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ORIGINAL ARTICLE

Thermosensitive mucoadhesive gel formulation loaded with 5-Fu: cyclodextrin complex for HPV-induced cervical cancer

Erem Bilensoy · Yasemin Çırpanlı · Murat Şen · A. Lale Doğan · Sema Çalış

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Abstract Human Papilloma Virus (HPV) infections are the major cause of cervical cancers. To achieve a better therapeutic efficacy and patient compliance in the treatment for HPV-induced cervical cancers, anticancer agent 5-fluorouracil has been formulated in a vaginal gel using the thermosensitive polymer Pluronic® F127 together with alternative mucoadhesive polymers e.g., hyaluronic acid, Carbopol 934 and hydroxypropylmethylcellulose. To increase its aqueous solubility and to achieve the complete release of 5-FU from the gel, the drug was incorporated as its inclusion complex with 1:1 molar ratio with either β-cyclodextrin or hydroxypropyl-β-cyclodextrin. Following the characterization of drug:CD complexes, thermosensitive gel formulations containing different mucoadhesive polymers and the drug in free or complexed form were characterized in vitro by determining the gelation temperature and the rheological behavior of different formulations along with the in vitro release profiles of these formulations in pH 5.5 citrate buffer. It was observed that complexation with cyclodextrin accelerated the release of 5-FU with the exception of formulation containing Carbopol 934 as mucoadhesive polymer. As far as rheological properties are concerned, favorable thermosensitive in situ gelling properties were obtained with formulations containing HPMC as mucoadhesive polymer. Complete release of 5-FU from gels were obtained with both complexes of β-CD and HP-β-CD and cytotoxicity studies against HeLa human cervical carcinoma cells demonstrated that 1% 5-FU:CD complexes were equally effective as 1% free 5-FU indicating better therapeutic efficacy with lower dose.

Keywords 5-Fluorouracil · β-Cyclodextrin · Hydroxypropyl-β-cyclodextrin · Thermosensitive · Mucoadhesive · Cyclodextrin · Vaginal gel · Cervical cancer · HPV

E. Bilensoy (✉) · Y. Çırpanlı · S. Çalış
Faculty of Pharmacy, Department of Pharmaceutical Technology, Hacettepe University, 06100 Ankara, Turkey
e-mail: eremino@hacettepe.edu.tr

M. Şen
Department of Chemistry Polymer Chemistry Division, Hacettepe University, 06532 Beytepe, Ankara, Turkey

A. L. Doğan
Department of Basic Oncology, Hacettepe University Oncology Institute, 06100 Ankara, Turkey

Introduction

Cervical cancer which is mainly associated with Human Papilloma Virus (HPV) infection is the second most common cause of cancer death among women worldwide affecting approximately 1% of all female population [1–4]. In women under the age of 50 years, cervical cancer is reported to be the most common cause of death [5–6]. Genital HPV infections are widespread in the general population. Anogenital warts are the most common clinical disease however the majority of HPV-infected individuals have only sub-clinical disease or latent infection. Genital warts are caused in almost all cases by HPV 6 and 11 although a proportion of 40% will be co-infected with high oncogenic risk HPV types 16 and 18 [3]. These latter HPV subtypes usually cause subclinical or latent

infections and are predominant viral types associated with squamous intraepithelial lesions and in particular cervical or other anogenital carcinomas [3, 7]. As many as 95% of cervical carcinomas contain HPV DNA, particularly types 16 and 18 [8].

Briefly, therapy regimens for HPV-related genital diseases include the administration of antiprophylactic agents such as podophyllin/podophyllotoxin [9], cidofovir [10] or 5-fluorouracil (5-FU) [11, 12] in the form of gels, creams or pessaries either topically or by intralesional injection. Destructive therapy modes include cryotherapy, application of trichloroacetic acid, scalpel, curette or scissor excisions, electrosurgical techniques, laser therapy and photodynamic therapy [13]. Another recent approach is the administration of immunomodulators or vaccines among which interferons and imiquimod are widely studied and were proved to be significally indifferent than ablation or cryotherapy techniques [6, 13–16]

Vaginal drug delivery systems comprise creams, gels, tablets, foams, pessaries and irrigations, which are believed to be of limited efficacy depending on the residence time at genitourinary tract. Conventional vaginal delivery systems are removed rather rapidly by the self-cleansing action of the vaginal tract [17, 18]. Moreover, the physiology arising from the protective mechanism of the genitourinary tract limiting residence time and impairing therapeutic efficacy of the drug make multiple and frequent administration necessary for treatment. Another important challenge in vaginal delivery is patient compliance when administering the dosage forms and following the repeated dose therapeutic regimen. Patients are generally reported to tolerate gels better than other dosage forms [19] however it is believed that vaginal therapy can be significantly improved if a delivery system can retain the drug at site of administration for a prolonged period compared to conventional dosage forms.

