and PC-CYS6 (0.856 ± 0.051 Nmm) formulations exhibited the highest adhesiveness (Table II).

Mucoadhesion Studies

The mucoadhesion test was performed to measure the adhesive strength of gel formulations to the vaginal mucosa. Importantly, factors such as the type, molecular weight and architecture of polymer and the polymer concentration in the gel can influence the mucoadhesive performance of the formulation (5,21). PC was reported to possess good bio-adhesive properties (40). Chang *et al.* (47) developed the mucoadhesive thermosensitive gel formulations composed of poloxamers 407, 188 and PC for the effective treatment of vaginal candidiasis and reported that the formulations with higher contents of PC had shown stronger mucoadhesiveness. CHI was also reported to be a linear polycation that readily adheres to negatively charged surfaces at acidic pH simulating vaginal conditions (48).

In our study, when the polymer concentration was increased from 2% to 4% for PC gels and from 2% to 3% for CHI gels, the mucoadhesion ability was also increased (Table II, Fig. 6). The type of polymer used in the preparation of gels influenced the mucoadhesion ability of gels and PC gels were found more mucoadhesive than CHI gels.

These findings were in accordance with literature (32,49). It was reported that due to the existence of more than 90% of the acid groups of polyacrylic acids in non-ionized form at pH 5 and below, polymer cannot be charged electrostatically and exhibits lower swelling ability. Hence, the polymer binds directly to polysaccharides or proteins with

hydrogen bonds and increases the mucoadhesion (32). On the contrary, Bonferoni *et al.* found that the higher lactic acid content reduce ionisation of the acidic moieties of sialic acids of the mucin chains which are usually known to contribute to strengthening the mucoadhesive bonds with cationic polymers such as CHI and correspond to poor mucoadhesiveness (49). In this study, gels were prepared in lactate buffer and this could be the reason of limited interaction between CHI and vaginal mucosa.

The addition of increasing amount of thiol conjugates significantly enhanced the mucoadhesion ability of parent gels. For example, the work of mucoadhesion for the 4% polymer gels containing PC alone, PC plus 0.2 g conjugate (PG-CYS3) and PC plus 0.5 g conjugate (PG-CYS6) were 0.092 ± 0.007 mJ cm⁻², 0.118 ± 0.009 mJ cm⁻² and 0.209 ± 0.010 mJ cm⁻², respectively ($p < 0.05$). Similar results were obtained with gels containing CHI. Upon addition of CHI-TG conjugates at increasing amounts, the work of mucoadhesion of gel formulations containing CHI were also increased from 0.038 ± 0.002 mJ cm⁻² (CHI-TG3) to 0.075 ± 0.006 mJ cm⁻² (CHI-TG6; $p < 0.05$), albeit higher than that of CHI alone (CH3: 0.023 ± 0.001 mJ cm⁻²; $p < 0.05$). The addition of thiol conjugate was found to have a higher effect on mucoadhesion ability in PC gels than in CHI gels. Since the presence of thiol groups were reported to enhance the mucoadhesive ability of the parent polymer (25,27,28), it was believed that the higher amounts of thiol groups per gram of polymer in the PC conjugates (148.42 ± 4.16 μmol) compared to that of CHI conjugates (41.17 ± 2.34 μmol) may have enhanced the mucoadhesive ability of the former. PC-CYS6 formulation has exhibited the highest mucoadhesive property (0.209 ± 0.010 mJ cm⁻²) in all formulations analysed (Fig. 7).

Drug Release Studies

According to the *in vitro* drug release study results, the release of CLM from gels was found to decrease with increasing concentrations of polymer in PC and CHI gel formulations.

