Preparation and In Vitro Evaluation of Methotrexate Mucoadhesive Nanogel proposed for Cervical Cancer Therapy

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Abstract

Cervical cancer disease is the second most prevalent cause of cancer-related deaths in females. Systemically administered anticancer drugs have difficulty in reaching the cervix with effective concentration. Fortunately, local access to the cervix is possible through the vagina. The present study was designed to formulate and evaluate Methotrexate mucoadhesive nanogel to prolong residence time in addition to improving its efficacy against the cervical tumor. Methotrexate O/W nanoemulsion (consisting of 5 % Eucalyptus oil, 30 % S-mix, and 65 % deionized water) was formulated. Then, this nanoemulsion was incorporated in Carbopol 934 (mucoadhesive gelling agent) dispersion to produce a homogenous Methotrexate mucoadhesive nanogel with a dose of 0.3 mg / 1g.Methotrexate o/w nanoemulsion formulation displayed globules size (57.45 nm) PDI (0.19), zeta potential (-23.67), pH (4.1), electrical conductivity (189 µs/cm), %T (99.57) and % drug content (99.7). Then used to produce a homogenous Methotrexate mucoadhesive nanogel with easy spreading (2.4 ± 0.05 cm) and acceptable Methotrexate content (99.35±0.2). In addition to compatible cervical application as indicated by pH and viscosity values of 4.5 ± 0.1 and 4695.5 mPa.s respectively. The results of Methotrexate release from Methotrexate mucoadhesive nanogel demonstrated a significant ($p \leq 0.05$) higher cumulative drug released percent with 4 fold increment than that of pure Methotrexate gel. The overall data obtained revealed the feasibility of preparing Methotrexate as mucoadhesive nanogel for the treatment of cervical cancer.

Keywords: Methotrexate, Mucoadhesive, Nanogel, Cervical cancer

الخلاصة

يعد مرض سرطان عنق الرحم ثاني أكثر أسباب الوفيات المرتبطة بالسرطان انتشارًا بين الإناث. تواجه الأدوية المضادة للسرطان التي يتم تناولها بشكل فموي او وريدي صعوبة في الوصول إلى عنق الرحم بتركيز فعال. لحسن الحظ ، يمكن الوصول إلى عنق الرحم موضعيا من خلال المهبل .

صممت الدراسة الحالية لصياغة وتقييم مادة الميثوتريكسات اللاصقة النانوية لإطالة مدة الإقامة وكذلك تحسين فعاليتها ضد ورم عنق الرحم .تمت صياغة مستحلب النانو ميثوتريكسات يتكون من 5٪ زيت أوكالبتوس ، 30٪ S-mixو 65٪ ماء منزوع الأيونات. بعد ذلك ، تم دمج مستحلب النانو هذا في Carbopol 934(عامل التبلور المخاطي) لإنتاج مادة متناهية الصغر متجانسة من Methotrexate بجرعة 0.3 مجم / 1 جرام .عرضت تركيبة مستحلب الميثوتريكسات النانوي حجم الكريات (57.45 نانومتر PDI (0.19)، جهد زيتا (-23.67) ، الرقم الهيدروجيني (4.1) ، الموصلية الكهربائية (189 ميكرو ثانية / سم (،٪ T (99.57)و٪ محتوى المخدرات (99.7). ثم يتم استخدامه لإنتاج مادة نانوية لاصقة متجانسة من مادة الميثوتريكسات سهلة الانتشار (2.4 ± 0.05 سم) ومحتوى ميثوتريكسات مقبول (99.35 ± 0.2) بالإضافة إلى تطبيق عنق الرحم المتوافق كما هو موضح بواسطة قيم الأس الهيدروجيني واللزوجة 4.5 ± 0.1 و 4695.5 ملي باسكال. ق على التوالي .أظهرت نتائج إطلاق الميثوتريكسات من مادة النانوجيل اللاصق المخاطي الميثوتريكسيت ارتفاعًا ملحوظًا و تم إطلاق الدواء التراكمي بنسبة 4 أضعاف من هلام الميثوتريكسات النقي. كشفت البيانات الإجمالية التي تم الحصول عليها عن جدوى تحضير الميثوتريكسات باعتباره مادة نانوجيل لاصقة مخاطية لعلاج سرطان عنق الرحم.