Mucoadhesive vaginal gels containing bioadhesive polymers e.g., polycarbophil, carbopol, hydroxypropylcellulose and polyvinylpyrrolidone have been incorporated into in situ-gelling thermosensitive systems prepared with Pluronic® F127 [20–24]. Different techniques are used in thermosensitive drug delivery systems including dispersing the drug in the gel with a concentration exceeding the drug's solubility value or by dispersing a colloidal carrier system and drug: cyclodextrin complexes which help solubilize and stabilize active ingredients [25–28].

Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch. The special structure of cyclodextrins composed of a truncated cone with an apolar cavity and a hydrophilic external part conveys inclusion forming capability to natural and modified cyclodextrins. Hydrophobic molecules can be included in the apolar cavity. By the formation drug:CD complex, the physicochemical properties of the drug such as aqueous solubility, stability unwanted side effects, taste or odor [29, 30]. Cyclodextrins are also reported to convey controlled release properties to certain active ingredients. Natural cyclodextrin, β-cyclodextrin (β-CD) and its most frequently used derivative hydroxypropyl-β-cyclodextrin (HP-β-CD) are used to solubilize and stabilize active ingredients by the formation of drug:CD inclusion complexes [31].

The objective of this study was to design a vaginal gel formulation loaded 5-FU with thermosensitive and mucoadhesive properties to ensure longer residence at HPV-infection site, the vagina and the genital tract. At the same time, providing a favorable release profile for the anticancer drug 5-FU via complexation to cyclodextrins to reduce side effects associated with 5-FU application and to provide an efficient therapy for HPV-related diseases such as cervical cancer or genital warts with a lower dose was considered an onjective in this study.

Material and methods

Materials

Anticancer active ingredient 5-fluorouracil (5-FU) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) were supplied from Sigma-Aldrich, St. Louis, USA. β-cyclodextrin (β-CD) (Kleptose®) was supplied by Roquette Frères, Lestrem, France. 5-FU, HP-β-CD and β-CD are depicted in Fig. 1a, b and c, respectively. Pluronic® F127 (PF127) was purchased from Sigma-Aldrich, St. Louis, USA, Carbopol 934PH (CP) was of pharmaceutical grade purchased from BF Goodrich, Cleveland, USA and Hydroxypropylmethylcellulose (HPMC) USP was purchased as Metolose® 90SH 15,000 from Seppic, Paris, France. Hyaluronic acid sodium salt (HA) was supplied from Sigma Chemicals, Steinheim, Germany. All other reagents were of analytical grade and used without further purification.

HeLa An1 cervical carcinoma cell line (catalogue no: 92 12 30 01) was purchased from Foot-and-Mouth Disease Institute (Ankara) of Ministry of Agriculture and Rural Affairs of Turkey Cell Culture and Virus Bank.

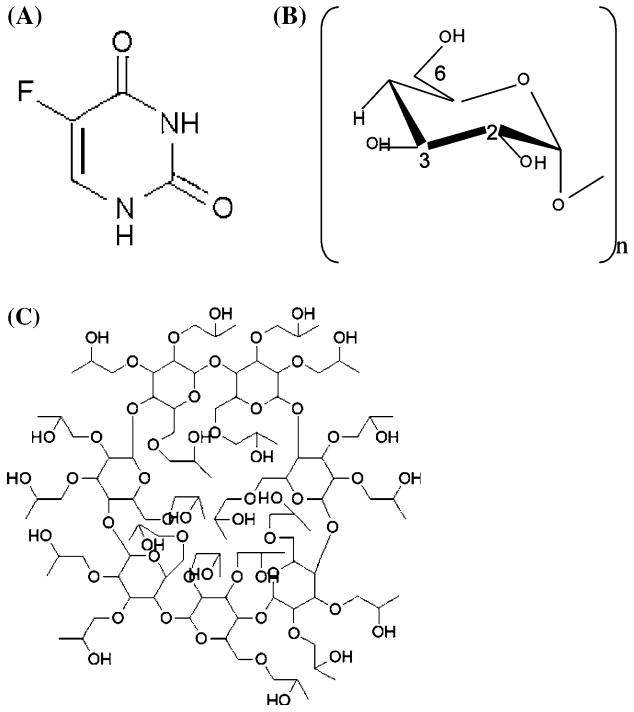

Fig. 1 Structure of anticancer drug 5-FU (**a**), β-cyclodextrin glucopyranose unit (**b**) and HP-β-CD (**c**)