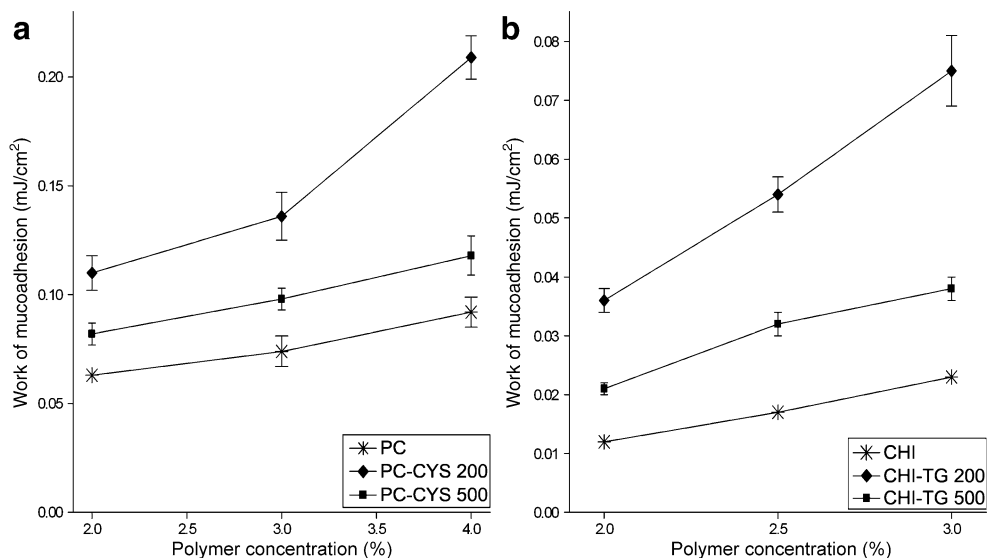


Fig. 7. Work of mucoadhesion of the gel formulations ($n=6$)

PC and its thiol conjugate were found to prolong the release of CLM longer than 72 h unlike gel formulations prepared using CHI and CHI-TG which were found to release CLM in only 9–12 h. 49.8%, 39.2% and 30.5% CLM release were achieved in 72 h for the formulations composed

of 2%, 3% and 4% PC (PC1-PC3), respectively (Fig. 8a). The amount of CLM released from formulations including CHI was 100% at 8th h for CHI1, 100% at 10th h for CHI2 and 92.1% at 12th hour for CHI3 (Fig. 8b). In comparison of the *in vitro* drug release profiles, a significant difference was