Introduction

Cervical cancer is the second most common cancer among females globally, of which about 275 000 women die per year [1]. For patients suffering from cervical cancer, it is difficult for systemically administered drugs to reach the target sites, which reduced the drug efficacy to a large extent. Fortunately, for cervical cancer, it is possible to get access to the cervix through the vagina, without the hepatic first-pass effect. Also, the vagina has a wide surface area, which contributes to high drug adsorption [2]. It is also necessary to overcome multidrug resistance (MDR) to improve drug efficiency. The major mechanism of MDR in tumor cells is the expression of an efflux pump that expels chemotherapy drugs, known as P-glycoprotein (P-gp) [3].

Therefore, we predict that with the combination of nanotechnology, mucoadhesive systems can potentially serve as promising delivery systems to treat cervical cancer and overcome MDR.

Nanotechnology is a promising approach to overcoming MDR attributed to the advantages including strengthening the accumulation and internalization of drugs within tumors and stimuli-responsive intracellular release. Mucoadhesive systems can tightly adhere to the mucosa, improving the local concentration of a drug, consequently, prolonged residence time and enhanced efficacy in cervical cancer therapy will be developed.Methotrexate (MTX) is an antimetabolite and antifolate agent with antineoplastic and immunosuppressant activities. Methotrexate binds to and inhibits the enzyme dihydrofolate reductase, resulting in inhibition of purine nucleotide and thymidylate synthesis and, subsequently, inhibition of DNA and RNA syntheses [4]. MTX had been used in advanced squamous cell cervical cancer [5].

Materials and Methods

Materials: MTX was bought from YIBAI biotechnology (China), Tween 20 was purchased from Sigma ch. (USA), Eucalyptus oil and Carbopol 934 were provided from HIMEDIA chemicals in India, Dimethyl sulfoxide (DMSO was bought from Merck (Germany). Franz cell was purchased from SES GmbH (Germany).

Methods

Formulation and evaluation of MTX nanoemulsion

The selected dose of MTX was added to the required amount mixture (previously mixed and heated to 45◦C) Eucalyptus oil, tween 20, and DMSO with continuous stirring. After cooling to room temperature, with gentle mixing, the aqueous phase was gradually titrated, drop by drop, until an isotropic transparent nanoemulsion was formed, with a dose of 0.6 mg MTX per 1 g of nanoemulsion.

The prepared MTX nanoemulsion was then evaluated by measuring globules size, polydispersity index (PDI), zeta potential, pH, electrical conductivity, percent of light transmittance (%T), and % drug content.

Preparation of MTX mucoadhesive nanogel

MTX nanoemulsion has high water content and thus demonstrates low mucosal retention after cervical application. Therefore to prolong the contact time of MTX nanoemulsion with the mucosa and for convenience of cervical application, MTX nanoemulsion formulation was selected for the development of MTX mucoadhesive nanogel [6]. This would circumvent the leakage of MTX nanoemulsion from the cervical mucosa and improve its stability. Mucoadhesive gel base was firstly prepared by Carbopol 934 (gelling agent), in which 1g of Carbopol 934 powder was dispersed in a small quantity of distilled water by continuous stirring, then 50 g of MTX nanoemulsion was added to the dispersion with continuous stirring by magnetic stirrer then 100 mg of Benzoic acid was added as a preservative [7]. After that, the total weight was completed to 100 grams by distilled water. The dispersion was then made viscous by the addition of ten drops of Triethanolamine (TEA) resulting in the formation of clear homogenous viscous MTX nanogel with a strength of 0.3 mg MTX in each 1 g of mucoadhesive nanogel [8]. The prepared mucoadhesive nanogel was left in a dark place at room temperature for 24 h and then inspected visually for appearance, color and consistency.

Table 1: Components of the prepared MTX mucoadhesive nanogel

Carbopol 934	**MTX nanoemulsion**	**Benzoic acid**	**TEA**	**Distilled water**
1 g (1%)	50 g (50%)	100 mg (0.1%)	10 drops	Up to 100 g

Characterization of the prepared MTX mucoadhesive nanogel

1. Homogeneity of MTX mucoadhesive nanogel

By visual observation, the homogeneity and organoleptic properties such as appearance, color and clarity of the prepared MTX mucoadhesive nanogel were determined. The homogeneity was validated by putting a small amount of the prepared MTX mucoadhesive nanogel between the thumb and index finger then pressing to sense the consistency of the nanogel if it is homogenous or not and to predict the existence of any coarse particles in the nanogel [9].