Methods

Preparation of 5-FU:β-CD and 5-FU:HP-β-CD complexes

Inclusion complexes of 5-FU with β-cyclodextrin (1:1 molar ratio) and with HP-β-CD (25% drug content) were prepared according to co-lyophilization technique [32]. According to this technique, fixed amount of 5-FU was weighed and transferred to round-bottomed flask. β-CD/HP-β-CD solution in distilled water was added onto 5-FU and the powder was dispersed evenly. Upon ensuring that all drug powder is wetted by the β-CD/HP-β-CD aqueous solution, the dispersion then was left to equilibrate for 7 days at room temperature under constant stirring. At the end of equilibrium time, dispersion was filtered and filtrate containing soluble 5-FU:β-CD complex (1:1) or 5-FU:HP-β-CD (25%) was lyophilized using a HETO Lyolab Freeze Dryer, UK to obtain the complex in dry powder form.

Characterization of 5-FU:β-CD and 5-FU:HP-β-CD complexes

FT IR spectrophotometry FT IR spectra of 5-FU, β-CD, HP-β-CD and 5-FU:β-CD and 5-FU:HP-β-CD complexes were taken with a Nicolet 520 FTIR Spectrophotometer, USA using discs of each sample and previously prepared KBr containing 0.01 g sample in 0.1 g of potassium bromide between the wavelengths 400–4,000 cm^{-1}.

DSC analysis DSC analyses were performed on lyophilized samples of 5-FU, β-CD and HP-β-CD and corresponding complexes with a DuPont 910 model Differential Scanning Calorimeter, USA. Each sample weighing approximately 3 mg was heated in hermetically sealed aluminum pans at a rate of 10°C/min up to 200°C in a dynamic nitrogen atmosphere.

Preparation of 5-FU gel formulations

Thermosensitive and mucoadhesive 5-FU gel formulations were prepared according to the cold method [33]. Briefly, mucoadhesive polymer (CP, HA or HPMC) (0.2% w/v) was slowly added to citrate phosphate buffer (0.1 M, pH 4.0) at 4°C with gentle mixing. Pluronic® F127 (20% w/v) was then added to CP, HA or HPMC solution and allowed to dissolve overnight at 4°C. About 5-FU in free form (1%) or as 5-FU:β-Cd and 5-FU:HP-β-CD complexes were added and dissolved by gentle mixing into cold PF127 solution containing 0.2% mucoadhesive polymer.

Characterization of 5-FU gel formulations

Gelling temperature Gelling temperature of the formulations were determined as follows; a 20 ml transparent vial containing a magnetic bar in 5 ml of Pluronic®F127 gel was placed in a water bath. A thermometer connected to a thermistor was immersed into the gel, which was heated at a rate of 2°C/min with constant (150 rpm) stirring. When the magnetic bar stopped moving due to gelation, the temperature displayed on the thermistor was determined as the gelation temperature [34, 35].

In vitro drug release The in vitro release of free and complexed 5-FU was determined from different vaginal gel formulations using a dialysis bag placed in a sealed glass vial under constant magnetic stirring. The gel formulations (0.75 g) were packed into the dialysis bags (Spectra/Por Cellulose Ester Membrane MWCO: 100,000 Da, Spectrum Labs, Rancho Dominguez, CA) sealed with closures of 50 mm (Spectrum Labs). The release medium was 100 ml pH 5,5 citrate buffer providing sink conditions for 5-FU. The medium was

maintained at 37°C and stirred at 100 rpm. At various time intervals, 5 ml of dissolution fluid was collected. Levels of 5-FU in the samples were analyzed with a Shimadzu UV–VIS 160A spectrophotometer at λ = 266 nm. The exact amount of 5-FU released from the formulation was calculated with a calibration curve obtained through an analytically validated method (r^2: 0.9993).