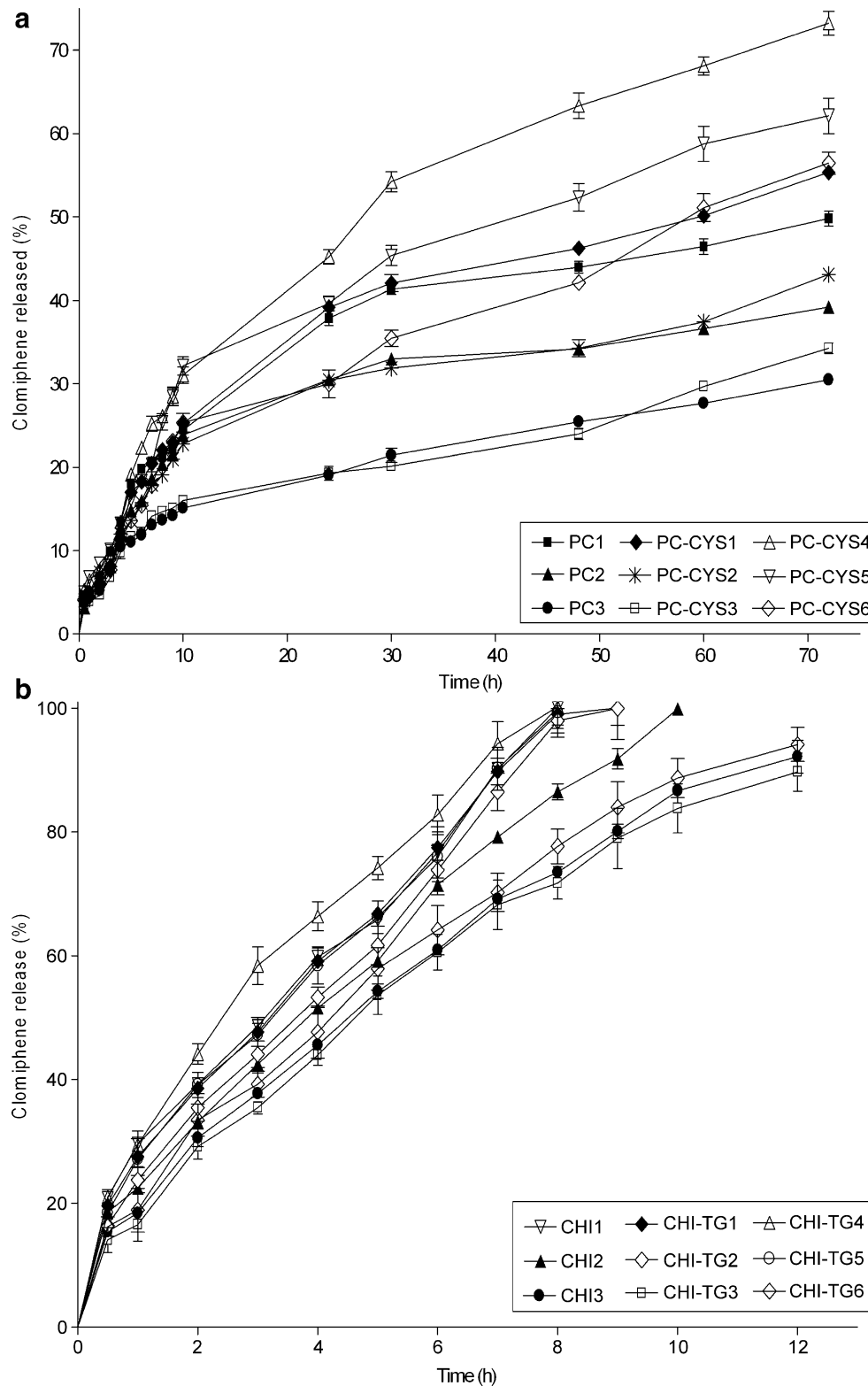


Fig. 8. *In vitro* drug release profiles of CLM from the gel formulations including **a** polycarbophil and polycarbophil–cystein conjugate and **b** chitosan and chitosan–thioglycolic acid conjugate ($n=3$)

determined between PC and CHI gel formulations ($p < 0.05$). Upon addition of conjugate at increasing amounts, the drug release rate from PC gels (PC-CYS1–PC-CYS6; Fig. 8a) and CHI gels (CHI-TG1–CHI-TG6; Fig. 8b) was increased accordingly ($p < 0.05$).

The drug release from gels formulated using 0.2 g PC-CYS conjugate (PC-CYS1–PC-CYS3) and from gels formulated using 0.5 g conjugate (PC-CYS4–PC-CYS6) was, respectively, 10.0–12.5% ($p < 0.05$) and 47.0–85.5% ($p < 0.05$) higher than formulations including the same amount of the polymer without conjugate (PC1–PC3). However, when the formulations including CHI-TG conjugate (CHI-TG1–CHI-TG6) were compared with formulations including the same amount of the parent polymer (CHI1–CHI3), no significant difference was seen between the drug release profiles ($p > 0.05$).

Stability Test

The stability of CLM was observed by keeping all gel formulations in stability cabinet at $25 \pm 2^\circ\text{C}$, $60 \pm 5\%$ RH; $30 \pm 2^\circ\text{C}$, $65 \pm 5\%$ RH; $40 \pm 2^\circ\text{C}$, $75 \pm 5\%$ RH for 3 months. According to the stability test, no significant difference in the CLM concentrations of all formulations ($p > 0.05$) were observed and the amount of CLM was determined between

$98.76 \pm 2.15\%$ and $103.07 \pm 2.48\%$ at the end of the stability study. Stability data of CLM in PG-CYS6 and CHI-TGA6 formulations was given in Fig. 9 (Data belong to other gel formulations are not shown).

CONCLUSION

HPV is a sexually transmitted disease and the infected patients may develop venereal warts in the genital region. CLM is a non-steroidal ovulation-promoting substance and acts by competitively inhibiting the binding of HPV to the estrogenic receptors in cervix glandular cells, metaplastic cells and the basal cells of the squamous epithelium. Due to this effect, CLM is highly effective in the treatment of HPV infections. The clinical efficiency of CLM in HPV treatment could be increased by delivering CLM *via* mucoadhesive vaginal delivery systems.