2. Spreadability of MTX mucoadhesive nanogel

The spreadability was measured by placing 0.5 g of the prepared MTX mucoadhesive nanogel within a pre-marked circle of (1cm) diameter on a glass slide, then a second glass slide was placed over the first slide and thus sandwiching the nanogel between two slides. Later, a 50-gram weight was set for 5 min over the upper slide, so the nanogel would squeeze and spread. The increments in diameter were documented [10]. This experiment was carried out three times.

3. Determination of pH of MTX mucoadhesive nanogel

The pH of MTX mucoadhesive nanogel was determined by a digital pH meter, in which the glass electrode was dipped into the nanogel directly without dilution and when the reading was stable it was recorded [10]. The measurement was performed in triplicate.

4 Determination of viscosity of MTX mucoadhesive nanogel

The viscosity of MTX mucoadhesive nanogel was measured by Brookfield digital viscometer. The viscometer spindle was immersed into 100 mL of sample, then rotated with different speeds of 6, 12, 30, and 60 rpm at room temperature. The viscosity values at each speed were recorded to describe the rheological properties of the prepared MTX mucoadhesive nanogel [11]. The experiment was performed in triplicate.

5. MTX content determination in mucoadhesive nanogel

The actual amount of MTX loaded within the prepared nanogel was determined by dissolving 2 g of the nanogel (supposed to have 0.6 mg of MTX) in 20 ml of DMSO with sonication until a solution was obtained. The solution was filtered by a 0.45 µm filter syringe and then analyzes by a UV spectrophotometer at 302 nm [8]. The measurement was performed in triplicate.

6. *Ex vivo* mucoadhesive force determination

A modified manual balance method was used for determining the *Ex vivo* mucoadhesive strength of the prepared MTX mucoadhesive nanogel. In which freshly excised sheep cervical mucosa was obtained and kept in phosphate buffer pH 4.5 and used within 2 hours of slaughter. The prepared MTX mucoadhesive nanogel was laid on a glass slide and then positioned onto the mucosal membrane under manual pressure of 5 min. The mucoadhesive force was measured in terms of weight in grams required to detach the nanogel from the sheep cervical mucosa [12]. The measurement was performed in triplicate.

7. *In vitro* release study of MTX mucoadhesive nanogel

Franz diffusion cell was used to evaluate the in vitro release of MTX from the prepared mucoadhesive nanogel through a semipermeable dialysis membrane

(M.W8000 – 14000 Da) fixed between donor and receptor compartments of the Franz cell [13]. 1 g of nanogel was placed on the diffusional membrane in the donor part, while the receptor part was filled with 40 ml of phosphate buffer pH 4.5. The temperature was kept at 37 ± 0.5 ◦C and continuous stirring by a magnetic stirrer.

Samples of 1ml were withdrawn at specified time intervals of 0,5,10, 15, 30, 45, 60, 90, 120 and 150 min and replaced by fresh phosphate buffer to keep sink condition, and the content of MTX in each sample was quantified by UV spectrophotometer at 305 nm using phosphate buffer pH 4.5 as blank. The cumulative amount percent of MTX released was plotted versus time [14]. The experiment was repeated three times. Under the same conditions, the experiment was employed for MTX ordinary gel as a control for *in vitro* release comparative study with MTX mucoadhesive nanogel.

8. Microscopic morphology studies by Atomic Force Microscopy

AFM can scan surfaces in controlled environmental conditions and can measure the particle size of the nanoparticles accurately [15].

9. Effect of temperature during storage

Several samples of 1g of MTX mucoadhesive nanogel were stored in sealed glass containers at different temperatures (4, 25, and 40°C) for 12 weeks. Samples were taken every two weeks for assessment of phase separation, change in color, pH, and viscosity were measured at 60 rpm by digital viscometer, then dissolved in DMSO for MTX content determination using UV- spectrophotometer at 302 nm [16].

Statistical analysis

One-way analysis of variance (ANOVA) was used for data analysis in this research, using ($P \leq 0.05$) for the significant difference. The results were studied in an average of triplicate (n=3).

Results and Discussion

Evaluation of MTX nanoemulsion

Methotrexate o/w nanoemulsion formulation displayed globules size (57.45 nm) PDI (0.19) and zeta potential (-23.67) which indicate the stability of the prepared nanoemulsion. The pH was (4.1) and the electrical conductivity was (189 µs/cm) which confirm the preparation of the O/W type of nanoemulsion. The %T was (99.57) and the % drug content was (99.7) which means transparent nanoemulsion was formulated with good drug content.