Cytotoxicity studies of 5-FU in free and complexed form

HeLa An1 human cervical carcinoma cells were maintained in DMEM supplemented with 10% Fetal Calf Serum at 37°C in a humidified incubator containing 5% CO_2. Confluent cell monolayers were trypsinized and cells in exponentially growing phase were used in cytotoxicity experiments. The cytotoxicity of free and complexed 5-FU against HeLa cells was assessed using MTT assay. Free 5-FU was of 1% solution (0.1 g 5-FU in 10 ml distilled water). However, 5-FU:β-CD and 5-FU:HP-β-CD complexes were of 1% complex concentration in aqueous solution e.g., 0.1 g complex in 10 ml distilled water. MTT assay measures the ability of viable cells to reduce a water-soluble, yellow tetrazolium salt (MTT) into a purple, insoluble formazan product. The color reaction is used as a measure of cell viability and proliferation. HeLa An1 cells in culture medium were seeded in 96-well plates and following incubation for adherence at 37°C in 5% CO_2, 20 μl of drug solution or formulations were added into the wells.

After 48 h incubation, 20 μl MTT solution (5 mg/ml) was added to each well and the plates were incubated for further 2 h. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 100 μl of DMSO was added to each well. The plates were then shaken and the optical density (OD) was read on Σ960 ELISA reader at test wavelength of 570 nm. Cells incubated in culture medium alone served as a control for cell viability (untreated wells).

Results and discussion

The formation of a total or partial inclusion complex between 5-FU and the cyclodextrins; β-CD or HP-β-CD, were evaluated by different techniques such as DSC, FTIR, SEM and XRD. An indicative for the formation of a drug:CD complex is the changes observed for the FTIR spectra of the drug in complex form. Figure 2a and b give the FTIR spectra of 5-FU, β-CD and 5-FU:β-CD complex and 5-FU, HP-β-CD and 5-FU:HP-β-CD complex respectively. FTIR spectra of the complexes show different characteristics than the free drug. In particular, characteristic peaks of C–F bond at 1,100 cm^{-1} and aromatic ring regions at 500–600 cm^{-1} and 2,500–3,000 cm^{-1} together with C=C stretching bond at 1,300–1,550 cm^{-1} have disappeared in the spectra of both complexes. However, the C–F singlet is present in the 5-FU:HP-β-CD suggesting a partial inclusion of the drug or the presence of some free drug in the lyophilizate. For the demonstration of a drug:CD inclusion complex, results should be confirmed with different and independent techniques. DSC thermograms shown in Fig. 3a and b indicate the thermal behavior of the lyophilized free drug, the cyclodextrins and the co-lyophilizates in a range of 20–300°C. It can be observed from the thermograms that sharp melting endotherm of 5-FU at approximately 285°C followed by decomposition is not present in the DSC thermograms of the complexes. However, the complexes reflect the decompositions of β-CD and the wide endotherm of decomposition of HP-β-CD statistical mixture.

After the characterization of inclusion complex between 5-FU and the two CD derivatives, thermosensitive gel formulations containing different mucoadhesive polymers were prepared and evaluated for their in vitro properties. All gel formulations were prepared with the cold method, however, the tested formulations contained the drug either in free form or complexed to β-CD or HP-β-CD with the presence of a mucoadhesive polymer HA, CP or HPMC. It was observed that formulation components such as mucoadhesive polymer type had a significant influence on gelling properties and rheological behavior.

Table 1 displays the gelling temperatures of different formulations for 5-FU loaded thermosensitive mucoadhesive gels. It is observed that gels prepared with 0.2% hyaluronic acid did not display thermosensitive properties. The expected in situ gelling behavior over room temperature was not observed in hyaluronic acid formulations, which were already in gel state at 8°C.