Mucoadhesive systems could increase the retention time of the CLM at the application site and reduce clinically beneficial dose. These sustained drug delivery systems may also improve patient compliance by reducing the frequency of administration. In this study, the mucoadhesive gel formulations of CLM using PC and CHI and their conjugates including thiol groups were prepared and their mechanical,

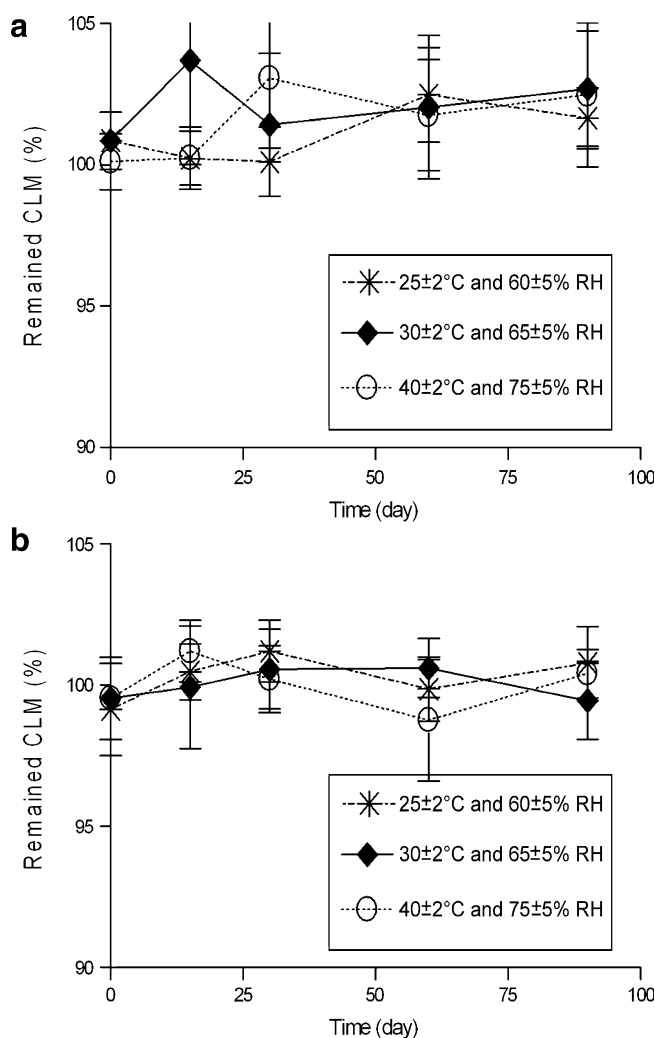


Fig. 9. Stability graph of CLM in **a** PG-CYS6 and **b** CHI-TGA6 formulations

mucoadhesive properties, drug release characteristics were evaluated. By increasing the polymer concentrations, gels with higher hardness, compressibility, adhesiveness and mucoadhesion ability can be achieved. Furthermore, a slower rate of CLM release from gels was achieved when the polymer concentrations were increased in the gel formulations. PC and its thiol conjugates were found to prolong the release of CLM longer than 72 h unlike gel formulations prepared using CHI and its thiol conjugates which were able to release CLM up to 12 h. The increasing in the amount of the thiol conjugates was found to enhance the elasticity, cohesiveness, adhesiveness and mucoadhesion of the gel formulations but not its hardness, compressibility when compared to gels prepared using their respective parent formulations. The type of polymer used to prepare gels had a profound effect on the mechanical properties of gels. PC gels are harder, more elastic and adhesive than CHI gels, although PC gels have roughly similar cohesiveness as CHI gels. Stability of CLM was preserved in all the formulations tested after incubation in a stability cabinet at $25 \pm 2^\circ\text{C}$, $60 \pm 5\%$ RH; $30 \pm 2^\circ\text{C}$, $65 \pm 5\%$ RH; $40 \pm 2^\circ\text{C}$, $75 \pm 5\%$ RH for 3 months. On the basis of the obtained data PC-CYS6 formulation was found as the most appropriate formulation in the vaginal treatment of HPV infection due to its suitable mechanical properties, exhibiting high cohesion and mucoadhesion and releasing the drug *in vitro* longer than 72 h.

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