Characterization of the prepared MTX mucoadhesive nanogel

The elegant appearance of clear white-yellowish colored nanogel and suitable consistency with no separated particles or phase separation was observed upon visual inspection. Furthermore, clear white-yellowish MTX nanogel was prepared with no aggregate or coarse particles sensed by thumb pressing [17].

The spreadability was found to be 2.4 ± 0.05 cm and this indicates easily spreading nanogel by applying small shear with maximum slip and drag. The high spreadability of the prepared MTX mucoadhesive nanogel indicates ease of application and enables a faster release of MTX over a large mucosal surface area after nanogel cervical application with improved permeation flux through the mucosal barrier [18].

The pH of MTX mucoadhesive nanogel was found to be 4.5 ± 0.1 and which is comparable with the vaginal pH range which is 3.5-4.5 indicating compatible cervical application without irritation to the mucosal surface [19].Adequate MTX content of 99.35±0.2 was measured within MTX mucoadhesive nanogel, and that indicates successful construction of MTX nanoemulsion formulation into stable MTX mucoadhesive nanogel form.The *ex vivo* mucoadhesive strength of the prepared MTX mucoadhesive nanogel was measured using sheep cervical mucosa and the results were found to be 15.5 ± 0.5 g, this indicates the mucoadhesive potential of carbopol

934 used in the formulation of MTX mucoadhesive nanogel. The mucoadhesion was attributed to the formation of a hydrogen bonding between carbopol 934 due to swelling and mucin of the cervical mucosa [20].

Determination of viscosity of MTX mucoadhesive nanogel

The results showed decreased viscosities values with the increase in shearing rate related to rotation speed (rpm). MTX mucoadhesive nanogel, revealed viscosity values of 4695.5, 2507.9, 1287.5 and 695.8 mPa.s under corresponding shearing rates of 6, 12, 30 and 60 rpm. As shown in figure 1. The rheological performance is an essential parameter for gel performance of MTX mucoadhesive nanogel, which manages its flowability, spreadability, and MTX release from nanogel [21].

MTX mucoadhesive nanogel displays pseudo-plastic performance with shear-thinning viscosity, proposing the formation of colloid network structure due to Carbopol 934 polymer chain that align itself in direction of shear and hence, viscosity decrease as shear rate increase and delays the free flow, thus ensure no self-flow upon mucosal application [22].

The pseudo-plastic performance of MTX mucoadhesive nanogel is the desired behavior in pharmaceutical formulation due to high apparent viscosity at low shear and hence, low mobility of dispersed phase thus keeping the nanogel components in a homogenous distribution. While upon shear stress service, the MTX mucoadhesive nanogel would exhibit free-flowing with decreased viscosity that enables easy spreading without washout or drug loss upon its application on cervical mucosa [23].

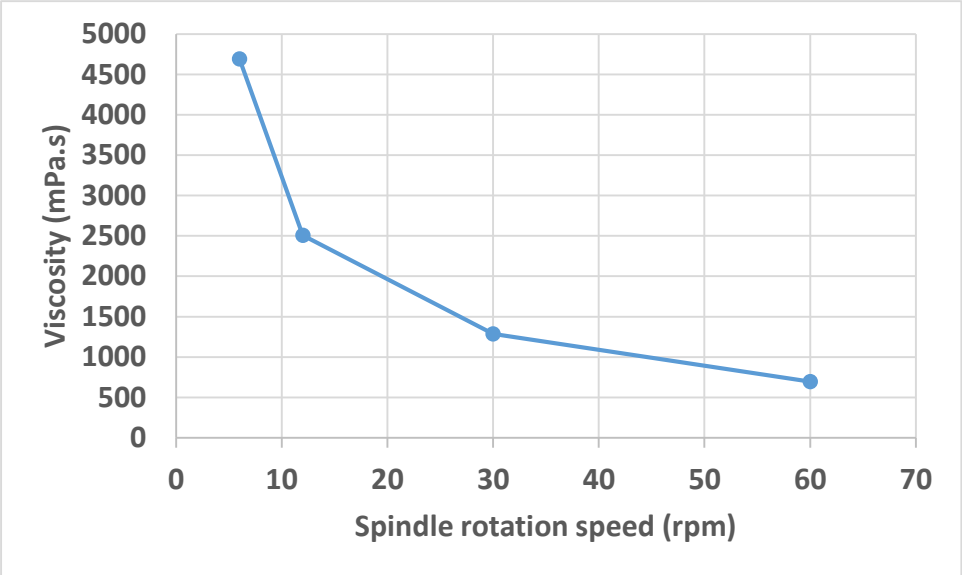

Figure 1: Rheogram of MTX mucoadhesive nanogel

In vitro release study of MTX mucoadhesive nanogel

The *in vitro* release study was achieved as a comparative revision between the *in vitro* release profiles of MTX mucoadhesive nanogel and MTX ordinary Carbopol 934 gel as a control, as presented in figure 2.