Table 1 displays the significant differences in gelation temperature for different formulations. Gelation temperature is dependent largely on polymer content. Together with the high percentage of Pluronic® (20%) known to lower gelation temperature [28] excess amount of polymer content with the presence of mucoadhesive polymers cause the formation of gels at even 4°C. On the other hand, as seen in Table 1, mucoadhesive polymer type seems to have a significant effect on gelation temperature. Hyaluronic acid having a higher viscosity forms gels at 4°C, however, HPMC

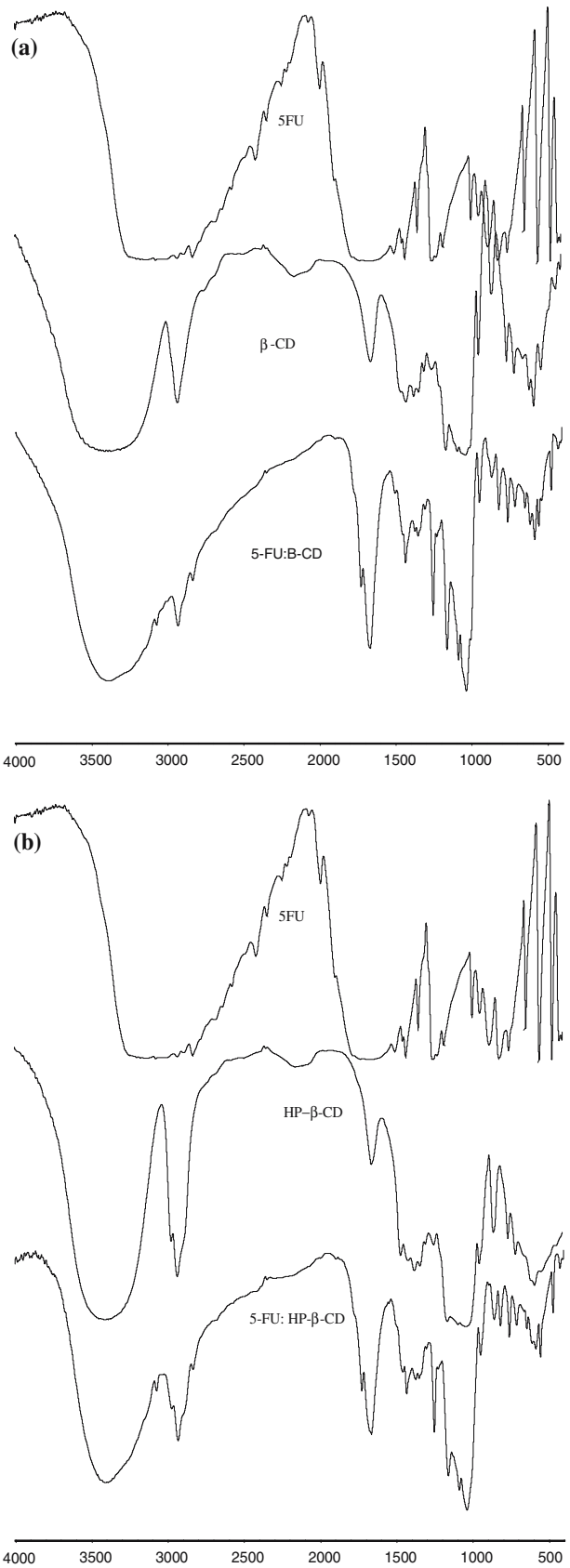

Fig. 2 FT IR spectra of (**a**) 5-FU, β-CD and 5-FU:β-CD complex and (**b**) 5-FU, HP-β-CD and 5-FU:HP-β-CD complex

Table 1 Gelation temperatures (°C) of different 5-FU gel formulations

Formulation	Gelation temperature (°C)
HPMC-5-FU	25
HPMC-5-FU:β-CD	28
HPMC-5-FU:HP-β-CD	23
CARBOPOL-5-FU	20
CARBOPOL-5-FU:β-CD	23
CARBOPOL-5-FU:HP-β-CD	25
HA-5-FU	8
HA-5-FU:β-CD	4
HA-5-FU:HP-β-CD	4

or Carbopol is shown to give more favorable thermosensitive properties. The physicochemical status of the drug whether in free form or in complex with cyclodextrins do not affect the gelling temperature significantly. In previous studies, Carbopol was reported to interact strongly with low molecular weight drugs [35]