The results of MTX release from MTX mucoadhesive nanogel reveal a significant ($p \leq 0.05$) higher cumulative percent of drug released with fourfold increment than that of ordinary MTX gel, in which the MTX mucoadhesive nanogel demonstrates nearly complete MTX release after 90 min, while only 25.3 % of MTX was released from ordinary MTX gel within the same period.

The similarity factor (f 2) was 24.14 and which indicate a dissimilar release profile for MTX mucoadhesive nanogel and MTX ordinary Carbopol 934 gel (control).

The nanodroplet size of MTX mucoadhesive nanogel could provide high effective surface area that is exposed to the releasing medium and therefore enhance the release rate of MTX. Furthermore, MTX was present in dispersed form within nanoemulsion

oil phase droplets of Eucalyptus oil within Carbopol 934 gel context that additionally contribute to the faster passage through gel matrix and dialysis membrane [24].

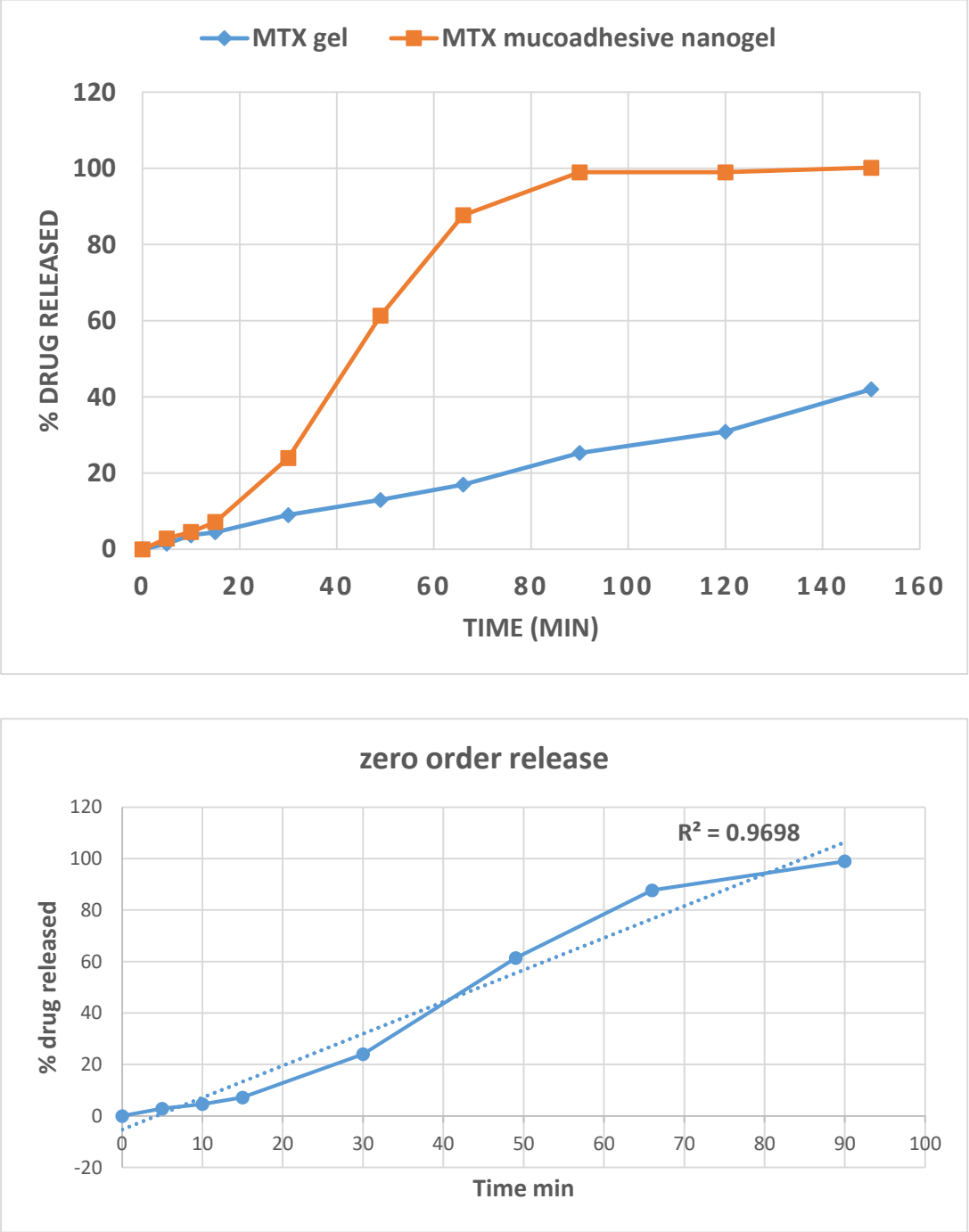

Figure 2: Comparative *in vitro* release study between MTX mucoadhesive nanogel and MTX gel as a control (upper), Zero-order release mechanism of MTX from MTX mucoadhesive nanogel (lower).

Microscopic morphology studies by Atomic Force Microscopy

The noticed results of AFM images confirm the formation of MTX mucoadhesive nanogel with nanosized uniformly distributed droplets [25], as displayed in figure 3.

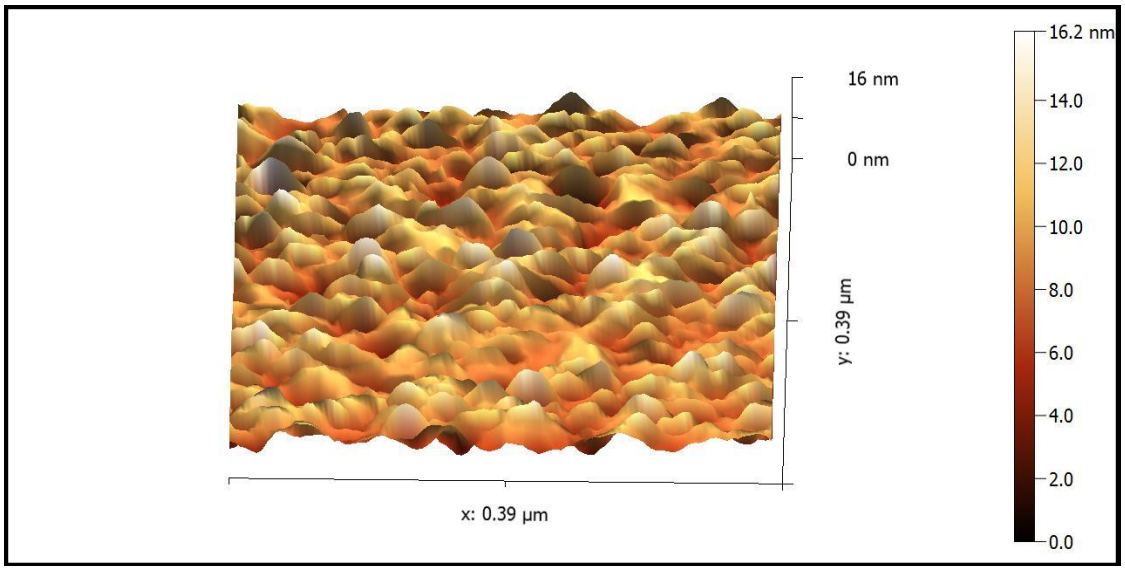

Figure 3: AFM image of MTX mucoadhesive nanogel

Effect of temperature during storage

The stability of MTX mucoadhesive nanogel was evaluated for MTX content, viscosity, pH, color-changing, and phase separation. Visual inspection indicated no color changing nor phase separation during all test periods. Additionally, there was a non-significant ($p > 0.05$) change in MTX content, viscosity, and pH of MTX nanogel after each withdrawal period.

The non-significant variant in pH values during the experiment duration and at different temperatures indicated the stability of MTX nanogel against chemical reactions [26]. Carbopol 934 increase the viscosity of the MTX nanogel formulation, the high viscosity value might limit Brownian movement and retard the nanoemulsion droplet aggregation to improve its stability [27].

Conclusion

Methotrexate (MTX) was formulated successfully into o/w nanoemulsion and mucoadhesive nanogel with globule size (< 200 nm), as indicated by DLS technique as well as AFM imaging microscope. Additionally, the PDI reveal uniform particle size distribution of MTX formulations.

The characterization techniques and in vitro release studies of the prepared MTX mucoadhesive nanogel approve their relevance for effective, safe and practical vaginal application.

Zero-order release kinetic has better clarified the in vitro release mechanism of MTX mucoadhesive nanogel.

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