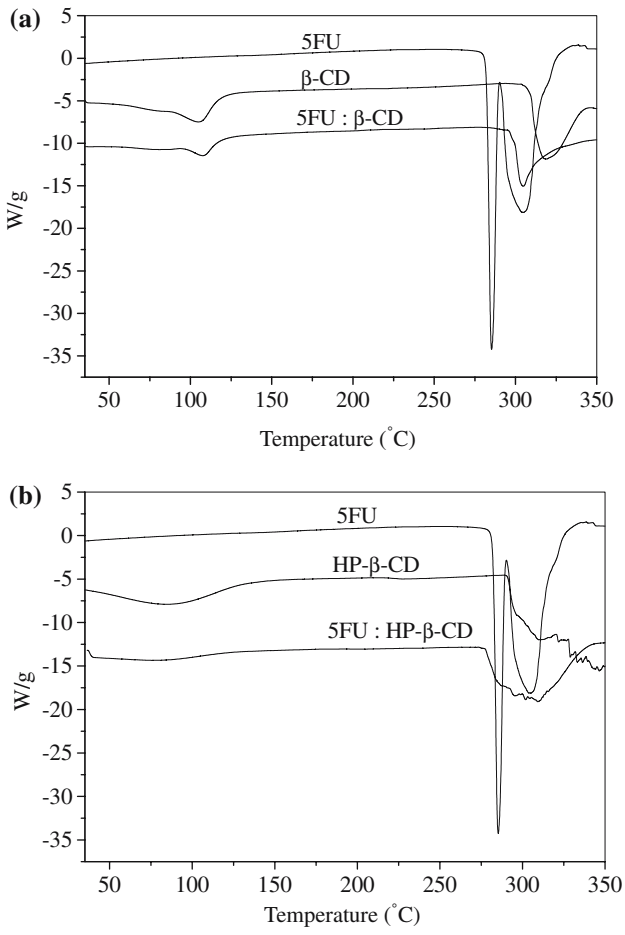

Fig. 3 DSC thermograms of (**a**) 5-FU, β-CD and 5-FU:β-CD complex and (**b**) 5-FU, HP-β-CD and 5-FU:HP-β-CD complex

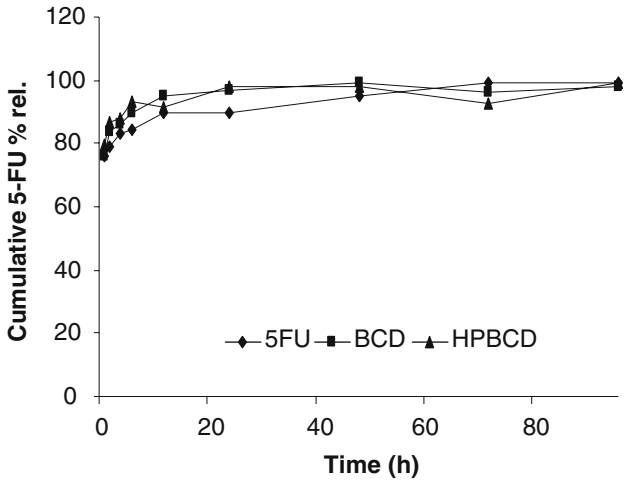

Fig. 4 In vitro release profiles of hyaluronic acid containing 5-FU vaginal gels at 37°C1 in pH 5.5 citrate buffer

however this was not the case for 5-FU complexed to β-CD or HP-β-CD as seen in Table 1.

Rheological properties of gels are largely affected by their polymer content and the thickening behavior of the mucoadhesive polymer used in the formulation. It can also be observed that the presence of macromolecules β-CD and HP-β-CD contributed significantly to the overall viscosity and the flow properties of the formulations causing an increase in the viscosity of gels containing the drug complexed to cyclodextrins as seen in Table 1.

In vitro release experiments were realized on all gel formulations. Figures 4, 5 and 6 represents the in vitro release profiles of 5-FU thermosensitive gel formulations prepared with HPMC, hyaluronic acid and Carbopol, respectively. Complexation to β-CD and HP-β-CD was performed in order to enhance the aqueous solubility of anticancer drug 5-FU. It was

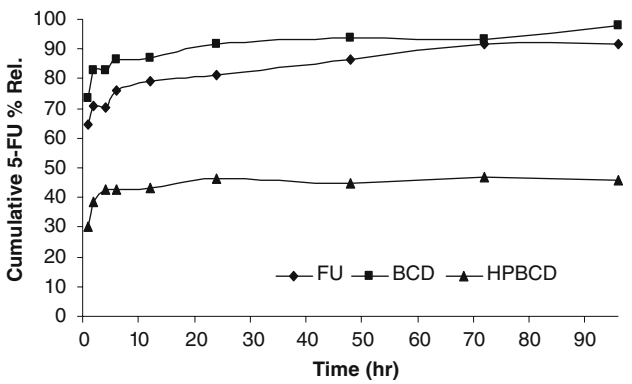

Fig. 5 In vitro release profiles of carbopol containing 5-FU vaginal gels at 37°C1 in pH 5.5 citrate buffer

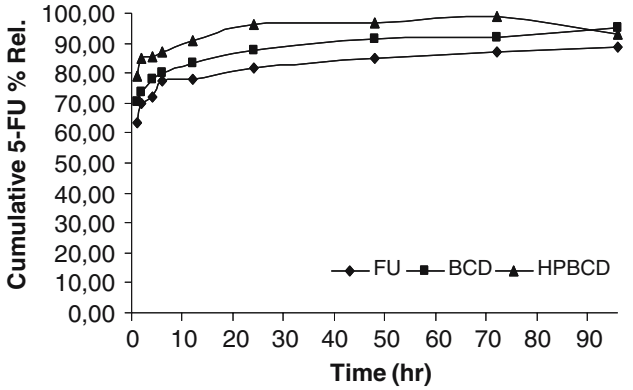

Fig. 6 In vitro release profiles of hydroxypropylmethyl cellulose containing 5-FU vaginal gels at 37°C in pH 5.5 citrate buffer

reported preveiously that although the rate of dissolution of Pluronic® gel is actually the controlling factor in drug release, it is not the only effect. Because the interface is eroding at a constant rate, surface concentration of the drug is held relatively constant and drug diffuse out at a constant rate [36], which is the case in our study. More concentrated gels were reported to dissolve at a slower rate than less concentrated ones because of the decreased water diffusion coefficient for the rate of water diffusing into the gel [36]. Release from the complex is, on the other hand, a competitive process controlled by diffusion of the drug upon dilution and competitive displacement of the drug by components in the dissolution media [37]. These two factors may have contributed to the controlled release of 5-FU from the gel in complexed and free form within a period of 12 h continuing until 92 h. As an exception, 5-FU:HP-β-CD complex formulated in Carbopol resulted in a slow release profile which could be due to the reported interaction of cyclodextrins with carbopol [34, 36]

Cytotoxic activity of 5-FU in free form and in complex form were evaluated against human cervical epithelial carcinoma cells (HeLa An1) with an MTT assay. Figure 7 represents the anticancer efficacy of 1% 5-FU solution and 1% 5-FU:β-CD or 1% 5-FU:HP-β-CD solution against HeLa cells. As can be seen from the figure, all three tested formulations have significantly indifferent cytotoxic effects. This presents an advantage for the 5-FU complexed to cyclodextrins because equivalent cytotoxic effect is obtained with a much less drug dosage meaning that a 10-fold increase in the anticancer activity is observed through complexation to cyclodextrins which is also confirmed by the enhanced release profile of 5-FU through complexation as seen in Figures 4, 5 and 6.

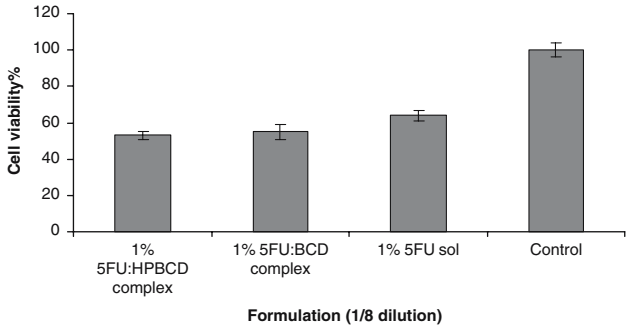

Fig. 7 Cell viability % of HeLa An1 cervical carcinoma cells treated with free 5-FU, 5-FU complexed to β-CD and 5-FU complexed to HP-β-CD (n = 3) (control: untreated cells)

Conclusion

In the light of the current findings, it is possible to conclude that the formulation of an anticancer drug in free form or in complex with natural or synthetic cyclodextrins may exert favorable properties such as thermosensitive characteristics, controlled and prolonged release profile and high anticancer activity. It is also rational to suggest that if a poorly soluble anticancer drug is formulated in such as mucoadhesive thermosensitive gel in complex with highly soluble cyclodextrins including β-CD and HP-β-CD, it is possible to obtain higher anticancer efficacy with much lower doses avoiding unwanted side effects of the drug with controlled release and prolonged residence time in administration site.

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